Received: 15 November 2021 | Revised: 16 September 2022 | Accepted: 11 October 2022

DOI: 10.1111/mec.16757

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

DNA methylation in clonal duckweed (*Lemna minor* L.) lineages reflects current and historical environmental exposures

Morgane Van Antro[1] | Stella Prelovsek[1] | Slavica Ivanovic[1] | Fleur Gawehns[1] | Niels C. A. M. Wagemaker[2] | Mohamed Mysara[3] | Nele Horemans[3] | Philippine Vergeer[2,4] | Koen J. F. Verhoeven[1]

[1]Department of Terrestrial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands

[2]Plant Ecology and Physiology, Radboud University, Nijmegen, The Netherlands

[3]Biosphere Impact Studies, Belgian Nuclear Research Centre (SCK CEN), Mol, Belgium

[4]Wageningen University and Research (WUR), Plant Ecology and Nature Conservation Group, Wageningen, The Netherlands

Correspondence
Koen J. F. Verhoeven, Department of Terrestrial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands.
Email: k.verhoeven@nioo.knaw.nl

Funding information
H2020 Marie Skłodowska-Curie Actions, Grant/Award Number: 764965

Handling Editor: Victoria L. Sork

Abstract

Environmentally induced DNA methylation variants may mediate gene expression responses to environmental changes. If such induced variants are transgenerationally stable, there is potential for expression responses to persist over multiple generations. Our current knowledge in plants, however, is almost exclusively based on studies conducted in sexually reproducing species where the majority of DNA methylation changes are subject to resetting in germlines, limiting the potential for transgenerational epigenetics stress memory. Asexual reproduction circumvents germlines, and may therefore be more conducive to long-term inheritance of epigenetic marks. Taking advantage of the rapid clonal reproduction of the common duckweed *Lemna minor*, we hypothesize that long-term, transgenerational stress memory from exposure to high temperature can be detected in DNA methylation profiles. Using a reduced representation bisulphite sequencing approach (epiGBS), we show that temperature stress induces DNA hypermethylation at many CG and CHG cytosine contexts but not CHH. Additionally, differential methylation in CHG context that was observed was still detected in a subset of cytosines, even after 3–12 generations of culturing in a common environment. This demonstrates a memory effect of stress reflected in the methylome and that persists over multiple clonal generations. Structural annotation revealed that this memory effect in CHG methylation was enriched in transposable elements. The observed epigenetic stress memory is probably caused by stable transgenerational persistence of temperature-induced DNA methylation variants across clonal generations. To the extent that such epigenetic memory has functional consequences for gene expression and phenotypes, this result suggests potential for long-term modulation of stress responses in asexual plants.

KEYWORDS
clonal reproduction, DNA methylation, epigenetic memory, *Lemna minor*, temperature stress, transgenerational

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2022 The Authors. *Molecular Ecology* published by John Wiley & Sons Ltd.

temperature-sensitive to begin with. This supports the idea that the Phase 1 memory effect is caused by transgenerational stability, over several clonal generations, of temperature-induced DNA methylation changes that were triggered during the Phase 1 treatment. Consistently, a strong signal is detected when testing the DNA methylation difference between t24-t24 and t30-t24 lineages (1356 DMCs in CHG context, tested as an a priori contrast within the Phase 1 factor of the full factorial model, see Table 1), which is expected if the Phase 1 memory effect is caused by long-term persistence of DNA methylation modifications that were induced during Phase 1 30°C treatment. No significant interaction effect of Phase 1 × Phase 2 temperature treatment was detected at the individual cytosine level.

The majority of DMCs induced by the Phase 2 temperature treatment (~50%) were found within intergenic regions, with around 10% landing in TEs, and only a small fraction landing in or within a gene. The hypogeometric test revealed significant enrichment of CG and CHG Phase 2 DMCs as well as CHG Phase 1 DMCs in TEs only (Table 1). Filtering DMCs to include only the strongest-responding cytosines (a minimum of 20 percentage point cytosine methylation difference between any of the experimental groups) drastically reduced the number of DMCs. Interestingly, of the DMCs showing a Phase 1 temperature effect in CHG context, a relatively large proportion belonged to this category of strongly responding cytosines (249 out of 985 significant Phase 1 DMCs; see Table 1) indicating that long-lasting transgenerational stress memory in CHG methylation is associated with strong methylation differences. Furthermore, within these strongly responding DMCs, enrichment was observed within TEs.

3.4 | Differentially methylated epiGBS loci

Consistent with the DMC results, most of the epiGBS loci that contained 10 or more DMCs ("putative DMRs") were observed in CHG context, in response to the current Phase 2 temperature treatment and within intergenic or unannotated genome regions (Figure S5). Four putative DMRs were observed that showed a Phase 1 temperature effect (transgenerational memory) and these were all very strongly responding DMRs, with all DMCs within these DMRs being 20 percentage points or more differentially methylated. A total of 483 putative DMRs were observed in response to current (Phase 2) temperature treatments (30 DMRs consisting of solely CG DMCs, 245 of CHG DMRs, zero of CHH DMRs and 204 for DMRs consisting of combined cytosine contexts; Figure S5). Of these, 15 landed within or near a gene (five CG, five CHG and five all cytosine context combined). A functional annotation of these 15 genes was conducted, (Table S2), with four genes of these 15 encoding proteins either involved in gene expression regulation or involved in response to heat stress. *MED33A* and *BFA2* code for proteins involved in the regulation of RNA polymerase II, a multiprotein complex required for gene transcription (Bonawitz et al., 2012). *SDR1* codes for a protein that is involved in the abscisic acid biosynthesis process. Abscisic acid is a plant hormone important for the plant's response to environmental stresses, including heat stress (Islam et al., 2018) and *BG2* is involved in the response to cold (Amme et al., 2006).

4 | DISCUSSION

In this study, we present DNA methylation and phenotypic data acquired from a highly clonal aquatic species, the common duckweed *Lemna minor*, after experimental exposure to either control, high or fluctuating temperature growing conditions. Using epiGBS we showed that high temperature induces many changes in DNA methylation in both CG and CHG context. More importantly, in a subset of the responsive CHG cytosines, DNA methylation levels showed a memory of temperature treatments experienced several clonal generations ago. Structural annotation of the epiGBS loci showed that methylation changes are enriched within TEs. The observation of an epigenetic footprint of environments experienced many generations ago is in contrast to what has been reported previously in several sexually reproducing plant species (Pecinka et al., 2009; Wibowo et al., 2016), but in accordance with our hypothesis under asexual reproduction, and suggests that a subset of environment-induced DNA methylation variants is be transgenerationally stable for multiple clonal generations.

Numerous studies have shown that changes in the environment can influence DNA methylation patterns. However, there seems to be high variation between species and stresses in the exact nature of the DNA methylation response (Mirouze & Paszkowski, 2011; Niederhuth et al., 2016; Sahu et al., 2013). In *Arabidopsis*, which has been the model species for many DNA methylation studies in plants, gene body-like DNA methylation occurs primarily in the CG context, while repeats and TEs can show DNA methylation in all three types of cytosine context (Chan et al., 2005; Dubin et al., 2015). When exposed to environmental stressors, DNA methylation changes often occur in the CHH context, with less frequent and extreme variation occurring in the CG and CHG contexts (Dubin et al., 2015; Sun et al., 2021; Xu et al., 2018; Yaish et al., 2018). The patterns observed in duckweed are different: CHG and CG are the primary cytosine contexts whose methylation level responds to stress exposure, not CHH. In the closely related *Spirodela polyrhiza*, partial and complete loss of genes involved in the DNA methylation pathway have been demonstrated (Harkess et al., 2020). This loss has led to a drastic decrease in genome-wide methylation in all three cytosine contexts, and especially in CHH context (~10% CG, ~3.3% CHG and ~0.1% CHH) as well as a loss in CG gene-body methylation compared to what is normally observed in land plants. In *L. minor*, the methylation levels obtained in this experiment show much higher levels (78.9% CG, 30.4% CHG and 4.76% CHH), very much comparable to ranges measured in other plant species (Niederhuth et al., 2016). Furthermore, upon blasting the protein sequence of the main proteins involved in the DNA methylation pathway (MET1, DCL1, DCL3, AGO4, CMT3, DRM1, DRM2, NRPB1, CMT1, RDM1, RDR1), we found strong homology with all of the targeted protein sequences

within the transcriptome of *L. minor* (Table S3). All of these factors imply a unique loss of DNA methylation levels in *S. polyrhiza* only, and thus the absence of an *L. minor* stress response in CHH context is not due to absence of CHH methylation. The observed specific patterns of CG- and CHG-biased DNA methylation response to environmental changes in *L. minor* have rarely been documented in other plant species (Bewick & Schmitz, 2017; Takuno et al., 2016). We speculate that in the case of this experiment the duration of the multigenerational stress might explain these observed differences: CHH may show a rapid response to environmental change, but after continued, multigenerational exposure to the altered environment CHH might no longer respond to the new and now constant environment. A multigenerational stress experiment in *Arabidopsis* exposed to different gamma radiation levels for three generations found similar results, with DMRs predominantly found in CG context and to a lesser extent in CHG context, with no effect observed in CHH cytosine context (Laanen et al., 2021).

The main aim of this study was to determine if a transgenerational memory of heat stress could be detected in *L. minor*'s DNA methylation profiles, even once the stress is removed. Such a legacy effect was observed in CHG DNA methylation (as seen in Table 1) 3 weeks after removal of the heat stress in a subset of heat-affected cytosines, with partial but incomplete reversal of induced patterns towards control 24°C patterns. This pattern is consistent with the hypothesis that plants which reproduce without a germline undergo reduced epigenetic resetting, allowing for strong transgenerational stability of spontaneous and environmentally induced DNA methylation variants (Kinoshita & Jacobsen, 2012; Verhoeven & Preite, 2014). Similar results were observed in the clonal alligator weed *Alternanthera philoxeroides* (Shi et al., 2019). In this experiment, using vegetative cuttings, Methylation-Sensitive-AFLP revealed that stress-induced DNA methylation patterns were maintained for over 10 clonal generations, with progressive reversal of these patterns back towards a common state. We point out that our experimental design did not track DNA methylation changes during the course of the experiment, so no direct evidence is provided at the level of individual loci that stress exposure in Phase 1 of the experiment triggered DNA methylation modifications that were subsequently stably transmitted into Phase 2. Instead, we measured DNA methylation at the end of Phase 2 and detected evidence of Phase 1 temperature effects on DNA methylation. This proves that a Phase 1 treatment effect has carried over to the end of Phase 2. We suggest that stable transmission of Phase 1 treatment-induced DNA methylation variants (i.e., absence of resetting between clonal generations) is a probable explanation. Several observations from our data support this hypothesis of stable transgenerational inheritance. First, the PCA patterns based on CHG methylation show that continuous exposure to heat stress induces strong and directional changes in the current methylation profiles. Yet when placing lineages grown at 30°C (during Phase 1) into 24°C (during Phase 2), the methylome profile of the lineages are intermediate compared to lineages constantly grown at 24 or 30°C. Thus, after removing the high temperature stress, the methylome profiles are shifting back towards the methylation state of lineages constantly grown at 24°C. This suggests reversal of methylation level of individual loci from the 30°C-induced state to the 24°C state across a large number of clonal generations. Second, we showed that cytosines that express a significant memory effect of past Phase 1 temperatures are a subset of the cytosines that are temperature-sensitive during Phase 2. This observation is most easily explained by stable inheritance across generations of 30°C-induced DMCs. Nevertheless, to confirm whether these observed patterns indeed show stable inheritance of DNA methylation marks across a clonal generation, a time-series approach would be required, where methylation levels of individual loci can be tracked across the different generations.

Our study was not designed to demonstrate functional consequences of DNA methylation variants; this would require, at the very least, insight into gene expression effects of the treatments. However, the absence of significant long-lasting transgenerational memory at the level of frond number and frond area could indicate that transgenerational epigenetic inheritance does not play a role in adaptive phenotypic plasticity. Yet, a true lack of phenotypic effects is difficult to confirm based on our results. Previous studies have shown that the adaptive interpretation of phenotypic variation in *L. minor* is quite complex (Vasseur & Aarssen, 1992a, 1992b; Wilkinson, 1963). This complex interpretation is also the case in our study, with frond area and number showing dynamic changes over time, with individuals increasing or decreasing in frond number and area depending on the duration of the high-temperature stress. Furthermore, frond number and area represent performance traits, whereas adaptive phenotypic plasticity may target underlying functional traits (such as root length, biomass or chlorophyll fluorescence) whose regulation may function to maintain performance homeostasis across environments (Ruprecht et al., 2014). Screening for molecular phenotypes, such as gene expression, would be a logical subsequent step to evaluate if inherited methylation variants have functional relevance, as gene expression should show the most direct link to DNA methylation effects.

One other striking result from our study is the large proportion of cytosines affected by the temperature stress. For instance, 28.7% of the cytosines in CHG context were significantly differentially methylated due to the current Phase 2 temperature treatment. While DNA methylation responses to heat stress have been reported in other species to target specific functional pathways that are relevant to stress responses (Korotko et al., 2021), consistent with the hypothesis that induced DNA methylation variants can mediate phenotypic plasticity via gene expression regulation, in the case of *L. minor* these genome-wide changes in methylation levels are clearly not restricted to a few functional loci. We note that much of the DNA methylation variation in plant genomes is not expected to have functional consequences for gene expression, for instance because DNA methylation can build-up as a by-product of gene expression itself (Secco et al., 2015) or as presumably neutral epimutations in transcribed genes (Wendte et al., 2019). Unravelling a potential functional role of induced DNA methylation would therefore involve a search for a (potentially small) subset of loci that matter among

many changes that could be nonfunctional. While we identified few putative DMRs near or within a gene which might hint at functional regulation of processes related to temperature tolerance (in particular *MED33A*, *SDR1*, *BFA2* and *BG2*), a clear result of our study is that methylation responses are enriched in TEs; it is unclear if this has functional consequences for gene expression (Baduel & Colot, 2021). Nevertheless, this enrichment of TEs is consistent with previous studies in other plant species which have shown that environmental stresses influence the DNA methylation state of TEs (Baduel & Colot, 2021; Matzke & Mosher, 2014; Wibowo et al., 2016). Possibly, the cumulative effect of methylation changes at a large number of TEs might explain the genome-wide methylation response that we observed.

Our study clearly demonstrates that a subset of environmentally induced CHG DNA methylation variants can show strong memory effects of a stress that was experienced many clonal generations ago. This stable and long-lasting memory provides evidence that some form of molecular information has been inherited across clonal generations, with transgenerational stability of induced DNA methylation variants a strong candidate mechanism to explain our observations. It is an open question if, or to what extent, such stable methylation variants have functional consequences for gene expression. If some do, this could hint at the role DNA methylation has in mediating long-term transgenerational plastic responses. One can speculate that adding such a transgenerational dimension to the plant's repertoire of plastic responses might be of evolutionary benefit to clonal lineages, which in the absence of genetic adaptation need to rely heavily on phenotypic plasticity to cope with environment heterogeneity (Baker, 1965; Parker et al., 1977).

AUTHOR CONTRIBUTIONS

KJFV and SP designed the experiment. SP, SI and MVA conducted the greenhouse and laboratory aspect of the experiment. NH provided stock *L. minor* individuals. CAMW provided training and necessary material for epiGBS. Statistical analysis was performed by MVA, MM and PV. MVA, KJFV and PV drafted the manuscript, with input and approval of the final version by all authors.

ACKNOWLEDGEMENTS

Maarten Postuma, Bernice Sepers, Cristian Peña, Wim van der Putten and Anupoma Niloya Troyee are acknowledged for their critical questions and discussions and contributions to the initial phases of the data analysis. Thanks go to Verónica Noé Ibañez and Mark W. Schmid for sharing their scripts and help in answering questions on the data analysis. Christa Mateman and colleagues at NIOO-KNAW are acknowledged for their critical thinking and contribution during the laboratory phase of the experiment. Gregor Disveld and other NIOO-KNAW caretakers are acknowledged for maintenance and up-keep of the greenhouse facilities. The data analysis and journal submission were enabled by the European Training Network "EpiDiverse," which received funding from the EU Horizon 2020 programme under Marie Skłodowska-Curie grant agreement No. 764965. All experiments, data analysis and writing was conducted at NIOO-KNAW. This publication is filed under NIOO-KNAW publication no. 7492.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at [https://doi.org/10.5281/Zenodo.5680942].

DATA AVAILABILITY STATEMENT

The raw sequencing data and demultiplexed sequencing data have been deposited in NCBI (BioProject no. PRJNA883550). The filtered methylation data, epiGBS de novo reference, as well as all phenotypic data we deposited in Zenodo (Van Antro et al., 2022a, 2022b; DOI 10.5281/Zenodo.5680942). All analysis R scripts and the epiGBS pipeline scripts corresponding to the pipeline version used to obtain the methylation data have been made publicly accessible via gitlab: https://gitlab.bioinf.nioo.knaw.nl/MorganeA/script-for-l.minor-paper.git.

ORCID

Morgane Van Antro https://orcid.org/0000-0002-7788-4866
Fleur Gawehns https://orcid.org/0000-0002-9236-966X
Niels C. A. M. Wagemaker https://orcid.org/0000-0002-8968-4546
Mohamed Mysara https://orcid.org/0000-0001-5746-4330
Nele Horemans https://orcid.org/0000-0002-6241-0342
Philippine Vergeer https://orcid.org/0000-0003-3978-2313

REFERENCES

Aliferis, K. A., Materzok, S., Paziotou, G. N., & Chrysayi-Tokousbalides, M. (2009). *Lemna minor* L. as a model organism for ecotoxicological studies performing 1H NMR fingerprinting. *Chemosphere*, *76*(7), 967–973. https://doi.org/10.1016/J.CHEMOSPHERE.2009.04.025

Amme, S., Matros, A., Schlesier, B., & Mock, H.-P. (2006). Proteome analysis of cold stress response in *Arabidopsis thaliana* using DIGE-technology. *Journal of Experimental Botany*, *57*(7), 1537–1546. https://doi.org/10.1093/JXB/ERJ129

Baduel, P., & Colot, V. (2021). The epiallelic potential of transposable elements and its evolutionary significance in plants. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *376*(1826), 20200123. https://doi.org/10.1098/RSTB.2020.0123

Baker, H. G. (1965). Characteristics and modes of origin of weeds. In H. G. Baker & G. L. Stebbins (Eds.), *The genetics of colonizing species* (pp. 147–168). Academic Press.

Bewick, A. J., & Schmitz, R. J. (2017). Gene body DNA methylation in plants. *Current Opinion in Plant Biology*, *36*, 103–110. https://doi.org/10.1016/J.PBI.2016.12.007

Bonawitz, N. D., Soltau, W. L., Blatchley, M. R., Powers, B. L., Hurlock, A. K., Seals, L. A., Weng, J. K., Stout, J., & Chapple, C. (2012). REF4 and RFR1, subunits of the transcriptional coregulatory complex mediator, are required for phenylpropanoid homeostasis in *Arabidopsis*.

Journal of Biological Chemistry, *287*(8), 5434–5445. https://doi.org/10.1074/jbc.M111.312298

Bošković, A., & Rando, O. J. (2018). Transgenerational epigenetic inheritance. *Annual Review of Genetics*, *52*, 21–41. https://doi.org/10.1146/Annurev-Genet-120417-031404

Brain, R. A., & Solomon, K. R. (2007). A protocol for conducting 7-day daily renewal tests with *Lemna gibba*. *Nature Protocols*, *2*(4), 979–987. https://doi.org/10.1038/nprot.2007.146

Calarco, J. P., Borges, F., Donoghue, M. T. A., van Ex, F., Jullien, P. E., Lopes, T., Gardner, R., Berger, F., Feijó, J. A., Becker, J. D., & Martienssen, R. A. (2012). Reprogramming of DNA methylation in pollen guides epigenetic inheritance via small RNA. *Cell*, *151*(1), 194–205. https://doi.org/10.1016/J.CELL.2012.09.001

Carroll, S. P., Hendry, A. P., Reznick, D. N., & Fox, C. W. (2007). Evolution on ecological time-scales. *Functional Ecology*, *21*(3), 387–393. https://doi.org/10.1111/j.1365-2435.2007.01289.x

Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A., & Cresko, W. A. (2013). Stacks: An analysis tool set for population genomics. *Molecular Ecology*, *22*(11), 3124–3140. https://doi.org/10.1111/mec.12354

Chan, S. W.-L., Henderson, I. R., & Jacobsen, S. E. (2005). Gardening the genome: DNA methylation in *Arabidopsis thaliana*. *Nature Reviews Genetics*, *6*(5), 351–360. https://doi.org/10.1038/nrg1601

Dong, B. C., Yu, F. H., & Roiloa, S. R. (2019). Editorial: Ecoepigenetics in clonal and inbreeding plants: Transgenerational adaptation and environmental variation. *Frontiers in Plant Science*, *10*, 622. https://doi.org/10.3389/fpls.2019.00622

Douhovnikoff, V., & Dodd, R. S. (2014). Epigenetics: A potential mechanism for clonal plant success. *Plant Ecology*, *216*(2), 227–233. https://doi.org/10.1007/s11258-014-0430-z

Dubin, M. J., Zhang, P., Meng, D., Remigereau, M. S., Osborne, E. J., Casale, F. P., Drewe, P., Kahles, A., Jean, G., Vilhjálmsson, B., Jagoda, J., Irez, S., Voronin, V., Song, Q., Long, Q., Rätsch, G., Stegle, O., Clark, R. M., & Nordborg, M. (2015). DNA methylation in *Arabidopsis* has a genetic basis and shows evidence of local adaptation. *eLife*, *4*, 1–23. https://doi.org/10.7554/eLife.05255

Feng, S., Jacobsen, S. E., & Reik, W. (2010). Epigenetic reprogramming in plant and animal development. *Science*, *330*(6004), 622–627. https://doi.org/10.1126/science.1190614

Gallego-Bartolomé, J. (2020). DNA methylation in plants: Mechanisms and tools for targeted manipulation. *New Phytologist*, *227*(1), 38–44. https://doi.org/10.1111/nph.16529

Gawehns, F., Postuma, M., Antro, M., Nunn, A., Sepers, B., Fatma, S., Gurp, T. P., Wagemaker, N. C. A. M., Mateman, A. C., Milanovic-Ivanovic, S., Groβe, I., Oers, K., Vergeer, P., & Verhoeven, K. J. F. (2022). epiGBS2: Improvements and evaluation of highly multiplexed, epiGBS-based reduced representation bisulfite sequencing. *Molecular Ecology Resources*, *22*, 2087–2104. https://doi.org/10.1111/1755-0998.13597

Hairston, N. G., Ellner, S. P., Geber, M. A., Yoshida, T., & Fox, J. A. (2005). Rapid evolution and the convergence of ecological and evolutionary time. *Ecology Letters*, *8*(10), 1114–1127. https://doi.org/10.1111/j.1461-0248.2005.00812.x

Harkess, A., Bewick, A. J., Lu, Z., Fourounjian, P., Messing, J., Michael, T. P., Schmitz, R. J., & Meyers, B. C. (2020). Unusual predominance of maintenance DNA methylation in *Spirodela polyrhiza*. *bioRxiv*, 2020.12.03.410332. https://doi.org/10.1101/2020.12.03.410332

Hauser, M.-T., Aufsatz, W., Jonak, C., & Luschnig, C. (2011). Transgenerational epigenetic inheritance in plants. *Biochimica et Biophysica Acta (BBA) – Gene Regulatory Mechanisms*, *1809*(8), 459–468. https://doi.org/10.1016/j.bbagrm.2011.03.007

Heard, E., & Martienssen, R. A. (2014). Transgenerational epigenetic inheritance: Myths and mechanisms. *Cell*, *157*(1), 95–109. https://doi.org/10.1016/j.cell.2014.02.045

IPCC. (2021). Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press. https://doi.org/10.1017/9781009157896

Islam, M. R., Feng, B., Chen, T., Tao, L., & Fu, G. (2018). Role of Abscisic acid in thermal acclimation of plants. *Journal of Plant Biology*, *61*(5), 255–264. https://doi.org/10.1007/S12374-017-0429-9

Ito, T., Nishio, H., Tarutani, Y., Emura, N., Honjo, M. N., Toyoda, A., Fujiyama, A., Kakutani, T., & Kudoh, H. (2019). Seasonal stability and dynamics of DNA methylation in plants in a natural environment. *Genes*, *10*(7), 544. https://doi.org/10.3390/genes10070544

Kawashima, T., & Berger, F. (2014). Epigenetic reprogramming in plant sexual reproduction. *Nature Reviews Genetics*, *15*(9), 613–624. https://doi.org/10.1038/nrg3685

Kenchanmane Raju, S. K., Ritter, E. J., & Niederhuth, C. E. (2019). Establishment, maintenance, and biological roles of non-CG methylation in plants. *Essays in Biochemistry*, *63*(6), 743–755. https://doi.org/10.1042/EBC20190032

Kinoshita, T., & Jacobsen, S. E. (2012). Opening the door to epigenetics in PCP. *Plant and Cell Physiology*, *53*(5), 763–765. https://doi.org/10.1093/pcp/pcs061

Korotko, U., Chwiałkowska, K., Sańko-Sawczenko, I., & Kwasniewski, M. (2021). DNA demethylation in response to heat stress in *Arabidopsis thaliana*. *International Journal of Molecular Sciences*, *22*(4), 1555. https://doi.org/10.3390/IJMS22041555

Kronholm, I., & Ketola, T. (2018). Effects of acclimation time and epigenetic mechanisms on growth of Neurospora in fluctuating environments. *Heredity*, *121*(4), 327–341. https://doi.org/10.1038/s41437-018-0138-2

Kuehdorf, K., & Appenroth, K. J. (2012). Influence of salinity and high temperature on turion formation in the duckweed *Spirodela polyrhiza*. *Aquatic Botany*, *97*(1), 69–72. https://doi.org/10.1016/j.aquabot.2011.10.003

Laanen, P., Saenen, E., Mysara, M., van de Walle, J., van Hees, M., Nauts, R., van Nieuwerburgh, F., Voorspoels, S., Jacobs, G., Cuypers, A., & Horemans, N. (2021). Changes in DNA methylation in *Arabidopsis thaliana* plants exposed over multiple generations to gamma radiation. *Frontiers in Plant Science*, *12*, 611783. https://doi.org/10.3389/FPLS.2021.611783

Langmead, B., Trapnell, C., Pop, M., & Salzberg, S. L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology*, *10*(3), 1–10. https://doi.org/10.1186/gb-2009-10-3-r25

Lee, H., Depuydt, S., Shin, K., Choi, S., Kim, G., Lee, Y. H., Park, J. T., Han, T., & Park, J. (2021). Assessment of various toxicity endpoints in duckweed (*Lemna minor*) at the physiological, biochemical, and molecular levels as a measure of diuron stress. *Biology*, *10*(7), 684. https://doi.org/10.3390/biology10070684

Liu, J., & He, Z. (2020). Small DNA methylation, big player in plant abiotic stress responses and memory. *Frontiers in Plant Science*, *11*, 1977. https://doi.org/10.3389/fpls.2020.595603

Lobet, G. (2017). Image analysis in plant sciences: Publish then perish. *Trends in Plant Science*, *22*(7), 559–566. https://doi.org/10.1016/j.tplants.2017.05.002

Lu, X., Wang, X., Chen, X., Shu, N., Wang, J., Wang, D., Wang, S., Fan, W., Guo, L., Guo, X., & Ye, W. (2017). Single-base resolution methylomes of upland cotton (*Gossypium hirsutum* L.) reveal epigenome modifications in response to drought stress. *BMC Genomics*, *18*(1), 1–14. https://doi.org/10.1186/S12864-017-3681-Y/FIGURES/7

Luo, C., Hajkova, P., & Ecker, J. R. (2018). Dynamic DNA methylation: In the right place at the right time. *Science (New York, N.Y.)*, *361*(6409), 1336. https://doi.org/10.1126/SCIENCE.AAT6806

Mardanov, A. V., Ravin, N. V., Kuznetsov, B. B., Samigullin, T. H., Antonov, A. S., Kolganova, T. V., & Skyabin, K. G. (2008). Complete sequence of the duckweed (*Lemna minor*) chloroplast genome: Structural

organization and phylogenetic relationships to other angiosperms. *Journal of Molecular Evolution*, *66*(6), 555–564. https://doi.org/10.1007/s00239-008-9091-7

Mathieu, O., Reinders, J., Čaikovski, M., Smathajitt, C., & Paszkowski, J. (2007). Transgenerational stability of the *Arabidopsis* epigenome is coordinated by CG methylation. *Cell*, *130*(5), 851–862. https://doi.org/10.1016/J.CELL.2007.07.007

Matzke, M. A., & Mosher, R. A. (2014). RNA-directed DNA methylation: An epigenetic pathway of increasing complexity. *Nature Reviews Genetics*, *15*(6), 394–408. https://doi.org/10.1038/nrg3683

McCaw, B. A., Stevenson, T. J., & Lancaster, L. T. (2020). Epigenetic responses to temperature and climate. *Integrative and Comparative Biology*, *60*(6), 1469–1480. https://doi.org/10.1093/icb/icaa049

Mirouze, M., & Paszkowski, J. (2011). Epigenetic contribution to stress adaptation in plants. *Current Opinion in Plant Biology*, *14*(3), 267–274. https://doi.org/10.1016/j.pbi.2011.03.004

Mounger, J., Boquete, M. T., Schmid, M. W., Granado, R., Robertson, M. H., Voors, S. A., Langanke, K. L., Alvarez, M., Wagemaker, C. A. M., Schrey, A. W., Fox, G. A., Lewis, D. B., Lira, C. F., & Richards, C. L. (2021). Inheritance of DNA methylation differences in the mangrove Rhizophora mangle. *Evolution & Development*, *23*(4), 351–374. https://doi.org/10.1111/EDE.12388

Muyle, A. M., Seymour, D. K., Lv, Y., Huettel, B., & Gaut, B. S. (2022). Gene body methylation in plants: Mechanisms, functions, and important implications for understanding evolutionary processes. *Genome Biology and Evolution*, *14*(4), evac038. https://doi.org/10.1093/GBE/EVAC038

Niederhuth, C. E., Bewick, A. J., Ji, L., Alabady, M. S., Kim, K. D., Li, Q., Rohr, N. A., Rambani, A., Burke, J. M., Udall, J. A., Egesi, C., Schmutz, J., Grimwood, J., Jackson, S. A., Springer, N. M., & Schmitz, R. J. (2016). Widespread natural variation of DNA methylation within angiosperms. *Genome Biology*, *17*(1), 1–19. https://doi.org/10.1186/s13059-016-1059-0

Niederhuth, C. E., & Schmitz, R. J. (2017). Putting DNA methylation in context: From genomes to gene expression in plants. *Biochimica et Biophysica Acta. Gene Regulatory Mechanisms*, *1860*(1), 149–156. https://doi.org/10.1016/J.BBAGRM.2016.08.009

OECD. (2006). *OECD/OCDE 221 OECD guidelines for the testing of chemicals*. OECD.

Park, Y., & Wu, H. (2016). Differential methylation analysis for BS-seq data under general experimental design. *Nature Reviews Molecular Cell Bioinformatics*, *32*(10), 1446–1453. https://doi.org/10.1093/bioinformatics/btw026

Parker, E. D., Selander, R. K., Hudson, R. O., & Lester, L. J. (1977). Genentic diversity in colonizing parthenogenetic cockroaches. *Evolution*, *31*(4), 836–842. https://doi.org/10.1111/j.1558-5646.1977.tb01076.x

Pecinka, A., Rosa, M., Schikora, A., Berlinger, M., Hirt, H., Luschnig, C., & Scheid, O. M. (2009). Transgenerational stress memory is not a general response in *Arabidopsis*. *PLoS One*, *4*(4), e5202. https://doi.org/10.1371/journal.pone.0005202

Pinheiro, J. C., & Bates, D. M. (Eds.). (2006). Extending the basic linear mixed-effects model. In *Mixed-effects models in S and S-PLUS* (pp. 201–270). Springer.

Quinlan, A. R., & Hall, I. M. (2010). BEDTools: A flexible suite of utilities for comparing genomic features. *Bioinformatics*, *26*(6), 841–842. https://doi.org/10.1093/BIOINFORMATICS/BTQ033

Rendina González, A. P., Preite, V., Verhoeven, K. J. F., & Latzel, V. (2018). Transgenerational effects and epigenetic memory in the clonal plant *Trifolium repens*. *Frontiers in Plant Science*, *9*, 1677. https://doi.org/10.3389/fpls.2018.01677

Richards, E. J. (2006). Inherited epigenetic variation – Revisiting soft inheritance. *Nature Reviews Genetics*, *7*(5), 395–401. https://doi.org/10.1038/nrg1834

Ruprecht, E., Fenesi, A., & Nijs, I. (2014). Are plasticity in functional traits and constancy in performance traits linked with invasiveness? An experimental test comparing invasive and naturalized plant species. *Contributions to Mineralogy and Petrology*, *167*(4), 1359–1372. https://doi.org/10.1007/S10530-013-0574-0/FIGURES/3

Saban, J. M., Watson-Lazowski, A., Chapman, M. A., & Taylor, G. (2020). The methylome is altered for plants in a high CO_2 world: Insights into the response of a wild plant population to multigenerational exposure to elevated atmospheric [CO_2]. *Global Change Biology*, *26*(11), 6474–6492. https://doi.org/10.1111/GCB.15249

Sahu, P. P., Pandey, G., Sharma, N., Puranik, S., Muthamilarasan, M., & Prasad, M. (2013). Epigenetic mechanisms of plant stress responses and adaptation. *Plant Cell Reports*, *32*(8), 1151–1159. https://doi.org/10.1007/s00299-013-1462-x

Schield, D. R., Walsh, M. R., Card, D. C., Andrew, A. L., Adams, R. H., & Castoe, T. A. (2016). EpiRADseq: Scalable analysis of genome-wide patterns of methylation using next-generation sequencing. *Methods in Ecology and Evolution*, *7*(1), 60–69. https://doi.org/10.1111/2041-210X.12435

Schmid, M. W., Giraldo-Fonseca, A., Rövekamp, M., Smetanin, D., Bowman, J. L., & Grossniklaus, U. (2018). Extensive epigenetic reprogramming during the life cycle of *Marchantia polymorpha*. *Genome Biology*, *19*(1), 1–17. https://doi.org/10.1186/s13059-017-1383-z

Schmid, M. W., Heichinger, C., Coman Schmid, D., Guthörl, D., Gagliardini, V., Bruggmann, R., Aluri, S., Aquino, C., Schmid, B., Turnbull, L. A., & Grossniklaus, U. (2018). Contribution of epigenetic variation to adaptation in *Arabidopsis*. *Nature Communications*, *9*(1), 4446. https://doi.org/10.1038/s41467-018-06932-5

Schmitz, R. J., Lewis, Z. A., & Goll, M. G. (2019). DNA methylation: Shared and divergent features across eukaryotes. *Trends in Genetics: TIG*, *35*(11), 818–827. https://doi.org/10.1016/J.TIG.2019.07.007

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*(7), 671–675. https://doi.org/10.1038/nmeth.2089

Secco, D., Wang, C., Shou, H., Schultz, M. D., Chiarenza, S., Nussaume, L., Ecker, J. R., Whelan, J., & Lister, R. (2015). Stress induced gene expression drives transient DNA methylation changes at adjacent repetitive elements. *eLife*, *4*, e09343. https://doi.org/10.7554/ELIFE.09343.001

Shi, W., Chen, X., Gao, L., Xu, C. Y., Ou, X., Bossdorf, O., Yang, J., & Geng, Y. (2019). Transient stability of epigenetic population differentiation in a clonal invader. *Frontiers in Plant Science*, *9*, 1851. https://doi.org/10.3389/fpls.2018.01851

Smit, A. F. A., Hubley, R., & Green, P. (2015). RepeatMasker Open-4.0. 2013-2015. http://www.repeatmasker.org

Sommer, R. J. (2020). Phenotypic plasticity: From theory and genetics to current and future challenges. *Genetics*, *215*(1), 1–13. https://doi.org/10.1534/GENETICS.120.303163

Sun, R.-Z., Liu, J., Wang, Y.-Y., & Deng, X. (2021). DNA methylation-mediated modulation of rapid desiccation tolerance acquisition and dehydration stress memory in the resurrection plant *Boea hygrometrica*. *PLoS Genetics*, *17*(4), e1009549. https://doi.org/10.1371/JOURNAL.PGEN.1009549

Takuno, S., Ran, J.-H., & Gaut, B. S. (2016). Evolutionary patterns of genic DNA methylation vary across land plants. *Nature Plants*, *2*(2), 1–7. https://doi.org/10.1038/nplants.2015.222

Trucchi, E., Mazzarella, A. B., Gilfillan, G. D., Lorenzo, M. T., Schönswetter, P., & Paun, O. (2016). BsRADseq: Screening DNA methylation in natural populations of non-model species. *Molecular Ecology*, *25*(8), 1697–1713. https://doi.org/10.1111/mec.13550

Van Antro, M., Prelovsek, S., Ivanovic, S., Wagemaker, N., Mysara, M., Horemans, N., Gawehns, F., Vergeer, P., & Verhoeven, K. (2022a). DNA methylation in clonal Duckweed lineages (*Lemna minor* L.) reflects current and historical environmental exposures. https://doi.org/10.5281/zenodo.5680942

Van Antro, M., Prelovsek, S., Ivanovic, S., Wagemaker, N., Mysara, M., Horemans, N., Gawehns, F., Vergeer, P., & Verhoeven, K. (2022b). DNA methylation in clonal Duckweed lineages (*Lemna minor* L.)

reflects current and historical environmental exposures. https://dataview.ncbi.nlm.nih.gov/object/PRJNA883550

van der Graaf, A., Wardenaar, R., Neumann, D. A., Taudt, A., Shaw, R. G., Jansen, R. C., Schmitz, R. J., Colomé-Tatché, M., & Johannes, F. (2015). Rate, spectrum, and evolutionary dynamics of spontaneous epimutations. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(21), 6676–6681. https://doi.org/10.1073/pnas.1424254112

van Dooren, T. J. M., Silveira, A. B., Gilbault, E., Jiménez-Gómez, J. M., Martin, A., Bach, L., Tisné, S., Quadrana, L., Loudet, O., & Colot, V. (2020). Mild drought in the vegetative stage induces phenotypic, gene expression, and DNA methylation plasticity in *Arabidopsis* but no transgenerational effects. *Journal of Experimental Botany*, *71*(12), 3588–3602. https://doi.org/10.1093/jxb/eraa132

van Gurp, T. P., Wagemaker, N. C. A. M., Wouters, B., Vergeer, P., Ouborg, J. N. J., & Verhoeven, K. J. F. (2016). EpiGBS: Reference-free reduced representation bisulfite sequencing. *Nature Methods*, *13*(4), 322–324. https://doi.org/10.1038/nmeth.3763

Van Hoeck, A., Horemans, N., Monsieurs, P., Cao, H. X., Vandenhove, H., & Blust, R. (2015). The first draft genome of the aquatic model plant *Lemna minor* opens the route for future stress physiology research and biotechnological applications. *Biotechnology for Biofuels*, *8*(1), 188. https://doi.org/10.1186/s13068-015-0381-1

Vasseur, L., & Aarssen, L. W. (1992a). Phenotypic plasticity in *Lemna minor* (Lemnaceae). *Plant Systematics and Evolution*, *180*(3–4), 205–219. https://doi.org/10.1007/BF00941151

Vasseur, L., & Aarssen, L. W. (1992b). Interpretation of adaptive plasticity in *Lemna minor*. *Oikos*, *65*(2), 233. https://doi.org/10.2307/3545014

Verhoeven, K. J. F., & Preite, V. (2014). Epigenetic variation in asexually reproducing organisms. *Evolution*, *68*(3), 644–655. https://doi.org/10.1111/evo.12320

Vymazal, J. (2008). Constructed wetlands, surface flow. In S. E. Jørgensen & B. D. Fath (Eds.), *Encyclopedia of ecology* (pp. 765–776). Academic Press.

Wendte, J. M., Zhang, Y., Ji, L., Shi, X., Hazarika, R. R., Shahryary, Y., Johannes, F., & Schmitz, R. J. (2019). Epimutations are associated with CHROMOMETHYLASE 3-induced de novo DNA methylation. *eLife*, *8*, e47891. https://doi.org/10.7554/ELIFE.47891

West, B. T., Welch, K. B., & Galecki, A. T. (2006). *Linear mixed models: A practical guide using statistical software*. Chapman & Hall/CRC.

Wibowo, A., Becker, C., Marconi, G., Durr, J., Price, J., Hagmann, J., Papareddy, R., Putra, H., Kageyama, J., Becker, J., Weigel, D., & Gutierrez-Marcos, J. (2016). Hyperosmotic stress memory in *Arabidopsis* is mediated by distinct epigenetically labile sites in the genome and is restricted in the male germline by DNA glycosylase activity. *eLife*, *5*, e13546. https://doi.org/10.7554/eLife.13546

Widman, N., Feng, S., Jacobsen, S. E., & Pellegrini, M. (2014). Epigenetic differences between shoots and roots in *Arabidopsis* reveals tissue-specific regulation. *Epigenetics*, *9*(2), 236–242. https://doi.org/10.4161/EPI.26869

Wilkinson, R. E. (1963). Effects of light intensity and temperature on the growth of Waterstargrass, Coontail, and duckweed. *Weeds*, *11*(4), 287. https://doi.org/10.2307/4040533

Wilschut, R. A., Oplaat, C., Snoek, L. B., Kirschner, J., & Verhoeven, K. J. F. (2016). Natural epigenetic variation contributes to heritable flowering divergence in a widespread asexual dandelion lineage. *Molecular Ecology*, *25*(8), 1759–1768. https://doi.org/10.1111/mec.13502

Xu, J., Zhou, S., Gong, X., Song, Y., van Nocker, S., Ma, F., & Guan, Q. (2018). Single-base methylome analysis reveals dynamic epigenomic differences associated with water deficit in apple. *Plant Biotechnology Journal*, *16*(2), 672–687. https://doi.org/10.1111/pbi.12820

Yaish, M. W., Al-Lawati, A., Al-Harrasi, I., & Patankar, H. V. (2018). Genome-wide DNA methylation analysis in response to salinity in the model plant caliph medic (*Medicago truncatula*). *BMC Genomics*, *19*(1), 1–17. https://doi.org/10.1186/S12864-018-4484-5

Zhang, H., Lang, Z., & Zhu, J.-K. (2018). Dynamics and function of DNA methylation in plants. *Nature Reviews Molecular Cell Biology*, *19*(8), 489–506. https://doi.org/10.1038/s41580-018-0016-z

Zhang, M., Xu, C., von Wettstein, D., & Liu, B. (2011). Tissue-specific differences in cytosine methylation and their association with differential gene expression in sorghum. *Plant Physiology*, *156*(4), 1955–1966. https://doi.org/10.1104/PP.111.176842

Zhang, X., Yazaki, J., Sundaresan, A., Cokus, S., Chan, S. W. L., Chen, H., Henderson, I. R., Shinn, P., Pellegrini, M., Jacobsen, S. E., & Ecker, J. R. R. (2006). Genome-wide high-resolution mapping and functional analysis of DNA methylation in *Arabidopsis*. *Cell*, *126*(6), 1189–1201. https://doi.org/10.1016/J.CELL.2006.08.003

Zhao, P., Ma, B., Cai, C., & Xu, J. (2022). Transcriptome and methylome changes in two contrasting mungbean genotypes in response to drought stress. *BMC Genomics*, *23*(1), 1–16. https://doi.org/10.1186/S12864-022-08315-Z/FIGURES/8

Ziegler, P., Adelmann, K., Zimmer, S., Schmidt, C., & Appenroth, K. J. (2015). Relative in vitro growth rates of duckweeds (Lemnaceae) – the most rapidly growing higher plants. *Plant Biology*, *17*(s1), 33–41. https://doi.org/10.1111/plb.12184

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Van Antro, M., Prelovsek, S., Ivanovic, S., Gawehns, F., Wagemaker, N. C. A. M., Mysara, M., Horemans, N., Vergeer, P., & Verhoeven, K. J. F. (2022). DNA methylation in clonal duckweed (*Lemna minor* L.) lineages reflects current and historical environmental exposures. *Molecular Ecology*, *00*, 1–16. https://doi.org/10.1111/mec.16757