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1 **The methylome of *Biomphalaria glabrata* and other mollusks:**
2 **enduring modification of epigenetic landscape and phenotypic**
3 **traits by a new DNA methylation inhibitor**

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24 **Abstract**

25 5-methylcytosine (5mC) is an important epigenetic mark in eukaryotes. Little information about its
26 role exists for invertebrates. How 5mC contributes to phenotypic variation in invertebrates can be
27 investigated by experimental alteration of methylation patterns. Here, we apply new non-nucleoside
28 DNA methyltransferase inhibitors (DNMTi) to introduce global changes into the methylome of
29 mollusk species. Flavanone inhibitor Flv1 was highly efficient in reducing 5mC in the freshwater snails
30 *Biomphalaria glabrata* and *Physa acuta*, and to a lesser degree, probably due to lower stability in sea
31 water, in the oyster *Crassostrea gigas*. Flv1 has no toxic effects and significantly decreased the 5mC
32 level in the treated *B. glabrata* generation and in its untreated offspring. Drug treatment triggers
33 significant variation in the morphometric traits in both generations. An epigenotyping by sequencing
34 method corroborates hypomethylation effect of Flv1 in both *B. glabrata* generations and identifies
35 one Differential Methylated Region (DMR) out of 8, found both in Flv1-exposed snails and its

36 progeny, demonstrating a multigenerational effect of an induced epimutation. By targeted bisulfite
37 sequencing, we confirmed hypomethylation in a *locus* associated with reduced gene expression.

38 **Keywords:** DNMT inhibitors, 5-methylcytosine, mollusks, Invertebrates, epigenetic inheritance,
39 epimutation.

40 **Background**

41 DNA methylation is an epigenetic mark that can be associated with changes in gene function without
42 changes in the underlying DNA sequence (Ganesan, Arimondo, Rots, Jeronimo, & Berdasco, 2019).

43 Modifications in DNA methylation can be induced by the environment and some changes can be
44 mitotically and/or meiotically heritable and/or are reversible (Dupont, Armant, and Brenner 2009,

45 Nicoglou and Merlin 2017). Some of these modifications can influence gene function by providing
46 differential access to the underlying genetic information in cells, and thus may alter their

47 phenotypes. Epigenetic marks such as DNA methylation may provide an additional dimension to
48 inheritance, linked to but different from genetic inheritance. Epimutations can be provoked directly

49 by environmental stresses and contribute to rapid evolutionary changes but unlike genetic variation,
50 epimutations have higher rates and are reversible (Bossdorf, Richards, and Pigliucci 2008, Cosseau et

51 al. 2017). Biochemically, DNA methylation is the modification of a DNA base, and is present in a
52 diverse range of eukaryotic organisms, ranging from *fungi* to mammals (Chen 2011). One type of

53 DNA methylation is cytosine methylation that is catalyzed by the DNA methyltransferases (DNMTs),
54 enzymes that transfer the methyl group (-CH₃) from the co-substrate S-adenosyl-L-methionine (SAM)

55 to the carbon-5 of the cytidine, to form 5-methylcytidine (5mC) (Moore, Le, and Fan 2013). In
56 vertebrates, DNA methylation occurs on cytosines in a CpG context (cytidine followed by a

57 guanosine) (Li and Zhang 2014) whereas, DNA methylation can also occur in CHH and CHG (H=A, T, C)
58 context (Meng et al. 2015) in plants. Less is known about the methylation in invertebrates, though

59 many species present DNA methylation in a CpG context (Glastad et al. 2011).

60 DNA methylation is assumed to be evolutionary ancient, but its function and pattern is very
61 diversified. This is consistent with the notion of a dynamically evolving mechanism that can adapt to
62 perform various functions (Zilberman 2008), but having a common origin and being always part of an
63 inheritance system (Aliaga et al. 2019). Major differences in DNA methylation are observed among
64 phyla (Keller, Han, and Yi 2016). In the animal kingdom, vertebrates have one of the highest levels of
65 DNA methylation that is uniformly spread all over the genome and found in all sorts of genomic
66 contexts such as gene bodies, gene promoters, intergenic regions and repetitive DNA such as
67 transposons (Suzuki and Bird 2008) (“global methylation”). Only promoter sequences are generally
68 unmethylated and methylation here has been demonstrated to modulate gene expression in cis.
69 Methylation also affects DNA repair stability, splicing, imprinting, development, germ cell
70 pluripotency and cell fate (Schübeler 2015). In contrast, in many invertebrates, a common type of
71 DNA methylation is the “mosaic” pattern consisting in large domains of methylated DNA separated
72 by large domains of unmethylated DNA (Hendrich and Tweedie 2003). Another pattern observed is a
73 very low level (Gowher, Leismann, and Jeltsch 2000) or a total absence of DNA methylation (Capuano
74 et al. 2014, Aliaga et al. 2019). When methylation is of mosaic type, 5mC is often found in genes (in
75 exons and sometimes to a lesser degree in introns), a type of methylation also called Gene Body
76 Methylation (GBM). GBM is considered as the ancestral form of DNA methylation (Feng et al. 2010).
77 Higher GBM is believed to be associated with active transcription in vertebrates and invertebrates,
78 while promoter methylation in vertebrates is associated with repression of gene expression (Sarda et
79 al. 2012).

80

81 An important aspect of epigenetic marks is their inheritance. There is evidence in model species,
82 mainly plants (Johannes et al. 2009), that heritable variation in ecologically important traits can be
83 generated through changes in DNA methylation and that these changes may be inherited to future
84 generations. Nevertheless, in contrast to plants and vertebrates, there is little evidence of

85 transgenerational stability of DNA methylation in invertebrates. Consequently, more evidence is
86 needed about whether environmental-based DNA methylation changes can be inherited across
87 generations in invertebrates and in here we focused on this question in mollusks. DNA methylation
88 has been relatively little investigated in mollusks as discussed in (Fallet, Luquet, David, & Cosseau,
89 2020), where information is essentially based on data from two species: the pacific oyster
90 *Crassostrea gigas* and the freshwater snail *Biomphalaria glabrata*. In the abovementioned work,
91 authors distinguish the terms multigenerational and transgenerational. Multigenerational effect
92 results from a direct exposure of the germline, gametes or embryos to the environmental stress,
93 while a transgenerational effect involves a germ line transmission between generations without
94 direct exposure to the environmental stress (Fallet et al. 2020). In this work, we investigated these
95 two mollusks species and added the previously unstudied *Physa acuta*, i.e. three molluscan models of
96 medical, economic and ecological importance.

97

98 The snail *B. glabrata* is the intermediate host of *Schistosoma mansoni*, the causative agent of
99 schistosomiasis, a parasitic disease affecting 200 million people in 78 countries (McManus, 2019).
100 The interaction of these species is characterized by a phenomenon called compatibility
101 polymorphism, meaning that some snail phenotypes can be infected by a specific parasite phenotype
102 while others cannot (Theron et al. 2014). It has been demonstrated that epigenetic alterations are
103 involved in the *B. glabrata* parasite compatibility phenotype (Knight et al. 2016), even though
104 contrasting results have been obtained by others (Sullivan 2018, Allan et al. 2020). It remains,
105 therefore, an open question whether epigenetic mechanisms play a role in the capacity of *B. glabrata*
106 to produce phenotypic plasticity or variability. DNA methylation machinery components in *B.*
107 *glabrata* include a maintenance DNMT (*BgDNMT1*), a DNA/tRNA methyltransferase (*BgDNMT2*) and
108 a methyl-CpG-binding domain protein (*BgMBD2/3*), *BgDNMT1* and *BgDNMT2* being probably
109 responsible for the 5mC modifications (Fneich et al. 2013, Geyer et al. 2017).

110 *Crassostrea gigas* is a mollusk of commercial importance and its phylogenetic position and life traits
111 make this bivalve an ideal model to study the physiological, ecological and evolutionary implications
112 of DNA methylation (Rivière 2014). *In silico* analysis revealed that genes predicted to be
113 hypermethylated are generally involved in DNA and RNA metabolism and genes predicted to be
114 sparsely methylated are involved in cell adhesion (Roberts and Gavery 2012a). Similar results were
115 found in *B. glabrata*: genes predicted to be methylated are associated with housekeeping functions
116 and genes predicted to be poorly methylated are associated with inducible functions (Fneich et al.
117 2013). These findings suggest that DNA methylation has regulatory functions in genes implicated in
118 stress and environmental responses meaning it could contribute to increase phenotypic plasticity in
119 mollusks and/or produce potentially heritable phenotypic variation (Roberts and Gavery 2012b).
120

121 *Physa acuta* is one of the most widespread freshwater snail invaders (Vinarski 2017) and is an
122 occasional host of several human trematode diseases, including echinostomiasis and fasciolosis
123 (Dreyfuss et al. 2002, Kanev 1994). Besides it has been demonstrated to be a bioindicator species for
124 its sensitivity to environmental contaminants (Müller et al. 2016, Bal, Kumar, and Nugegoda 2017). *P.*
125 *acuta* has a short generation time that makes it a good model for multigenerational studies (Seeland
126 et al. 2013). Studies about the impact of toxic compounds in the global DNA methylation of *P. acuta*
127 and in its phenotypic traits (Bal, Kumar, and Nugegoda 2017) suggest that DNA methylation can play
128 a role in the phenotypic plasticity of this snail, however, further work is needed to explore this
129 hypothesis.

130

131 We reasoned that to investigate the role of DNA methylation in mollusks, we must modify its
132 methylation. We borrowed an approach from cancer biology in which the use of DNMT inhibitors
133 (DNMTi) has brought considerable advancements in the understanding of DNA methylation
134 mechanism but also in therapeutic approaches (Gnyszka, Jastrzebski, and Flis 2013, Lopez, Halby, and

135 Arimondo 2016, Geyer et al. 2011(Pechalrieu, Etievant, & Arimondo, 2017). The most used DNMTi in
136 invertebrates is 5-azacytidine (5-AzaC) (Athanasio et al. 2018, Maharajan et al. 1986, Geyer et al.
137 2018), nevertheless important advancements in the design of DNMTi have been done in the last
138 years, notably in decreasing the toxicity and improving the specificity of these compounds (Gros et al.
139 2012, Pechalrieu et al 2017). Further, 5-AzaC induces unstable and major side-effects, *e. g.* it caused
140 malformations and apoptosis in the fetal nervous system when administered into pregnant mice
141 (Ueno et al. 2002). We therefore used to alter DNA methylation in mollusks by using the
142 commercially available non-covalent nucleoside inhibitor, zebularine (Champion et al., 2010) and
143 novel generation of non-nucleoside DNMT inhibitors that do not incorporate into DNA and therefore
144 induce minimal side effects (Erdmann et al. Arimondo, 2015). In addition, we evaluate if DNMTi-
145 induced DNA methylation modifications are inherited to offspring. For global DNA methylation
146 screening, we developed a simple, low cost, antibody-based method to measure DNA methylation
147 levels over large sample numbers and requiring only small amounts of DNA. Our dot blot method and
148 a commercial ELISA-based kit showed equivalent results. For genome-wide methylation profiling we
149 used epi-genotyping-by-sequencing method (epiGBS) (van Gurp et al. 2016, Gawehns et al. 2020) and
150 we compared the results with a previous methylation information obtained by Whole Genome
151 Bisulfite Sequencing (WGBS) (Adema et al. 2017).

152

153 We tested two types of DNMTi with different mechanisms of action (Supplementary file 1: Figure S1).
154 We used zebularine, a nucleoside analogue of cytidine that has proven to be an inhibitor of DNA
155 methylation in human cancer cells (Cheng et al. 2004) but differently from 5-AzaC, it does not form
156 an irreversible covalent complex with the DNMTs (Champion et al. 2010) and two custom made
157 compounds (nitroflavanones) that showed *in vitro* inhibition activity against DNMT1 and DNMT3a-c
158 in human cancer cell lines (Pechalrieu et al. 2020).

159 In summary, our results showed that flavanone Flv1 significantly decreased the 5mC level in the
160 exposed generation and its progeny, it triggered variation in the morphometric traits in both
161 generations and it did not show toxic effects. EpiGBS sequencing confirmed the genome-wide effect
162 caused by Flv1 and allowed us to find Differential Methylated CpG sites (DMCs) between treatment
163 and control samples. Furthermore, a parental effect was demonstrated by the presence of a
164 Differential Methylated Region (DMR) in Flv1 exposed snails and its offspring. Flv1-induced
165 hypomethylation in the BGLTMP010125 *locus* was associated with reduced gene expression. Since
166 Flv1 inhibitor demonstrated efficiency as DNMTi in *B. glabrata*, it was also tested in the two other
167 mollusk species: the oyster *C. gigas* and the freshwater snail *P. acuta*, where it triggered also
168 significant decrease of 5mC, suggesting that Flv1 can be used to modify methylation in other mollusk
169 species and possibly other invertebrate's taxa. Our results also indicate that induced DNA
170 hypomethylation is associated with increased phenotypic variance.

171

172 **Methods**

173 **Ethics statement**

174 *B. glabrata* albino Brazilian strain (BgBRE) was used in this study. *P. acuta* juvenile individuals were
175 raised in the Centre d'Ecologie Fonctionnelle et Evolutive CEFE UMR 5175 in Montpellier, France. *C.*
176 *gigas* juveniles' oysters were a generous gift of Bruno Petton from the Marine Mollusks Platform
177 IFREMER in Bouin, France. *B. glabrata* mollusks were maintained at the IHPE laboratory facilities;
178 they are kept in aquariums and fed with lettuce *ad libitum*. *C. gigas* and *P. acuta* mollusks were
179 maintained during the 10 days of drug exposure in the quarantine room at the IHPE laboratory to
180 avoid contact with the home breeding species (*B. glabrata* strains). The Direction Départementale de
181 la Cohésion Sociale et de la Protection des Populations (DDSCPP) provided the permit N°C66-136-01
182 to IHPE for experiments on animals. Housing, breeding, and animal care were done following the
183 national ethical requirements.

184 **DNA methyltransferase inhibitor (DNMTi) treatments in *B. glabrata***

185 Three types of DNMT inhibitors were tested in the snail *B. glabrata*, the cytidine analogue zebularine
186 (Sigma, France, Cat. No. 3690-10-6) and custom-made inhibitors, previously selected for their
187 inhibitory activity against *hDNMT1* and *hDNMT3-c* (Ceccaldi et al. 2011, , Pechalrieu et al. 2020).

188 The custom-made compounds consist in the active flavanones: Flv1, Flv2, and Flv-neg corresponding
189 to compounds MLo1507 (3b), DD880 (880) and MLo1607 (19) in Pechalrieu et al. 2020. Stock
190 solution at 10 mM were made in ultrapure Milli-Q water and aliquoted and stored at -20°C for all
191 compounds.

192 For each condition, 100 snails *B. glabrata* Brazilian strain (*Bg BRE*) of approximately the same age (8
193 weeks) and size (5-7 mm) 7mm) were randomly assigned to treatment groups and control groups,
194 the treatments were done with the drug at a final concentration of 10 µM in 1000 mL of well water in
195 a plastic container, a single aquarium was made within each treatment. The *Bg BRE* strain is not an
196 inbred strain, it can show concomitant genetic variability (Carvalho et al. 2001). The water was
197 replaced once with fresh drug-containing water at the same concentration, the replacement was
198 performed after 3 days and 22 h. After 10 days of exposure, the drug was removed and replaced by
199 drug-free water. Snails were then raised in the plastic tank for 70 days, during which different life
200 history traits were measured. Mortality was measured at days 3, 4, 6, 8 and then each week. The
201 egg-capsules laid by the snails of the generation F0 were separated each week to raise the F1
202 generation in another plastic container, the fecundity was reported as a single measure of number of
203 juveniles and total number of eggs per treatment. At day 70, snails of the generation F0 and F1 were
204 collected, the shell width, shell height and weight of each snail were recorded to compare
205 morphometric trait variations between treatments. Finally, snails were stored wrapped in aluminum
206 sheets individually at -20 °C.

207

208 **Flv1 treatment in *C. gigas* and *P. acuta***

209 Thirty individuals of *P. acuta* and thirty of *C. gigas* were raised as the control groups. Thirty
210 individuals of *P. acuta* and thirty of *C. gigas* were exposed to the Flv1 inhibitor at a concentration of
211 10 µM. The water was replaced once with fresh water for *P. acuta* and with filtered sea water for *C.*
212 *gigas* both containing Flv1 inhibitor at the same concentration, the replacement was done after 3
213 days and 22 h. After 10 days of exposure, snails and oysters were collected and stored in aluminum
214 sheets individually at -20°C.

215 **Genomic DNA extraction**

216 Zirconia/Silica beads and the NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany) a method
217 developed to extract DNA from the Pacific oyster (de Lorgeril et al. 2018) were used for DNA
218 extraction from whole body without shell of *B. glabrata* (n=300, 30 per treatment), *P. acuta* (n=60)
219 and *C. gigas* (n=60). Briefly, for the lysis phase, 180 µL of lysis buffer, 25 µL of Proteinase K (20
220 mg/mL) and 100 µg of zirconia/silica beads (BioSpec, USA, Cat. No. 11079110z) were added to
221 samples that were submerged in liquid nitrogen and then shaken in a Mixer Mill (Retsch MM400) at a
222 frequency of 30 Hz for 12 min. Then an incubation in water bath at 56 °C during 1 h 30 was done.

223 After lysis, the NucleoSpin® Tissue Kit protocol was applied according to the manufacturer
224 instructions. Elution was performed into a final volume of 100 µL elution buffer. The samples were
225 stored at -20°C. DNA concentrations of all samples were quantified using a Qubit® 2.0 fluorometer
226 (Invitrogen) and a fluorescence-based Qubit™ dsDNA BR Assay Kit (Invitrogen, Q32853).

227 **DNA methylation screening**

228 Detection and quantification of DNA methylation in genomic DNA were performed by dot blot assays
229 using an antibody against 5mC. Before large screening, we optimized the dot blot method with DNA
230 extracted from HeLa cells as a positive control and unmethylated PCR products as negative control.

231 Different concentrations of HeLa cells were spotted to test the sensitivity and linearity of the
232 method. After standardization of the method, genomic DNA of the control and treated mollusks (100
233 ng in 5 μ L per replicate for equal loading) were denatured with 0.3 M NaOH at 42 °C for 10 min and
234 spotted on nitrocellulose membranes (Hybond®). The membranes were blocked in 5% powdered
235 milk diluted in 1×TBS containing 0.05% Tween 20 (TBST) for 1 h 30 at room temperature. Then, the
236 membranes were incubated with a 1:500 dilution ratio of anti-5mC antibody (Abcam, #ab73938) and
237 5% powdered milk in TBST for 1 h 30, followed by 3×10 washes with TBST and elliptical agitation.
238 Then incubation with a 1:500 dilution ratio of HRP-conjugated Goat anti-mouse IgG secondary
239 antibody (ClinicSciences, #AS111772) was done.

240 The antibody mixture was then removed, and the membrane was washed with TBST under elliptical
241 agitation during 3×10 min. Lecture of the signal was performed using the SuperSignal™ West Pico
242 Chemiluminescent system (Thermo Fisher Scientific, USA) and the ChemiDoc MP Imaging System.
243 Finally, the densitometry of the 5mC was analyzed with the software ImageLab5.1. Detailed protocol
244 of this method is found in our preprint (Luviano et al. 2018).

245 **ELISA-based 5mC quantification**

246 Methylated DNA Quantification Kit (Colorimetric) (Abcam, ab117128) was used to determine global
247 5mC level in isolated genomic samples of mollusk controls (n=15 for *B. glabrata* and n=10 for *P.*
248 *acuta* and *C. gigas*) and Flv1 treated (n=15 for *B. glabrata* and n=10 for *P. acuta* and *C. gigas*)
249 according to manufacturer instructions. To quantify the absolute amount of methylated DNA, a
250 standard curve was generated plotting the OD values versus the amount of positive control at each
251 concentration point.

252

253

254 **Statistical analyses**

255 The data of mean spot densitometry provided by the software ImageLab5.1 was normalized by the
256 DNA amount to obtain a relative measure of the 5mC level. Then, we calculated the 5mC % using the
257 following equation:

$$258 \text{ 5mC \%} = [\text{sample densitometry/ng}] / [\text{positive control densitometry/ng}] \times \text{Positive control 5mC \%}$$

259 where the positive control densitometry corresponds to 6.9 ± 1.2 per ng of HeLa cells and the
260 positive control 5mC % corresponds to 2.3% of cytosines methylated in HeLa cells (Diala and Hoffman
261 1982).

262 Rstudio was used for statistical analysis. When data displayed normal distribution, Student's T test
263 was used to compare means and when data did not display a normal distribution, then the Wilcoxon
264 Mann-Whitney test was applied to test significance of differences in means. The survival curves were
265 compared by a Mantel-Cox test and the fecundity was measured as the number of offspring and the
266 number of eggs laid by the snails. A contingency table was elaborated with the number of offspring,
267 the non-hatched eggs and the total of eggs laid, then a Fisher's exact test was done to test for
268 significant differences between the treatments. PCA analyses with the three morphometric measures
269 (shell width, shell height and weight) were done to examine variability in all treatments.

270 **Library preparation and high throughput bisulfite sequencing**

271 We used an existing protocol called epiGBS (van Gurp et al. 2016, Gawehns et al. 2020), a reduced
272 representation bisulfite sequencing method for cost-effective exploration of DNA methylation and
273 genetic variation designed for multiplexed high-throughput sequencing to maximize sample size
274 while losing *loci*. epiGBS sequencing was performed with the snails exposed to the DNMTi that
275 showed the most significant changes in the global 5mC % (Supplementary file 2, Figure S3). Eight
276 samples per treatment were sequenced from control group, Flv1-treated, and from the progeny of

277 control and the Flv1-treated group. 32 DNA isolated samples were quantified with Qubit fluorometer
278 with the dsDNA HS Assay Kit (Invitrogen). The concentration was homogenized in all samples to 10
279 ng/ μ L in a total volume of 35 μ L. epiGBS library preparation was applied as described in the step- by-
280 step most recent protocol (Gawehns et al. 2020). Paired-end sequencing (2 \times 150 bp) using an
281 Illumina NextSeq™550 instrument at the Bio-Environment NGS Platform at the University of
282 Perpignan.

283 **Bioinformatics epiGBS pipeline**

284 We used the epiGBS2 pipeline (<https://github.com/nioo-knaw/epiGBS2>) to remove PCR duplicates,
285 and demultiplex samples. We took the filtered and demultiplexed reads from epiGBS2 pipeline to use
286 another adapted pipeline (Meröndun, Murray, and Shafer 2019). Adapter removing was done using
287 TrimGalore! V06.5 (Krueger 2012), 30 nucleotides were removed from 3' and 5' end. Single-end
288 reads were aligned to *B. glabrata* genome v BglaB1 from
289 <https://www.vectorbase.org/organisms/biomphalaria-glabrata> without scaffolds < 5kb with BSMAP
290 Mapper (Xi and Li 2009). Then mapped reads were merged and used as input in BSMAP Methylation
291 Caller to get a tabular file with cytosine and thymine counts that was used as input to calculate
292 coverage and Frequency of C and T for subsequent analysis.

293 After alignment, we filtered the CpG sites covered by 8 or more reads and pairwise comparisons and
294 differential methylated analyses were done between control and treated samples in individuals of
295 the same generation (F0 and F1) using MethylKit (Akalin et al. 2012). The parameters to calculate the
296 Differentially Methylated Cytosines (DMCs) in MethylKit were q-value < 0.01 and > 15% methylation
297 difference. The visualization of DMCs was done in Integrative Genomics Viewer (IGV). Reference
298 transcriptome of *B. glabrata* was uploaded with bigwig files to see the location of DMCs. Genomic
299 feature annotation was done by visualizing each differential methylated DMCs. Promoter was
300 arbitrarily defined as the region 2 Kb upstream of transcription start site (TSS).

301 **Bisulfite conversion**

302 300 ng of DNA from 8 control snails and 8 Flv1-treated snails were bisulfite converted as described
303 previously (Boyd and Zon 2004, Frommer et al. 1992, Grunau, Clark, and Rosenthal 2001). 2 µg of
304 tRNA from baker's yeast (*S. cerevisiae*) were added to each sample, 3 M NaOH was added to a final
305 concentration of 0.3 M, and DNA was denatured at 42 °C for 20 min. Then, 240 µL of freshly prepared
306 bisulfite solution (5.41 g of sodium metabisulfite + 7 mL of distilled water + 0.5 mL of diluted
307 Hydroquinone [0.022 g/10 mL]) were added to the denatured DNA samples and incubated in the
308 dark during 4 h at 55 °C. After that, 200 µL of distilled water were added to the samples, and the total
309 volume was transferred to an Amicon column (UFC501024, Millipore), and centrifugation was done
310 at 12 000 g during 5 min. The column was washed 3 times with 350 µL of distilled water and
311 centrifugation at 12 000 g during 5 min was done each time. Following this, 350 µL of 0.1 M NaOH
312 was added to the DNA in the Amicon column, centrifuged at 12 000 g during 5 min, subsequently 350
313 µL of distilled water were added and a centrifugation at 12 000 g for 5 min was done. 50 µL of 10 mM
314 TRIS/Cl pH 7.5-8.0 was added to the DNA in the Amicon column and it has been incubated at room
315 temperature during 5 min. Finally, the tube was inverted, and the DNA was collected by
316 centrifugation at 1000 g for 3 min. DNA was stocked at -80 °C.

317 **Nested PCR amplification of bisulfite converted DNA**

318 Primers were designed for PCR amplification in a CpG rich region of the first intron of the
319 BGLTMP010125 gene using MethPrimer (Li and Dahiya 2002). The external primers (forward
320 ATTGTGTTTTTATTTTGATGGTTATGATA and reverse CCCCAAACTTACAAAACCTTAC) were used to
321 amplify a region spanning 861 bp in the BGLTMP010125 gene (Scaffold 4692: 13866-14343). The
322 internal primers used in the nested PCR were the forward primer AGTTTTTTTTATTTGTATGTAGAGT
323 and the reverse primer ATCCTTTCAAAAACAAATCATATATC; that amplify an amplicon of 565 bp. The
324 initial PCR amplification was performed using 1 µL of the bisulfite converted gDNA samples as

325 templates with external primer set as follows: 94 °C for 2 min, 5 cycles of 94 °C for 1 min, 50 °C for 2
326 min and 72 °C for 3 min, followed by 30 cycles of 94 °C for 30 secs, 50 °C for 2 min and 72 °C for 1:30
327 min and finally 72 °C for 10 min. The nested PCR was performed on a 10-fold dilution of the first PCR
328 product using the internal primer set in the same conditions as for the first PCR. PCR products were
329 separated by electrophoresis through 2% agarose gels to check for the specific amplification of each
330 target gene. PCR products were sequenced by Sanger sequencing (Genoscreen, Lille, France).
331 Sequence chromatograms were analysed as previously described (Jiang et al. 2010) to measure T-
332 peaks heights for unmethylated cytosines converted to thymines, and C-peaks heights for
333 methylated cytosines, providing an estimate for the degree of methylation.

334 **Dual DNA and RNA extraction and RT-qPCR**

335 DNA and RNA were extracted from the same samples (n=8 per treatment) with TRIzol reagent (Sigma
336 Life Science) according to manufacturer's instructions. DNA was subsequently bisulfite converted as
337 described previously and RNA was reverse transcribed to first strand cDNA using Maxima H Minus
338 First Strand cDNA Synthesis Kit with dsDNase to remove contaminating genomic DNA and following
339 manufacturer's instructions (Cat. Num. K1682, ThermoFisher, Scientific). Real-time RT-qPCR analyses
340 were performed using the LightCycler 480 System (Roche) in a 10 µL final volume comprising 5 µL of
341 No Rox SYBR Master Mix blue dTTP (Takyon), 1.75 µL of ultrapure MilliQ water, and 1 µL of each
342 primer at a concentration of 1 µM. The primers used for the RT-qPCR are shown in the Table 1. Two
343 housekeeping genes were used to normalize the results, the 28S ribosomal protein gene and the
344 αTubulin protein gene, the primers efficiencies were previously evaluated by amplifying four
345 different dilutions of each couple of primers at the RT reaction (1:1, 1:10; 1:100 and 1:1000), a
346 standard curve was generated and the efficiency was calculated with the equation (Efficiency of the
347 amplification= $10^{(1/-slope)}$, as earlier described (Jozefczuk and Adjaye 2011). The cycling program was:
348 denaturation step at 95 °C for 2 min, 40 cycles of amplification (denaturation at 95 °C for 10 secs,

349 annealing at 58 °C for 20 secs, and elongation at 72°C for 30 secs), with a final elongation step at 72°C
 350 for 5 min. For each reaction, the cycle threshold (Ct) was determined using the second derivative
 351 method of the LightCycler 480 Software release 1.5.0 (Roche). Reactions without RT served as
 352 negative control for each sample (in duplicate) to exclude amplification of DNA. None of these
 353 negative RT reactions amplified the target. All PCR experiments were performed in duplicates
 354 (technical replicates). The mean Ct value of each reaction was calculated and the $2^{-\Delta\Delta Ct}$ method was
 355 applied to calculate relative gene expression, the geometric mean of the Ct values of two
 356 housekeeping genes (28S and α -Tubulin) were used to normalize gene expression. Corrected melting
 357 curves were checked using the Tm-calling method of the LightCycler 480 Software release 1.5.0.

358 **TABLE 1.** *Biomphalaria glabrata* gene-specific primers used to amplified gene fragments used in the
 359 RT-qPCR.

Gene	Primer Sequence	Amplicon lenght	Primer efficiency
28S ribosomal protein	F : GCTGGCACGACCGCTCCTTT	100 bp	2.01
	R : TTTGAACCTCGCGACCCGGC		
α -Tubulin	F : CGACATCTGCCGCGTAACCT	112 bp	2.04
	R : GGCGCCATCAAACCTGAGGGA		
BGLTMP010125-RA:	F : TTGCTGTGACTGTCAGTGTC	95 bp	1.90
	R : TAGACTCAATGGACGGTGGAC		

360

361 Nuclear fraction extraction

362 Nuclear fractions were prepared by collecting *Bge* cells (the embryonic cell line of our model *B.*
 363 *glabrata*) by centrifugation, then cell pellet was lysed with a dounce homogenizer (7 mL) for 10 min
 364 at room temperature with cold 10 mM HEPES pH 7.7, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 0.4%
 365 IGEPAL CA-630 in the presence of protease inhibitors. The lysed cells were centrifuged at 15,000 x g
 366 for 3 min and the soluble fractions removed. The pellet was resuspended in 20 mM HEPES pH 7.7, 0.4
 367 M NaCl, 10% glycerol, 1 mM DTT in the presence of protease inhibitors by vortexing for 2 h at 4 °C,
 368 followed by centrifugation at 15,000 x g (5 min, 4 °C) to provide the nuclear fractions (supernatant)

369 and a membrane pellet. The nuclear fractions were quantified with the 2D Quant Kit (GE Healthcare
370 Life Sciences, USA) and then stored at -80 °C until use.

371 **Chemical stability measurement**

372 The flavanone compounds stability was measured by High Performance Liquid Chromatography
373 (HPLC) by the method described in Pechalrieu *et al.*, 2020. HPLC analysis were done using an X-terra
374 column (100 × 4.6 mm; 5 µm) with 1 mL/min flow and the following gradient: H₂O acetonitrile 95:5
375 for 2 min then up to 0:100 in 10 min and maintained at 0:100 for 2 min with H₂O and acetonitrile
376 containing 0.1% of trifluoroacetic acid. First, flavanone compounds (Flv1, Flv2 and Flv-neg) were
377 injected in solution at 100 µM in 100% DMSO to check its purity. Then 50 µL of solution at 10 µM of
378 tested compound was prepared by dilution in DNMT3A-c enzyme buffer (Hepes 20 mM pH 7.2, KCl
379 50 mM, EDTA 1 mM final concentration), in freshwater used in the aquariums of *B. glabrata* or in
380 filtered sea water used in the aquariums of *C. gigas*. The percentages of remaining compound were
381 determined with the area of the corresponding HPLC peak on the 250 nm chromatogram.

382 **DNMT inhibition assays**

383 Compound activities were determined with a fluorescence-based assay (Ceccaldi *et al.* 2011). In brief,
384 a double-strand DNA with a unique CpG site overlaying an endonuclease restriction site for
385 methylation-sensitive enzyme was used. This oligonucleotide comprises a 6-carboxyfluorescein (6-
386 FAM) at one end and biotin on the other end allowing immobilization into a 384-well plate
387 (PerkinElmer) pre-coated with avidin. Compounds to be evaluated and SAM as methyl donor were
388 added followed by DNMT3A-c to start the methylation reaction, which was prolonged 1 h at 37 °C.
389 After several washing, with PBS tween (0.05%) containing NaCl (0.5 M) and PBS tween (0.05%).
390 Restriction step was performed with HpyCH4IV (New England, BioLabs) to hand on only the specific
391 fluorescence signal. Fluorescence was quantified on a spectrofluorometer SAFAS FLX-Xenius.

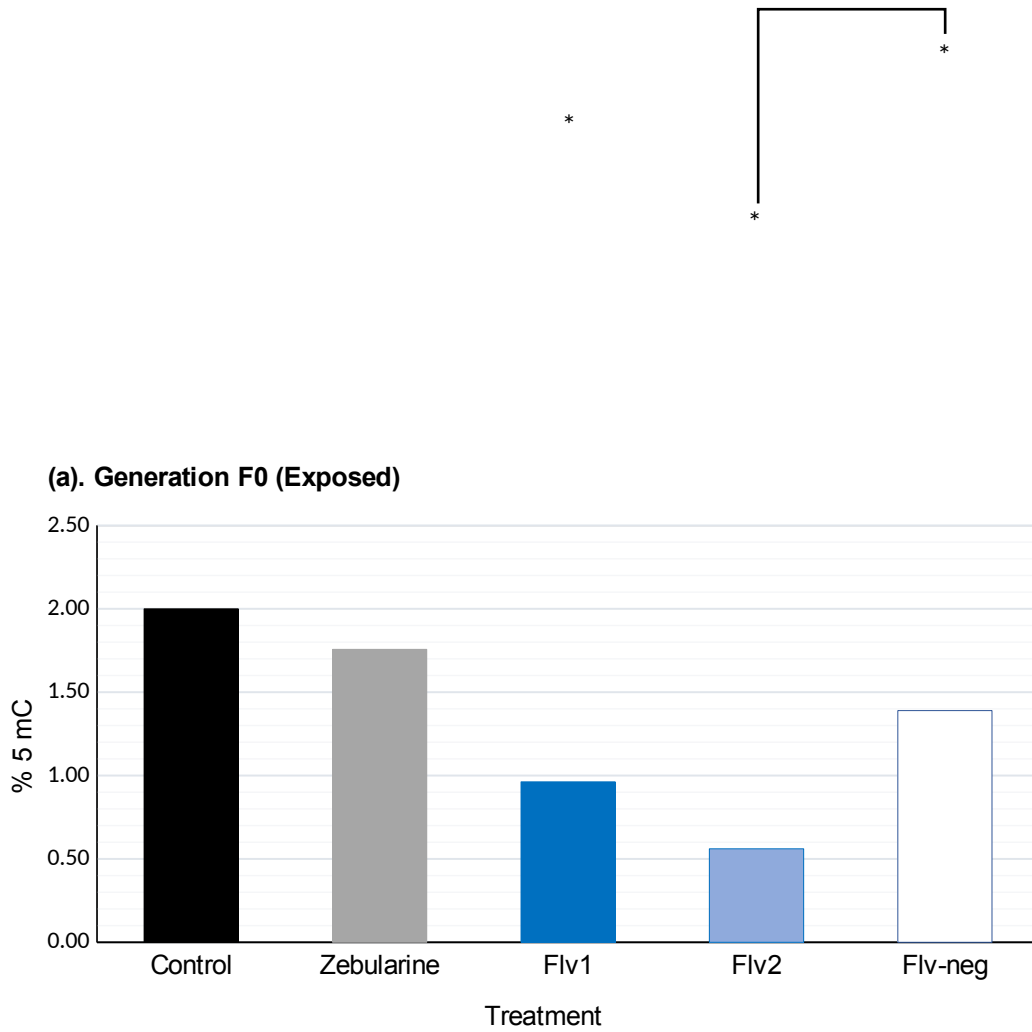
392 Methylation activities are defined as $[(X_{\text{meth}}-X_{\text{restr}})/(X_{\text{DNA}}-X_{\text{restr}})] \times 100$, where X_{meth} , X_{restr} and X_{DNA} are
393 respectively the fluorescence signals of the compound methylation, restriction and DNA controls.

394 **Results**

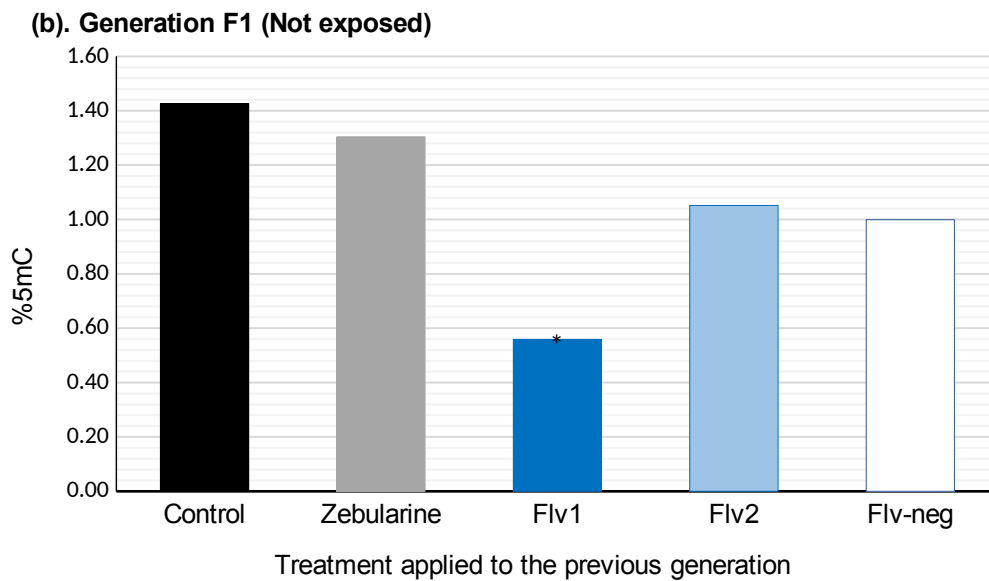
395 **New DNMTi influence global DNA methylation *in vivo* over two consecutive generations** 396 **(multigenerational effect)**

397 Using a newly developed population epigenetics screening method that delivered results comparably
398 to ELISA (Supplementary file 2, Figure S4 and S5) but at much lower costs (Luviano et al. 2018) we
399 showed that, in the F0 generation, zebularine did not produce a statistically significant difference in
400 5mC % compared to the control group (W=383, p=0.32). However, 5mC % was significantly different
401 in the groups treated with Flv1 (W=365 p<0.0001) and Flv2 (W=445, p<0.0001) compared to control.
402 The reduction in 5mC % between control (2%) and Flv1 (0.96%) was 2-fold (Figure 1a). Unlike Flv1,
403 Flv2 showed a significant difference compared to the inactive flavanone Flv-neg (W=361, p<0.0001).

404 In the F1 generation, offspring snails of the Flv1 exposed generation presented a significantly lower
405 5mC % (W=713, p<0.0001) than the control group (Figure 1b).



406



407

408 **FIGURE 1.** 5mC % of *B. glabrata* snails upon DNMTi treatments at a concentration of 10 μ M, error
 409 bars represent SD, n=30 per treatment. (a) 5mC % in the F0 generation (exposed), black bar for
 410 control, grey bar for zebularine, blue bars represent the flavanone inhibitors (blue bar for Flv1 and
 411 light blue bar for Flv2) and the white bar with blue outline represent the inactive Flv-neg. (b) 5mC %
 412 in the non-exposed F1 generation. Compounds are the ones used in F0. Mann-Whitney Wilcoxon test
 413 was applied, if not otherwise indicated, between treatment and control significant differences are
 414 marked as * for $p < 0.0001$. 5mC ng was normalized to the 5mC global percentage present in the
 415 genome of *B. glabrata*.

416 **Flv1 blocks DNMT activity *in vitro***

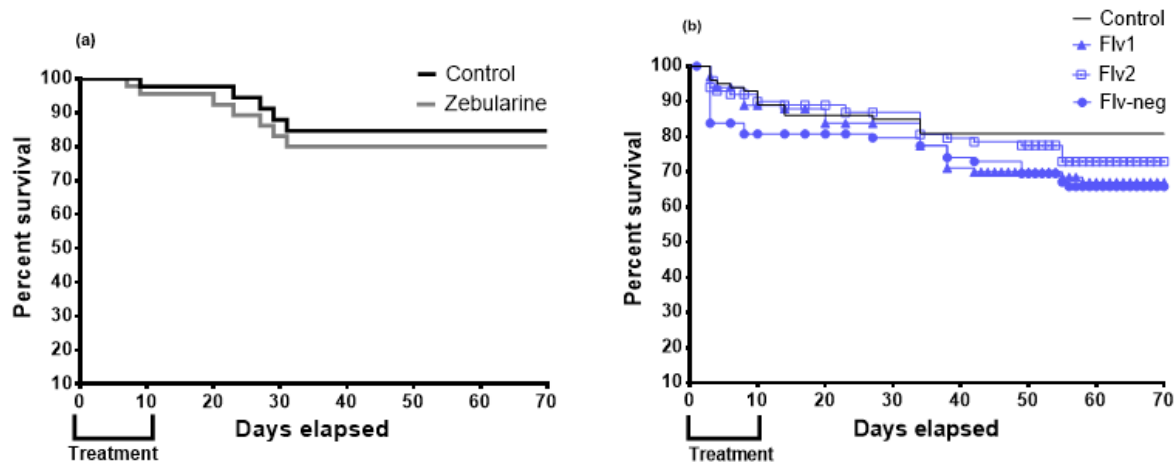
417 After having firmly established that Flv1 inhibits DNA methylation *in vivo* in *B. glabrata* we wondered
 418 if this effect was due to a direct action on DNMT or whether it was indirect by influencing upstream
 419 pathways. To verify this, we extracted soluble nuclear proteins from *Bge* cells and performed an *in*
 420 *vitro* enzyme inhibition assay. We showed that methylation activity of *Bge* nuclear protein extract
 421 was inhibited by 55% and 78% after treatment with 32 μ M and 100 μ M of Flv1, respectively
 422 (Supplementary data 1, Figure S2).

37

38

423 **DNMTi influence survival, fecundity and morphometric traits**

424 Since our findings had clearly indicated that Flv1 had an *in-vivo* and *in-vitro* demethylating activity *i.e.*
425 probably due to a direct effect on the DNMT we wondered if this epimutagenic activity had
426 phenotypic consequences. Therefore, we measured survival, fecundity and morphometric traits in
427 the DNMT-treated snails. We used as pharmacological reference molecule zebularine. Zebularine
428 induced the lowest mortality with no significant difference compared to control group (Mantel-Cox
429 test $\chi^2=0.3$, $p=0.56$). This compound followed a similar trend as the control and the snail final survival
430 rate reached 80% compared to 84% in the control. (Figure 2a). The mollusks treated with Flv1, Flv2
431 and the inactive Flv-neg had a survival rate of 68%, 73%, and 69%, respectively (Figure 2b), and none
432 of these rates were statistically different compared to the control group ($\chi^2=3.5$, $p=0.06$ for Flv1,
433 $\chi^2=1.16$, $p=0.2$ for Flv2 and $\chi^2=5.9$, $p=0.1$ for Flv-neg).



434

435 **FIGURE 2.** Kaplan-Meier survival curves upon treatment with the two types of DNMTi. (a) Cytidine-
436 analogue zebularine (grey line). (b) Flavanones Flv1 (blue line with triangles) and Flv2 (blue line with
437 squares) and their inactive derivative Flv-neg (blue line with circles).

438 The fecundity of snails was affected by the treatment with Flv2. With zebularine the number of the
439 offspring was significantly lower than the control group (Fisher's exact test, $p<0.0001$) and the

440 number of eggs was very high compared to the other treatments (Table 2). Mollusks treated with
 441 Flv2 presented a low number of offspring (n=20) and it was significantly different compared to
 442 control group (p=0.004); the treatments with Flv1 and Flv-neg showed no significant difference in the
 443 number of offspring against control group.

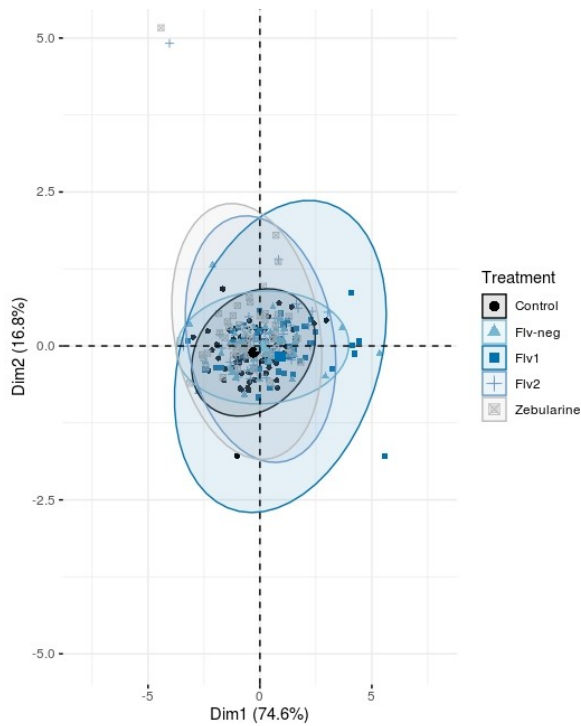
444 **TABLE 2.** Contingency table of fecundity of the snails exposed to different DNMTi. Total number of
 445 laid eggs (first row), number of non-hatched eggs (second row) and number of offspring snails (third
 446 row). Fisher's exact test was applied, significant differences with control group are marked with * for
 447 p<0.005 and ** for p<0.0005.

	Control	Flv1	Flv2	Flv-neg	Zebularine
<i>Total number of laid eggs</i>	191	183	199	188	326
<i>Number of non-hatched eggs</i>	152	147	179	147	301
<i>Number of offspring</i>	39	36	20*	41	25**

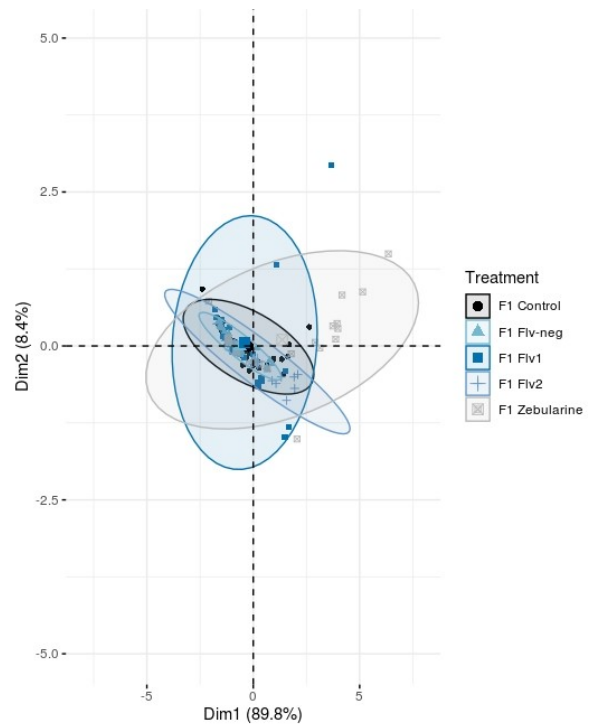
448

449 To visualize the variation of morphometric traits induced by DNMTi treatment in the F0 generation
 450 and in its respective offspring, we performed PCA with three morphometric measures. Flv1
 451 treatment induced the largest variance (the highest range of values in the axis of the PCA plot) in
 452 both the F0 and F1 generations (Figure 3). In other words, the inhibition of DNMT activity by Flv1 led
 453 to a decrease of global DNA methylation and resulted in a higher diversity of morphometric traits in
 454 the Flv1-treated population and its offspring (Figure 3 e-f).

(a) PCA morphometric traits F0 generation

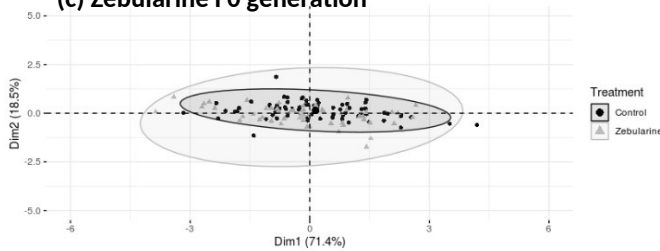


(b) PCA morphometric traits F1 generation

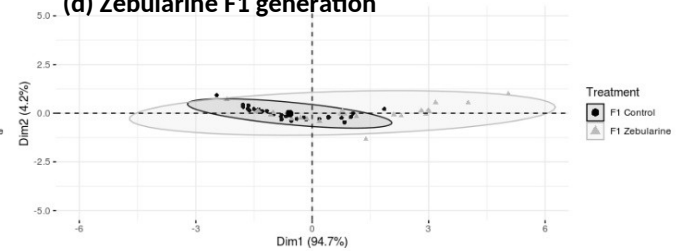


455

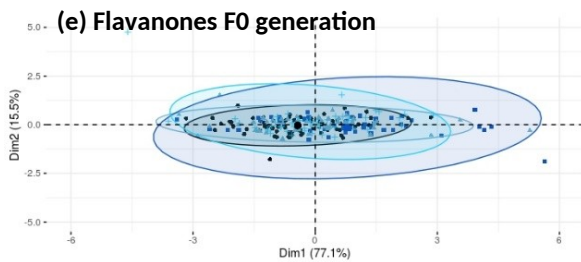
(c) Zebularine F0 generation



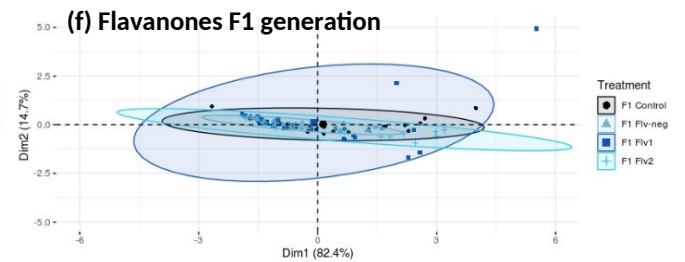
(d) Zebularine F1 generation



(e) Flavanones F0 generation



(f) Flavanones F1 generation



456

457 **FIGURE 3.** PCA of morphometric traits of all treatments of F0 (a) and F1 generation (b). PCA analyses
458 splitted by treatment, (c) PCA of morphometric traits after zebularine treatment and (d) its offspring
459 (e) PCA of morphometric traits of snails exposed to flavanone derivatives F0 generation and (f) the
460 non-exposed F1 generation. The confidence ellipses show a confidence interval of 95%. The axis 1
461 includes the three morphometric measures (shell width, height and weight) and the axis 2 include
462 shell width and height.

43

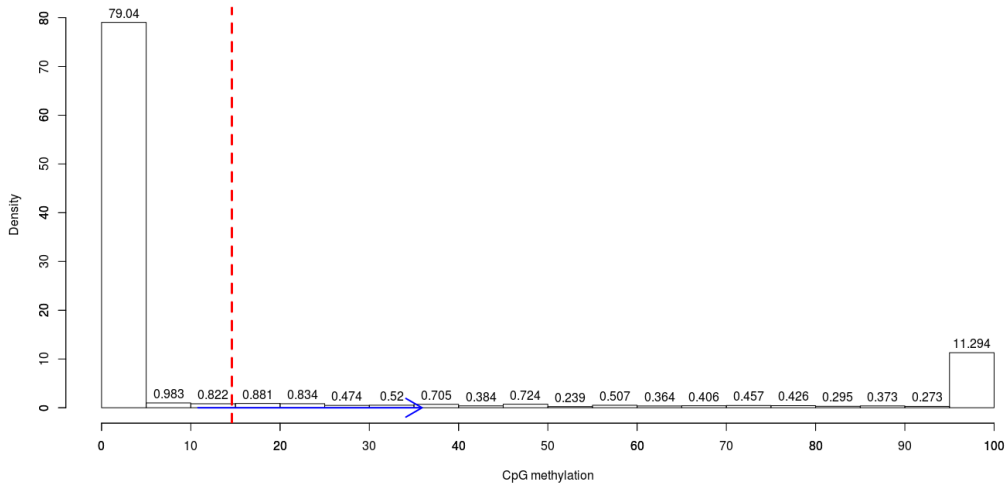
44

463 **epiGBS reduces sequencing effort roughly 10x but allows for reliable evaluation of global 5mC level**
464 **and identification of differentially methylated sites and regions**

465 To obtain a clearer picture of where hypomethylation occurred in the epigenomes of the DNMTi
466 exposed populations and their offspring we adapted a reduced representation technique that was
467 originally developed for mosaic methylation of plants: epigenotyping by sequencing (epiGBS). Since it
468 was the first time epiGBS was used on mollusks, we first had to make sure that it delivers reliable
469 results here. We used our previously obtained WGBS data (Adema et al. 2017) to compare the WGBS
470 to epiGBS results on *B. glabrata*. We reanalyzed the WGBS data with updated pipeline analysis and
471 generated a new reference methylome of *B. glabrata*. Using the BSMAP Mapper, 46.2% of reads
472 mapped unambiguously to the *B. glabrata* reference genome. Paired-end sequencing of the 32
473 pooled epiGBS libraries (8 per treatment) resulted in a total of 140,751,495 filtered and
474 demultiplexed reads. After quality control and alignment, an average of 34% of unique reads per
475 sample mapped to the *B. glabrata* reference genome using BSMAP Mapper (Supplementary file 1,
476 Table S1). After methylation calling, 6 samples per treatment with CpG sites covered by ≥ 8 reads
477 were retained for further analysis, the removed samples showed very low number of CpG sites
478 (< 4200). After filtering; we obtained an average of 47,715 \pm 31,774 methylated CpG methylation
479 positions per sample (Supplementary file 1, Table S1).

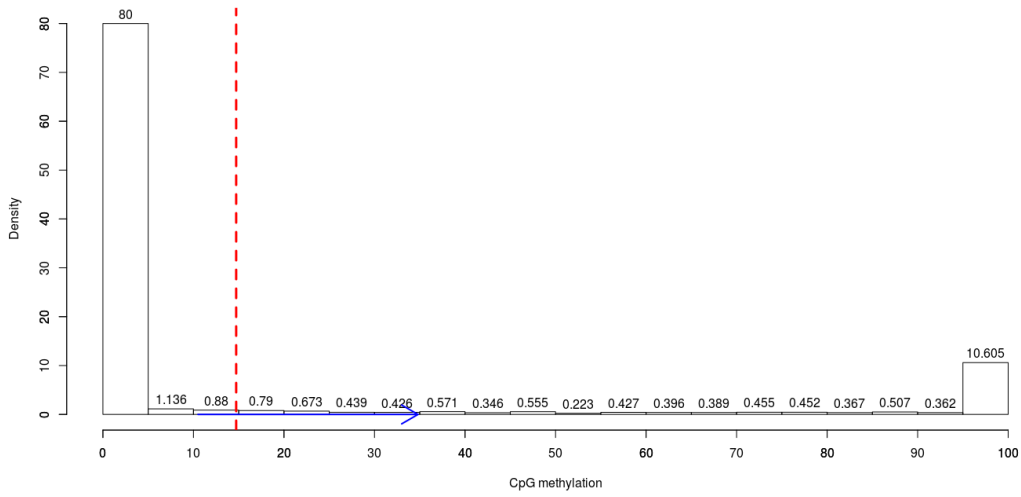
480 To analyze the distribution of methylated CpG over the entire genome we represented its frequency
481 distribution. We found a characteristic distribution of two peaks for both WGBS and epiGBS
482 indicating the majority of the CpG sites being either unmethylated or completely methylated, as
483 expected for a species that displays a mosaic distribution type of DNA methylation pattern (Figure 4
484 a-c). The peak of methylated CpG sites was higher in the epiGBS sequencing results compared to
485 WGBS, but mean CpG methylation values and confidence interval (CI) of 95% were highly similar in
486 both methods (Figure 4 a-c).

(a) Histogram of CpG methylation F0 Controls epiGBS



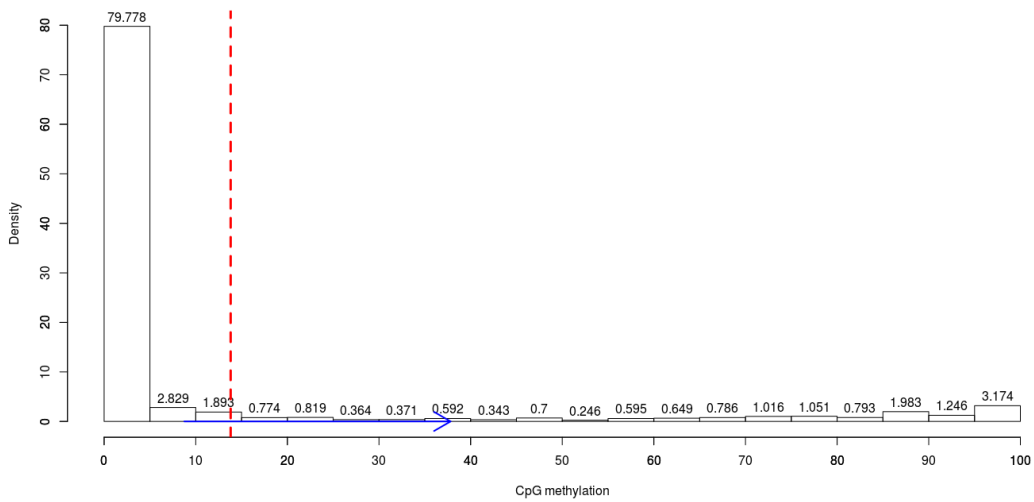
487

(b) Histogram of CpG methylation F1 Controls epiGBS



488

(c) Histogram of CpG methylation WGBS



489

47

48

490 **FIGURE 4.** Histograms of CpG methylation distribution, (a) histogram of F0-control epiGBS libraries
491 (b) histogram of F1-control epiGBS libraries and (c) histogram of WGBS library. The abscissa
492 represents the CpG methylation % (0-100) and the ordinate showed the density of CpG positions. The
493 dashed red line indicates the mean CpG methylation value and the blue arrow indicates the
494 confidence interval (CI) of 95%.

495 A direct comparison was done to examine the data obtained for CpG methylation from epiGBS library
496 versus WGBS (Supplementary file 1, Table S1). We chose the best covered control samples from each
497 generation of epiGBS libraries to compare them with WGBS. WGBS data had a higher mapping
498 efficiency than epiGBS (46.2% compared to 32.8%). The number of CpG sites with a minimum read
499 coverage of 8x was of 34,646 and 63,892 for epiGBS libraries and 4,061,906 for WGBS. epiGBS
500 represents 0.8% (epiGBS F0) and 1.6% (epiGBS F1) of the CpG sites covered by WGBS. However, the
501 average levels of CpG methylation percentage were very similar between both methods (Table 3).

502

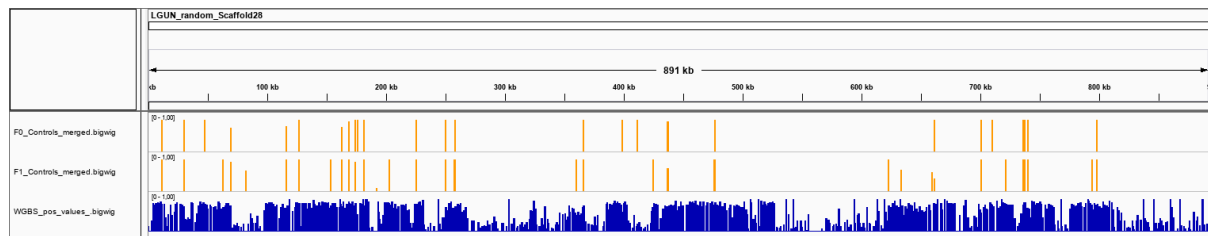
503 **TABLE 3.** Mapping efficiencies, CpG coverage and average genome-wide methylation levels resulting
 504 from epiGBS and WGBS libraries.

Samples	Mapping efficiency%	Sequence reads	Total no. CpGs	CpGs \geq 8x coverage	Methylated CpG sites	Methylated CpG %
epiGBS F0	34.3	707 010	95 369	34 646	7 621	22.0
epiGBS F1	34.5	1 299 293	180 852	63 892	13 982	21.9
WGBS	46.5	152 842 929	17 493 207	4 061 906	855 624	21.1

505

506 To evaluate concordance of epiGBS and WGBS, a correlation was done with the methylation values
 507 of CpG positions covered by both methods. A high correlation was found between WGBS and epiGBS,
 508 Spearman correlation, $R=0.74$, $p<2.2e^{-16}$. We also visualized the CpG methylation profile of epiGBS
 509 samples compared to WGBS in IGV in a wide-ranging Scaffold. Visual inspection showed that both
 510 epiGBS libraries of F0 and F1-controls have similar methylation profiles (Figure 5 yellow bars) while,
 511 naturally, epiGBS results represent a small fraction of the information found with WGBS (Figure 5,
 512 blue bars).

513

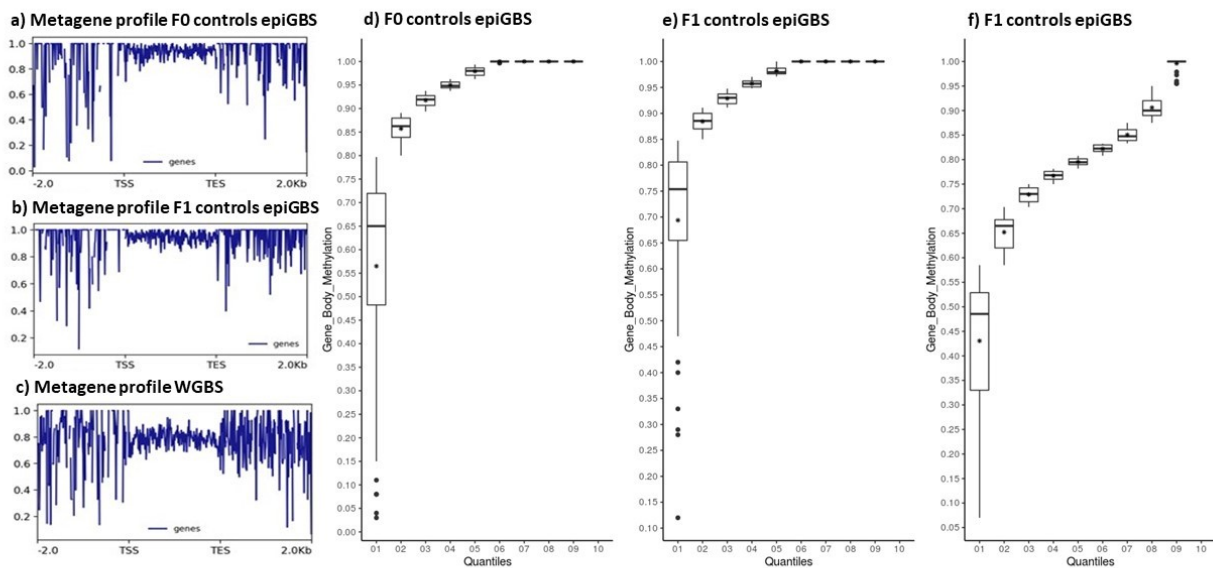


514 **FIGURE 5.** Screenshot of IGV of the region LGUN_random_Scaffold28: 1-800 Kb, that showed the
 515 regions covered by epiGBS libraries (in yellow) versus the regions covered by WGBS library (blue).

516 We then produced the CpG methylation metagene profiles across gene bodies from 2kb upstream of
 517 the transcription start sites (TSS) and 2kb downstream of the transcription end sites (TES). The CpG
 518 sites used for these profiles were those covered by both methods. We found that CpG methylation
 519 levels remained a plateau after TSS and along the gene bodies and then showed a high range of

520 methylation before TSS and after TES in both methods. The range of GBM levels were different in
 521 epiGBS libraries (0.9-1) (Figure 6a-b) than in WGBS (0.7-0.9) (Figure 6c).

522 The quantile distribution of GBM was different between epiGBS and WGBS. In the epiGBS libraries of
 523 F0 and F1 controls, the highest quantile is the first one and comprises CpG values of 0.15-0.80 and
 524 0.47-0.85 (Figure 6d-e). In the WGBS library, the highest quantile is also the first one but comprises
 525 values of 0.07 to 0.58 (Figure 6f). When all CpG sites covered by WGBS are compared to epiGBS
 526 libraries, the metagene profiles and the quantiles distribution are, as expected, more marked
 527 (Supplementary file 2 Figure S6).



528

529 **FIGURE 6.** CpG methylation ratio profile across the bodies of genes and quantiles distribution of
 530 epiGBS and WGBS libraries. (a) Metagene profile of CpG methylation ratio of F0 control epiGBS
 531 libraries, (b) F1-control epiGBS libraries and (c) WGBS library. -2.0 kb indicates the upstream 2,000
 532 bp of TSS, and 2.0 kb indicates the downstream 2,000 bp of TES. Quantiles (deciles) distribution of
 533 Gene body methylation of (d) F0-control epiGBS (e) F1-control epiGBS and (f) WGBS.

534 The global distribution of CpG methylation sites displayed a two-peak histogram in all epiGBS
 535 samples, with most of the CpG sites being either unmethylated or completely methylated. The

536 percentage of CpG sites which displayed no methylation or complete methylation for each sample
 537 are indicated in Table 4.

538 In summary, epiGBS mirrors WGBS on a global scale but has necessarily a lower resolution (at our
 539 sequencing depth about 1% of the CpG sites are captured) and it also has a slight bias towards
 540 methylated regions of the epigenome.

541 **TABLE 4.** Percentage of CpG methylation sites which display an unmethylated or complete
 542 methylated pattern.

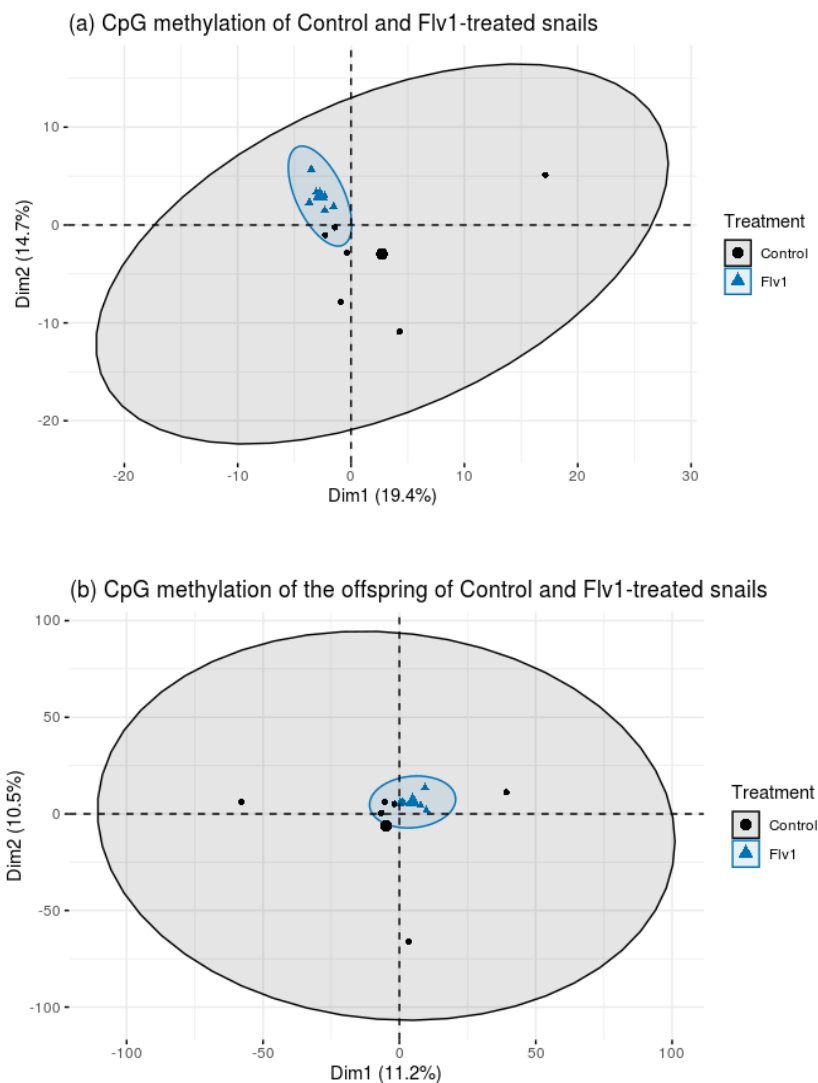
Generation F0			Generation F1		
Samples	CpG methylation frequency		Samples	CpG methylation frequency	
	Unmethylated %	Completely methylated %		Unmethylated %	Completely methylated %
Control 1	78	9.9	F1-control 1	81.2	13.6
Control 2	77.6	10.4	F1-control 2	78.6	9.7
Control 3	79.7	10.5	F1-control 3	82.4	13.5
Control 4	79.6	12.5	F1-control 4	82.4	13.5
Control 5	77.6	11.7	F1-control 5	81.5	12.5
Control 6	79.2	11	F1-control 6	82.3	12.6
Flv1-1	80.9	9.4	F1-Flv1-1	80.4	10.6
Flv1-2	77.1	8.7	F1-Flv1-2	80.9	10.8
Flv1-3	80.4	11.9	F1-Flv1-3	81	10.6
Flv1-4	82	13.4	F1-Flv1-4	82.9	11.8
Flv1-5	80.9	12.2	F1-Flv1-5	81.2	11.1
Flv1-6	81.9	12.7	F1-Flv1-6	81.7	11.2

543

544 **epiGBS corroborates multigenerational hypomethylation by Flv1**

545 We considered epiGBS a reliable method that allows for epigenome-wide analysis of DNA
 546 methylation changes in populations at reasonable costs and we used it to capture regional
 547 methylation differences in Flv1 -treated samples. The mean percentage of CpG methylation was 15.8
 548 ± 0.8 % in control snails and 13.5 ± 0.6 % in Flv1-exposed snails, 13.5 ± 0.3 % in offspring of control
 549 snails and 13.1 ± 0.1 % in the offspring of Flv1-exposed snails. There was significant difference in
 550 global percentage of CpG methylation between control and Flv1-exposed snails ($t= 6.0$, $df= 9.4$, $p=$
 551 0.0001) and significant difference was also found in their offspring ($t= 3.0$, $df= 6.0$, $p=0.023$).

552 PCA analysis of CpG methylation was performed on controls and Flv1-treated samples (Figure 7).
553 Interestingly, Flv1-treated samples clustered tightly, while control samples were spread out (Figure
554 7a). PCA analysis of CpG methylation in F1 generation showed the same tendency, F1-Flv1 samples
555 were grouped and F1-control samples dispersed (Figure 7b). PCA of both generations displayed the
556 same pattern, indicating an impact in the CpG methylation at the genome-wide level and a decrease
557 of CpG methylation variability/diversity in both generations.



558

559

560 **FIGURE 7.** PCA of CpG methylation of the Flv1-treated and control groups in F0 generation (a) and in
561 its offspring (b). The ellipses represent the 95% confidence interval.

57

58

562 **One out of eight of Flv1-induced hypomethylated DMR is heritable**

563 Differential Methylated CpG sites (DMCs) were analyzed between control and Flv1-treatment in both
 564 generations. We found multiple DMCs concentrated in DMRs, one DMR consists in 2 or more DMCs
 565 found in the same genomic region (within 3.5 Kb). We found 25 DMCs in the F0 generation between
 566 control and Flv1-exposed samples, comprising 23 hypomethylated CpG sites and two
 567 hypermethylated CpG sites (Table 5). The higher content in hypomethylated CpG sites further
 568 confirmed the genome-wide effect of the Flv1 inhibitor. Interestingly, these DMCs are not isolated
 569 and rather concentrated in some closed regions: the 25 DMCs comprise eight DMRs (Supplementary
 570 file 1, Table S2).

571 **Table 5.** DMCs in Flv1-treated and control group for each generation. The parameters to calculate
 572 the DMCs were q-value < 0.01 and > 15% methylation difference.

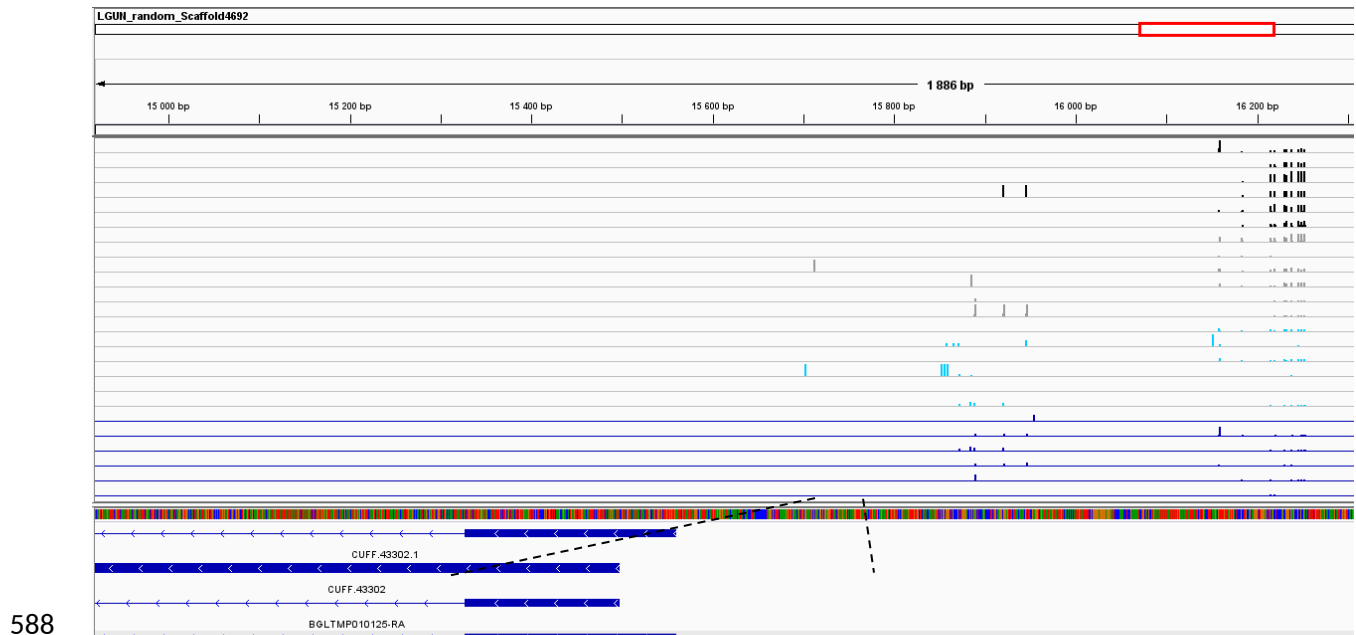
Treatment	Generation	Total DMCs	Total DMRs	Hypomethylated DMCs	Hypomethylated DMRs	Hypermethylated DMCs	Hypermethylated DMRs
Flv1	F0	25	8	23	6	2	1
Flv1	F1	325	51	203	38	120	13
Flv1	F0 and F1	6	1	6	1	0	0

573

574 In the generation F1, 323 DMCs were found between F1-control and F1-Flv1 samples, 203
 575 hypomethylated and 120 hypermethylated (Table 5). The majority of hypomethylated DMCs
 576 demonstrates a hypomethylated genome wide effect. 325 DMCs represent a considerably higher
 577 amount of DMCs than in the generation F0. The context of each DMC was examined, the majority of
 578 hypomethylated DMCs were found in the intergenic region (42.8%), 19.7% in the promoter region,
 579 28.1% in introns and 9.4% in exons. In the case of hypermethylated DMCs, 32.5% were found in the
 580 intergenic region, 11.7% in the promoter region, 30% in introns and 25.8% in exons (Supplementary
 581 file 1, Table S3). The 203 hypomethylated DMCs are concentrated in 38 DMRs and the 120
 582 hypermethylated DMCs are concentrated in 13 DMRs.

583 Six DMCs were common between both generations, being hypomethylated, five of these DMCs were
584 found in one DMR, which was visualized in the Integrative Genomics Viewer (IGV) using the *B.*
585 *glabrata* genome (Assembly GCA_000457365.1) and the reference transcriptome for annotation
586 (Figure 8). The DMR is close to transcript BGLTMP010125.

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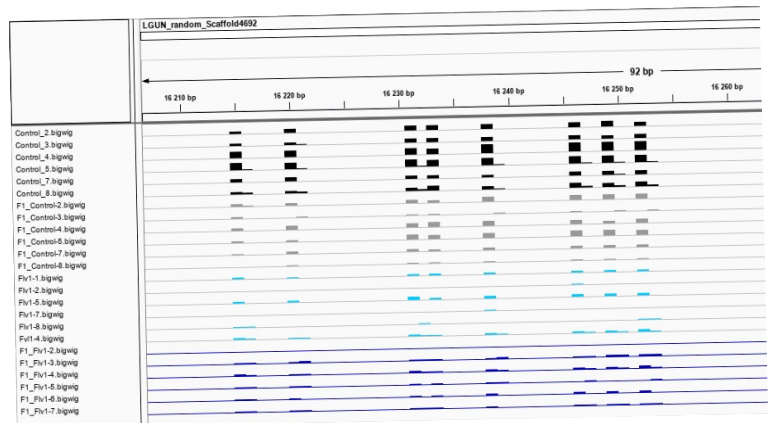
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595 **Figure 8.** IGV screenshot of the LGUN_random_scaffold4962:16175-16266 of *B. glabrata* genome

596 assembly (GCA_000457365.1). Each bar indicates the position of a methylated CpG site for the

597 different samples: F0-control F0 (black), F1-control (gray), Flv1-treated samples (light blue) and its

598 offspring (dark blue). Hypomethylated DMCs have been detected in this region and are in common

599 between F0 and F1 generation.

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602 **Gene BGLTMP010125 that is hypomethylated by Flv1 shows decreased transcription**

603 One of the identified DMR was particularly intriguing. This DMR that was hypomethylated in Flv1-
604 treated snails and in their offspring and was close to BGLTMP010125. However, no epiGBS sites were
605 located within the BGLTMP010125 so that we could not evaluate gene body methylation (GBM) by
606 this method. We therefore decided to resort to targeted bisulfite sequencing (TBS). We chose a
607 region in the first intron of the transcript, roughly 2kb upstream of the DMR and spanning 9 CpG to
608 further explore the relationship between GBM and gene expression. Our TBS results showed that
609 control snails had five methylated CpG sites in the targeted region of the transcript BGLTMP010125-
610 RA and that the Flv1-treated snails showed a decrease of the 5mC level in three of the five CpG sites
611 (Table 6), in the CpG 4 of the control snail 6, the decreased of CpG methylation percentage was from
612 83.2 to 0%. GBM was significantly lower in Flv1-treated snails ($t= 10.58$, $df= 8.18$, $p= 4.673e-06$) than
613 in controls (Figure 9a) and the transcript was significantly lower in Flv1-treated samples compared to
614 controls ($t= 6.53$, $df= 10.02$, $p=6.477e-05$) (Figure 9b).

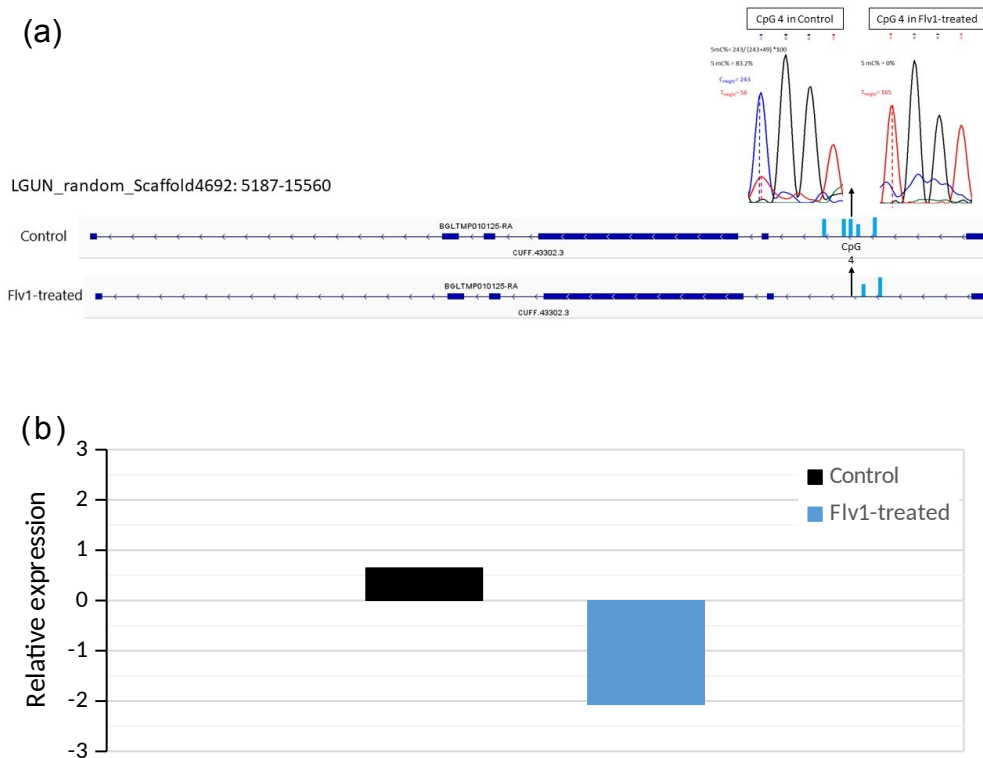
615

616 **TABLE 6.** 5mC % per CpG sites in the bisulfite converted sequence of the transcript BGLTMP010125.

CpG sites	<i>CpG 1</i>	<i>CpG 2</i>	<i>CpG 3</i>	<i>CpG 4</i>	<i>CpG 5</i>	<i>CpG 6</i>	<i>CpG 7</i>	<i>CpG 8</i>	<i>CpG 9</i>
Position on Contig LGUN_random_Scaffold4692	<i>13866</i>	<i>13976</i>	<i>14024</i>	<i>14042</i>	<i>14059</i>	<i>14061</i>	<i>14317</i>	<i>14331</i>	<i>14343</i>
Control 1	100	0	100	100	0	63.4	0	100	0
Control 2	88.4	0	94.2	83.3	0	62.9	0	95.2	0
Control 3	100	0	97.8	100	0	87.8	0	100	0
Control 4	100	0	100	100	0	40	0	100	0
Control 5	100	0	100	83.1	0	73.4	0	100	0
Control 6	100	0	100	100	0	60	0	100	0
Control 7	89.5	0	95.3	100	0	81.1	0	100	0
Control 8	100	0	100	83.2	0	63.0	0	100	0
Flavanone 1	0	0	0	0	0	79.6	0	76.0	0
Flavanone 2	0		0	0		80.4		100	
Flavanone 3	0	0	0	0	0	54.3	0	93.7	0
Flavanone 4		0		0	0	59.4	0	100	0
Flavanone 5	0	0	0	0	0	60.	0	100	0
Flavanone 6	0	0	0	0	0	71.9	0	100	0
Flavanone 7	0	0	0	0	0	60	0	100	0
Flavanone 8	0	0	0	0	0	68.4	0	100	0
Control mean	97.2	0	98.4	93.7	0	66.4	0	99.4	0
Flavanone mean	0	0	00	0	0	66.7	0	96.2	0

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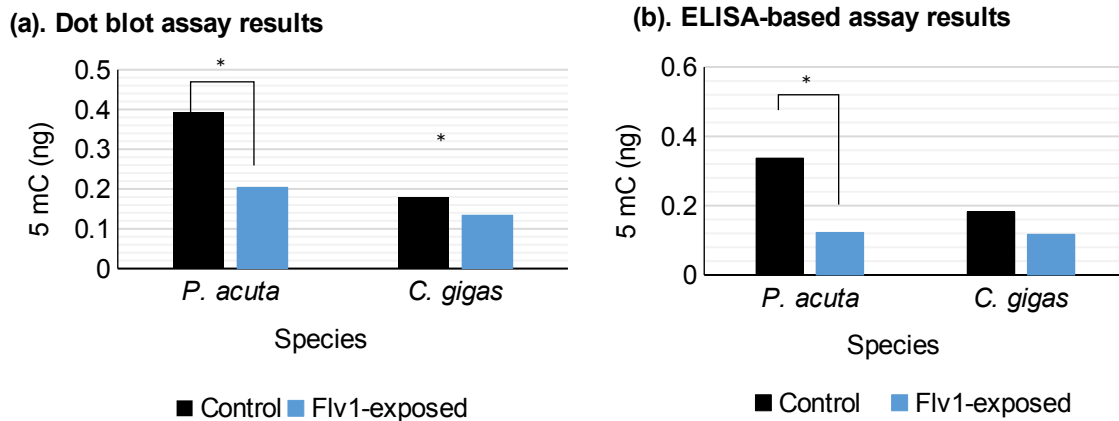
621

622 **Figure 9.** (a) Position and level of methylation of the five CpG positions, which has been studied by
 623 TBS within the first intron of the transcript BGLTMP010125-RA. (b) Relative expression of the
 624 transcript BGLTMP010125-RA compared to two housekeeping genes (28S and α -Tubulin), the
 625 ordinate show the logarithm of the values obtained with the $2^{-\Delta\Delta CT}$ method.

626 Flv1 reduces global 5mC in other mollusks

627 Since Flv1 showed efficiency as DNMTi in *B. glabrata* we wondered if it would be active also in other
 628 mollusk species and used *P. acuta* and *C. gigas*. The dot blot results (Figure 10a) displayed that Flv1
 629 exposed *P. acuta* snails have a significantly decreased of the 5mC (ng) concentration compared to
 630 controls ($t = 5.90$, $df = 52.23$, $p = 2.76 \text{ e-}07$). For *C. gigas* the decreased in 5mC (ng) by the Flv1
 631 treatment was also significantly different compared to control ($t = 2.18$, $df = 47.946$, $p = 0.0342$). The
 632 ELISA results (Figure 10b) showed that the Flv1 compound decreased significantly the 5mC
 633 concentration (ng) in *P. acuta* snails compared to controls ($t = 4.80$, $df = 12.33$, $p = 0.0004$), for *C.*

634 *gigas* we did not find a significantly decrease of the 5mC (ng) ($t = 1.48$, $df = 12.11$, $p = 0.16$) in ELISA-
 635 based results but we found a tendency to decrease. The reason for this is probably the lower stability
 636 of Flv1 in sea water (Supplementary file 2, Figure S7).



637

638 **FIGURE 10.** (a) 5mC % measures obtained by the dot blot method for *P. acuta* and *C. gigas*. (b) 5mC
 639 % measures obtained by the ELISA-based assay. The bars represent the 5mC (ng) mean, the error
 640 bars represent the standard deviation (SD), $n = 30$ per group per specie for dot blot and $n = 10$ for
 641 ELISA. Significant differences between treatment and control are marked as * for $p < 0.05$.

642 **Discussion**

643 An extension of the concept of inheritance system includes the genotype, the epigenotype, the
 644 heritable cytoplasmic elements and the microbiome that interacts with the environment to shape
 645 and transmit the phenotype (Cosseau et al. 2017). The epigenotype and the microbiome can be
 646 altered by environmental factors and these modifications can be inherited, at least in some systems,
 647 to later generations, potentially facilitating genetic adaptation. One of the most-studied epigenetic
 648 mark is DNA methylation. It has been widely studied in vertebrates and plants but remains poorly
 649 understood in invertebrates, one of the largest phyla of invertebrates are mollusks, that include
 650 several species that are commercially, ecologically and medically important. It was hypothesized that
 651 DNA methylation in mollusk can be a mechanism to produce phenotypic variation and potentially

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652 adaptation to new environments (Roberts and Gavery 2012a), but experimental proof is lacking. DNA
653 methylation in mollusks is likely to be an important element of the inheritance system. One way to
654 analyze its role is to expose the inheritance system to external perturbations that target specifically
655 the DNA methylation, *e.g.* by using DNMTi. Such specific inhibitors were synthesized to be used in
656 human cell lines and they were applied to invertebrates assuming they would have the same effect.
657 This strategy already led to important advances in other invertebrate species where treatments with
658 the most used DNMTi, 5-AzaC, were correlated with demethylation and phenotypic changes
659 (Athanasio et al. 2018, Maharajan et al. 1986, Geyer et al. 2018). Nevertheless, this drug has shown
660 low response rates, low stability in aqueous solutions and a high toxicity. New DNMTi have been
661 developed to overcome the weaknesses mentioned above. The aim of this work was to find an
662 efficient DNMTi for mollusks that (i) provoked minimal side-effects and (ii) allowed the study of the
663 DNA methylation contribution to phenotypic variability and the heritability of environmental DNA
664 methylation changes. We tested new generation DNMTi in the snail *B. glabrata* to evaluate their
665 inhibition potency in a mollusk-like DNA methylation.

666 We used here an antibody-based assay as a screening method of global 5mC % modifications. We
667 determined a linear correlation between DNA amount and mean spot density in the dot blot assay,
668 and we demonstrated that it showed comparable results to ELISA-based commercial kit but allowing
669 the screening of a larger number of samples at a lower price (Supplementary file 2, Figure S5).
670 Furthermore, we used the epiGBS method for the first time in a mollusk species, providing evidence
671 that this method can be used to analyze environmental DNA methylation changes genome-wide. This
672 method allowed the analysis of DNA methylation changes at the nucleotide level of numerous
673 replicates, that is a prerequisite for ecological studies, at an affordable price and giving results that
674 represent the same global pattern as WGBS, as shown by the high correlation found between the
675 methylation ratios of the CpG positions covered by both methods (Spearman correlation, $R=0.74$,

676 $p < 2.2e^{-16}$). Besides, epiGBS laboratory protocol and bioinformatics analysis are very flexible and can
677 be further improved to obtain higher coverage.

678 **Zebularine is not suitable for DNA methylation modification in *B. glabrata***

679 Zebularine has been reported as an efficient DNMT inhibitor in vertebrates, especially in human
680 cancer cell lines (Tan et al. 2013). In this work we set out to evaluate its effect on the DNA
681 methylation and phenotypic variation on the snail *B. glabrata*. We decided to use this drug as it is
682 associated with lower cytotoxicity than the nucleoside analogs (5-AzaC and 5-Aza-deoxycytidine) due
683 to a different mechanism of action and higher stability in aqueous media (Flotho et al. 2009,
684 Champion et al. 2015). Nevertheless, the decrease of DNA methylation was not significant following
685 zebularine treatment. Moreover, we observed an increase in the oviposition of snails treated with
686 Zebularine (Table 2). This phenomenon was also observed in snails exposed to the parasite *S.*
687 *mansoni* (Thornhill, Jones, and Kusel 1986), where oviposition is increased during the first days of
688 parasite exposure. This response may be a fecundity compensatory strategy for expected future
689 suppression of egg-laying and it is caused by environmental stress and the toxicity of zebularine
690 possibly triggered this response. Zebularine has demonstrated a transient hypomethylation effect in
691 plants (Baubec et al. 2009), we cannot exclude that the same could happen in *B. glabrata*, since we
692 observed some phenotypic effects, especially in the fecundity, presenting the lowest percentage of
693 hatching rate and a tendency to decrease in the global methylation level. Moreover, zebularine is not
694 a specific inhibitor of DNMTs, it also inhibits cytidine deaminase, an important enzyme in the
695 biosynthesis of nucleotides, and most of the compound can be sequestered by this enzyme and
696 therefore lowering its effective concentration. This is concordant with other studies showing that, in
697 order to have an effective inhibition of DNMTs, a high concentration of this compound was required
698 ($\geq 100 \mu\text{M}$) (Cheng et al. 2004).

699 As a nucleoside analogue, the mechanism of action of zebularine requires its incorporation into DNA
700 after phosphorylation and its conversion to the deoxy-zebularine triphosphate. The new DNMTi
701 tested in this work are non-nucleoside analogues that do not incorporate into DNA being potentially
702 more specific to DNMTs (Gros et al. 2012).

703 **Flavanone-type inhibitor has no toxic effects and reduces 5mC level in two subsequent generations**
704 **associated with variation in morphometric traits**

705 No significant differences were found in the survival and fecundity between Flv1 and its negative
706 analogue (Flv-neg) against control group. Flv1 triggered a significant decrease on 5mC % in F0
707 generation and in the F1 generation. Since we found an inhibitory efficiency of the Flv1 in the
708 mollusks *B. glabrata*, *P. acuta* and *C. gigas*, we decided to test the stability of the flavanone
709 compounds (3-halo-3-nitroflavanones) in freshwater and in sea saltwater that was used to raise our
710 mollusks models. We found differences in the chemical stability of Flv1 between freshwater and sea
711 salt water, the compound was ~3 times more stable in freshwater than it was in sea-salt water
712 (Supplementary file 2, Figure S7), this can explain the results for *C. gigas* (raised in seawater) were
713 diminution of global DNA methylation was lower than in the freshwater snails *P. acuta* and *B.*
714 *glabrata*. Furthermore, we demonstrated an *in vitro* DNMT inhibition activity of the Flv1 compound
715 in a nuclear extract from *Bge* cells. We concluded that for Flv1, 5mC modulation was most likely due
716 to direct inhibition of DNMT activity.

717 We corroborated hypomethylation effect in *B. glabrata* by high-throughput bisulfite sequencing with
718 the epiGBS method, confirming that the average of overall percentage of CpG methylation was
719 significantly lower in Flv1-exposed snails compared to control and the same trend was found in its
720 offspring.

721 A total of 26 DMCs (25 hypomethylated and 1 hypermethylated) were found in the Flv1-exposed
722 snails compared to controls, and its progeny showed 325 DMCs (203 hypomethylated and 120

723 hypermethylated) compared to F1-controls. The higher number of DMCs in the F1 generation, might
724 be due to an indirect exposure of the germline to the inhibitor. In mollusks, germ cells appear early in
725 the embryonic development (Luchtel 1972). It has been demonstrated that exposure of the germline
726 to DNMTi affects epigenetic programming in sperm and oocytes and are likely to affect outcomes and
727 offspring development principally in vertebrates (Western 2018, Prokopuk, Hogg, and Western
728 2018). Additionally, the morphometric traits variation was higher in the Flv1-exposed snails and its
729 offspring. These results are similar to those published in *S. mansoni* (Cosseau et al. 2010), where we
730 found significant differences in the body length of the parasite larvae between control group and
731 group treated with the epimutagenic TSA, a histone deacetylase inhibitor. Both results showed that
732 modification of epigenetic marks by specific drugs can have effects on the phenotype variability of
733 organisms. Indeed, our results in *B. glabrata* goes in line with the idea that the absence of DNA
734 methylation could contribute to stochastic transcriptional opportunities and thus be a way to
735 produce (heritable) phenotypic variability/diversity in mollusks (Roberts and Gavery 2012a).
736 Nevertheless, more work is needed to verify if epimutations at multiple loci are causing the observed
737 phenotypic variability through post-transcriptional or gene expression changes or if phenotypic
738 variability is independent of these induced epimutations. Morphometric traits variation is indicative
739 of growth in mollusks and the heterogeneity of these traits in Flv1-exposed snail's offspring is
740 coherent with our hypothesis that the germline was indirectly exposed to Flv1.

741 Furthermore, one DMR was observed in Flv1 exposed snails and in its progeny, demonstrating a
742 multigenerational effect, resulting from a direct exposure of the germline to the inhibitor. Few
743 examples of multigenerational effect have been reported in mollusks (Fallet et al. 2020), one is our
744 previous study in *C. gigas* showing that a parental herbicide exposure strongly affected the offspring
745 DNA methylation pattern (Rondon et al. 2017). Another example was found in *P. acuta*, where
746 exposure to prednisolone, a steroid hormone evacuated from hospital wastewater, negatively

747 affected the phenotypic traits of the snail, exhibited multigenerational toxicity and affected global
748 DNA methylation of adult progeny (Bal, Kumar, and Nugegoda 2017).

749 The DMR found in both *B. glabrata* generations mapped to the putative promoter region of
750 transcript BGLTMP010125 coding for a thump domain-containing protein 3-like. A protein BLAST
751 (blastp) with the amino acids sequence of this protein showed 66.4% of identity with the THUMP
752 domain containing protein 3-like of *Aplysia californica* (NCBI reference sequence XP_012941090) and
753 52.8% of identity with the THUMP domain protein 3 of the brachiopod *Lingula anatina*
754 (XP_013378720.1), these proteins are part of AdoMet_MTases superfamily, enzymes that use S-
755 adenosyl-L-methionine (SAM or Adomet) as a substrate for methyltransfer, creating the product S-
756 adenosyl-L-homocysteine. TBS in the first intron showed that the Flv1 inhibitor treatment decreased
757 significantly the GBM level in this transcript. qPCR indicted reduced gene expression in Flv1 treated
758 F0. Our results are in agreement with earlier results in the invertebrates *Nematostella vectensis* and
759 *Bombyx mori* (Xiang et al. 2010, Zemach et al. 2010), where a positive linear correlation was found
760 between GBM and mRNA levels.

761 Interestingly, the gene impacted by the inhibitor is coding for a SAM-dependent methyltransferase
762 whose decreased expression could have leverage effects on 5mC level at multiple loci by influencing
763 SAM homeostasis.

764 In conclusion, Flv1 is a good candidate to perform multigenerational DNMTi experiments: it did not
765 impact fecundity neither survival and it induce a DMR found in two consecutive generations. Since
766 DNMTs are a conserved family of cytosine methyltransferases and since we showed that Flv1
767 inhibitor is efficient in another two mollusk species *P. acuta* and *C. gigas*, we conclude that this new
768 DNMTi can be used to pharmacologically modify 5mC level in mollusks species and possibly other
769 invertebrates, providing a tool to study the inheritance of 5mC environmental modifications.

770 In neo-Darwinian theory, genetic variation is considered a pre-requisite for hereditary phenotypic
771 variation and as the primary material of adaptation by natural selection. Nevertheless, it has been
772 demonstrated that the epigenetic inheritance system allows the environmentally induced
773 phenotypes to be transmitted between generations, which can constitute the basis of adaptative
774 phenotypic plasticity (Jablonka and Lamb 1999, Jablonka and Lamb 1998). Moreover, epigenetic
775 changes can be behind rapid adaptive changes observed in scenarios such as climate change,
776 biological invasions and coevolutionary interactions. However, we need to disentangle the epigenetic
777 variation from the genetic one and for that we need approaches that allow us to decrease genetic
778 background and introduce epigenetic changes.

779 Our results hint at epimutations being a source of phenotypic variance that can be induced by
780 chemicals that disrupt normal mechanisms of methylation control. And this disruption may act on
781 the germline, with phenotypic expression in the form of heightened phenotypic and epigenetic
782 variance in the next generation. But we have no proof that variation in methylation patterns are the
783 only source of the variance in the phenotype found in F0 and F1 generations and we cannot formally
784 exclude concomitant genetic variation. However, it can now be envisaged to use our new Flv1 DNMTi
785 to induce epimutations in inbred self-fertilization lines and cross epimutant snails with contrasting
786 epigenomes (*e.g.* hypomethylated vs hypermethylated snails) allowing to create epigenetic
787 recombinant inbred lines (epiRILs). In this way one can evaluate if, in the absence of genetic
788 variation, epimutations and phenotypic variation induced in the exposed generations are transmitted
789 across multiple generations and produce phenotypes having a selective advantage.

790

791 **Figure and table legends.**

792 **FIGURE 1.** 5mC % of *B. glabrata* snails upon DNMTi treatments at a concentration of 10 μ M, error
793 bars represent SD, n=30 per treatment.

794 **FIGURE 2.** Kaplan-Meier survival curves upon treatment with the two types of DNMTi.

795 **FIGURE 3.** PCA of morphometric traits of all treatments of F0 (a) and F1 generation (b).

796 **FIGURE 4.** Histograms of CpG methylation distribution, (a) histogram of F0-control epiGBS libraries
797 (b) histogram of F1-control epiGBS libraries and (c) histogram of WGBS library.

798 **FIGURE 5.** Screenshot of IGV of the region LGUN_random_Scaffold28: 1-800 Kb, that showed the
799 regions covered by epiGBS libraries (in yellow) versus the regions covered by WGBS library (blue).

800 **FIGURE 6.** CpG methylation ratio profile across the bodies of genes and quantiles distribution of
801 epiGBS and WGBS libraries

802 **FIGURE 7.** PCA of CpG methylation of the Flv1-treated and control groups in F0 generation (a) and in
803 its offspring (b).

804 **Figure 8.** IGV screenshot of the LGUN_random_scaffold4962:16175-16266 of *B. glabrata* genome
805 assembly (GCA_000457365.1).

806 **Figure 9.** (a) Position and level of methylation of the five CpG positions, which has been studied by
807 TBS within the first intron of the transcript BGLTMP010125-RA. (b) Relative expression of the
808 transcript BGLTMP010125-RA compared to two housekeeping genes (28S and α -Tubulin), the
809 ordinate show the logarithm of the values obtained with the $2^{-\Delta\Delta CT}$ method.

810 **FIGURE 10.** (a) 5mC % measures obtained by the dot blot method for *P. acuta* and *C. gigas*. (b) 5mC
811 % measures obtained by the ELISA-based assay.

812 **TABLE 1.** *Biomphalaria glabarata* gene-specific primers used to amplified gene fragments used in the
813 RT-qPCR.

814 **TABLE 2.** Contingency table of fecundity of the snails exposed to different DNMTi.

815 **TABLE 3.** Mapping efficiencies, CpG coverage and average genome-wide methylation levels resulting
816 from epiGBS and WGBS libraries.

817 **TABLE 4.** Percentage of CpG methylation sites which display an unmethylated or complete
818 methylated pattern.

819 **Table 5.** DMCs in Flv1-treated and control group for each generation.

820 **TABLE6.** 5mC % per CpG sites in the bisulfite converted sequence of the transcript BGLTMP010125.
821

822 **Abbreviations**

823 6-FAM: 6-Carboxyfluorescein

824 THUMP: THioUridine synthases, RNA Methyltransferases and Pseudo-uridine synthases

825 AdoMeth or SAM: S-adenosyl-L-methionine

826 5mC: 5-methylcytosine

827 DNMTs: DNA methyltransferases

828 DNMTi: DNA methyltransferase inhibitors

829 5-AzaC: 5-azacytidine

830 BgBRE: *Biomphalaria glabrata* strain Brazil BRE

831 Bge: *Biomphalaria glabrata* embryonic

832 **Ethics approval and consent to participate**

833 The Direction Départementale de la Cohésion Sociale et de la Protection des Populations (DDSCPP)

834 provided the permit N°C66-136-01 to IHPE for experiments on animals.

835 **Consent for publication**

836 All authors read and endorsed the manuscript.

837 **Availability of data and materials**

838 All Flv substrates are available on request. Raw data is available at NCBI SRA XXXXXX (provided on

839 accepted version)

840 **Competing interests**

841 The authors declare no conflict of interest.

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849 **Authors' contributions**

850 CG and CC designed the study, analyze data and writing the manuscript. NL performed the
851 experimental work, processed and analyses data and wrote the manuscript. ML and PBA synthesized
852 the chemical compounds used in this study, elaborate the chemical stability and *in vitro* inhibition
853 assays and participate in the writing of the manuscript. SI helped in the elaboration of epiGBS
854 libraries. KV and FG help in the analysis of epiGBS sequencing reads and participate in the writing of
855 the manuscript. PD participated in the experimental design and in the writing of the manuscript. CrC
856 help with the bioinformatics analysis.

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865

866

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