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

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Abstract

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

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

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

Background

DNA methylation is an epigenetic mark that can be associated with changes in gene function without changes in the underlying DNA sequence (Ganesan, Arimondo, Rots, Jeronimo, & Berdasco, 2019). Modifications in DNA methylation can be induced by the environment and some changes can be mitotically and/or meiotically heritable and/or are reversible (Dupont, Armant, and Brenner 2009, Nicoglou and Merlin 2017). Some of these modifications can influence gene function by providing differential access to the underlying genetic information in cells, and thus may alter their phenotypes. Epigenetic marks such as DNA methylation may provide an additional dimension to inheritance, linked to but different from genetic inheritance. Epimutations can be provoked directly by environmental stresses and contribute to rapid evolutionary changes but unlike genetic variation, epimutations have higher rates and are reversible (Bossdorf, Richards, and Pigliucci 2008, Cosseau et al. 2017). Biochemically, DNA methylation is the modification of a DNA base, and is present in a diverse range of eukaryotic organisms, ranging from *fungi* to mammals (Chen 2011). One type of DNA methylation is cytosine methylation that is catalyzed by the DNA methyltransferases (DNMTs), enzymes that transfer the methyl group (-CH_3) from the co-substrate *S*-adenosyl-L-methionine (SAM) to the carbon-5 of the cytidine, to form 5-methylcytidine (5mC) (Moore, Le, and Fan 2013). In vertebrates, DNA methylation occurs on cytosines in a CpG context (cytidine followed by a guanosine) (Li and Zhang 2014) whereas, DNA methylation can also occur in CHH and CHG (H=A, T, C) context (Meng et al. 2015) in plants. Less is known about the methylation in invertebrates, though many species present DNA methylation in a CpG context (Glastad et al. 2011).

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

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

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

The snail *B. glabrata* is the intermediate host of *Schistosoma mansoni*, the causative agent of schistosomiasis, a parasitic disease affecting 200 million people in 78 countries (McManus, 2019). The interaction of these species is characterized by a phenomenon called compatibility polymorphism, meaning that some snail phenotypes can be infected by a specific parasite phenotype while others cannot (Theron et al. 2014). It has been demonstrated that epigenetic alterations are involved in the *B. glabrata* parasite compatibility phenotype (Knight et al. 2016), even though contrasting results have been obtained by others (Sullivan 2018, Allan et al. 2020). It remains, therefore, an open question whether epigenetic mechanisms play a role in the capacity of *B. glabrata* to produce phenotypic plasticity or variability. DNA methylation machinery components in *B. glabrata* include a maintenance DNMT (*Bg*DNMT1), a DNA/tRNA methyltransferase (*Bg*DNMT2) and a methyl-CpG-binding domain protein (*Bg*MBD2/3), *Bg*DNMT1 and *Bg*DNMT2 being probably responsible for the 5mC modifications (Fneich et al. 2013, Geyer et al. 2017).

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

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

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

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

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

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

Methods

Ethics statement

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

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

Three types of DNMT inhibitors were tested in the snail *B. glabrata*, the cytidine analogue zebularine (Sigma, France, Cat. No. 3690-10-6) and custom-made inhibitors, previously selected for their inhibitory activity against *h*DNMT1 and *h*DNMT3-c (Ceccaldi et al. 2011, , Pechalrieu et al. 2020).

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

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

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

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

Genomic DNA extraction

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

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

DNA methylation screening

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

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

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

ELISA-based 5mC quantification

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

Statistical analyses

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

5mC % = [sample densitometry/ng]／[positive control densitometry/ng] × Positive control 5mC %

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

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

Library preparation and high throughput bisulfite sequencing

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

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

Bioinformatics epiGBS pipeline

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

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

Bisulfite conversion

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

Nested PCR amplification of bisulfite converted DNA

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

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

Dual DNA and RNA extraction and RT-qPCR

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

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

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

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

Nuclear fraction extraction

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

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

Chemical stability measurement

The flavanone compounds stability was measured by High Performance Liquid Chromatography (HPLC) by the method described in Pechalrieu *et al.*, 2020. HPLC analysis were done using an X-terra column (100 × 4.6 mm; 5 µm) with 1 mL/min flow and the following gradient: H_2O acetonitrile 95:5 for 2 min then up to 0:100 in 10 min and maintained at 0:100 for 2 min with H_2O and acetonitrile containing 0.1% of trifluoroacetic acid. First, flavanone compounds (Flv1, Flv2 and Flv-neg) were injected in solution at 100 µM in 100% DMSO to check its purity. Then 50 µL of solution at 10 µM of tested compound was prepared by dilution in DNMT3A-c enzyme buffer (Hepes 20 mM pH 7.2, KCl 50 mM, EDTA 1 mM final concentration), in freshwater used in the aquariums of *B. glabrata* or in filtered sea water used in the aquariums of *C. gigas*. The percentages of remaining compound were determined with the area of the corresponding HPLC peak on the 250 nm chromatogram.

DNMT inhibition assays

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

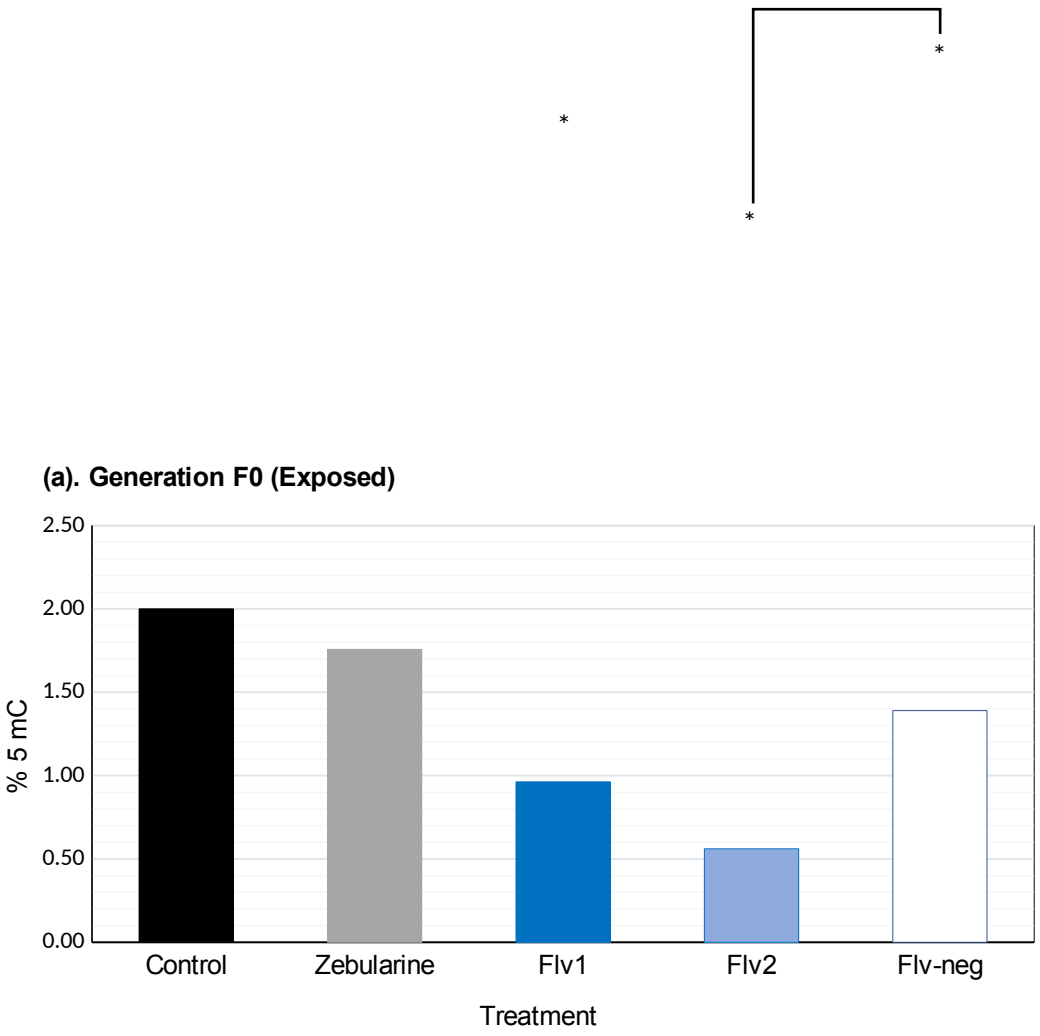
Methylation activities are defined as $[(X_{meth}-X_{restri})/(X_{DNA}-X_{restri})]x100$, where X_{meth}, X_{restri} and X_{DNA} are respectively the fluorescence signals of the compound methylation, restriction and DNA controls.

Results

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

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

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



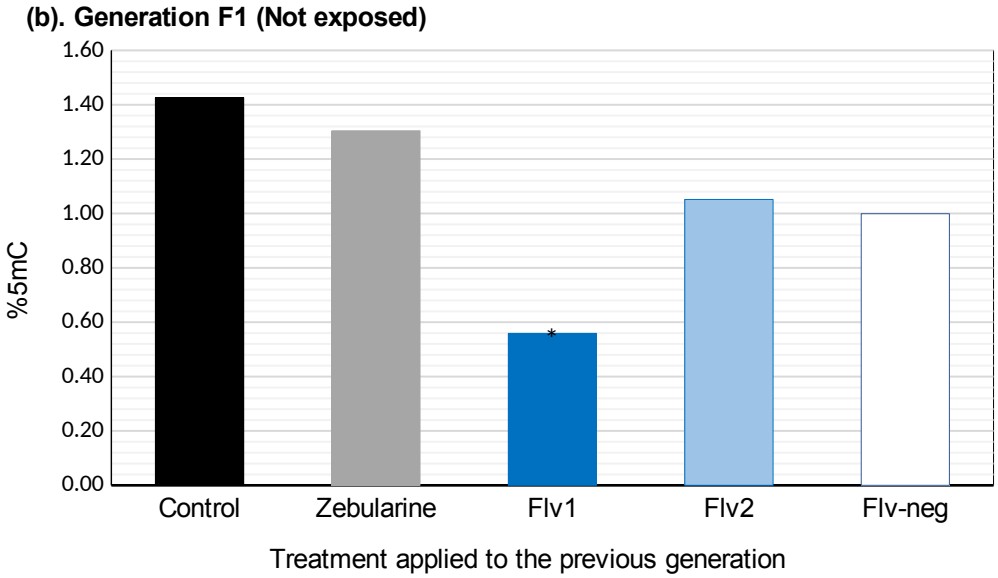


FIGURE 1. 5mC % of *B. glabrata* snails upon DNMTi treatments at a concentration of 10 µM, error bars represent SD, n=30 per treatment. (a) 5mC % in the F0 generation (exposed), black bar for control, grey bar for zebularine, blue bars represent the flavanone inhibitors (blue bar for Flv1 and light blue bar for Flv2) and the white bar with blue outline represent the inactive Flv-neg. (b) 5mC % in the non-exposed F1 generation. Compounds are the ones used in F0. Mann-Whitney Wilcoxon test was applied, if not otherwise indicated, between treatment and control significant differences are marked as * for p<0. 0001. 5mC ng was normalized to the 5mC global percentage present in the genome of *B. glabrata*.

Flv1 blocks DNMT activity *in vitro*

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

DNMTi influence survival, fecundity and morphometric traits

Since our findings had clearly indicated that Flv1 had an *in-vivo* and *in-vitro* demethylating activity *i.e.* probably due to a direct effect on the DNMT we wondered if this epimutagenic activity had phenotypic consequences. Therefore, we measured survival, fecundity and morphometric traits in the DNMT-treated snails. We used as pharmacological reference molecule zebularine. Zebularine induced the lowest mortality with no significant difference compared to control group (Mantel-Cox test $\chi2=0.3$, $p=0.56$). This compound followed a similar trend as the control and the snail final survival rate reached 80% compared to 84% in the control. (Figure 2a). The mollusks treated with Flv1, Flv2 and the inactive Flv-neg had a survival rate of 68%, 73%, and 69%, respectively (Figure 2b), and none of these rates were statistically different compared to the control group ($\chi2=3.5$, $p=0.06$ for Flv1, $\chi2=1.16$, $p=0.2$ for Flv2 and $\chi2=5.9$, $p=0.1$ for Flv-neg).

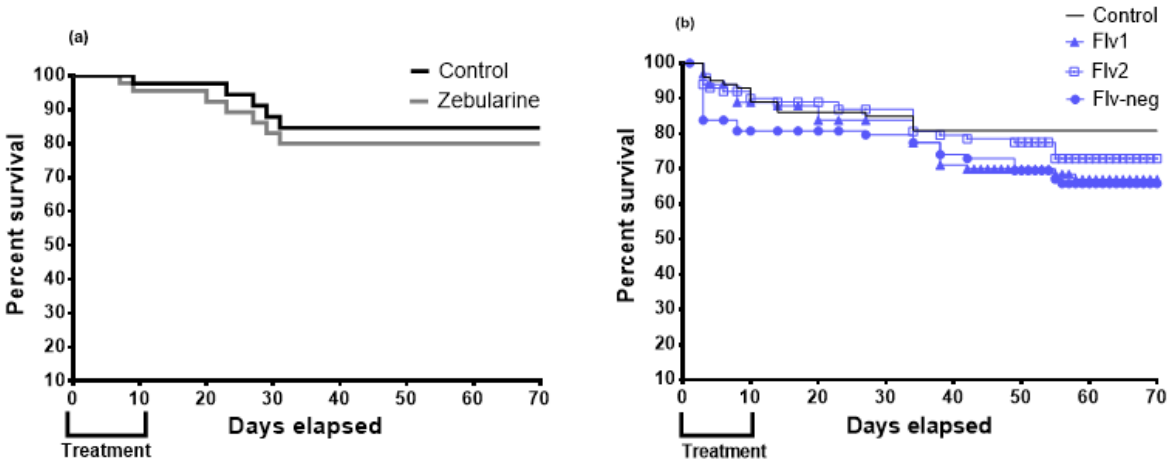


FIGURE 2. Kaplan-Meier survival curves upon treatment with the two types of DNMTi. (a) Cytidine-analogue zebularine (grey line). (b) Flavanones Flv1 (blue line with triangles) and Flv2 (blue line with squares) and their inactive derivative Flv-neg (blue line with circles).

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

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

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

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

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

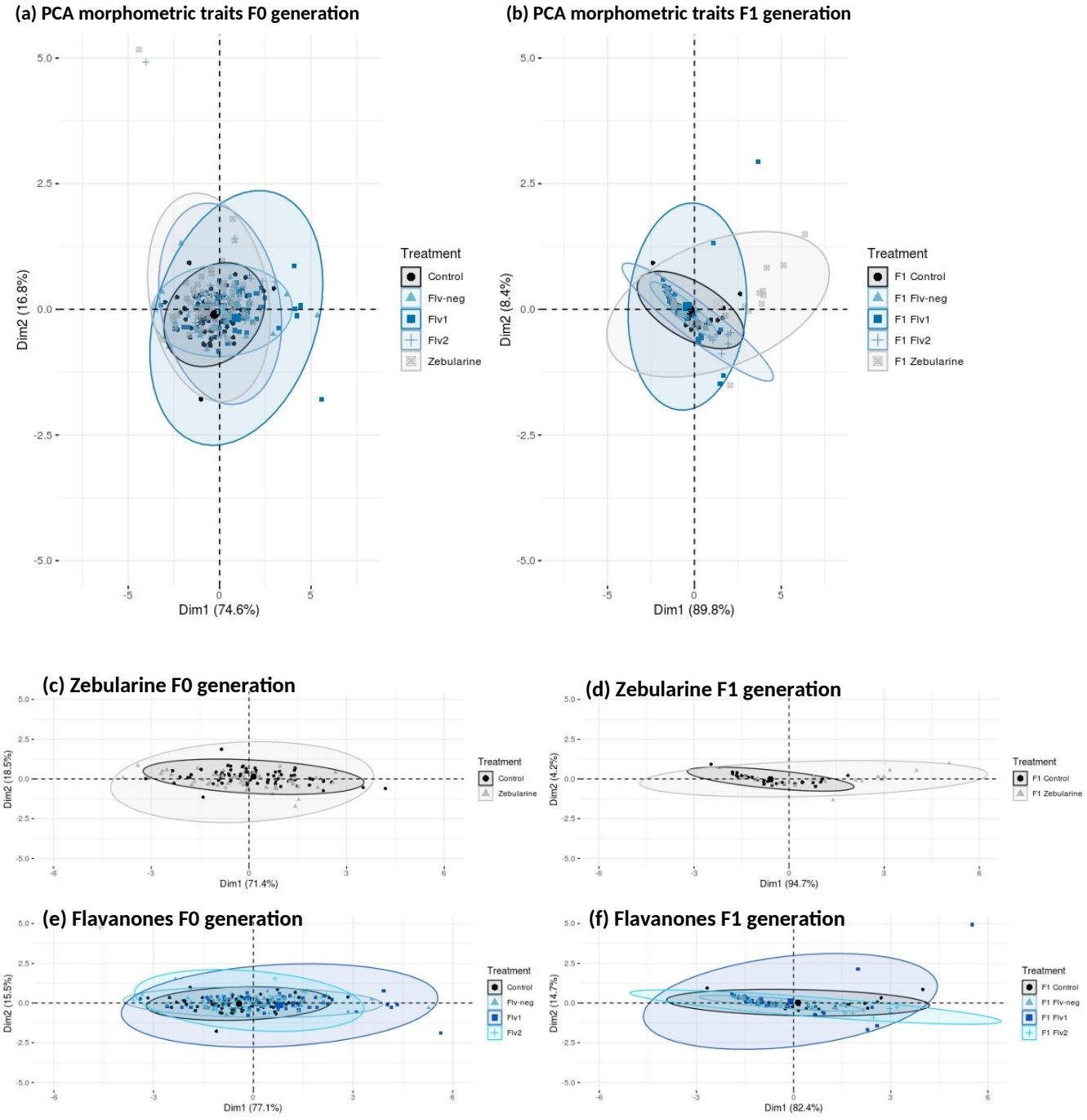
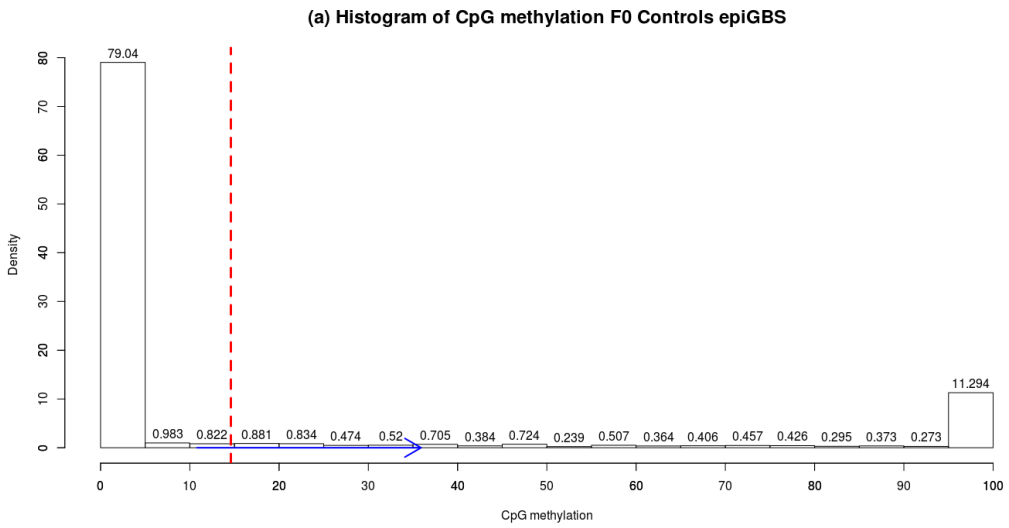


FIGURE 3. PCA of morphometric traits of all treatments of F0 (a) and F1 generation (b). PCA analyses splitted by treatment, (c) PCA of morphometric traits after zebularine treatment and (d) its offspring (e) PCA of morphometric traits of snails exposed to flavanone derivatives F0 generation and (f) the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

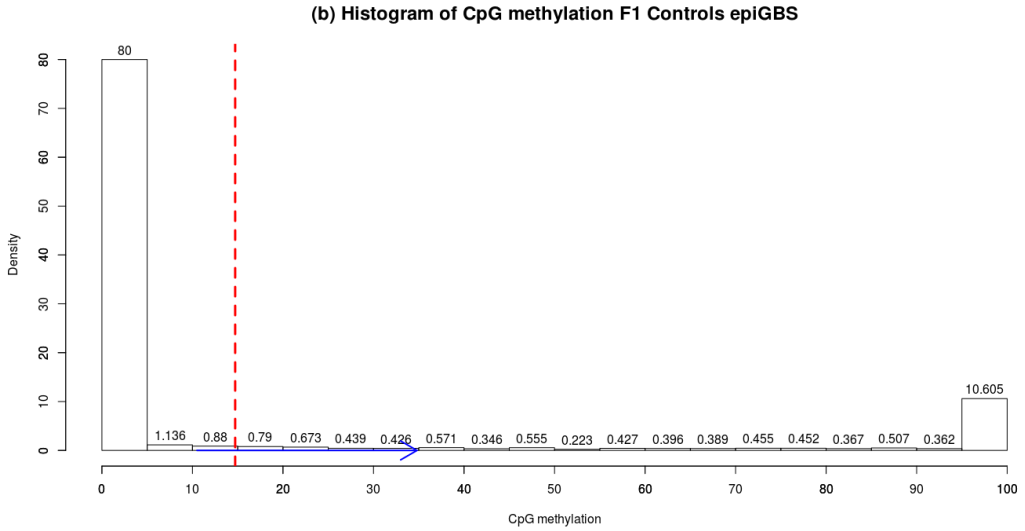
epiGBS reduces sequencing effort roughly 10x but allows for reliable evaluation of global 5mC level and identification of differentially methylated sites and regions

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

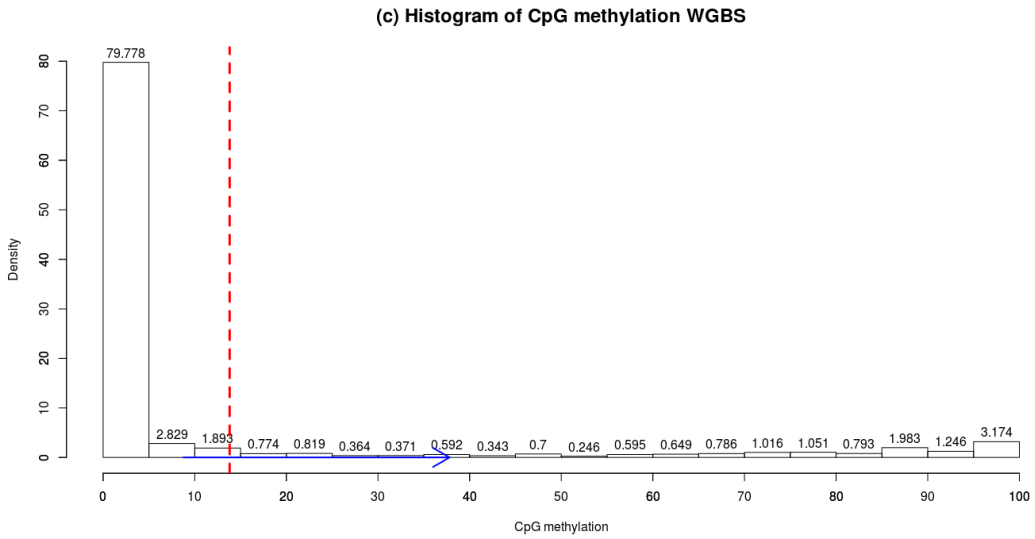
To analyze the distribution of methylated CpG over the entire genome we represented its frequency distribution. We found a characteristic distribution of two peaks for both WGBS and epiGBS indicating the majority of the CpG sites being either unmethylated or completely methylated, as expected for a species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


(a) Histogram of CpG methylation F0 Controls epiGBS
Density
CpG methylation
79.04
0.983
0.822
0.881
0.834
0.474
0.52
0.705
0.384
0.724
0.239
0.507
0.364
0.406
0.457
0.426
0.295
0.373
0.273
11.294

487


(b) Histogram of CpG methylation F1 Controls epiGBS
Density
CpG methylation
80
1.136
0.88
0.79
0.673
0.439
0.426
0.571
0.346
0.555
0.223
0.427
0.396
0.389
0.455
0.452
0.367
0.507
0.362
10.605

488


(c) Histogram of CpG methylation WGBS
Density
CpG methylation
79.778
2.829
1.893
0.774
0.819
0.364
0.371
0.592
0.343
0.7
0.246
0.595
0.649
0.786
1.016
1.051
0.793
1.983
1.246
3.174

489

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

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

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

Samples	*Mapping efficiency%*	*Sequence reads*	*Total no. CpGs*	*CpGs ≥ 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

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

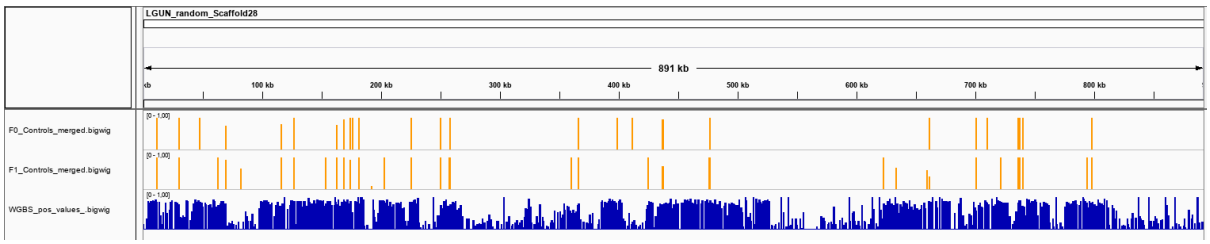


FIGURE 5. Screenshot of IGV of the region LGUN_random_Scaffold28: 1-800 Kb, that showed the regions covered by epiGBS libraries (in yellow) versus the regions covered by WGBS library (blue).

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

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

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

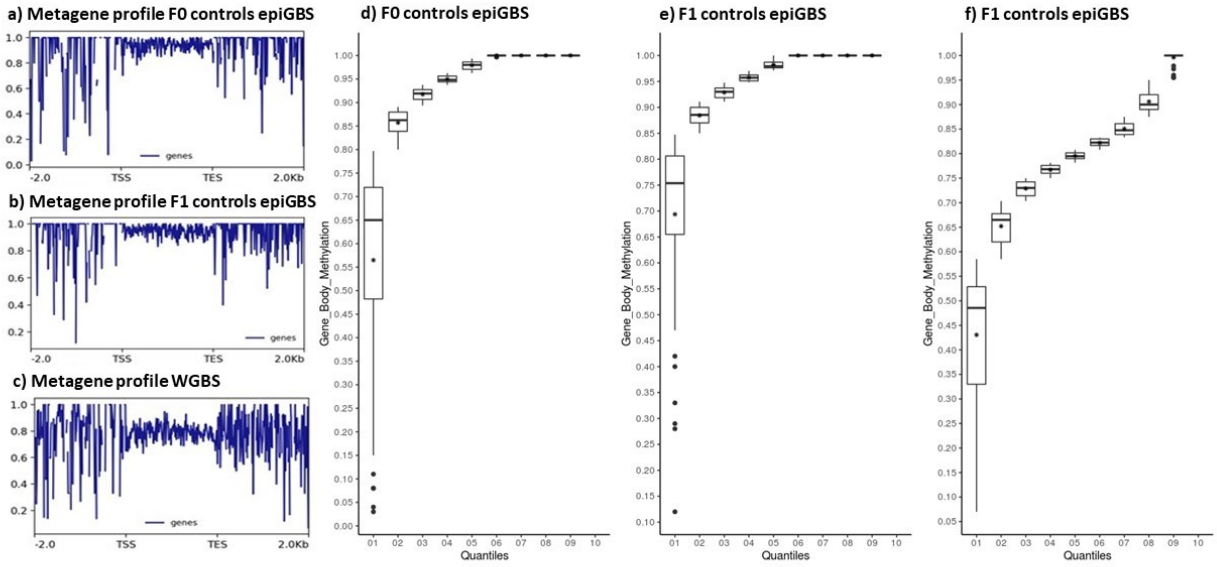


FIGURE 6. CpG methylation ratio profile across the bodies of genes and quantiles distribution of epiGBS and WGBS libraries. (a) Metagene profile of CpG methylation ratio of F0 control epiGBS libraries, (b) F1-control epiGBS libraries and (c) WGBS library. −2.0 kb indicates the upstream 2,000 bp of TSS,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

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

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

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

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

Generation F0	CpG methylation frequency		Generation F1	CpG methylation frequency	
Samples	Unmethylated %	Completely methylated %	Samples	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

epiGBS corroborates multigenerational hypomethylation by Flv1

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

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

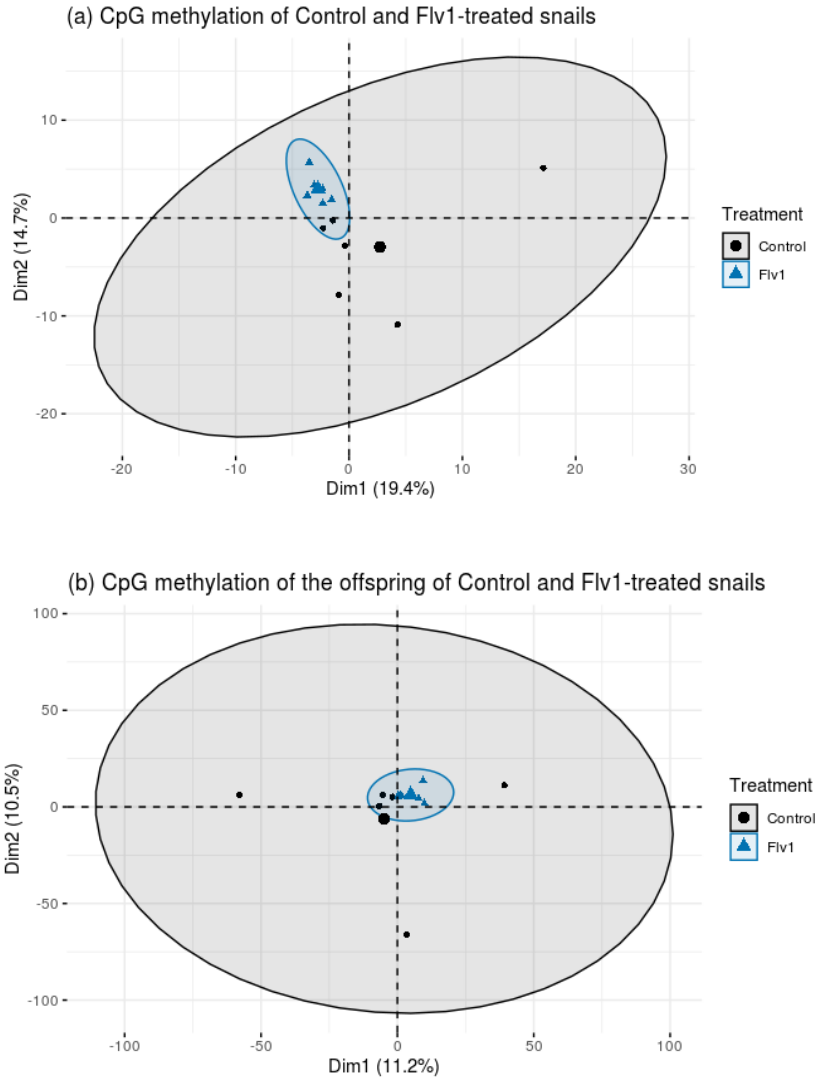


FIGURE 7. PCA of CpG methylation of the Flv1-treated and control groups in F0 generation (a) and in its offspring (b). The ellipses represent the 95% confidence interval.

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

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

Table 5. DMCs in Flv1-treated and control group for each generation. The parameters to calculate 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

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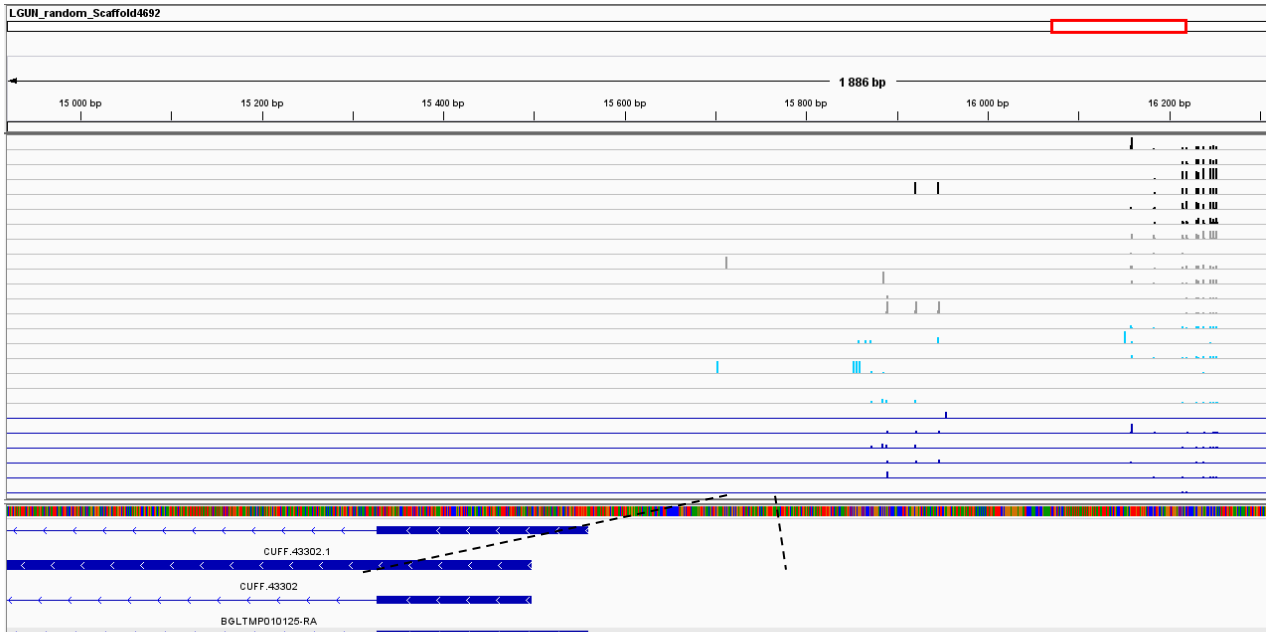


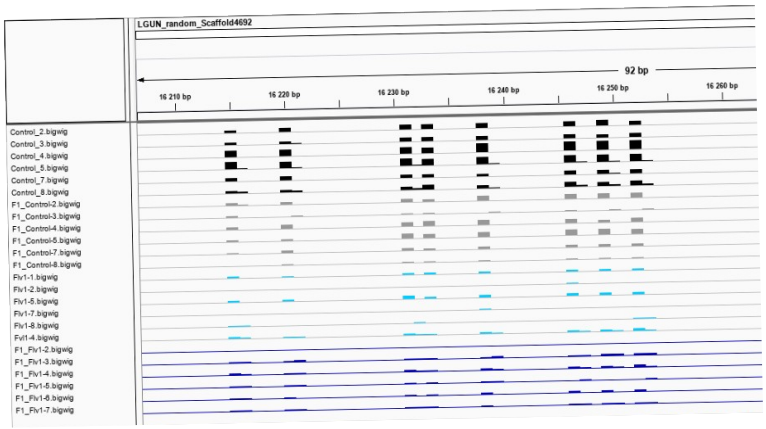


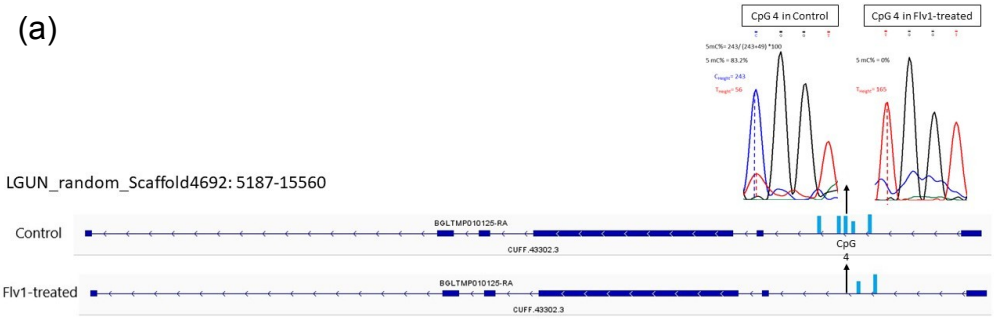
Figure 8. IGV screenshot of the LGUN_random_scaffold4962:16175-16266 of *B. glabrata* genome assembly (GCA_000457365.1). Each bar indicates the position of a methylated CpG site for the different samples: F0-control F0 (black), F1-control (gray), Flv1-treated samples (light blue) and its offspring (dark blue). Hypomethylated DMCs have been detected in this region and are in common between F0 and F1 generation.

Gene BGLTMP010125 that is hypomethylated by Flv1 shows decreased transcription

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

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

CpG sites	*CpG 1*	*CpG 2*	*CpG 3*	*CpG 4*	*CpG 5*	*CpG 6*	*CpG 7*	*CpG 8*	*CpG 9*
Position on Contig LGUN_random_Scaffold4692	*13866*	*13976*	*14024*	*14042*	*14059*	*14061*	*14317*	*14331*	*14343*
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		804		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



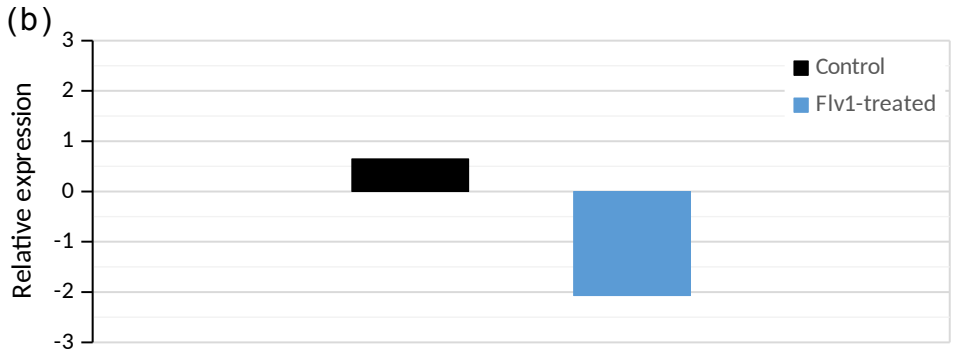


Figure 9. (a)Position and level of methylation of the five CpG positions, which has been studied by TBS within the first intron of the transcript BGLTMP010125-RA. (b) Relative expression of the transcript BGLTMP010125-RA compared to two housekeeping genes (28S and α-Tubulin), the ordinate show the logarithm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

Flv1 reduces global 5mC in other mollusks

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

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

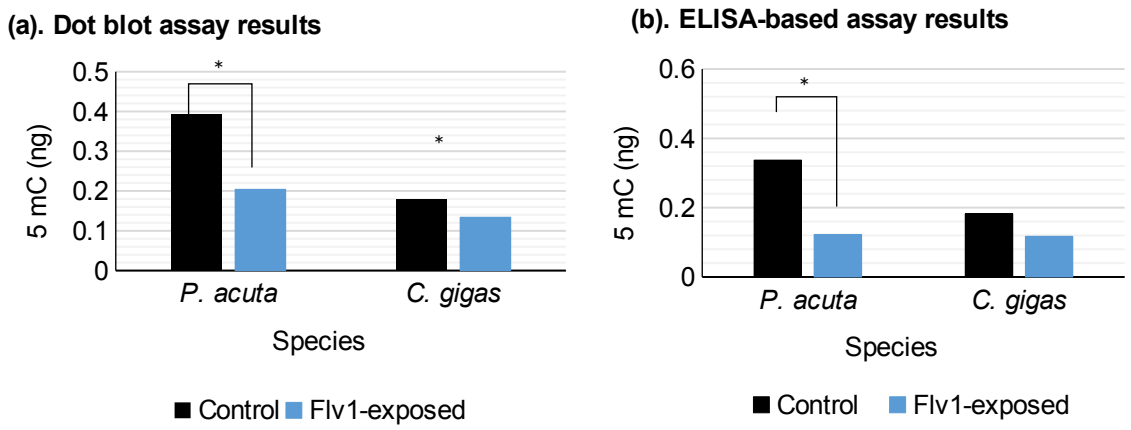


FIGURE 10. (a) 5mC % measures obtained by the dot blot method for *P. acuta* and *C. gigas*. (b) 5mC % measures obtained by the ELISA-based assay. The bars represent the 5mC (ng) mean, the error bars represent the standard deviation (SD), n = 30 per group per specie for dot blot and n = 10 for ELISA. Significant differences between treatment and control are marked as * for p<0. 05.

Discussion

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

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 synthetized 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 (≥100 µM) (Cheng et al. 2004).

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

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

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

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

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

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

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

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

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

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

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

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

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

Figure and table legends.

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

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

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

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

FIGURE 5. Screenshot of IGV of the region LGUN_random_Scaffold28: 1-800 Kb, that showed the regions covered by epiGBS libraries (in yellow) versus the regions covered by WGBS library (blue).
FIGURE 6. CpG methylation ratio profile across the bodies of genes and quantiles distribution of epiGBS and WGBS libraries

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

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

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

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

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

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

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

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

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

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

Abbreviations

6-FAM: 6-Carboxyfluorescein

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

AdoMeth or SAM: S-adenosyl-L-methionine

5mC: 5-methylcytosine

DNMTs: DNA methyltransferases

DNMTi: DNA methyltransferase inhibitors

5-AzaC: 5-azacytidine

BgBRE: *Biomphalaria glabrata* strain Brazil BRE

Bge: Biomphalaria glabrata embryonic

Ethics approval and consent to participate

The Direction Départementale de la Cohésion Sociale et de la Protection des Populations (DDSCPP) provided the permit N°C66-136-01 to IHPE for experiments on animals.

Consent for publication

All authors read and endorsed the manuscript.

Availability of data and materials

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

Competing interests

The authors declare no conflict of interest.

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Authors’ contributions

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

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