

# Within-plant heterogeneity in fecundity and herbivory induced by localized DNA hypomethylation in the perennial herb *Helleborus foetidus*

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**PREMISE:** Phenotypic heterogeneity of reiterated, homologous structures produced by individual plants has ecological consequences for plants and their animal consumers. This paper examines experimentally the epigenetic mosaicism hypothesis, which postulates that within-plant variation in traits of reiterated structures may partly arise from different parts of the same genetic individual differing in patterns or extent of genomic DNA methylation.

**METHODS:** Leaves of paired ramets borne by field-growing *Helleborus foetidus* plants were infiltrated periodically over the entire flowering period with either a water solution of the demethylating agent zebularine or just water as the control. The effects of the zebularine treatment were assessed by quantifying genome-wide DNA cytosine methylation in leaves and monitoring inflorescence growth and flower production, number of ovules per flower, pollination success, fruit set, seed set, seed size, and distribution of sap-feeding insects.

**RESULTS:** Genomic DNA from leaves in zebularine-treated ramets was significantly less methylated than DNA from leaves in control ones. Inflorescences in treated ramets grew smaller and produced fewer flowers, with fewer ovules and lower follicle and seed set, but did not differ from inflorescences in untreated ramets in pollination success or seed size. The zebularine treatment influenced the within-plant distribution of sap-feeding insects.

**CONCLUSIONS:** Experimental manipulation of genomic DNA methylation level in leaves of wild-growing *H. foetidus* plants induced considerable within-plant heterogeneity in phenotypic (inflorescences, flowers, fecundity) and ecologically relevant traits (herbivore distribution), which supports the hypothesis that epigenetic mosaicism may partly account for within-plant variation.

**KEY WORDS** demethylating agent; DNA methylation; epigenetic mosaicism; herbivory; inflorescence growth; leaf infiltration; Ranunculaceae; seed production; subindividual variation; zebularine.

A consequence of the modular construction of plants by continual organogenesis, which involves the reiteration of homologous structures, is the appearance of subindividual variation in the traits of the multiple copies of a given structure (e.g., leaves, inflorescences, flowers, fruits, seeds; Herrera, 2009). In recent years, an increasing number of studies have shown that continuous variation among homologous structures produced by individual plants has a number of ecological effects on both the plants themselves and their animal consumers (see Herrera, 2017, for review). The consequences of subindividual variation for plants include enhancing the exploitation of

biotically and abiotically heterogeneous environments through “division of labor” effects; widening the ecological breadth of species and individuals; increasing the functional diversity of populations; and modifying the outcome of interactions with animal antagonists and mutualists (Herrera, 2009, 2017; Sobral et al., 2014; Herrera et al., 2015; Dai et al., 2016; Wetzel et al., 2016; Arceo-Gómez et al., 2017; Palacio et al., 2019; Wetzel and Meek, 2019). The actual evolutionary and ecological significance of the effects of within-plant variation, however, will ultimately depend on its underlying causes and maintenance mechanisms (Herrera, 2017).

Epigenetic mosaicism, in which different parts of the same genetic individual differ in the pattern (distribution across specific sites or regions in the genome) or level (proportion of total cytosines that are methylated) of genomic DNA methylation, has been recently suggested as one possible mechanism contributing to the appearance of within-plant variation in organ traits (Herrera and Bazaga, 2013; Herrera, 2017; Alonso et al., 2018). This hypothesis was initially motivated by results of a handful of studies showing that the genomes of homologous, yet phenotypically distinct organs in the same genetic individual may differ in pattern or level of DNA cytosine methylation (Bitonti et al., 1996, 2002; Gao et al., 2010; Bian et al., 2013; Herrera and Bazaga, 2013). For example, prickly and nonprickly leaves borne by the same individual of the heterophyllous tree *Ilex aquifolium* differed in DNA methylation, the genomes of nonprickly leaves being significantly more methylated than those of prickly ones (Herrera and Bazaga, 2013). In the shrub *Lavandula latifolia*, leaves from different branchlets in the same individual differed significantly in global DNA cytosine methylation, and such epigenetic mosaicism was correlated with within-plant variation in seed number and size (Alonso et al., 2018). Cytosine methylation is a major epigenetic mechanism in plants with significant roles in gene expression, transposon activity, and plant growth and development (Finnegan et al., 2000; Cokus et al., 2008; Lister et al., 2008), and DNA methylation variants independent of DNA sequences are causally related to individual differences in continuous traits (Zhang et al., 2013; Cortijo et al., 2014; Hu et al., 2015; Kooke et al., 2015). On this basis, Alonso et al. (2018) formulated the hypothesis that subindividual heterogeneity in pattern and/or level of DNA methylation could be partly responsible for within-plant variation in organ traits.

Within-plant association between DNA cytosine methylation and seed size and fecundity reported by Alonso et al. (2018) for *Lavandula latifolia* shrubs, albeit consistent with the hypothesized causal link between epigenetic mosaicism and within-plant variation, could also reflect a coordinated response of the two variables to some unmeasured factor. Stronger evidence for a causative connection between within-plant variation in DNA methylation and subindividual variation in organ traits would be obtained if artificial epigenetic mosaicism created experimentally were able to induce concomitant subindividual variation in organ traits. This paper reports an experiment of this sort, in which we artificially produced within-plant epigenetic heterogeneity in adult plants of the perennial herb *Helleborus foetidus* by localized infiltration of a DNA methylation inhibitor into leaves, and then followed its effects on inflorescence and flower traits, seed fecundity, and insect herbivore distribution. Adult *H. foetidus* plants consist of several ramets arising from a single small rootstock (Werner and Ebel, 1994), each of which might function as an autonomous physiological subunit (sensu Watson, 1986). We took advantage of this evident modular construction to test directly under field conditions the hypothesis that epigenetic heterogeneity among the modules of a genetic individual could induce within-plant heterogeneity in phenotypic and ecologically relevant traits.

## MATERIALS AND METHODS

### Study species

*Helleborus foetidus* L. (Ranunculaceae) is a perennial herb widely distributed in western and southwestern Europe, occurring from

sea level to 2100 m a.s.l. in habitats ranging from open scrub to conifer and broad-leaved forests (Mathew, 1989). Adult plants bear one or a few reproductive ramets per season arising from a small superficial rootstock, each of which has 10–15 leaves and differentiates one terminal inflorescence in late autumn, which grows and produces flowers during the following months (Werner and Ebel, 1994). Each inflorescence produces 25–75 hermaphrodite flowers, which are predominantly pollinated by bumble bees. In our southern Spanish study area (see below) flowering mostly takes place during February–April. Flowers are apocarpous, each generally bearing 1–3 distinct carpels. For convenience, the term “follicle” will be used here to designate either flower carpels (gynoecium) or true follicles (developing fruits). Follicle maturation and seed shedding mostly takes place in June. Detailed information on the natural history and reproductive biology of *H. foetidus* is provided, among other, by Vesprini and Pacini (2000, 2010), Herrera et al. (2001), and Herrera (2002).

### Field experiment

This study was carried out in a large population of *H. foetidus* in the understory of a mature *Pinus nigra* forest in the mountain habitat of the Sierra de Cazorla, Jaén Province, southeastern Spain (Las Navillas site; Herrera et al., 2013; Herrera and Medrano, 2017). The experimental layout consisted of a randomized complete block design (Mead, 1988). Individual plants were treated as blocks, and two ramets within each plant were the experimental units randomly assigned to treatment and control levels. Twenty-five widely spaced plants (separation between plants  $\leq 340$  m), each bearing at least two reproductive ramets with incipient inflorescences, were chosen in December 2016 for experimentation. In each plant, one ramet was randomly selected for subsequent treatment of their leaves with the DNA methylation inhibitor zebularine (“treated” ramet hereafter), while the other ramet was set as a paired “control” (Fig. 1). Zebularine can transiently induce genome-wide hypomethylation levels similar to those obtained using genetic means (Baubec et al., 2009). It was also chosen because its minimal cytotoxicity in vitro and in vivo allows the repeated delivery of a low dose to maintain demethylation over an extended period (Champion et al., 2010). Leaves of selected ramets were subjected to a total of 16 experimental sessions evenly spaced from 23 December 2016 to 5 April 2017 (about one session per week on average). Occasional departures from the planned weekly schedule were due to snowfalls sometimes precluding access to the study site. The experimental period encompassed the whole flowering season of marked plants.

For each experimental session, syringe infiltration was used to deliver 0.5 mL of a 0.1 mM sterile solution of the DNA methylation inhibitor zebularine (Sigma-Aldrich, St. Louis, MO, USA) in Milli-Q water (Millipore Corp., Darmstadt, Germany) to the intercellular spaces of the parenchyma in leaves near the inflorescence borne by each treated ramet. The infiltration method consisted of pressing a needleless 1-mL syringe against the underside of leaves and gently forcing the solution through the stomata into the leaf parenchyma (e.g., Jelly et al., 2014; Zhao et al., 2017) as illustrated in Fig. 2. The volume of infiltrated solution was evenly distributed among 3–4 leaves and, within each leaf, among 2–4 different segments. In the same experimental session, leaves of control ramets were infiltrated with 0.5 mL of sterile Milli-Q water in the same way as for treated ramets. Experimental sessions were



**FIGURE 1.** One of our experimental *Helleborus foetidus* plants, bearing two reproductive ramets arising from the common rootstock (left, zebularine-treated; right, control). The photograph was taken at the mid-point of the flowering period.

conducted late in the morning and only on days when leaves were not frozen and air temperature was  $>5^{\circ}\text{C}$ . In this way, leaf damage was avoided, infiltration was easier, and the infiltrated solution was more readily redistributed within the plant. The infiltrated solution was mobilized from the points of application within a few hours of delivery, as evidenced by quick disappearance of the dark green areas in the leaf blade that arose around infiltration points following application (Fig. 2). To verify the effectiveness of



**FIGURE 2.** Field application of the syringe infiltration method to leaves of experimental *Helleborus foetidus* plants.

our zebularine treatment at inducing within-plant variation in genomic methylation, two leaf samples, each consisting of one leaf segment from different leaves, were collected from each treated and control ramet 2 weeks before the end of experimental sessions. They were placed in paper envelopes, dried immediately at ambient temperature in sealed containers with silica gel, and stored at ambient temperature until analysis.

Growth and flower production of inflorescences in treated and control ramets was monitored throughout the flowering period by measuring the length of the inflorescence from tip to basal bracts and counting the number of open and withered flowers, on five and seven occasions, respectively. The sum of withered and open flowers borne by an inflorescence at a given time provided information on the cumulative number of flowers produced up to that date. Number of leaves and basal diameter of the inflorescence were also recorded for treated and control ramets at the mid-point of the flowering period, with the purpose of statistically accounting for their

possible influence on inflorescence growth and flower production.

The pollination success of flowers in treated and control ramets was assessed by counting the pollen grains deposited on the stigma and the pollen tubes in the style. A random sample of styles was collected from withered flowers 1 month after completion of flowering (5–6 styles per inflorescence,  $N = 280$ ), and stored in microcentrifuge tubes filled with 2.5–2.5–95% formaldehyde–acetic acid–ethyl alcohol. Pollen grains and pollen tubes were counted in individual styles using the differential staining technique described by Herrera and Medrano (2017), which involves a combination of bright-field and fluorescence microscopy.

To compare the realized reproductive output of treated and control ramets, fruits were collected in late May–early June, shortly before follicles would have dehisced and dispersed seeds. The total number of follicles and the number of these that eventually set seeds was determined for each fruit. All follicles were dissected, and the number of sound seeds and undeveloped, shrivelled ovules were counted. The sum of these two values was used to estimate the total number of initial ovules per flower. Follicle set was computed as the proportion of the follicles in a fruit which eventually set seed. For the subset of fruits that produced some seed, seed set was computed as the proportion of initial ovules in the flower that eventually set seeds. All seeds collected were stored at ambient temperature and weighed individually to the nearest 0.1 mg after removal of the elaiosome.

Some developing fruits became infested by aphids and lygaeid bugs around mid-May, shortly before ripening. Aphids tended to congregate on the adaxial surface of the persistent, photosynthetically active sepals (Herrera, 2005), while lygaeids generally stood on the surface of developing follicles. The possible effect of the zebularine treatment on the within-plant distribution of these sap-feeding insects was assessed on two separate occasions in late May. The number of aphids per fruit, mostly winged and wingless forms of *Macrosiphum hebebori* (Aphididae), was counted in a random sample of 10 fruits per ramet. The total number of lygaeid bugs on

each inflorescence, mostly adult *Melanocoryphus albomaculatus* (Lygaeidae), was also counted.

### Genomic methylation

Effectiveness of experimental zebularine infiltration at inducing within-plant variation in genomic methylation level was assessed in this study by analyzing the leaf samples collected from treated and control ramets near the end of the experimental sessions. Total genomic DNA was obtained from each sample using the Bioline ISOLATE II Plant DNA Kit and digested with DNA Degradase Plus (Zymo Research, Irvine, CA, USA), a nuclease mix that degrades DNA to its individual nucleoside components. Digested samples were stored at  $-20^{\circ}\text{C}$  until analyzed. The HPLC procedure described by Alonso et al. (2014, 2016) was used to determine DNA cytosine methylation separately for 2–3 independent technical replicates for each leaf sample. Replicates consisted of subsamples from the original DNA hydrolyzate that were processed independently. Global DNA cytosine methylation was determined for each replicate by reversed-phase HPLC with spectrofluorimetric detection and estimated as  $100 \times 5\text{mdC}/(5\text{mdC} + \text{dC})$ , where 5mdC and dC are the integrated areas under the peaks for 5-methyl-2'-deoxycytidine and 2'-deoxycytidine, respectively. The position of each nucleoside was determined using commercially available standards (Sigma Aldrich).

### Data analyses

Statistical significance of the effects of leaf infiltration with zebularine was tested using linear or generalized linear mixed effects models, and Cohen's  $d$  was used as a measure of treatment effect size (Nakagawa and Cuthill, 2007). Zebularine treatment was included as a two-level (control, treated) fixed effect in all models. Individual plants (i.e., blocks in the randomized block design) were always treated as a random effect, which ensured that any possible influence of among-plant heterogeneity in local environment (e.g., soil fertility, insolation regime) or genetic background were adequately accounted for by the models (i.e., blocked; Mead, 1988). Furthermore, to account for potential pretreatment disparity between control and treatment ramets in the same plant (e.g., slight age differences between ramets), inflorescence basal diameter and number of leaves per ramet were included as fixed-effect covariates in models having inflorescence length and flower production as response variables.

All statistical analyses were carried out using the R environment (R Core Team, 2017). Functions `lmer` and `glmer` in the `lme4` library (Bates et al., 2015) were used to fit linear mixed models (global cytosine methylation, inflorescence growth, flower production, ovules per flower, individual seed mass) and generalized linear mixed models (follicle and seed set, pollen grain and pollen tube counts, herbivore counts), respectively. In generalized linear models, proportions (follicle set, seed set) were modelled as binomial processes, and counts (pollen grains, pollen tubes, number of sap-feeding insects) as Poisson processes, and the corresponding canonical link function was used in each case (logit and log, respectively). In each analysis, estimated marginal means (sensu Searle et al., 1980) and associated standard errors for the response variable at each treatment level were obtained with the `emmeans` function of the `emmeans` library (Lenth, 2018). Marginal means from generalized linear models involving proportions and counts were back-transformed to the original scale of measurement. Statistical significance of the effect

of zebularine treatment on response variables was determined in all cases with ordinary likelihood ratio tests using the `anova` function from the R stats library (Zuur et al., 2009). Adequacy of model specification was assessed by examination of scaled residuals using the package `DHARMA` (Hartig, 2018). Weak overdispersion of a few models was corrected by adding observation-level random effects to the data (Bolker, 2015). Cohen's  $d$  measures for effect sizes of zebularine treatment were obtained with the `lme.dscore` function in the `EMAtools` package (Kleiman, 2017).

## RESULTS

As intended by our experimental design and expected from previous studies on zebularine effects, localized infiltration of leaves throughout the flowering period with zebularine solution had induced discernible within-plant mosaics in genomic methylation level by the end of the experimental period. Averaged over all plants, DNA from leaves in treated ramets (33.6% of cytosines methylated) was less methylated than DNA from control ones in the same plant (33.8%). On average ( $\pm\text{SE}$ ), there were  $0.17 \pm 0.07\%$  fewer methylated cytosines in genomic DNA of zebularine-treated leaves than in control leaves from the same individual (Cohen's  $d = -0.330$ ), the treatment effect being statistically significant ( $\chi^2 = 5.09$ ,  $P = 0.012$ , one-tailed likelihood ratio test).

Leaf infiltration with zebularine solution had statistically significant effects on the size and flower production of inflorescences. Inflorescences in control and treated ramets were statistically indistinguishable in basal diameter ( $\chi^2 = 0.32$ ,  $P = 0.57$ ) and number of subtending leaves ( $\chi^2 = 1.66$ ,  $P = 0.20$ ), yet inflorescences borne by zebularine-treated ramets were shorter and produced fewer flowers than did inflorescences of paired ramets in the same plants whose leaves were treated with water alone (Table 1). These differences between inflorescences in treated and control ramets were already apparent shortly after the initiation of the experiment, at the early stages of inflorescence growth and development, and persisted until the end of the flowering period (Fig. 3). In some plants, the smaller size of inflorescences in treated ramets was already apparent by the mid-point of the flowering period (Fig. 1). The zebularine treatment had a strong, highly significant negative effect on the number of ovules in individual flowers (Table 1). Flowers from inflorescences in treated ramets had 13.6 ovules on average, in comparison with the 20.7 ovules of flowers in control ramets (Table 1). Flowers from treated and control ramets did not differ significantly with regard to number of pollen grains on the stigma and number of pollen tubes in the style, the two variables used to assess pollination success (Table 1).

The zebularine treatment induced significant declines in follicle set and seed set, the two complementary measurements of relative reproductive success considered here (Table 1). Follicle set decreased from 88.6% in control inflorescences to 23.7% in treated ones. In those fruits that produced some seed, the proportion of initial ovules eventually setting seed declined from 83.0% in control inflorescences to 73.0% in treated ones (Table 1). There were no significant differences between control and treated inflorescences in the size of individual seeds produced (Table 1).

Leaf infiltration with zebularine had significant effects on the numbers of aphids and, particularly, lygaeids feeding on developing fruits, although the sign of the effect differed between groups: aphids were significantly more abundant on the developing fruits in

**TABLE 1.** Effects of leaf infiltration with a water solution of the demethylating agent zebularine. The control treatment involved infiltration with water.

Response variable	Treatment mean ( $\pm$ SE) <sup>a</sup>		$\chi^2$	P	Effect size (Cohen's <i>d</i> )
	Control	Zebularine			
Inflorescence and flower traits					
Inflorescence length (cm) <sup>b</sup>	27.9 $\pm$ 0.7	26.5 $\pm$ 0.7	40.5	2.0e-10	-0.952
Cumulative flowers per inflorescence <sup>b</sup>	11.5 $\pm$ 0.8	9.9 $\pm$ 0.8	12.3	4.5e-04	-0.359
Ovules per flower	20.7 $\pm$ 0.8	13.6 $\pm$ 1.0	62.9	2.2e-15	-0.994
Pollination success					
Pollen grains per stigma	80.2 $\pm$ 6.1	76.2 $\pm$ 5.8	0.8	0.36	-0.089
Pollen tubes per style	12.1 $\pm$ 0.6	11.6 $\pm$ 0.6	1.0	0.32	-0.153
Seed fecundity					
Follicle set (%) <sup>c</sup>	88.6 $\pm$ 1.6	23.7 $\pm$ 2.7	676.9	2.2e-16	-0.598
Seed set (%) <sup>d</sup>	83.0 $\pm$ 2.7	73.0 $\pm$ 4.6	8.3	0.004	-0.382
Individual seed mass (mg)	13.8 $\pm$ 0.2	13.6 $\pm$ 0.2	1.6	0.20	-0.041
Herbivores					
Aphids per fruit	0.045 $\pm$ 0.032	0.113 $\pm$ 0.078	16.7	4.5e-05	+0.335
Lygaeids per inflorescence	1.37 $\pm$ 0.31	0.22 $\pm$ 0.07	66.5	3.5e-16	-1.233

<sup>a</sup>Model-adjusted marginal means.<sup>b</sup>Means estimated at the mid-point of the flowering period. See Fig. 3 for the comparative temporal dynamics of these traits over the flowering period.<sup>c</sup>Computed from data for all fruits.<sup>d</sup>Computed only for those fruits that produced some seed.

inflorescences of zebularine-treated ramets, while the reverse was true for lygaeids (Table 1).

## DISCUSSION

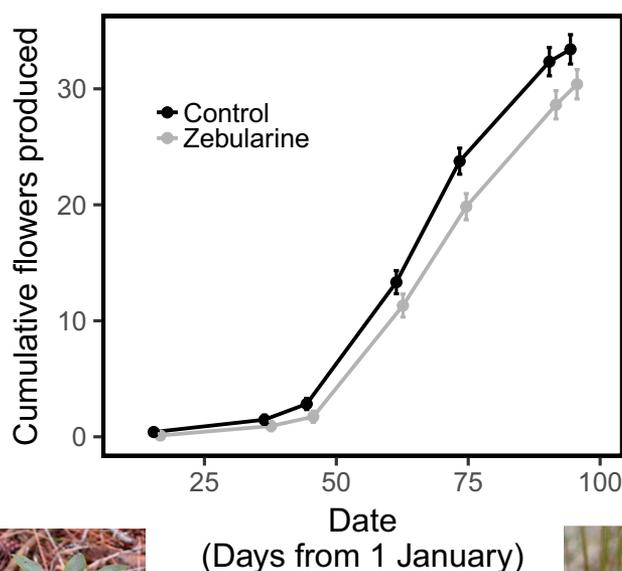
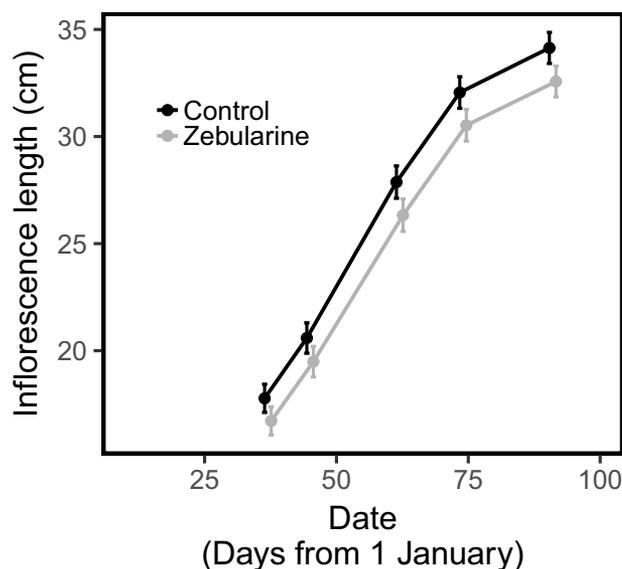
Experiments designed to reveal possible epigenetic effects on ecologically relevant plant traits have often relied on interfering with developmental processes by using some DNA demethylating agent (Fieldes et al., 2005; Kondo et al., 2007; Bossdorf et al., 2010; Verhoeven and Van Gurp, 2012; Kellenberger et al., 2016; Alonso et al., 2017). The demethylating agent chosen in most of these earlier investigations was the cytidine analogue 5-azacytidine, applied to germinating seeds or young seedlings using immersion or, less often, spraying procedures (Puy et al., 2018). The present study differs from most of these earlier experiments with regard to the demethylating agent chosen and the application procedure, both of which were tailored to suit the specific needs of a test of the epigenetic mosaicism hypothesis on adult plants conducted in the field.

Zebularine, the demethylating agent used here (see also Verhoeven and Van Gurp, 2012; Herman and Sultan, 2016; Baker et al., 2018), is a cytidine analogue that covalently binds methyl transferases to DNA, decreasing the dissociation, temporally trapping the enzyme, and thereby preventing methylation and turnover even at other sites (Baubec et al., 2009; Champion et al., 2010; Griffin et al., 2016). It has the advantage for field experimentation of fewer side effects and longer half-life in aqueous solution, which reduces the probability of artifacts due to cell toxicity effects unrelated to DNA methylation interference and presumably confers longer in vivo activity in plants (Baubec et al., 2009) and mammals (Champion et al., 2010). Our results have effectively shown that weekly application over the flowering period of a small volume of zebularine water solution to leaves of adult *H. foetidus* plants was sufficient to induce (1) a moderate, statistically significant reduction in genomic methylation level relative to control leaves in the same plants; and (2) significant, moderate-to-strong effects on inflorescences, flowers,

seed fecundity, and herbivore distribution. The intended test of the epigenetic mosaicism hypothesis required controlling the spatial distribution of zebularine, which should be applied only to some parts of the plant. In this respect, syringe infiltration of leaves with a zebularine solution was a more reliable application method relative to spraying (Puy et al., 2018) because it allowed closer control of the spatial distribution of the treatment and the actual volume of solution delivered to plants. Syringe infiltration is widely used in transient gene expression assays and other molecular transformation studies (Jelly et al., 2014; Zhao et al., 2017), but we are not aware of any prior application in ecological epigenetic experiments with wild plants. The results obtained here with *H. foetidus* illustrate the potential of the syringe infiltration technique in epigenetic experiments that require spatially well-defined delivery of demethylating agents to specific parts of adult plants.

By the end of the experimental period, genomic DNA from zebularine-treated leaves was significantly less methylated than DNA from control ones in the same plant, thus demonstrating that our experimental procedure efficaciously produced the intended within-plant epigenetic mosaics. Inflorescences whose subtending leaves received the zebularine solution and had less-methylated DNA were smaller and produced fewer flowers, and their flowers had fewer ovules and a lower follicle and seed set, than control inflorescences whose leaves received only water. These results are consistent with two central elements of the epigenetic mosaicism hypothesis of subindividual variation (Alonso et al., 2018), namely, that (1) epigenetic mechanisms related to changes in DNA cytosine methylation were involved in inflorescence, flower, and fruit development in *H. foetidus*, and (2) adult *H. foetidus* plants were organized sectorially, i.e., distinct reproductive ramets represented relatively independent physiological subunits that behaved semi-autonomously (“integrated physiological units”; Watson, 1986).

Zebularine delivered to leaves eventually affected inflorescences, flowers and fruits that were up to 30–40 cm away from the points of infiltration. This result may be interpreted as a consequence of the demethylation of DNA in subtending leaves disturbing the physiological control of inflorescence, flower and fruit development



A



B

**FIGURE 3.** Growth and cumulative flower production of *Helleborus foetidus* inflorescences in control and zebularine-treated ramets during the whole flowering season. The control treatment involved leaf infiltration with water. Dots are model-adjusted marginal means, and vertical segments extend  $\pm 1$  SE. In the early experimental sessions most inflorescences were still small and scarcely developed (A), while at the late sessions most inflorescences had already finished flower production and had unripe fruits at different stages of development (B).

through alteration of leaf-provided hormones or photosynthates. In addition, a mutually non-exclusive interpretation is that the demethylating agent also reached the actively growing tissues in the inflorescence and altered developmental processes in situ. This

mobilization could result from source-to-sink translocations linking leaves and developing fruits and inflorescences through vascular transport (Marshall, 1996; Aloni et al., 1999; Orians, 2005). Experiments with model plants have shown that zebularine inhibits plant growth and development through dose-dependent induction of genome-wide hypomethylation and transient reactivation of transcriptionally inactive loci (Baubec et al., 2009; Griffin et al., 2016). The detrimental effects of the zebularine treatment found in this study could thus be parsimoniously interpreted as evidence that inflorescence size, at least some floral traits (ovule number), and seed fecundity (follicle set, seed set), are developmentally controlled in *H. foetidus* through DNA methylation-related epigenetic mechanisms as found in other plants (e.g., Grover et al., 2018; Kottler et al., 2018).

It is unlikely that our results are mostly a consequence of zebularine effects other than genomic demethylation. On one side, previous experimental studies on several plants with zebularine concentrations comparable to that used here have not reported toxicity or side effects (e.g., malformations) associated with zebularine treatments (Baubec et al., 2009; Verhoeven and Van Gurp, 2012; Herman and Sultan, 2016). In particular, the short-term reversibility of the zebularine effects documented by Baubec et al. (2009) points to the innocuousness of zebularine beyond its transient demethylating effect. The fact that leaf or stem damage and flower, fruit, or seed malformations were never recorded in the treated ramets of this study despite weekly infiltration of zebularine over a 3.5-month period effectively points to innocuousness. On the other hand, cryptic interference of the zebularine treatment on plant-animal interactions influential on fecundity can also be safely ruled out. Differences between treated and control ramets in fecundity cannot be attributed to the action of sap-feeding insects, since fruits were infected when they had nearly completed development. In addition, the two insect groups responded in opposite ways to the zebularine treatment. Flowers from treated and control ramets had similar pollination success (pollen grains on stigmas, pollen tubes in styles), which rules out the possibility that the impact of zebularine on fruit and seed set was mediated by some detrimental effect on pollinator visitation. This result contrasts with those obtained by Kellenberger et al.

(2016) for *Brassica* plants treated with 5-azacytidine, in which the demethylation treatment reduced the attractiveness of plants to pollinators. Experimental insect exclusion and hand-pollination, combined with experimental localized demethylation, would be

valuable to elucidate the possible relationships between biotic interactions and the plant epigenome which may affect plant fitness (Alonso et al., 2019)

The lack of effect of zebularine on the mean size of seeds produced by treated ramets was a somewhat puzzling result of this study, given that (1) significant associations between genome-wide methylation and seed size across modules of the same plant were previously found in *Lavandula latifolia* shrubs (Alonso et al., 2018) and (2) variation in seed size has been related to patterns or extent of DNA methylation in parental genomes in several species including *H. foetidus* (Xiao et al., 2006; FitzGerald et al., 2008; Amoah et al., 2012; Herrera et al., 2014; Grover et al., 2018). Robustness of seed size to zebularine treatment could have arisen if methylation changes induced by zebularine in the genome of reproductive tissues were transitory and could be successfully reset during female gametogenesis and/or early embryo growth (Kawashima and Berger, 2014), thus cancelling or buffering any potential disruption of the epigenetic mechanisms involved in seed growth regulation (Xiao et al., 2006; Li et al., 2013). In contrast, relationships between the level of naturally occurring, genome-wide methylation of leaves, and seed size and fecundity found previously in *H. foetidus* and other species (Alonso et al., 2014, 2018) would reflect the influence of permanent, transgenerationally stable methylation marks that are not reset at gametogenesis (Quadrana and Colot, 2016). These interpretations must remain speculative until further data become available, yet the lack of effect of zebularine on seed size found in this study supports Herrera's (2017, p. 59) contention that "links between epigenetic mechanisms and subindividual variation in seed mass are probably far more complex than envisaged by the simple epigenetic mosaicism hypothesis."

The zebularine treatment induced a significant shift in within-plant distribution of sap-feeding insects, with aphids tending to prefer, and lygaeids to avoid, the fruits on treated inflorescences. This result points to an epigenetic basis of the host-related cues governing microhabitat selection by these insects and suggests the existence of contrasting response to the zebularine treatment of the specific cues used by different groups of insects. Nutrients and secondary compounds play key roles in food site selection by sap-feeding insects (Powell et al., 2006); hence, alterations in their concentration and/or spatial distribution within plants brought about by localized DNA hypomethylation may ultimately account for observed shifts in small-scale patterns of herbivore distribution. The proximate mechanisms responsible for zebularine-induced shifts in within-plant herbivore distribution cannot be ascertained with the data available, but our experiment does suggest that within-plant heterogeneity in epigenetic characteristics related to DNA methylation can ultimately account for small-scale patchiness in the within-plant distribution of insect herbivores so frequently reported (Herrera, 2009, table 8.1; Wetzel and Meek, 2019).

## CONCLUSIONS

Localized genomic hypomethylation of leaves in *H. foetidus* plants induced significant within-plant heterogeneity in phenotypic (inflorescences, flowers, fecundity) and ecologically relevant (herbivore distribution) features. These results support the epigenetic mosaicism hypothesis of within-plant variation, and suggest that within-plant patchiness in genomic methylation patterns arising from localized epigenetic responses to environmental factors could

be preserved by the sectoriality of phloem transport, which will constrain horizontal transfer of the phloem-mobile molecules that regulate genomic methylation (Molnar et al., 2010; Lewsey et al., 2016). Epigenetic heterogeneity within individual plants could represent an additional, hitherto unrecognized layer of epigenetic diversity boosting the phenotypic and functional diversity of plant populations.

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