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RESEARCH

Effects of DNA-demethylation on the expression of plastic and transgenerational responses to nitrogen availability in annual plant species

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Abstract
The parental environment can influence the phenotype of the offspring generation. Such parental environmental effects may be transmitted via epigenetic modifications, such as DNA methylation. In a set of annual plant species, exposure of the parental generation to disparate nitrogen availability was previously shown to effect trait expression of the progeny. In this study, we explore whether these elicited transgenerational responses are associated with inherited DNA methylation. We exposed a parental generation of six annual plant species to nitrogen-limitation (N-limitation) or balanced nutrient supply (balanced). Their offspring were exposed to increasing amounts of the demethylation agent zebularine during germination aiming to alter the inheritance of DNA methylation, or a control treatment. The two offspring types were grown again under N-limited and balanced conditions. When germinated in the absence of zebularine and grown under N-limitation, offspring of N-limited parents showed longer and thinner roots, as well as larger leaves, compared to the offspring of balanced-parents. Such transgenerational effects, known to be advantageous under N-limitation, were removed by demethylation; i.e., demethylated offspring of N-deficient parents produced roots of similar length (in *Epilobium ciliatum*) and leaves of similar size (in *Lolium temulentum*) as demethylated offspring of balanced parents when grown under similar conditions. These results demonstrate that transgenerational plasticity (TGP) to N-limitation can potentially be adaptive in some annual plants and support that the inheritance of adaptive traits can be mediated by DNA methylation. For other annual plants, however, our results indicate non-adaptive trait responses to the nutrient treatments and mediation of TGP without involvement of DNA methylation.

Keywords Adaptive transgenerational plasticity · Epigenetics · Non-genetic inheritance · Nutrient limitation · Parental effect · Phenotypic plasticity

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Introduction

Plants are sessile organisms and exposed to the biotic and abiotic conditions at their specific location, including the supply of resources and their temporal fluctuations. As plants cannot escape from the environmental conditions at their site, at least not within one generation, they have evolved mechanisms to cope with fluctuating resources, for example via phenotypic plasticity (i.e., the capacity of a single genotype to produce different phenotypes in different environments, Fox et al. 2019; Gomulkiewicz and Holt 1995; Sultan 2000). Plasticity is the variation of a trait in response to environmental variation and a plastic trait-response may have positive repercussions on a plant's performance, in which case plasticity is considered adaptive (Harmon and Pfennig 2021), whereas it can also be neutral or maladaptive. As such, plasticity may serve as the adaptive solution over non-plastic phenotypes in heterogeneous environments and/or may help bridging the time-lag until genetic evolution has adapted the population to new conditions (Fox et al. 2019; Harmon and Pfennig 2021). Phenotypic trait plasticity can be expressed within one generation (within-generational plasticity, Bradshaw 1965) or between generations. Transgenerational plasticity (TGP, Holeski et al. 2012; Lamarck 1809), also called parental/maternal effects, describes the phenomenon when the environmental cues experienced by the parent plant translate into a modified phenotype of the offspring individual, independently of genetic inheritance (Herman and Sultan 2011; Waterman and Sultan 2021). For example, González et al. (2017) exposed a parental generation of *Trifolium pratense* L. to drought stress and ambient water regime and found that total biomass was higher in offspring plants that experienced water-stress and with drought-treated parents than offspring grown under ambient water regime and from parents that experienced water-stress. In this context the environmental information can be transmitted, for example, by changes in seed provisioning or seed hormone content (Herman and Sultan 2011). Furthermore, gene expression can be transgenerationally altered by epigenetic regulations which refer to chemical modifications at the molecular level, such as histone modifications, small non-coding RNAs, and/or DNA-methylation (Banerjee and Roychoudhury 2017; Bruce et al. 2007; Rajpal et al. 2022). In particular, methylation of cytosines is a prominent epigenetic DNA modification in most eukaryotic organisms, which in plants is sensitive to environmental stimuli coming from biotic interactions such as pathogen infections or herbivory, or abiotic stressors such as salinity, nitrogen-limitation or water-stress (Akkerman et al. 2016; Boyko et al. 2010; Dowen et al. 2012; Kou et al. 2011; Pastor et al. 2013) and can be transmitted to the offspring (Fitz-James and Cavalli 2022). The role of DNA-methylation in mediating TGP is suggested by observations that the expression of TGP can be nullified by experimental demethylation with specific chemical agents (see e.g. Baubec et al. 2009 for the agent zebularine). For instance, Herman and Sultan (2016) demonstrated parental environmental effects on offspring phenotypes in the annual herb *Polygonum persicaria* L. Offspring of parent plants grown under dry conditions, i.e., under water-stress, produced higher biomass compared to offspring of well-watered parents, when both were grown under moist conditions. However, when DNA-methylation was altered under germination due to the use of zebularine, no such parental effect on offspring biomass production was observed.

Both within-generational and transgenerational plasticity are regarded as relevant for organisms to adapt to novel environmental conditions, including those induced by anthropogenic actions (Matesanz and Gianoli 2010; Richards et al. 2006). Human activities are

accelerating global environmental changes, e.g. by releasing greenhouse gasses or by changing bioavailability of nitrogen (N) and phosphorus (P) (Stevens 2016; Wassen et al. 2021). Growth of terrestrial plants or plant communities are frequently limited by N (Elser 2011), and is negatively affected by changes on the stoichiometric level caused by shifts in, for example, P, or both N and P availabilities (Fujita et al. 2014; Minden and Olde Venterink 2019; Olde Venterink and Güsewell 2010). These stoichiometric shifts may impact on plant competitiveness, accelerating for example loss of endangered species (Wassen et al. 2021), and potentially affect human society because of their consequences on both food production and security (Peñuelas et al. 2013; Peñuelas and Sardans 2022). The physiological effects of N-limitation, and the underlying molecular basis are well studied (Marschner 2012; Richard-Molard et al. 2008; Yan et al. 2006). Functional traits associated with N-uptake are for example long and thin roots with a large surface area, a large root-shoot ratio and a higher transpiration rate to increase the mass flow of the soil solution (White et al. 2013a, b). The effects of N:P imbalances on transgenerational trait plasticity have been studied by Minden et al. (2024) who found enhanced tolerance in offspring of some species with parents exposed to different N:P ratios, when again exposed to the same nutrient condition as in their parental environment. Similarly, Latzel et al. (2010) showed that *Plantago lanceolata* L.-plants produced more leaf biomass when they experienced the same nutrient deficiencies as in their parent generation. These results show that the parental nutrient environment can have profound effects on offspring performance, however, knowledge of the underlying mechanisms of the TGP is still pending.

Here we present the results of a greenhouse study testing the involvement of DNA-methylation in transgenerational trait expression induced by variation in N-availability conducted with six different annual plant species. A parental generation was grown either under N-limitation or balanced nutrient supply, with the offspring growing in both conditions (N-limited and balanced). Inherited effects of parental N-availability on offspring performance (i.e., biomass production and relative growth rate) and functional traits related to growth and N-uptake (e.g., total leaf area, root surface area, average root diameter, White et al. 2013b) were measured. We assume plant traits to show TGP when the parent and offspring environments match, e.g., when both generations were grown under N-limitation. In this case, TGP is adaptive if it manifests in a better performance of the offspring compared to offspring of non-exposed parents, i.e., parents grown under balanced nutrient conditions. This should translate in a higher SLA (Fenesi et al. 2014), longer and thinner roots (Minden et al. 2025; White et al. 2013b), as well as higher growth rate and higher biomass production (Latzel et al. 2010, 2014), assuming to be a good indicator of fitness (Laughlin et al. 2020; Younginger et al. 2017). Under germination, seeds of the offspring generation were exposed to the DNA demethylation agent zebularine in different doses. If DNA-methylation mediates transgenerational trait plasticity, experimental demethylation of DNA during germination should affect (and potentially nullify) the expression of TGP in offspring plants. Thus, our study tests the hypothesis that DNA-methylation is involved in the expression of TGP in response to variation in N-availability.

Materials and methods

Parent generation

Six plant species were grown in the greenhouses of the Vrije Universiteit Brussels (VUB) in Belgium. Three forb species belonged to the genus Epilobium (Onagraceae) whereas three grass species (Poaceae) belonged to different genera, and were selected based on their ability for sexual reproduction, autonomous self-pollination, as well as being phylogenetically related (Ackerly 2009). The species were *Epilobium anagallidifolium* Lam., *Epilobium ciliatum* Raf. and *Epilobium fleischeri* Hochst., as well as *Avena sterilis* L., *Hordeum murinum* L. and *Lolium temulentum* L. This species choice does not permit for a comprehensive forbs versus grasses comparison, but does contribute to some insight in how general or variable measurable TGP effects are, compared to including, for instance, either only forbs or grasses. Being conscious of the distinct degree of phylogenetic diversity included in the two groups, we should expect a higher consistency in the results of the three congeneric forbs. Seeds were purchased at B&T Seeds (France), Weber Seeds (The Netherlands) as well as at Jelitto Samen, Botanical Garden Konstanz and Rieger-Hofmann (Germany), with no particular estimate of the genetic variation contained in the stocks. To exclude the possible influence of environmental differences between the seed production sites (Latzel 2015), all species were grown for new seed production in the same standard potting soil in the greenhouse at VUB. The seeds were harvested and stored in a dry and dark place until they were used to grow the experimental parental generation.

The parental generation was grown in the growing period of 2021 at VUB greenhouse (14 h light, 24 °C). Seeds were sterilized with ethanol (70%) and sodium hypochlorite (5%) and then rinsed in de-ionized water. This was done to prevent potential seed-borne plant diseases. Subsequently, seeds were germinated in a climate chamber and seedlings were transplanted when radicula and cotyledons were clearly developed. Each individual was planted in a pot of 10 × 8 cm and filled with 500 ml quartz sand from Sibelco Benelux (Dessel, Belgium, type M31), containing neither N nor P.

Transplantation to the experimental pots was between end of April and mid-June 2021, earliest for *A. sterilis* and latest for *E. fleischeri* due to differences in germination rates. A total of 120 pots were used, comprising two nutrient treatments and 10 replicates per treatment and species (2 treatments × 6 species × 10 pots). Following Güsewell (2005), Güsewell (2004) and Olde Venterink and Güsewell (2010), we applied two nutrient treatments in which we manipulated both the N:P ratio and the supply level of N and P. The two treatments were defined as N-limitation (N:P ratio 1.7, with a weekly dose of 1.7 mg N and 1 mg P per individual) and balanced (N:P ratio 15, with a weekly dose of 5.1 mg N and 0.34 mg P per individual), provided that the previous studies showed strongest negative responses of biomass production at N:P ratio of 1.7 and strongest positive growth response at N:P ratio of 15 (Güsewell 2004; Minden et al. 2023). We applied N and P as $NaNO_3$ and $NaH_2PO_4.2H_2O$, respectively. Details on concentrations of all macro- and micronutrients applied are given in Table S1. The adjusted pH of nutrient solutions was 5.5, and each individual received 5 ml of nutrient solution per week until seed production was terminated (see Table S2 for average days until start of seed harvesting per species and treatment). Pots were watered with de-ionized water when the upper layer of sand was dry and saucers were empty, which also prevented leaching off nutrients from the pots.

When plant individuals entered the generative stage, they were wrapped in Aratubes© (Arasystem, Belgium) to prevent foreign pollen to fertilize the flowers (see photo in Figure S1). Each parent plant was both the maternal and paternal parent, hence uniparental seeds were produced for F1. Seeds were earliest produced by *E. anagallidifolium* in the N-limited treatment, after 68 days, and latest by *H. murinum* after 262 days in the same treatment (see Table S2 for all species and treatments). Collected seeds were stored in paper bags in a dry, dark space. When individuals of the parent generation did not produce any more flowers and/or biomass was dying off, they were disposed.

Offspring generation

The treatments of the offspring generation comprised the same N-limitation and balanced nutrient supply which were applied to the two parental treatments. In total, this created four treatments: N-limited-parental/N-limited-offspring, N-limited-parental/balanced-offspring, balanced-parental/N-limited-offspring, balanced-parental/balanced-offspring. Each of these four treatment combinations comprised six zebularine treatments, i.e., 0µM (control), 10, 20, 30, 40 or 50µM zebularine, respectively. The whole experiment comprised 1152 pots (6 species × 2 parental treatments × 2 offspring treatments × 6 zebularine treatments × 8 replicates).

Seeds of the parental generation were used to grow the offspring generation on November 4th 2022. For this, seeds from the parental plants of each species and parental treatment were pooled and sterilized as described above. They were subsequently spread on solidified 0.8% agar plates containing the aforementioned different zebularine-concentrations, respectively. Zebularine ($C_9H_{12}N_2O_5$, molar weight 228.2 g/mol) was purchased from Merck Life Science BV, Hoeilaart, Belgium. Preparation of agar plates and placing of seeds was done in the Netherlands Institute of Ecology (NIOO), Wageningen, The Netherlands. Seeds were kept in the agar plates in the climate chambers for 10 days (at 25 °C, appr. 46% humidity and 16/8 h day/night rhythm) before transplantation to the experimental pots and application of nutrient solutions.

Transplantation into experimental pots took place on November 14th 2022, growing of plants took place in VUB's greenhouse. Experimental pots were filled with quartz sand in the same manner and with the same size as in the parental generation. Pots were randomly arranged in blocks of species, comprising 192 pots each (1 species × 2 parental treatments × 2 offspring treatments × 6 zebularine treatments × 8 replicates). Concentrations of all macro- and micronutrients, light and temperature conditions, as well as watering of plants with de-ionised water, were the same as in the parental generation. We decided to end the experiment after five weeks just before plants were expected to transition to the generative phase according to data recorded for the parental generation (see Table S2), in order to avoid trait expressions being influenced by the plants entering their generative stage at different ages.

Plant trait measurements

We studied individual performance (RGR (Relative Growth Rate), final biomass, total leaf area), leaf physiology (SLA (Specific Leaf Area), SPAD (light absorption, strongly correlated to leaf chlorophyll) and root morphology (length, diameter, area) to characterize above

and below-ground phenotypic responses to N-limitation. At the beginning of the experiment, when 10-day old offspring seedlings were transplanted into the experimental pots, approximately 20 seedlings per species, parental treatment and zebularine treatment were harvested to measure initial biomasses, which was used to evaluate effects of zebularine on early biomass production and to determine relative growth rate of each offspring individual (RGR, mg/mg*day). RGR for each plant individual of the offspring generation was calculated with the averaged initial biomass of seedlings of the same parental treatment (W) at the start of the experiment (t1) and the biomass of each harvested plant individual at the end of the experiment (t2), as RGR = (logW2 – logW1) / (t2 – t1) (Hunt 1990).

All plants of the offspring experiment were harvested between the 19th and 23rd of December 2022. On each day the same amount of each combination species × parental treatment × offspring treatment × zebularine treatments × replicates were harvested to terminate the experiment evenly over all combinations, instead of harvesting, e.g., species by species. Plants were taken out of the pots, sand was washed off the roots, samples were dried at 70 °C for 72 h and weighted on a precision balance to determine total biomass (mg).

Two fully developed leaves were clipped off from each plant individual and their area was measured with a flatbed scanner and the software Image J (Schneider et al. 2012). From the same leaves, three measurements per leaf with a chlorophyll-meter were taken to obtain a measure of chlorophyll content ('SPAD'-values, Chlorophyll Meter 502- SPAD Plus, Konica Minolta, Germany) and subsequently specific leaf area (SLA, mm^2/mg) and total leaf area (cm^2) were calculated. SLA is the area of a leaf divided by its dry weight and total leaf area is based on the area and the weight of the two clipped leaves extrapolated to the biomass of all living leaves (Pérez-Harguindeguy et al. 2013). Leaf blades were considered leaves, whereas the petioles were considered as part of the stem.

Belowground traits comprised total root length (m), root surface area (cm^2) and root average diameter (mm). For each plant individual the root was cut in two parts, with the cutting point at the 'top' part, i.e. close to the insertion point to the stem. The fresh mass of both parts was determined. One part of the root was then stored in 70% ethanol until it was scanned (LA2400 scanner for WinRhizo®, Regent Instruments Inc. 2013, Canada) and the image was processed with WinRhizo® software (Regent Instruments Inc. 2013). The other part was dried at 70 °C for 48 h and its dry mass was measured. By offsetting both fresh mass of the two root parts per individual with the dry mass of one part of the root, total root weight could be calculated as part of total biomass.

Determination of methylation levels

At the same moment when seedlings of the offspring generation were transplanted into single pots, biomass of seedlings not used for the main experiment were harvested, from each species and from each parental treatment and zebularine treatment separately. This material was used to determine the global cytosine methylation level (%) as a measure of the effect of zebularine on methylation levels. There was not enough seedling biomass available to determine DNA-methylation levels separately for the two parental nutrient treatments, i.e., N-limitation and balanced nutrient supply, and we therefore pooled seedlings from both parental treatments to obtain an overall estimate of the effect of zebularine concentration on global DNA-methylation levels. Further, whereas the effect of zebularine on methylation levels could be tested for all treatments in *A. sterilis* and *H. murinum*, some treatments had

to be merged for the other species due to insufficient plant material (in *E. anagallidifolium* Z0&Z10, Z20&30, Z40&Z50, in *E. ciliatum* Z10&Z20, Z30-Z50, in *E. fleischeri* Z0&Z10, Z20-Z50, in *L. temulentum* Z0&Z10, Z20&Z30, biomass for Z40 and Z50 was not available). Seedlings were airdried and kept in silica gel until processing at Estación Biológica de Doñana, CSIC, Spain. Samples were homogenized to a fine powder using a Retsch MM 200 mill. Total genomic DNA was extracted using Bioline ISOLATE II Plant DNA Kit and quantified using a Qubit fluorometer 2.0 (Thermo Fisher Scientific, Waltham, MA, USA). A 100 ng aliquot of DNA extract was digested with 3 U of DNA Degradase PlusTM (Zymo Research, Irvine, CA, USA), a nuclease mix that degrades DNA to its individual nucleoside components. Digestion was carried out in a 40 µl volume at 37°C for 3 h, and terminated by heat inactivation at 70 °C for 20 min. Two DNA extracts and three independent replicates of digested DNA per sample were processed in randomized order to estimate global cytosine methylation more precisely. Selective derivatization of cytosine moieties with 2-bromoacetophenone under anhydrous conditions and subsequent reverse phase HPLC with spectrofluorimetric detection, were conducted at LEQ-EBD. The percentage of total cytosine methylation on each replicate was estimated as 100*5mdC/(5mdC + dC), where 5mdC and dC are the integrated areas under the peaks for 5-methyl-2'-deoxycytidine and 2'-deoxycytidine, respectively (see Alonso et al. 2016 for further details).

Data analyses

Effects of zebularine on global methylation levels and on biomass and plant traits

For each species we determined the effects of the zebularine treatments on the cytosine methylation levels at seedling stage with univariate ANOVA and subsequent Tukey-test. In order to detect possible toxic effects of the chemical agent on early biomass (i.e. seedling biomass), especially of the highest zebularine treatment (Z50 µM) a linear model was conducted with initial biomass responding to zebularine treatments (6 levels, Z0 – Z50 µM on a continuous scale), parental environment (2 levels, N-limitation and balanced) and species (6 levels, all species in this study). Initial biomass was standardised prior to analyses as biomass differed between forbs and grasses by factor 10.

Effects of parental and offspring environments, and presence and absence of zebularine (Z0 versus Z50) on biomass production and plant traits

After confirming that there were no toxic zebularine-effects and that initial biomass was not largely impaired at Z50µM, we ran ANOVA analyses for all measured traits, across all species and for each species separately (*across-species analysis* and *single-species analysis*, respectively). The ANOVA analyses included phenotypic data from just two levels of demethylation treatment, i.e. the highest zebularine concentration and the control, as our results confirmed that cytosine methylation levels were lowest in the highest zebularine treatment (50 µM), for those species for which they could be determined. For the across-species analysis, we analysed the fixed effects of the parental treatments (2 levels, N-limitation and balanced), the offspring treatments (2 levels, N-limitation and balanced), the zebularine treatments (2 levels, Z0µM and Z50µM) and the functional groups (2 levels,

forbs and grasses as these species groups may respond differently to variation in both N and P fertilization, Nelson et al. 2025) and their interactions. Species was included as random factor (6 levels, each species of this study).

To evaluate if results of the across-species analyses were driven by single plant species, we analysed the fixed effects of parental treatments (2 levels), offspring treatments (2 levels) and zebularine treatments (2 levels) and their interactions on traits of each species separately (*single-species analysis*). For both types of ANOVA analyses, a significant interaction of parental treatment x zebularine treatment would indicate that demethylation modified the expression of TGP (after Herman and Sultan 2016). A significant three-way interaction between parental treatment × offspring treatment × zebularine treatment would indicate that the aforementioned modification of TGP would differ additionally if the offspring environments were N-limited or balanced. For each combination of parental and offspring treatment separately, Tukey pairwise comparisons using estimated marginal means (R-package emmeans, Lenth 2023) were performed.

All statistical analyses were performed with the computer software R (The R Foundation for Statistical Computing 2023). Homogeneity of variances of all dependent variables as well as the normal distribution of residuals were tested (levene-test and R-package nortest, Fox 2016; Gross and Ligges 2015) and when necessary variables were either square-root, log- or Box Cox-transformed, the latter with the R-packages car (Fox and Weisberg 2019) and geoR (Ribeiro et al. 2022). All dependent variables and their type of transformation are presented in Table S3. For all ANOVA analyses and for each trait, p-values were corrected to control for type I error via implementation of controlling the false discovery rate, with significance indicated at FDR-adjusted p-value < 0.05 (Benjamini and Hochberg 1995).

Results

Effects of zebularine on DNA-methylation and early biomass production

To confirm if the zebularine treatments caused hypomethylation, we evaluated the quantitative effect of zebularine dose on cytosine methylation levels at seedling stage. We found that in the two grass species with better replication (*A. sterilis* and *H. murinum*), methylation levels of cytosine gradually decreased with increasing levels of zebularine (Fig. 1a). Low concentrations of zebularine, i.e. 10 µM and 20 µM, showed no difference to the control treatment, whereas 30 µM and 40 µM significantly reduced cytosine methylation levels, with the strongest effects found at 50 µM. In particular, methylation of cytosine was on average 20% in the control treatment for *H. murinum* and 17% in treatment Z50 µM, whereas the effect was less strong for *A. sterilis* with, on average, 19.3% methylation in Z0 µM and 18.8% methylation in Z50 µM. A significant reduction between the control and Z30-50 µM-treatments was recorded in *E. ciliatum*, with 14% methylation in Z0µM and 10% methylation in Z30-50 µM, and a less strong reduction in *E. fleischeri*, *with* 16% methylation in Z0&10 and 15% methylation in Z20-40, suggesting that the treatment was effective also in forbs (Fig. 1a). Altogether, our results indicate that the highest doses of zebularine treatment assayed were successful in reducing DNA-methylation of the offspring seedlings, although the dosage effect could not be appropriately evaluated in some cases because grouping enlarged variance across replicates (see e.g., *E. anagallidifolium*) or mate-

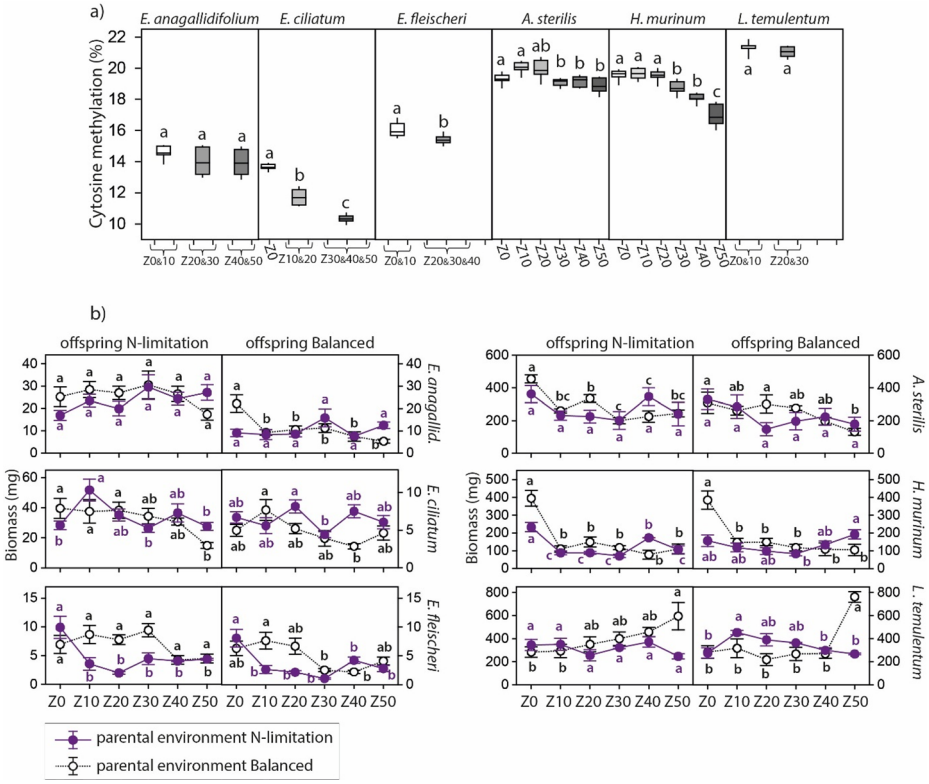

Fig. 1 (**a**) Mean cytosine methylation levels at seedling stage with standard error for each species and for each zebularine treatment (Z0 µM to Z50 µM). In some species, biomass in different treatment levels were joined to provide sufficient plant material for analysis. (**b**) Mean biomass at the day of harvest and for each combination of parental and offspring environment, with standard error and for each species and for each zebularine treatment (Z0µM to Z50µM). For each a) and b), differences between zebularine levels were determined with ANOVA and subsequent Tukey test with $p < 0.05$

rial from the highest doses was not available for analysis (see e.g., *L. temulentum* and *E. fleischeri*).

We further analysed the effects of the zebularine treatments on early biomass production, i.e. in seedlings harvested 10 days after germination, to evaluate potential toxic side-effects, expressed as strong growth reduction, of the zebularine treatments. Growth reduction of zebularine treatment can be caused by effects that are mediated by de-methylation (which may compromise optimal growth) or by toxic side-effects that act independently from hypomethylation. In the case of toxic side-effects, we expect a dose-dependent and strong seedling biomass decrease with increasing levels of zebularine treatment. In some species and for some treatments, there were no differences across the gradient from control to the highest zebularine treatment, for example in N-limited offspring plants of N-limited parents of *E. anagallidifolium*, *A. sterilis* and *L. temulentum* and in balanced offspring plants of N-limited parents in *E. anagallidifolium* and *A. sterilis* (see purple lines in Fig. 1b). The same could be found in N-limited offspring plants of balanced parents in *E. anagallidifolium* and *E. fleischeri* (black lines in Fig. 1b). Other species-treatment combinations showed a

decrease of biomass towards the higher zebularine treatments, for example in *E. ciliatum* (offspring N-limited/parent balanced) and *H. murinum* (offspring N-limited/parent balanced and offspring N-limited/parent-limited). Along the 24 combinations of species-parental-offspring treatments, 9 showed significantly lower biomass in the highest zebularine treatment compared to the control, whereas two combinations showed higher biomass in Z50 µM compared to Z0 µM (*L. temulentum* offspring N-limited/parental balanced and offspring balanced/parental balanced) and 13 showed no differences.

The linear models for each species separately yielded non-significant effects of both the parental environment and the zebularine treatments on seedling-biomass for all species. Only biomass of *E. fleischeri* responded significantly to the parental treatment with $p=0.03$ (Table S4). Across-species analysis resulted in non-significant effects of zebularine treatments on biomass ($p=0.12$), or in any of the interactions between zebularine with parental treatment ($p=0.98$), with species ($p=0.32$) or both ($p=0.99$) (Table S4).

Overall, while some growth effects were observed in some species, these results do not suggest strong toxic side-effects of the high zebularine treatment. We therefore interpret the zebularine effects as hypomethylation-mediated effects, and we tested the effects of experimental demethylation on trait expressions and DNA methylation can silence TGP to N-availability in each of the species by comparison of the control treatment and the highest zebularine treatment (i.e., Z0 versus Z50 µM).

Phenotypic consequences of N-limitation: across-species analysis and zebularine interactions

Across species, the parental nutrient environment affected relative growth rate, root average diameter, SLA and SPAD (Table 1 shows results of RGR, leaf area root length and root diameter, following main results of Minden et al. 2024; results for remaining traits are in Table S5), suggesting that TGP involved traits with individual performance, root morphology and leaf physiology. Nutrient availability in the offspring generation affected all traits but root diameter and SPAD, whereas zebularine-treatments affected all traits but root diameter and SLA. Functional group, i.e. whether plants were forbs or grasses, exerted strongest effects in terms of F-values and significantly affected all measured traits.

The effects of parental environment were modified by the presence of zebularine in several traits. Demethylation modified the expression of TGP in SLA, SPAD, root length and root surface area (cf. two way interaction between parental and zebularine treatment). The same effects were apparent in root length, SLA and root surface area, but depended on whether offspring was grown in similar or different conditions than their parents (cf. significant three-way interaction between parental x offspring environment x zebularine treatment).

If we focus on the three species with a stronger response to zebularine treatment (*Avena sterilis*, *Epilobium ciliatum* and *Hordeum murinum*), we found that the two-way interaction PT × ZT is significant for the two traits more directly related to fitness (RGR and biomass). The three three-way interaction PT × OT × ZT exerted significant effects on root length, root average diameter, SLA and root surface area (see Table S6 for details).

Table 1 Results of ANOVA (F-values) of the effects of parental and offspring treatments, as well as zebularine (control versus Z50µM) and functional group (forbs, grasses) and their multiple interactions on several plant traits across all species of this study. Presented p-values are FDR-adjusted p-values after benjamini and Hochberg (1995)

	Df	RGR	Leaf area	Root length	Root average diameter
PT	1	**7.67***	0.01	0.31	**26.08*****
OT	1	**24.41*****	**56.06*****	**69.32*****	1.26
ZT	1	**6.93***	**28.53*****	**32.3*****	1.51
FG	1	**31.12*****	**1154.26*****	**860.85*****	**43.22*****
PT × OT	1	0.05	1.24	0.96	**9.92****
PT × ZT	1	2.54	0.03	**6.72***	0.08
OT × ZT	1	0.14	0.32	1.48	4.98
PT × FG	1	1.19	**12.43****	4.41	**18.48*****
OT × FG	1	**14.42*****	**24.82*****	**18.42*****	1.57
ZT × FG	1	0.28	0.50	**21.25*****	0.30
PT × OT × ZT	1	0.32	0.38	**8.59****	0.04
PT × OT × FG	1	0.01	0.54	0.89	4.73
PT × ZT × FG	1	0.18	1.02	1.41	0.14
OT × ZT × FG	1	1.23	3.47	**9.76****	1.02
PT × OT × ZT × FG	1	0.24	4.39	0.17	**7.65***
Residuals	315				

FDR procedure was applied over all p-values of one trait with significance indicated at P_{FDR} <0.05. * P_{FDR} < 0.05, ** P_{FDR} < 0.01, *** P_{FDR} < 0.001. PT: parental treatments, OT: offspring treatments, ZT: zebularine treatments, FG: functional group. ANOVA results for the remaining plant traits can be found in Table S5

Single-species analysis: effects of parental environment and zebularine treatments on plant traits

The parental nutrient environment affected each offspring plant trait measured in at least some of the plant species regardless of the offspring environment (significant main effect of parental treatments, Table 2 and Table S7). For example, total biomass and RGR were significantly affected by parental treatments in *H. murinum* and *L. temulentum*, with, on average, 31% and 41% lower biomass, and 13% and 9% lower growth rate under parental N-limitation, irrespective of offspring environment, see Figure S2. Leaf area responded significantly to parental treatments in *A. sterilis*, *E. ciliatum* and *E. fleischeri*, but whereas it was higher under parental balanced nutrient supply in *A. sterilis*, it was higher under parental N-limitation in the two *Epilobium* species. Likewise, whereas both root length and surface area were on average greater under parental N-limitation in *L. temulentum* (50% for root length and 46% for surface area) compared to parental balanced nutrient supply, roots were on average 50% shorter and had a 51% lower area under parental N-limitation in *E. fleischeri.*

Both root length and surface area were both reduced under zebularine, in all species but *E. anagallidifolium* and *E. ciliatum* (single-species analysis, Table 2). For example, *A. sterilis*-individuals produced roots that were on average 65% shorter when exposed to zebularine 50µM (*H. murinum* 62% and *L. temulentum* 32% shorter) compared to offspring of the control treatments (see Figure S3). Similarly, average root surface area was reduced by 60% in *A. sterilis*, 60% in *H. murinum* and 44% in *L. temulentum*.

Table 2 Results of ANOVA (F-values) of the effects of parental and offspring treatments, as well as zebularine (control versus Z50μM) on several plant traits of the study species

	Df	RGR	Leaf area	Root length	Root av. diameter	Df	RGR	Leaf area	Root length	Root av. diameter
Epilobium anagallidifolium						*Epilobium ciliatum*				
PT	1	0.98	0.11	1.51	**12.94****	1	**9.42****	**14.93*****	**5.49***	**141.91*****
OT	1	**31.95*****	**46.86*****	**63.17*****	**58.20*****	1	**211.55*****	**148.67*****	**483.57*****	**19.14*****
ZT	1	**6.32***	0.44	0.74	1.11	1	**12.79****	1.54	0.14	0.57
PT × OT	1	0.03	**24.42*****	3.23	**10.57****	1	0.33	**26.14*****	**38.84*****	**10.12****
PT × ZT	1	**24.07*****	**6.05***	**23.16*****	**22.96*****	1	0.71	1.31	1.15	0.02
OT × ZT	1	3.99	2.70	2.12	**5.79***	1	2.30	0.27	5.87*	**18.77*****
PT × OT × ZT	1	1.85	4.21	0.02	1.70	1	4.91	0.37	**5.66***	**11.55****
Residuals	55					54				
	Df	RGR	Leaf area	Root length	Root av. diameter		RGR	Leaf area	Root length	Root av. diameter
Epilobium fleischeri						*Avena sterilis*				
PT	1	0.01	**17.08*****	**29.41*****	4.12	1	0.06	**9.41***	1.42	1.25
OT	1	2.04	**14.23****	2.85	0.63	1	**6.62***	**8.60***	0.06	**9.16***
ZT	1	**10.90***	**124.95*****	**15.35*****	**21.69*****	1	**7.25***	**20.81*****	**40.6*****	**20.45*****
PT × OT	1	0.74	**11.30****	0.58	**69.34*****	1	1.38	1.37	6.14	1.35
PT × ZT	1	2.28	**8.03****	**10.86****	**20.72*****	1	0.66	1.98	3.25	4.62
OT × ZT	1	0.18	0.17	**5.28***	0.81	1	0.05	0.01	0.96	0.11
PT × OT × ZT	1	0.02	2.12	4.28	1.63	1	0.01	0.26	0.05	**19.47*****
Residuals	49					40				
		RGR	Leaf area	Root length	Root av. diameter		RGR	Leaf area	Root length	Root av. diameter
Hordeum murinum						*Lolium temulentum*				
PT	1	**22.29*****	4.40	3.54	0.07	1	**11.60****	1.19	**31.05*****	0.83
OT	1	0.04	0.33	**18.72*****	**14.14****	1	0.31	0.31	1.16	0.15
ZT	1	**13.35****	**30.35*****	**24.07*****	0.69	1	**8.37***	1.42	**11.87****	**6.37***
PT × OT	1	0.25	3.84	0.50	**12.78****	1	1.14	**7.49***	1.07	**24.24*****
PT × ZT	1	**16.33*****	**14.90****	3.12	2.43	1	**13.17****	2.98	**14.06****	0.52
OT × ZT	1	2.58	2.30	**5.63***	3.34	1	2.51	5.60	**16.97*****	**53.52*****

Table 2 (continued)

	Df	RGR	Leaf area	Root length	Root av. diameter	Df	RGR	Leaf area	Root length	Root av. diameter
PT × OT × ZT	1	3.12	1.39	**8.30***	**30.92*****	1	0.13	**7.61***	3.91	3.02
Residuals	43					42				

Presented p-values are FDR-adjusted p-values after benjamini and Hochberg (1995). FDR procedure was applied over all p-values of one trait with significance indicated at $P_{FDR}<0.05$. * $P_{FDR} < 0.05$, ** $P_{FDR} < 0.01$, *** $P_{FDR} < 0.001$. PT: parental treatments, OT: offspring treatments, ZT: zebularine treatments. Results for remaining traits are presented in table S7

Single-species analysis: interactive effects of parental and offspring environments on plant traits

Parental environments affected some plant traits conditionally on offspring treatments, mainly leaf area, SLA and root diameter (see interaction parental x offspring treatments, Table 2 and Table S7). For example, the average total leaf area in *L. temulentum* was 28 cm² in offspring of N-limited parents grown under N-limitation and 18 cm² (approximately 38% lower) in offspring of N-limited parents grown under balanced supply (see means and standard errors in Table S8). In contrast, plants whose parents grew under balanced nutrient supply and experienced N-limitation had a total leaf area of 22 cm², which was approximately 27% smaller than in offspring grown under balanced nutrient supply (30 cm², equally in *L. temulentum*).

The effects of parental environment on root diameter were expressed differently in the different offspring environments in all species apart from *A. sterilis*. In several species the parental environment significantly affected root diameter of offspring grown under balanced nutrient supply: roots had lower diameter, i.e. were thinner, in offspring from N-limited parents which were grown under N-limitation (e.g. average root diameter in *E. ciliatum* 0.50 mm, or *H. murinum* 0.34 mm) than in individuals from N-limited parents grown under balanced nutrient supply (*E. ciliatum* 0.61 mm, or *H. murinum* 0.42 mm, Table S8).

Zebularine effects on expression of transgenerational plasticity

Effects of demethylation on the expression of TGP could be detected in biomass and RGR in *E. anagallidifolium*, *H. murinum* and *L. temulentum* (cf. significant interaction between parental environment × zebularine treatment, Table 2 and Table S7). Further, leaf related traits significantly responding to this two-way interaction included leaf area (*E. anagallidifolium*, *E. fleischeri*, *H. murinum*), SLA (*E. anagallidifolium*, *E. ciliatum*) and SPAD (*E. ciliatum*). Root length showed significant interactive responses in *E. anagallidifolium*, *E. fleischeri*, *L. temulentum*, root surface area in *E. anagallidifolium*, *L. temulentum* and root diameter in *E. anagallidifolium*, *E. fleischeri*.

Under control germination conditions, *E. anagallidifolium*-offspring of balanced-parents produced on average 44% more biomass compared to offspring of N-limited parents (24 mg and 13 mg when averaged over the two offspring environments, respectively, $p = 0.0125$, Table S9). When germinated with zebularine, the effect was reverse: offspring of balanced-parents produced 43% less biomass than offspring of N-limited parents (11 mg and 20 mg, $p = 0.0008$, respectively, Table S9). This trend was also apparent in *H. murinum*, but less in *L. temulentum*, as well as for RGR for the three species (compare Table S9). Root length showed a similar trend in *L. temulentum*: Under control germination conditions *L. temulentum*-individuals from N-limited parents produced on average longer roots than offspring from balanced parents (19 m versus 16 m, $p = 0.68$, difference of 17%). Germinated in the presence of zebularine, this effect was much stronger, with a difference of 504%, mainly driven by very short roots of an average of 3 m in offspring of balanced parents and longer roots of 20 m in offspring of N-limited parents ($p < 0.001$, Table S9).

As these results showed that there was detectable modification of TGP-expression via demethylation per se, they did not inform about the type of offspring environment in which these modifications manifested, which in our study comprised N-limitation and balanced

nutrient supply. This information could be inferred from the three-way interaction parental environment × offspring environment x zebularine treatments. Traits that responded to the three-way interaction were leaf area (*L. temulentum*), SLA (*E. anagallidifolium*, *E. ciliatum*, *H. murinum*), SPAD (*E. anagallidifolium*, *E. ciliatum*, *L. temulentum*), root length (*E. ciliatum*, *H. murinum*), root surface area (*E. fleischeri*, *H. murinum*) and root diameter (*A. sterilis*, *E. ciliatum*, *H. murinum*).

For some traits, zebularine modulated the expression of the parental effect conditioned on the offspring treatments, which manifested in a three-way interaction (PT × OT × ZT, Table 2). For example, under control germination, leaf area of *L. temulentum* was on average 114% higher in offspring of N-limited parents grown under N-limitation than in offspring of balanced parents grown under N-limitation (on average 42 cm^2 versus 20 cm^2, $p = 0.005$, see Fig. 2b, orange bar graph). When germinated in the presence of zebularine, the parental effect was removed and leaf area was similar between N-limited offspring plants with parents grown either under N-limitation or balanced supply (on average 16 cm^2 versus 20 cm^2, $p = 0.13$, see Fig. 2b, yellow bar graph). Other traits responding significantly to the three-way interaction showed less clear patterns: for example, whereas SLA-values in N-limited offspring of *E. anagallidifolium* followed the patterns described for leaf area in *L. temulentum*, offspring of *E. anagallidifolium* grown under balanced nutrient supply showed opposite responses to the parental environment and the zebularine treatments (see Fig. S4b).

Root length of *E. ciliatum*-offspring with N-limited parents that were germinated under control conditions and were grown under N-limitation (Fig. 2c, orange left bar graph), produced significantly longer roots than offspring of balanced-parents that were grown under N-limitation ($p = 0.04$, difference of 38%). Demethylation removed this effect: root length of offspring from N-limited parents which were grown under N-limitation was similar to offspring from parents grown under balanced nutrient supply when grown under N-limitation (Fig. 2c, yellow bar graph, $p = 0.50$, difference of 0.03%). A similar pattern was found for SLA in *L. temulentum* and *E. anagallidifolium* (Fig. S4, orange and yellow bar graphs). A pattern of parental effects in the Z0 treatment, but reversed parental effects under demethylation was found in average root diameter in *A. sterilis*. Offspring of N-limited parents grown under balanced nutrient supply and germinated under control conditions produced roots that were significantly and on average 30% thinner than when their parents were grown under balanced nutrient supply (Fig. 2d, dark-blue bar graph, $p = 0.004$). When demethylated the pattern was equally significant and reverse: roots were on average 42% thicker in offspring of N-limited parents compared to offspring of balanced parents, when both were grown under balanced nutrient supply (Fig. 2d, light-blue bar graph, $p < 0.001$).

Discussion

In a previous study we demonstrated that offspring of annual plants that experienced N-limitation show modified traits that can have beneficial effects on offspring when the offspring is also exposed to N-limiting growing environments (Minden et al. 2024). Here, we show that DNA-methylation can be involved in the expression of such adaptive transgenerational plasticity, but also that zebularine effects were erratic and did not consistently remove TGP-effects in the two nutrient environments investigated. In the following paragraphs, we will

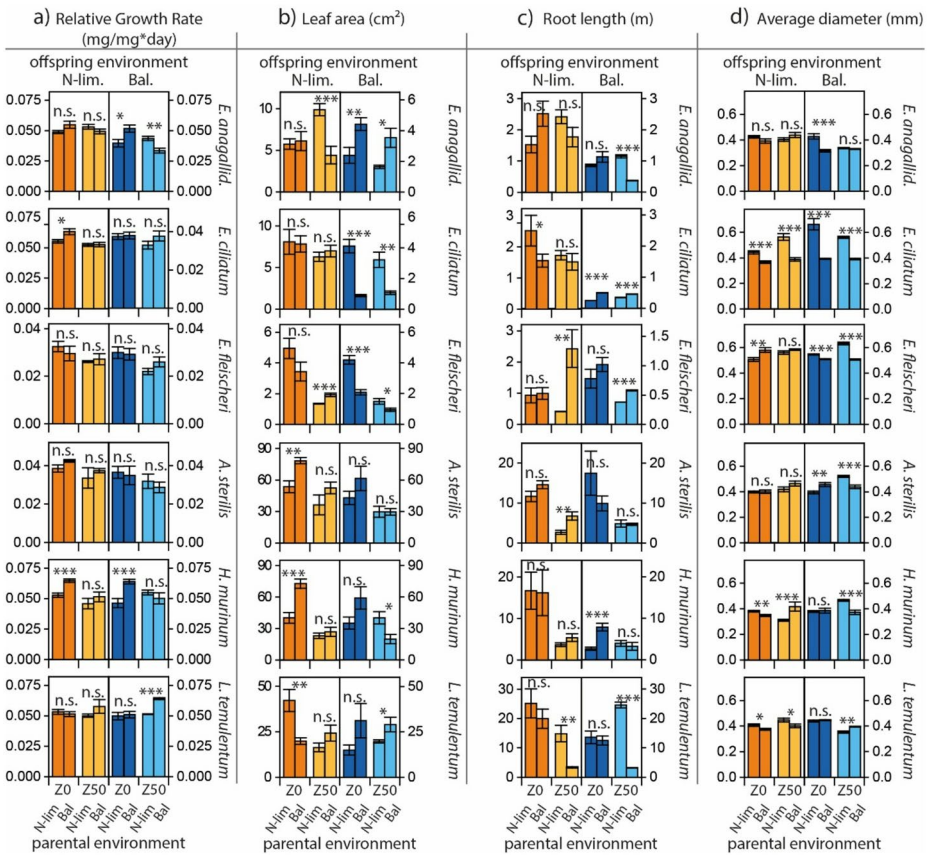

Fig. 2 Output of the effects of zebularine treatment at seed germination on plant traits showing transgenerational plasticity (i.e., with a significant PT effect or interaction PT × OT). Means and ± standard errors for plant traits and for each species separately. Parental generation was either grown under N-limitation or balanced nutrient supply, offspring generation either under N-limitation (left columns, orange and yellow graphs) or balanced nutrient supply (right columns, blue graphs). Orange bars and dark-blue bars show trait values for Control-zebularine treatments (0µM), yellow bars and light-blue bars for zebularine 50 µM. Asterisks indicate significance of parental nutrient treatment, for each zebularine treatment and parental/offspring treatment-combination separately (i.e. for bar graphs of the same colour) and done with Tukey test using marginal means, with *$p<0.05$, **$p<0.01$, ***$p<0.001$ and n.s. at $p>0.05$. Exact p-values for each trait are given in Table S10

discuss in more detail the consequences of seed demethylation and the species-specific effects we found across the six annual plant species studied.

Demethylation can remove potentially adaptive transgenerational plasticity of parental N-limitation

When germinated under control condition, i.e., in the absence of zebularine, and grown under N-limitation, offspring of N-limited parents produced either larger leaves or longer roots than progeny from balanced parents in *L. temulentum* and *E. ciliatum*, respectively. The parental effect expressed under N-limitation in these two traits was removed

by demethylation, consistent with a role of DNA-methylation in mediating the expression of the parental effect. In *L. temulentum*, the offspring of N-limited parents and germinated under 50 µM of zebularine produced leaves of similar size than offspring of parents from balanced nutrient conditions and likewise, root length was similar between offspring of *E. ciliatum* with N-limited and balanced parents, when both species were grown under offspring N-limitation.

The effects of zebularine can be dose-dependent and different levels of cytosine methylation levels have been reported for different species. Griffin et al. (2016) report cytosine methylation levels of 3.8, 3.3 and 3.1% in 8 days old *Arabidopsis thaliana*-plants treated with 0 µM, 25 µM and 50 µM zebularine. In 6 days old *Triticum aestivum* L. seedlings cytosine methylation levels of 26.9% were measured in control treatments and 25.4% in Z100 µM (Finnegan et al. 2018; Fig. 1 of corrigendum). Lastly, Baubec et al. (2009) measured global 5-methyldeoxycytidine (5-mdC) levels to determine the effects of zebularine on epigenetic regulation in 21 days old *A. thaliana* seedlings. 5-mdC-levels ranged between 6.2% in the control, 5.6% under 20 µM and 5.1% under 40 µM zebularine. The highest average cytosine methylation level in our experiment was 21.4% in Z20&30 µM in *L. temulentum* and 10.2% in Z30-50 µM in *E. ciliatum*, the strongest reduction of 3.6% was observed in *E. ciliatum* Z40 µM compared to the control, which is similar to the abovementioned studies. Similarly, our results indicate that the highest zebularine treatment did not have toxic effects on seedling biomass production, with 13 out of 24 species-treatment combinations showing no difference between the control and Z50 µM and two even higher biomass in Z50 µM. However, we cannot rule out other zebularine-effects, as we only measured biomass. For example, Prochazkova et al. (2022) found a strong reduction of root length in *A. thaliana*-plants of grown in the presence of 10 µM zebularine, and related this response to a damaging effect on DNA and subsequent cell death. Other adverse growth-effects were reported for some species, for example for concentrations higher than 40 µM in *Arabidopsis thaliana* L. (Baubec et al. 2009), higher than 50 µM in *Polygonum persicaria* (Herman and Sultan 2016), or toxic at concentrations of 50–100 µM in *Taraxacum officinalis* L. (Verhoeven and van Gurp 2012). In our six study species, highest reduction of cytosine methylation levels were reached at 50µM, and effects on some plant traits were apparent at concentrations levels of 30 µM. When *E. ciliatum* and *H. murinum* were germinated under gradual increase of zebularine, the potentially adaptive effects of parental N-limitation on root length and root average diameter gradually disappeared and were removed when the demethylation agent was applied at a concentration equal to/higher than 40µM, respectively.

Altogether, our results add to a growing number of studies that show that DNA-methylation can mediate both transgenerational trait plasticity (e.g., to shade, Baker et al. 2018; heavy metals, Cong et al. 2019; or drought, Zheng et al. 2017) and within-generational traits plasticity (e.g., to nutrient stress, Bossdorf et al. 2010; Verhoeven and van Gurp 2012). For example, both the studies by Alsdurf et al. (2016) and Herman and Sultan (2016) found drought-induced TGP. While Alsdurf et al. (2016) detected drought-induced changes in DNA-methylation correlating with increased drought tolerance, the study by Herman and Sultan (2016) demonstrated that adaptive positive effects of parental drought were removed by demethylation.

Studies show that plants grown under the influence of zebularine show continued hypomethylation in organs directly exposed to the agent and those not in direct contact, such as internodes. Further, hypomethylation continued also in a recovery phase, i.e. when plants

were grown in zebularine-free medium. For example, both internode length and internode number of *Salix purpurea* L.-plants were significantly reduced after two and three weeks of recovery period, after plants were exposed to a zebularine 50 µM and compared to a control treatment (Khodaeiaminjan et al. 2024). The same study reports strong hypomethylation effects of zebularine, which varied in different tissues and time intervals. Over a total of 11 weeks of recovery time, demethylation in internodes decreased, but both increased and decreased in roots (Khodaeiaminjan et al. 2024). Similarly, hypomethylation continued when *Salix purpurea*-plants were transplanted in recovery for 3 and 7 weeks both in internodes and roots, after being treated with Z50 µM (Pagano et al. 2024; Supplementary Table S1). We determined cytosine methylation levels in the aboveground biomass of 10-day old seedlings which were completely exposed to zebularine in the agar-medium during germination. Further, we can neither directly deduct changes of methylation levels over the course of the 5-week recovery period nor zebularine-effects on other plant organs, such as roots. However, the findings of the studies on *S. purpurea* suggest that hypomethylation affects various plant organs and continues also in the recovery phase. Whereas our study and the aforementioned include the parent and one offspring generation, some indications for methylation-mediated stress-tolerance across multiple generations exists. For example, Kou et al. (2011) exposed *Oryza sativa* L.-plants to N-limitation and recorded alteration of cytosine methylation levels in leaf tissue of the stressed parent plants. They were subsequently able to detect altered methylation patterns in the same tissue in the offspring generation, although they were themselves not stressed. When the grandchildren generation descending from N-stressed parents were again exposed to the same stress, they showed higher tolerance to N-deficiency than another group of plants with unmodified cytosine methylation levels. Other examples include modified methylation patterns and enhanced tolerance towards heavy metal stress across three generations (Ou et al. 2012), drought-induced genome wide-alterations of methylation levels across six generations (Zheng et al. 2013), and improved drought adaptability accompanied with altered methylation status across eleven generations (Zheng et al. 2017).

Although our study supports that DNA-methylation can mediate effects of parental N-variation with the potential to positively affect fitness, it also shows some stochasticity in the magnitude and direction of the effects, both on the plant trait level, the species level and the environment in which the offspring was grown. For instance, whereas we could identify leaf area, root length and root diameter as the most responsive traits to parental N-variation and most of them were affected by demethylation in four out of six species, a clear signal in other traits, such as SLA, could not be identified. This is in agreement with other studies on the effects of nutrient availability on trait expressions (Minden and Olde Venterink 2019; Olde Venterink and Güsewell, 2010) and on TGP (Minden et al. 2024).

Moreover, in some traits of some species parental effects were apparent in the zebularine treated plants, but were absent in the controls, for example total biomass in *E. anagallidifolium* and *L. temulentum*, leaf area in *E. anagallidifolium* and *E. fleischeri*, or root surface area in *L. temulentum*. This is not the expected pattern under the hypothesis that DNA-methylation is causally involved in the expression of TGP: under this hypothesis, we would predict to see TGP in the absence of zebularine and a modified, potentially even nullified, TGP-effect in the presence of zebularine. Although the interaction parental effect x zebularine treatment was significant in those traits, this cannot be unambiguously be interpreted as the parental effects were mediated by the zebularine treatment, which suggests that the

expression of the parental environment may be operated by more complex epigenetic mechanisms than just DNA methylation. For example, DNA methylation can silence activated transposable elements (TE's) in the genome, which affects gene expression. This effect can be reversed by the zebularine treatment and TE may become active, and may be a source for unexpected phenotypic differences between control and zebularine-treated plants (Lin et al. 2024; Vatov and Gechev 2025). Further, alteration of the DNA structure by the demethylation agent may have phenotypic consequences beyond the targeted effects, as zebularine may remove methylated cytosine at random (Prochazkova et al. 2022; Tomastíková and Pecinka 2025). In this study, we increased the zebularine treatments in a stepwise manner, from low (10 μM) to the highest (50 μM) concentration, to evaluate possible toxic effects that were not detected at early developmental stages. Also, species-specific responses were pronounced. Variation of resources, in our study of N, may lead to strong parental effects in some species and mild effects in others (Verhoeven and van Gurp 2012), which may lead to questioning of the broader significance of the detected phenotypic transgenerational-effects. Thus, our study could not differentiate if annual grasses or forbs are more prone to trait plasticity across generations and further studies are necessary to better predict which other species features that have been associated to global DNA-methylation in angiosperms (e.g., genome size, habitat specialization, distribution range, Alonso et al. 2019) could determine plant adaptive/non-adaptive responses to a changing soil environment, partially caused by anthropogenic nutrient input. Lastly, growing of different genotypes within each study species may have been a source of phenotypic variation. Seeds of different parental plants were pooled to grow the offspring generation, which could have resulted in an averaging of phenotype-responses of different directions. Considerable effects of genotype on the expression of TGP has been shown by previous studies, e.g., Herman and Sultan (2016) and Earley et al. (2023).

Parental N-limitation can induce species-specific transgenerational-effects in offspring

Our study confirmed that N-limitation in the parental generation can potentially prime the offspring generation to the same nutrient deficiency. Transgenerational-effects were observed in several plant traits, amongst which some are related to improved N-uptake, such as long and thin roots, as well as a large leaf size to increase transpiration rate and N-uptake when water is not limited (White et al. 2013a, b). In offspring plants of three species, we found longer roots and larger leaves when parents were exposed to N-limitation than if parents were grown under balanced nutrient supply (in *L. temulentum*, *E. ciliatum* and *E. fleischeri*). However, these patterns did not manifest and/or were reversed in other species, for example in *A. sterilis*, which produced smaller leaves under parental N-limitation. We also found higher biomass in *E. fleischeri* and *L. temulentum*-progeny with N-limited parents that were grown under N-limited conditions, than if parents were grown under balanced conditions. Also, root surface area was higher under the same conditions, though not significantly different (*E. ciliatum*, *H. murinum*, *L. temulentum*, Fig. S4d). Both these results, i.e., transgenerational-effects and species-specific responses, are in line with other studies. For example, Latzel et al. (2009) showed that leaf number and length were increased in *Plantago lanceolata* L.-offspring when parents experienced nutrient deficiency. Likewise, *Polygonum persicaria*-offspring from drought-stressed parents showed longer roots than

offspring from non-stressed parents (Sultan 1996). Species-specific patterns were found by Fenesi et al. (2014), who tested congeneric pairs of invasive and non-invasive herb species for the effects of parental N and water availability on shoot/root ratio and SLA and observed TGP only in the invasive species (see also Latzel and Klimesova 2010; Sultan et al. 2009).

When offspring plants of *L. temulentum* with N-limited parents were grown under N-limitation they produced larger leaves than offspring from parents grown under balanced nutrient supply and grown under N-limitation. Similarly, root surface area was higher in *E. ciliatum*-plants with N-limited parents and resubjected to N-limitation, than in plants with balanced parents and grown under N-limitation. The same pattern was found in average root diameter in *E. ciliatum*, *H. murinum* and *L. temulentum*. A large leaf area, high root surface area and low root diameter are seen as advantageous under N-deficiency, as its acquisition can be accelerated by increasing transpiration rate and by a root-system constituted of thin roots that sum up to a large root surface without an increase in carbon allocation to the root (Garnett et al. 2009; Lynch 2013; Marschner 2012). Although these patterns were not consistent across all our study-species, these examples show TGP-effects in plant traits related to enhanced uptake of N under recurrent N-deficiencies across two generations that potentially positively affect their fitness. Other studies found parental environmental stress pre-adapting the offspring and enhancing plant performance under the same stress (Galloway and Etterson 2007; Herman and Sultan 2016; Sandner et al. 2018). Under a similar experimental setup, Minden et al. (2024) identified lowest average root diameter in N-deficient offspring with N-deficient parents and highest root phosphatase activity in P-deficient offspring with P-deficient parents (high root phosphatase activity is advantageous under P-limitation, cf. Olde Venterink 2011), each in comparison to non-deficient parent and offspring treatments, respectively. These results combined with those of the present results identify the aforementioned plant traits, leaf area, root surface area and root average diameter to pre-adapt plants to recurrent N-limitation across two plant generations. Our study also revealed that closely related species, such as the three *Epilobium* species in our study, expressed dissimilarities in their TGP-responses, which is accordance with other studies (Fenesi et al. 2014; Latzel et al. 2010; Xiao et al. 2023) and altogether suggest that species-specific effecting phenotypic and behavioural differences also occur in congeneric comparisons (Sultan et al. 2009). To conclude, this study shows that parental nutrient deficiency can induce transgenerational-effects in offspring plants, especially when parental and offspring environments match. Epigenetic changes, such as reduced methylation levels, can have significant effects on plasticity of ecologically relevant plant traits, with potential effects on species performance, although again the magnitude of the effect sems to be species-specific.

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Author contributions Vanessa Minden, Harry olde Venterink and Koen J.F. Verhoeven conceived the ideas and designed methodology; Vanessa Minden collected the data; Vanessa Minden and Conchita Alonso analysed the data; Vanessa Minden led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Data availability Data will be publicly available through the Dryad repository upon publication.

Code availability No custom codes or software applications were developed or used specifically for this study.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval Not applicable.

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