The DNA methylation of the snail Biomphalaria glabrata, role and impact on the generation of phenotypic plasticity

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Abstract

The understanding of the molecular mechanisms that allows the rapid adaptation of mollusks that are vector of parasites, to new environments is important for disease control. Rapid adaptation is difficult to explain by traditional Mendelian genetics and there is strong evidence supporting that epigenetic mechanisms, are behind rapid adaptations in other species. I studied one epigenetic mark called DNA methylation that has demonstrated to be environmentally modulated and to play a role in phenotypic plasticity in many species, principally plants and vertebrates. Nevertheless, the role of DNA methylation in generating phenotypic variation in invertebrates has been poorly studied. I addressed the question of the role of DNA methylation in the generation of phenotypic plasticity and its heritability in the snail *Biomphalaria glabrata*, the intermediate host of the parasite *Schistosoma mansoni*, the causal agent of schistosomiasis, a neglected tropical disease. DNA methylation in *B. glabrata* has been found to be modulated by the infection of the parasite *S. mansoni* and by environmental stress, furthermore, it was demonstrated that DNA methylation affects its gene expression, suggesting that DNA methylation can affect phenotypic variation and therefore the adaptation of the snail to new environments. To study the role of DNA methylation in the generation of phenotypic variation, experimental manipulation of the DNA methylation in the snail was necessary. Therefore, two approaches were proposed in this thesis to introduce epimutations in the snail *B. glabrata*: 1) Random epi-mutagenesis using chemical DNA methyltransferase (DNMT) inhibitors and by consequent segregation of epimutations in self-fertilization lines and 2) Methylate the cytosines of a targeted locus with a targeted epigenome editing tool consisting in the use of the DNA methyltransferase (DNMT3) construct fused to the nuclease-inactivated dCas9. For the random epi-mutagenesis approach, a novel DNMT inhibitor has shown methylation inhibiting effects in two subsequent generations, showing a

multigenerational epigenetic effect and without showing toxic effects in either survival nor fecundity of the snail *B. glabrata*. In addition, the inhibitor Flv1 has been shown to be effective in other two mollusk species, the freshwater snail *Physa acuta* and the pacific oyster *Crassostrea gigas*, which suggests that this inhibitor represents a molecular tool to modulate the methylation of DNA in other mollusks. In the case of the targeted epimutagenesis approach, I used a transfection method that allows introducing two plasmid vectors with an SV40 viral promoter *in vivo* in embryos of the snail *B. glabrata*. The transfection was performed at the gastrula stage, which resulted in mosaic incorporation of the vector into the transfected cells. However, the method was able to methylate some CpG sites of the targeted gene.

Résumé

La compréhension des mécanismes moléculaires qui permettent l'adaptation rapide des mollusques vecteurs de parasites à de nouveaux environnements est importante pour le contrôle des maladies. L'adaptation rapide est difficile à expliquer par la génétique mendélienne traditionnelle et il existe des preuves solides qui soutiennent que les mécanismes épigénétiques sont à l'origine des adaptations rapides chez plusieurs espèces. Je me suis focalisée sur une marque épigénétique appelée la méthylation de l'ADN, qui est modulée par l'environnement et joue un rôle dans la plasticité phénotypique chez de nombreuses espèces, principalement les plantes et les vertébrés. Néanmoins, le rôle de la méthylation de l'ADN dans la génération de variations phénotypiques chez les invertébrés a été très peu étudié. J'ai abordé la question du rôle de la méthylation de l'ADN dans la génération de la plasticité phénotypique et de son héritabilité chez l'escargot *B. glabrata*, l'hôte intermédiaire du parasite *Schistosoma mansoni*, l'agent pathogène de la schistosomiase, une maladie tropicale négligée. La méthylation de l'ADN chez *B. glabrata* est régulée par l'infection

du parasite *S. mansoni* et par le stress environnemental, de plus, il a été démontré que la méthylation de l'ADN affecte son expression génique, suggérant que la méthylation de l'ADN peut affecter la variation phénotypique et donc l'adaptation de l'escargot à de nouveaux environnements. Pour étudier le rôle de la méthylation de l'ADN dans la génération de la variation phénotypique, une manipulation expérimentale de la méthylation de l'ADN chez l'escargot était nécessaire. Par conséquent, deux approches ont été proposées dans cette thèse pour introduire des épimutations chez l'escargot *B. glabrata*: 1) Épi-mutagenèse aléatoire en utilisant des inhibiteurs chimiques des enzymes ADN methyltransferases (DNMT) et par ségrégation conséquente des épimutations dans des lignées d'autofécondation et 2) Par la méthylation des cytosines d'un locus ciblé avec un outil d'édition épigénétique qui consiste à l'utilisation d'une vecteur plasmidique codant pour l'ADN méthyltranférase (DNMT3) fusionnée avec l'enzyme dCas9 (Cas9 avec l'activité nucléase désactivé). Pour l'approche d'épimutagenèse aleatoire, un nouvel inhibiteur des enzymes DNMT a montré des effets d'inhibition de la méthylation dans deux générations consécutives, en montrant un effet épigénétique multigénérationnelle et sans montrer d'effet toxique ni dans la survie ni dans la fécondité de l'escargot *B. glabrata*. De plus l'inhibiteur Flv1 a montré être efficace dans deux autres espèces de mollusques, l'escargot d'eau douce *Physa acuta* et l'huître creuse *Crassostrea gigas*, ce qui suggère que cet inhibiteur représente un potentiel outil moléculaire pour moduler la méthylation de l'ADN chez d'autres mollusques. Dans le cas de l'approche ciblée, j'ai utilisé une méthode de transfection qui permet d'introduire deux vecteurs plasmidiques avec un promoteur viral SV40 de façon *in vivo* dans des embryons de l'escargot *B. glabrata*. La transfection a été effectuée au stade gastrula, ce qui a entrainé une incorporation mosaïque du vecteur dans les cellules transfectées. Toutefois, la méthode a permis de méthyler certains sites CpG du gène ciblé.

The DNA methylation of *Biomphalaria glabrata*, role and impact on the generation of phenotypic plasticity

General introduction

I. Phenotypic plasticity

Phenotypic variation is the total variation of a trait in a population regardless the cause (genetics, environment, etc.). Ecologists and evolutionary biologist study phenotypic variation across fluctuating environments or between individuals from different populations (Coleman, McConnaughay et al. 1994). When phenotypic variation is caused in response to an environmental stimulus it is called phenotypic plasticity (Pigliucci 2001). Therefore, phenotypic plasticity, is defined as the capability of a genotype to express different phenotypes as a consequence of an exposure to different environments, those different phenotypes can be changes in morphology, physiological state, biochemistry, behavior or life-history traits (Pigliucci 2001).

Plasticity is a characteristic of all living beings; evidence of plasticity is found among all taxa, from phage, bacteriophages and bacteria to plants and animals (Willmore and Hallgrímsson 2005). Though phenotypic plasticity, the phenotype of individuals can be altered without changing the genetic variation of the population, and these phenotypic changes can be adaptive or not (Grenier, Barre et al. 2016). In the case of an adaptive phenotypic plasticity, this last can influence the fitness of individuals contributing to the evolution of organisms by being the target of selection (Grenier, Barre et al. 2016). In the case of non-adaptive plasticity, this last is the product of the physicochemical nature of organisms, all organisms are sensitive to temperature, pressure, pH, etc. and individuals that do not buffer against those environmental changes will present phenotypic plasticity, this type of plasticity is limited by the organism's chemistry (Ghalambor, McKay et al. 2007). Non adaptive phenotypic plasticity causes phenotypes that are *"away from the local optimum"* and that have a negative association with the direction of adaptive evolution (Ghalambor, McKay et al. 2007). Non adaptive phenotypic plasticity can have temporary impacts in the life traits, a typical example is the toxicity induced by a pollutant which result in negative effects in growth, survival and fecundity (Levis, Schooler et al. 2016). Adaptive and non-adaptive plastic phenotypes could be randomly expected but traits displaying adaptive plasticity would be

under feebler directional selection than the non-adaptive plasticity (Ghalambor, McKay et al. 2007).

Different concepts of phenotypic plasticity are found in the literature and they can be confounded, one is that the phenotypic plasticity is a quantitative trait of a given genotype that can be the object of selection and evolve (Scheiner 1993, DeWitt and Scheiner 2004) and the other concept describes it as a developmental process that can facilitate evolution (Pigliucci, Murren et al. 2006, West-Eberhard 2008). The first concept attends to respond to how the phenotypic plasticity can evolve, while the second one attends to understand the role of phenotypic plasticity as an agent of evolution (Gibert 2012). In this introduction I will focus on the first concept. The assessment of phenotypic variability is crucial for study the ecological and evolutionary consequences of the adaptive character of phenotypic plasticity (Coleman, McConnaughay et al. 1994).

1. Plasticity and reaction norms

The overall effect of the environment on phenotypic variation is denoted as phenotypic plasticity, which includes an absence of effect and norm of reaction refers to a particular form of environmental effect (Pigliucci, Murren et al. 2006). The norm of reaction is the representation of a curve that connects a genotype with the phenotypic variation through the contribution of environmental variation, allowing the illustration of phenotypic plasticity (Pigliucci 2001). The reaction norm measures environmental sensitivity as a continuously variable response, discrete plasticity such as binary traits (e.g. queen and worker morphs in ants) cannot be described with reaction norms (Scheiner 1993, West-Eberhard 2008).

One genotype can produce a range of phenotypes depending on the environment encountered. The reaction norm is represented in a plot in which the environmental parameter is on x-axis and the measure of the phenotype on the y-axis. The line traced in the diagram represents the reaction norm (Figure 1a) and the dashed line is the average phenotypic value of the genotype across environments. Observing at an illustration of a reaction norm give us an idea of how sensitive a trait is to changes in the environment (Figure 1b): if we don't see a relation between a trait and the environment (a slope is not observed) this trait is not plastic and if we see a slope it means that the trait changes in function of the environment encountered and that the trait is plastic. The slope expresses the degree of plasticity if the slope is steep, the organisms measured are very sensitive

to the environment, if its the slope is less pronounced, so the plasticity is lower. Genotypic means and plasticity depend on the range of environments used in an experiment. If we submit a genotype to different environments (Figure 1c, d and e) the reaction norms will be different. The absence of association between the plasticity and the mean of a curve suggests that plasticity is genetically independent (Figure 1c). Genetic variation for plasticity is inferred when different genotypes have different curvature and slopes (Figure 1e) (Pigliucci 2001).

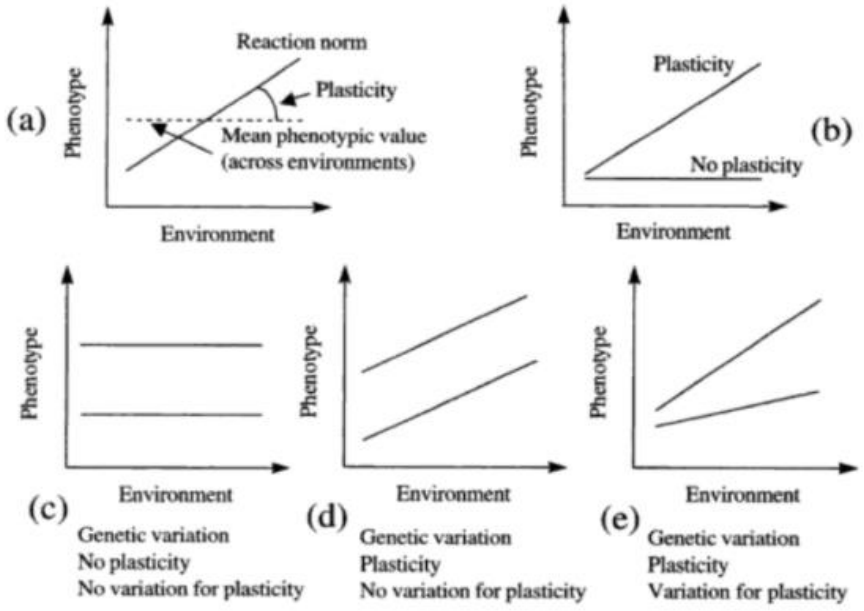

Figure 1. a) Environment-phenotype plot representing one reaction norm traced as a solid line; b) Difference between plasticity and reaction norm showing that when there is no a slope it means there is not plasticity. c) two genotypes showing no plasticity, d) two genotypes showing the same plasticity and e) two genotypes showing different norms of reaction denoting genetic variation. Image taken from Pigliucci et al 2001.

Some examples of reaction norms are found in the shell morphology of the gastropods such as the snail *Physa virgata* (Figure 2). Predation signals induce snails to produce less elongated and rotund shells, it has been demonstrated that snails of the genus *Physa* displayed shell morphometric plasticity when exposed to fluctuating environments (DeWitt, Robinson et al. 2000, Langerhans and DeWitt 2002, Gustafson, Kensinger et al. 2014).

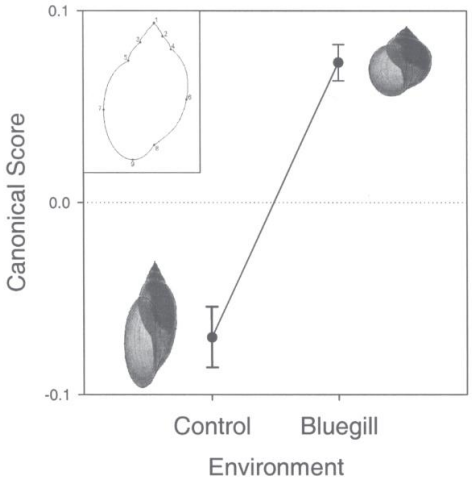

Figure 2. Reaction norm example in shell morphology. The y-axis describes the canonical score of shells, elongate shells appeared in a no inducing (control) environment while rotund shells are found in the presence of a fish predator (bluegill). Image taken from Gustafson et al 2014 based on the study of Langerhans et al. 2002.

The reaction norm concept is not limited to morphology; gene expression can have reaction norms (Chen, Nolte et al. 2015). An example has been showed in the analysis of the gene expression in *Drosophila melanogaster* through a range of developmental temperatures from 13°C to 29°C. 9,995 of 18,764 *D. melanogaster* genes were expressed at all temperatures corresponding to 70% of the protein-coding genes. They distinguished four categories of gene expression variations, the genes whose expression decreased with temperature represent the 34.3% (Figure 3A) and the genes whose expression increased represent the 33.5% (Figure 3B). 8.2% of the genes did not show change in their expression (Figure 3C) Furthermore, in this work they identified that diverse patterns of plasticity were revealed by their functional description, showing that *"reaction norms are a good approach to recognize how natural population respond when exposed to fluctuate environments"* (Chen, Nolte et al. 2015).

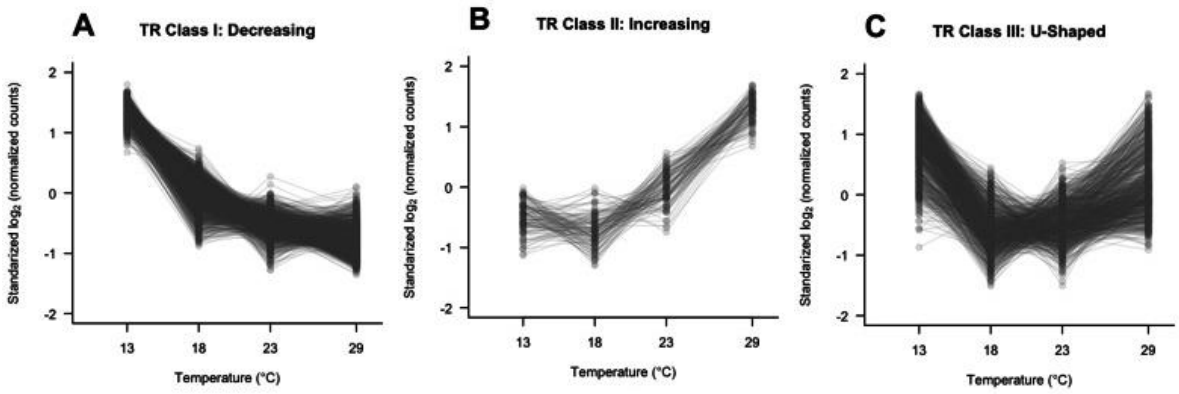

Figure 3. Classification of genes according to their expression plasticity. A) Genes whose expression decreased with temperature. B) Genes with expression increasing with temperature. C) Genes that did not show a monotonous change in gene expression. Image taken from Chen et al. 2015.

2. Adaptive phenotypic plasticity

Phenotypic plasticity was assumed to result from developmental accidents, but newly evidence shows that environmentally induced phenotypic variation exhibited by an organism can be selectively advantageous (Robinson and Wilson 1996, Svanbäck and Eklöv 2006). An adaptation is defined as a modification in the phenotype caused by a particular environmental stimulus and that has a strong enhancement in the fitness of the organism (Stearns 1989).

There are multiple examples of adaptive phenotypic plasticity such as the prey-induced, the predator-induced, the parasite-induced and the polyphenism (Stearns 2015).

Prey-induced phenotypic plasticity

Prey-induced phenotypic plasticity refers to variations in the predator's phenotype that allow it to ingest more efficiently one or more preys depending on the availability of those preys in the environment, this is also known as "inducible offenses" (Mougi, Kishida et al. 2011). A much studied example are amphibians, such as the north american spadefoot toads of the genus *Spea* that displayed two alternative phenotypes prey-induced, an omnivore morph and a carnivore morph. The omnivore morph that feed in detritus and microorganisms has small jaw muscles, smooth mouthparts, abundant denticle rows and a lengthy gut (Figure 4A). Otherwise, the carnivore morph

whose prey is the shrimp (Figure 4B and C), has larger jaw muscles, a keratinized beak, lesser number of denticle rows and a smaller gut (Figure 4D) (Levis and Pfennig 2019).

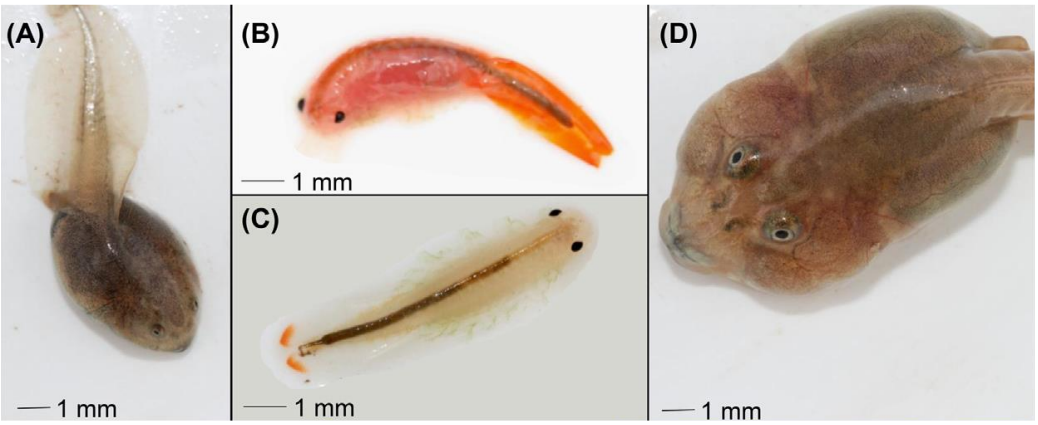

Figure 4. Example of prey-induced plasticity in the north american spadefoot of the genus *Spea*. Anuran tadpoles normally develop into an omnivore morph (A). If a juvenile tadpole consumes an Anostracan shrimp prey (B and C), it displayed a new different carnivore morph (D). Image taken from Levis and Pfenning 2019.

Predator-induced plasticity

Predator induce phenotypic plasticity or "inducible defenses" are phenotypic modifications in prey that are triggered by predation danger and that reduce the mortality rate of the prey by predation (Tollrian and Harvell 1999). An example is found in gastropods that can alter their shell form in response to the predation stress, this alteration of shell form affects the adaptability of the gastropods. The freshwater snail *Radix balthica*, showed a thin shell with a long spire when is in a fish-free pond and when it coexisted with fish predators its shell is more rotund with a low spire. The plasticity showed in the shell morphology is an antipredator trait, the change in morphology increase survival rate in those snails that coexisted with the fish predators (Brönmark, Lakowitz, and Hollander 2011).

R. balthica has another plastic trait: the mantle pigmentations as its shell is translucent. If the snails are exposed to ultraviolet radiation, the shell becomes darker and if the snails are exposed to a fish predator, the shell becomes spotted, as a strategy of camouflage to escape from predation, and if the snail is exposed to fish and UV radiation, the spots and the dark color appear in the shell (Figure 5). The same trait is altered with two different environmental factors, one biotic and another

abiotic, and these changes are different between them (spots vs darkness). It was also found that snails have to trade-off their cryptic patterns when exposed to both UV radiation and predator, because it becomes darker and the complexity of the camouflage is lower so still have their photoprotection but their camouflage might not be optimal (Ahlgren, Yang et al. 2013). This example shows how phenotypic plasticity can have an impact on the ecology and evolution of a species by directly affecting the fitness of individuals.

Figure 5. Predator-induced plasticity example in the snail *Radix balthica*. The first image is a snail not exposed to environmental stress, the second one is a snail exposed to a fish predator, the third one a snail exposed to UV radiation and the fourth one a snail exposed to UV radiation and a fish predator. Image taken from Ahlgren et al. 2013.

Parasite-induced plasticity

Parasites provoked the reduction of the life span of their host, and some parasites such as the trematodes, castrate their hosts. The snail *Biomphalaria glabrata* increases reproduction early in life when exposed to the parasite *Schistosoma mansoni*, infection is unnecessary, the reaction in the snail is induced just by water in which the parasites have been, not by the parasites themselves (Minchella and Loverde 1981) and this strategy assures reproduction of the snail before being castrated by the parasite. Another parasite-induced phenotype variation is that provoked by the parasite *Cercaria batillariae* to the snail intermediate host *Batillaria cumingi*, the snails infected by the trematode move to the subtidal zone, and they are bigger and displayed more changes in shell morphology than those in the intertidal zone potentially affecting the dynamics of the snail population (Miura, Kuris et al. 2006).

Polyphenism

Polyphenism is the occurrence of two or more alternative phenotypes in response to an environmental stimulus from a single genotype (Simpson, Sword et al. 2011). Polyphenisms are highly found in insects, in the conversion from larva to pupa and adult in many insect's taxa such as Lepidoptera (butterflies) and Hymenoptera (ants, bees and wasps). Polyphenisms offer the chance for insects *"to arrange the same genome to a screen of life history stages from nourishing larvae to reproductive adults*" (Simpson, Sword et al. 2011), they also adopt phenotypes that allow them to survive better to environmental changes through seasonal morphs, e.g. seasonal polyphenism in the butterfly *Junomia almana* (Brakefield and LARSEN 1984), whose dry-season morph has less marks in the underside wings than the wet-season morph, that has additional eyespots and lines. These differences in pigmentation patterns offer suitable camouflage and modify heat retention according to the season (Figure 6 A-B).

Polyphenism are found also in cephalopods, in the peculiar behaviours of octopuses. Rapid neural polyphenism in cephalopods is a term introduced to describe how *Octopus cyanea* generated multiple body patterns for defence and camouflage, changing them instantaneously according to the visual and behavioural background. The polyphenism is based in the diverse body patters that each individual can show (Figure 6 C-D), a body pattern consists of a combination of chromatic, textural and locomotor components that are combined at a given time to provide camouflage in response to environmental stimulus such as predation or prey presence (Chiao and Hanlon 2019).

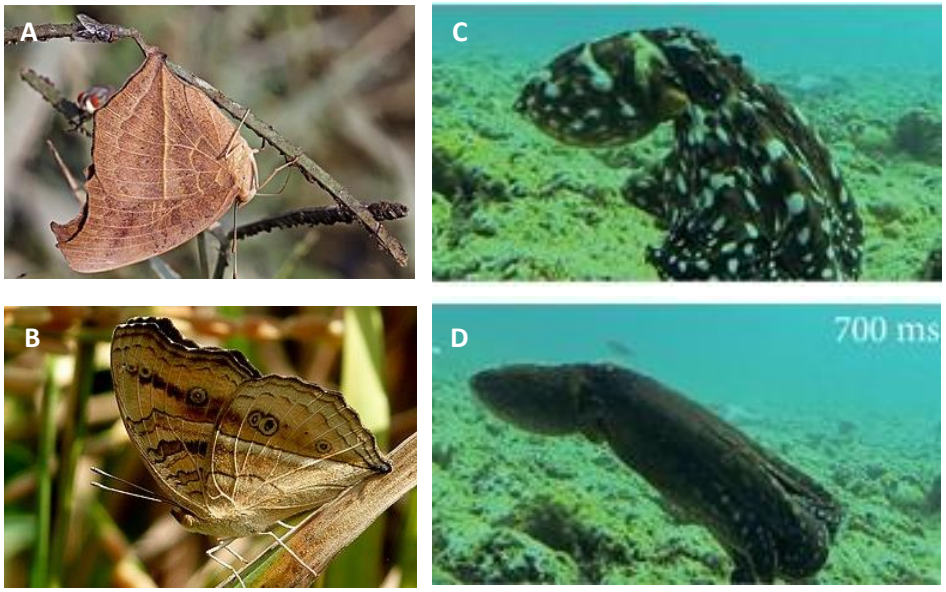

Figure 6. Seasonal polyphenism in the butterfly *Junomia almana*, a) dry-season morph has a brown colour and a few marks in the underside wings and b) the wet-season morph has additional colourful eyespots and lines in the underside wings. Image taken from the website "Sharp photography, wildlife portrait photography". C) *Octopus cyanea* with mottle camouflage use for primary defense, d) secondary defense of deimatic behaviour comprising skin texture, colour and papillae morph changes. Image taken from Chiao and Hanlon, 2019.

3. Genotype by Environment Interaction

Genetically, phenotypic plasticity is expected as a consequence of dissimilarities in allelic expression across different environments and to fluctuations in interactions between loci (Scheiner 1993). Multiple genotypes are present in nature, and the reaction norms are likely to have different slopes for the different individuals with different genotypes, therefore intersect on a graph (Figure 7) (Stearns 1989, Conner and Hartl 2004). When the slope of the reaction norms is positively correlated with the fitness of the individuals, there will be an increased phenotypic plasticity in that population for a particular trait (Conner and Hartl 2004). The phenotypic plasticity evolves differently in separate populations and species, subject to the selection forces (Schlichting and Pigliucci 1998, Aubin-Horth and Renn 2009).

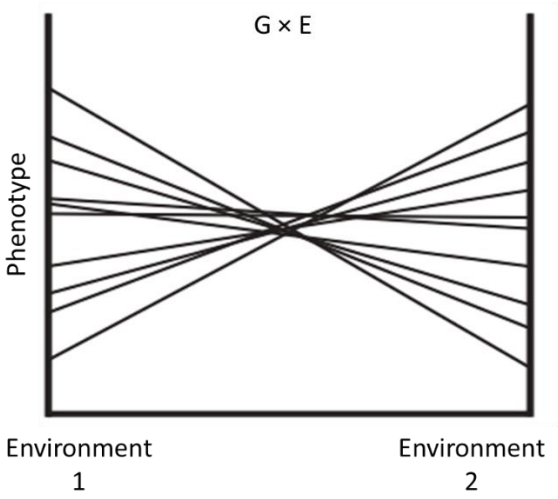

Figure 7. Different slopes denote differences among families in plasticity, there is genetic variation for plasticity, the crossing of reaction norms is called a genotype-environment interaction (G × E). Image taken from Conner et al. 2004.

Quantitative genetics is a classical method that links phenotypic variation and genotypes by combining environment and genetic components on phenotypic variation. The phenotypic variance (VP) has a genetic component (VG) and an environmental component (VE). VP= VG+VE (Falconer 1996).

Quantitative genetics study how phenotypes are affected by the action of many genes (Hill 2010). The detection of numerous molecular markers, the access to cost-effective genomic sequencing and the improvement of statistical approaches such as quantitative trait loci (QTL) mapping, have transformed the field of quantitative genetics. QTL can be identified through genetic markers such as single nucleotide polymorphisms (SNPs), polymorphic insertions or deletions and microsatellites influencing a quantitative trait (Mackay 2009, Mackay, Stone et al. 2009).

There exist genetic mapping techniques developed to identify loci causing quantitative trait variation in organisms. Correctly mapping a genotype to a particular phenotype necessitates integration of additive and epistatic effects and identify how these fluctuate between different environments (Benfey and Mitchell-Olds 2008). Additive gene effect happens when multiple genes interact to determine a trait (Etterson and Shaw 2013, Joseph 2013). Epistatic gene effects is the effect of a particular genetic variant that is masked by a variant at another locus or the combination of two genes that produce a new trait (VanderWeele 2010).

In addition, a difference is made in quantitative genetics between the inheritance of a simple Mendelian trait or complex genetic traits. In quantitative genetics, inheritance is defined as the transmission of genetic information from parents to offspring (Graf, van Schaik et al. 1992). A simple Mendelian trait is controlled by a single-locus with two alleles, and the pattern of inheritance follow mendelian laws: law of segregation, law of independent assortment and law of dominance (Gautam 2018). The phenotypic variation of a complex trait is affected by complex interactions of multilocus genetic architecture and environmental factors and do not follows a Mendelian inheritance pattern (Sherman 1997).

Two approaches are used to understand genetic architecture, the unmeasured and the measured genotype approach (Templeton 2000). The unmeasured genotype approach associated the phenotypes of individuals with a pedigree, a representation of the relationships between family members, allowing to infer the genetic architecture. It is based on the analysis of the phenotypes distribution across the individuals of the same pedigree but do not associate the phenotypes to any direct measure of DNA dissimilarity (Pérusse and Bouchard 2000).

The measured genotype approach allows to evaluate the incidences and effects of alleles at specific loci and the polygenetic variance, and it is separated in linkage marker and candidate locus methods (Templeton and Johnston 1988). Linkage analysis is a method for mapping the genes for inherited traits to their genomic regions (Cantor 2019). The genomic markers are genotyped and verified in a sample of pedigrees, and the markers displaying the highest statistical evidence of linkage localize the trait gene to the corresponding genomic region (Cantor 2019). The candidate locus approach uses previous information about the basis of a phenotype and make possible to identify plausible causative loci. Genetic dissimilarity at candidate loci can be evidenced for association with phenotypic variability, or the candidate genes can be added as markers in a linkage study, this method is largely used in human health research (Kwon and Goate 2000).

Population genetics comprise the genetic composition of the individuals and the inheritance of the genes through generations. The genetic constitution of a population would be designated by the percentage of individual belonging to a particular genotype; these proportions are called genotype frequencies. Genotype frequencies can change by process such as migration, mutation, recombination and selection. Genetic mutations gradually increases genetic variability; under

fluctuating environments, new mutations can contribute to adaptation (Carja, Liberman et al. 2014).

The study of population genetics has been helpful to understand how genetic diversity can be controlled by environment, but the molecular mechanisms causing swift adaptation responses to complex environments are under current study. Responses to the environmental stimuli are controlled at the molecular level, and there is the hypothesis that other non-genetic mechanisms may also induce phenotypic variants (Salinas, Brown et al. 2013). Recent insights in molecular biology along with the advance of genomic sequencing has generated evidence that concentrating only on the DNA sequence cannot totally explain the adaptive phenotypic plasticity observed in fluctuate environments (Keller 2014).

4. Genetic assimilation and accommodation concepts

Whether the process of genetic assimilation and accommodation can explain evolutionary change is a debated issue among evolutionary biologists (Braendle and Flatt 2006). Waddington experiments with *Drosophila melanogaster* in 1953, showed that certain phenotypes can be obtained at low frequency in a population by an environmental input, e. g. the temperature stress in early stages of development (Pigliucci, Murren et al. 2006). Waddington selected the flies that showed the new phenotype increasing their frequency, after some generations, he observed that the frequency of the novel phenotype augmented and that the environmental input was no longer essential to induce the new phenotype, that is how he introduced the term "genetic assimilation" (Pigliucci, Murren et al. 2006, Noble 2015).

Genetic assimilation or canalization happens when phenotypic plasticity becomes genetically fixed. Numerous authors have highlighted that genetic assimilation may have evolutionary consequences through the integration of phenotypic variants. *"If plasticity is costly, selection can eradicate it and induced the canalization of the preferred phenotype"* (Levis and Pfennig 2019). The mechanisms that produce genetic assimilation of a phenotype are unknown, the question of how environmental impacts on phenotypes turn into genetic mutations still open, are other non-genetic elements involved in this process? The recognition of the molecular mechanisms allowing conversion from "environmentally induced" to "genetically encoded" is a major subject of current

research (Ehrenreich and Pfennig 2016). Genetic assimilation is a kind of genetic accommodation (Braendle and Flatt 2006). In genetic accommodation a new phenotype produced by mutation or environmental changes can turn into established as an adaptive phenotype by genetic modifications (Pigliucci, Murren et al. 2006). Selection can stimulate an adaptive alteration in the regulation of an environmentally induced phenotype that can: 1) remain phenotypically plastic or 2) can become genetically fixed by genetic assimilation (West-Eberhard 2003, Sommer 2020). The dissimilarities between genetic assimilation and accommodation is that the assimilation describes only the genetic fixation of a trait while genetic accommodation describes two possibilities, the evolution of environmentally insensitive or sensitive trait expression. Another difference is that genetic assimilation assumes only an environmental cause of phenotypic variation while genetic accommodation assumes that the cause can be either genetic or environmental (Braendle and Flatt 2006).

A model for the role of phenotypic plasticity and the origin of new phenotypes was recently proposed, where genetic accommodation and assimilation are included in the steps (Sommer 2020). First environmental fluctuations or genetic mutations generate plastic traits, then developmental switches regulate the phenotype during development, after that, independent and unique expression of plasticity in distinct individuals produces and adaptation in response to selection (Sommer 2020). Finally, one of the plastic traits may become genetically encoded or continue to be plastic (Figure 8).

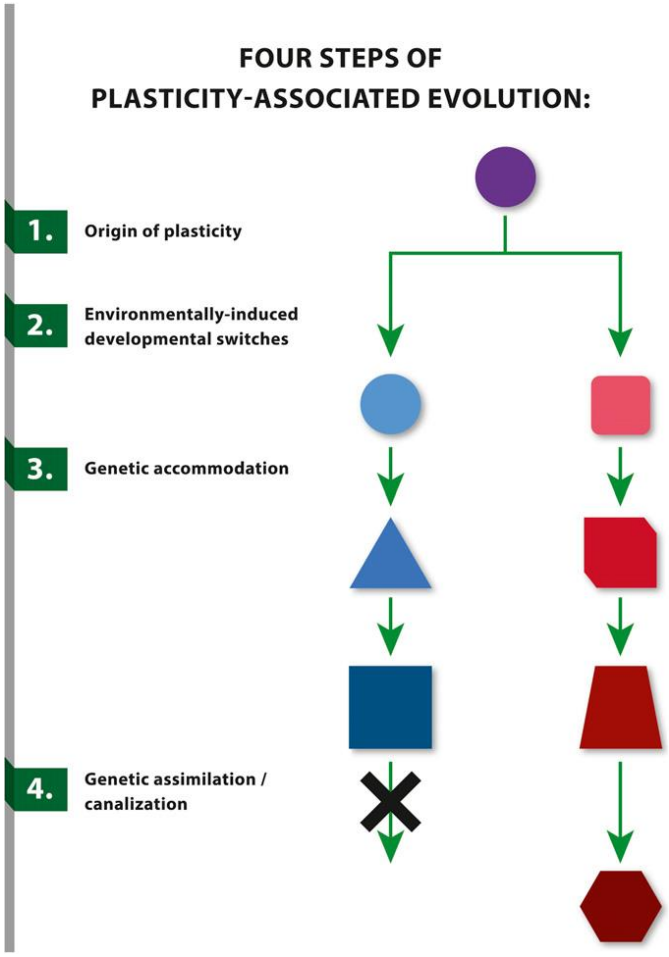

Figure 8. Model for the role of phenotypic plasticity and the beginning of novelty comprising four stages (Sommer 2020). 1) Environmental changes induced new phenotypes (change of color or form) through phenotypic plasticity, 2) then different genotypes differ in their response patters and developmental plasticity can appeared (change of form) 3) Natural selection acts on these response patterns and result in adaptation and more phenotypic variability 4) a phenotype can become genetically fixed (X) or continue to be plastic (change of color and form) and still being the target of selection. Image taken from Sommer 2020.

But how can we explain phenotypic plasticity? whether phenotypic plasticity is explained by the genetic information and whether is not explained by genetics? This is currently one of the most debated subjects in evolutionary biology.

II. Modern Synthesis and the Extension of the Modern Synthesis

Evolutionary biology describes why populations variates in precise directions over time. The central problem for evolutionary biologists has been to recognize how selection forces causes adaptations and diversifications (Kutschera and Niklas 2004). Evolution by natural selection implicates phenotypic variation, differential fitness through reproduction advantage and heredity (Conner and Hartl 2004)

The Modern Synthesis (Huxley 1942) which regroups the Darwin's theory of natural selection and Mendelian genetics, is the evolutionary theory that explains the origin of biological diversity. Recent findings of new phenomena suggest that not everything is explained by this theory. Modern synthesis (MS) focuses, primarily on genetic variation through random mutations, inheritance through DNA and, natural selection of genes as the sole cause of adaptation (Orr 2005).

The biological sciences have advanced widely, consequently, new research fields have arisen, e.g. molecular ecology and systems biology. Additionally, our understanding of evolution has importantly extended, giving rise to the extended evolutionary synthesis (EES) (Pigliucci 2007, Pigliucci and Muller 2010). EES is a distinct framework for understanding evolution, and its consideration is inexact as a consequence of the different interpretations of the most recent discoveries derived from inclusive inheritance, developmental plasticity, genomics and other research fields (Pigliucci and Muller 2010, Laland, Uller et al. 2015).

The EES preserves the bases of MS but it changes in the inclusion of other processes in evolution. One of these processes is inclusive inheritance, the MS exclude non genetic inheritance of the heredity system, genetic information is considered explanatorily enough for the evolution of adaptations. In EES heredity includes other mechanisms by which progeny resemble their parents and non-genetic elements are considered in the heritability, foundation and dispersion of environmentally induced phenotypes (Danchin, Charmantier et al. 2011, Uller 2018).

MS theory does not explain some consequences of phenotypic evolution like novelty, modularity, homology, etc. and the EES considers research field that address certain aspects of these processes. In the EES, developmental plasticity is considered to have a crucial role in defining which genetic variants will produce selectable phenotypic alterations under certain environments through spreading or reducing the range of the phenotypic response capability of a population (Uller, Feiner

et al. 2020). Fixation of environmentally produced phenotypic variations can occur through genetic accommodation, producing swiftly adaptations. In addition, developmental plasticity produces phenotypic variation that can turn into adaptations, accounting in evolutionary processes (Uller, Feiner et al. 2020).

Genomics is the science that studies the whole genome of an organism, allowing to observe that the genome changes across the evolutionary time by different mechanisms such as duplication, deletions or mutations. Today we know that genetic information can be acquired otherwise than by inheritance from the parents, the horizontal transfer of genes has been documented in prokaryotes (Eisen 2000) and eukaryotes (Keeling and Palmer 2008) and horizontal transfer can exist between species phylogenetically distant having functional implications. In addition, the transposable elements (TEs) were found to be the source of genetic pronounced fluctuations in the genotypes, being powerful facilitators of evolution instead of "junk DNA" and demonstrating that genomic evolution is dynamic and non-gradual as early assumed (Oliver 2012). Moreover, TEs are also important epigenetic regulators of the genome, TEs cause chromosome changes and influence neighboring genes by altering splicing patterns (Slotkin and Martienssen 2007). These new evidences in the genomics field differ substantially from the explanations delivered by the initiators of the MS that suggested random replacement of single alleles as the only mechanism in evolution (Müller 2017).

EES establishes a different evolutionary background, its predictions allow the origin of new hypothesis and encourage novel research in evolutionary biology and nearby fields. A new theory is required to englobe the new ideas of the contemporary evolutionary biology, and EES attempts to provide this structure (Müller 2017).

As we can see EES is very large and multiple research fields and concepts contribute to its understanding, the aspect of the EES in which I will focus on this introduction is on the concept of inclusive inheritance and in the inclusion of epigenetic inheritance, as one of my thesis objectives is to determine if environmental epigenetic changes can be inherited through generations and if they contribute to the generation of phenotypic plasticity/variability.

Inclusive inheritance

Modern evolutionary theory relates inheritance only with the transference of genetic information from parents to progeny focusing on genetic inheritance. Recent evidence demonstrates that genetic and non-genetic inheritance can participate in the heritability of traits. The inclusive inheritance concept comprises genetic and non-genetic inheritance elements. The non-genetic inheritance includes parental effects, ecological inheritance, cultural inheritance and epigenetic inheritance (Danchin, Charmantier et al. 2011).

1.1. Parental effects

Parental effects refer to the impact that parental environment induce on the phenotype of their progeny and that are independent of the progeny's own genotype. They can occur when the expression of progenitor's genes turns into an environmental component disturbing the development of the progeny (Danchin, Charmantier et al. 2011). An example is found in *D. melanogaster*, where maternally derived mRNA accumulated in the egg and regulate oogenesis and early embryo development (Barckmann and Simonelig 2013).

Parents have significant impacts in their progeny beyond birth through resource provisioning and behavioral interactions, playing important roles in the ontogeny and individual phenotypic variation. Parental effects can also provide a source for expression of novel phenotypic variation influencing population dynamics, it can preserve transgenerational transmission of developmental variability and rise phenotypic resemblance between progeny and their parents, modifying development to previously accumulated variability (West-Eberhard 2003, Uller 2012).

There are three general categories of transmission between parents and progeny; transmission from germ cells to germ cells, transmission from somatic tissues to germ cells and transmission from somatic tissues to somatic tissues (Uller 2012).

Germ cells to germ cells transmission occurs during genomic imprinting, genes can have different epigenetic marks between eggs and sperms which causes gene expression in a parental manner in the progeny. Conflicts between maternal and paternal genes can happen playing an important function in the development of embryos, this encounters can repress sexual development of the

progeny resulting for example in sterile castes in some insect species such as the termites (Matsuura 2020).

An example of somatic tissues to germ cells transmission is the organelles inheritance. Cell wall formation in the diatom *Cyclotella meneghiniana*, is affected by the shape of the parent cell, each cell has two siliceous valves and one is inherited from the parent while the other is newly formed within offspring cells (Shirokawa and Shimada 2016).

A type of somatic to somatic tissues transfer is the transmission of symbionts from parents to offspring. Symbionts transmitted from parent to offspring can be divided in germline lineages and somatic lineages, this decrease antagonism between symbionts (Frank 1996). An example of symbionts transmission through the soma is found in the stinkbug *Megacopta punctatissima* that is obligatorily associated with the gut bacterium '*Candidatus* Ishikawaella capsulata'. The symbionts parent to offspring transmission is intermediated by an exclusive mechanism called "symbiont capsule" that is produced by mothers to transfer symbionts to multiple eggs (Hosokawa, Kikuchi et al. 2007). The capsules with symbionts inside are deposited at the bottom of the egg mass, and when offspring hatches from egg they eat the capsule (Hosokawa, Kikuchi et al. 2005). If the transmission is not assured, the offspring has negative effects such as retarded development and mortality (Fukatsu and Hosokawa 2002)

Parental effects are accountable for a wide variety of plastic responses through generations, e.g. predator defenses, acclimation to abiotic factors and disease resistance. An example of parental effect is the transgenerational immune priming (TGIP), which occurs when parents improve immune defense of their progeny by the transmission of their immunological experiences (Roth, Beemelmanns et al. 2018).

An examples of TGIP in vertebrates is found in the teleost fish *Sparus aurata*, where mothers transfer antibodies directly into egg increasing the body weight of their progeny and providing lysozyme activity and anti-protease activity in the egg's yolk (Hanif, Bakopoulos et al. 2004). And in fish with parental care, father can also deliver antimicrobial immune elements into eggs which protects the progeny from pathogens (Giacomello, Marchini et al. 2006)

Examples of TGIP in invertebrates are principally found in insects (Sadd, Kleinlogel et al. 2005, Freitak, Heckel et al. 2009), e.g. in the mealworm beetle *Tenebrio molitor*, antimicrobial activity

was higher in progeny when their parents received a microbial immune challenge during the larval stage (Moret 2006).

1.2. Ecological inheritance

Niche construction is defined as the environmental ecological alterations made by organisms and its inheritance is called ecological inheritance (Odling-Smee, Erwin et al. 2013). Niches are modified by the organisms through their metabolism, behaviors and actions, numerous examples are found in animal species that construct nests, holes, webs, etc. and modify nutrient cycles and bacterial composition that affects the environment (Laland, Odling-Smee et al. 2009).

Some of the environmental alterations shaped by organisms in a niche continue longer than the individual's lifetime thereby affecting succeeding generations of the population. To qualify if a niche modification as a niche construction, it should cause ecological changes that modify selection forces with an evolutionary consequence (Badyaev and Uller 2009).

Ecological inheritance is transferred by organisms through the alterations of an outdoor environment and not by reproduction. Therefore, ecological inheritance occurs in organisms that are not necessarily genetically related but that are ecologically associated by sharing the same niche. If a selection force is altered by niche construction, it will be inherited to the descendants through the genetic information and it will change the fitness value of the population (Odling-Smee and Laland 2011).

An example of ecological or niche inheritance is the construction of a mound made by termites, which involves modifying the ecosystem in order to get used and modifying some parameters such as humidity and temperature to allow their larvae to develop, therefore the habitat modification is transmitted to the offspring to assure its survival and then to allow them to have an environment to grow up and this habitat construction is transmitted to the subsequent generations (Odling-Smee 2007).

1.3.Cultural inheritance

Cultural inheritance is the transference from one generation to other of information that includes behavior, social customs, language, etc. Cultural inheritance is transferred by communication, instruction, imitation, social learning, etc. and it is inherited from parent to offspring showing greater formal resemblance to biological evolution and it is thought to have evolved by epigenetic mechanisms (Peedicayil 2001).

As cultural information is inherited, it can evolve. The wide presence of social learning proposes that intergenerational cultural inheritance could exist among all taxa being not limited to mammals or vertebrates. Culture inheritance can increase organism's fitness allowing organisms to take and transfer adaptive behavior that they could not have learned without cultural inheritance, thus providing a faster adaption to environmental change than genetic inheritance alone (Danchin, Charmantier et al. 2011).

Cultural selection can interact with natural selection though genetic information, genetic information produces the template on which behavior can develop and vary during development. Cultural inheritance has the essential conditions for natural selection can happen: i) cultural variation existence, ii) there is a link between this variation and the organism's fitness and iii) the information is transmitted to the progeny. Therefore, cultural evolution should be considered in the evolution of species (Creanza, Kolodny et al. 2017).

Evidence of cultural inheritance in vertebrates is found in cetaceans, cultural transmission of migratory routes is functional in guiding naïve individuals to locations that are important for the individual fitness, young humpback whales have been observed to follow their mothers from low-latitude to distant feeding grounds, and they follow her back the next year to memorize the route, this locations are very distant and may be difficult to discover by oneself (Baker, Palumbi et al. 1990), moreover migratory routes transmission is found in other taxa, such as birds (Palacín, Alonso et al. 2011) and fishes (Helfman and Schultz 1984). Evidence of social learning and cultural inheritance in invertebrates is poorly studied but evidence is found in insects, e. g. *Drosophila melanogaster* females express strong social learning that allow to stabilize a preference for a male phenotype at the population scale. The female mating preferences can be culturally transmitted across generations leading to a constant tradition in mating preference (Danchin, Nöbel et al. 2018).

III. Epigenetic information and epigenetic inheritance

Epigenetic information consists on the status of gene function that do not involve any changes of the DNA sequence. Epigenetic modifications are reversible and it can take place in the cells following an environmental change, they are mitotically transmitted and some of them can be meiotically transmitted (Nicoglou and Merlin 2017). Epigenetic modifications are different configurations of the chromatin state or differences in the level of biochemical marks that are present on the histones, the proteins that keep DNA compacted, or on some nucleotides such as the cytosine and adenine, that binds to an epigenetic mark consisting in a methyl group. These changes are called epimutations, and it has been demonstrated that the rate of epimutations is significantly higher than genetic mutations (Horsthemke 2006) consequently, it is important to study the contribution of epimutations in the heritability and phenotype variability.

There exist three classes of epigenetic variation: facilitated, obligatory, and pure (Richards 2006).

Facilitated epigenetic variation is when the genotype conditions an allele to stochastically fall under epigenetic control (Richards, 2016). An example is the epigenetic modification at the agouti locus in mice, where the transcription originated in a retrotransposon introduced upstream the agouti gene. This transposon insertion results in induction of ectopic expression of agouti protein, occasioning the phenotype that presents yellow hair, obesity, diabetes and bigger vulnerability to tumors (Morgan, Sutherland et al. 1999). When mice are fed with a diet complemented with extra methyl groups the progeny is not obese and has brown hair as a result of the hypermethylation in the promoter that represses gene expression (Sharma and Aazmi 2019).

Obligatory epigenetic variation is totally dependent on genetic variation; epigenetic variation is associated with specific mutation(s). An example of obligatory epigenetic variation is found in the plant *Sinapsis alba*, where transposon insertions in four alleles of the fatty acid elongation 1 gene (FAE1) produced epigenetic modifications that are involved in modulating the expression of FAE1, alleles become epialleles with different DNA methylation levels in its promoter region (Zeng and Cheng 2014).

Pure epigenetic variation are the epigenetic changes that result from inaccuracies in the establishing and preservation of the epigenotype, being a variation independent of environmental conditions and genetic background. Pure epigenetic variation has been observed in clonal

individuals raised in a certain environment. This “errors” in the epigenotype can produce phenotypic variants and this epimutations have a significantly higher rate than mutations, being an important source of phenotypic variation, independently of its adaptive value (Angers, Perez et al. 2020).

The pure epigenetic variation is subcategorized in stable (contributes to heritability) or metastable (does not contributes to heritability). An example of pure epigenetic variation is found at the *Lcyc* locus of the plant *Linaria vulgaris*, an active *Lcyc* gene results in unilaterally symmetrical flowers, while a repressed *Lcyc* gene, silenced by the spread of DNA methylation from an upstream transposable element (TE), causes radially symmetrical flowers, a phenotype variant. The phenotype can spontaneously revert back by pure epigenetic change (Pecinka, Abdelsamad et al. 2013).

These classification does not consider the epigenetic variation due to the environment. Another classification of epimutations is provided by Roquis et al. 2016 that divided in three categories: induced, random and genotype dependent. Induced epimutations are those triggered by an environmental condition. Random epimutations are spontaneous and independent from the genotype or the environment and genotype dependent epimutations are totally dependent of the genotype and independent from the environmental condition (Roquis, Rognon et al. 2016).

The possible independence of epimutations from their genotypic background together with the persistence and stability of epigenetic marks constitutes an inheritance system that operates at the boundary of the genetic information and the molecular mechanisms (Eichten, Swanson-Wagner et al. 2011, Aliaga, Bulla et al. 2019).

1. Epigenetic inheritance

Epigenetic inheritance is the transmission of epigenetic marks from parents to the progeny, and these epigenetic marks are originated by an initial environmental stimulus and can became stable over time (Nicoglou and Merlin 2017). There are two classes of transmission of epigenetic marks between different generations called intergenerational epigenetic effects: multigenerational and transgenerational effects (Skinner 2008).

Multigenerational effect occurs when the generational effect result from a direct exposure of the germ cells (Figure 9), gametes or embryos to the environmental stress that induce the epigenetic change (Fallet et al. 2020). e.g. the epigenotype of mice offspring displayed changes in cytosine methylation profiles according to the different parental food regimes, that modify the lipid metabolism of the offspring (Carone, Fauquier et al. 2010) another example is found in the oyster *Crassostrea gigas*, where an exposure to a chemical pollutant affects the epigenome of the offspring of exposed oysters (Rondon, Grunau et al. 2017).

Transgenerational effects, are the effects transmitted across more than three consecutive generations and that are not related to a direct exposure to an environmental stress on the affected organism (Figure 9), e.g. transgenerational inheritance of lifespan longevity in *Caenorhabditis elegans*, where changes in chromatin modifier histone H3 lysine 4 trimethylation (H3K4me3) of the parental generation stimulate an elongation of the lifespan of offspring on the second and third generation (Greer, Maures et al. 2011).

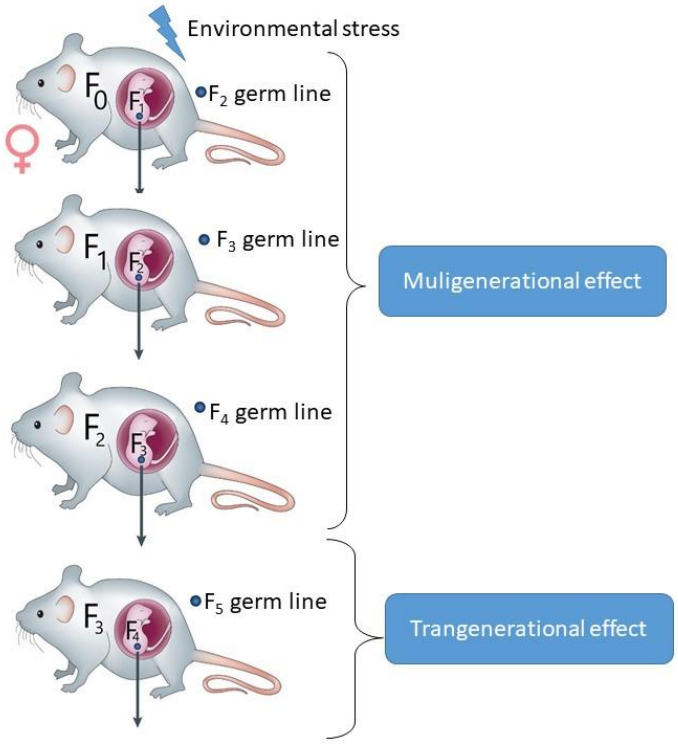

Figure 9. Scheme of a multigenerational effect and a transgenerational effect in a female mouse. A female mouse pregnant carry three generations: the mother, the fetus and the germ line of the fetus (F_0-F_1-F_2). Therefore, transmission until the F_2 generation is a multigenerational effect and if transmission continues after the unexposed F_3 generation then is considered a transgenerational effect. Image taken and modified from Danchin et al. 2011.

There is evidence that parental environment induced epigenetic alterations are transmitted through the germline, but the response of the gamete epigenome to the parental experience remains unclear (Wang, Liu et al. 2017). Epigenetic patterns are passed from cells to daughter cells during mitosis but it remains unknown whether epigenetic states can be transferred from one generation to another, it is assumed that the epigenome is reestablished during gametogenesis (Doerfler and Böhm 2006), thus, the inheritance of epigenetic modifications that occurs in parental gametes involves evading reprogramming. However, transgenerational epigenetic inheritance has been demonstrated in invertebrate models such as *Drosophila melanogaster* (Wang, Lu et al. 2013) and *Caenorhabditis. elegans* (Kishimoto, Uno et al. 2017). Nevertheless, the transgenerational epigenetic inheritance has been not enough studied in other invertebrate groups such as the mollusks.

Transgenerational epigenetic inheritance is present in two forms: the germline epigenetic inheritance and the experience-dependent epigenetic inheritance (Danchin, Charmantier et al. 2011).

In the germline epigenetic inheritance, the epigenome of germline cells is transferred unchanged to the progeny through multiple generations meaning that epigenetic marks are not always reestablished between generations. Incomplete resetting at genes associated with a phenotype variation can change the patterns of inheritance (Chong and Whitelaw 2004). There is evidence that this occur in plants, e.g. the hypermethylation and consequent non-expression of a transcription factor *Lcyc* is correlated with the floral symmetry of *Linaria vulgaris* (Figure 10). The epimutation and the radially symmetrical phenotype (peloric) was transmitted to subsequent generations authentically (Cubas, Vincent et al. 1999).

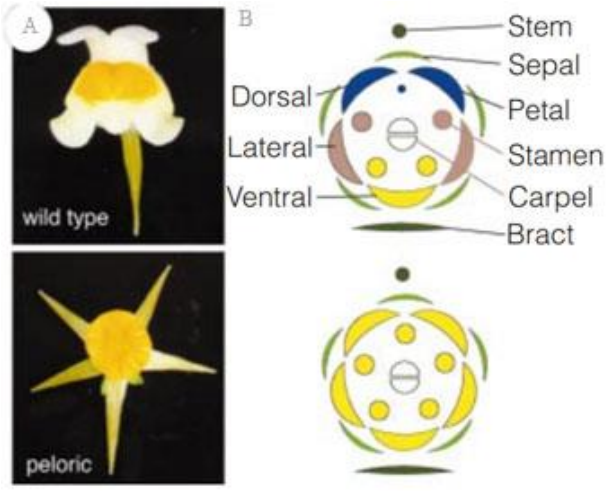

Figure 10. A) Frontal view of a wild-type (top) *Linaria vulgaris* flower compared to a radially epimutant (bottom). B) Flower schemes of a the wild-type and peloric flowers showing the location of organs specified by colors: blue (dorsal) brown (lateral) and yellow (ventral); the wild-type flower (top) has an axis of dorsoventral asymmetry while the peloric epimutant (bottom) is radially symmetrical, with all petals similar to the ventral petal of the wild-type. Image taken from Cubas et al. 1999.

In the experience-dependent epigenetic inheritance, an epigenetic profile disturbs parental behavior that causes the same epigenetic profile in its progeny (Figure 11A) (Fradin and Bougnères 2011). An integrated system possibly exists between soma and germline. In behavioral epigenetics,

antagonistic experiences produce modifications of the epigenetic profile in soma, for example, an absence of maternal care activates aberrant methylation profiles in genes of the nervous system, miRNAs and hormones are controlling systems that flow epigenetic information from the soma to the germline; the germline acquires epigenetic information from soma and transmitted to the progeny (Figure 11B) (Pang, Lu et al. 2019).

Parental effects and epigenetics interact, if maternal behavior is altered by the environment there could be a disruption of the transgenerational continuity; this reversibility of epigenetic marks when environmental conditions change, constitutes the key difference between genetic and epigenetic inheritance that has capital implications for adaptation (Danchin, Charmantier et al. 2011). Environmental stress exposure can induce epigenetic modifications in the germline through disturbing the soma, this contradicts the conventional view that heriditary information is only transmitted from germline to soma and not inversely, however, there is evidence of soma-germline molecular comunication (Sharma 2013).

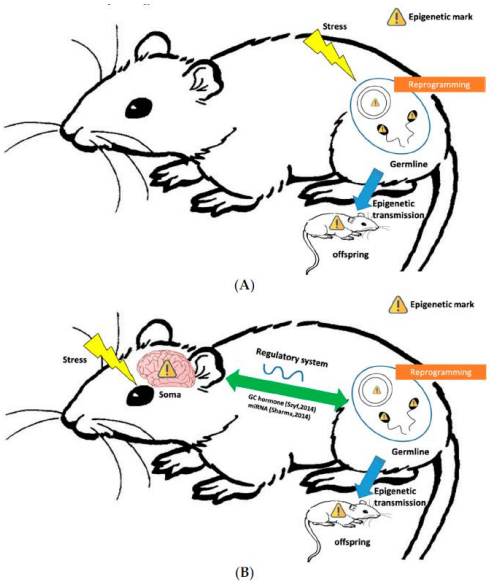

Figure 11. Potential inheritance pathways in the experience-dependent epigenetic inheritance. A) Germline-mediated epigenetic inheritance. When mice confronted environmental variations, their epigenetic profile can be modified, besides reprogramming events can happen in the germline and epigenetic modifications retained during reprogramming can be transmitted to the progeny through germline-mediated epigenetic inheritance. B) A combined organisation may exist between the soma and the germline. Hostile experiences generate alterations of the epigenetic profile in soma,

e. g. an absence of maternal care causes a variation in methylation patterns of genes located in the nervous system. Germline is capable of obtain epigenetic information from soma and transfer it to the progeny through information shippers (miRNAs and hormones). Image taken from Pang et al. 2019

Epigenetic modifications can potentially produce phenotypic variations that are transmitted to subsequent generations of cells or organisms. Notwithstanding, genetic variation is recognized as indispensable for heritable phenotypic variants, but some inherited adaptive phenotypes cannot be explained by selection through sporadic genetic mutations, which involved long periods of time to happen. Some hypothesis propose that populations may adapt to the environment through epigenetic variation long before genetic mutations appeared (Thorson, Smithson et al. 2017), suggesting that when natural selection acts on epigenetic and genetic variation, adaptive phenotypes can emerge rapidly (Vogt 2018). Epigenetics represent an extension to the Modern Synthesis, and the classical genetics by recognizing the possibility of a new system of rapidly emergent transgenerational phenotypic variation and that under particular situations certain acquired traits could be heritable in organisms (Jablonka 2013). Evolution based on genetic variation alone is difficult to understand in scenarios where species are facing rapid environmental changes such as global climate change (Hoffmann and Sgrò 2011). Epigenetic and genetic variation can contribute to speciation, when two populations are geographically disconnected, they collect different epigenetic marks that are followed by the fixation of DNA mutations (Pál and Miklós 1999). Recently, a new conceptual framework that uses systems biology approaches to reconcile genetic and epigenetic inheritance was developed (Cosseau, Wolkenhauer et al. 2017).

There exists evidence that heritable variation in ecologically important traits can be shaped through epigenetic mechanisms, even in the lack of genetic variation, besides epigenetic changes are stably inherited through generations (Johannes, Porcher et al. 2009). Furthermore, epigenetic processes possibly provide an alternative inheritance system, (Figure 12), epigenetic variation, unlike genetic variation, is provoked directly by environmental pressures and could consequently deliver an additional, rapid and reversible way for evolutionary change (Bossdorf, Richards et al. 2008, Cosseau, Wolkenhauer et al. 2017).

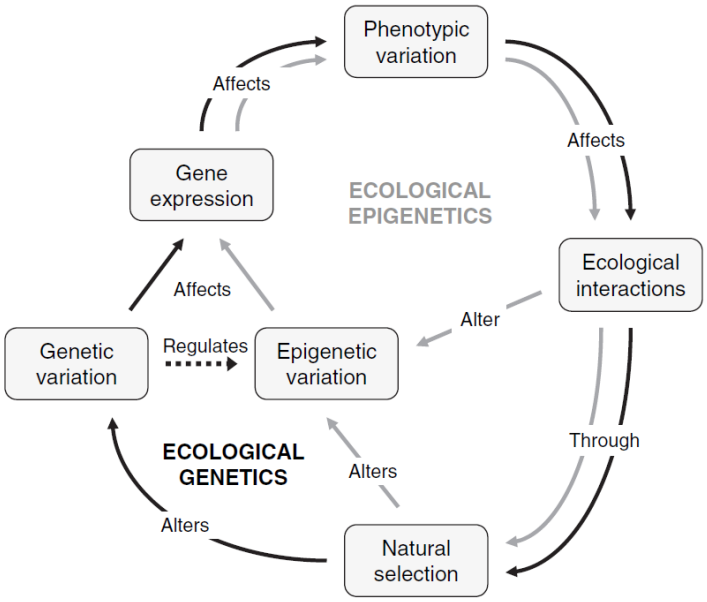

Figure 12. Comparison between ecological genetics (black arrows) and ecological epigenetics (grey arrows). Image taken from Bossdorf et al. 2008.

2. Carriers of epigenetic information

There are four molecular carriers of epigenetic information (also referred as chromatin regulators) described that can interfere at several levels (transcriptional, post-transcriptional and translational): Post-translational modifications of histones, Non-coding RNAs, nuclear localization of the loci and DNA methylation (Keung, Joung et al. 2015).

1.1 Post-translational modifications of histones

Chromatin structure has a function in the control of gene expression and is regulated by histone post-translational modifications. The histones are highly conserved proteins separated in two categories, the core histones and the linker histones (Baake, Bäuerle et al. 2001). The core histones are H2A, H2B, H3 and H4 and the four histones are bringing together into an octamer to constitute the central particle of a nucleosome with the strands of DNA wrapped around it (Figure 13) (Luger, Mäder et al. 1997).

The H1 family histones constitute the linkers and these proteins are situated on the entrance and exit sites of the DNA to keep the DNA attached to core histones. Histones have lysine and arginine

residues that are available for post-translational modifications (PTMs) denoted as the "histone code" (Smith and Denu 2009). Specific histone modification activates proteins involved in transcriptional process by specific recognition of the modified histone sites. The transcription of genetic information coded in DNA sequence is in part regulated by histone PTMs (Li, Ding et al. 2014).

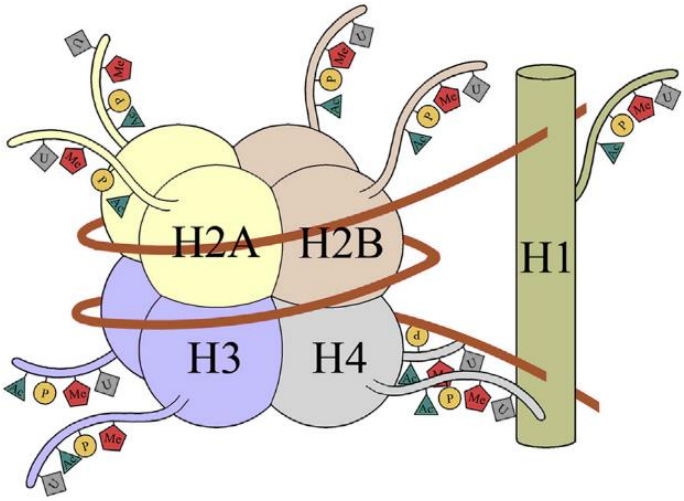

Figure 13. Histones and modifications of histone tails. Core and linker histones can be marked by covalent post-translational modifications (PTMs) such as methylation (Me), acetylation (Ac), phosphorylation (P), ubiquitylation (U) and sumoylation (S). Image taken from Li et al., 2014.

There are core histones variants that incorporate on chromatin modifying the properties of the nucleosomes. Histone variants can regulate the dynamic of the nucleosome to provide the necessary for nuclear metabolic mechanisms such as transcription, replication and repair (Ishibashi, Li et al. 2010). The histone variants have different deposition modes that makes them sensitive to environmental inputs. H2A variants are involved in DNA repair, the phosphorylation of the histone variant H2A.X occurs in response to double stranded breaks before recruitment of repair proteins and H2A.Z is a variant of the H2A family that mediates responses to environmental changes in eukaryotes (Talbert and Henikoff 2014).

Core histones variants have been recognized in a few mollusk species, e.g. two H2A.Z variants (H2A.Z.1 and H2A.Z.2) were reported in the mussel of the genus *Mytilus*, these variants displayed gene expression differences suggesting that they possess different and specialized functions. The

high diversity of histone variants across eukaryotes indicates an ancestral nature, nevertheless further research is needed to know the specialized functions they display; specially in understudied invertebrate taxa such as mollusks (Rivera-Casas, González-Romero et al. 2016).

Histone acetylation caused the partial deactivation of the positive charge on histones, decreasing the DNA binding attraction of histones altering the interaction between nucleosomes and recruiting transcription factors and transcription proteins to DNA. In some organisms such as the fungus *Neurospora* and *Aspergillus,* histone acetylation is related to active transcription and deacetylation with inactive transcription (Grimaldi, Coiro et al. 2006, Shwab, Bok et al. 2007).

Histone methylation has been shown to play a role in DNA repair, cell cycle, stress response and transcription in eukaryotes (Greer and Shi 2012). Methylated histones are recognized by chromatin as readers causing the recruitment of other molecules to alter the chromatin and transcription state. Four core histones can be methylated at numerous sites, the proteins HTM methylate different sites of a histone and histones can be mono, di or tri-methylated, on a single residue (Guo, Nady et al. 2009). Nevertheless, it is difficult to find a consensus of the function of histone methylation, it varies depending on species, on tissues and on developmental stages and on the combination with another marks (Cosseau personal communication).

Histone phosphorylation in mammals is associated with chromosome remodeling linked to other nuclear processes such as transcription and chromatin organization. Phosphorylation of H2A is an histone modification responsible for DNA reparation by non-homologous end joining (NHEJ) and homologous recombination (HR) (Rossetto, Avvakumov et al. 2012).

Histone ubiquitylation is a key player in numerous process in the nucleus including transcription, DNA repair and maintenance of chromatin structure in human cells (Meas and Mao 2015), transcription termination in mouse, gene silencing in *Drosophila* and gene expression on the yeast (Osley, Fleming et al. 2006).

There is an interplay between histone modifications, a cross talk among them, one histone modification can inhibit or activate another one, e. g., H3K9 inhibits phosphorylation of H3S10 and the ubiquitin mark on H2B induce H3K4 and K79 tri-methylation in human cells (Meas and Mao 2015).

Histone modifications and DNA methylation influence each other during development. Changes in histone modification are prerequisite for DNA methylation, e.g. in *Arabidopsis thaliana*, a mutation in the gene of H3K9 methyltransferase KRYPTONITE results in diminution of CNpG methylation levels (N, is A, C, G or T). Histone methylation can contribute to the direction of DNA methylation patterns that can be a secondary event after histone modification that provides long term stability and contributes to the reconstruction of histone modifications after DNA replication (Cedar and Bergman 2009).

Chromatin was seen as either an open an accessible form called euchromatin or as a compacted and repressed form called heterochromatin (Murakami 2013). Recently findings demonstrated that PTMs interact with each other's and with distinctive combinations of proteins and histone modifications, having different biochemical properties, transcriptional activities and structural characteristics that constituted complexes called chromatin colors (Delandre et al. 2019). In *Drosophila* for instace, 5 chromatin colors were described (Figure 14) it should be noted that the number of colors might depend on the number of chromatin marks analyzed (Filion, van Bemmel et al. 2010).

In *Drosophila*, blue chromatin is the Polycomb or PcG associated chromatin that is involved in developmental gene regulation. Green chromatin or HP-associated heterochromatin is prominent in pericentric regions with a high level of H3K9me2 mark and is involved in reactivation of pluripotent genes during the formation of pluripotent stem cells (Delandre and Marshall 2019). Black chromatin is the most abundant type and has poor gene content with some genes with reduced transcription indicating that this type of chromatin has a role in transcriptional silencing, having also a role in neuronal development (Fillion et al 2010; Delandre et al. 2019). Red and yellow chromatin are two types of euchromatin, the permissive chromatin state, and genes inside this chromatin colors are highly transcribed. The yellow chromatin regulates housekeeping metabolic genes and the red one developmental active genes (Serra, Baù et al. 2017).

Figure 14. Representation of the structural properties of the five chromatin colors of *Drosophila*. The idea of chromatin colors is that combinations of different marks are necessary to determine diferent types (colors) of chromatin. In *Drosophila* five colors were determined but the number can be different in other species. Image taken from Serra et al. 2017.

2.2. Non-coding RNAs

Non-coding RNAs (ncRNAs) are RNAs that are not translated into a protein and have an important function in gene regulation. ncRNAs comprise microRNAs measuring between 18 to 28 nucleotides (miRNAs), long noncoding RNAs measuring several Kb (lncRNAs), piwi-interacting RNAs (piRNAs) and short interfering RNAs (siRNAs) (Carthew and Sontheimer 2009). ncRNAs bind to a target mRNA and can cause inhibition of translational expression of a target gene. They are key regulator in life cycle of the organism, they operate in the nucleus or cytoplasm while targeting the mitochondria. Several ncRNAs have been identified in chloroplasts. The transcription of ncRNAs is done by RNA polymerase II and the transcripts are capped and polyadenylated. Small RNAs such as siRNAs, piRNAs and miRNAs controlled endogenous genes and protect the genome from intrusive nucleic acids in eukaryotes (Tiwari, Gupta et al. 2019).

They are ncRNAs with essential biological roles, e.g. the ribonuclease P that is indispensable for the biosynthesis of the 5’ terminus of tRNA molecules in *Escherichia coli* (Stark, Kole et al. 1978), the small nuclear ribonucleic acid (snRNA) that is involved in RNA splicing (Ohshima, Itoh et al. 1981) required for splicing of adenoviral early RNA sequences (Legoff, d'Cruz et al. 2019) and the eukaryotic signal recognition particle RNAs (SRP RNAs) that recognizes peptide signals and binds to the ribosome, ending protein synthesis (Rosenblad, Larsen et al. 2009).

lncRNAs can present secondary configurations, providing plasticity for target recognition and protein interactions (Legoff, d'Cruz et al. 2019) and they can also function as scaffold to identify

higher order organization in ribonucleic-protein (RNP) complexes influencing the regulation of gene expression complexes and chromatic states. DNA microarray sequencing demonstrated that the genome encodes as many lncRNAs as known protein coding genes (Rinn and Chang 2012).

microRNAs have a significant gene-regulatory function in eukaryotes by inhibiting translation of mRNAs via imprecise antisense base-pairing (Bartel 2009). *C. elegans* contains approximately 100 distinct miRNAs genes, 30% are conserved across vertebrates. These miRNAs control developmental timing in *C. elegans*, as these miRNAs are phylogenetically conserved, probably their primary sequences can have strong evolutionary pressure. Numerous miRNAs have been recognized in eukaryotes, nevertheless, information about which miRNAs are evolutionary conserved and the spectrum of these miRNAs in many species is unknown. In *Schizosaccharomyces pombe*, the production of small endogenous RNA and associated proteins is related with inactive transcription, small RNAs could be employed in eukaryotic cells to control gene regulation. The miRNA and piRNA processing pathway genes were characterized in the mollusc *Biomphalaria glabrata* (Queiroz, Silva et al. 2017) and deep sequencing of small RNAs, reveals that this mollusk possess numerous miRNAs and piRNAs (Queiroz, Portilho et al. 2020). Nevertheless, to understand the mechanisms of this small RNAS in an organism we required the characterisation of the number, the diversity and the function of these miRNAS (Ambros, Lee et al. 2003).

2.3 Location of loci in the cell nucleus

The cell nucleus is an extremely complex and organized organelle where nuclear constituents are in a precise nuclear architecture. There are two forms of subnuclear sections, the nuclear bodies and the chromosome territories including related chromatin domains. The chromatin bulk consisting in each specific chromosome occupies a precise position known as chromosome territory or domain. Chromosome territories are permeated by nucleoplasmic channels, forming a permeable and broaden surface area which is available to different nuclear features. Extensive chromatin domains having its place in chromosome territories are defined as heterochromatic, that are highly compacted regions or as euchromatic, less compacted regions (Espada and Esteller 2007).

Many gene loci have a tendency to concentrate inside a specific chromosome territory rather than in the central nuclear compartment. There exist large loops of protuberant chromatin that contain actively transcribed genes, suggesting that these loops are relocated to the exterior surface of chromosome territories proximate to transcription factors. Oppositely, genes are silenced when they are placed inside transcriptionally heterochromatin domains, heterochromatin is a condensed chromatin arrangement in which transcription is repressed. Nevertheless, the location of deactivated genes into heterochromatin domains could be a consequence instead of a cause of gene repression, recent evidence showed a relationship among chromatin structure and gene density, exposed chromatin areas are enriched in active and inactive gene loci and condensed chromatin are associated to poor gene content (Gilbert, Boyle et al. 2004).

Non-random positioning of the genes within the nucleus has been found in the mollusc *Biomphalaria glabrata* (Figure 15). The parasite *Schistosoma mansoni* stimulate a response in the nuclear architecture of the host snail *B. glabrata*. There is a specific temporal repositioning of genes within interphase nuclei after *in vitro* schistosome exposure, and the gene repositioning was strongly correlated with gene expression (Knight, Ittiprasert et al. 2011).

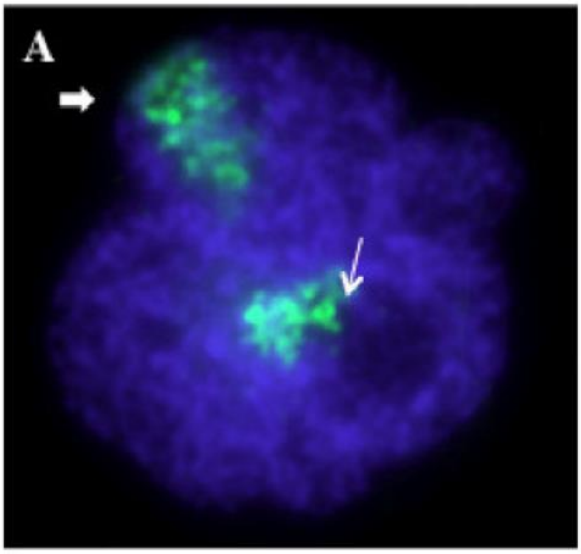

Figure 15. The interphase chromosomes of the *B. glabrata* embryonice cells (*Bge* cells) nuclei occupy chromosome territories similar to those found in mammals. Chromosomes have interactions with the nuclear membrane and the nucleolus. A) Two chromosome territories (green) in the nucleus of a *Bge* cell (blue). Image taken from Knight et al. 2011.

The chromosomes of animals are organized into domains of special chromatin interactions known as topologically associating domains (TADs), which are implicating in functional chromosomal organization. TADs are divided into smaller domains, similar to mammals, *Drosophila* chromosomes are portioned into TADs, this suggests that the partitioning of genomes into TADs is evolutionary conserved (Ulianov, Khrameeva et al. 2016). The internal structure of TADs changes in response to environmental stress during cell differentiation in mammals (Dixon, Jung et al. 2015). TAD margins correspond to those of replication domains, genes located in the same TAD tend to be co-regulated (Szabo, Bantignies et al. 2019). However, TAD function are not completely understood, there are some studies that suggest there is a decoupling between gene expression and TAD organization (Ghavi-Helm, Jankowski et al. 2019)

IV. DNA methylation

DNA methylation is a mechanism through which methyl groups can bind to the cytosine or adenine nucleotides, adenine methylation is the binding of a methyl group to the N (Nitrogen)-6 position of adenine and cytosine methylation consist in the addition of a methyl group to the C(Carbon)-5 position of the pyrimidine cytosine nucleotide. Adenine methylation can modify the interactions of proteins-DNA, the enzymes responsible for adenine methylation are DNA adenine methylase (Dam) that methylates adenine in the sequence GATC and the cell cycle-regulated methylase (CcrM) which methylates adenine in GAnTC sequences. These enzymes contribute in cellular controlling actions principally those that regulate bacterial virulence and the expression of certain genes in bacteria (Low, Weyand et al. 2001). In prokaryotes, DNA methylation protects the integrity of prokaryotic genomes and potentially other functions that remain unknown, as suggested by the recent recognition of several DNA Methyltransferases (MTases) in the genome of 230 bacterial and archaeal species (Blow, Clark et al. 2016).

In some multicellular eukaryotes DNA methylation exists on cytosines in CpG context and cytosine methylation varies importantly between taxa, however, there are four DNA methylation categories: ultra-low gene body methylation, low gene body methylation, gene body methylation and mosaic type DNA methylation (Figure 16) that are constant within clades (Aliaga et al. 2018). Among animals, the DNA methylation levels are very variable. DNA methylation can be absent like in the nematode *Caenorhabditis elegans*, whose genome does not encode a DNA methyltransferase. Or sometimes, it is present at very low level, such as in *Drosophila melanogaster*, which has a very low DNA methylation level (Zemach, McDaniel et al. 2010).

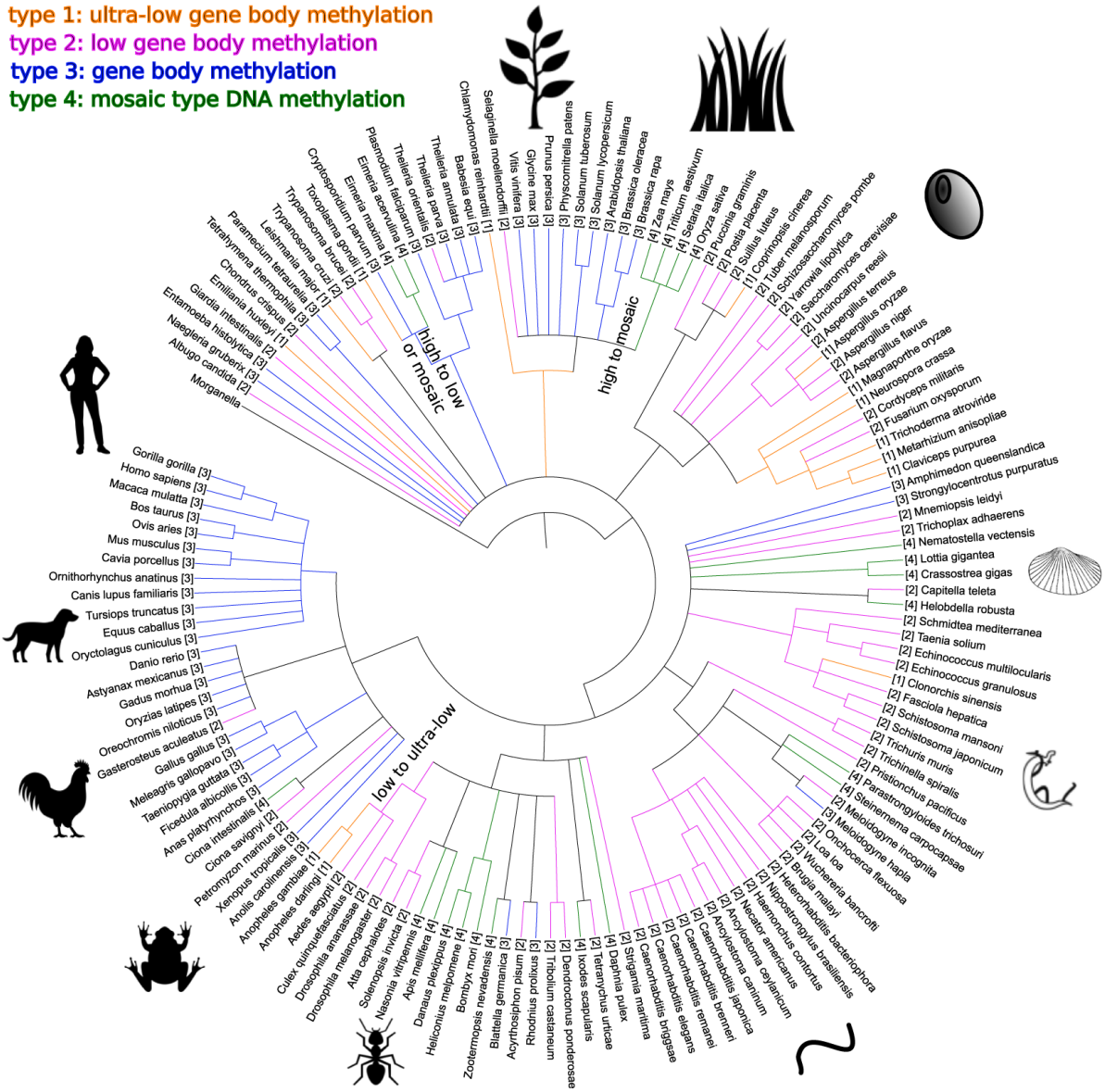

Figure 16. Representation of the four different types of DNA methylation across taxa. Colors correspond to methylation types. Image taken from Aliaga et al. 2018.

Many invertebrate exhibits a mosaic-type DNA methylation pattern consisting in broad domains of methylated DNA separated by broad domains of unmethylated DNA (Hendrich and Tweedie 2003). Intragenic methylation is a general attribute of invertebrate genomes and methylation of transposons (TE) and repetitive elements is only moderated.

Sequencing technologies have improved our knowledge on DNA methylation in arthropods, especially insects (Lyko, Ramsahoye et al. 2000, Lyko, Foret et al. 2010), bringing understandings

into the evolution of DNA methylation functions, but many invertebrate taxa are still poorly studied, such as lophotrochozoans (worms and mollusks).

DNA methylation in invertebrates is predominantly found in gene bodies (introns and exons), this type of methylation is called Gene Body Methylation (GBM), GBM is consensually considered as the ancestral form of DNA (Suzuki, Kerr et al. 2007, Feng, Cokus et al. 2010) and is observed in invertebrates, vertebrates and plants. The function of gene body methylation on gene expression is different from that of promoter methylation, promoter methylation is associated with repression of gene expression and GBM is related with active transcription in animals (Zemach, McDaniel et al. 2010, Sarda, Zeng et al. 2012). However, DNA methylation in invertebrates is less studied than in vertebrates, some studies have shown an association between the transcription level and the GBM (Rivière 2014), and have suggested a role of intragenic methylation in alternative splicing (Lyko, Foret et al. 2010), but functional effects of intragenic DNA methylation are barely addressed in many invertebrate groups such as the mollusks.

Multiple studies have documented the existence of DNA methylation in mollusks such as *Donax trunculus* (Petrović, Pérez-García et al. 2009), *Chlamys farreri*, *Patinopecten yessoensis* (Wang, Bao et al. 2008), C*rassostrea gigas* (Gavery and Roberts 2010, Rivière 2014) and *Biomphalaria glabrata* (Fneich, Dheilly et al. 2013). Nonetheless, the methylomes are only available for *C. gigas* (Gavery and Roberts 2013) and *B. glabrata* (Adema, Hillier et al. 2017).

The great variability of animal DNA methylation profiles reflects the existence of diverse roles for the DNA methylation among taxa. DNA methylation analyses of chordate genomes suggest that the evolution from mosaic to global methylation pattern happened at an early phase of vertebrate evolution. The lancelet *Branchiostoma lanceolatum* that is phylogenetically positioned at the base of the chordates (Theodosiou, Colin et al. 2011) has a typical invertebrate methylation pattern, whereas, primitive vertebrates such as the lamprey *Lampetra planeri* have a global methylation pattern that is characteristic of vertebrates (Tweedie, Charlton et al. 1997).

The pattern and the role of the DNA methylation function evolved and changed and its functions are not the same in invertebrates and vertebrates. Contrary to invertebrates, DNA methylation in vertebrates is present much more in the genome, a pattern known as global methylation and it occurs in the gene bodies, and repetitive DNA such as transposons or transposable elements (TEs) (Bird 1993). Methylation of promoter sequences has shown to moderate gene expression, and

methylation affects DNA repair stability, inheritable genome imprinting, development, germ cell pluripotency, maintenance and fate of cells (Schübeler 2015). DNA methylation in vertebrates is crucial for the development, aberrant methylation patterns are found in many diseases, like cancer, which displays an overall decrease on DNA methylation level and a hypermethylation of CpG islands in the promoter regions of the genes implicated in DNA repair, cell cycle regulation and apoptosis (Jones and Baylin 2007).

In plants, DNA methylation can occur in CpG, CHH and CHG contexts (H=A, T or C) and it is highly found in transposable elements (TEs) where it plays a role in the repression of its transcription (Gallego-Bartolomé 2020). DNA methylation in CG context is present in gene bodies in several plants species but its role remains poorly known, there is evidence that it suppresses intragenic antisense transcripts (Choi, Lyons et al. 2020).

Cytosine methylation has been the most-studied epigenetic mark in ecology with effects on trait plasticity. Variance in 5 methyl cytosine (5mC) levels has been demonstrated to increase in response to ecologically stress (Verhoeven, Jansen et al. 2010, Flores, Wolschin et al. 2013). DNA methylation can provide a pathway of rapid response for organisms living in highly fluctuating environments (Bossdorf, Richards et al. 2008), influence the evolutionary rate and trajectory of natural populations (Klironomos, Berg et al. 2013) and potentially lead to genome-wide epigenetic signatures. DNA methylation can be a biomarker of physiological conditions of individuals for past or present environmental pressure events as well as biomarkers of physiological conditions of individuals (Rey, Eizaguirre et al. 2020).

1. DNA methylation machinery in animals

The enzymes that add, identify and eliminate DNA methylation in animals are classified in three categories: writers, erasers and readers. Writers start the reaction of the binding of the methyl groups into cytosine. Erasers change and eliminate methyl groups and readers identify and fix to methyl groups to modify gene expression (Biswas and Rao 2018).

1.1. The writers of DNA methylation

The DNA methyltransferase (DNMT) enzymes catalyze the transfer of the methyl group from the co-substrate S-Adenosyl methionine (SAM) (Chen and Pellegrini 2006), in the case of cytosine methylation, the methyl group binds to the fifth carbon of a cytosine that is followed by a guanine (CpG motifs), transforming SAM into S-Adenosyl homocysteine (SAH) (Lyko 2018).

DNMT1 is the main DNMT founded in stem cells, its function is the conservation of methylation (Chen and Li 2004). Its target is the hemimethylated DNA at CpG sites. Hemimethylated DNA consists in only one strand of the double-stranded DNA (dsDNA) that is methylated. DNMT1 methylates the newly synthesized strands that are non-methylated, in this way it maintains CpG methylation patterns through mitosis (Edwards, Yarychkivska et al. 2017). DNMT1 has also the ability to repair DNA methylation, it conserves the pattern of DNA methylation that plays a major role in cellular differentiation (Moore, Le et al. 2013). The carboxy-terminal region of DNMT1 is responsible for transfer of methyl groups from SAM to cytosine. The carboxy terminal (C-terminal) region is the catalytic domain common in all cytosine DNA methyltransferases (Figure 17) (Robertson 2002).

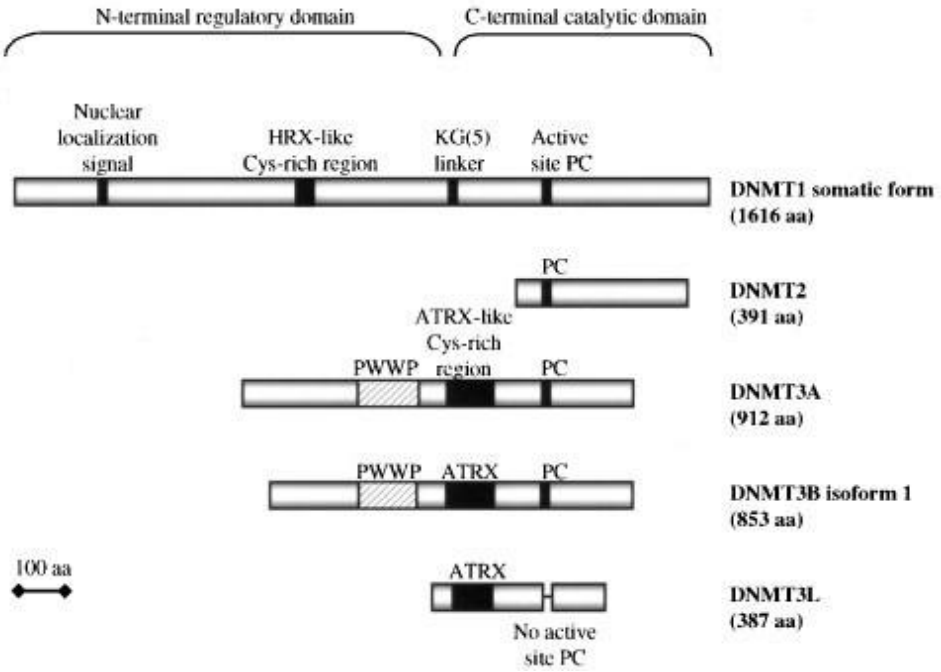

Figure 17. DNA methyltransferases found in mammals. C-terminal catalytic domain is found in the four DNMTs. Image taken from Robertson et al. 2002.

DNMT3 is a family of DNA methyltransferases that regulate the *de novo* CpG methylation, essential for forming genomic methylation patterns during development and reproduction of mammals and some invertebrates (Xie, Wang et al. 1999). DNA methylation patterns are established and preserved during development by DNMT1 and DNMT3 family methyltransferases (Hata, Okano et al. 2002).There are three members of the DNMT3 family : DNMT3A, DNMT3B and DNMT3L that share significant homology with DNMT1 being able to methylate hemymethylated and unmethylated DNA. DNMT3 family members share the ATRX domain (Figure 17) that is member of a family of chromatin remodeling enzymes suggesting that DNMT3 enzymes can be associated to chromatin changes (Bhattacharyya, De et al. 2020). DNMT3A and 3B have in common the PWWP domain (Figure 17) whose role remains largely unknown, there is a report suggesting that this domain is essential to target DNMT3A and 3B to a particular region of the heterochromatin (Chen, Tsujimoto et al. 2004).

Human DNMT2 is essentially known as a RNA methyltransferase since it methylates tRNA and not DNA (Lyko 2018). Its function as a DNA methyltransferase remains controversial because no clear finding about its DNA methyltransferase activity *in vivo* has been reported. DNMT2 uses the

catalytic mechanisms of DNMTs to move the methyl group from SAM to cytosine 38 of the aspartic acid transfer RNA (tRNA-Asp), the reaction results in methylation at the cytosine 38 in the anticodon loop and adenosylhomocysteine (AdoHcy) as a product (Goll, Kirpekar et al. 2006). Human DNMT2 methylates tRNA isolated from DNMT2 knock-out mutants of *Drosophila melanogaster*, *Dictyostelium discoideum* (Jurkowski, Meusburger et al. 2008) and *Arabidopsis thaliana* (Goll, Kirpekar et al. 2006).

DNMT2 is possibly the most highly conserved of all DNMTs across species, it is basically composed of the carboxy terminal region found in all cytosine DNMT enzymes (Figure 14). DNMT2-like proteins have been found in the insect *D. melanogaster* (Lyko, Ramsahoye et al. 2000) in the yeast *S. pombe* (Wilkinson, Bartlett et al. 1995) and in the plant *A. thaliana* (Song, Wu et al. 2010).

1.2. The readers of DNA methylation

There is a category of proteins with a high attraction for 5 methylcytosine (5mC) that inhibit transcription factor binding. These proteins are composed of three families: MBD, UHRF and zinc-finger proteins (Buck-Koehntop and Defossez 2013).

The MBD proteins are a highly conserved family of DNA-binding proteins that possess the common methyl CpG binding domain (MBD) that translates the information coded by methylation patterns into the appropriate function. Five members of the MBD family have been described in mammals, the primary structure of these proteins is not similar between them apart the MBD motif (Figure 18). Four members of the MBD family (MeCP2, MBD1, MBD2, and MBD3) are thought to have a function in transcriptional repression and the fifth member MBD4, has a DNA N-Glycosylase enzymatic activity and a possible role in DNA repairing (Fatemi and Wade 2006).

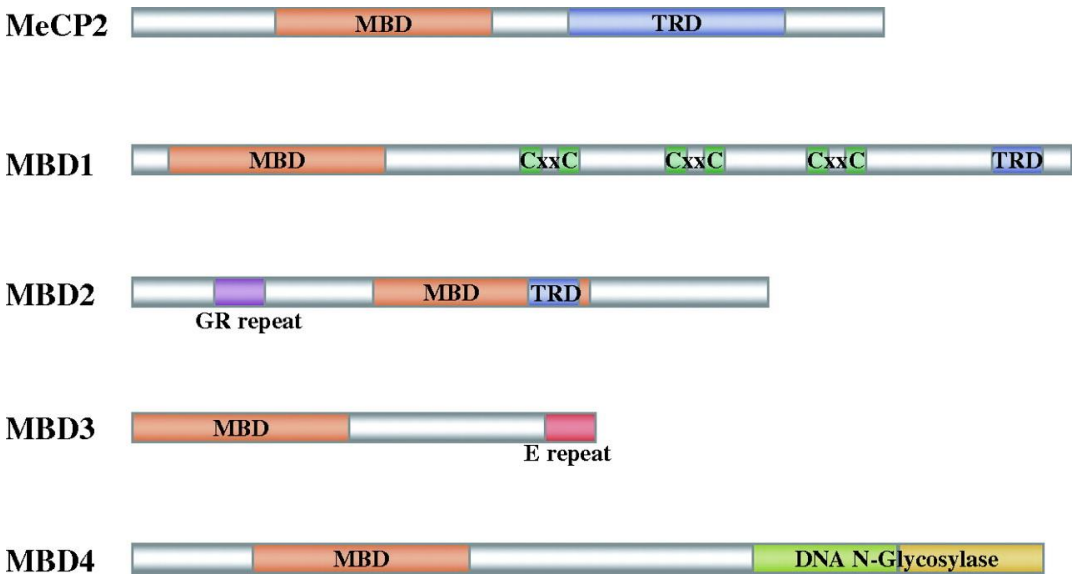

Figure 18. The five members of the MBD protein family. The 5 proteins share the methyl-binding domain (MBD). MBD1, MBD2 and MeCP2 have in common the TRD domain. MBD4 have a N-Glycosylase domain. MBD1 possess a cysteine-rich domain (CxxC). MBD2 possess a glycine and arginine repeat (GR repeat), and MBD3 possess a glutamate repeat (E repeat). Image taken from (Fatemi and Wade 2006)

MeCP2, MBD1 and MBD2 bind directly to methylated DNA and have a transcriptional repression domain (TRD) that permits to MBD proteins to bind to a variety of repressor complexes (Sarraf and Stancheva 2004). Moreover, MeCP2 recruits DNMT1 to hemimethylated DNA to maintain DNA methylation patterns (Kimura and Shiota 2003).

The UHRF protein family binds to DNMT1 and targets it to hemimethylated DNA to maintain DNA methylation. The UHRF proteins includes UHRF1 and UHRF2 that are multidomain proteins that bind methylated cytosines. There is a direct interaction between UHRF1 and DNMT1 but it is necessary to evaluate if the UHRF1 affects the specificity of DNMT1 to the hemi-methylated DNA (Hashimoto, Horton et al. 2009).

The zinc-finger domain proteins can inhibit transcription in a methyl-dependent way. Kaiso is a zinc finger protein member that intermediates methyl-CpG-dependent transcriptional repression (Buck-Koehntop, Martinez-Yamout et al. 2012). In *Xenopues laevis* it has been shown that Kaiso bind to a large number of methylated promoters to repress transcription, which is required during early development of this amphibian (Ruzov, Dunican et al. 2004). In human cells Kaiso also bind

to some methylated promoters and regulate transcriptional repression in a methylated locus together with N-CoR deacetylase complex that bring histone acetylation and methylation changes (Yoon, Chan et al. 2003).

1.3. The erasers of DNA methylation

DNA demethylation is the elimination of a methyl group from DNA, an essential mechanism for epigenetic reestablishment of genes. DNA demethylation occurs in recently synthesized DNA via DNMT1, active demethylation take place by the deletion of 5mC via the oxydation of cytosine by the ten-eleven translocation enzymes (TETs). There exists three TET-types (TET1, TET2 and TET3), these enzymes promote the DNA demethylation pathway (Figure 19). The TET-proteins remove the methyl group from the 5 methylcytosine (5mC) converting 5mC to 5 hydroxy methylcytosine (5 hmC), then 5 hmC to 5-formylcytosine (5 fC) and 5 fC to 5-carboxylcytosine (5-caC); modifications from 5mC to 5-caC are due to deamination and/or oxidation reactions (Kohli and Zhang 2013).

The existence of DNA demethylation suggests that DNA methylation is not always fixed but requires continues regulation, especially in mammalian cells (Bhutani, Burns et al. 2011). In mammals, TET enzymes have a function in transcriptional activation and repression (TET1), tumor suppression (TET2) and DNA methylation reprogramming processes (TET3). TET1 binds to CpG islands in human embryonic stem cells potentially maintaining the consistency of DNA methylation patterns in cells by maintaining hypomethylation at the CpG islands and TET2 is essential for the expression of genes involved in cell pluripotency (Neidhart 2015).

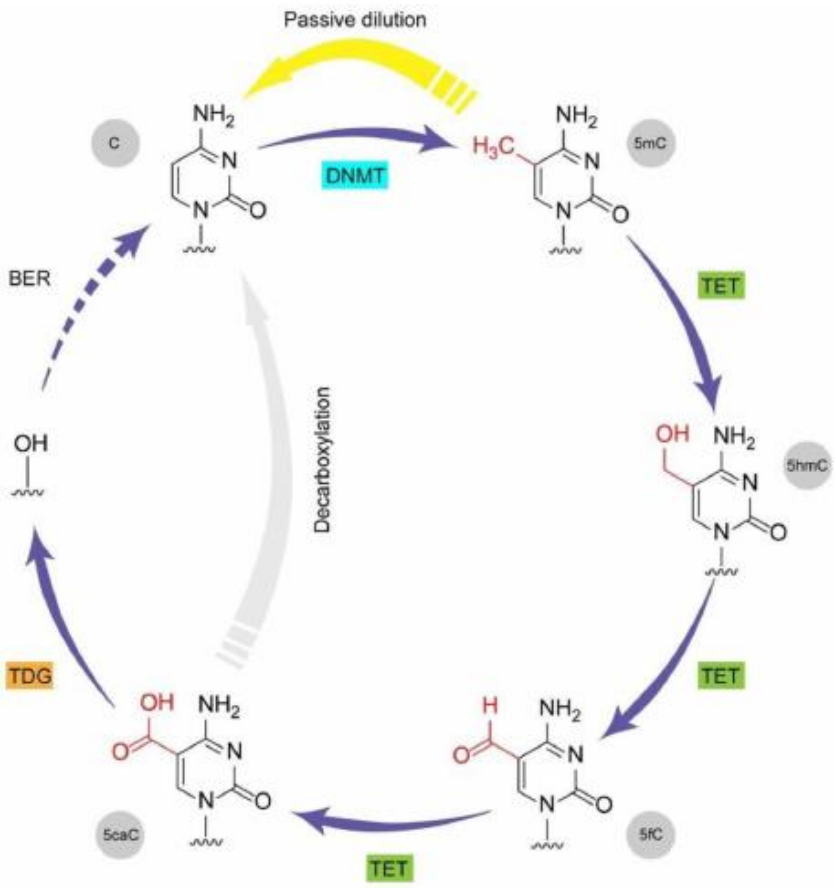

Figure 19. DNA methylation and demethylation pathways. DNMTs catalyze the methylation of cytosine. TET enzymes catalyze the demethylation by oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5 hmC). Additional TET-dependent oxidative reactions lead to the successive conversion of 5 hmC into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Image taken from (Xu and Walsh 2014).

2. Methods to measure DNA methylation.

2.1. Assessment of whole genome methylation

The first techniques to quantify DNA methylation involved the separation of methylated and unmethylated deoxynucleosides, such as the separation of purines and pyrimidines via paper chromatography (Vicher and Chargaff, 1948). Then 5-methylcytosine was measured quantitatively by reversed-phase high performance liquid chromatography (RP-HPCL). Then this method was improved with the incorporation of mass spectrometry with standard HPLC and later, restriction endonucleases, *MspI* and *TaqI* were added to distinguish between methylated and unmethylated CpG residues and digested DNA was labeled with isotopes to hydrolyzed DNA and then by separation in two dimensions through thin-layer chromatography (TLC) (Harrison and Parle-McDermott 2011).

A high-sensitivity approach to HPCL methods requiring smaller quantities of hydrolysed DNA sample is liquid chromatography combined with tandem mass spectrometry (LC-MS/MS). This technique has been validated to detect low levels of methylation and another advantage is that it is not negatively impacted by degraded DNA which makes the utilization of this method in a wide-range type of conserved samples (Kurdyukov and Bullock 2016).

If was found that methyl cytosine could bind to particular antibodies (Adouard, Dante et al. 1985). The use of anti-5mC monoclonal antibodies coupled with secondary antibodies labeled with a fluorescent marker is an effective method to investigate DNA methylation global changes. The most known and used immunological DNA methylation assay is the ELISA-based, which enables quick assessment of DNA methylation status and there are several commercially available kits, but they are suitable for the rough estimation of global DNA methylation as it showed high sample variability. Besides, the bases of this method can be applied to effectuate a practical and cost-effective method called dot blot used mainly in biomedical research (Jia, Liang et al. 2017).

Currently, next generation sequencing (NGS) procedures are applied to DNA methylation measuring, allowing for massive analysis of the methylation status of nearly each CpG site and the capture of the methylome, that is the pattern of DNA methylation marks across the entire genome. There exist three genome-wide approaches for DNA methylation profiling: a) Affinity

Enrichment-based methods, b) Restriction enzymes-based methods and c) Bisulfite conversion-based methods (Pajares, Palanca-Ballester et al. 2020).

a) Affinity Enrichment-based approaches

These methods use antibodies and methylated-CpG binding proteins to capture the methylated genomic regions for subsequent sequencing (Yong, Hsu et al. 2016). The method Methylated DNA immunoprecipitation (MeDIP) consist in sonicating and fragment genomic DNA (in 300-1000 bp segments) and subsequent incubation with 5mC antibody. Immunoprecipitations is then applied by magnetic beads with a secondary antibody, then DNA is recovered from beads. MeDIP technique can be combined with array-based hybridization (MeDIP-chip) or high-throughput sequencing (MeDIP-seq). Nevertheless, MeDIP-seq replaced the use of microarrays by NGS techniques that have become affordable, and this technique is useful for characterizing enriched CpG regions (Yong, Hsuet al. 2016). The principal inconvenience of MeDIP-seq is that methylation at single base-pair resolution and hemi-methylated sites cannot be recognized (Neary and Carless 2020). MeDIP-seq data is presented as differentially methylated regions (DMRs) there are peaks of methylation enrichment through the genome, has a bias toward hypermethylated regions, sparse methyl-CpG regions can be underrepresented and unmethylated regions must be indirectly recognize by a lack of reads (Neary and Carless 2020).

b) Restriction enzyme-based methods

This approach uses principally the restriction enzymes *MspI* and *HpaII*, a combination of enzymes that was used in the earliest DNA methylation approaches (Cedar, Solage et al. 1979), both enzymes identify the same CCGG motif but have distinct sensibilities to DNA methylation enabling discrimination between methylated and unmethylated DNA sequences. Then combined with high throughput sequencing (HELP assay and Methyl-Seq) this approach provides wide-ranging coverage of DNA methylation positions (Oda, Glass et al. 2009).

The major disadvantage of this approach is the inability to cover regions of interest and therefore low coverage as a consequence of the dependency on the location of restriction positions in the genome, which impedes the application of this approach in genomes with scarce restriction sites (Yong, Hsu et al. 2016).

c) Bisulfite conversion-based methods

The genome –scale DNA methylation analysis with nucleotide resolution rely mostly in bisulfite sequencing. DNA sequencing technologies cannot distinguish methylcytosine from cytosine, and the bisulfite treatment of DNA makes possible to disentangle methylated cytosines from non-methylated ones by deaminating unmethylated cytosines that are converted to uracil and will be sequenced as a thymine whereas methylated cytosine is unaffected by the treatment (Frommer, McDonald et al. 1992, Grunau, Clark et al. 2001). This approach can be extended to the whole genome with next generation sequencing. The "gold standard" method to measure DNA methylation is the whole genome bisulfite sequencing (WGBS), which assesses methylation level of each cytosine in the genome but this method is limited to species with high-quality reference genome and is prohibitively expensive for large sample numbers (Kurdyukov and Bullock 2016), making this epigenomic technique accessible for only a few model species. A prerequisite for ecological studies is to episequence large sample numbers, and recently, several approaches have been developed based on reduced-representation of the genome by combining the method with restriction enzyme digestion, providing a cost-effective alternative to WGBS such as Reduced Representation Bisulfite Sequencing (RRBS) (Meissner, Gnirke et al. 2005, Gawehns, Postuma et al. 2020).

RRBS allows the sequencing of a representative fraction of the genome, this approach is scalable for ecological experimental designs. Nevertheless, RRBS is designed to enriches for CpG islands, which is a common feature in mammalian genomes. CpG islands are genomic regions with numerous nonmethylated CpG sites, these regions represent around 1% of the genome and cover promoters and DNA replication origins (Antequera 2007). RRBS method limits in terms of enzyme choice, multiplexing level and necessitates a reference genome for effective mapping and variant calling (Gu, Smith et al. 2011, van Gurp, Wagemaker et al. 2016).

There exist other methods alternative to RRBS that have adapted protocols and bioinformatics analysis to the species lacking a reference genome and that have different patterns of DNA methylation. Some of these techniques are epigenotyping by sequencing (epiGBS), bisulfite-converted restriction site associated DNA sequencing (BsRADseq) and bisulfited RAD-seq (EpiRADseq), whose principally differences are described in Table 1.

Table 1. Differences between reduce representation bisulfite sequencing techniques: RRBS, epiGBS, BsRADseq and EpiRADseq.

	RRBS	**epiGBS**	**BsRADseq**	**EpiRADseq**
Restriction enyme(s)	Methylation insensitive *MspI*, cuts at C/CGG site	Methylation insensitive multiple combinations possible*:* *Csp6I-NsiI* *AseI-NsiI* *PacI-NsiI*	Methylation insensitive *SbfI*	Methylation sensitive *HpaII* and another methylation insensitive: *PstI*
Adapters	Contained methylated cytosines so they are not converted to uracil in the bisulfite conversion step	Non-phosphorylated adaptors and a control non methylated Cytosine is added in the adapters for Watson/Crick annotation of reads and to calculate bisulfite conversion rate	Methylated P1 and P2 barcoded adapters and a short version of the P1 sequences is chosen	Double-stranded sequencing adapters
Size selection	Adaptor-ligated DNA is size-selected on a gel	Adaptor-ligated DNA is purified by column-based and SPRI bead cleanup	Non purification step is done after adapters ligation and before bisulfite conversion	Purification using AMPure beads before adapters ligation, and after adapters ligation, samples are size selected in a electrophoresis platform
Sequencing	Single-end sequencing in Illumina HiSeq with 50 bp read	Paired-end sequencing (2 × 150 bp) using an Illumina NextSeq550 or HiSeq instrument	Illumina HiSeq as 100 bp paired-end reads	Illumina Miseq using 168-bp paired-end reads.
Pipeline workflows	Raw reads are filtered and trimmed. After quality metrics, reads are mapped to the reference genome followed by methylation calling with Bismark in a single step which discriminated the three context of Cytosine methylation. Several alignment tools can be used for RRBS analysis such as BS-Seeker, bwa-meth, BSMAP and GSNAP (Sun, Han et al. 2018).	The bioinformatics epiGBS pipeline was embedded into snakemake workflow with conda environments. The first step is the identification of PCR clones identified. Then the reads are demultiplexed, only the reads with expected restriction enzymes overhang are retained and a Cytosine is added as a control nucleotide (CN) in the adapters, allowing Watson/Crick annotation of reads. Mapping is performed with the alignment program STAR (version 2.5.3). and SNP and methylation sites are called by custom scripts (van Gurp et al. 2016; Gawehns et al. 2020).	Demultiplexing and filtering is done with Stacks. Then reduced reference genome is constructed using custom Python scripts, BSRADseq libraries are mapped and methylation information is extracted using Bismark. Results across individual samples are summarized using custom Python scripts (Trucchi, Mazzarella et al. 2016).	EpiRADseq bioinformatics analysis consist in filtering of PCR clones and demultiplexing using Stacks v.1.19. Reads are then quality-filtered and trimmed using TRIMMOMATIC v0.32. A pseudo-reference genome can be created by clustering reads and assembling contigs using RAINBOW v.2.02 and CD-Hit v 4.6. Then BWA is used to map reads for each sample to the pseudo-reference contigs (Schield, Walsh et al. 2016).

epiGBS is a technique appropriated for model and non-model organisms due to the free choice of restriction enzymes and to the possibility to sequence the genetic and epigenetic information even in species without reference genome. epiGBS extends genotyping by sequencing (GBS) combined with bisulfite conversion allowing an alternative method to RRBS that is usually high efficient in vertebrate genomes, besides this method has a substantial reduction in per-sample costs (van Gurp, Wagemaker et al. 2016). epiGBS allows to obtain DNA methylation polymorphisms and single nucleotide polymorphisms (SNPs) by the reconstruction of the *de novo* consensus sequences. Furthermore, recent improvements in the computational analysis were done to do more user friendly the reproducibility of the bioinformatics analysis (Gawehns, Postuma et al. 2020).

BsRADseq is a RRBS method that combines restriction site associated DNA sequencing (RADseq) with bisulfite sequencing. This technique can be used in non-model organisms lacking a published genome, but for that, single loci construction is done from standard RADseq library taking an aliquot of digested DNA with the restriction enzyme *SbfI* before bisulfite conversion (Trucchi, Mazzarella et al. 2016).

EpiRADseq is another reduced representation of bisulfited DNA sequencing to assess the methylation level of multiple genes. This method allows to quantify changes in methylation, DNA digestion is done with the enzyme (*HpaII*) that enriched genes lacking 5mC. If a locus is methylated, no EpiRADseq reads will be acquired, while if a locus is unmethylated, epiRADseq reads will be sampled (Schield, Walsh et al. 2016).

DNA methylation marks are less stable than DNA sequence information and they are limited to few adjacent genomic elements that may be undetected in RRBS methods, and if a trait is controlled by few functional loci, these may be missed in RRBS and this problem is intensified in the long and difficult genomes. Differentially Methylated Regions are hard to delimit in whole genome studies, and the short fragments sequenced with RRBS (< 500 bp) will cover a limited number of cytosines, therefore DMRs calling will be difficult, most RRBS studies will be limited to identify differentially methylated cytosines (DMCs) with their inherent statistical probability (Paun, Verhoeven et al. 2019).

The RRBS practices are flexible allowing for numerous methodical ways to improve them, the selection of restriction enzymes defines the regions of the genome that will be sampled an *in silico* digestions can be can be done to determine the best option. Enhancements can also be done by

sequencing in platforms that yield longer reads e.g. MiSeq as a substitute of HiSeq. The emergence of several RRBS methodologies permits to study epigenetics in non-model species, however for a better understanding and to identify genomic features that have differential methylation, a reference genome continues to be desirable (Paun, Verhoeven et al. 2019).

2.2. Targeted DNA methylation analysis

Several methods exist to measure targeted DNA methylation: pyrosequencing, targeted bisulfite sequencing, quantitative methylated DNA immunoprecipitation (qMeDIP), methylation-sensitive high-resolution melting (MS-HRM) (Cheishvili, Petropoulos et al. 2017) and combined bisulfite restriction analysis (COBRA) (Xiong and Laird 1997).

Pyrosequencing is a real-time DNA sequencing-by-synthesis, that quantify pyrophosphate ion discharge and uses a biotinylated primer that is integrated into PCR product. This method allows the measurement of DNA methylation for thousands of samples. This method is suitable for the sequence of short length reads ranging from 25 to 100 bp (Elahi and Ronaghi 2004).

Targeted bisulfite sequencing combines DNA sequencing and bisulfite conversion allowing the quantitative recognition of methylcytosine at a nucleotide resolution. Bisulfite converted DNA can be examined by sanger sequencing, a method that have a sequence read length of about 500 bp, this method involves the amplification of the target DNA sequence by a chain-termination PCR that adds dideoxyribonucleotides (ddNTPs) (Sanger, Nicklen et al. 1977). 5 methylcytosine level can be assessed by direct measure of the fluorescent peak of each non converted and converted cytosines in the chromatogram output (Jiang, Zhang et al. 2010).

With the advent of NGS technologies, cytosine methylation of targeted loci can be assessed in significant numbers of samples, one of these methods is the Bisulfite Amplicon Sequencing (BSAS) which uses bisulfite conversion and amplicon sequencing combined with next-generation library construction, allowing high-throughput sequencing providing a method with high quantitative accuracy to examine cytosine methylation in targeted genomic regions (Masser, Stanford et al. 2015).

qMeDIP combines the MeDIP and qPCR techniques allowing to measure the relative enrichment of DNA methylation in particular loci targeted by specific primers, this technique provides the methylation status of the target loci but unlike bisulfite sequencing cannot distinguish between the number of CpGs in a methylated region (Cheishvili, Petropoulos et al. 2017).

MS-HRM combines bisulfite conversion and high-resolution melting, bisulfite DNA is input for a PCR amplification with the melting phase integrated. Double-strand disassociation of the melting phase is quantified by fluorescence; the amount of methylated DNA is determined by comparison of the melting profiles derived from controls with known methylation level (Cheishvili, Petropoulos et al. 2017).

COBRA combines bisulfite converted DNA amplification with restriction digestion, the percentage of methylation can be assessed by calculating the ratio between the digested PCR product and the total amount of PCR product (Xiong and Laird 1997)

V. Mollusk species used as biological models.

1. The freshwater snail *Biomphalaria glabrata*

Biomphalaria glabrata is a gastropod mollusk of the family Planorbidae that distributes from the Greater Antilles to Brazil, in the Lesser Antilles and in Venezuela (Paraense 2001, Mavárez, Steiner et al. 2002). *B. glabrata* is the intermediate host of the parasite trematode *Schistosoma mansoni*, the causal pathogen of the disease called schistosomiasis (Toledo and Fried 2010). As intermediate host, *B. glabrata* has a main role in the transmission of the schistosomiasis. Currently the most used method to combat the snail is the use of molluscicides, but they are very toxic for aquatic organisms and have only limited efficiency since snails become rapidly resistant (Souza 1995). An alternative approach to combat the disease is through controlling the mollusk (Geyer et al. 2017).

The parasite *S. mansoni* has a complex life cycle, it needs two obligatory hosts, the intermediate host that is the snail *B. glabrata*, where it multiplies asexually and the definitive host, a human or rodent, where it effectuates the sexual reproduction. The parasite develops into adult inside the vertebrate host, it reproduces sexually in the venous system, the females produce 200-300 eggs per day. The eggs possess a spike that allows them to perforate the intestine wall and then they are released in the feces while many other eggs are transported to the liver where they accumulate and provoke inflammations and the hepatic fibrosis (Nelwan 2019). The eggs that are liberated by the urine (urine schistosomiasis) or feces (intestine schistosomiasis) hatch when they are in contact with the freshwater, they release the larval stage called miracidium, this miracidium needs to infest a planorbid snail of the genus *Biomphalaria*, and inside the mollusk the parasite effectuates its asexual multiplication where a miracidium transforms into primary sporocysts and then into secondary sporocysts. From the sporocysts it develops into new larvae named cercariae which are released from the snail into freshwater ecosystems such as lakes. Cercariae are free swimming larvae able to infect the definitive host: they penetrate the skin of humans (or rodents) who are present in those infested lakes (Nelwan 2019).

Biomphalaria snails differ in their compatibility to schistosomes, some are resistant to infection while others are susceptible, this is due to a phenomenon called compatibility polymorphism with the parasite *S. mansoni*: snail and parasite populations are either compatible (infection is possible) or incompatible, with variant degrees of compatibility in between (Theron, Rognon et al. 2014,

Mitta, Gourbal et al. 2016). In a compatible snail the larva miracidium is able to penetrate the snail and infection begins in the foot and then develops into a primary sporocyst. On the other hand, an incompatible snail will encapsulate the sporocyst by haemocytes and will destroy the parasite by a cytotoxic reaction involving free radicals (Hahn, Bender et al. 2001).

The term 'resistant' can be applied to those individuals within a single snail species that are capable to avoid infection by a species or strain of schistosome that is compatible, meaning able to infest that species of snail (Lockyer, Spinks et al. 2007). Compatibility is strain specific, one snail strain that is very vulnerable to one particular strain of schistosome could be relatively or totally unsusceptible to another one (Webster and Davies 2001, Theron and Coustau 2005).

The study of the molecular basis of susceptibility and resistance phenotypes in snails could open promising alternatives to current schistosomiasis control measures. Traditionally, genetic mutations were considered the sole basis for heritable phenotypic variants. Indeed, genetic loci for snail incompatibility/resistance have been mapped but they seem to be strain dependent. We know today that heritable phenotypes can be produced without modifications in the genotype and that behind these variations are the epigenetic mechanisms. The relative contribution of genetic and epigenetic components of the *B. glabrata* compatibility to the parasite is the topic of a lively academic debate (Knight, Ittiprasert et al. 2016, Sullivan 2018).

Interestingly, immune defense has been shown to be improved when snails are early exposed to the parasite, after the first infection, *B. glabrata* is able to generate an anticipatory response for a future infection (Pinaud 2017), in the second exposure to the parasite, the snail is able to generate an better immune response, as it has been observed in vertebrates, a phenomena which is called the innate immune memory (or trained immunity) (Netea, Joosten et al. 2016). The trained immunity suggests that a somatic mechanism carried by epigenetic processes improves the immune response of an individual, these modifications could affect germ cells and thus participate in the transmission of protection to offspring (Moret and Siva-Jothy 2003).The contribution of epigenetics in immunity priming of the snail *B. glabrata* is under current investigation at the IHPE laboratory.

Beyond medical perspectives, *B. glabrata*, is an interesting biological model to study its population biology (Toledo and Fried 2010). These snails live in temporal ponds of freshwater that can be

desiccated in the summer season, implying an ephemeral habitat with metapopulational dynamics (Jarne and Théron 2001), nevertheless the contribution of epigenetic and genetic marks to the population dynamics has not been addressed. Furthermore, the mating system can be studied in this species because as a self-fertile hermaphrodite constitute a model to study the evolution of the self-fertilization (Jarne et al. 2010). Self-fertilization in this snail allows snails to reproduce when they are isolated (Coyne and Orr, 2004) and this can be used in laboratory studies to decrease their genetic background and study the bases of phenotypic variation when genotypic variation is absent.

DNA methylation machinery was found to be present in the snail *B. glabrata*, BgDNMT1, BgDNMT2 and BgMBD2/3 are the enzymes responsible of cytosine methylation that is principally found in a CpG context (Fneich et al. 2013). The methylome of this species showed a classical mosaic-like invertebrate pattern with a bimodal distribution (Adema et al. 2017). It was recently found that the trematode parasite *S. mansoni* is able to modify the transcript abundance of the DNA methylation machinery enzymes of the snail *B. glabarata* during infestation, suggesting that the DNA methylation of the snail could have a role in their interaction with the parasite (Geyer, Niazi et al. 2017).

Nevertheless, the contribution of DNA methylation to the phenotypic variability of *B. glabrata* has not been assessed. There are some studies showing how biotic and abiotic stress can impact DNA methylation of the snail but neither of them have evaluated the stability of the impacts across generations nor the effect of DNA methylation changes in the phenotypic variability of the snail.

2. The freshwater snail *Physa acuta*

Physa acuta is an aquatic pulmonate snail originating from North America. It is a highly successful invader worldwide. The high expansiveness of this species allows its distribution on various continents that was probably originated from the accidental dispersal mediated by humans through the transportation of exotic plants (Vinarski 2017). This snail is a biomonitoring organism due to its high rate of metal accumulation and its sensitivity to metal contaminants (Spyra, Cieplok et al. 2019).

P. acuta has a short generation time of 35 days that makes it a good model for multigenerational studies. Moreover, *P. acuta* is self-fertile freshwater but it is preferentially outcrosser with strong inbreeding depression and it showed partial selfing in natural populations, making this snail a good model to study the consequences of inbreeding depression in the fitness of organisms (Jarne, Perdieu et al. 2000). Indeed, studies about the selfing impact have been done, finding that repeated selfing reproduction decreases the adaptive potential of population and that selfing provides reproductive assurance but only during a few generations because when prolonged, it increases the possibility of losing genetic variation and the capacity to adapt. It has been also found that phenotypic variance is higher under selfing and that it improves the response to selection at the beginning but then it becomes less responsive to selection (Noël, Jarne et al. 2017), these results inferred that the phenotypic variance under selfing increase in non-genetic components of variance and reduced developmental homeostasis provoking a "decanalization" that is the loss of a stabilized well-adapted phenotype, but the evolutionary forces causing decanalization are still unkown. *P. acuta* allows experimental evolution studies by evaluating the influence of self-fertilization on adaptive responses to environmental variation, genetic variance is believed to be the sole source of phenotypic variation and this model allows the opportunity to evaluate if, in the absence of genetic variance, phenotypic plasticity is observed.

One of the non-genetic aspects that has begun to be studied in *P. acuta* is the environmental responsiveness of its DNA methylation. It has been demonstrated that *P. acuta* showed DNA methylation changes in response to the exposure to environmental chemical pollutants such as the glucocorticoids (Bal, Kumar et al. 2017) and the fungicide vinclozolin (Müller, Charaf et al. 2016, Sánchez-Argüello, Aparicio et al. 2016). Furthermore, these chemical pollutants not only change their DNA methylation, but they also triggered phenotypic effects, e.g. in the shell structure and

size of exposed snails. DNA methylation measurement has been reported in *P. acuta* in the three studies mentioned previously (Muler et al. 2016, Sanchez-Arguello et al. 2016 and Bal et al. 2017) that allow to infer that DNA methylation in a CpG context is present in its genome. The methylome has not been sequenced, but the presence of 5mC has been detected through immunoassay-based methods and since the reference genome has been recently sequenced (GenBank: RDRX00000000.1), this could allow the application of bisulfite sequencing approaches in the time to come.

P. acuta is also a good model to study phenotypic plasticity, Physidae snails develop adaptive phenotypes in response to predation risk, e. g. crayfish induce within-generational plasticity on life-history traits such as a delay of age at maturity, a larger size, escape behavior and changes in shell thickness and size. The physa snails exposed to the predation risk inherits phenotypic changes provoked by this stress to the two subsequent generations, nevertheless it is still unknown for how many generations transgenerational effects of anti-predator responses can persist (Tariel, Plénet et al. 2020).

Phenotypic plasticity and DNA methylation has begun to be studied in *P. acuta* but an experimental approach allowing to distinguish the contribution of DNA methylation and genetic variation to the phenotypic plasticity of this species has not been applied.

3. The marine oyster *Crassostrea gigas*

The pacific oyster *C. gigas* is an estuarine species and it can be also inhabiting in intertidal marine zones, it has a wide-ranging salinity tolerance from 20 to 25‰ and can live in temperatures between -1.8 to 35°C. Due to its rapid development and extensive ranging tolerance to environmental conditions, *C. gigas* has become the oyster of choice for cultivation in various regions of the world. The species is very fecund with long length females (15 cm) producing 50-200 million eggs in a single spawning. Nevertheless, *C. gigas* suffers from a lot of disease with high mortality rates. One of this disease is currently studied in the IHPE laboratory: The Pacific Oyster mortality syndrome (POMS) which affect juveniles, and which has a high incidence in littoral regions of many countries principally France. It is provoked by the virus OsHV-1, which induces an immunosuppression in the oyster. The suppression induces a dysbiosis (microbiota imbalance) which finally results into septicemia (de Lorgeril, Petton et al. 2020). Many research efforts have been done recently to understand the causes of this disease, and biotic and abiotic factors contributing to the disease outbreak are currently under investigation into the IHPE laboratory.

C. gigas has been one of the few mollusks species whose DNA methylation has been intensively studied, it is an interesting model to study this epigenetic mark due to its particular life traits, and its economic and ecological importance. The DNA methylation in *C. gigas* is intragenic and display variations depending on physiological contexts. Furthermore, DNA methylation influences gene expression and is essential for oyster development probably by regulating the homeobox genes expression (Riviere, Wu et al. 2013). The three enzymes implicated in established and maintenance of DNA methylation (DNTM1, DNMT2 and DNMT3) are present in the oyster *C. gigas* (Wang, Li et al. 2014). In fact, this oyster is one of the few invertebrates that possess the three methylation writer's enzymes since in other invertebrates, including mollusks, DNMT3 is absent (e.g. *B. glabrata*).

In *C. gigas* methylome, the majority of the methylated cytosines are found in a CpG context, and the methylated CpGs are highly found in gene bodies, particularly exons and high methylation preferentially occurs in highly and stably expressed genes (Wang Li et al. 2014). Furthermore, it was found that CpG methylation in *C. gigas* preferentially targeted genes originated in the last common ancestor (LCA) of eukaryotes rather than the oldest genes of the LCA of cellular

organisms, this finding suggests that the appearance of methylation regulation in the most recent genes was essential for the origin and radiation of eukaryotes (Wang, Li et al. 2014).

Environmental exposure to chemical pollutants was shown to change the levels of DNA methylation in *C. gigas*, it was demonstrated that the diuron herbicide triggered methylcytosine changes in the offspring of exposed oysters. This exposure has been performed during gametogenesis demonstrating a multigenerational effect (Rondon et al. 2017).

Recently, the methylation landscape of two groups of oysters inhabiting in different temperatures (temperate and high) was assessed, they found that irrespective of the temperature, oysters inhabiting the intertidal zone exhibited hypomethylation and high plasticity of methylation in response to heat shock while oysters occupying subtidal zone showed hypermethylation and low plasticity, these results suggest that DNA methylation intermediates phenotypic divergence in oysters adapting to different environments suggesting that DNA methylation can produce phenotypic plasticity in response to rapid climate change (Wang, Li et al. 2020).

Thesis Objectives

There is wide evidence that phenotypic plasticity observed in organisms is due to the interaction between genetic and non-genetic information. Nevertheless, for a long-time only gene-centered approaches were used to explain phenotypic plasticity and adaptation in species. The objective of my thesis is to analyze if non-genetic information has an impact in phenotypic plasticity and if this is transmitted through generations. The understanding of the molecular mechanisms that allows the rapid adaptation of mollusks that are vectors of parasites, to new environments is important for disease control. Rapid adaptation is difficult to explain by traditional Mendelian genetics and there is strong evidence supporting that epigenetic mechanisms are behind rapid adaptations in other species.

I focused on a particular non genetic information called epigenetics, which consist in a layer of information that defines how the genes are going to be used by a cell, this information consists in biochemical modifications that do not change the nucleotides of the DNA sequence and can be transmitted through mitosis and sometimes also through meiosis. I studied one epigenetic mark called DNA methylation that has demonstrated to be environmentally modulated and to play a role in phenotypic plasticity in many species, principally plants and vertebrates. Nevertheless, the role of DNA methylation in generating phenotypic variation in invertebrates has been less studied.

We addressed the question of the role of DNA methylation in the generation of phenotypic plasticity and its heritability in an invertebrate model, the snail *B. glabrata*, the intermediate host of the parasite *S. mansoni*, the causal agent of schistosomiasis, a neglected tropical disease. DNA methylation in *B. glabrata* has been found to be regulated by the infection of the parasite *S. mansoni* (Knight, Ittiprasert et al. 2016) and by environmental stress (Geyer, Niazi et al. 2017), furthermore it was demonstrated that DNA methylation affects its gene expression (Ittiprasert, Miller et al. 2015), suggesting that DNA methylation can affect phenotypic variation and possibly the adaptation of the snail to new environments.

To respond to the principal question of this thesis, experimental manipulation of the DNA methylation in the snail was necessary to effectuate changes in its DNA methylation, and then, study the effects of these modifications in its phenotypic variation.

Therefore, two approaches were proposed in this thesis to induce epimutations in the snail *B. glabrata*:

1) Random epimutagenesis using chemical DNMT inhibitors and by consequent segregation of epimutations in self-fertilization lines and

2) Methylate the cytosines of a targeted locus with an epigenetic engineering editing tool consisting in the use of plasmids coding for the DNA methyltranferase DNMT3 fused to the nuclease-inactivated dCas9.

For the first approach,

- An efficient DNA methyltransferase inhibitor (DNMTi) was necessary to be able to pharmacologically modify the DNA methylation of the snails. I tested new DNMTi based on those used in cancer research and that were synthesized by our collaborators from the Institute of Biomolecules Max Mousseron (IBMM) in Montpellier, France; from the Institute Pasteur in Paris, France and from the Laboratory Epigenetic Targeting of Cancer (ETaC), CNRS-FRE3600 in Toulouse, France.

- To test whether the new DNMTi that showed inhibitory efficiency in human cancer cells were efficient also in the snail *B. glabrata*, a large-scale screening method of global DNA methylation that allowed us to measure 5 methylcytosine (5mC) in multiple samples at a low cost was optimized and compare to a commercial 5mC DNA ELISA-based Kit.

- Once the method of screening was optimized, and a DNMTi efficient was found, a cost-effective method that allows us to explore genomic regions impacted by the DNMTi treatments was required. I tested an epigenotyping sequencing method called epiGBS for the first time in a mollusk species and I compared it to WGBS to evaluate if it is a reliable method to identify differential methylated regions following a pharmacological manipulation of the DNA methylation.

- A multigenerational experimental approach was proposed to study the contribution of genetic and epigenetic information to the phenotypic variability of the snail *B. glabrata*.

For the second approach,

- The optimization of a transfection technique was done to be able to introduce targeted epimutations to the embryo of the snail *B. glabrata*.
- An epigenetic engineering tool validated in human cancer cell lines was tested *in vivo* in the embryo of the snail *B. glabrata*.

Chapter I. Pharmacological inhibition of DNA methylation to study the contribution of this epigenetic mark to the phenotypic variation of a mollusk species.

Pharmacological manipulation of DNA methylation

DNA methylation is an epigenetic carrier with significant therapeutic value as this mark is involved in cancers, neurological and cardiovascular disorders in humans and mammals (Kulis and Esteller 2010). As an example of that, the repression of certain tumor-suppressor genes arises as a result of hypermethylation in their promoter regions, several studies have proved an extensive variety of genes repressed by DNA methylation in cancers (reviewed in Kullis and Esteller 2010). For this reason, DNA methyltranferase inhibitors (DNMTi) have been developed to be used as epigenetic modulators for their application in cancer therapy. The most successful DNMTi are 5 azacytidine (5-AzaC) and decitabine (5-azadC) the only two DNMTi approved by the Food Drug Administration (FDA) for the treatment of acute myelodysplastic syndrome and chronic myelomonocytic leukemia (Edlin, Connock et al. 2010). However, these molecules display toxicity as they are nucleoside analogues that require to integrate into DNA/RNA to start their mechanism of action, and this integration is not cell-specific leading to undesired side effects and poor chemical stability. This has raised the necessity to find more efficient DNMTi, especially non-nucleoside analogues that present less toxicity and potentially more specificity for DNA methylation (Lopez, Halby et al. 2016, Pechalrieu, Etievant et al. 2017). Another cytidine analogue is Zebularine that is more stable that 5-AzaC and 5-azadC and has been shown to be effective inhibitor of DNA methylation in cancer cell lines (Cheng, Matsen et al. 2003).

The cytidine analogues 5-AzaC, 5-azadC and Zebularine have been widely used to pharmacologically modify DNA principally in mammals (Cashen, Schiller et al. 2010, Fenaux, Gattermann et al. 2010) and some plants species (Griffin, Niederhuth et al. 2016), and only in few invertebrate species (Table 1). In invertebrates, this has been made mainly in insects (e. g. *Nasonia vitripensis*, *Apis mellifera*, *Achroia grisella, Apis cerana, Drosophila* cell line*),* in mollusks *(Aplysia californica, Crassostrea gigas, Biomphalaria glabrata* and *Lymneaea stagnalis),* in crustaceans *(*e.g. *Daphnia magna),* in platyhelminthes *(*e.g. *Schistosoma mansoni),* in echinoderms *(*e.g. *Sphaerechinus granularis, Paracentrotus lioidus* and *Psammechinus miliaris*) and in ascidians (e.g. *Phallusia mammilata*). The effect of this DNMT inhibitors has been tested on the phenotype and epigenotype level although sometimes only one of these both information was evaluated, e.g. in *A. grisella* and in *B. glabrata* the effects of the DNMT inhibitor were only evaluated at the phenotypic level but the effect at the epigenotype was not assessed.

Table 1.1. Invertebrate species where the DNMTi 5-azacytidine, 2'-deoxy-5-azacytidine and Zebularine have been tested.

Species	**DNMTi used**	**Epigenotype effect**	**Phenotypic Effects**	**Referece**
Insect: *Drosophila* embryonic cell culture,	5-AzaC	Not assessed	Obstructs cellular differentiation of primary embryonic cells and induced the production of a subset of heat-shock proteins, acting as a teratogen (that can produce anomalies on physiological embryonic development). *Drosophila* embryonic cell line is a reference model for detection of teratogens such as demonstrated by the in vitro assay of 100 chemicals.	(Bournias-Vardiabasis, Buzin et al. 1983)
Echinoderms : *Paracentrotus lioidus*, *Sphaerechinus granularis* and *Psammechinus miliaris* Ascidian*: Phallusia manzmilata*	5-AzaC	Total genomic DNA showed a diminution by 5-azac in a depending dose way.	Stops the early embryo development of eggs at the blastula stage	(Maharajan, Maharajan et al. 1986)
Crustacean: *Daphnia magna*	5-AzaC, 5-azadC, vinclozolin, genistein & biochanin A	Only the treatments with high concentrations of vinclozin and 5-AzaC caused a reduction in overall DNA methylation.	Effects on length (reduced length) and reproduction (decrease of produced eggs)	(Vandegehuchte, Lemière et al. 2010)
Crustacean: *Daphnia magna*	5-AzaC	CpG methylation reduction at the gene level was demonstrated in six genes. 5-AzaC diminishes the production of substrates required for DNA methylation intensifying the de-methylating effect.	Significant decrease of body length in treated individuals. Impact in the concentration of metabolites related to the one-carbon pathway	(Athanasio, Sommer et al. 2018)

Species	**DNMTi used**	**Epigenotype effect**	**Phenotypic Effects**	**Referece**
Crustacean: *Daphnia magna*	5-AzaC	An overall reduction of DNA methylation in the exposed generation and no changes on histones modifications. Global reduction of DNA methylation and changes in histone modifications in the offspring.	In F0 generation significantly reduction of the fecundity, differential expression of genes involved in the one-carbon-cycle. In the F1 offspring generation, changes in gene expression	(Lindeman, Thaulow et al. 2019)
Mollusk: *Aplysia californica*	RG108	Induce decrease of DNA methylation in the CREB2 gene	Inhibition of DNMT activity in neurons strongly increase CREB2 gene levels, this gene is a transcriptional repressor of memory. *Aplysia* treated with R108 abolished the enhanced serotonin dependent memory and synaptic plasticity	(Rajasethupathy, Antonov et al. 2012)
Mollusk: *Crassostrea gigas*	5-AzaC	Induce a strong decrease in DNA methylation	Modify the mRNA expression of some *homeobox* genes during embryogenesis, treated oysters showed phenotypes with a delayed cell differentiation. Lethal effect after zygotic genome reinitiating	(Riviere, Wu et al. 2013)
Mollusk: *Lymneaea stagnalis*	5-azadC	Not measured	Treatment with 5-azadC prevents methamphetamine induced extended memory. Meth exposures enhance memory persistence, and this persistent memory is blocked by 5-azadC suggesting that changes in DNA methylation partially mediate the enhance memory persistence.	(Lukowiak, Heckler et al. 2014)
Mollusk*: Biomphalaria glabrata*	5-AzaC	Not assessed	Inhibits oviposition	(Geyer, Niazi et al. 2017)
Platyhelminthe; *Schistosoma mansoni*	5-AzaC and 5-azadC	Decrease of DNA methylation with 5aza but not 5-azadC	Inhibits egg production, egg maturation and ovarian development. It modulates transcription and translation and affected stem cell proliferation.	(Geyer, Munshi et al. 2018)
Insect: *Achroia grisella*	5-azadC	Not assessed	Negative effect on fecundity of females and on the development of the progeny	(Sak 2018)

Species	**DNMTi used**	**Epigenotype effect**	**Phenotypic Effects**	**Referece**
Insect: *Nasonia vitripennis*	5-azadC	They proved by WGBS that multiple genes showed significantly altered methylation in response to the treatment with 5azadC and that some genes exhibited hypomethylation and some other hypermethylation	Demethylation leads to variations in sex allocation	(Cook, Pannebakker et al. 2015, Cook, Parker et al. 2019)
Insect: *Apis mellifera*	Zebularine	Upregulation of the *de novo* DNMT3, increase of the levels of DNMT3 mRNA	Reduces the acquisition memory retention, affects formation of the extinction memory showing a role of cytosine methylation in learning and memory.	(Lockett, Helliwell et al. 2010)
Insect: *Apis mellifera*	Zebularine, RG108	RG108 and zebularine showed significant overall decrease of DNA methylation in the brain.	DNMT inhibition was associated with upregulation of 9 memory-associated genes. *DNMT3* was upregulated after DNMT inhibition and DNA methylation levels fluctuate in its promoter after learning. suggesting a feedback loop in *DNMT3* controlled by DNA methylation.	(Biergans, Giovanni Galizia et al. 2015)
Insect: *Apis cerana* and *Apis mellifera*	Zebularine	No assessed assumed to inhibit DNA methylation as previously showed by Lockett et al. 2010	Alteration of extinction learning in both species. The treatment had opposing effects on extinction memory retention in the two species reflecting probably differences in the recovery of olfactory memories.	(Gong, Wang et al. 2016)

One of the few non-nucleoside DNMTi already tested in an invertebrate species is RG108 that showed inhibitory efficiency against DNA methylation in human cancer cell lines without showing significant toxicity (Schirrmacher, Beck et al. 2006). RG108 was tested in the mollusk *A. californica* were it induces a reduction in DNA methylation of a gene involved in memory process and its increasing expression level. It was also tested in the bee *Apis mellifera* where it induced a reduction of the global DNA methylation on the brain and the upregulation of 9 memory-associated genes (Biergans, Giovanni Galizia et al. 2015).

An example of phenotype and epigenotype assessment after treatment with 5azadC is found in the wasp *Nasonia vitripennis* where they conclude that demethylation leads to variations in sex allocation (Cook, Pannebakker et al. 2015), the DNMT inhibitory efficiency of 5azadC was not properly confirmed in this publication. Therefore, the demethylation effect of 5azadC was later adressed by WGBS and it was demonstrated that 8500 genes showed significantly altered methylation level in response to the treatment with 5azadC and that some genes exhibit hypomethylation and some other hypermethylation (Cook, Parker et al. 2019). Another example is found in the crustacean *Daphnia magna*: the assessment of the impact of 5 azaC has been evaluated three different times, a correlation of DNA methylation and histone modifications is done and intergenerational effects of the drug begin to be evaluated helping to pave the way to transgenerational evaluation of the epigenetic marks modulated in response to an epidrug treatment.

As we can see the epigenetic drugs 5aza and 5azadC have been shown to be either effective or not depending on the species where it was tested, (Table 1). It can display non-target and toxic effects in some invertebrate models, as was previously shown in mammal species (Ueno, Katayama et al. 2002), the model where this drug is the most effective. Consequently, the current research efforts of new DNMTi aimed at identifying non-nucleoside analogues. Our collaborators from the Laboratory of Epigenetic Targeting of Cancer from Center Pierre Fabre, developed a screening of 3-chloro-3-nitroflavanones as potent inhibitors of murine DNMT3A/3L complex (Ceccaldi, Rajavelu et al. 2011). These flavonoid compounds were not potent enough and they were unstable encouraging the research to find new flavonoid DNMTi developing a synthesis pathway to improve potency and stability of the compounds. The new compounds are 3-halo-3-nitroflavaones and they showed an improved inhibition activity in purified human DNMT3A-c compare to the 3-choro-3 nitroflavanones (Pechalrieu, Dauzonne et al. 2020). Another non cytidine analogues recently synthesized are the quinolone derivates that showed inhibitory activity against DNMT3A and DNMT1 in colon carcinoma HCT116 cells (Halby et al. 2017).

Principal results of the Pharmacological inhibition of DNA methylation

Phenotypic plasticity in response to the environment offers a mechanism for producing rapid variability in the phenotype that influence the adaptation of species. Epigenetic modifications can play a role in intermediating gene regulatory response to environmental factors and are important processes affecting phenotypic plasticity and adaptation. The most studied epigenetic mark is DNA methylation. The function of DNA methylation is well-known in vertebrates but in invertebrates its role remains poorly known. To study the contribution of DNA methylation to the phenotypic plasticity of a mollusk species we wished to introduce alterations in this epigenetic mark to evaluate the effect of those alterations on the phenotype variability. To introduce 5 methylcytosine (5mC) alterations, new chemical DNMT inhibitors used in cancer research were tested, they displayed high potency and low toxicity in murine model compared to the cytidine analogues 5-AzaC and 5-azadC that are highly toxic, which can induce undesirable side effects in the phenotypes that do not have any correlation with DNA methylation changes. To minimize or avoid undesired side effects, we tested new DNMT inhibitors that are non-cytidine analogues that do not require their incorporation into DNA to be active, being potentially less toxic and more specific to DNMT enzymes. We tested two kind of non-cytidine analogues compounds, the 3-halo-3- nitroflavanones (Pechalrieu et al. 2020) and the bisubstrate analogues (Halby et al. 2017).

For assessed the effect of those DNMT inhibitors in the global 5mC level of the snails *B. glabrata* treated, I developed a screening high throughput and cost-effective method for global 5mC changes. There exist multiple methods to measure DNA methylation, the choice of the method will depend on the question addressed. Before investing time and money in a costly sequencing technique, we used an immunological assay method to screen in the first place if global 5 methylcytosine (5mC) changes were induced in the snails treated with different DNMT inhibitors. The method was optimized and is based in the immunolabeling of the epitope 5mC by the monoclonal antibody anti-5MC, then a second antibody aids in the detection by chemiluminescence. Immunolabeling is widely used to screening 5mC levels changes in multiple species exposed to an abiotic or biotic stress and the commercially available ELISA-based Kit is the method of choice in many publications addressing this topic. I have evaluated the dot blot technique in terms of sensitivity and reliability, demonstrating that it displays good results and a sensitivity comparable to Kit ELISA but for a lower price which enables to assess a larger

number of samples. The optimization and detailed protocol of this technique is described in the **manuscript 1** entitled "A simple Dot Blot Assay for population scale screening of DNA methylation".

Once we optimized this screening technique this allow us to have preliminary encouraging results about the effect of the DNMT inhibitors at the genome wide level. Then we decided to use a sequencing method to analyze the regions of the genome impacted by the most efficient DNMT inhibitors. We used the epigenotyping by sequencing (epiGBS) method, a technique that allows the DNA methylation assessment at the nucleotide level of multiple samples and for simultaneous detection of genetic and epigenetic variations at high resolution, but at reasonable cost since only a small, informative part of the genome is sequenced. epiGBS is thus a method suited to perform genotyping and epigenotyping of large populations. The principal of epiGBS is that it allows for an easier protocol and a cost-effective and high-throughput method comparing to whole genome bisulfite sequencing (WGBS).

epiGBS libraries from controls snails were compared to a WGBS library from an snail of the same strain BgBRE, previously published (Adema et al. 2017). epiGBS libraries represented in average 1.4% of the CpG sites covered by WGBS, but the average levels of CpG methylation percentage were very similar between both methods (~22%). Furthermore, a high correlation was found between the CpG values covered by both methods (Spearman coefficient of correlation, R=0.74 and p<2.2e-16). Additionally, epiGBS results confirmed a mosaic-like pattern of DNA methylation in *B. glabrata* and a bimodal distribution of the CpG methylation as found with WGBS.

The compound Flv1 was the most efficient DNMT inhibitor that decreased 5mC levels in the treated generation and its offspring without toxic effect in its fecundity and survival. epiGBS allows us to identify differential methylated cytosines (DMCs) between controls and Flv1-treated snails, 25 DMCs were found in F0 generation, comprising 23 hypomethylated and 2 hypermethylated. In the F1 generation, 325 DMCs were found, 204 hypomethylated and 121 hypermethylated. One differential methylated region (DMR) comprising 5 DMCs within the 100 bp was found in both generations demonstrating a multigenerational effect. The higher number of DMRs in the F1 generations than in F0 generation suggest that the germline was indirectly exposed to the Flv1 inhibitor treatment. This DMR was further analyzed by evaluating if the

hypomethylation produced an effect in its gene expression. Indeed, the hypomethylation of the first intron was identified by bisulfite targeted sequencing and this was associated with its gene expression, the less methylated, the less expressed. This shows empirical evidence that gene body methylation is associated with gene expression.

Furthermore, the Flv1 treatment induced a higher phenotypic variability in the shell morphological traits also in both generations. This is consistent with the hypothesis that the germline was exposed to the DNMTi.

Once we identified the most efficient DNMT inhibitor in *B. glabrata* we decided to test this inhibitor in two other mollusk species of medical and economical importance: the freshwater snail *P. acuta* and the marine oyster *C. gigas*. We showed that the inhibitor Flv1 was also efficient in inhibit the DNA methylation is these mollusk species, providing evidence that this chemical inhibitor can be used as epigenetic modulator in other mollusks.

The bisubstrate analogue BA1compound showed medium toxicity by impacting significantly the survival but not the fecundity of treated snails. It induces a decrease of global DNA methylation in the F0 generation and an increase of global DNA methylation in the F1 generation. This was further validated by epiGBS results where we found the same trend. The hypothesis is that the hypermethylation observed in F1 generation is a compensatory effect due to the exposure of the germline to the toxicity of the BA1 compound. A common DMR was identified between BA1 and Flv1 treatment, this is probably a hotspot of environmental modifications.

These results are described in the **manuscript 2** named “The methylome of *Biomphalaria glabrata* and other mollusks: enduring modification of epigenetic landscape and phenotypic traits by new DNA methylation inhibitors.”

Then, a multigenerational experiment was done with the offspring of the snails treated with the new DNMTi (flavanones and bisubstrate-analogues). I crossed the snails hypomethylated (offspring of those treated with the flavanone compound Flv1) with the hypermethylated snails (offspring of those treated with the bisubstrate-analogue BA1). Then the snails of the generation F2 were isolated to reproduce them by self-fertilization, this experiment was done to evaluate if epimutations can be segregated in selfing lines. The F3 generation is expected to have a decreased on the genetic variation and an increase on epigenetic variation.

I exposed the snails of the F3 generation to the trematode parasite *Schistosoma mansoni* to verify if the snails coming from the multigenerational experiment with DNMTi treated snails, presented a difference in the parasite compatibility phenotype. I measured the fecundity, the prevalence of infestation and the intensity of infestation in the snails of the F3 generation and I found that the three phenotypes showed more variability in the snails coming from the crossing of the DNMTi-treated snails. The prevalence and intensity of infestation were highly homogenous in control lines, while the offspring of the divergent methylated crossing snails, a first attempt of epigenetic recombinant inbreed lines (epiRILs) showed high variation, with some individuals with significant lower prevalence and intensity of infestation, suggesting that epimutations originated in F0 generation could be segregated in consequent generations affecting the phenotype variability of the snails. The phenotypic results of this approach are described and discussed in the **manuscript 3** "Epigenetic and genetic contribution to the phenotypic plasticity of the mollusk *Biomphalaria glabrata*".

Manuscript 1

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A simple Dot Blot Assay for population scale screening of DNA methylation

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Abstract

The study of epigenetic changes in natural and experimental populations has increased the need to find a cost-effective and high throughput method to analyze multiple samples to effectuate a population-wide screening to study epigenetic changes triggered by biotic or abiotic stress. One of the most studied epigenetic marks is global DNA methylation, its measurement is used as a first step to differentiate methylation between individuals. There is a wide range of methods designed to detect genome-wide 5 methyl-cytosine (5mC) that differ in sensitivity, price, level of expertise required, but as a general rule, require large amounts of DNA and are relatively expensive. This is a limit for the analysis of 5mC in a large number of individuals as a prerequisite to population-wide testing of methylation markers. In this work, we evaluated a method based on antibody recognition of 5mC to measure the DNA methylation level of individuals of the species *Biomphalaria glabrata*, the intermediate host of schistosomiases, a neglected tropical disease. We validated the method to complete a large screening in the genome of *B. glabrata* snails treated with a chemical inhibitor of DNA methylation; however, the method can be applied to any species containing 5mC. The dot blot assay is a suitable method to perform a large-scale screening of global DNA methylation to compare 5mC levels between individuals from different natural or experimental populations. The dot blot method compares favorably with methods with an equivalent sensitivity such as the Enzyme Linked Immunosorbent Assay (ELISA) kit since it requires a smaller amount of DNA (30 ng) is less expensive and allows many more samples to be analyzed.

Keywords: DNA methylation, 5-methylcytosine, screening, dot blot

Introduction

Epigenetic mechanisms refer to heritable and reversible alterations in gene expression or cellular phenotype originated by changes other than modifications in the underlying DNA sequence (Nicoglou and Merlin, 2017). There are at least four carriers of epigenetic information: histone modification, non-coding RNA, location of genes in the nucleus and DNA methylation. The latter consists of the addition of a methyl group to a nucleotide, usually in the carbon 5 of the cytosine pyrimidine ring forming 5-methylcytosine (5mC). It is present in protists, plants, fungi and animals. DNA methylation is catalyzed by a family of conserved DNA methyltransferases (DNMTs). In most animals studied, DNA methylation occurs principally at CpG dinucleotides (Colot and Rossignol, 1999, Sarda et al., 2012) but methylation can occur in CHH and CHG (H= A, T, C) contexts in plants.

The types and levels of genomic DNA methylation varies significantly between species from undetectable DNA methylation (e. g. nematode *Caenorhabditis elegans*, the yeast *Schizosaccharomyces pombe* and *Saccharomyces cerevisae*) to very high levels in vertebrates (60-90% of all CpGs methylated) and most plants (Hendrich and Tweedie, 2003).
The earliest methods to measure DNA methylation were based on the separation of methylated and unmethylated deoxynucleosides. One of the first techniques to measure 5-mC quantitatively was the reversed-phase high performance liquid chromatography (RP-HPLC). The quantitative measurement of DNA methylation with this method is based on the relative intensity between cytosine and 5 methylcytosine (5mC) fractions of hydrolyzed DNA (Kuo et al., 1980). HPLC was useful to compare global DNA methylation amongst different species, but has limitations (Harrison and Parle-McDermott, 2011), in particular, due to the high amount of DNA (~2.5 µg) necessary to quantify 5mC. Liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) improved sensitivity and requires much smaller amounts of hydrolyzed DNA sample (50-100 ng of DNA sample) in addition, this technique is not affected by poor-quality DNA (Song et al., 2005). High performance capillary electrophoresis (HPCE) is another alternative method that is faster, low-cost and more sensitive than HPLC (Fraga et al., 2000). Nevertheless, a certain level of expertise and sophisticated equipment is necessary to perform such analyses not always available in research laboratories.
Bisulfite genomic sequencing is recognized as the gold standard method that allows for single-base resolution measurement of DNA methylation. Bisulfite treatment (Frommer et al., 1992) transforms the non-methylated cytosine into deoxy-uracil that will be read as thymine when sequenced, while 5-methylcytosine (5mC) remains intact and is still read as cytosine. Furthermore, the bisulfite genomic sequencing method works as a fundamental principle to several derived methods to quantify DNA methylation, i. e. Methylation Specific PCR (MSP), Combined Bisulfite Restriction Analysis (COBRA), and many other techniques depending on the application (Li and Tollefsbol, 2011). Complete bisulfite conversion is essential in order to have reliable quantitative methylation analysis, if the total conversion is not accomplished, unmethylatedcytosines can be mistaken for methylated residues and result in partial methylation

profiles. The method also requires PCR and often sequencing and is therefore time-consuming and relatively expensive

Global DNA methylation can be quantified also by 5-methylcytosine-specific antibody combined with fluorescence staining. The analyses of quantitative DNA methylation can be accomplished by the analysis of an image with a charge-coupled device camera and sampled results need to be compared with results acquired from cells with known methylation levels (Veilleux et al., 1995). Another method to screen the global 5mC level is the Enzyme Linked Immunosorbent Assay (ELISA), there are several commercially available kits, the procedure involves the DNA manipulation in a well plate followed by consecutive incubation periods. Firstly, with a primary antibody against 5mC, then a labelled secondary antibody and finally with colorimetric/fluorometric detection reagents. However, only large variations in DNA methylation (~1.5-2 times) can be determined using this method due to the high level of inter and intra-assay variability and therefore this method is only suitable for the rough estimation of DNA methylation (Kurdyukov and Bullock, 2016).

More recently, all techniques have been applied to more technically advanced systems such as DNA cleavage by methylation-sensitive restriction enzymes combined with polymerase extension assay by Pyrosequencing, called LUminometric Methylation Assay (LUMA) (Karimi et al., 2006); or combined with genomic microarrays (Schumacher et al., 2006, Weber et al., 2005). Another system is Methylated DNA immunoprecipitation (MeDIP) that separates methylated DNA fragments by immunoprecipitation with 5mC-specific antibodies, the enriched methylated DNA can be evaluated in a genome-wide approach by comparative genomic hybridization against a sample without MeDIP enrichment (Vucic et al., 2009)
With the emergence of population-wide epigenetic screens, global DNA methylation measurements are often used as a first step to differentiate methylation between individuals. Consequently, this increases the need to find a cost-effective and high throughput method that allows analyzing multiple samples to study DNA methylation changes triggered by e.g. biotic or abiotic stress. There exists a wide range of methods designed to detect genome-wide 5mC that differ in sensitivity, price, level of expertise required, but as a rule, require either large amounts of DNA or are relatively expensive (Sant et al., 2012, Laird, 2010, Kurdyukov and Bullock, 2016). This is a limit to the analysis of 5mC in a large number of individuals as a prerequisite to population wide testing of methylation markers.

In this work, we evaluated a method based on antibody recognition of 5mC to measure the DNA methylation level of individuals of the species *Biomphalaria glabrata*. We believe that the method can be used with any methylated DNA.

B. glabrata is a mollusk, intermediate host of the human parasite *Schistosoma mansoni*, the causative agent of schistosomiasis, the second most severe parasitic disease in terms of morbidity just after malaria (Walker, 2011). About 2% of cytosines are methylated in the genome of *B. glabrata* (Fneich et al., 2013) and DNA methylation machinery plays probably a role in parasite-host interaction (Geyer et al., 2017). But as in most invertebrates, the precise function of 5mC in its genome remains enigmatic. We developed our method to measure changes of DNA methylation upon exposure of snail populations to chemical stress and to see if these modifications in DNA methylation produced changes in phenotypic traits (Luviano et al. manuscript in preparation).

Our method consists of immunological detection of 5mC and allows for fast screening of changes in DNA methylation in a large number of samples. It is also a simpler and less expensive screening strategy that compares favorably with methods with an equivalent level of sensitivity.

Results

5mC methylation of DNA samples of *B. glabrata* extracted by different methods was measured. The NucleoSpin Kit improved with zirconium/silica beads method presented the most reproducible results and it was the only method that allows discriminating clearly the denaturated, the naturated and the renaturated samples (Fig.1). Since the methyl group (CH_3) is located inside the double DNA helix, it can only be detected by the antibody against 5mC if DNA is properly denaturated.

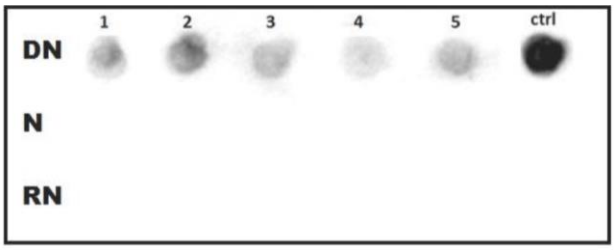

Fig.1. Methylated Cytosine Dot Blot of five tissue samples of *B. glabrata* obtained with the NucleoSpin kit and zirconium/silica beads method, with 120 ng of DNA. Exposure time: 35 sec. Control: HeLa DNA (200 ng). Top DN: denatured with NaOH at 42°C, middle N: non-denatured, bottom RN: renatured after 1 h at room temperature means it returns to the non-denatured form. As expected, there is no signal for the non-denatured and renatured samples.

To standardize our method, we used HeLa DNA as positive control and PCR products as negative one. Methylation level in HeLa cells is 2.3%± 0.22 of 5mC of total cytosines (Diala and Hoffman, 1982) and 0% in the case of DNA amplified in vitro by PCR and thus unmethylated. In order to test the linear range of the method, a correlation was calculated between the mean spot densitometry obtained from membrane imager and the amount of input sample 5mC in pg. The results showed a strong linearity between 5 mC amount and mean spot densitometry, therefore we decided to measure by this method a large number of *B. glabrata* samples (Fig.2). Each ng of HeLa cells contains 5.1 pg of 5mC, this value was obtained by calculating the molecular weight of 2.3% of cytosines in the human genome composed by 3.2×10^9 nucleotides and a 40% GC content (Li, 2011).

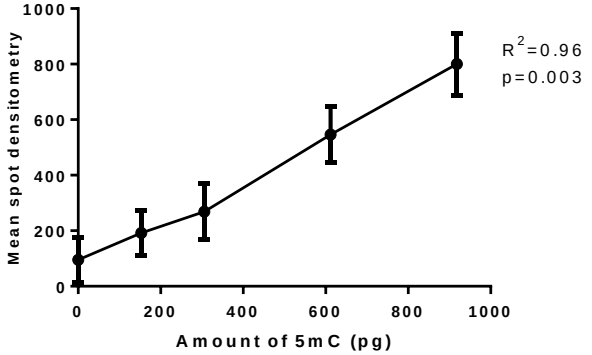

Fig.2. Linearity of the mean spot densitometry of the HeLa cells obtained from dot blot assay and the amount of 5mC in picograms in input sample. Each ng of HeLa cells contains 5.1 pg of 5mC, five points showed in the graphic correspond to 0, 30, 60, 120 and 180 ng of HeLa DNA

When we used as reference the densitometry/nanogram value of HeLa cells (Positive Control densitometry/nanogram value =6.92±1.2 and Positive Control 5mC%= 2.3%) to calculate by the next equation the 5mC% from *B. glabrata* samples:

$$5mC\% = \frac{\text{Sample densitometry/ng value}}{\text{Positive Control densitometry/ng value}} * \text{Positive Control 5mC\%}$$

We obtained 1.97 % (Fig. 3b), which is concordant with the known methylation level in *B. glabrata* whose genome has 2% of cytosines methylated (Fneich et al. 2013). As expected, when we calculated the 5mC% in zebularine treated snails we detected a decrease in DNA methylation, the mean 5mC% decreased from 1.97% to 1.74% however, this decrease is statistically not significant (t=1.15, df=45.29, p=0.25).

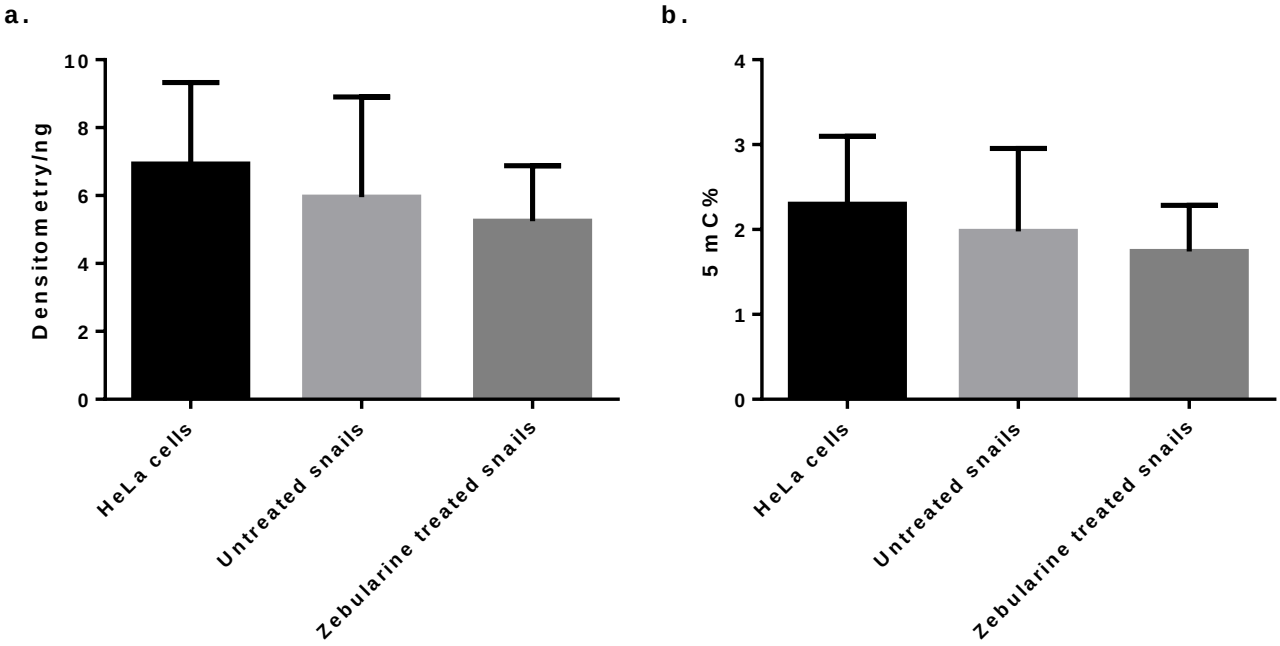

Fig.3.a) Densitometry/nanogram obtained by the dot blot assay of HeLa cells ($\bar{x}$=6.92±1.2), untreated snails ($\bar{x}$=5.95±0.53) and zebularine treated snails ($\bar{x}$=5.24±0.29). b) 5mC% calculated for HeLa cells ($\bar{x}$=2.3%), Untreated snails ($\bar{x}$=1.97%) and treated snails ($\bar{x}$=1.74%).

Discussion

Three different methods to perform DNA purification before DNA methylation detection were tested in order to optimize the DNA extraction method adapted to the methylated cytosine dot blot assay. The method phenol/chloroform is widely used and requires reagents that most laboratories possess and is not expensive, however, it is a time-consuming method as it requires a lysis phase overnight. The E.Z.N.A kit is a simple, rapid and cost-effective method for the isolation of DNA, with the spin-column based technology, multiple samples can be processed in parallel. However, the overnight lysis is still required when applying this method. The NucleoSpin kit uses the same spin-column based approach as E.Z.N.A kit, but in order to improve this method zirconia/silica beads can be used to effectuate a mechanical cell lysis to speed up lysis phase comparing with other methods. In this work, the NucleoSpin kit improved with zirconia/silica beads was the method that gave us the more reproducible results. Furthermore, it was the only method that allowed us to differentiate between the denatured and non-denatured samples after dot blot assay was applied. The other two methods showed a binding of the antibody anti-5 methyl cytosine even in the non-denatured samples which is not possible since the CH_3 groups of the cytosine are inside the double helix of DNA and therefore it can be only detected if we open the double-strand DNA (denaturation). For this reason, the signals from non-denatured samples were interpreted as non-specific binding of the antibody.

The exposure time suitable for our membranes in the CCD imager was 200 secs to avoid signal saturation, the exposure time can vary depending on experimental conditions but have to be always below the point of saturation of the most concentrated spot, detected by imager software. Otherwise a plot between signal and exposure time must be done in order to identify the linear range and select an exposure inside this range. Once the experimental conditions were established for the dot blot assay, we did not change the input DNA sample, membrane size, denaturation time, transfer method, transfer time, antibody solution, antibody incubation time, temperature or exposure time in all experiments as these factors can alter significantly the detection signals. All the steps in the protocol were homogeneous to avoid high intra assay-variability. In addition, positive and negative controls have to be present in all membranes to be able to compare results between them.

Dot blot method compares favorably to methods with an equivalent sensitivity like ELISA. The sensitivity of ELISA-based global DNA methylation assays vary from 0.1 ng to 10.5 ng of 5-mC DNA, our Dot Blot method showed a sensitivity of 0.15 ng, very similar to the better detection limit of ELISA. The Methylated Cytosine Dot blot assay allows for high throughput samples; it provides a measure of global 5-methylcytosine in the genome in a short period of time. In our hands, 288 samples with replica per day as multiple membranes can be incubated at the same time, and at a price (3€ per sample approximately) that compares favorably to other methods like the commercially available ELISA kit that allow to do 96 samples with replica per day if two plates are done at the same time and at 14€ per sample approximately.

After applying the methylated cytosine dot blot assay to our samples, we can conclude that it is a method adequate to perform a large-scale screening of global DNA methylation to compare 5mC levels between individuals of different natural or experimental populations. We validated the method to complete a large screening in the genome of *B. glabrata* snails treated with a chemical inhibitor of DNA methylation. This method requires a very small amount of DNA material (30-180 ng).

Materials and Methods

Ethics statements

B. glabrata Brazilian strain (Bg BRE) was used in this study. The mollusks are maintained at the IHPE laboratory facilities; they are kept in aquariums and fed with lettuce *ad libitum*. 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.

Zebularine treatment

One hundred *B. glabrata* BRE snails (5-7 mm in size) were maintained in 1L of freshwater in the presence or absence of the demethylating agent zebularine (Sigma, France, Cat. No. 3690-10-6) at a concentration of 10 µM. The water and the fresh zebularine were replaced once at the same concentration, the replacement was performed after 3 days and 22 hours. After 10 days of exposure, the drug was removed and replaced by only water. Snails were then raised in the plastic tank during 70 days. At day 70, snails were collected, individually wrapped in aluminum sheets, and stored at -20°C.

Optimization of DNA extraction

Three DNA extraction methods were optimized in terms of cost, scale, effectiveness and time. For this purpose, 5 samples of *B. glabrata* were isolated by phenol-chloroform method, 5 samples by the E.Z.N.A ®Tissue DNA Kit (Omega Bio-Tek, Ref D3396) and 5 with the NucleoSpin® Tissue Kit (Macherey-Nagel, Ref #740952) combined with zirconium/silica beads.

For the phenol/chloroform method, tissue samples of *B. glabrata* were incubated overnight at 55°C with 1 ml of lysis buffer (20 mM TRIS pH 8; 1 mM EDTA; 100 mMNaCl; 0.5% SDS) and 20 µl (0.3 mg) of Proteinase K (Invitrogen, Cat. No. 11578916) The DNA of samples were extracted adding twice equal volumes of phenol/chloroform followed by 2× extractions with identical volumes of chloroform. DNA was precipitated with the same volume of isopropanol/sodium acetate. After centrifugation and washing with 1 ml of 70% ethanol, the pellet was dissolved in 200 µl of 1 mMTris/HCl, pH 8, and stored at -20°C.

Other tissue samples of *B. glabrata* were isolated using an E.Z.N.A Tissue DNA Kit (Omega Bio-Tek, Denmark), lysis, binding, washing and elution of the DNA was done according to the manufacturer's protocol. This kit is based on proteinase K digestion overnight and in spin column-based technology.

The NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany) combined with the use of zirconia/silica beads, a method developed to extract DNA from the pacific oyster *Crassostrea gigas* (de Lorgeril et al., 2018) was applied in tissue samples of *B. glabrata*. The zirconia/silica beads perform a mechanical cell lysis while lysis buffer acts chemically to break the cells. Briefly, for the lysis phase, samples were transferred into180 µl of lysis buffer and 25 µl (0.32 mg) of Proteinase K (Macherey-Nagel, Ref 740506.30) in 2 ml screw cap microtubes containing 100 µg of zirconia/silica beads (BioSpec, USA, Cat. No. 11079110z). This mix was vortexed. Tubes containing samples were submerged in liquid nitrogen and then shacked in a Mixer Mill (Retsch MM400) at a

frequency of 30 Hz for 12 minutes. After that, incubation in a water bath at 56°C during 1 h 30 minutes was done.

After the lysis phase, the NucleoSpin® Tissue Kit protocol was applied according to the manufacturer instructions for binding, washing and elution of the DNA. Elution was performed into a final volume of 100 µl in 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).

Dot Blot assay

Once the DNA extraction method was optimized, 30 samples from the control group and 30 from the group treated with zebularine were isolated for the 5mC dot blot assay. DNA extracted from HeLa cells served as positive control and DNA of *B. glabrata* amplified by PCR was used as negative one. HeLa cells were a kind gift of Albertina De Sario INSERM U827 (IURC). A linear regression was done using the mean spot densitometry of HeLa cells obtained from the imaging system and the input of 5mC in picograms. After confirmation of our positive and negative controls, the dot blot assay was applied to the all samples of genomic DNA extracted from *B. glabrata* individuals.

Denaturation of DNA samples

Since the 5mC moiety that is detected by the antibody resides inside the DNA double helix and is therefore inaccessible, DNA must be denatured to expose the methylated site. The amount of DNA that allowed for optimal 5mC detection range was 30ng-180 ng in our case, and to effectuate dot blot we took 180 ng from each DNA sample. DNA was adjusted with MilliQ water to 10.8 µL in 0.2 mL tubes and 1.2 µl NaOH 3M was added (total volume= 12 µl). Tubes were incubated at 42°C for 12 minutes. After incubation, the samples were rapidly transferred by spotting each sample into the membrane of nitrocellulose (Hybond®). This step is important because samples can renaturate within a couple of hours. Each spot consists of 6 µl of denatured DNA so that each sample can be spotted in duplicates. The samples and their replicates must be spotted in a random way, for this purpose a paper grid template must be created with the label of each sample in the appropriate grid space. This grid template can be used to spot the samples into the nitrocellulose membrane (13 × 9 cm for 96 samples) using a white light transilluminator or a UV transilluminator with a white light conversion screen, an ultraviolet blocking cover and by wearing ultraviolet protections eyewear.

When the transfer of DNA to the membrane was finished, the membrane was introduced into a Stratalinker® UV crosslinker and the Autocrosslink setting was run to fix DNA to the membrane. After this step, the blocking of the membrane can be done or the membrane can be stored in plastic cover sheets at -20°C for further use.

Blocking of the membrane

A TBS 10X solution (500mM Tris/Cl, 1.5 M NaCl, pH 7.5) was prepared. 30.25g of Tris/Cl, and 43.8 g of NaCl were added to a glass laboratory bottle, then 400 mL of MilliQ water was added, pH was adjusted to 7.5 and 500 mL of water were added to a final NaCl concentration of 500 mM. After this, a solution of 1×TBS-0.05% Tween20 can be prepared. The preparation consists of 100 mL of 10×TBS pH 7.5 and 0.5 mL of Tween20 added to 900 mL of Milli-Q ultrapure water.

The blocking solution was prepared with 1×TBS-0.05% Tween20 and 5% powdered milk (2.5 g of powdered milk for 50 mL of 1×TBS-0.05% Tween20).
Blocking of the membrane was done during 1h at 37°C under saturation solution and elliptical agitation.

Preparation of primary antibody solution and incubation

1/500 dilutions of the anti-5mC antibody (Abcam, Cat. No. ab73938, Lot: GR278832-3) were produced in blocking solution and the membrane was incubated in this solution under elliptical agitation during 1h30 at room temperature. After that, the membrane was washed three times with TBS-Tween20 for10 minutes under elliptical agitation.

Preparation of secondary antibody solution and incubation

1 µl of HRP-conjugated Goat anti-mouse IgG secondary antibody (Agrisera, Ref. AS11 1772, Lot: 1612) was diluted 500-fold in blocking solution.
Incubation of the membrane under elliptical agitation was done during 1h10 at room temperature. Then the antibody was removed by washing the membrane 3 times for 10 minutes in elliptical agitation

Reading of the signal

SuperSignal™ West Pico chemiluminescent (Thermo Fisher Scientific, Cat. No. 34580) substrate was used to detect signal intensity, a solution of 750µl of Luminol/Enhancer and 750µl of Stable Peroxide was prepared for each membrane. The solution was added to the membrane to cover it completely, then the membrane was placed on a glass plate and introduce it to the ChemiDoc™ MP Imaging System. Luminescence was captured with a CDD camera with an exposure time of 200 secs (1 photo each 10 sec) and quantified with the Image Lab™ 5.1 software. In order to determine the appropriate exposure time, the membrane was exposed to 40 secs, as DNA spots appeared too light, exposure was incremented to 3 minutes and photos were taken each 10 secs. All images were saved and the most concentrated spot in the membrane was identified, the selected exposure time was the exposure time below the point of saturation of this spot, detected by the software Image Lab (highlighting in red the saturated pixels). Normalization of the measures was performed by dividing the mean spot densitometry by the input sample DNA in ng, this provides a densitometry value per ng of DNA.

End Matter

Author Contributions and Notes

C.G and C.C. designed research, N. L. and S. D. P. performed research, N. L analyzed data; and N.L., C.G., C.C. and S.D.P. wrote the paper.
The authors declare no conflict of interest.

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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) were randomly assigned to treatment and control groups, the treatments were done with the DNMTi 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). WGBS raw data was analyzed with the same pipeline for direct comparison with epiGBs libraries. CpG sites with 8X coverage were used to created bigwig profiles for visualization in IGV (data available at: https://zenodo.org/record/4277533)

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= $10^{\wedge(1/\text{-slope}),}$ 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 comparable 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).

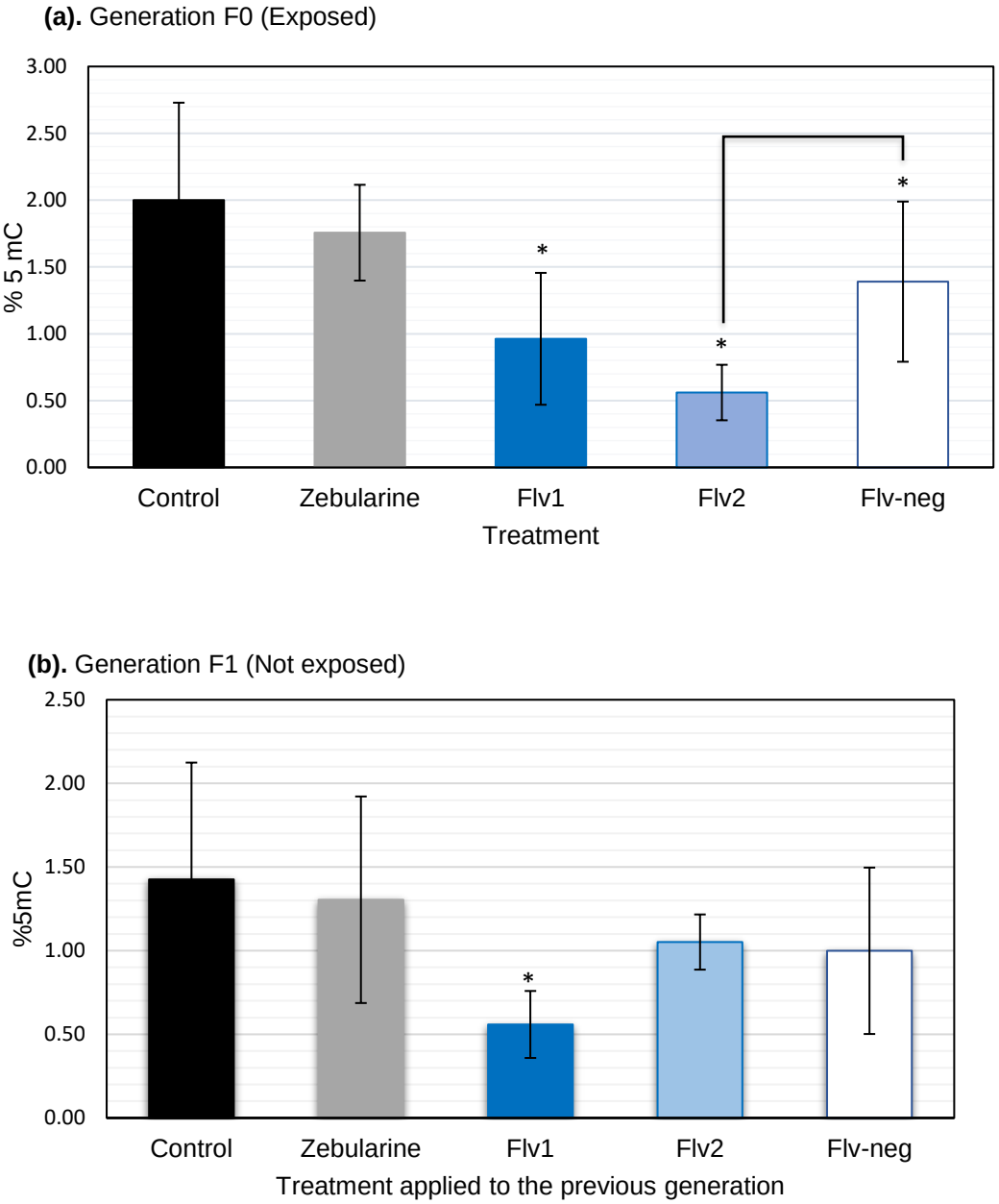


FIGURE 1. 5mC % of *B. glabrata* snails upon DNMTi treatments at a concentration of 10 µM, error bars represent SD, n=30 per treatment. (a) 5mC % in the F0 generation (exposed), black bar for

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).

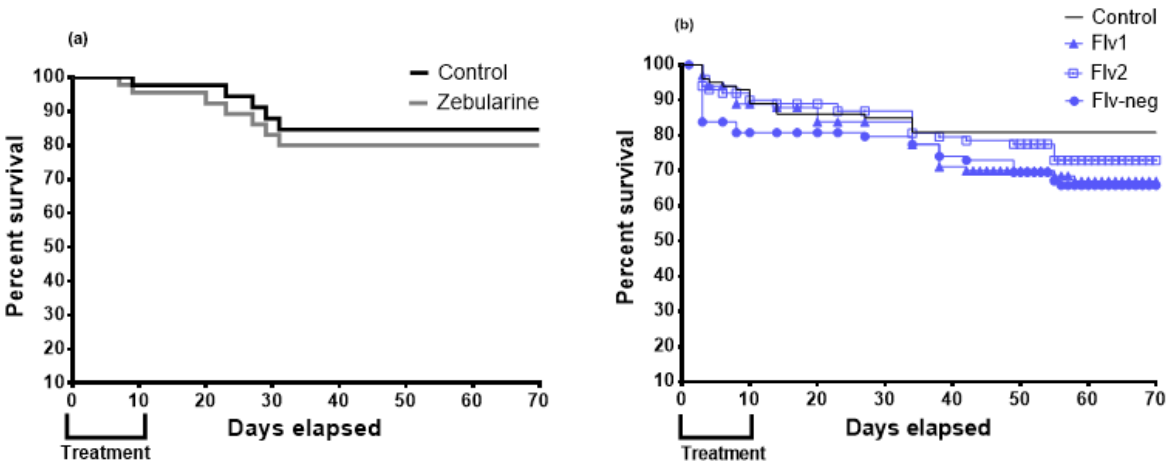


FIGURE 2. Kaplan-Meier survival curves upon treatment with the two types of DNMTi. (a) Cytidine-analogue zebularine (grey line). (b) Flavanones Flv1 (blue line with triangles) and Flv2 (blue line with squares) and their inactive derivative Flv-neg (blue line with circles).

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).

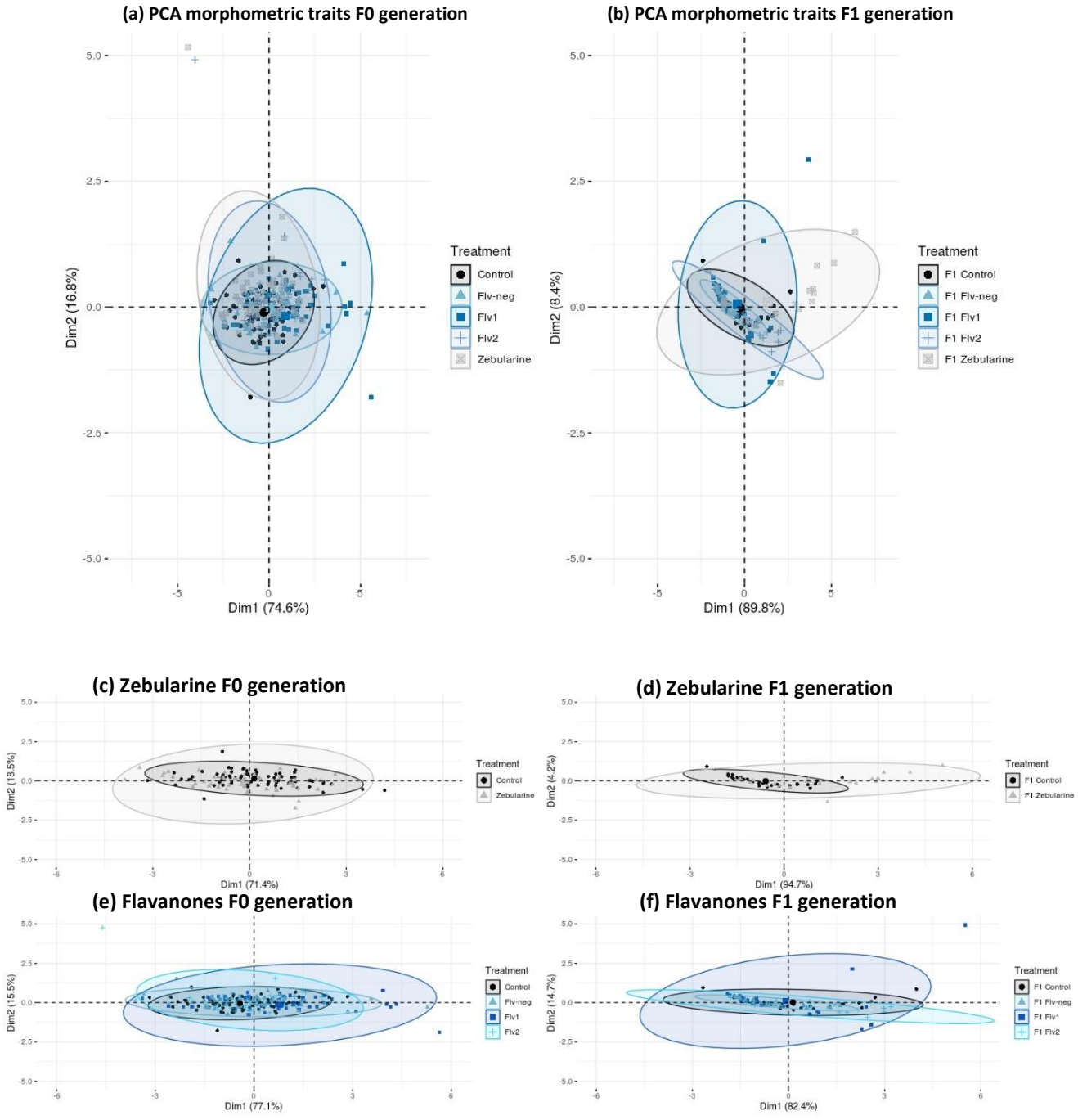


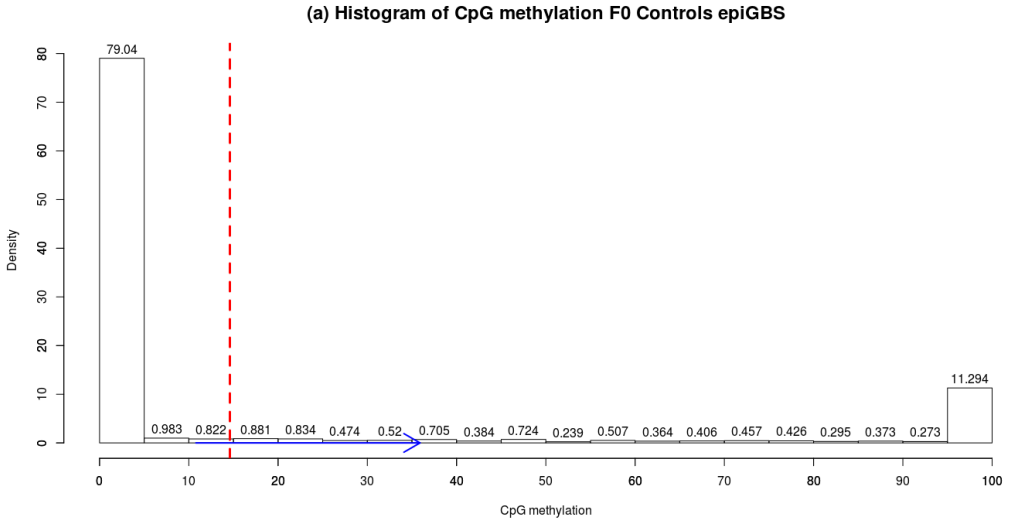
FIGURE 3. PCA of morphometric traits of all treatments of F0 (a) and F1 generation (b). PCA analyses splitted by treatment, (c) PCA of morphometric traits after zebularine treatment and (d) its offspring (e) PCA of morphometric traits of snails exposed to flavanone derivatives F0 generation and (f) the 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.

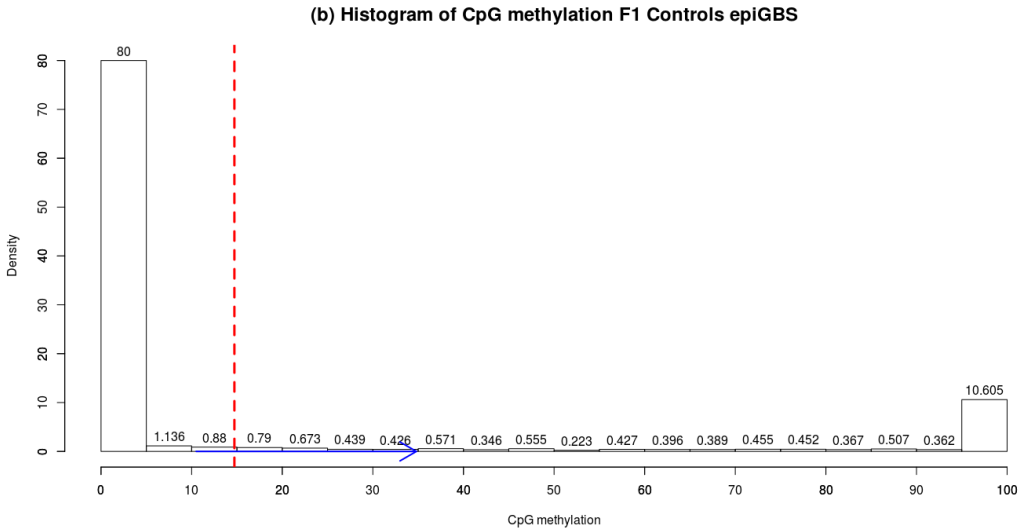
epiGBS reduces sequencing effort roughly 10x but allows for reliable evaluation of global 5mC level and identification of differentially methylated sites and regions

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).





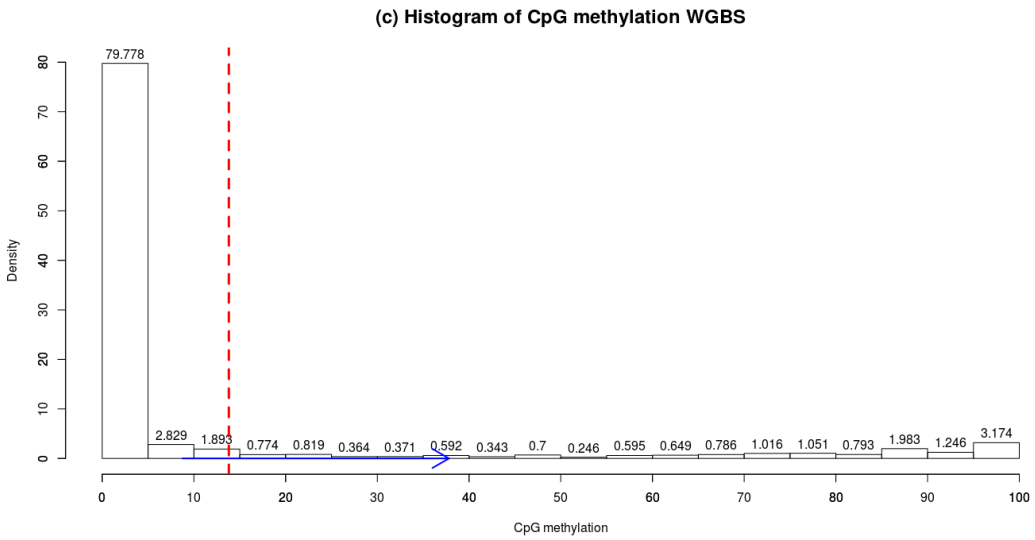


FIGURE 4. Histograms of CpG methylation distribution, (a) histogram of F0-control epiGBS libraries (b) histogram of F1-control epiGBS libraries and (c) histogram of WGBS library. The abscissa represents the CpG methylation % (0-100) and the ordinate showed the density of CpG positions. The dashed red 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).

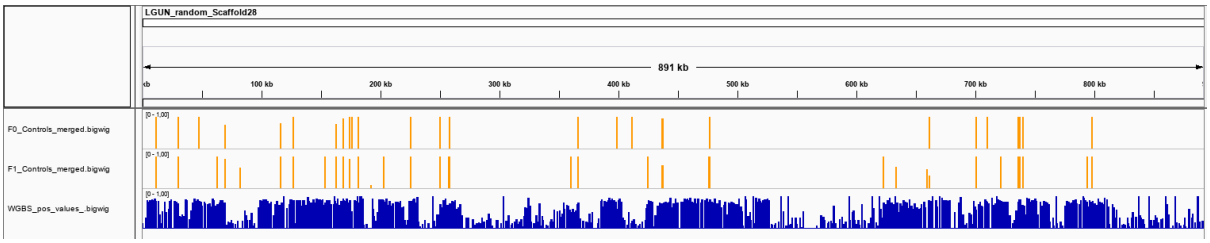


FIGURE 5. Screenshot of IGV of the region LGUN_random_Scaffold28: 1-800 Kb, that showed the regions covered by epiGBS libraries (in yellow) versus the regions covered by WGBS library (blue).

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).

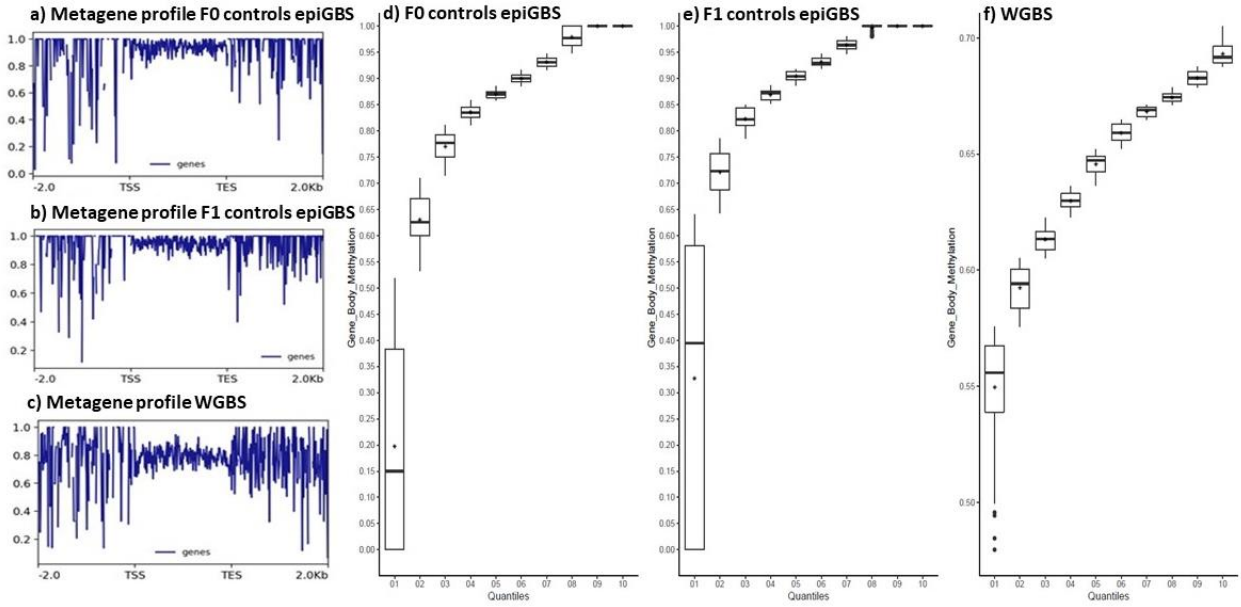


FIGURE 6. CpG methylation ratio profile across the bodies of genes and quantiles distribution of epiGBS and WGBS libraries. (a) Metagene profile of CpG methylation ratio of F0 control epiGBS libraries, (b) F1-control epiGBS libraries and (c) WGBS library. −2.0 kb indicates the upstream 2,000 bp of TSS,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

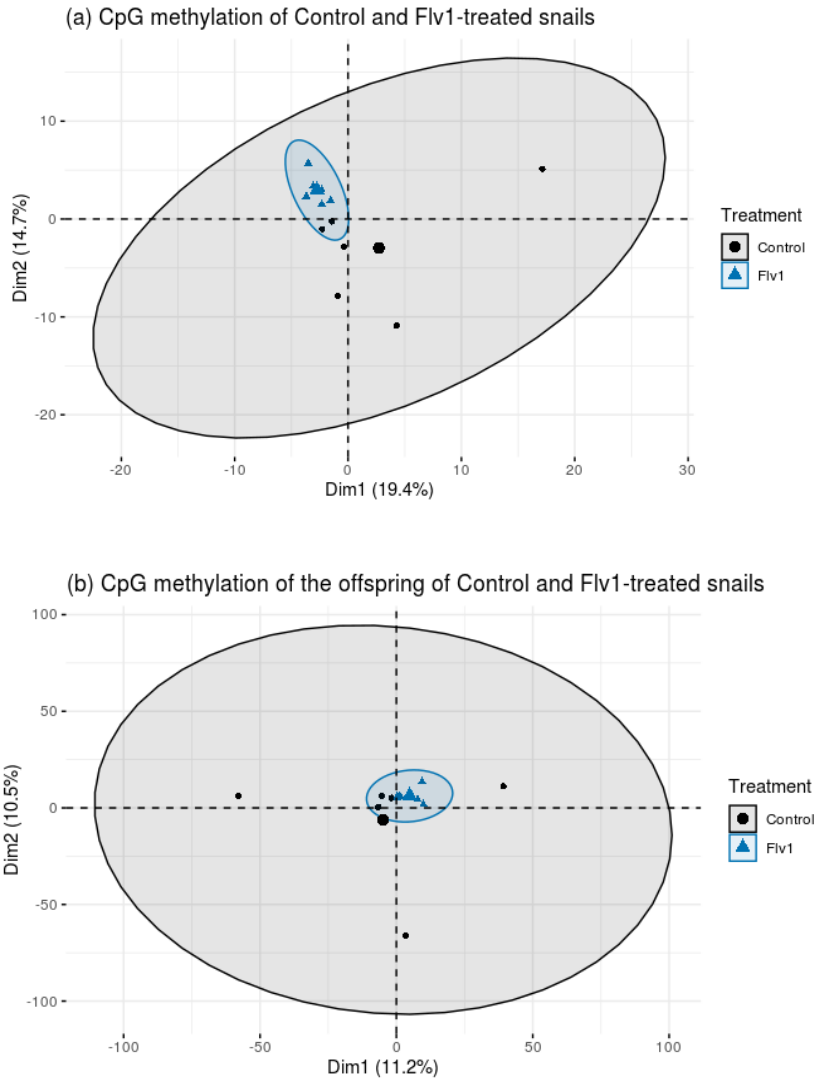


FIGURE 7. PCA of CpG methylation of the Flv1-treated and control groups in F0 generation (a) and in its offspring (b). The ellipses represent the 95% confidence interval.

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.

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

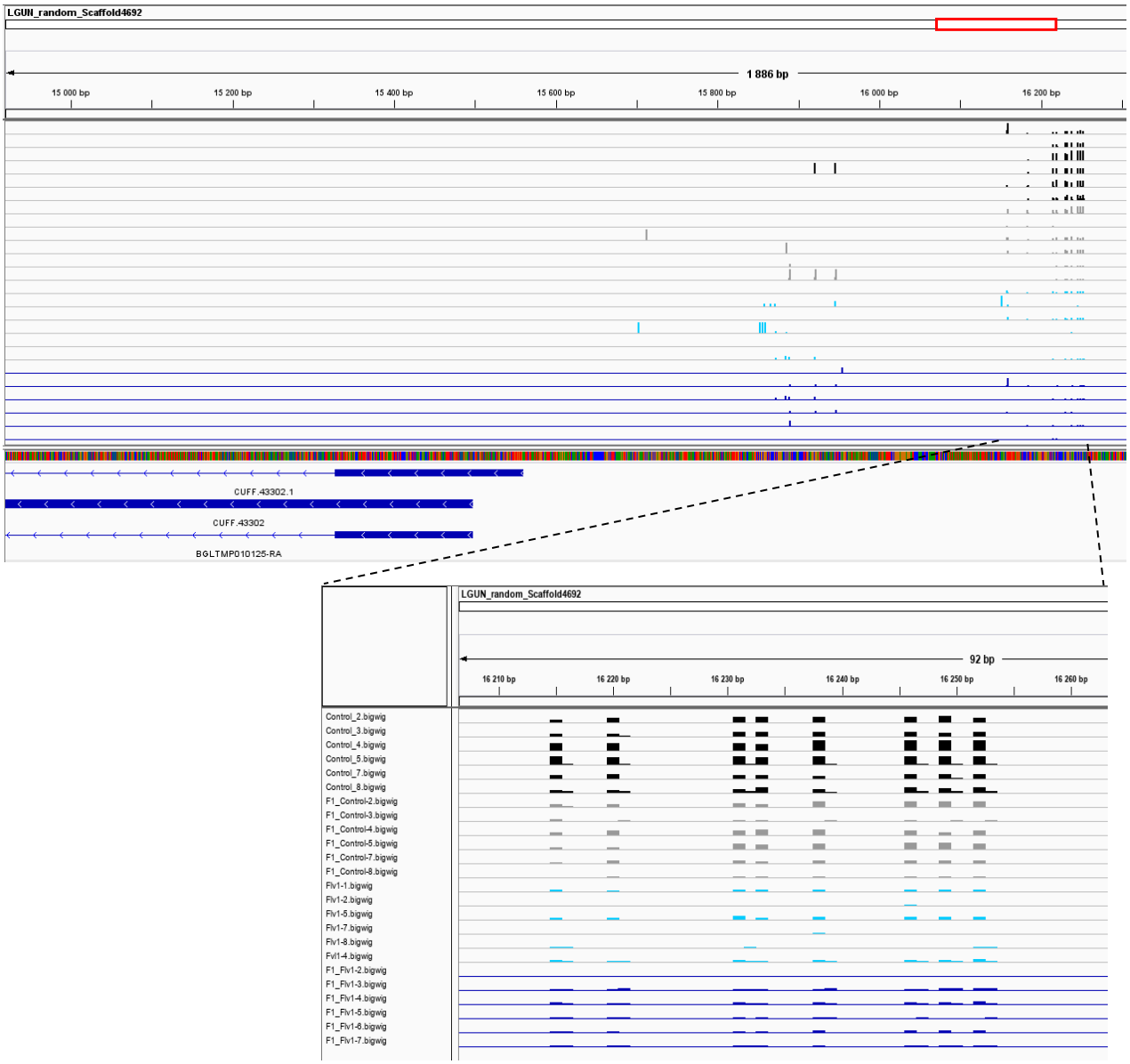


Figure 8. IGV screenshot of the LGUN_random_scaffold4962:16175-16266 of *B. glabrata* genome assembly (GCA_000457365.1). Each bar indicates the position of a methylated CpG site for the different samples: F0-control F0 (black), F1-control (gray), Flv1-treated samples (light blue) and its offspring (dark 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

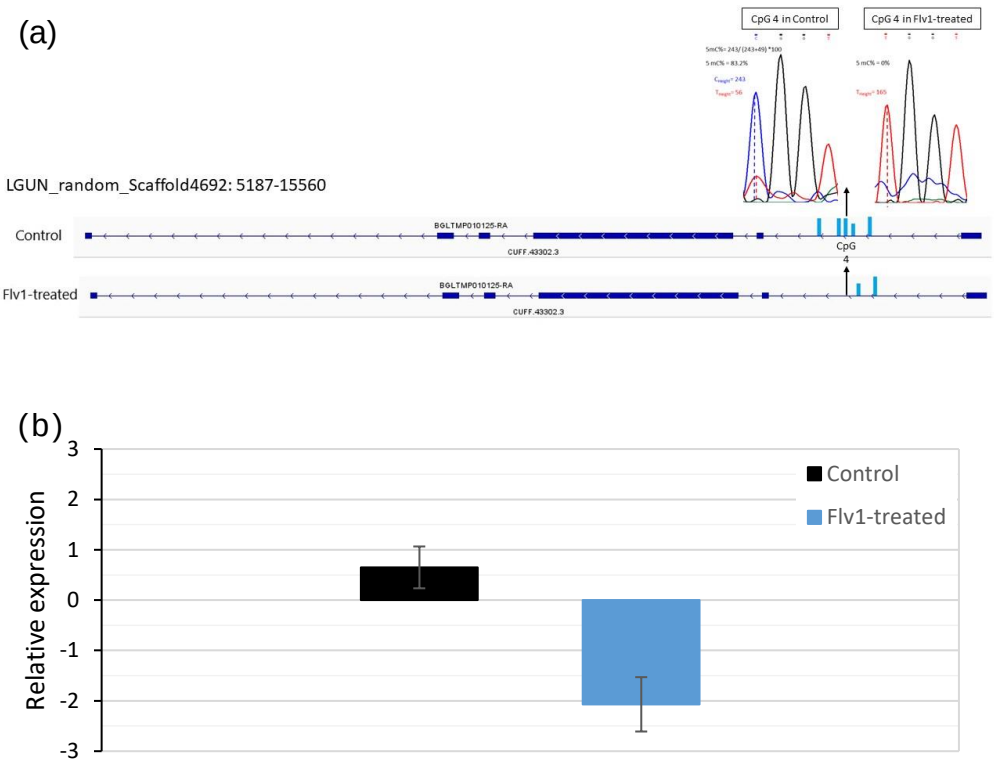


Figure 9. (a)Position and level of methylation of the five CpG positions, which has been studied by TBS within the first intron of the transcript BGLTMP010125-RA. (b) Relative expression of the transcript BGLTMP010125-RA compared to two housekeeping genes (28S and α-Tubulin), the ordinate shows the logarithm 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).

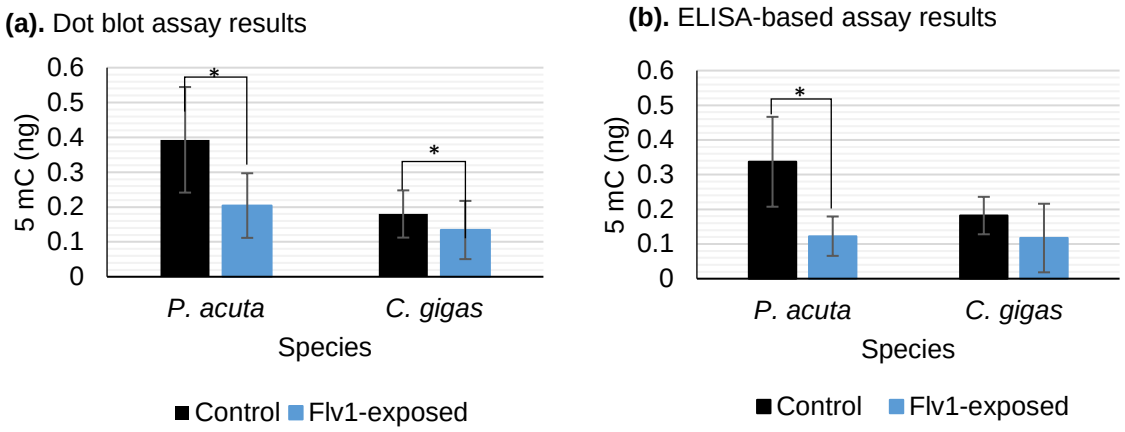


FIGURE 10. (a) 5mC % measures obtained by the dot blot method for *P. acuta* and *C. gigas*. (b) 5mC % measures obtained by the ELISA-based assay. The bars represent the 5mC (ng) mean, the error bars represent the standard deviation (SD), *n* = 30 per group per species for dot blot and *n* = 10 for ELISA. 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 adaptation to new environments (Roberts and Gavery 2012a), but experimental proof is lacking. DNA methylation in mollusks is likely to be an important element of the inheritance system. One way to

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

We used here an antibody-based assay as a screening method of global 5mC % modifications. We determined a linear correlation between DNA amount and mean spot density in the dot blot assay, and we demonstrated that it showed comparable results to ELISA-based commercial kit but allowing the screening of a larger number of samples at a lower price (Supplementary file 2, Figure S5). Furthermore, we used the epiGBS method for the first time in a mollusk species, providing evidence that this method can be used to analyze environmental DNA methylation changes genome-wide. This method allowed the analysis of DNA methylation changes at the nucleotide level of numerous replicates, that is a prerequisite for ecological studies, at an affordable price and giving results that represent the same global pattern as WGBS, as shown by the high correlation found between the methylation ratios of the CpG positions covered by both methods (Spearman correlation, R=0.74, p<2.2e^{-16}). Besides, epiGBS laboratory protocol and bioinformatics analysis are very flexible and can be further improved to obtain higher coverage.

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

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

5mC: 5-methylcytosine

5-AzaC: 5-azacytidine

6-FAM: 6-Carboxyfluorescein

AdoMeth or SAM: S-adenosyl-L-methionine

BgBRE: *Biomphalaria glabrata* strain Brazil BRE

Bge: Biomphalaria glabrata embryonic

DNMTs: DNA methyltransferases

DNMTi: DNA methyltransferase inhibitors

DMR: Differential methylated reion

DMC: Differential methylated cytosine

EDTA: Ethylenediaminetetraacetic acid

EpiGBS: Epi-Genotyping by Bisulfite Sequencing

epiRILs: epigenetic recombinant inbred lines

Flv1: Flavanone 1

GBM: Gene Body Methylation

IGV: Integrative Genome Viewer

mRNA: messenger Ribonucleic acid

PCA: Principal component analysis

SAM: S-Adenosyl-Methionine

TBS: Targeted Bisulfite Sequencing

TSA: Trichostatin A

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

WGBS: Whole Genome Bisulfite Sequencing

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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Supplementary figures and tables

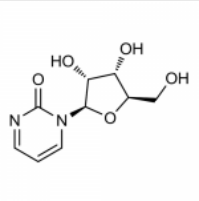
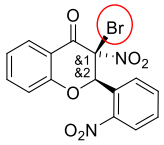
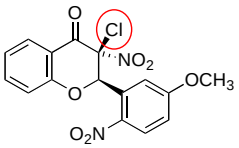
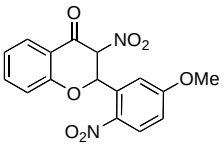
Molecule ID	Structure	M_w ($g.mol^{-1}$)	Molecular formula	Mechanims of action
Zebularin		228.20	$C_9H_{12}N_2O_5$	Zebularine incorporates into the DNA in the place of cytosine and impedes the methylation reaction by forming a reversible covalent complex at the carbon-6 with the DNMTs that induces a steric obstruction and inhibits the methylation at the carbon-5 of the cytosine (Zhou et al. 2002, Champion et al. 2010).
Flv1		393.15	$C_{15}H_9BrN_2O_6$	Flv1 compound is a 3-halo-3 nitroflavananone that possesses a bromine atom in α-position of the carbonyl group. The nitro group is crucial for inhibition activity of both flavanones, although the interactions remain unclear between this electron-withdrawing group (NO2) and the mainly hydrophobic cavity of Dntm3a.
Flv2		378.72	$C_{16}H_{11}ClN_2O_7$	Flv2 compound is a 3-halo-3 nitroflvananone that possesses a chlorine atom in α-position of the carbonyl group.
Flv-neg		344.28	$C_{16}H_{12}N_2O_7$	Halide atom elimination leads to the inactive flavone, the molecule undergo β-elimination being less stable that the active flavanones.

FIGURE S1. Structure, molecular weight (Mw) and molecular formula of DNMT inhibitors.

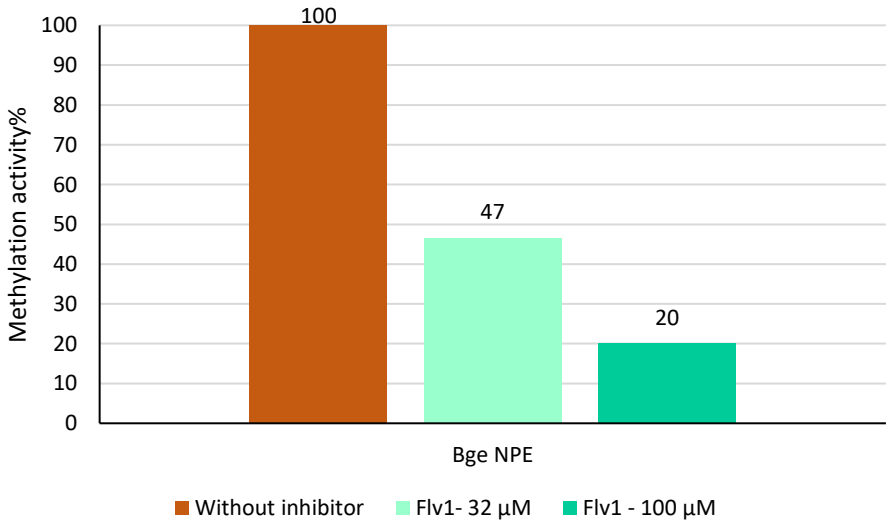


FIGURE S2. Methylation activity% of the nuclear protein extract of *Biomphalaria glabrata* embryonic cell line (Bge SNE) is inhibited after treatment with Flv1 at a concentration of 32 µM and 100 µM. Control DNA is 100% methylated, recombinant hDNMT3-A-c displayed 34% of methylation and Bge SNE displayed 15% of methylation, recombinant hDNMT3-A-c is completely inhibited by Flv1. Bge SNE is inhibited in a dose dependent way by Flv1.

Table S1. Mapping efficiencies of paired-end reads, total number of CpG sites, CpG covered by at least 8 reads, number of CpG sites methylated, %CG methylated and sequencing depth										
Samples	Mapping R1%	Mapping R2%	Mapping reads R1	Mapping reads R2	Total Mapping reads	Total no. CpGs	CpGs ≥ 8x coverage	Methylated CpG sites	CG% methylated	% of WGBS
WGBS	48.2	46.5	78465140	74377789	152842929	17493207	4061906	855624	21.06	
F0 Control 2	35.8	32.8	370126	336884	707010	95369	34646	7621	22.00	0.85
F0 Control 3	35.9	32.7	246836	224218	471054	92071	22732	5075	22.33	0.56
F0 Control 4	34.9	31.9	166061	150779	316840	74206	15761	3163	20.07	0.39
F0 Control 5	35.5	32.6	76190	69684	145874	37891	6315	1271	20.13	0.16
F0 Control 7	35.9	33	167990	153402	321392	73474	15402	3351	21.76	0.38
F0 Control 8	35.9	32.9	142421	129663	272084	61012	13115	2687	20.49	0.32
F1 Control 2	35.8	31.3	370125	326323	696448	128730	35311	7140	20.22	0.87
F1 Control 3	36.2	32.8	683605	615688	1299293	180852	63892	13983	21.89	1.57
F1 Control 4	34.3	31	169216	152041	321257	77200	15499	3015	19.45	0.38
F1 Control 5	34.2	31.6	112027	102837	214864	54728	10712	2248	20.99	0.26
F1 Control 7	36.1	32.6	401112	360362	761474	135092	39999	8345	20.86	0.98
F1 Control 8	36.8	33.2	361054	323899	684953	121021	35826	6959	19.42	0.88
F0 FM1-1	34.6	31.6	349937	318316	668253	133334	35918	6952	19.36	0.88
F0 FM1-2	33.8	30.8	1190349	1078459	2268808	256560	97312	23837	24.50	2.40
F0 FM1-4	34	31.3	767770	702422	1470192	199222	71403	15992	22.40	1.76
F0 FM1-5	33.8	31.4	228397	210666	439063	101427	23363	4701	20.12	0.58
F0 FM1-7	35.7	32.7	641237	583992	1225229	180803	62807	13598	21.65	1.55
F0 FM1-8	34.3	31.2	402234	363131	765365	138811	40778	8028	19.69	1.00
F1 FV1-2	35.5	32.2	1426071	1282986	2709057	231607	110000	25186	22.90	2.71
F1 FV1-3	35.3	32	1156570	1043048	2199618	211126	97790	20908	21.38	2.41
F1 FV1-4	35.5	32.2	1074960	969469	2044429	204943	95457	20057	21.01	2.35
F1 FV1-5	34.7	31.4	440029	395994	836023	140446	47366	8860	18.71	1.17
F1 FV1-6	35.5	32.4	859858	781371	1641229	184485	80703	16838	20.86	1.99
F1 FV1-7	35.4	31.8	744750	665157	1409907	176434	73059	14805	20.26	1.80

Table S2. Differential Methylated Cytosines (DMCs) of the F0 generation of snails treated with Flv1.

Chromosome	Start	End	Differential methylation	Context	Transcripts near
LGUN_random_Scaffold1294	50014	50015	-38.99	Intergenic	No transcript near before 3.5 Kb
LGUN_random_Scaffold1294	50019	50020	-38.03		
LGUN_random_Scaffold1294	50030	50031	-43.17		
LGUN_random_Scaffold226	75484	75485	-18.99	Intergenic	No transcript near before 3.5 Kb
LGUN_random_Scaffold2491	21139	21140	-32.16	Intergenic	539 bp downstream the only exon of the CUFF.31291
LGUN_random_Scaffold2491	21145	21146	-39.59		
LGUN_random_Scaffold3890	33057	33058	-16.04	Promoter	2.5 Kb upstream the CUFF.39715.1
LGUN_random_Scaffold3939	8047	8048	16.64	Intergenic	No transcript near before 3.5 Kb
LGUN_random_Scaffold3939	8048	8049	-24.59		
LGUN_random_Scaffold3939	8072	8073	25.29		
LGUN_random_Scaffold3939	8073	8074	-23.12		
LGUN_random_Scaffold3939	8105	8106	-27.58		
LGUN_random_Scaffold3939	8122	8123	-15.5		
LGUN_random_Scaffold4692	16213	16214	-24.98	Promoter	643 bp upstream the firs exon of the transcript CUFF.43302.1
LGUN_random_Scaffold4692	16218	16219	-28.59		
LGUN_random_Scaffold4692	16229	16230	-29.35		
LGUN_random_Scaffold4692	16231	16232	-32.01		
LGUN_random_Scaffold4692	16236	16237	-31.34		
LGUN_random_Scaffold4692	16244	16245	-36.44		
LGUN_random_Scaffold4692	16247	16248	-35.05		
LGUN_random_Scaffold4692	16250	16251	-33.82		
LGUN_random_Scaffold5434	21440	21441	-18.93	Promoter	200 bp upstream the CUFF.46937
LGUN_random_Scaffold5434	21442	21443	-18.52		
LGUN_random_Scaffold6378	12375	12376	-27.72	Intergenic	1.1 Kb downstream the CUFF.53692.1
	12390	12391	-23.40		

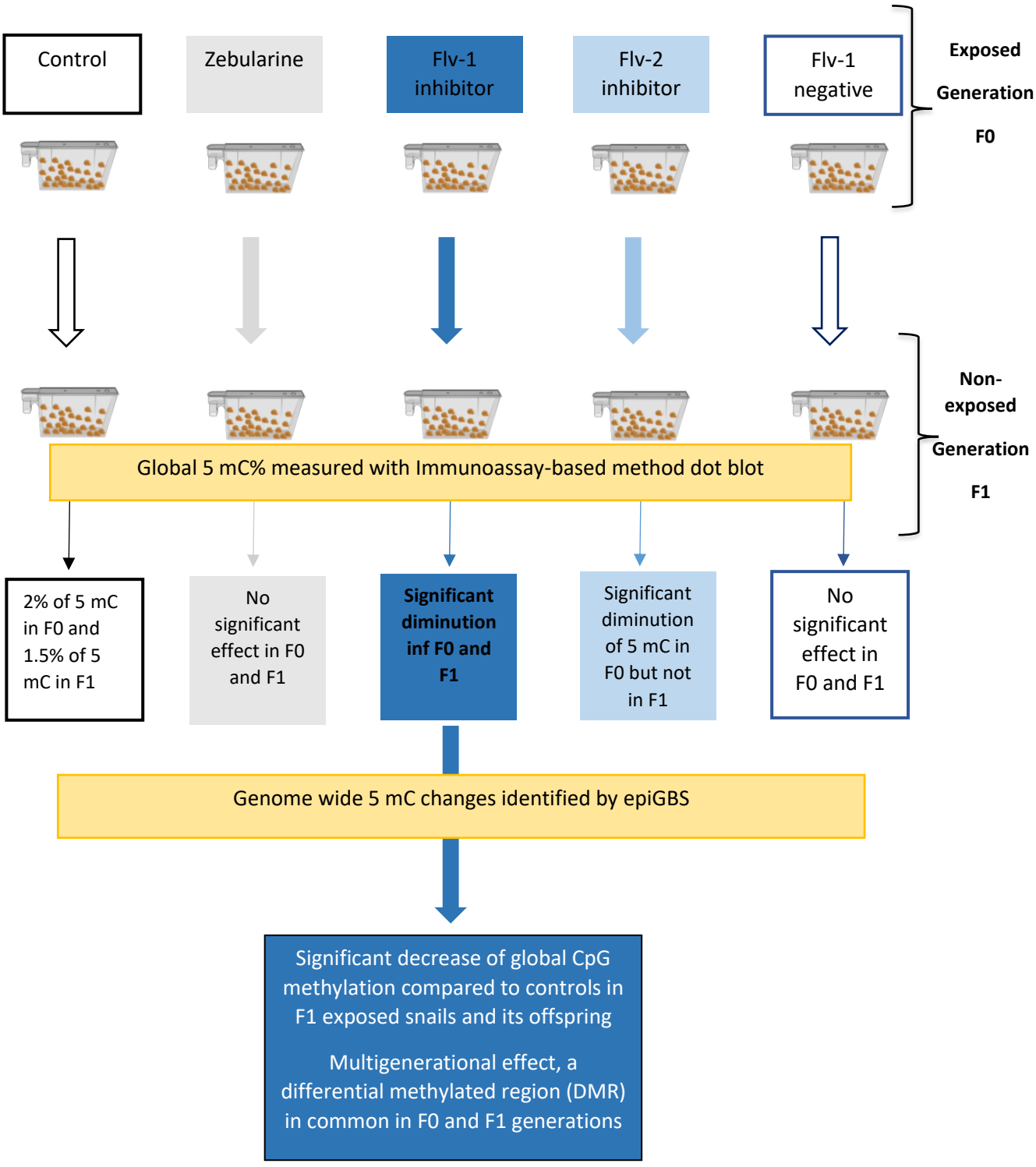


FIGURE S3. Experimental scheme of the screening of 5mC changes induced by the DNMTi. Immunoassay-based method to identify global 5mC changes and subsequent epiGBS analysis of the samples treated with the inhibitor that showed the most significant global changes.

Dot blot densitometry allows for cost efficient screening of 5mC global level in large sample numbers.

In this work we used a low-cost immunological fluorescence-based method to carry out a large sample screening to measure 5mC levels in the genome of the mollusk *B. glabrata* with an antibody against 5mC. A standard curve was done with different concentrations of DNA from HeLa cells. We found a linear positive correlation between the densitometry measure of each Hela cell's DNA spots and the 5mC amount (Figure 1, R^2=0.96, p=0.003).

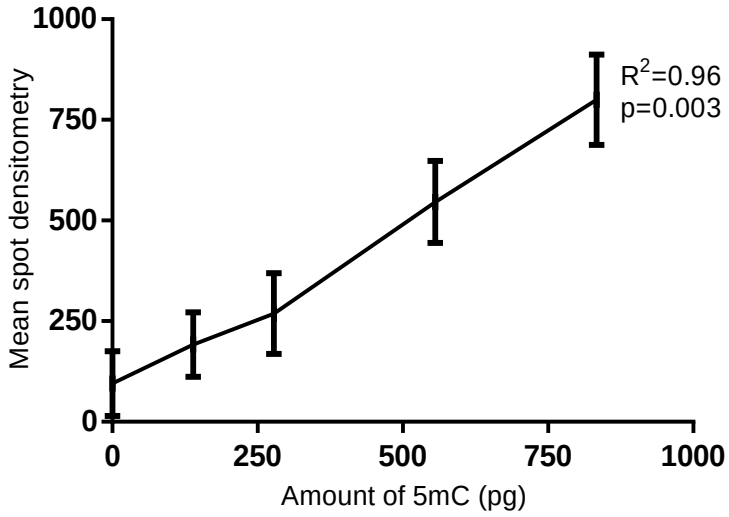


FIGURE S4. Linearity of the mean spot densitometry of the HeLa cells obtained from dot blot assay and the amount of 5mC in pg in each sample. Each ng of HeLa cells contains 4.63 pg of 5mC, five points showed in the graphic correspond to 0, 30, 60, 120 and 180 ng of HeLa DNA. Error bars represented the standard deviation (SD). Simple linear regression is displayed with its *p* value; the linear equation is Y = 0.8592*X + 70.16.

Dot blot and ELISA-based Kit showed equivalent results.

Control and Flv1-treated *B. glabrata* snails (n=15 per group) were used to measure their 5mC % by our dot blot method and simultaneously we measured the 5mC % with a commercial ELISA-based Kit. We found that there was significant difference between the control and the Flv1-exposed snails in the measures obtained by the dot blot assay (*t*= 5.88, *df*=32.70, *p*= 1.389e-06) and the same was found with the ELISA-based commercial kit (*t*=3.98, *df*=34.53, *p*=0.003273). Furthermore, the values obtained were similar between control samples in both methods, 2.05 ± 0.74 and 2.3 ± 1.4 respectively, and for Flv1-treated samples, they showed a 5mC % of 0.75 ± 0.47 with dot blot assay and 0.94 ± 0.81 with the ELISA-based kit (Figure 5). The fold change between control and Flv1-treated snails was similar in both methods (2.7-fold change with dot blot assay and 2.4-fold change with ELISA assay).

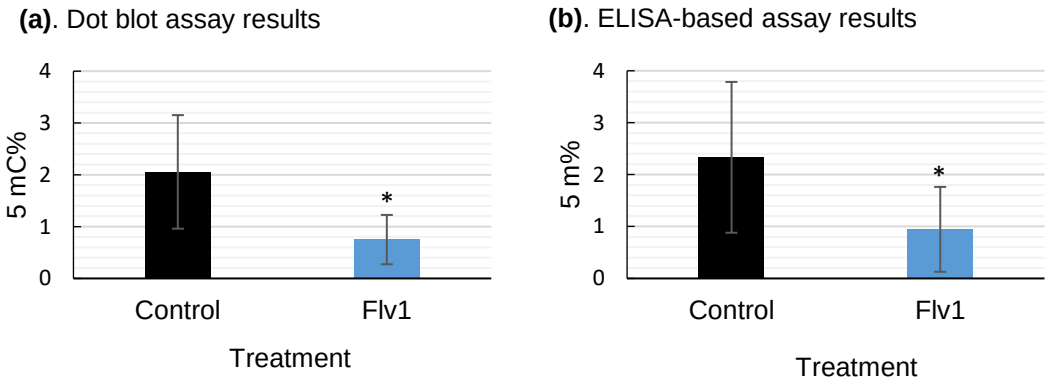


FIGURE S5. (a) 5mC % measures obtained by the dot blot method. (b) 5mC % measures obtained by the ELISA-based assay. Mann-Whitney Wilcoxon test was applied, if not otherwise indicated, between treatment and control significant differences are marked as * for p<0. 0001. 5mC ng was normalized to the 5mC global percentage present in the genome of *B. glabrata*.

Our results showed that dot blot assay delivered comparable results to established ELISA techniques. The dot blot assay allowed medium high throughput (about 200 samples per day) at low cost (about 3 € per sample compared to 19 € per sample with ELISA Kit) and required small amounts of DNA (30-100 ng).

WGBS and epiGBS comparison

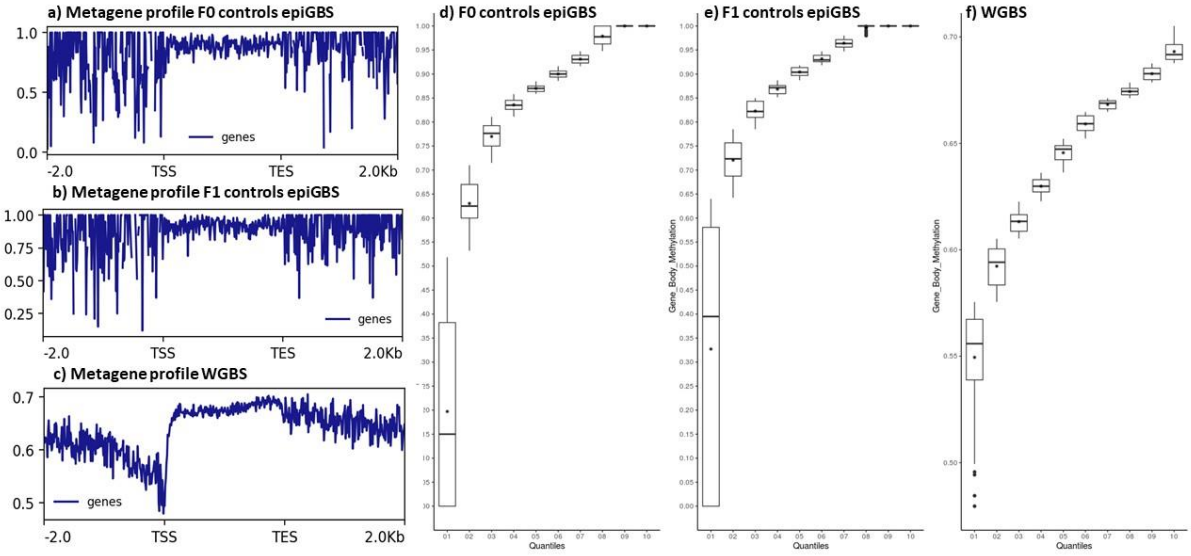


FIGURE S6. CpG methylation ratio profile across the bodies of genes and quantiles distribution of epiGBS and WGBS libraries. (a) Metagene profile of CpG methylation ratio of F0 control epiGBS libraries, (b) F1-control epiGBS libraries and (c) WGBS library. −2.0 kb indicates the upstream 2,000 bp of TSS, 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.

Increase of CpG methylation level after TSS is more evident in WGBS, where it showed a maximum level of 0.7 relative methylation, while in epiGBS showed a maximum level of 1.0. Moreover, the range of methylation changes was higher in epiGBS results, the levels of CpG methylation ratio varied from 0 to 1 before TSS and after TES (Figure S6a-b) while the levels of methylation varied from 0.5 to 0.6 in WGBS before TSS and from 0.6 to 0.7 after TES (Figure S6c). The quantile distribution of GBM was also different between epiGBS and WGBS. In the epiGBS libraries of F0-controls, the highest quantile is the first one and comprises CpG values of 0 to 0.52 (Figure S6d). In the epiGBS libraries of F1-controls, the highest quantile is the first one and includes values from 0 to 0.64 (Figure S6e). And in the WGBS library, the highest quantile is also the first one and comprises the values of 0.50 to 0.57 (Figure 6f). EpiGBS showed a bias towards methylated regions of the epigenome.

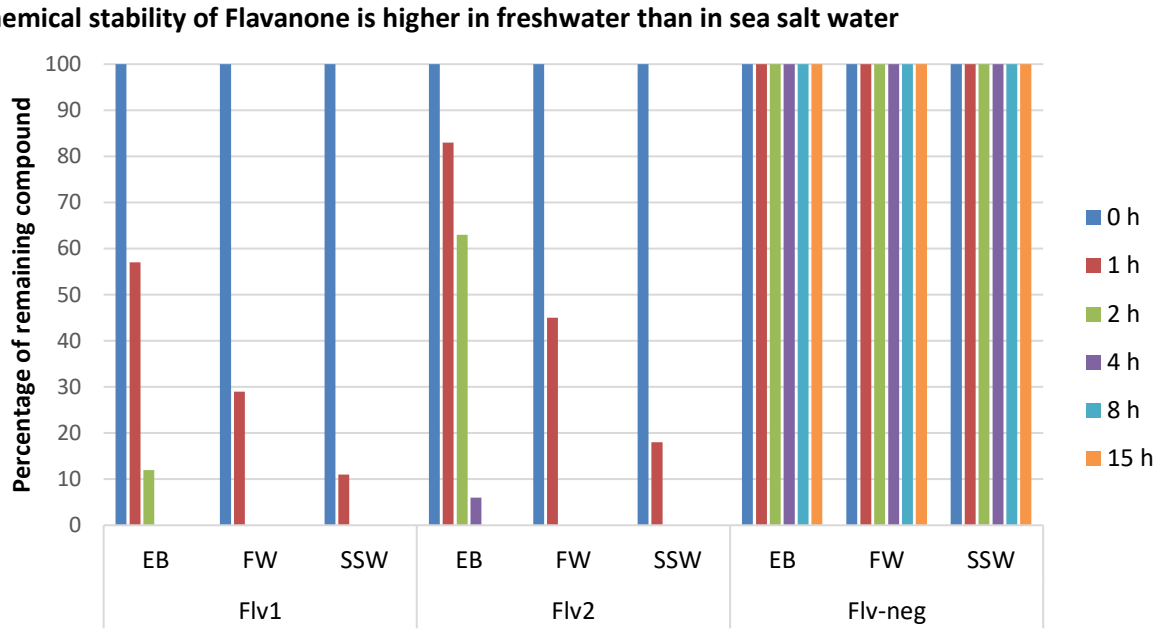


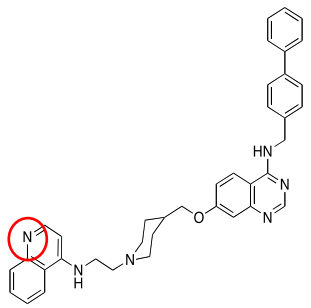
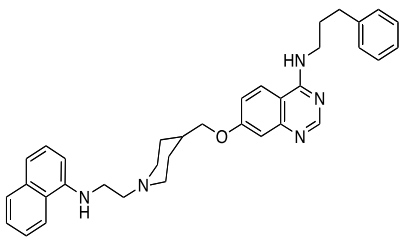
FIGURE S7. Flavanone chemical stability in three different media, enzymatic buffer (EB), freshwater (FW) and sea salt water (SSW). Stability was measured by HPLC at 100 µM of each compound in the three media.

The two active flavanones (Flv1 and Flv2) are moderately stable in the enzymatic buffer at pH 7.2 (EB), there was 12% of remaining compound after 2h for the Flv1 and 6% of remaining compound after 4 h for the Flv2. Their stability decreased in freshwater at pH 7.8 (FW), with 29% of remaining compound after 1 h for Flv1 and 45% of remaining compound after 1 h for Flv2 (Figure 7). The lowest stabilities were found in sea salt water at pH 8 (SSW), 11% of remaining compound after 1 h for Flv1 and 18% of remaining compound after 1h for Flv2 (Figure S6). On the other hand, the negative flavanone compound (Flv-neg) was highly stable in the three conditions (EB, FW and SSW).

Unpublished additional results

Bisubstrate analogue compounds (BA compounds), the BA active compound (BA1) and the inactive analogue (BA-neg), (Halby et al. 2017) were used in an exposition experiment during 10 days to *B. glabrata* mature adult individuals. Stock solution at 10 mM were made in ultrapure Milli-Q water and aliquoted and then applied to 1 L of water in an aquarium with 100 snails, another aquarium without the inhibitor was maintained as the control group with another 100 snails. BA1 is a quinazoline -quinoline derivate showing potent *in vitro* inhibition activity agaisnt DNMT1 and DNMT3A-c. BA1 act by mimicking both substrates of the DNA methyltranferase, SAM and deoxycytidine. The inactive BA-neg bearing a naphtyl moiety instead of a quinoline group, is inactive against DNMTs but maintains comparable cytotoxicity and is thus used as a control (Halby et al. 2017).

TABLE 1. Structure, molecular weight (Mw) and molecular formula of Bisubstrate analogues compounds. The red circle showed the chemical difference between BA1 and BA-neg.

Name	**Structure**	**Mw**	**Formula**
BA1		594.75	$C_{38}H_{38}N_6O$
BA-neg		545.72	$C_{35}H_{39}N_5O$

Global 5mC changes induced by BA compounds.

The bisubstrate analogue BA1 produced a significantly lower 5mC % ($W=126$, $p<0.0001$) compared to control group, but the supposedly inactive bisubstrate analogue BA-neg also led to a significant difference in 5mC % compared to control group ($W=168$, $p<0.0001$), no difference found between BA1 and inactive BA-neg ($W= 39$, $p=0.68$) (Figure 1a).

Unexpectedly, the offspring snails coming from F0 exposed to BA1 showed a 2-fold higher 5mC % (1.42% vs 2.9%) than the control group ($W=126$, $p<0.0001$) (Figure 1b).

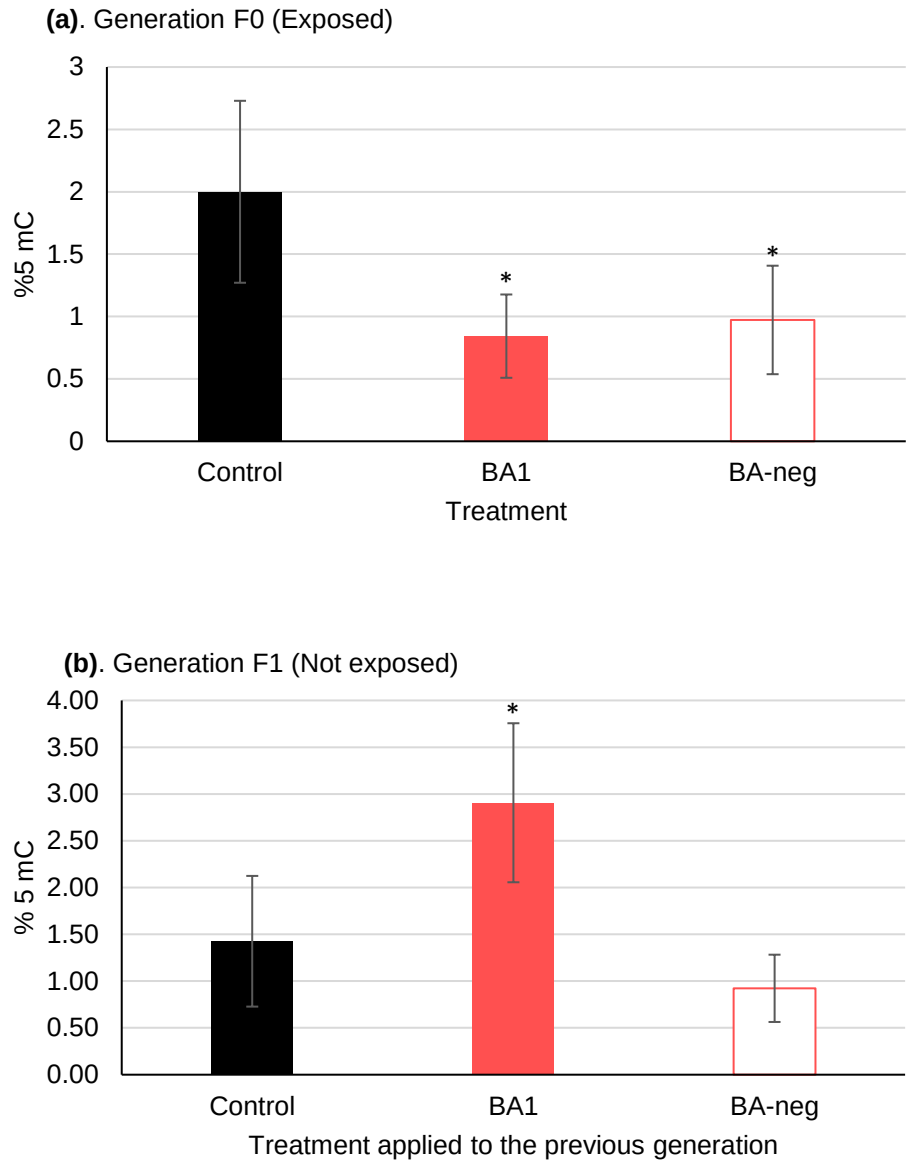


FIGURE 1. 5mC % of *B. glabrata* snails upon treatments with BA compounds at a concentration of 10 µM, error bars represent SD, n=30 per treatment. (a) 5mC % in the F0 generation (exposed), black bar for control, red bar corresponds to the bisubstrate analogue BA1 and the white bar with red outline represents the inactive BA-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 represented by *.

Phenotypic effects of BA compounds

Compound BA1 triggered a significant mortality showing a 55% survival at the end of the 70 days' period (Figure 2), and significant difference was found in survival percentage between snails treated with both bisubstrate analogues, BA1 and BA-neg, and the control group ($\chi 2=14.17$, $p=0.0002$ for BA1 and $\chi 2=7.54$, $p=0.006$ for BA-neg).

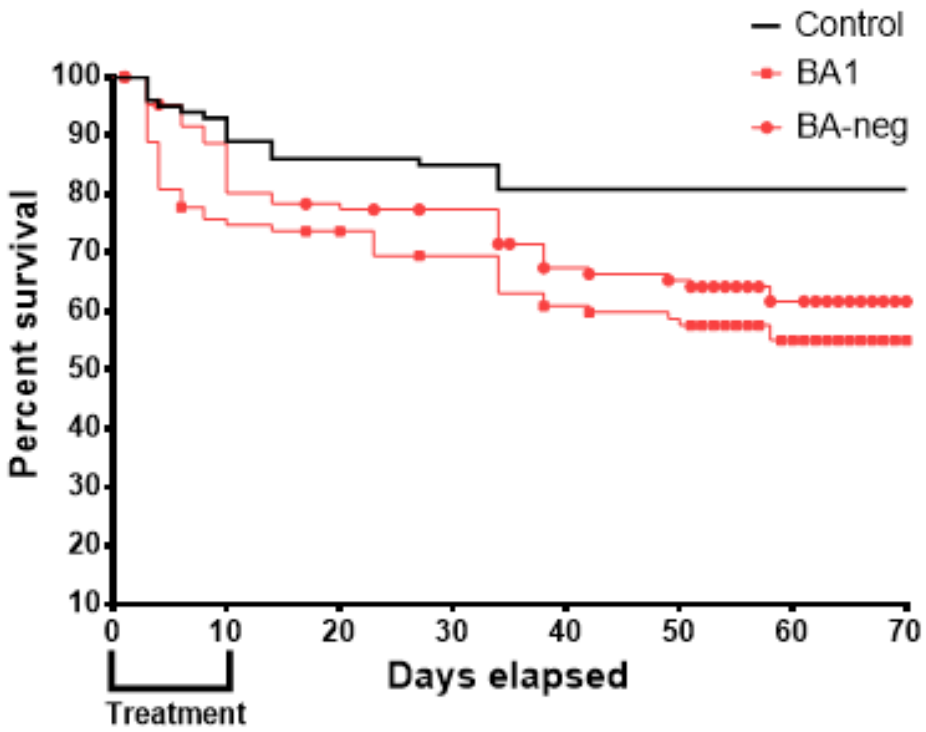


FIGURE 2. Kaplan-Meier survival curves upon treatment with Bisubstrate analogues, BA1 (red line with squares) and the inactive BA-neg (red line with circles).

Bisubstrate analogue BA1 had no significant effect in the fecundity (number of offspring) but BA-neg presented a significant difference in the number of offspring compared to control group ($p=0.0002$).

TABLE 2. Contingency table of fecundity of the snails exposed to BA compounds. 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	BA1	BA-neg
Total number of laid eggs	191	170	208
Number of non-hatched eggs	152	129	192
Number of offspring	39	41	16**

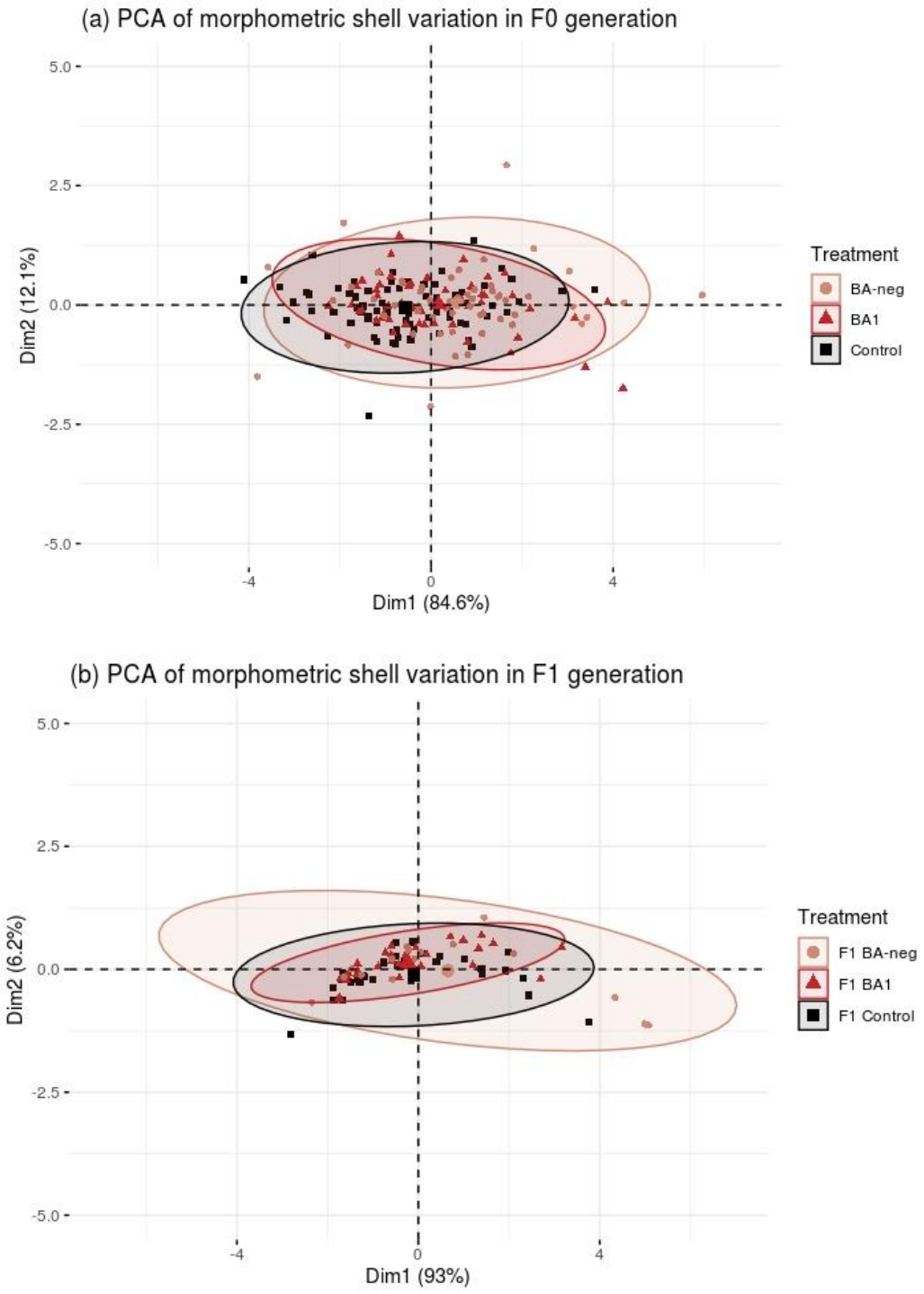


FIGURE 3. PCA of morphometric traits of BA treatments of F0 (a) and F1 generation (b). The confidence ellipses show a confidence interval of 95%. The axis 1 includes the three morphometric measures (shell width, height and weight) and the axis 2 include shell width and height.

Epigenomic profile of BA treated snails

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 BA1-treated and controls snails. 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 3, some differences are observed between control and BA-treated snails.

TABLE 3. Percentage of CpG methylation sites which display an unmethylated or complete methylated pattern in controls and BA1-treated samples.

Generation F0	CpG methylation frequency	Generation F1	CpG methylation frequency	Generation F0	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
BA1-1	81.7	12.6	F1 BA1-1	83.2	13.9
BA1-2	81.3	12.4	F1 BA1-2	82.4	14.9
BA1-3	80.4	10.4	F1 BA1-3	80.7	11.3
BA1-4	79.6	11.8	F1 BA1-4	83	14.8
BA1-5	79.3	11.4	F1 BA1-5	81.4	12
BA1-6	81.4	12.3	F1 BA1-6	80.3	12.4

epiGBS confirms hypomethylation by BA1 in F0 generation and identifies hypermethylated regions in F1 generation

The mean percentage of CpG methylation was 18.11 ± 0.89 % in control snails and 15.81 ± 0.68 % in BA1-exposed snails, 11.1 ± 0.6 % in the offspring of control snails and 13.0 ± 2.7 % in the offspring of BA1-exposed snails. There was significant difference in global percentage of CpG methylation between Control and BA1-exposed snails (t=5, df= 9.3, p=0.0006) and no significant difference was found in their offspring (t= -1.7, df= 5.6 p=0.13).

PCA analysis of CpG methylation showed that BA1 treated samples clustered strongly and control samples are spread out (Figure 4a). In the F1 generation, PCA of CpG methylation showed that F1-BA1 samples are not grouped as in the previous generation, conversely, they are spread out and F1-control samples are more grouped than in the generation F0 (Figure 4b).

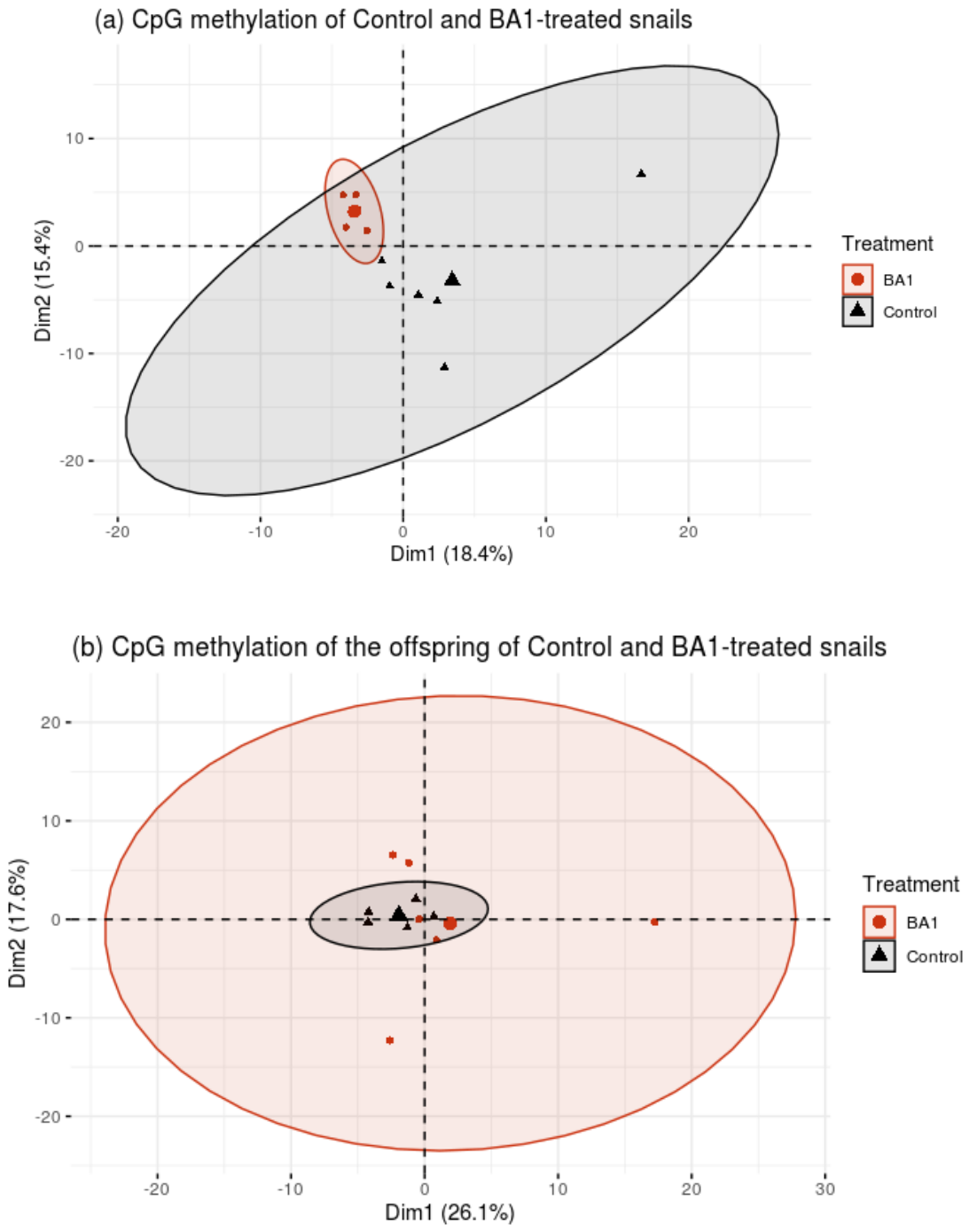


Figure 4. PCA of CpG methylation of (a) control and BA1-treated samples and of (b) the progeny of control and BA1-treated samples.

BA1 induced hypomethylated DMRs in F0, one DMR was inherited to F1 generation but shifts from hypomethylated to hypermethylated

We found 26 DMCs between control and BA1-treated samples, 24 hypomethylated and 2 hypermethylated. The higher amount of hypomethylated DMCs is consistent with the global hypomethylation effect found with dot blot in BA1-exposed snails. Among these DMCs, the majority was found in the intergenic region (14 DMCs), 9 DMCs were found in the promoter region and only 3 DMCs in exons. From the 26 DMCs found, 19 DMCs were concentrated in 4 DMRs (Table S4).

19 DMCs, 3 hypomethylated and 16 hypermethylated were found between F1-control and F1-BA1 samples (Table 5). The higher number of hypermethylated DMCs reestablished the global CpG methylation level since no significant differences were found at the global level between the offspring of the control group and the offspring of BA-treated snails. Among these DMCs, most of them were found in an intergenic context (11 DMCs), 3 DMCs were found in a promoter region, 4 DMCs in exons and 1 DMC in an intron (Table S5). From the 19 DMCs, 8 DMCs were concentrated in 2 DMRs. 1 DMR was found in common in both generations, being hypomethylated in the generation F0 and hypermethylated in the generation F1 (Figure 5).

Table 5. DMCs per inhibitor treatment vs control samples 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
BA1	F0	26	4	24	4	2	0
BA1	F1	19	2	3	0	16	2
BA1	F0 and F1	4	1			4	1

Figure 5. 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), BA1-treated samples (blue) and its offspring (magenta).

Manuscript 3 in preparation

An approach to study the relative contributions of epigenetics and genetics to phenotypic plasticity in the mollusk *Biomphalaria glabrata,* vector of the human parasite *Schistosoma mansoni*

An approach to study the relative contributions of epigenetics and genetics to phenotypic plasticity in the mollusk *Biomphalaria glabrata,* vector of the human parasite *Schistosoma mansoni*

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Abstract

An approach to determine the contribution of DNA methylation and genetic information to the phenotypic plasticity in the snail *Biomphalaria glabrata*, the vector of the parasite *Schistosoma mansoni*, is proposed in this work to find out if DNA methylation plays a role in this host-parasite interaction. A multigenerational experiment was done by crossing snails with contrasting global DNA methylation profiles induced by the use of chemical DNMT modulators (DNMTm) and by subsequent self-fertilization reproduction to evaluate if we can induce epimutations and if these are segregated in self-fertilization lines, we inspired us in the epigenetic recombinant inbred lines (epiRILs) approach used in plants. Our phenotypic results showed that our epiRILs showed more phenotypic variability in the hatching rate, the prevalence and intensity of infection by the parasite *S. mansoni* than control recombinant inbred lines. We hypothesized that this wider phenotypic variability can be due to segregated epimutations. Epigenotyping by sequencing will be done to assessed genetic and DNA

methylation polymorphisms of the epiRILs that presented the most divergent phenotypes to analyze the contribution of both informations to the phenotypic variability observed.

Introduction

DNA methylation plays a role in the generation of phenotypic plasticity in many species (Johannes, Porcher et al. 2009, Herrera and Bazaga 2013). One of the bearers of epigenetic information is DNA methylation. In invertebrate species, there is evidence for a role of DNA methylation in the generation of developmental plasticity in the bee *Apis mellifera* (Elango, Hunt et al. 2009*)* and it also plays a major role in the development of the oyster *Crassostrea gigas* (Riviere, Wu et al. 2013). DNA methylation is involved in behavioral plasticity in the bumble bee *Bombus terrestris* (Amarasinghe, Clayton et al. 2014) and in the size variation of worker ants *Camponotus floridanus* (Alvarado, Rajakumar et al. 2015). DNA methylation is considered to influence alternative splicing in genes that are responsive to environmental stress, allowing to display transcript variability that in turn, will generate phenotypic variability (Flores, Wolschin et al. 2012). In the case of the mollusk *Biomphalaria glabrata*, a methylome has been generated (Adema, Hillier et al. 2017). DNA methylation pattern is of the mosaic type and occurs predominantly in CpG pairs (Fneich, Dheilly et al. 2013). Methylation show responsiveness to parasite infection by *Schistosoma mansoni* (Knight, Ittiprasert et al. 2016, Geyer, Niazi et al. 2017). Furthermore, we demonstrated previously that DNMT inhibitors induce phenotypic diversity in the morphometric traits of treated *B. glabrata* snails and its offspring. However, we did not formally exclude an impact of genotype diversity of the snails in this work (Luviano, Lopez et al. 2020). Here we set out to evaluate the relative contributions of genetic and epigenetic variance on phenotypic variance.

In model plants there are experimental approaches which were very successful in the characterization of the contributions of DNA methylation to the phenotypic variability. These approaches permitted also to disentangle the genetic and the epigenetic contributions to the

phenotypic variation. One of these methods relies on the generation of epigenetic recombinant inbred lines (epiRILs) in *Arabidopsis thaliana*, allowing for the discovery of epialleles that contribute to phenotypic variation in this species. The basis of this approach consists in reducing genetic diversity by crossing genetically very similar individuals that possess large DNA methylation differences. In *A. thaliana* this was achieved by crossing wild-type plants with mutants of the *DDM1* gene. The gene encodes an ATPase chromatin remodeler involved in the maintenance of DNA methylation and the mutant *DDM1* plants exhibit a ~70% reduction of global DNA methylation (Kakutani, Jeddeloh et al. 1995). Crosses with these mutant *DDM1* plants introduced epigenetic diversity while keeping the genetic diversity low. The epigenetic recombinant inbred lines (epiRIL), were obtained by repeated backcrossing over six generations and subsequent self-fertilization for four generations (Johannes, Porcher et al. 2009). The epiRILs realized in *A. thaliana* showed variation and high heritability in multiple phenotype traits in addition to stable inheritance of parental DNA methylation "epialleles" over eight consecutive generations (Johannes et al, 2009). These epiRILs allowed to find that epigenetic information have an important role in the generation of phenotypic variability for some traits but less for others, and that epigenetic marks can be inherited across multiple generations having the potential to participate in adaptation of species to changing environments.

Similar approaches have been performed in some animal species, *e.g.* the "epilines" obtained from genistein-injected eggs in the quail, (Leroux, Gourichon et al. 2017) but these approaches have, at our knowledge, not been applied to invertebrate species. There are invertebrates that can reproduce by self-fertilization, meaning that this approach can be used to study the contribution of epigenetic marks to the phenotypic variation in this group whose DNA methylation has been poorly studied so far.

We decided to adapt the epiRILs approach to our model, the snail *Biomphalaria glabrata*. This snail has a generation time of 70 days and it can reproduce by cross-fertilization and self-

fertilization. To introduce epimutations we treated the snails with DNMT modulators (DNMTm). Two new DNMTm were used: Flv1 (Luviano, Lopez et al. 2020) and BA1 allowing us to generate snails with contrasting DNA methylation profiles: hypomethylated snails offspring of group treated with Flv1, and hypermethylated snails that are offspring of those treated with BA1. By producing crosses between hypo- and hypermethylated snails and pursuing the subsequent generation by self-fertilization we evaluated the phenotypic variability of the snails regarding its fecundity and its response to the parasite infection. We focused on these two phenotypic traits because they are among most important for parasite transmission.

Material and methods

Ethics statement

Biomphalaria glabrata Brazilian strain *(Bg BRE)* was used in this study. The IHPE laboratory maintains multiple strains stocks of *B. glabrata*. 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.

DNMTm treatments.

In a previous work, treatments with a new flavanone-type DNMT inhibitor (Pechalrieu, Dauzonne et al. 2020) named Flv1 were performed in the snail *B. glabrata* and it showed high efficiency to reduce global DNA methylation in two consecutive generations (Luviano et al. 2020). In this work, we decided to use this flavanone compound and a second type of DNMTm called bisubstrate-type compound BA1 that have a different mechanism of action (Halby, Menon et al. 2017). Flv1, and BA1 and BA-neg were used to treat *B. glabrata* (Brazilian strain *BgBRE*) mature adults of 5-7 mm of shell diameter for 10 days. Stock solutions at 10 mM were made for each compound in ultrapure Milli-Q water and added to 1L of water (final concentration 10 µM) in an aquarium with 100 snails for each compound (Flv1, BA1, and BA-neg). Another aquarium without any compound was maintained as the control group with 100 snails.

DNA extraction and global DNA methylation assays

The NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany) combined with the use of zirconia beads (BioSpec, USA, Cat. No. 11079110z) as described previously (de Lorgeril, Lucasson et al. 2018) was used for DNA extraction from whole body without shell of 30 *B. glabrata* snails per treatment group. The global 5mC of the 30 snails per treatment was measured by the dot blot method based on the recognition of 5 methylcytosine (5mC) by anti-

5mC antibody (Abcam, Cat. No. ab73938, Lot: GR278832-3) as described previously (Luviano, Diaz-Palma et al. 2018).

The global 5mC level of the 4 control couples and the 15 couples with altered epigenomes were quantified using a commercial Kit ELISA Methylated DNA Quantification Kit (Colorimetric) (Abcam, ab117128) for detection of 5mC following manufacturer's instructions. To quantify the absolute amount of methylated DNA, a standard curve was made plotting the OD values versus different concentrations of the positive control provided in the Kit.

Crossing experiment

A crossing experiment was done with the offspring of the group treated with the compound Flv1, and the offspring of snails treated with the compound BA1. 15 couples were formed with one snail offspring of those treated with Flv1 and one snail offspring of those treated with BA1, these couples were named epi-lines and another four couples with the offspring of controls snails were formed and correspond to the control lines. Then 3 snails per control line and per epi-line were reproduced in self-fertilization for a screening of divergent phenotypes. The 6 epi-lines with the most contrasting phenotypes were selected to realize a larger snail phenotyping, with 10 self-fertilization snails per epi-line and 10 snails in self-fertilization per control line (2 control lines). The snails obtained by self-fertilization from the epi-lines were named epigenetic recombinant inbred lines (epiRILs) and the snails obtained by self-fertilization from control lines were named recombinant inbred lines (RILs), epiRILs and RILs correspond to the F3 generation (Figure 1).

Snail phenotyping

The fecundity of the epi-lines and the control lines were analyzed by recording the number of eggs and the number of hatched snails, to then calculate the hatching rate (number of hatched

snails/ total number of eggs ×100). Then to screen the most divergent phenotypes, a screening of the 19 lines was done by placing three snails per control line and per epi-line in self-fertilization (Figure 1). In the screening of divergent phenotypes, 15 snails per epiRIL and RIL were exposed to the parasite *S. mansoni*.

The experimental infections were done by exposing each snail to five parasite miracidia larvae. *S. mansoni* strain Bresil (*SmBRE*) and the *B glabrata* strain Brazil (*BgBRE*) were used in this study. The prevalence (number of infected snails/number of total snails exposed to parasite ×100) and the intensity of infection (number of parasite larvae developed inside the snail) was measured per snail and then compared between control lines and epi-lines. In the second phenotyping with more statistical power, with 10 self-fertilization snails per epi-line and 20 self-fertilization snails as controls, infections were done in ten snails per RIL and per epiRIL (Figure 1).

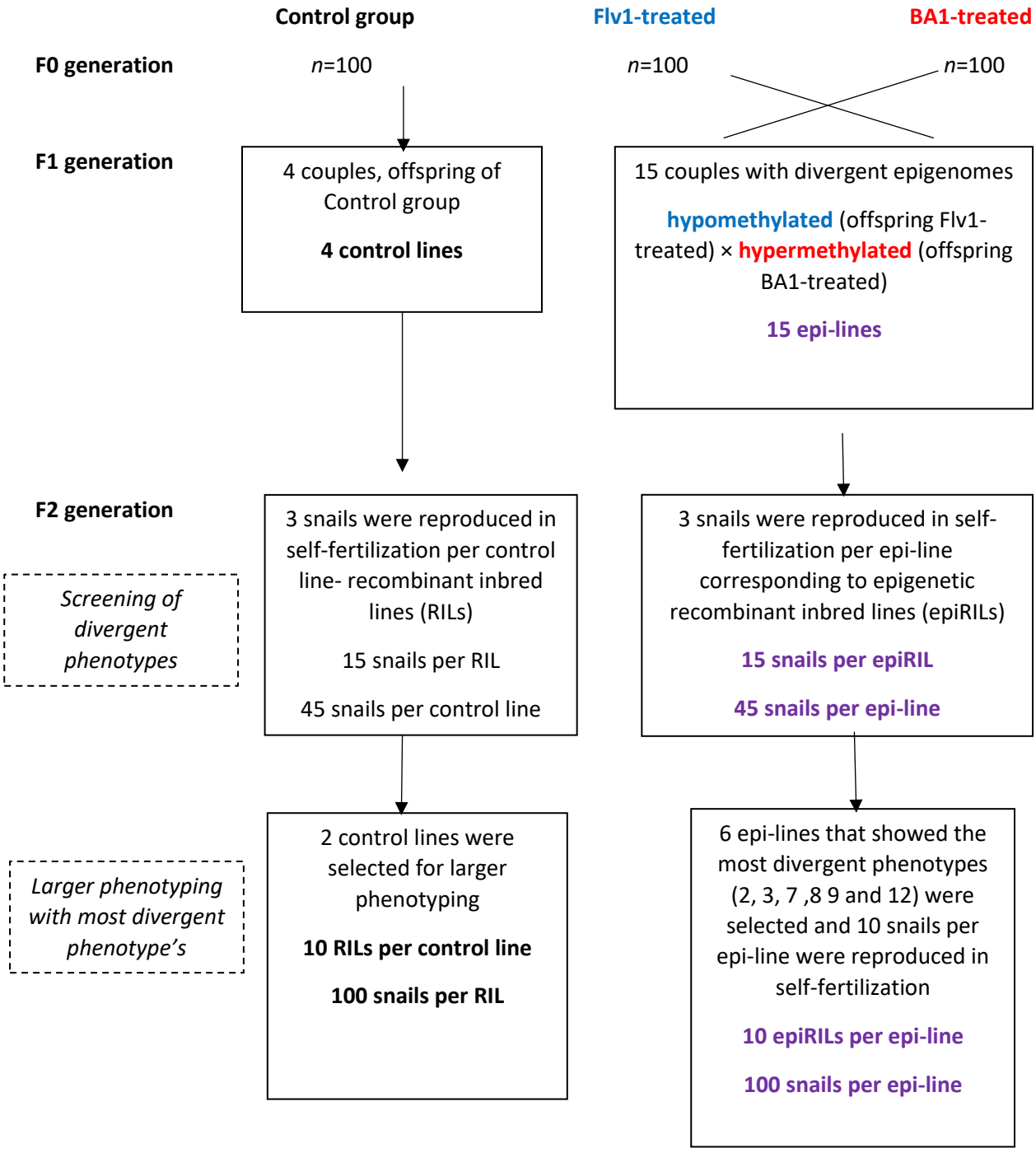


Figure 1. Experimental design of the multigenerational experiment. The snails of the F0 generation were exposed with the DNMTm Flv1 (in blue), BA1 (in red) or not exposed (control group, in black). In the F1 generation, four control couples and 15 couples with contrasting epigenomes (offspring of snails exposed with Flv1 and BA1, respectively) were formed. In the F2 generation, 3 snails were reproduced in self-fertilization for each control line and for each epi-line, 15 snails were used per snail in self-fertilization to measure their fecundity, the prevalence and intensity of infection when exposed to *S. mansoni*. The 6 epi-lines that showed the most divergent phenotypes were selected for a second phenotyping with higher statistical power, 10 snails in self-fertilization by epi-line (named epiRILs) and 10 snails in self-fertilization for 2 control lines (named RILs).

Results

DNMTm produce viable and fertile hyper- and hypomethylated snails in F1

The dot blot results showed that the Flv1 compound reduce the global 5mC% 2-fold in the F0 and F1 generation (Figure 2a-b, blue bars). BA1 reduces the global 5mC% 2-fold in the F0 generation but led to a 2-fold higher global 5mC% in the F1 generation compared to control (Figure 2a-b, red bars).

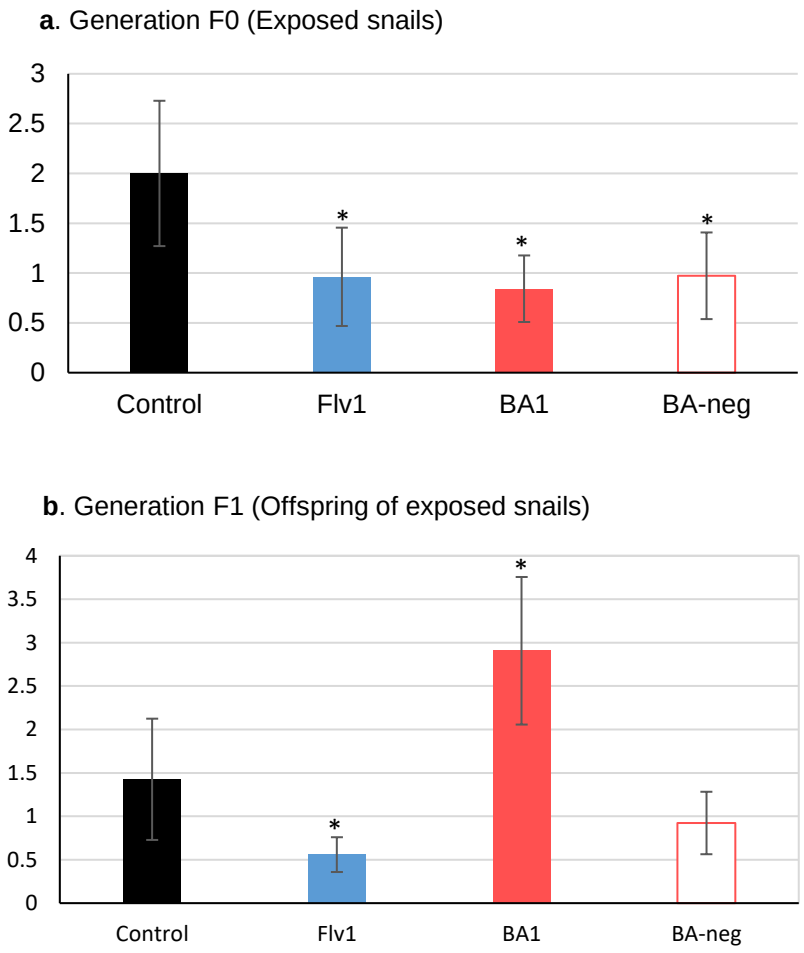


Figure 2. a) 5mC% of the Flv1, BA1 and BA-neg treated snails compared to control group. B) 5mC% of the F1 generation, offspring of control snails (black bar) and of snails treated with the Flv1 inhibitor (blue bar), the BA1 inhibitor (red bar) and the BA-negative compound (white bar with red outline). Mann-Whitney Wilcoxon test was applied, significant

differences between control and treatment are marked as * for p<0. 05. 5mC ng was normalized to the 2% 5mC global percentage present in the genome of *B. glabrata* (Fneich, Dheilly et al. 2013).

The results of the ELISA-based detection of 5mC%, showed that the snails paired to produce the epi-lines presented high global methylation level in the offspring of BA1-treated snails and low global methylation level in the offspring of Flv1-treated snails and control individuals and some treated snails showed ~2% of global 5mC% (Table 1). The lines 2, 3, 4, 7, 8, 9, and 12 present the most contrasting profiles of methylation as they showed the highest difference in their 5mC% between paired snails (Figure 3).

Table 1. 5mC% of the snails of the F1generation (offspring from F0 treated either with Flv1 or BA1) used to generate the epi-lines (F2 generation) and the eight control snails used to generate the four control lines. The snails paired to produce the epi-lines present either high methylation level (highlighted in blue), low methylation level (red) or 2% of 5mC% (grey).

Snail Flv1 (offspring from Flv1 treated snails)	5mC%	Snail BA1 (offspring from BA1 treated snails)	5mC%	Gradient in 5mC% in F1 couples (Figure 5)	Line
Control 1	2.11	Control 1	2.16	0.05	Control line 1
Control 2	2.0	Control 2	2.02	0.02	Control line 2
Control 3	2.20	Control 3	2.10	0.10	Control line 3
Control 4	2.13	Control 4	2.06	0.07	Control line 4
Flv1.1-1	1.99	BA1-1	3.36	1.37	Epi-line 1
Flv1.1-2	0.7	BA1-2	3.05	2.35	Epi-line 2
Flv1.1-3	1.8	BA1-3	4.82	3	Epi-line 3
Flv1.1-4	2.2	BA1-4	3.35	1.15	Epi-line 4
Flv1.1-5	2.31	BA1-5	3.9	1.59	Epi-line 5
Flv1.1-6	1.32	BA1-6	1.5	0.18	Epi-line 6
Flv1.1-7	1.54	BA1-7	4.82	3.28	Epi-line 7
Flv1.1-8	1.16	BA1-8	3.55	2.39	Epi-line 8
Flv1.1-9	1.5	BA1-9	3.84	2.34	Epi-line 9
Flv1.1-10	1.87	BA1-10	1.97	0.1	Epi-line 10
Flv1.1-11	1.83	BA1-11	3.4	1.57	Epi-line 11
Flv1.1-12	1.42	BA1-12	4.39	2.97	Epi-line 12
Flv1.1-13	1.27	BA1-13	1.36	0.09	Epi-line 13
Flv1.1-14	0.31	BA1-14	0.61	0.3	Epi-line 14
Flv1.1-15	0.54	BA1-15	1.08	0.54	Epi-line 15

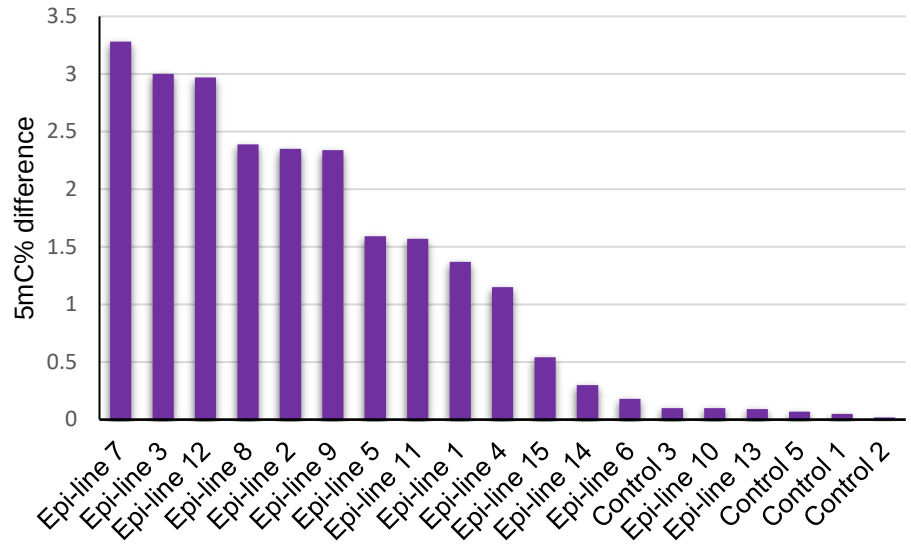


Figure 3. Difference in global 5mC% between paired snails (from F1 generation). The lines with the most contrasting 5mC levels are the lines 7, 3, 12, 8, 2, and 9.

Epi-lines and epiRILs have higher variation in hatching-rate compared to controls

The most divergent fecundities by cross-fertilization were found in the epi-lines 2, 3, 7, 8, 9 and 12 showing higher hatching rate % compared to control (Figure 4).

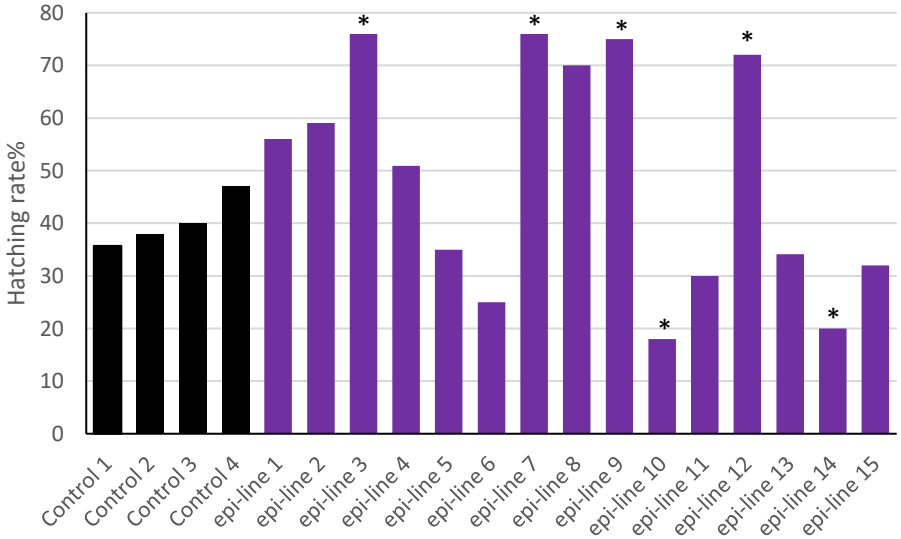


Figure 4. Hatching rate in cross-fertilization lines (F1 generation). In black the hatching rate of control lines. In violet, the hatching rate of the 15 epi-lines. The asterisks (*) indicate significant

differences compared to control lines (Fisher's exact test, $p<0.05$). Significant differences compared to control were found in line 2, 3, 7; 8; 9, 12, 13 and 14.

The epiRILs (Figure 5b, violet bars) showed higher variability in self-fertilization fecundities compared to control RILs (Figure 5a, black bars).

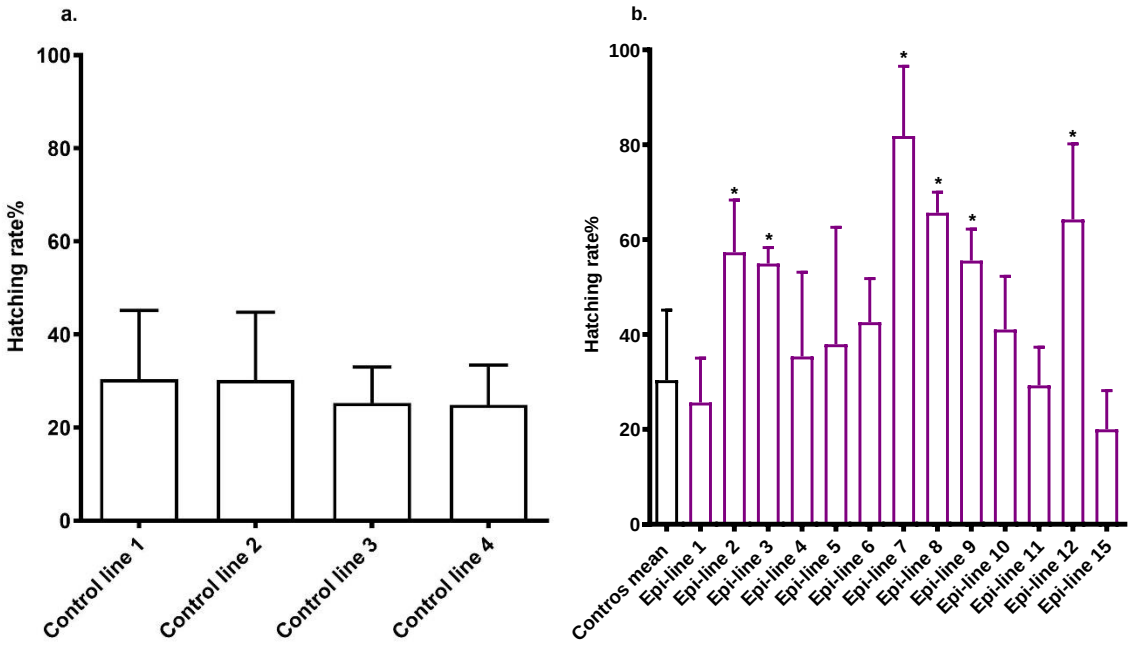
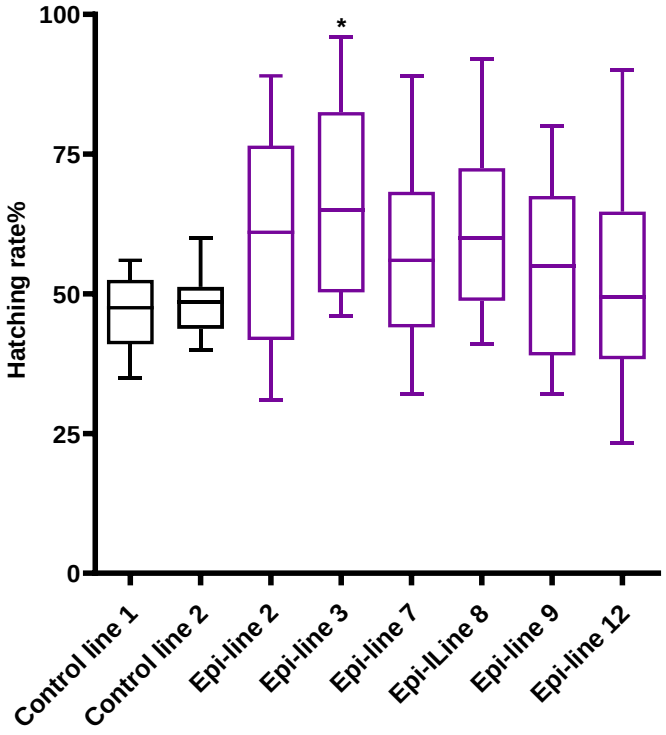


Figure 5. Hatching rate in self-fertilization lines (F2 generation) in the control lines (3 RILs per control line), black bars (a) and in epi-lines (3 epiRILs per epi-line), violet bars (b). The asterisks (*) indicate significant differences compared to control lines. The lines that presented significant differences in the hatching rate against control lines mean are 2, 3, 7, 8, 9 and 12. The epi-lines 13 and 14 were not included as some selfed snails died making unfeasible to compare fecundity with the other epi-lines.

The epi-lines 2, 3, 7; 8; 9 and 12 were selected since they showed significant differences in the hatching rate in cross-fertilization and self-fertilization compared to controls. Moreover, these epi-lines presented the highest differences in the 5mC% levels between paired snails. A larger phenotyping with more statistical power by increasing the number of snails in self-fertilization per epi-line to 10 was done. 10 epiRILs per epi-line (60 epiRILs) were compared to 20 control RILs from two control lines.

The epiRILs showed more variability in the hatching rate than control RILs. To simplify the graphic representation, we analyzed variability between RILs and epiRILs regrouped by line (10 RILs per control lines and 10 epiRILs per epi-lines), the coefficient of variation (CV%) was calculated. The CV% was higher in the six epi-lines than in the control lines. The median of the hatching rate was significantly different in epi-line 3 against controls line mean (Dunntt's test, $p<0.005$).

a)



b)

Line	RIL 1	RIL 2	Epi-line 2	Epi-line 3	Epi-line 7	Epi-line 8	Epi-line 9	Epi-line 12
CV%	14.52%	17.17%	32.80%	26.67%	29.32%	26.30%	30.61%	37.90%

Figure 10. **a)** Hatching rates of control lines (black boxplots) and epi-lines (violet boxplots). The asterisks (*) indicate significant differences compared to controls (One-way ANOVA and Dunnett's multiple comparisons test, $p<0.005$), data is displayed in Tukey boxplots, the middle lines represent the mean, whiskers represent 1.5 times the highest/lowest point

distance and the error bars represent the standard deviation (SD). **b)** Table with the percentages of coefficient of variation (CV%) per line.

epiRILs have higher variation in parasite prevalence compared to controls

The prevalence of parasite infection was homogeneous between RILs, ranging from 77% to 88% with not significant differences between them (Kruskal-Wallis test, p=0.1). In contrast, the prevalence of the parasite in epiRILs showed significant differences compared to controls (Kruskal-Wallis test, p<0.005). The epiRILs which showed significant lower prevalences were 3A (53%), 3B (70%), 7A (67%), 8A (58%) and 9A (70%) and the epiRILs that showed significate higher prevalence were 4B, 5A, 5B, 10A, 15B (100%).

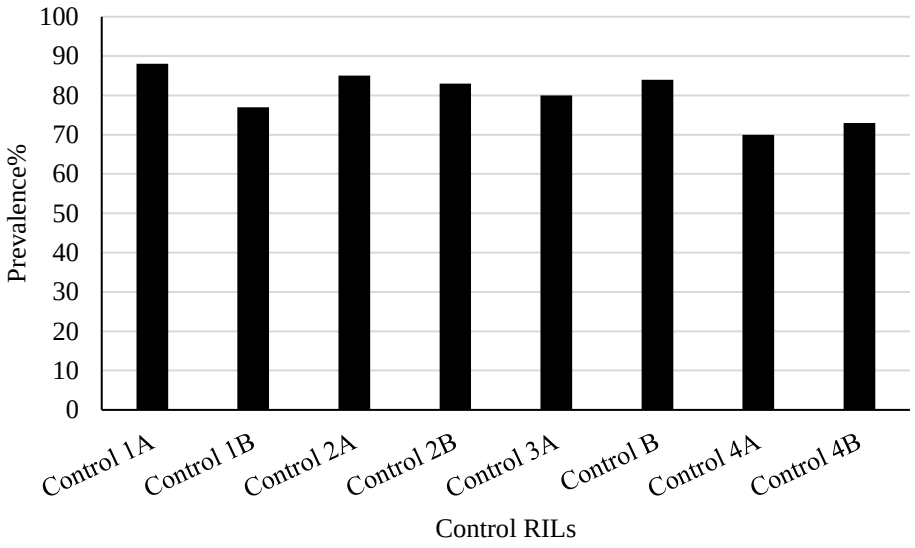


Figure 6. Prevalence of the parasite infection in control RILs (15 snails per RIL). The Fisher's exact test was applied; not significant differences were found.

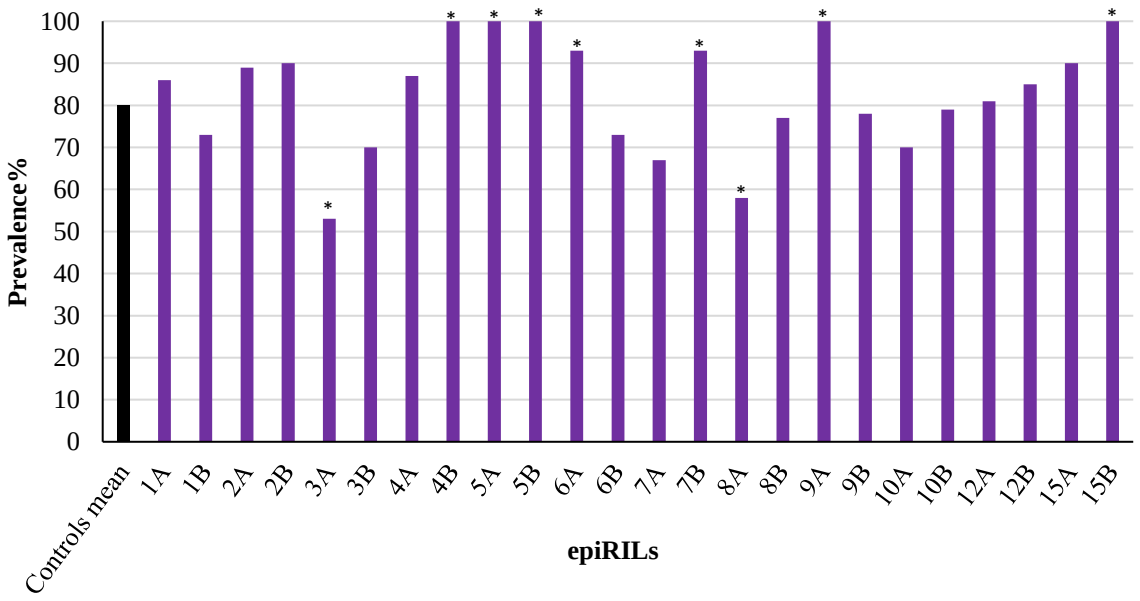


Figure 7. Prevalence of the parasite infection in epiRILs (15 snails per epiRILs) vs the mean of prevalence in control RILs (black bars). The Fisher's exact test was applied, the significant differences against control group are marked with ***** for $p< 0.05$. Significant lower prevalence compared to control were found in 3A, 3B, 7A, 8A, 9A. Significant higher prevalence were found in 4B, 5A, 5B, 10A, 15B. The epiRILs 11, 13 and 14 did not reproduce enough to have enough replicates for experimental infection (less than 15 snails per epiRIL).

Then a second phenotyping with more statistical power in the epi-lines 2, 3, 7, 8, 9 and 12 was done since some of them presented significant differences in prevalence compared to controls (3, 7, 8 and 9) and present higher fecundities that allows to have the necessary replicates for infections. With this higher statistical power, there were not significant differences in RILs regarding the prevalence. They showed a prevalence ranging from 80 to 90% with a mean of 86%±4. Again, the prevalence of the epiRILs presented significant differences against control. The epi-line 8 showed significant different prevalence, the epiRIL 8-7 presented a 20% prevalence and the epiRIL 8-6, 30%. Other epiRILs presented also significant differences in the prevalence, the epiRILs 3-6, 3- 9 and 9-3 displayed a prevalence of infection of 30%. Some epiRILs of the epi-lines 2, 7 and 12 showed significant differences in the prevalence compared to control, with prevalence ranging from 40%-60% (Figure 11).

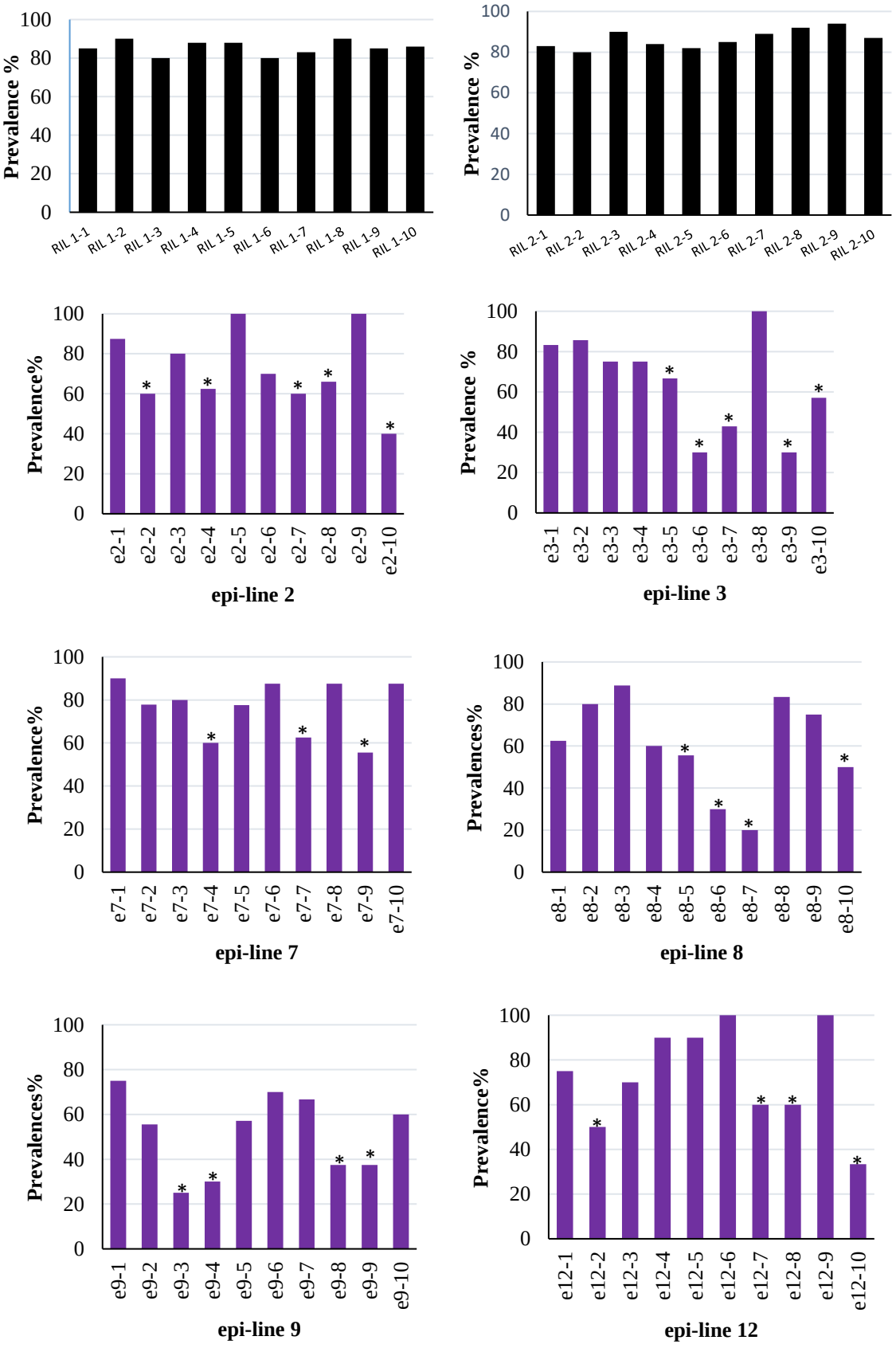


Figure 11. Prevalence of infection in control RILs (black bars). The Fisher's exact test was applied; not significant differences were found between control RILs. Prevalence of the

parasite infection in epiRILs (violet bars). Significant lower prevalence compared to control were found in epiRILs marked with ***** (Fisher's exact test, $p< 0.05$).

epiRILs have higher variation in parasite intensities of infection compared to controls

The intensity of infection is a measure of the number of parasites which penetrate the snails and successfully develop into primary sporocysts. Since the infestations were performed with 5 miracidia (infectious larvae) per snail, the maximum intensity of infection expected is 5 and the minimum is 0. The intensity of infection between control RILs was similar, showing a media between 1.93-2.20 and no significant differences were found (Kruskal-Wallis test, p=0.1).

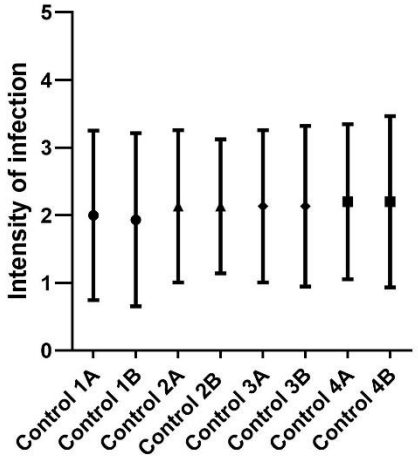


Figure 8. Intensity of infection in control RILs (2 RILs per control line). The Fisher's exact test was applied; not significant differences were found between RILs. 15 snails were infected by RIL.

The epiRILs 2B, 7A, 8A, 8B and 9B showed significant differences against the control RILs (Dunn's test, p>0.05), and epiRILs from the same epi-line presented significate differences, as in the case of 7A and 7B (Dunn's test, p=0.03), 9A and 9B (p=0.02) and between 12A and 12B (p=0.04).

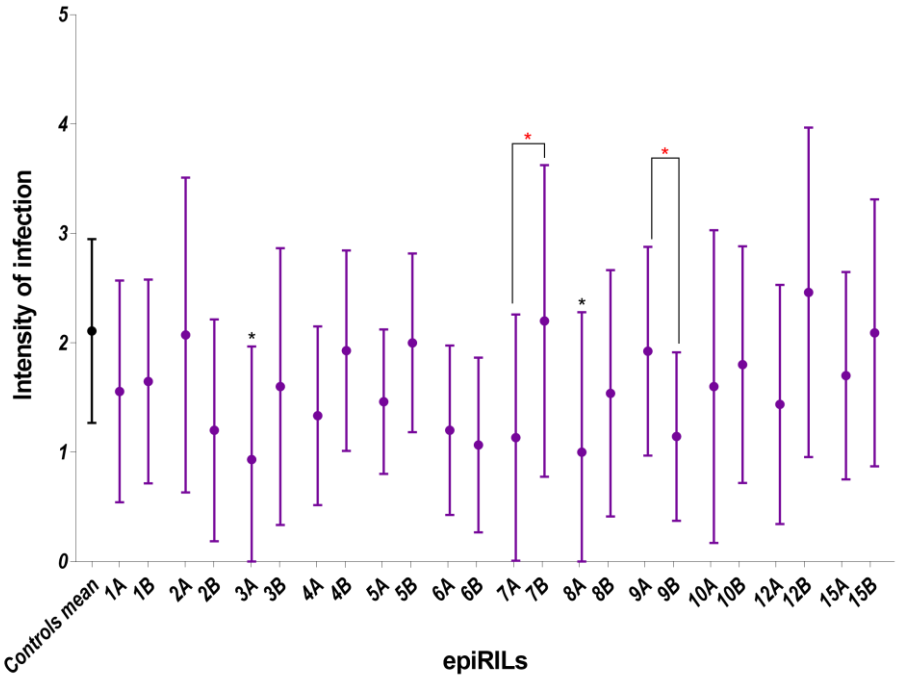


Figure 9. Intensity of infection in epiRILs (violet bars) vs the mean of prevalence in control RILs (black bars). The Kruskal-Wallis test was applied, the significant differences against control RILs mean are marked with ***** for $p< 0.05$. Significant lower intensities compared to control were found in 3A and 8A. Significant differences between epiRILs from the same epi-line are marked with * (Mann-Whitney test, $p<0.05$). 15 snails were infected by epiRIL.

Then a second phenotyping with more statistical power in the epi-lines 2, 3, 7, 8, 9 and 12 was done since they presented variability in their intensities of infection, and similarly, with this higher statistical power, there were no significant differences between intensities in the control lines (Kruskal-Wallis test, $p=0.3$)., In the epi-lines, significant differences were found in all the epi-lines (Kruskal-Wallis test, $p<0.05$). The variability inside each control line and each epi-line was analyzed, and we found significant differences in the epi-line 7 between epiRIL 7-4 and 7-6 (Dunn's test, $p=0.07$) and in the epi-line 12, between epiRIL 12-6 and 12-7 (Dunn's test, $p=0.01$). Significant differences were also found between RILs and epiRILs e2-3, e2-10, e3-3, e3-7, e3-10, e7-4, e8-1, e8-4, e8-5, e8-6, e8-7, e9-3, e9-4, e9-5, e9-8, e9-10 and e12-7 (Figure 12).

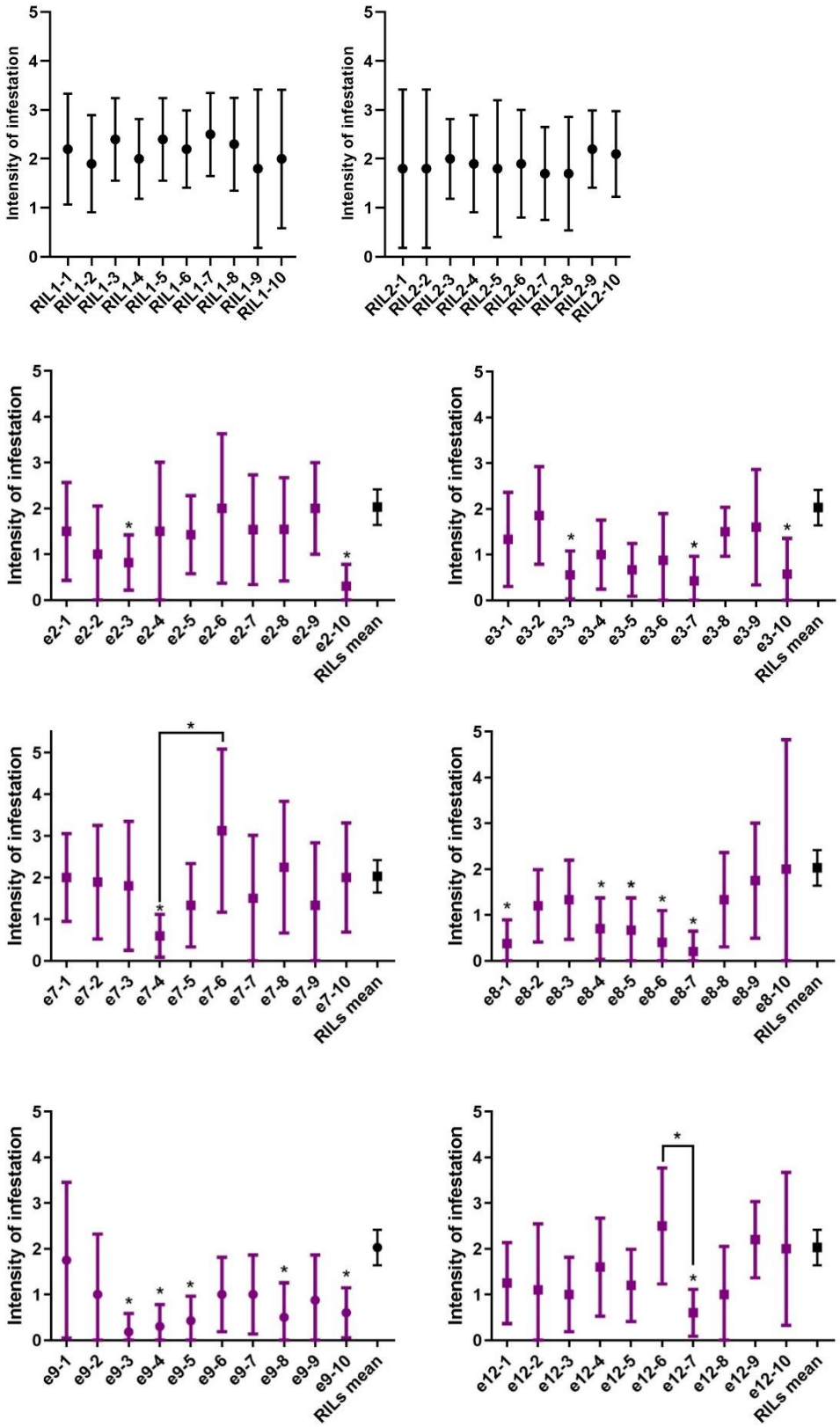


Figure 12. Intensity of infection in control RILs (black error bars) and in epiRILs (violet error bars). The Kruskal-Wallis test was applied; significant differences were found in all epi-lines ($p<0.05$). Then the Dunn's multiple comparison test was applied to see significant differences between epiRILs and RILs. Significant differences against control were found in the epiRILs marked with * ($p<0.05$).

Discussion

In this work, we used the compound Flv1 which showed high inhibitory efficiency against DNA methylation in *B. glabrata* (Luviano et al. 2020) and we tested the bisubstrate-analogue compounds, to evaluate if these new compounds could also change the global 5mC% in exposed snails and its offspring. We found that BA1 and BA-neg compound decrease the 5mC% in the exposed generation which suggests that BA-neg is not inactive and that the hypomethylation effect is not due to a specific inhibitory activity of DNMT. Even if it is not a specific DNMTi, it allows to modulate DNA methylation by triggering an hypermethylated aberrant effect in the offspring of exposed snails, this effect could be a compensatory effect due to the toxicity of the compound. Therefore, we called Flv1 and BA1 DNMT modulators (DNMTm) which allows to obtain snails with two divergent global epigenomes, hypo and hyper-methylated. By crossing these snails (hyper paired with hypomethylated) and by subsequent self-fertilization reproduction, we were able to generate epigenetic recombinant inbred lines (epiRILs). We found significant differences in the phenotypes of these epiRILs regarding the fecundity, the prevalence, and the intensity of infection compared to controls.

The fecundity showed high variability in the snails of the generation F2, that we called epi-lines. The six epi-lines with the highest differences in the global 5mC level between coupled snails (Figure 3) showed the highest hatching rates in cross-fertilization and self-fertilization (2, 3, 7, 8 ,9 and 12) suggesting an epi-hybrid-vigor effect similar to the one often observed in genetic hybridization (Geyer, Niazi et al. 2017).

The prevalence and intensities of infection showed high variability also in the epi-lines on the contrary to the control lines which didn't show significant differences neither in prevalence nor in intensity of infection. The sympatric combination used in this study, consisting in *Schistosoma mansoni* strain Bresil (*SmBRE*) and the *Biomphalaria glabrata* strain Bresil (*BgBRE*), is expected to display consistent prevalence of infection without significant

variations, this consistency in the prevalence has been observed in our laboratory for six generations (Fneich, Théron et al. 2016). Indeed, the infection traits displayed low variability in our control snails. Several epiRILs were significantly less infected than control RILs, displaying a significant decrease in the prevalence down to 20% and lower intensities of infection than control RILs. Not only the prevalence and intensity of infections were reduced, but the variability of this phenotype increase in the epiRILs, which suggests that the epiRILs present probably epigenetic variability resulting into a higher phenotypic variability when exposed to the parasite. The role of DNA methylation in the host-parasite interaction has been very poorly studied, however, there is evidence supporting that phenotypic variability of plasticity in parasite life-history traits is regulated by DNA methylation and that parasites induced DNA methylation changes in their hosts (Gómez-Díaz, Jordà et al. 2012).

The parasite *S. mansoni* has a high level of phenotypic plasticity in its life cycle traits regulated by posttranslational histone modifications which allow it to change its phenotype depending on the host it infects, on its sex and on the environment in which it develops (Augusto, Duval et al. 2019, de Carvalho Augusto, Cosseau et al. 2019, Picard, Vicoso et al. 2019). Simultaneously, the phenotype of the host snail *B. glabrata* is altered by the presence of the pathogen, there is a compensatory effect on fecundity when exposed to the parasite before being castrated by the parasitic infection (Thornhill, Jones et al. 1986), and probably the offspring of this compensatory effect or of the snails that resist infection can inherit epigenetic marks that allow them to show greater phenotypic plasticity when exposed to the parasite, nevertheless this aspect has never been reported in *B. glabrata*. Moreover, S. *mansoni* induced an alteration in the snail DNA methylation, e.g. the expression of DNMT1 and MBD enzymes increased in the snail when exposed to the parasite (Geyer, Niazi et al. 2017) and this probably alters the transcription of genes involved in the immune response or the response to environmental stress. Indeed, it has been found that *S. mansoni* control the cell

spatio-epigenetics of the snail to ensure parasitism. *S. mansoni* induces the relocation of the heat shock protein 70 gene (Bg-HSP70) to the nucleus to be transcribed and hypomethylation of the intergenic region of the gene Bg-HSP70 take place in temporal concordance with its relocation (Knight, Ittiprasert et al. 2016). However, the effects on the susceptibility of the snail triggered by the parasite-induced epigenetic alteration have not been explored and there is a lively debate whether or not epigenetics plays a role in the host-parasite interactions, therefore further investigation is necessary to observe if the host snail susceptibility variability can be due to epigenetic mechanisms such as DNA methylation.

Perspectives

To correctly evaluate if either DNA methylation or genetic polymorphism are involved in phenotypic plasticity of the snail, it will be necessary to establish both the methylome and genome profile of the snails in each of the epi- and control lines. This will allow to evaluate the contribution of both information for the observed phenotypic changes. The epigenotyping sequencing method epiGBS has already shown to be efficient to measure DNA methylation changes and single nucleotide polymorphisms in a percentage of the genome (van Gurp, Wagemaker et al. 2016, Gawehns, Postuma et al. 2020) . EpiGBS could be applied on the RILs and the epiRILs with the most divergent phenotypes regarding the prevalence and intensity of infection which were elaborated in this multigenerational study. Subsequent genetic and epigenetic distances can be calculated using a multi-matrix covariance method which will allow to evaluate the contribution of both information to the observed phenotypic variation (Thomson, Winney et al. 2018). Such analysis could allow to decipher key aspect of host-parasite interaction, especially if epigenetic mechanisms are key player involved in the immune defense of the snail *B. glabrata* and potentially other invertebrates against parasitic infections. This could also lead to mapping loci with epimutations linked to increased resistance, paving the way to subsequent functional studies and to establish parasite control strategies.

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Chapter II

Targeted epimutagenesis in the snail *Biomphalaria glabrata*

Epigenetic engineering tools

Recent approaches to manipulate DNA methylation consist mainly on random inhibition of DNA methyltransferases via hypomethylating drugs which induce large epigenetic changes and non-targeted effects such as activation of endogenous retroviruses (Chiappinelli, Strissel et al. 2017).

However, currently there is the possibility to modify DNA methylation at target loci by the engineering of programmable enzymes such as zinc finger proteins (ZFPs) and transcription activator-like effectors (TALENs). However, these techniques are difficult due to the process of protein engineering that limits large-scale applications (Pulecio, Verma et al. 2017).

A main advancement came from the arrival of clustered regularly interspaced short palindromic repeats (CRISPR) and the nuclease Cas9 that is programmed to target an exclusive DNA sequences by a guide RNA (gRNA) (Ran, Hsu et al. 2013)CRISPR-Cas9 is a microbial immune system that allows the prokaryotes to defend from viruses allowing the cleavage of foreign virus DNA with high precision. Charpentier and Doudna discovered this complex while they were studying how the bacterial immune system works, they determined that this complex can be used as an engineering tool to edit the genome (Jinek, Chylinski et al. 2012). There are three kinds of CRISPR Cas but the most used is the CRISPR Cas II since it is composed only of the enzyme endonuclease Cas9 and the other systems (CRISPR I and III) have a large complex of Cas proteins that become difficult to manipulate (Tian, Wang et al. 2017). The Cas9 used in most genome editing studies comes from *Streptococcus pyogenes* and this endonuclease can be programmed with single RNA molecules to cut precise DNA sequences, this allows to use this technology to generate target editing in the genome of any organism (Jinek, Chylinski et al. 2012).

Direct pronuclear injection of sgRNA and mRNA encoding Cas9 into zygotes enable rapidly and easily the generation of transgenic organisms (Nishimasu, Ran et al. 2014). Most of the genome editing applications by CRISPR Cas9 have been done *in vitro* in human culture cells, and it has been done *in vivo* in model species such as the vertebrates: *Mus* musculus (Platt, Chen et al. 2014), *Rattus norvegicus* (Shao, Guan et al. 2014)and *Danio rerio* (Li, Zhao et al. 2016) and in the plant models: *Arabidopsis thaliana* (Jiang, Yang et al. 2014), *Sorghum bicolor* (Char, Lee et al. 2020) and *Oryza sativa* (Romero and Gatica-Arias 2019).

In the case of invertebrate species, CRISPR-Cas9 has been used as genetic editing tool in the malaria mosquito vector *Anopheles gambiae* (Hammond, Galizi et al. 2016), the chikungunya mosquito vector *Aedes aegypti* (Kistler, Vosshall et al. 2015), the fruit fly *D. melanogaster* (Gratz, Rubinstein et al. 2015), the nematode *C. elegans* (Paix, Folkmann et al. 2017) and the yeast *Saccharomyces cerevisae* (DiCarlo, Norville et al. 2013). In mollusks, the use of CRISPR-Cas9 *in vivo* has been reported in the marine snail *Crepidula fornicata* (Perry and Henry 2015) and CRISPR-Cas9 induced a gene knock-out in the embryonic cell line of the snail *Biomphalaria glabrata* (*Bge cells*) (Coelho, Rodpai et al. 2020).

The CRISPR Cas9 has become the most applied genome editing tool, 7,206 scientific articles about CRISPR Cas9 genome editing are found in Pubmed database between 2012 and 2020 compare to 705 about ZFPs genome editing and 96 about TALENs genome editing in the same period.

Cas9 is widely used due to the high efficiency specificity and easy application, and the utility of Cas9 is further extended with the engineering of the dCas9, a Cas9 mutated without endonuclease activity that can be attached to epigenetic enzymes domains for targeted epigenome alterations (Pulecio, Verma et al. 2017). dCas9 has emerged as a malleable tool to control activation and inactivation of target genes following the same bases of Cas9 (Jensen, Ferreira et al. 2017).

The system dCas9 couple with a DNA methylation writer or a DNA methylation eraser enzyme, enables the study of the relationship between DNA methylation at a precise locus and its transcription level. Since 2016 it exists a generation of tools to specifically methylate or demethylate target sequences that have been applied *in vitro* to human culture cells and *in vivo* in mice showing that is possible to induce DNA methylation and demethylation in a specific sequence, allowing to study the relationship between DNA methylation and gene expression (Table 2.1).

Table 2.1. The efficiencies on targeted DNA methylation modification of different epigenetic engineering tools used in mouse and human models, the changes induce in CpG methylation and in gene expression.

Vector construction and transfection mode	**CpG methylation% effect**	**Genomic region, gene and model**	**Gene expression**	**Reference**
Lentiviral vectors dCas9-SunTag-DNMT3A1	Increase of 50-80% in 53 CpG sites of a CpG island along 600 bp	*Hox5* promoter in human embryonic kidney cells (HEK293T).	80% *Hox5* gene repression	Huang et al. 2017
dCas9-SunTag-DNMT3A	Increase of 60-95% at the 4-kb window	*HoxA5* gene promoter in HEK293T human cell line	Not assessed	Huang et al. 2017
dCas9—SunTag-DNMT3A	Increase of 13% over 62 CpG sites	*UNC5C GENE*	Not assessed	Huang et al. 2017
dCas9-DNMT3A	Increase of 20-40% at the 4-kb window	*HoxA5* gene promoter in HEK293T human cell line	Not assessed	Huang et al. 2017
dCas9-DNMT3A	Up to 30-50% in 3 CpG sites and up to 60% in 3 CpG sites	*BACH2* promoter in HEK293 human cell line	2 –fold decrease in gene expression	Votja et al. 2016
dCas9-MQ1 *in vitro*	Increase from 10% CpG methylation to 20-80% in 40 CpG sites of a CpG island along 600 bp	*Hox4*, *Hox5* and *RunX1* promoters in human HEK293T cells	40% reduction of Hox5 gene expression	Lei et al. 2017

Vector construction and transfection mode	**CpG methylation% effect**	**Genomic region, gene and model**	**Gene expression**	**Reference**
dCas9-MQ1 *in vivo*	Increase from 50% CpG methylation to 70% in 20 CpG sites along 500 bp	*Igf2/H19* locus in mice	Not significant changes in weight in transgenic mice in this study. Previous report showed that methylation of *IGF2* caused birth weight changes (Singh, Lee et al. 2012).	Lei et al. 2017
dCas9-Dnmt3a *in vitro*	Increase of 20-40% in 5 CpG sites with one sgRNA and increase of 60-80% in 13 CpG sites with 4 sgRNAs	*Snrpn* promoter in mice	Silencing of the Snrpn-GFP reporter expression in 12% of cells	Liu et al. 2016
dCas9-Tet1 *in vitro*	Decrease from 90% to 10-70% in 10 CpG sites from 29 CpG sites	*Snrpn* promoter in mice	Active Snrpn-GFP reporter expression in 26% of cells	Liu et al. 2016
dCas9-Tet1 *in vivo*	Not assesed	Methylation sensitive Snrpn-GFP reporter in skin dermal cells in mouse	GFP reporter activation in 85% in derma cells in vivo	Liu et al. 2016

The use of CRISPR-Cas9 allowed the modifications of DNA methylation at precise gene loci through the use of dCas9 couple with the catalytic domain of the mammalian *de novo* DNA methyltranferase DNMT3A (Oka, Rodić et al. 2006) and a gRNA. In a recent work it was

demonstrated that the dCas9-DNMT3A construct increase CpG methylation and silence gene expression in mammalian cells, methylation level increased from 0 to 25%-55% in 12 CpG sites at the *BACH2* gene promoter in transfected HEK293 human cells (Vojta, Dobrinić et al. 2016).

In a more recent work, dCas9 –DNMT3A construct (Figure 13) was used and, in order to increase the efficiency of the method, the plasmid express the SunTag system, which contains an amplifier constructed of numerous antibody epitopes of GCN4 identified by a single-chain variable fragment (scFv). The system dCas-SunTag-DNMT3A and the gRNA successfully targeted the *HOXA5* locus from human kidney cells (HEK293T) allowing increase efficiency in target DNA methylation compared to dCas9-DNMT3A, from 20-40% to 60-95% increase on CpG% methylation in a 4-kb window including a CpG island of the *Hox5A* gene promoter (Table 3). Furthermore, they demonstrated that this target methylation reduces the gene expression. (Huang, Su et al. 2017).

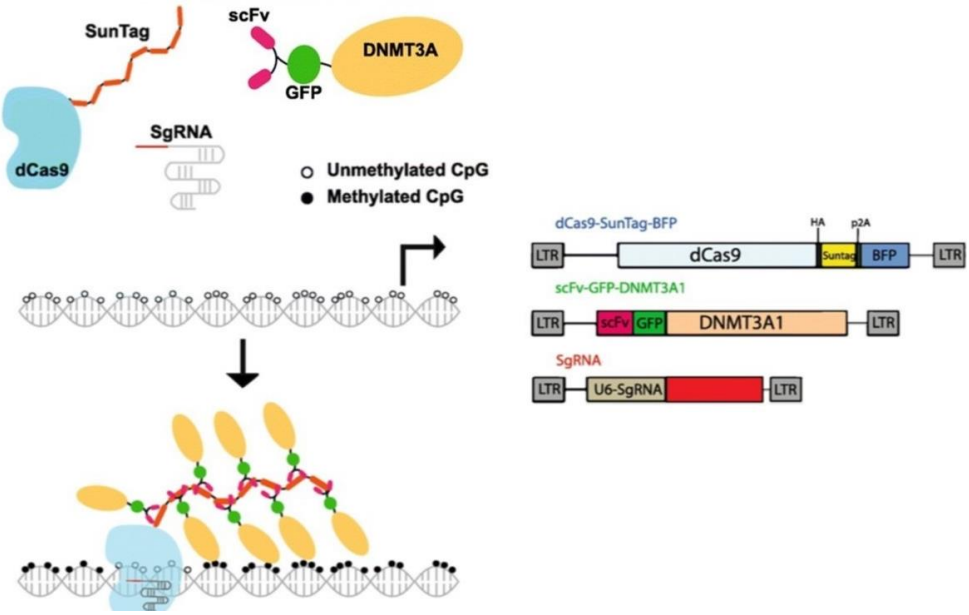

Figure 2.1. Image taken from Huang *et al.* (2017). The sgRNA transports dCas9, to the desired target sequence. Once the target sequence is found, dCas9 will leave above the desired sequence, the enzyme DNMT3A, a DNA methyltransferase that methylates the DNA of several organisms by inserting methyl groups to the cytosines (Oka, Rodić et al. 2006). The SunTag system, a network of repetitive peptides of GCN4, modulates transcription rates by increasing the local concentration of DNMT3A by recruiting multiple copies of this enzyme from the union with a fusion protein (scFv) associated with a fluorescent protein (GFP) improving the targeted *in vitro* DNA methylation. Besides, this system allows long-term

detection of DNMT3A protein in living cells because multiple GFP are recruited. The SunTag scaffold amplifies the intensity of the fluorescent signal and facilitates the tracking of protein expression. This scaffold consists in 10 copies of the short epitope GCN4 that recruits GFP fused to the scFV antibody (Tanenbaum, Gilbert et al. 2014).

The dCas9 fused to DNMT3A exhibited efficient targeted DNA methylation but requires a long incubation period in cells to accomplish its highest efficiency. Another alternative can be the fusion of dCas9 with MQ1, an engineered prokaryotic DNA methyltransferase that displayed a high efficiency *de novo* methylation function in DNA and is faster than DNMT3, which offers a good alternative for targeted methylation *in vivo* in other biological models (Lei, Zhang et al. 2017).

Alternative epigenetic engineering tool is the fusion of dCas9 to the catalytic domain of TET1 that is able to induce target demethylation. Higher targeted demethylation efficiency can be reached by combining dCas9-TET1 with a peptide repeat sequence to recruit multiple copies of TET1. dCas9–peptide repeat and scFv–TET1 achieved efficient targeted demethylation of CpG sites in promoter regions and up-regulation of the target genes *in vitro* and *in vivo* in mice (Morita, Noguchi et al. 2016).

In the case of invertebrate species, targeted DNA methylation tools have been only applied in the silkworm *Bombyx mori*, where dCas9-TET1 was used to demethylate the gene body of targeted genes *in vitro* and *in vivo* (Liu, Ma et al. 2019) There is a gap in the knowledge about the function of genome body methylation and its relationship with gene expression in the invertebrate's genomes. The poor studied taxa could benefit from the epigenetic engineering technology developed in other models, but many of this taxa are in its infancy in terms of study of their genome or epigenome and these aspects have to be optimized before the use of this tools becomes a possibility.

Principal results of the targeted epimutagenesis in the snail *Biomphalaria glabrata*

The epimutations triggered with chemical DNMT inhibitors and subsequent segregation by self-fertilization to produce epiRILs allows to study the implication of DNA methylation in phenotype variability, however, the inconvenience of this approach is that it requires long-time experiments and high throughput measures to eventually produce some epimutants and the changes in DNA methylation are totally random in the genome. Even if loci with epimutations

are finally discovered it remains to be shown the molecular phenotype of DNA methylation in the genes.

Nevertheless, it remains a major challenge to assign a causal relationship between DNA methylation at specific loci and its gene expression, this is due to the impossibility to target the chemical effect at specific loci. To better study the correlation between DNA methylation and gene expression, epigenetic engineering tools are necessary to introduce DNA methylation changes at a target locus to then measure the impact produced in its expression.

My host laboratory established a collaboration with Margaret Goodell from Baylor College of Medicine in Houston, Texas to test their plasmid constructions *in vivo* in the embryos of our model, the snail *B. glabrata*. These plasmid constructions were tested in the human embryonic kidney cells (HEK293T), where they demonstrate that the dCas9 protein fused to a scaffold of peptide epitopes called SunTag that recruits multiple DNMT3A increased the CpG methylation at the *Hox5A* locus and the targeted DNA methylation resulted in f repression of *HoxA5* gene expression (Huang, Su et al. 2017).

Before being able to effectuate targeted DNA methylation, the optimization of the transfection technique was necessary to introduce the plasmids and the sgRNA to the snail embryo. We found that the embryo size was very small compared to other species, the embryo measures only 50 µm and we realized that direct pronuclear injection will not be possible so we decided to test another transfection method such as the transfection with lipofectant reagents at different embryo stages, the *in vivo* electroporation and the juvenile transfection via the ovotestis using an *in vivo* polymer-based reagent.

The embryo yolk microinjection was further optimized with the collaboration of Genevieve Tavernier and Caroline Nevoit from Unité Mixte INSERM/UPS Centre Régional d'Explotation Fonctionnelle et de Ressources Expérimentales CREFRE in Toulouse, France and with the help of Ittiprasert Wannaporn from the George Washington University that communicate us that the use of an *in vivo* polymer-based reagent showed transfection efficiency by soaking in egg patches of the snail *B. glabrata*. We found that the microinjection at the embryo yolk beyond gastrula stage was not lethal for the embryos and showed the expression of the plasmid constructions, therefore we used this technique to transfect multiple embryos.

Additional optimisation of the procedure led to establish the following procedure: A first microinjection is performed with the plasmids coding for dCas9 fused with DNMT3A. Transfected embryos are screened using the fluorescence of the reporter genes of the system

Suntag 72 hrs later (when plasmids get expressed). sgRNA are then injected in plasmid positive embryos and three days later the hatched transfected snails were stocked for DNA purification. Using this procedure on a targeted homeobox Nkx 2.5-like gene, bisulfite amplicon sequencing BSAS results allow us to confirm the induction of CpG methylation changes in four of ten transfected snails and that the targeted DNA methylation changes were heterogeneous between transfected snails.

The results of the targeted epimutagenesis in *B. glabrata* are the object of the Manuscript 4 entitled: Hit-and-run epigenetic editing in the invertebrate parasite intermediate host snail *Biomphalaria glabrata*

Manuscript 4 in preparation

Hit-and-run epigenetic editing in the invertebrate parasite intermediate host snail *Biomphalaria glabrata*

Hit-and-run epigenetic editing in the invertebrate parasite intermediate host snail *Biomphalaria glabrata*

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Abstract

DNA methylation is an important epigenetic information carrier in eukaryotes that plays a major role in the regulation of gene expression. Epigenome editing tools have been instrumental to demonstrate functional importance of this mark in vertebrates. In invertebrates such tools have been poorly applied. Here we demonstrate that epigenetic engineering can be used to modify *in vivo* the CpG methylation level of a homeobox gene involved in embryogenesis in the freshwater snail *Biomphalaria glabrata*. The fusion of dCas9-SunTag-DNMT3A have induced hyper-cytidine methylation in *B. glabrata* Nkx2.5 homeobox gene. The increase of CpG methylation (40% of positive transfected embryos) was revealed after dCas9-SunTag-DNMT3A/sgRNA complex transfected in *B. glabrata* embryo cells.

Introduction

DNA methylation is an epigenetic mark that have been shown to play an important role in the control of gene expression, in genome imprinting, transposon silencing and X-chromosome inactivation in mammals (*1*). Nevertheless, the role of DNA methylation in invertebrate species is less known and its pattern is different from those found in vertebrates. In vertebrates, genomes are broadly and deeply methylated, besides, partial and local hypomethylation is found at the gene promoters, where hypermethylation induces gene silencing (*2, 3*). In contrast, in invertebrates, DNA methylation tends to occur at moderate levels, and is mostly found in the gene bodies (exons and introns) a genomic context of methylation known as gene body methylation (GBM) (*4*). GBM in invertebrates is probably associated with active transcription since it preferentially occurs in highly transcribed genes (*5, 6*). However, no mechanistic link to gene expression has been demonstrated for such type of DNA methylation and its function remains elusive for the time being.

Consequently, it appears essential to develop approaches that allow to study GBM and its relationship with gene expression. Currently, the method of choice for the modification of GBM in invertebrates is chemical treatment, but this approach does not allow to target specific genome loci (*7*) and an alternative is to repurpose the sequence specificity of the Cas9 enzyme to direct DNA methylation machinery to a desired location. The CRISPR-Cas type II system from *Streptococcus pyogenes* that consists of the nuclease Cas9 and a single guide RNA (sgRNA) (*8*) is the most used genetic editing tool at present. Both, can form a ribonucleoprotein complex (RNP) that recognizes and binds to a specific genomic region containing the protospacer adjacent motif (PAM.). The targeting of this region is due to the sgRNA that includes a sequence of 20 nucleotides upstream the PAM sequence, a NGG motif for the system coming from *S. pyogenes*. The sgRNA sequence will match the genomic sequence that is located upstream the PAM sequence, and Cas9 nuclease will cleave both

strands of the genomic DNA, leaving a DNA double stranded break (DSB) generally between the third and fourth nucleotides upstream PAM (Shan, Soltis et al. 2020). Then, the DSB will be repaired by non-homologous end-joining (NHEJ) that can introduce deletion or insertionof one or several desoxyribonucleotides. Alternatively, homology-directed repair (HDR), that is less efficient but more precise for introducing a DNA template, can occur (*8, 9*) .

Beyond gene editing, the sequence recognition capacity of Cas9 has been adapted to perform epigenome editing such as target DNA methylation. Cas9 without nuclease activity, deadCas9 (dCas9), and a sgRNA allowing the modifications of DNA methylation at precise gene loci when fused to the catalytic domain of the DNA methyltranferase DNMT3A which is the most active isoform of a *de novo* DNA methyltranferase in eukaryotes, it methylates CpG sites *in vivo* and *in vitro (10)*.

Epigenetic engineering tools have been mainly applied to various models such as mice or cells in culture, and multiple reviews discussed the advancements on epigenome editing in these models (*11-13*). In a recent work it was demonstrated that the dCas9-DNMT3A construct increases CpG methylation and silences gene expression in mammalian cells. Methylation level at the targeted loci varies from 25 to 55% after 7 days in transfected HEK293 cells and cultured cells lost the construct ten days after the transfection with the dCas9-DNMT3A plasmid (*14*). In contrast, *in vivo* transfection in “non-model” species this technique has proofed challenging and few studies have been conducted in invertebrates apart the two invertebrate models *Drosophila* (*15*) and *Bombyx mori (16)*. In the present work we used the epigenetic engineering system dCas9-SunTag-DNMT3A in a mollusk, the freshwater snail *Biomphalaria glabrata*, the intermediate host of the parasite *Schistosoma mansoni*. SunTag is a protein scaffold containing multiple epitopes able to recruit effector domains (*17*). The SunTag system consist in a repeating peptide array that can recruit multiple copies of an antibody-fusion protein (scFv) associated to DNMT3A, which increase the local

concentration of DNMT3A improving the targeted *in vitro* DNA methylation. Besides, the SunTag scaffold amplifies the intensity of the fluorescent signal due to the multiple associated GFP, facilitating the tracking of protein expression (*18*). dCas9-SunTag-DNMT3A has been successfully applied to target the HOXA5 *locus* in human kidney cells (HEK293T) where it showed a highly efficient increase in promoter methylation (60–95%) inducing a HOXA5 mRNA repression *(17)*.

The snail *B. glabrata* is indispensable for the life cycle of the Schistosome parasite and DNA methylation machinery of the snail has been showed to be affected by the parasite infection. The parasite *S. mansoni* is able to modified the transcript abundance of the DNA methylation machinery enzymes of the snail *B. glabrata* during infection, suggesting that the DNA methylation of the snail could have a role in their interaction with the parasite (*19*). Furthermore, the parasite modifies the DNA methylation of certain genes in the snail, which alters its gene expression (*20*). However, the causal relationship between DNA methylation and plasticity of gene expression in the snail and in invertebrates remains enigmatic, and epigenetic engineering tools are indispensable to establish clear causal relations between DNA methylation and gene expression.

We have microinjected two plasmids coding for the dCas9-SunTag-DNMT3A system and a sgRNA and we obtained fluorescent-positive snail embryos after *in vivo* transfection. Analysis of positive embryos demonstrated a successful increase in targeted GBM at CpG sites near the PAM motif of the sgRNA in the homeobox gene Nkx2.5 in the snail *B. glabrata.*

Results

Microinjection with *in vivo* jetPEI produces superior survival rates at gastrula stage and plasmid expression is observed 72 h after tranfection

Several *in vitro* transfection reagents (GeneCellin™HTC, Lipofertamine3000, jetOPTIMUS™) were tested at the two-and eight-cell stages. None of them displayed transfection efficiency and very low survival percentage was observed (1-2%) (Supplementary data, Table S1)). Therefore, we decided to microinject at five early stages of embryonic development (Table 1) with an *in vivo* transfection reagent (*in vivo*-jetPEI). The survival percentage was very low in the embryo snails transfected before gastrula stage (0-10%), while from gastrula stage the survival increases up to 50%. Moreover, GFP expression was not observed in any of the embryos injected before gastrula stage. The GFP expression was observed in the trocophore larvae of embryos transfected at the gastrula stage 72 hours after the microinjections.

The transcription level expression of GFP, DNMT3A, dCas9 and BFP was measured by RT-qPCR in trochophore larvae. mRNA of the reporter genes GFP (Figure 1a) and BFP (Figure 1b) and of the catalytic domain of the DNMT3A (Figure 1c) and the dCas9 (Figure d1d) were detected, while the non-transfected controls did not display the expression of any of the four mRNAs (Figure 1). The negative RT controls neither display the expression of the 4 mRNAs.

Table 11. Survival percentages of the transfections done at different embryo developmental stages with the *in vivo* jetPEI reagent.

Time after first cleavage	*Stage*	*Number of Injected snails*	*Number of surviving snails*	*Survival%*
During first cleavage	2-cell stage	50	0	0
160 min	8-cell stage	50	2	4
5 hours	Morula stage	50	4	8
18-20 hours	Blastula stage	50	5	10
26 hours	Gastrula	50	25	50

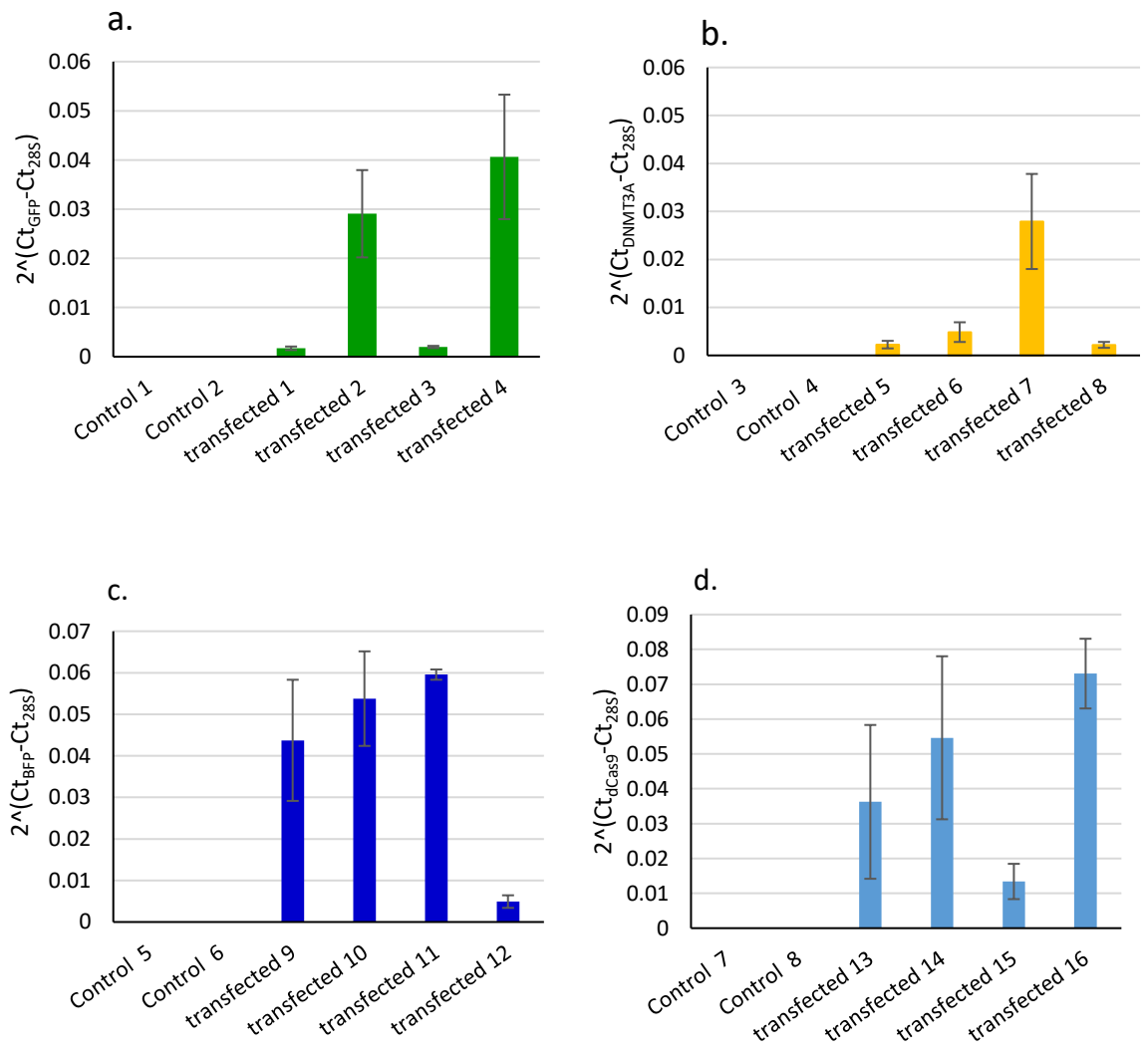

Figure 1 Relative expression of controls (microinjected with only *in vivo* jetPEI reagent) and transfected snails (microinjected with *in vivo* jetPEI and the two plasmids coding for the

dCas9-SunTag-DNMT3A system)**.** Two controls snails were compared to 4 transfected embryos per mRNA. Relative expression of a) GFP b) DNMT3A, c) BFP and d) dCas9 from transfected embryos. Error bars correspond to standard deviation of the two technical replicates from each larva.

No fluorescence signal was detected by microscopy until 72 hours after transfection. At this stage, the trochophore larvae started to show GFP fluorescence, the reporter gene of the plasmid coding for DNMT3A, and BFP fluorescence for the plasmid coding for dCas9 and the scaffold SunTag (Figure 2a-d). 96 hours after transfection the fluorescence of GFP and BFP continued to be visible (Figure 2e-h) and 110 hours later fluorescence was visible but weaker (Figure 2i-l). 168 hours post transfection the fluorescence was no longer visible, demonstrating that the plasmids were expressed transiently in the embryo snail (data not shown). Embryos at the veliger and hippo stage did not showed fluorescence expression after the microinjection with a control solution containing the *in vivo*-jetPEI reagent and 5% glucose but deprived of plasmids (Figure 3a-d).

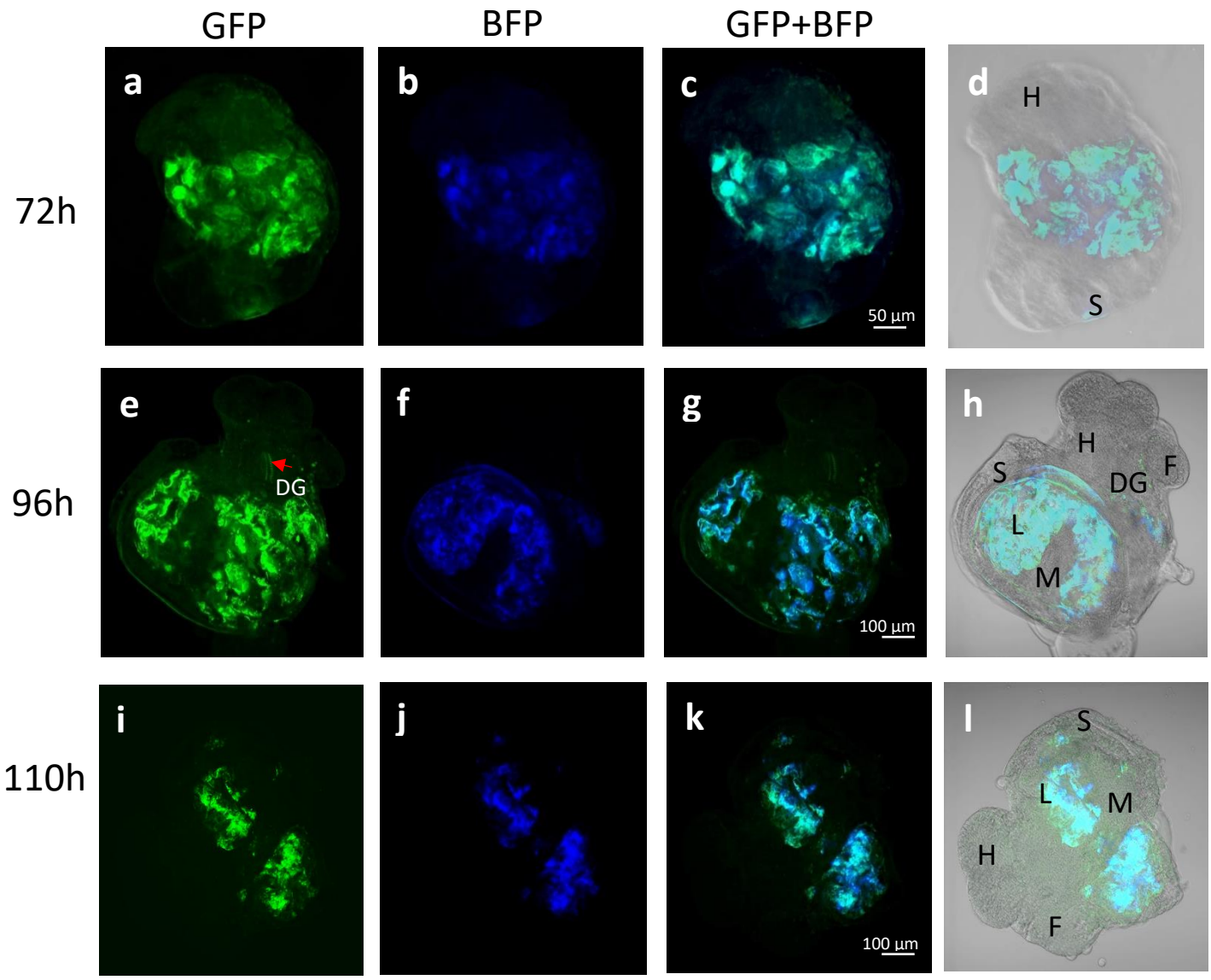

Figure 2. a-d) Trocophore larva 72 hpost transfection with dCas9-SunTag-BFP and scFv-DNMT3A plasmids. a) GFP expression b) BFP expression c) GFP and BFP co-localization. e-h) Veliger larva 96 h post transfection. i-l Snail in hippo stage 110 h post transfection. Anatomical annotations: H - head, F - foot, S - shell, L - lung, M - mantle, DG - digestive gland.

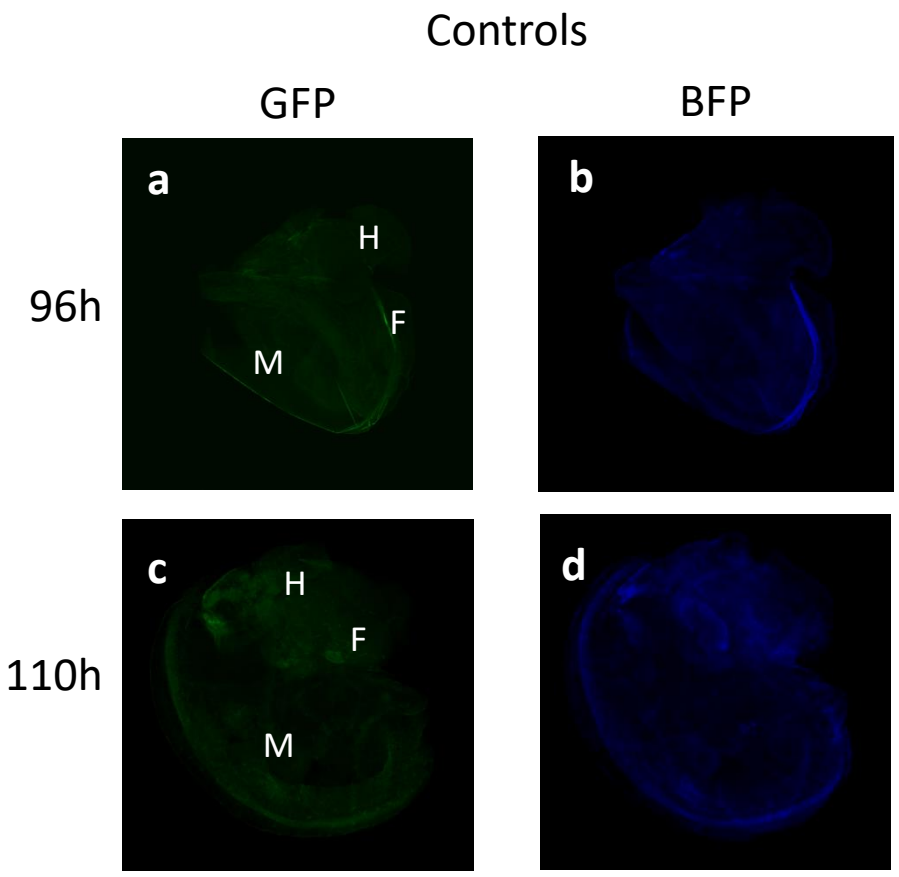

Figure 3. a-b) Veliger larva stage 96 h after microinjection with *in vivo* jetPEI deprived of plasmids and use as a control solution. c-d) Snail in hippo stage 110 h post microinjection with control solution.

The transfected embryos exhibited preferential fluorescence at the ectodermal tissues and at the nervous system. In some photographs we were able to distinguish nervous ganglia such as the pedal ganglia in an embryo at the hippo stage (Figure 4). Fluorescence expression was highly mosaic and limited to some cells in the nervous system, therefore mosaic integration is expected.

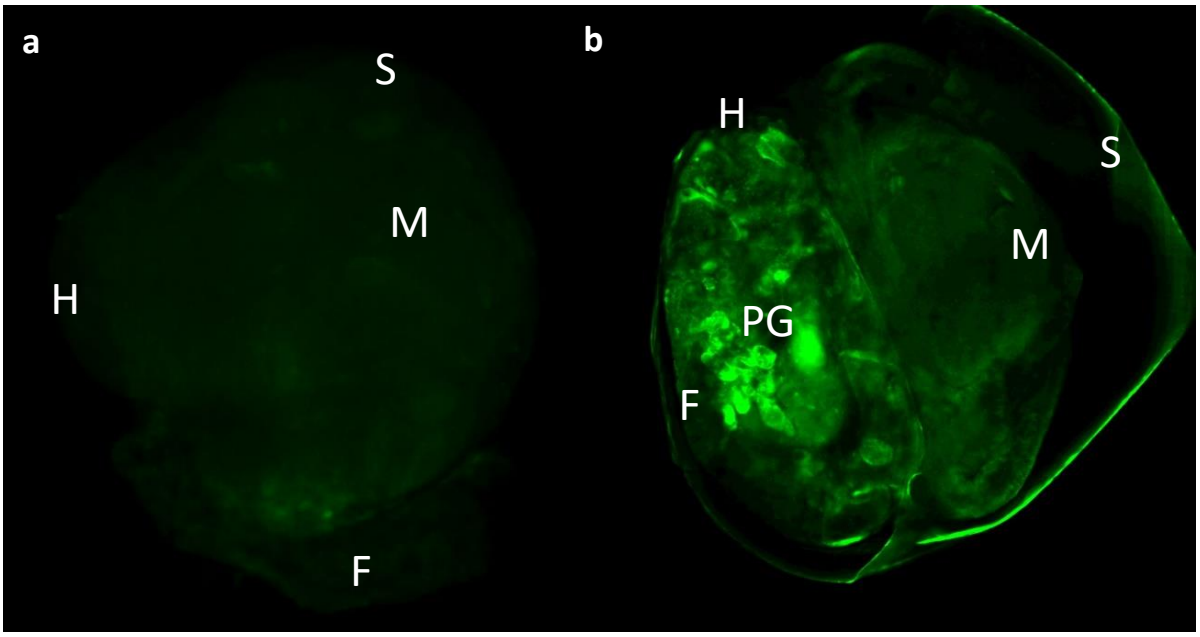

Figure 4 a). Control snail 96 after microinjection with the *in vivo* jetPEI reagent, showing only autofluorescence. b) Snail 96 hours after transfection with plasmids dCas9-Suntag and scFv—GFP-DNMT3A. H – head, S- shell, M - mantle, F – foot, PG – pedal ganglia.

In conclusion, transfection in *B. glabrata* is preferable from gastrula stage since earlier microinjection leads to high mortality rates. Expression of reported genes from plasmid of transfected snails was observed 72 hours after transfection and up to five days when embryos are at the end of the hippo stage. Approximately 12 h before hatching, the snails lost the fluorescence signal (Figure 4). This dynamic of expression was expected since a transient expression was also observed in transfected human embryonic kidney cells (Huang et al. 2017), in our hit-and-run approach, a constitutive expression of the DNMT activity would potentially lead to off-target effects and was not desirable. Total transfection efficiency was 4 % (10 replicates, from a total of 250 snails).

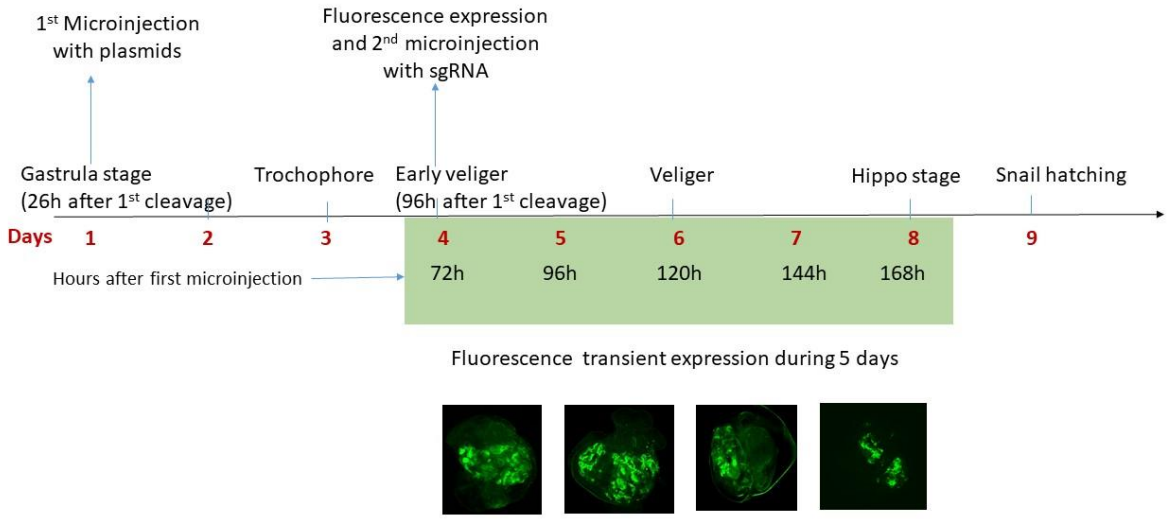

Figure 4. The microinjection is done at the gastrula stage, 26 h after the first cleavage of the zygote. 72 h after the first microinjection the plasmids are expressed in the embryo of *B. glabrata* and in the fluorescente positive embryos a second microinjection with the sgRNA was done. The fluorescence of the reporter genes (GFP and BFP) is observed for 5 days (from 4th to 8th day) and then is no longer visible, therefore the expression of reporter genes carrying by plasmids is transient. At 9th day, the snail hatches.

One sgRNA showed *in vitro* efficiency and targeted CpG hypermethylation from 3.4 to 76%; this was obtained in 40% of transfected snails in up to 5 CpG sites

After having established a time window in which both dCas9 and DNMT were expressed in the developing snail embryos, injections of guide RNA were performed. To verify the absence of mutations, the targeted *locus* was sequenced by Sanger methodology and to verify methylation level at the target gene, bisulfite Sanger sequencing was done. The target gene was not methylated at any of the 11 CpG sites. All three sgRNAs matched to the target sequence and are located at different positions (Figure 5). Cleavage efficiency was assayed *in vitro* and sgRNA3 was shown to be the unique tested guide able to direct Cas9 to cut the

targeted *locus* at the PAM sequence (Figure 6). Therefore, only sgRNA3 was used for transfection.

The sgRNA3 that showed an *in vitro* efficiency was microinjected in the positive fluorescent embryos to maximize dCas9 and sgRNA binding. Therefore, positive transfected snails were screened under the microscope at 72h using fluorescence of the GFP and BFP from the plasmids. At this time, we performed the microinjection of the sgRNA3 in those positive embryos. We then extracted genomic DNA from newly hatched snails and performed the bisulfite conversion. Amplification of the target gene was then performed and analyzed by Amplicon Bisulfited Sequencing (BSAS). This method allowed us to measure accurately the CpG methylation level of 9 controls and 10 transfected snails.

Also, two sets of microinjection, one containing the plasmids coding for dCas9-SunTag and DNMT3A-GFP, and another one with the synthetic sgRNA3, allowed us to methylate CpG sites in the targeted gene close to the guide RNA. BSAS of the target identify that four out of 10 transfected snails showed increased DNA methylation in some of the targeted CpG sites (Figure 7), while the controls showed a significant lower % of methylated CpG in all twelve CpG sites of the targeted region. One transfected snail (Transfected 6) showed a higher CpG% in 5 CpG sites with values ranging from 3.5 to 4.42 while the other positive transfected snails showed only one CpG site highly methylated (Transfected 1, 2, 3) with values ranging from 12-76%. A high heterogeneity was found in the targeted CpG methylation modifications in the transfected snails (Figure 8, Table S2).

Additionally, bisulfite conversion rate was calculated in all controls showing a high efficiency of the bisulfite conversion, 99% of the cytosines were converted to thymines in all controls (Table S2).

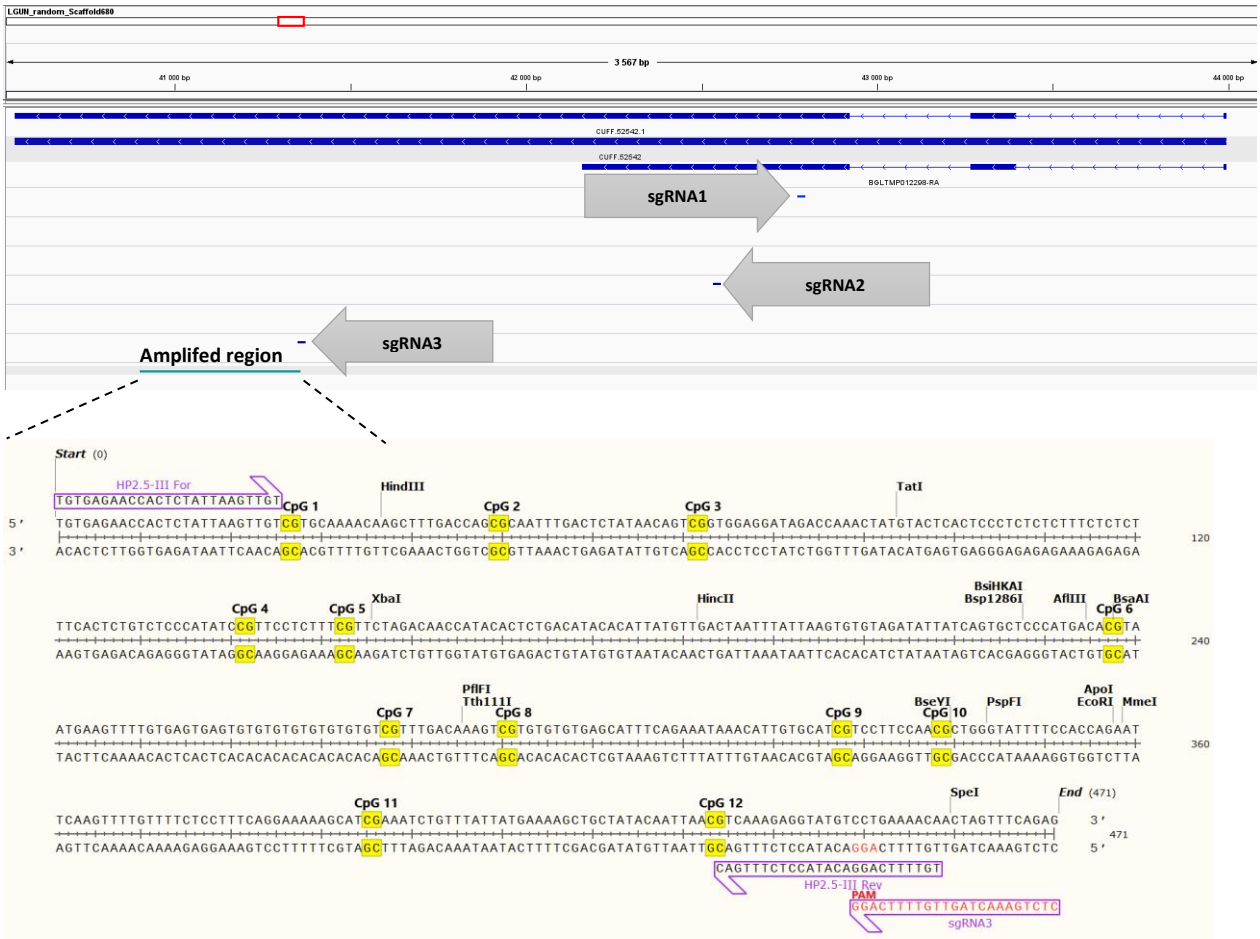

Figure 5. sgRNA position at the targeted locus, whose genomic location is: LGUN_random_Scaffold680:40914-41372. The target amplicon is within the gene body and contains twelve CpG sites. The complete sequence of each gRNA is found in the methods section.

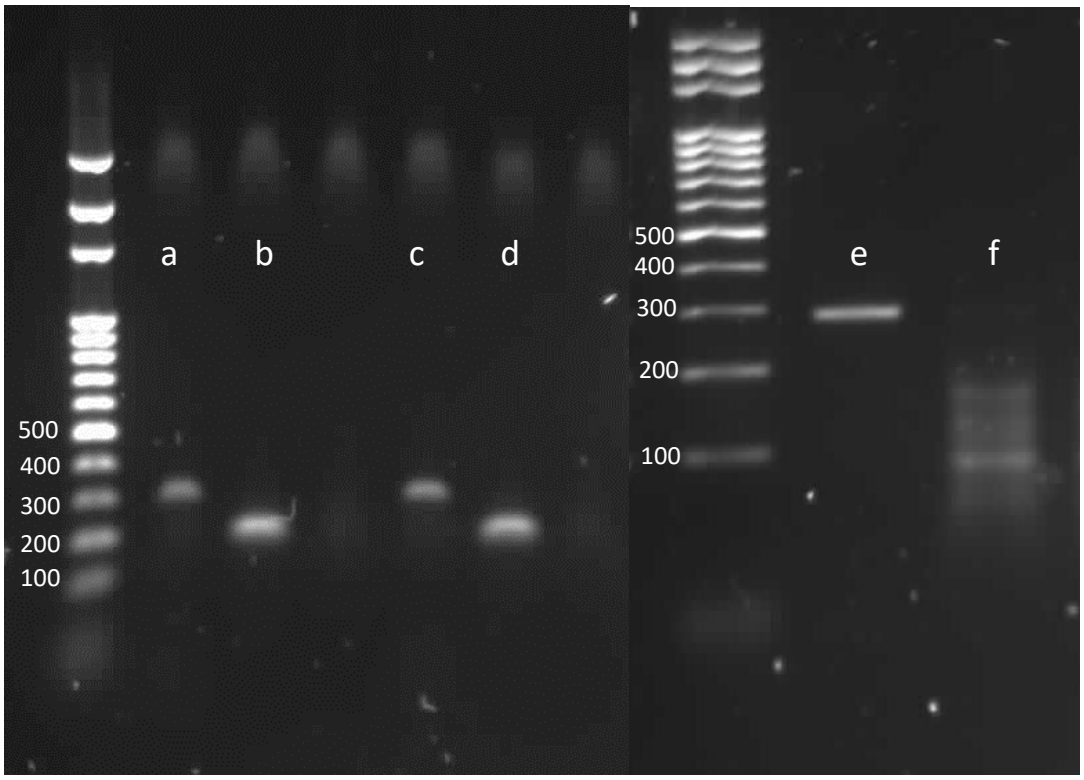

Figure 6. Electrophoresis gel of the *in vitro* efficiency of cleavage by RNP complex on targeted *loci*. a) PCR product with the primers HP2.5-1, amplicon length 315 bp, b) PCR product with the primers HP2.5-2, amplicon length 201 bp, c) PCR product with HP2.5-1 primers incubated with Cas9 and sgRNA1, d) PCR product with HP2.5-2 primers incubated with Cas9 and sgRNA2,, e) PCR product of the targeted gene with the couple of primers HP2.5-3, amplicon length 296 bp. f) PCR product of HP2.5-3 primers incubated with Cas9 and sgRNA3, cleaved fragments of the gene, two products one of ~188 bp and ~108 bp corresponding to the size fragments after DSB on the PAM sequence.

The % of CpG methylation in the transfected snails 6 was significantly higher than in controls (Mann-Whitney test, $p<0.05$) and the transfected snails 1, 2 and 3 showed a significant difference in variance compared to controls (*F*-test, $p<0.0001$) (Figure 7).

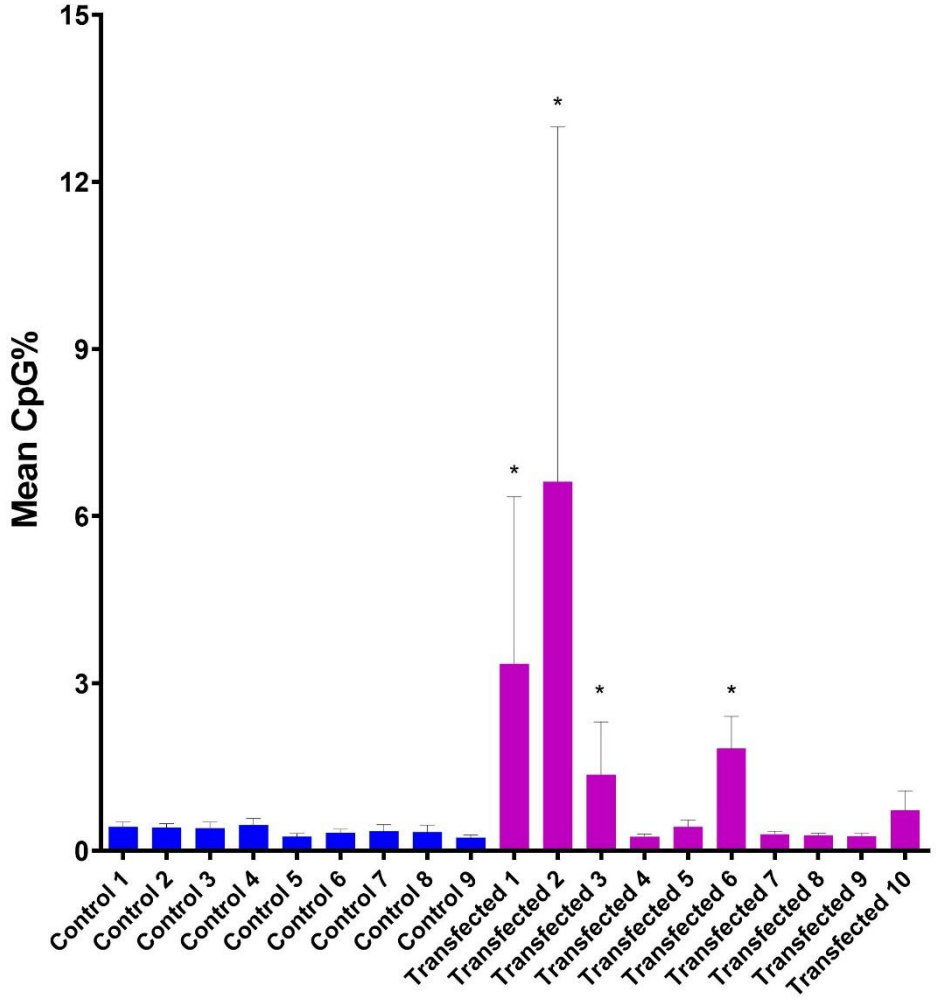

Figure 7. Mean CpG percentage of the controls snails (blue) and the transfected snails (magenta) (*n* = 9 and 10). % of Mann-Whitney test and F-test was applied, significate differences between transfected embryos and controls are marked by *. Bars represent the mean CpG% value (*n* =12 CpG sites), error bars represent the standard error of the mean (SEM).

Table 2. CpG methylation results from bisulfite amplicon sequencing CpG sites of the targeted gene BGLB032652 found in the genomic region: LGUN_random_Scaffold680: 40,914-41,372.

CpG sites	Bisulfite amplicon sequencing											
	CpG 1	**CpG 2**	**CpG 3**	**CpG 4**	**CpG 5**	**CpG 6**	**CpG 7**	**CpG 8**	**CpG 9**	**CpG 10**	**CpG 11**	**CpG 12**
Position on Scaffold 680	*40927*	*40950*	*40972*	*41042*	*41054*	*41138*	*41178*	*41191*	*41228*	*41239*	*41293*	*41334*
Control snail 1	0.9	0.4	0.3	0.2	0.3	0.2	0.4	0.0	0.4	0.5	0.5	1.0
Control snail 2	0.7	0.7	0.7	0.2	0.2	0.4	0.3	0.0	0.3	0.4	0.4	0.7
Control snail 3	0.3	1.0	0.5	0.1	0.1	0.3	0.3	0.0	0.4	0.3	0.4	0.3
Control snail 4	0.4	1.0	1.0	0.5	0.2	0.2	0.4	0.0	0.2	0.5	0.5	0.2
Control snail 5	0.7	0.4	0.3	0.1	0.1	0.3	0.2	0.0	0.2	0.3	0.4	0.3
Control snail 6	0.7	0.7	0.4	0.2	0.1	0.2	0.3	0.0	0.2	0.2	0.6	0.3
Control snail 7	0.6	0.4	0.4	0.2	0.1	0.2	0.2	0.0	0.1	0.2	0.5	0.3
Control snail 8	0.7	0.4	0.3	0.1	0.1	0.3	0.3	0.0	0.1	0.3	0.3	0.3
Control snail 9	0.5	0.5	0.3	0.1	0.1	0.2	0.3	0.0	0.1	0.2	0.4	0.3
Transfected snail 1	0.6	0.6	0.6	0.1	0.1	0.2	0.3	0.0	0.2	0.2	**36.3**	1.1
Transfected snail 2	0.7	**76.4**	0.3	0.1	0.1	0.2	0.3	0.0	0.2	0.3	0.4	0.3
Transfected snail 3	0.6	0.4	**11.6**	0.2	2.1	0.2	0.3	0.0	0.1	0.2	0.3	0.3
Transfected snail 4	0.6	0.4	0.3	0.1	0.1	0.2	0.4	0.0	0.2	0.2	0.3	0.3
Transfected snail 5	1.3	1.1	0.8	0.1	0.1	0.2	0.3	0.0	0.3	0.3	0.5	0.3
Transfected snail 6	**4.4**	0.4	**4.4**	0.2	0.3	**3.9**	0.3	0.0	**3.5**	**4.2**	0.3	0.2
Transfected snail 7	0.6	0.4	0.3	0.1	0.6	0.3	0.3	0.0	0.2	0.2	0.4	0.2
Transfected snail 8	0.4	0.5	0.3	0.1	0.2	0.3	0.3	0.0	0.2	0.3	0.4	0.3
Transfected snail 9	0.5	0.5	0.4	0.1	0.1	0.2	0.3	0.0	0.2	0.3	0.4	0.3
Transfected snail 10	4.4	0.5	0.5	0.2	0.1	0.3	0.3	0.0	0.2	0.4	0.4	1.4

The control snails (n = 9) displayed ≤1% of CpG methylation in the 12 CpG sites, while 4 of the 10 transfected snails (Transfected 1, 2, 3 and 6) showed an increase of ~4-76% of the CpG% in up to 5 CpG sites (Table 2). Since BSAS requires sequencing resources that are not always readily available we wondered if direct Sanger bisulfite sequencing would be used for quantification of DNA methylation of the transfected snails by measuring relative peak heights sequencing traces. As expected, for control embryos, the target region was completely unmethylated in eleven CpGs sites (CpG site 1 was missed in sanger sequencing), while 2 transfected embryos showed 1 methylated CpG site, displaying a methylation percentage of 30 and 60% (Table 3) concordant with the BSAS results and indicating that direct Sanger

sequencing represents a suitable alternative for rapid but much less sensitive screening of epigenetic engineering.

Table 3. CpG methylation results from Sanger sequencing CpG sites of the targeted gene BGLB032652found in the scaffold LGUN_random_Scaffold680: 40,914-41,372. CpG 1 was lost in the sanger chromatograms due to the noise background at first 26 nucleotides of the 5' end sequence.

	CpG 2	**CpG 3**	**CpG 4**	**CpG 5**	**CpG 6**	**CpG 7**	**CpG 8**	**CpG 9**	**CpG 10**	**CpG 11**	**CpG 12**
Position on Scaffold 680	*40950*	*40972*	*41042*	*41054*	*41138*	*41178*	*41191*	*41228*	*41239*	*41293*	*41334*
Control snail 1	0	0	0	0	0	0	0	0	0	0	0
Control snail 2	0	0	0	0	0	0	0	0	0	0	0
Control snail 3	0	0	0	0	0	0	0	0	0	0	0
Control snail 4	0	0	0	0	0	0	0	0	0	0	0
Control snail 5	0	0	0	0	0	0	0	0	0	0	0
Transfected snail 1	0	0	0	0	0	0	0	0	0	**30**	0
Transfected snail 2	**60**	0	0	0	0	0	0	0	0	0	0
Transfected snail 3	0	0	0	0	0	0	0	0	0	0	0
Transfected snail 4	0	0	0	0	0	0	0	0	0	0	0
Transfected snail 5	0	0	0	0	0	0	0	0	0	0	0
Transfected snail 6	0	0	0	0	0	0	0	0	0	0	0
Transfected snail 7	0	0	0	0	0	0	0	0	0	0	0
Transfected snail 8	0	0	0	0	0	0	0	0	0	0	0
Transfected snail 9	0	0	0	0	0	0	0	0	0	0	0
Transfected snail 10	0	0	0	0	0	0	0	0	0	0	0

Discussion

In vivo targeted epigenome modifications were reported in mouse (*21*), in the fish *Oryzias latipes* (*22*) and in the plant *Arabidopsis thaliana* (*23*). dCas9 fused to the transcriptional activator domain VPR (dCas9-VPR) was used to activate transcription of a target gene *in vivo* in *Drosophila* (*15*) and targeted DNA methylation changes with an epigenetic engineering tool in invertebrates has been only reported in *Bombyx mori* (*16*).

The use of epigenetic engineering tools and *in vivo* transfection of our biological model, the snail *Biomphalaria glabrata*, is technically challenging. One of the reasons is that the size of the embryo (50 µm diameter) is much smaller than in model species (e.g. zebrafish embryos

700 µm (*24*) and mice embryos 100 µm (*25*) . We developed a method of microinjection at the embryo yolk with an *in vivo* polymer-based reagent. Plasmids microinjected into the *B. glabrata* embryos produced transient expression of genes from the viral promoter SV40 during 5 days. These results suggest that plasmids were expressed without stable integration in the snail genome, SV40 promoter vectors can persist as nonreplicating extrachromosomal elements that are destroyed by nucleases, can be functionally inactivated by partitioning to non-nuclear compartments or can be diluted by cell division (*26*). Moreover, this study shown that dCas9-SunTag with DNMT3A-GFP transient expression (within 5 days' timeframe of post transfection) along with sgRNA complexity to dCas9 can perform site specific DNA methylation.

BSAS allowed to identify that the targeted methylation was achieved at low levels in some of the CpG sites of the transfected snails that were not detected in the Sanger sequencing. In one transfected snail, five CpG sites were methylated with CpG% of ~4%, the other 3 snails showed higher CpG% principally in one CpG site (11-76%). Our results showing low variants of DNA methylation in some CpG sites (~4%) are consistent with the idea that only some cells were transfected. It might now be conceivable to try to increase the targeted DNA methylation change by optimizing further the transfection at an earlier stage. 10 transfected embryos were compared to 9 control embryos (1 control bisulfite amplicon library yield very low number of reads) to confirmed that the methylation provoked was only achieved in the transfected embryos. Control snails displayed less than 1% of CpG% in the 12 CpG sites of the targeted gene. Bisulfite conversion percentage was of ≥98% making it unlikely that we observed non-conversion artefacts and this precisely at the target sequence.

The transfection technique presented in this work showed an efficiency of 4%, from 250 microinjected embryos, 10 embryos showed the fluorescence of the reporter genes GFP and BFP and from these 10 embryos, targeted methylation was demonstrated in 4 from 10 positive

transfected embryos, representing 2% of the total microinjected embryos and 40% of the fluorescent positive embryos. The efficiency of the targeted DNA methylation could depend also on the dynamic folding of the chromatin, which regulates the accessibility to the DNA, and possibly some regions were accessible to the dCas9-SunTag-DNMT3 complex while others were not. Mosaic DNA methylation patterns were found in the targeted gene, this was expected, since the transfection was done at the gastrula stage. Two-cell transfection was assayed but the embryos did not survive to the microinjection technique and to the toxicity of the polymer-based reagent (Table 1). Such mosaicism of Cas9 action was also observed in the mollusc *Crepidula fornicata*, the first mollusc species where genome editing has been achieved, even if the microinjection was there done at an earlier stage phase (at two cell stage) of the embryonic development (*27*). The high heterogeneity in targeted DNA methylation can be due to the dissimilar integration of vector constructions to the transfected cells, transfection is done at the embryo yolk and not at the embryo itself, and probably the integration of the vector to the embryo cells happens at different times. Additionally, the embryo development inside the eggs mass is not perfectly synchronized which could also play a role in the differential vector integration to cells.

The dCas9-SunTag-DNMT3A1 was earlier transduced by lentiviral vectors to the HEK293T human cell line where it showed a high efficiency in inginducing targeted DNA methylation, the increase was of 50-80% in 53 CpG sites of a CpG island along 600 bp of the gene *Hox5A*. The dCas9-SunTag-DNMT3A1 system was compared to the vector dCas9-DNMT3A, the first one showed an increase of 60-95% of the CpG methylation in a 4-kb window while the second one showed an increase of 20-40% at the same 4-kb window, these results showed that the dCas9-SunTag-DNMT3A1 system showed higher efficiency in inginducing a targeted DNA methylation than dCas9-DNMT3A (*18*).

The targeted modification *in vivo* of DNA methylation has been mostly addressed in mice model. E.g. the use of dCas9 fused to the prokaryotic methyltransferase MQ1 (dCas-MQ1) was tested *in vivo* in mouse zygotes where it induced an increase from 50% CpG methylation to 70% in 20 CpG sites along 500 bp of the locus *Igf2/H19 (21)*.

Comparisons of the results found *in vitro* in a human cell line, showed that, in our case, the targeted DNA methylation increase was weaker and highly heterogeneous. A major difference is that our transfections were performed *in vivo* at the gastrula stage and not at the one cell stage or in a cell culture. *In vivo* transfection efficiencies are not as high as viral vectors used *in vitro*, nucleic acids are more stable in cell culture and integrate more efficiently in one cell nucleus, but in a living organism, they can be degraded before cell integration.

It is more suitable to compare our increase targeted DNA methylation results to that obtained *in vivo* in mouse zygotes with the vector dCas9-MQ1 *(20)* . Indeed, our results are similar: in mice the global increase was of 10-60% in 7 CpG sites using a unique sgRNA while in our model the increase was of 4-76% in up to 5 CpG sites. In the mouse zygotes, the range of the CpG methylation increase was improved by using a pool of 4 sgRNAs, increasing the CpG% to 20- 50% in 12 CpG sites. In the case of dCas9-DNMT3 vector, co-transfection of multiple sgRNAs also revealed an increase in the range of the targeted CpG% modification compared to the use of a unique sgRNA (*14*); one sgRNA increases the methylation 30-60% in 6 CpG sites, and when 4 sgRNAs were cotransfected the methylation increases 25-55% in 12 CpG sites. Therefore, to increase the range of the DNA methylation effect multiple sgRNAs ingtargetingthe same *locus* should be used in the future. We estimate that at least 4 efficient sgRNAs must be developed for a target region of ~500 bp.

Another invertebrate in which targeted DNA methylation has been done is the silkworm *Bombyx mori*, where dCas9-TET1 was used to demethylate the gene body *in vitro* and *in vivo (16)*. The *in vitro* demethylation percentages were 30-60% in 9 of 9 CpG sites of the gene

BGIBMGA004109 and 10-42% in 8 of 9 CpG sites of the gene *BGIBMGA001471*. The *in vivo* demethylation was achieved in 5 of 9 CpG sites of the gene *BGIBMGA004109*, the 5 CpG sites displayed different DNA demethylation percentages from 20% to 50%. In the gene *BGIBMGA001471* the CpG demethylation *in vivo* was achieved in 7 of 9 CpG sites, with methylation decrease from 5% to 40%. The editing range was within 200 bp, and the editing efficiency was lower in embryo than in embryonic cell line. Furthermore, gene body demethylation was found to promote transcription in the *B. mori* embryonic cell line which is contradictory to the hypothesis that GBM is correlated with higher gene expression; nevertheless, further work is needed to explore the causal relationship between GBM and transcription, particularly in invertebrate taxa.

Our work showed that the use of epigenetic engineering tools *in vivo* in the snail *B. glabrata* and probably other molluscs species is now conceivable. The further development of transfection and epigenetic engineering tools in our biological model will bring insights about the role of GBM in invertebrate species, a subject which definitely requires experimental tools to address this question.

Methods

Ethics statement and plasmid transformation

B. glabrata albino Brazilian strain (*Bg*BRE) was used in this study. Snails were maintained at the IHPE laboratory facilities; 8 groups of 10 snails were kept in separate aquariums and fed with lettuce *ad libitum*, polystyrene pieces were introducing to the aquariums since the snails use it to laid their egg patches. 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. Plasmids were constructed as in Huang, et al. 2017. One Shot™ TOP10

Chemically Competent *Escherichia coli* cells (Invitrogen, Ref C404004) were transformed with the plasmids dCas9-SunTag and scFv-DNMT3-GFP**.** Subsequently, PCR was performed (Table 3) to confirm the transformation with the dCas9 and DNMT3A sequence inserts. Primers were elaborated in the web-interface Primer3Plus.

Selection of candidate genes

To identify suitable target genes for targeted DNA methylation epimutagenesis the following criteria were established:

1) Candidate genes were selected from known morphogenic genes of *Biomphalaria glabrata* or other mollusks, genes involved in embryogenesis in mollusks were identified by BLAST searches in the NCBI nucleotide collection database in order to find a similar sequence in the genome of *B. glabrata*. Homologues of *Hox* genes and homeobox proteins were used since we hypothesized that epimutations there would potentially provoke observable changes in the phenotype without lethal effects, but in this study, we did not evaluate phenotype changes.

2) The DNA methylation level of the selected genes were visually examined using our reference methylome (*28*) (https://zenodo.org/record/4277533) in the IGV-Integrative Genomics Viewer (http://software.broadinstitute.org/software/igv). The criteria to select a candidate gene was that it must be unmethylated.

3) The tissue expression of each gene was observed in a local RNA-Seq database in order to select those genes that are expressed in most tissues or at least in tissues that are easily accessible and, therefore, easy to manipulate (DNA/RNA extraction).

4) Local adult transcriptome data and a multi-stage transcriptome of *B. glabrata* (*29*) was used to align adult transcriptome reads with multi-embryo stage transcriptome reads, and we used HTseq-count in order to verify if genes were expressed at the embryo phase.

Elaboration of gRNAs

The homeobox Nkx 2.5-like gene was selected, a homologue of the *Has-Hox4* of the gastropod *Haliotis asinina* (GenBank: AAK11240.1), whose expression is restricted to the forming pleural ganglia and nerve cords, suggesting a role in the nervous system development (*30, 31*) . Three gRNAs targeting DNA methylation canyon on the target locus: LGUN_random_Scaffold680: 40871-42922 on *B. glabrata* gene database (https://legacy.www.vectorbase.org) were elaborated by a software for designing CRISPR/Cas9 guide RNAs called CHOPCHOP (*32*). The off-target were also predicted against *B. glabrata* genome by CHOPCHOP as previously described (*33*) and following considerations for dCas9-DNMT3 system described in (*34*). The three sgRNAs were designed in the CUFF52452. Oligonucleotides for the three sgRNAs (Table 3) targeting the gene were ordered from Sigma-Aldrich and Synthego.

Table 3. sgRNAs designed to target the homeobox 2.5 Nkx of *B. glabrata*.

ID	RNA Sequence [PAM]	DNA sequence [PAM]	Location
sgRNA 1	GGAGUAGUGAGGCUGCUGUG[AGG]	GGAGTAGTGAGGCTGCTGTG[AGG]	LGUN_random_Scaffold680:42773-42795
sgRNA 2	AACGACGGUUCAAGCAGCAG[AGG]	AACGACGGTTCAAGCAGCAG[AGG]	LGUN_random_Scaffold680:42533-42555
sgRNA 3	CUCUGAAACUAUUUGUUUUC[AGG]	CTCTGAAACTAGTTGTTTTC[AGG]	LGUN_random_Scaffold680:41352-41372

Efficiency of the *in vitro* DNA cleavage activity of the Cas9-sgRNA complex

To evaluate the cleavage efficiency of the Cas9-sgRNA complex, a PCR was performed to amplify the targeted region of the sgRNA. Primers designed to evaluate each sgRNA are shown

in Table 2. Forward and reverse primers were designed to amplify a region spanning the sgRNA target in the middle of the amplified sequence to be able to distinguish cleaved fragments from non-cleaved fragment in the electrophoresis gel. The PCR amplification was set as follows: 95°C for 1 min, followed by 30 cycles of 95 °C for 30 secs, Tm specific of the couple of primers for 30 secs, 72 °C for 2 min and lastly 72 °C for 10 min. The primers for each gene region are detailed in Table 4.

Table 4. Primers designed to verify sgRNA cleavage efficiency.

ID	sgRNA	Sequence	Tm	Amplicon lengthlength
HP2.5-1 For	sgRNA 1	GTCGCTGCTTCAGCAAAGTAC	51°C	315 bp
HP2.5-1 Rev		GTAAACTAACTTGCACTCAGC		
HP2.5-2 For	sgRNA 2	TCTTTCCTTTGTCTTTCTCGCT	52°C	201 bp
HP2.5-2 Rev		GCACTCAGCTTTCTTCACTTCA		
HP2.5-3 For	sgRNA 3	GTGTGTGTGTCGTTTGACAAAG	51°C	296 bp
HP2.5-3 Rev		CGACTGTCTACACAATTCTGTG		

The DNA cleavage activity of the previously amplified PCR fragment was assayed *in vitro* with Cas9-sgRNA complex as previously described (*35*). The cleavage reaction was performed by mixing, 1 µL of sgRNA (1 µg/LµL), 3 LµL 10X NEBuffer 3.1 (New England Biolabs, B7203S), 3 LµL Cas9 recombinant protein (200 ng/LµL), 1 LµL of the PCR product of the targeted gene and the volume was completed to 30 LµL with nuclease-free water. The reaction was incubated at 37°C for 2 h, then 1 LµL of Proteinase K (20 mg/LmL) was added to reaction and a final incubation was done during 10 min at room temperature. Reactions with all components except for sgRNA were used as negative controls. Recombinant protein Sp-Cas9-NLS-GFP-NLS was a generous gift of Jean Paul Concordet from the INSERM U1154, CNRS

UMR7196, Structure and Instability of Genomes, Sorbonne Universités, Museum National d'Histoire Naturelle in Paris, France. The amplicon digestion on the target site after sgRNA transfection was revealed by 2% agarose electrophoresis gel stained with Midori Green Advanced (Nippon Genetics Europe, catalog number MG04).

Microinjection tests

Tthe linear polyethylenimine derivative, *in vivo*-jetPEI™ (Polyplus Transfection, France) was used for 5 early stages of embryonic development. Two-cell, 8-cell, morula, blastula and gastrula stages were determined based on the descriptions and embryo illustrations in (*36*). Egg patches were collected from the aquarium and sorted under the microscope to identify the five developmental stages, fifty embryos per developmental stage were used to test *in vivo* jetPEI reagent. Microinjection of embryos yolk sacs with *in vivo* jetPEI reagent and the two plasmids coding for dCas9-SunTag and DNMT3-GFP has been performed. A pre-pulled glass micropipette of 1 mm of diameter and a tip of 100 µm of diameter was attached to a programmable nanoliter injector (Nanoject III, Drummond Scientific) to carry out microinjections in embryo's yolks under a stereoscopic microscope. The parameters used in the nanoliter injector were: injection volume 30 nanoliters and speed of the injection of 20 nanoliters per second.

Glucose solution and *in vivo* jetPEI were equilibrated at room temperature. The scFv-DNMT3A-GFP plasmid concentration was 78.8 ng/LµL and the dCas9-Suntag plasmid concentration was 88.8 ng/LµL. 21 LµL of each plasmid (42 LµL of total volume =3.5 µg total amount of plasmid DNA) were added to 21 LµL of 10% glucose in a tube (ttube A), and in another tube (ttube B), the quantity of *in vivo* jetPEI required for the amount of DNA was added (1 LµL) to 21 LµL of 5% glucose. After 15 min of incubation, tube A and tube B were mixed giving a total of 91 LµL of microinjection volume with a final concentration of plasmid DNA

of 41 ng/ LµL that were used to microinject 250 embryos. Another solution was prepared with 21 LµL of glucose 5% and 0.5 LµL of *in vivo* jetPEI and was used to microinject control eggs.

When transfection was done at the gastrula stage (26 h after the first cleavage of the zygote) we observed 72 h later, the fluorescent signals of BFP and GPF, indicating that dCas9-SunTag protein system was expressed in *B. glabrata* embryos. Hence, the mixture of *in vivo* jetPEI (0.5 LµL), 5% glucose (10 µL) and active sgRNA3 (20 ng in 10 µL) was injected into the fluorescent positive embryos. Three days after sgRNA3 transfection, newly hatched snails were collected and stored at -80°C for subsequent DNA and RNA extractions.

Confocal microscopy

Fluorescent larvae were washed in PBS solution and then fixed with 4% paraformaldehyde. Single larvae were put on a microscope slide with 2 drops of the fluorescence mounting medium Dako (Agilent Technologies) and the sample was covered with a coverslip. Slides mounted were stored at 4°C in the dark to preserve fluorescent signal. Slides were examined under an epifluorescence confocal microscope LSM700 (Zeiss).

***B. glabrata* embryo DNA isolation**

A DNA purification protocol used for zebrafish embryos was adapted to *B. glabrata* hatched snails (*37*) . Briefly, 40 LµL of lysis buffer containing 9.4 LmL of 10 mM Tris-HCL (pH 8.3), 50 mM KCl buffer, 300 LµL of NP40 (10% stock) and 300 LµL of Tween 20 (10% stock) was applied to single embryos. Incubation at 98°C for 10 min to lyse cells was applied and then 5 LµL of Proteinase K (10 mg/LmL) were added to each embryo. Incubation at 55°C for 2 hours were done followed by a final incubation of 5 min at 98°C to heat kill Proteinase K. DNA was directly use for PCR or bisulfite conversion. PCR mix, template DNA and primers (Table 2) were set as follows: 94 °C for 2 min, 5 cycles of 94 °C for 1 min, 46 °C for 2 min and 72 °C

for 3 min, followed by 25 cycles of 94 °C for 30 secs, 51 °C for 2 min and 72 °C for 1:30 min and finally 72 °C for 10 min. Then a 2% - -agarose gel was elaborated to run PCR reactions and identify if the target gene was amplified.

Isolation of poly (A+) mRNA from *B. glabrata* embryos and reverse transcription

For messenger RNA isolation, individual trochophore larvae were collected in RNAse-free tubes with 100 LµL of lysis buffer of the Dynabeads mRNA DIRECT Micro Purification Kit (Cat. Num. 6102, Invitrogen) and stored at -80°C. mRNA was purified using the Dynabeads mRNA DIRECT Micro Purification Kit according to the manufacturer's instructions. This method is based on base-pairing between the poly-A residues at the 3' end of the mRNA and the oligo-dT_{25} residues covalently couples to the surface of the paramagnetic beads. Reverse transcription to first strand cDNA was done using Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase (Cat. Num. K1682, ThermoFisher, Scientific). A DNase mixture (dsDNase, 10X dsDNase Buffer and water nuclease-free) was directly added to the bead-trapped mRNA, and then reaction was done in a final volume of 20 LµL (10 mM TPdNTP Mix, water nuclease-free, 5X RT Buffer and Maxima H Minus Enzyme Mix). An initial incubation step for 5 min at 50°C was done. Then the reaction was incubated for 10 min at 25°C followed by 15 min at 50°C. To terminate the reaction, an incubation at 85°C for 5 min was done.

RT-qPCR to check for reporter gene transcription

Real-time qPCR analyses were done on cDNA obtained from 8 controls and 16 transfected trocophore larvae 72h after their transfection.B. Before qPCR, cDNA was pre-amplified for each couple of primers (Table 3) when still attached to dynabeads as follows: 95°C for 5 min, 4 cycles of 95°C for 5 secs, 60°C for 30 secs and 72°C for 30 secs, then 72°C for 5 min and a final incubation at 94°C for 2 min to separate cDNA from dynabeads. Then qPCR was done using the LightCycler 480 System (Roche) in a 17.5 LµL final volume comprising 10 LµL of

No Rox SYBR Master Mix blue dTTP (Takyon), 3.5 LµL of ultrapure MilliQ water, and 1 LµL of each primer (Table 5) at a concentration of 10 µM and (final concentration of amorces was 500 nM) and the pre-amplified cDNA (1 ng). To ensure the absence of genomic DNA contamination, a reaction with all reagents except reverse transcriptase (RT) was done per samples (two replicates per sample) as a RT negative control.

Table 5. Primers designed to check for plasmid gene transcription.

ID	Sequence	Amplicon thlength
28S for	GCTGGCACGACCGCTCCTTT	100 bp
28S rev	TTTGAACCTCGCGACCCGGC	
GFP for	GAATTAGATGGTGATGTTAATGGG	254 bp
GFP rev	TTGAAAGATATAGTGCGTTCCT	
DNMT3A-1 for	TGATTGATGCCAAAGAAGTGTC	217 bp
DNMT3A-1 rev	AACACAGGAAAATGCTGGTCTT	
BFP-2 for	CAAGGAGGCCAACAACGAGA	80 bp
BFP-2 rev	CCAGTTTGCTAGGGAGGTCG	
dCas9-2 for	AAAGAAGGACTGGGACCCTAAG	233 bp
dCas9-2 rev	CAGAAAGTCGATGGGATTCTTC	

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. PCR experiments were performed in duplicate (technical replicates). The mean value of Ct and melting curves were checked using the LightCycler 480 Software release 1.5.0.

Bisulfite conversion

Bisulfite conversion was done as previously described in our preprint (*38*) 300 ng of DNA extracted from adult snails used as a control and 20 ng from embryo snails (10 control and 10 transfected) were denatured by adding 2 LµL of ribonucleic acid transfer from baker's yeast (*S. cerevisiae*) and 2.2 LµL of 3 M NaOH, and by incubating at 42°C for 20 min. Then 240 LµL of fresh prepared sodium bisulfite solution (5.41g Sodium metabisulfite in 7 L mL of distilled

water and 0.5 mL of diluted Hydroquinone (0.022g/10 mL)) were added to the denatured DNA samples. An incubation in the dark was done during 4 hours at 55°C. Then 200 µL of distilled water were added to the samples, and the total volume was transfered to a Amicon column (UFC501024, Millipore), a filter device to separate DNA from sodium bisulfite, a centrifugation was done at 12000 g during 5 min. The column was washed 3 times with 350 LµL of distilled water and centrifugation at 12000 g for 5 min was done each time. Following this, 350 LµL of 0.1 M NaOH was added to the DNA in the amicon column and a centrifugation at 12000 g during 5 min, subsequently 350 µL of distilled water were added and a centrifugation at 12000 g for 5 min was done. Twenty microliters of 10 mM TRIS/Cl pH 7.5-8.0 was added to the DNA in the amicon column and it incubated at room temperature for 5 min. Finally, the DNA was collected by centrifugation at 1000 g for 3 min. DNA was stocked at -80°C for further experiments.

Nested PCR on bisulfite converted gDNA

The initial PCR amplification was done with 5 LµL of the bisulfite converted gDNA as template with external primers (Table 6) set as follows: 94 °C for 2 min, 5 cycles of 94 °C for 1 min, 46 °C for 2 min and 72 °C for 3 min, followed by 25 cycles of 94 °C for 30 secs, 46 °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 2.5 LµL of the first PCR product using the internal primer set in the same condition as for the first PCR except for the annealing temperature which was increased to 50 °C. The subsequent PCR reaction was performed in 25 µL using 1.25 units of GoTaq DNA polymerase (Promega), dNTPs at 0.4 µM for each deoxynucleotide and primers at 0.4 µM. PCR products were separated by electrophoresis through 2% agarose gels to check for the specific amplification of each target gene. The house-keeping gene *Bg14.3.3* (BGLB005695, Scaffold 1582:42425–42875) was used as a control, this gene was previously showed to be highly methylated (*19*),

which allow us to evaluate the efficiency of the bisulfite conversion. Bisulfite PCR products were sequenced by Sanger sequencing (Genoscreen, Lille, France). Sequence chromatograms were analyzed as previously described to calculate Cytosine to Thymine conversion by measuring Cytosine and Thymine height peaks, this provides the degree of methylation of the targeted sequences (*39*) .

Table 6. Primers designed to check for efficiency of bisulfite conversion. Bisulfite conversion of C to T in forward primers is indicated by lowercase "t*"* and in reverse primers G to A conversion is indicated by lowercase "a".

ID	Type of primer	Sequence	Tm	Amplicon length
Bg14.3.3 BS 5	External	GAtTGAAtTTGAAGGTAAATTAAtAtAAGA	58.1	
Bg14.3.3 BS 5	External	aATaTCCACAaaaTAAaATTaTCAC	55.4	605 bp
Bg14.3.3 BS 1	Internal	tTGTtTGTTGtTTAtAAAAATGTTGTG	59.4	
Bg14.3.3 BS 1	Internal	CACTaATAaCCTCATCAAAaaCCTCTT	62.6	478 bp
HP2.5-II For	External	GAAGTtTTGGAGGTtAAAAAtTTTGt	60.6	
HP2.5-II Rev	External	aACTCAaCTCAaATCAaTAAATCCAC	61.4	838 bp
HP2.5-III For	Internal	TGTGAGAAttAtTtTATTAAGTTGT	56.3	
HP2.5-III Rev	Internal	TaTTTTCAaaACATACCTCTTTaAC	57	458 bp

Amplicon sequencing library construction obtained from PCR performed on bisulfite converted gDNA

For the amplicon sequencing library, the bisulfite converted DNA of 10 control and 10 transfected snails was used as template for a bisulfite PCR with the external bisulfite primers HP2.5-II (Table 4) and a nested PCR was performed with the internal bisulfite primers containing overhangs for subsequent indexing. For the HP2.5-III forward primer the overhang was: 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNN- [primer sequence]3' and

for the HP2.5-III reverse primer:

5’GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNN- [primer sequence]3’.

Later, the nested PCR was purified with 0.9X AMPure XP beads (Beckman Coulter) to be indexed. Dual indexing was done using the Illumina Nextera XT Index Kit following manufacturer’s instructions. After indexing, bead purification was done in amplified libraries with 0.9X AMPure beads and quantification of each indexed sample was done in Qubit fluorometer with the Qubit dsDNA HS (High Sensitivity) Assay Kit (Thermofisher Scientific). Then each sample was normalized to equal nanomolar concentration and then pooled. One LµL of the pooled libraries was analyzed on a Bioanalyzer (Agilent) and after checking broad size distribution, the libraries were sequenced in MiSeq system (Illumina) at the Bio-Environment NGS Platform at the University of Perpignan.

Bioinformatics and statistics

Raw reads were trimmed using Trim galore default parameters to remove Illumina adapters, then trimmed reads were mapped to the reference genome of *B. glabrata* with the alignment algorithm BWA-MEM that map long reads (>100 pb) to verify that the targeted gene was sequenced. After that, trimmed reads were used as input in the command line version of the software pipeline CRISPResso2 available at https://github.com/pinellolab/CRISPResso2. This pipeline allows to align reads to a reference sequence, and to obtain conversion of target bases around the sgRNA and in the entire amplicon which allows to calculate the conversion percentage of Cytosines transformed to Thymines in the target CpG sites (*40*)(Clement, Rees et al. 2019). Nucleotide frequency of the entire amplicon was obtained from this pipeline and CpG sites were searched manually to calculate the Cytosine to Thymine conversion. The normality of the data distribution was tested with the Shapiro-Wilk normality test. For comparing CpG%, between controls and transfected samples, aa Mann-Whitney test was

performed. To compare variance between two samples, *F*-test of equality of variances was applied.

Availability of data and materials

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

C.G and C.C designed the study and writing the manuscript. D.D. helps with the optimization of the microinjection technique, with the elaboration of the confocal microscopy photographs, and with the transfection protocol. C.Ch helps with the bioinformatics analysis. W.I helps in the optimization of the *in vivo* transfection in the embryos of *B. glabrata* and with the writing

of the manuscript. J.A elaborates and sequences the libraries of bisulfite amplicon sequencing. G.T optimizes the microinjection technique.

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Supplementary data

Table S1. *In vivo* transfection techniques were tested in the snail *B. glabrata*, four in the snail's embryos and one in the juvenile snails.

Technique	Survival%	Transfection efficiency
Microinjection in two cell and eight-cell embryos with GeneCellin™HTC	2%	No expression of plasmids
Microinjection in two cell and eight-cell embryos with Lipofertamine 3000	1%	No expression of plasmids
Microinjection in two cell and eight-cell embryos with jetOPTIMUS™	2%	No expression of plasmids
in vivo electroporation to egg patches	0%	No expression of plasmids
Soaking of egg patches with *in vivo* reagent	10%	Very low efficiency <0,5%
Soaking of juveniles with *in vivo* reagent	60%	Efficiency of 2%, expression of plasmids in ovotestis 72 h after transfection but have to wait long time to have offspring by self-fertilization (~1 month)
Microinjection to gastrula stage embryos with *in vivo* reagent	50%	Efficiency of 4%, expression of plasmids 72 hours after transfection

Video of the transfection protocol will be available at: zenodo.org

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Thesis general discussion

The need of an efficient and not toxic DNMT inhibitor

The role of DNA methylation in phenotypic plasticity has been demonstrated in many species, principally plants and vertebrates (Johannes, Porcher et al. 2009, Jones 2012) and this topic is under current study in invertebrate's species, but much less information is available, e.g. 61,016 articles in Pubmed are available for DNA methylation in vertebrates compared to 1,301 for DNA methylation in invertebrates. There is evidence of the role of DNA methylation in the generation of developmental plasticity on the bee *Apis mellifera*, e. g. DNA methylation changes are involved in caste differentiation (Elango, Hunt et al. 2009, Lyko, Foret et al. 2010) and in the regulation of reproductive status (Kucharski, Maleszka et al. 2008). Moreover, in the ant *Camponotus floridanus*, DNA methylation contributes to the size variation of the worker ants by regulating the transcription of the epidermal growth factor receptor (Alvarado, Rajakumar et al. 2015). DNA methylation has been found to be involved in behavioural plasticity, e.g. in the bumble bee *Bombus terrestris*, there are differences in the DNA methylation status between reproductive and non-reproductive workers, and a treatment with the DNMT inhibitor 5-aza-2'-deoxycytidine showed that the reproductive conflict among workers increased in < 1-year-old bees treated with the DNMTi (Amarasinghe, Clayton et al. 2014) and in the bee *A. mellifera*, the reversibility of the transition from nurse bee to forager is associated with reversible DNA methylation changes (Herb, Wolschin et al. 2012). In other invertebrates, there is some evidence of the role of DNA methylation in the development, e.g. DNA methylation plays a major role in the early development of the mollusk *Crassostrea gigas* (Riviere, Wu et al. 2013). Additionally, DNA methylation changes have been found as a response to changing environmental conditions, e.g. the DNA methylation of *Dahpnia magna* is altered by predation, diet changes and salinity alterations (Asselman, De Coninck et al. 2015).

One of the limitations on the study of DNA methylation in invertebrates, is the lack of molecular tools in non-model organisms, that allow the experimental manipulation of DNA methylation. One of these tools are the DNMT inhibitors (DNMTi), largely used in vertebrate models, especially mammals, in some plant species and in a lower number of invertebrate species as reviewed in the introduction of the chapter I (Table 1.1). Nevertheless, the use of DNMTi is not without difficulties due to possible undesired side effects. However, in the absence of knock out mutants with altered DNA methylation, this is the most accessible existing tool for experimental manipulation of DNA methylation for non-model species (Verhoeven, vonHoldt et al. 2016).

Currently, the use of DNMTi in invertebrates relies on three commercially available compounds that are efficient but show high toxicity, instability in aqueous solutions and low specificity (Ueno, Katayama et al. 2002), these compounds are the cytidine analogues 5 azacytidine, 2'-deoxy-5-azacytidine and zebularine. Recent advancements have been achieved in the synthesis of new generation DNMT inhibitors that are less toxic, more stable in aqueous solutions and more specific. These new compounds are non-cytidine analogues that do not incorporate to DNA to be active which induces lower toxicity (Gros, Fahy et al. 2012, Erdmann, Halby et al. 2015). Despite these enhancements, the *in vivo* inhibitory efficiency of these new compounds in an invertebrate model and the heritability of the DNA methylation changes that they induced, had not been tested prior to this work.

Validation of two methods to measure DNA methylation in a mollusk species

In order to screen for efficient DNMT inhibitors, I validated two cost-effective and high-throughput methods to measure DNA methylation, one at the global level and another at a nucleotide resolution, for use in a mollusk species. The first is an antibody based method called "dot blot" that is based on the recognition of 5 methylcytosine (5mC) by an antibody, this assay was used for a large-scale screening of multiple samples for global 5 mC changes at a lower price compared to commercially immunological-based kits such as ELISA kits. This method allowed us to produce first results to decide then which samples would be worth to be analysed by a more accurate method. I also used a new next generation sequencing method (epiGBS) to determine the DNA methylation changes at the nucleotide resolution, in a reduced part of the genome. Compared to whole genome bisulfite sequencing (WGBS), it allows to sequence multiple samples at a much lower price, and therefore it is more suitable to address population based studies.

Sequencing a small informative percentage of the genome after bisulfite treatment is a classical method which is used to address epigenetic population based studies. Several methods exist to address these questions and one of them is the reduced representation bisulfite sequencing (RRBS), a technique that has shown to be very informative for vertebrate genomes since it has a bias for methylated regions of the genome due to the restriction enzymes used (*Msp1* and *HpaI*). In the case of the mollusk I used as biological model, this technique is not suitable due to its mosaic methylation pattern, that is very different from the global methylation pattern found in vertebrates.

We used a technique suitable for non –model species that has been extensively used in plant species: epigenotype by sequencing (epiGBS), that has shown reliability and feasibility to sequence valuable informative DNA methylation. Plants present a "mosaic" methylation pattern as many invertebrate species. EpiGBS was already used to sequence the methylome of *Daphnia magna* showing that is was an efficient method to detect CG methylation information in an invertebrate species (van Gurp et al. 2016). Furthermore, the advantages of epiGBS are that is cost effective compared to RRBS and WGBS, this method allows to obtain DNA methylation polymorphisms and genetic polymorphisms from the same samples and the recent improvement of the pipeline makes accessible and use-friendly the analysis of DNA methylation and single nucleotide polymorphisms. For these reasons, we decided to test if epiGBS could be a successful method to sequence the epigenome and genome of the snails *B. glabrata* treated with the DNMTi.

Comparison of the sequencing depth between epiGBS and WGBS was done to evaluate the percentage of DNA methylation information obtained in epiGBS and to evaluate the correlation between both methods. I found that epiGBS allowed to obtain up to 4% of the sequencing depth obtained with WGBS. Moreover, the gene body methylation (GBM) values obtained by both methods were highly correlated and the quantiles distribution of GBM were also similar. Further optimizations of the technique are possible, such as the use of another restriction enzymes combinations, and *in silico* digestions analysis can be done to anticipate which genomic regions are going to be sequenced, allowing the improvement of the detection of differential methylated regions that is one of the difficulties of reduced representation bisulfite methods (Paun, Verhoeven et al. 2019). Nevertheless, this method allowed the detection of differential methylated cytosines (DMCs) between the control and the DNMTi-treated snails.

Pharmacological modification of DNA methylation in *B. glabrata*

I evaluated if it was possible to modulate DNA methylation with new generation chemical DNMT inhibitors (DNMTi). The inhibitory efficiency and the toxicity of 3 types of DNMTi was assessed in the freshwater snail *B. glabrata*, the intermediate host of *Schistosoma mansoni*. The three DNMTi types evaluated were: zebularine, flavanone compounds (Flv1, Flv2 and Flv-neg) (Pechalrieu et al. 2020) and bisubstrate analogue compounds (BA1 and BA-neg) (Halby et al. 2017). The toxicity was evaluated by measuring the effects in the

survival and fecundity of treated snails and in its non-treated progeny with the active DNMTi and its respective negative analogues.

I found that the three types of DNMTi displayed a different effect in the DNA methylation of the snail *B. glabrata*. Zebularine did not show a significant decrease of the global methylation in neither of the two generations, BA1 decrease significantly DNA methylation in F0 generation but displayed a tendency to increase DNA methylation in the F1 generation and Flv1 showed a significantly decrease of global DNA methylation in the two consecutive generations.

Zebularine did not significantly reduced overall 5mC level but showed a tendency to decrease in the exposed generation, this DNMT inhibitor was found to decrease global 5mC in the brain of the bee *Apis mellifera* (Biergans, Giovanni Galizia et al. 2015) where it was associated with the upregulation of memory-associated genes. Furthermore, zebularine induce the decrease of DNMT3 expression in the brain of *A. mellifera* (Lockett, Helliwell et al. 2010). A possible explanation of this contrasting results are the differences in the treatment administration, *A. mellifera* was treated topically on the thorax that is an efficient method for delivery to the brain and with a high concentration of zebularine (2 mM) and in my experiments, zebularine was administrated in water at a much lower concentration (10 µM) and the drug could be unstable in aqueous solution and the concentration could be insufficient to allow an hypomethylated global effect. The use of this drug in human cancer cells is usually done at high concentrations and continuous applications, e.g. continuous zebularine treatment at a concentration of 500 µM, induced efficient demethylation in T24 bladder carcinoma cells (Cheng, Weisenberger et al. 2004).

The presumably inactive bisubstrate derivative (BA-neg) caused a significant mortality, a significant decrease of the fecundity and a significant reduction of the 5mC level in the snails treated with this drug. These results indicate that bisubstrate analogue compounds (BA1 and BA-neg) have toxic effects in the snail. It has been demonstrated that DNA methylation level can be altered by toxic stress and exposure to environmental toxicants have shown to induce DNA methylation changes (Rondon et al. 2017, Reamon-Buettner, Mutschler, and Borlak 2008, Baccarelli and Bollati 2009). Moreover, the antiproliferative activity of the bisubstrate analogues was measured in five human cancer cell lines, showing a micromolar range cytotoxicity in all cell lines (Halby et al. 2017) meaning that cytotoxic effects in *B. glabrata* were not entirely unexpected. BA1 decreased the 5mC level in the F0 and increase the 5mC level in the F1 generation. The increased of the 5mC level in the non-treated progeny could be

a compensatory mechanism due to the toxic effect induced to the germline. Similar results were found in the oyster *Crassostrea gigas*, where an exposure to the herbicide diuron increase the DNA methylation level in the sperm of exposed genitors and in the offspring (Bachère, Barranger et al. 2017), DNA hypermethylation of exposed genitors sperm was transmitted to the offspring, diuron is a toxic pesticide that could induce a compensatory mechanism in the oyster's sperm global methylation.

It has been widely demonstrated that DNA methylation can be altered by toxic compounds, a review enlists several environmental chemicals such as metals, pollutants and endocrine-disrupting chemicals that are capable of modifying epigenetic marks (Bacarelli and Bollati, 2009), this is an important topic addressed in environmental toxicological studies, e.g. aquatic pollutants can influence DNA methylation level in invertebrate species such as the cladoceran *Daphnia magna* and the pacific oyster *C. gigas* and those changes can be inherited to subsequent generations (Vandegehuchte, Lemière et al. 2010, Rondon, Grunau et al. 2017). Moreover, some mollusks species such as the freshwater snail *Physa acuta* (Jeong, Patnaik et al. 2015, Bal, Kumar et al. 2017), are used as biomonitoring species to study chemical pollution in aquatic environments and DNA methylation changes in this mollusk and other species has been suggested to be used as a biomarker of environmental stress (Rey, Eizaguirre et al. 2020).

From the three types of compounds tested, the Flv1 showed the best efficiency as DNMT inhibitor in the snail, being a good candidate to pharmacologically reduce the DNA methylation without displaying toxicity, neither affect survival nor fecundity. Furthermore, a multigenerational effect was found to be induced by this compound, a differential hypomethylated region was found in common between F0 and F1 generation. Furthermore, a higher number of differential methylated regions was found in the offspring of the exposed generation. The most parsimonious explanation of this result, is that the germline was exposed to the Flv1 inhibitor when mature snails were exposed, since the enzyme DNMT1 is involved in DNA methylation maintenance during meiosis and embryonic development in other mollusk (e.g. *Patinopecten yessoensis*) (Li, Zhang et al. 2019), the DNMTi could impact this enzyme and induce changes in the development of the offspring which is reflected by the widening of the variance of morphometric shell traits observed in the F1 generation. Not only the morphometric shell variation was higher in the offspring, the hypomethylated global effect was also more pronounced in the offspring, which could impact the gametogenesis and the early development of the snails. These results are in agreement with the hypothesis that DNA

methylation plays an important role in mollusk development (Fallet, Luquet et al. 2020), e.g. DNA methylation is essential in the development of the oyster *C. gigas*, DNA methylation in proximal putative promoter may control the expression of homeobox genes, this cluster of genes are spatially and temporally expressed during embryonic development which is consistent with their changes of DNA methylation level observed across the stages of embryo development (Riviere, Wu et al. 2013).

Flv1 as a tool to study the inheritance of DNA methylation changes

Further analysis could allow to demonstrate a direct effect of the DNMTi Flv1 to the germline, by measuring directly the DNA methylation level of the germ cells (at least by dissecting the ovotestis) of a control snail and from a Flv1-treated one as previously done in the oyster *C. gigas*, where the global methylation level of the sperm of diuron-exposed oysters was measured (Bachère, Barranger et al. 2017). It could be also interesting to verify if epigenetic changes are inherited to a third generation to study if germline epigenetic marks are transgenerationally inherited. As I only analyzed two consecutive generations, I could not demonstrate a transgenerational effect. For that, it would have been necessary to do a multigenerational experiment with more than three generations, to analyze if the epimutations induced by the DNMTi treatments could be inherited until a generation that would not be directly exposed to the chemical treatment (Figure 3.1).

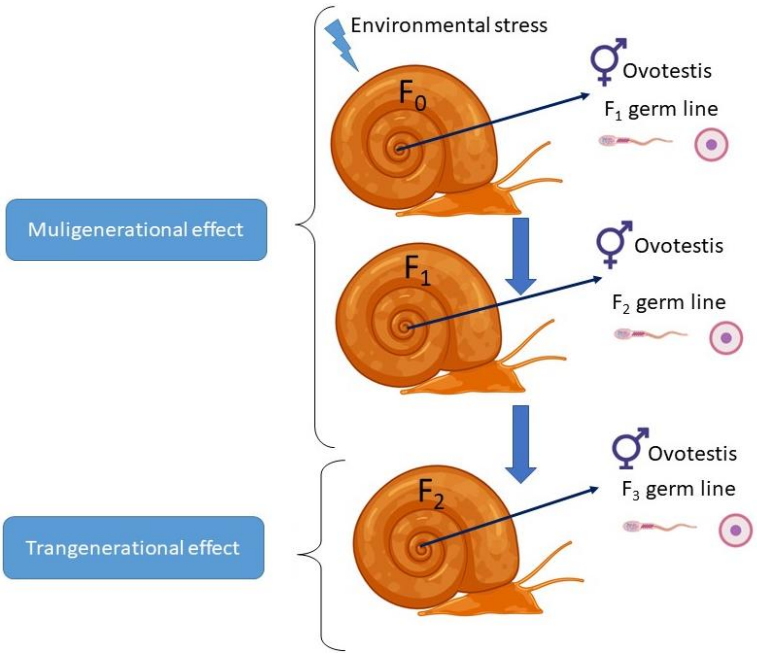

Figure 3.1. The freshwater mollusk *B. glabrata* possess an organ called ovotestis that contain the germline cells. Transmission of epigenetic marks caused by an environmental stress from F_0 to F_1 generation is considered a multigenerational effect because the germline can be exposed to the environmental stress, and if the transmission continues beyond F_2 generation, it is considered a transgenerational effect, since the F2 generation was not directly exposed to the environmental stress.

The study of the inheritance of epigenetic environmental marks through the germline has been very poorly explored in invertebrate species. This has been explored in vertebrates (Crews, Gore et al. 2007) and plants (Paszkowski and Grossniklaus 2011). Acquired epigenetic changes are seen with great scepticism due to the existence of epigenetic reprogramming in the germline in early mammal embryos (Hajkova 2011). However, evidence of germline epigenetic inheritance in mammals was demonstrated in mice, where the DNA methylation state of some genes escape epigenetic reprogramming in the germline, transferring an epigenetic signal to the offspring (Rakyan, Chong et al. 2003, Gapp and Bohacek 2018). In zebrafish it was also demonstrated that germline does not endure genome-wide erasure of DNA methylation during development, epigenetic marks are not completely erased and they can be inherited from parents to the progeny (Ortega-Recalde, Day et al. 2019), this shows that epigenetic reprogramming is not always present in vertebrates.

In the case of invertebrates, evidence of incomplete erasure of parental epigenetic marks during epigenetic reprogramming was found in *Drosophila*, where a mutation causing an increase of

DNA methylation in a gene promoter is transgenerationally inherited (Xing, Shi et al. 2007). Transgenerational epigenetic inheritance of longevity due to an incomplete reestablishment of the histone mark H3K4me3 during epigenetic reprogramming was found in *Caenohabditis elegans* (Greer, Maures et al. 2011). Nonetheless, more evidence of epigenetic marks transmission through the germline in invertebrates is lacking. Flv1 compound allowed me to induce a differential hypomethylated region in two subsequent generations. Since Flv1 demonstrated DNMT inhibitory efficiency in two other mollusk species, the oyster *Crassostrea gigas* and the freshwater snail *Physa acuta*, it would be now conceivable to study if a DNMTi induced epimutation can be transgenerationally inherited. Flv1 can be a tool to study epigenetic inheritance in mollusks to fill the gap that exists in the knowledge of the inheritance of DNA methylation marks in this phylum and probably other invertebrate groups.

DNA methylation and phenotypic variation

Another aspect I evaluated was if DNA methylation modification could induce phenotypic variability, and for that, I measured the morphometric shell traits of treated and control snails and from their respective offspring. I found that the compound Flv1 increased the variation of morphometric shell traits in the two generations analyzed. Hypomethylation effect induced by Flv1 was verified by high-throughput bisulfite sequencing in both generations, and *in vitro* DNMT inhibition activity of the Flv1 compound was verified in a nuclear extract from *Bge* cells, the embryonic cell line of the snail *B. glabrata*. These results suggested that the increase in phenotypic variability could be a consequence of the DNA methylation changes triggered by this Flv1. Variation in morphometric traits in treated snails indicated differences in growth, epimutations caused by Flv1 could provide a potential mechanism for inducing developmental phenotypic plasticity that acted also in the germline impacting the early developmental stages of the offspring resulting in higher morphometric differences.

Environmental stress can induce random modifications in DNA methylation patterns potentially resulting in the widening of phenotypic variance, therefore, DNA methylation is an important process even in the absence of inheritance because it could allow individuals to produce alternative phenotypes in response to environmental change (Angers, Castonguay et al. 2010). Random modification in DNA methylation were induced with the Flv1 inhibitor but surprisingly, a decrease of the DNA methylation variability in treated snails was observed compared to controls. The significant global DNA methylation decrease could be due to the

repression, induced by the demethylation on the gene body, of a gene coding for a SAM-dependent methyltransferase that uses the methyl donor S-adenosyl-L-methionine (SAM) as a cofactor to methylate numerous biomolecules (Struck, Thompson et al. 2012). The downregulation of this gene could produce a negative feedback loop that decreased the 5mC level at multiple loci by influencing SAM homeostasis, that altered global methylation in the exposed generation and its germline which induced a larger demethylation effect in the offspring. Furthermore, alteration of SAM homeostasis could have disturbed downstream cellular processes in both generations.

My results suggest that epimutations are a source of phenotypic variation that were induced by the DNMT inhibitor Flv1 that altered normal methylation control mechanisms, and this alteration could act on the soma and the germline, with phenotypic expression in the form of phenotypic variance increased in the exposed generation and its offspring.

On the other hand, I cannot confirm that DNA methylation changes are the only cause of the phenotypic effects observed, and an influence of genetic variability of the snail cannot be completely excluded. The snails used in our treatments were selected randomly from the same breeding stock and show very little genetic diversity, but they were not inbreed lines, and it has been demonstrated that even when the snails have been reared during many years in a laboratory they continue to present concomitant genetic variation (Carvalho, Caldeira et al. 2001). An approach to disentangle genetic and epigenetic contribution to the phenotypic plasticity could be to reduce genetic variability by elaborating inbred lines and combine this with the use of DNMTi. A similar approach exists in plants, the epigenetic recombinant inbred lines (epiRILs) allow increasing genetic similarity by self-fertilization and introducing epigenetic variation by crossing this inbred lines with a mutant for the *DDM1* gene, coding for an ATPase chromatin that is involved in the maintenance of DNA methylation, *DDM1* mutants present a reduced overall DNA methylation of 70% (Kakutani, Jeddeloh et al. 1995).

EpiRILs have been elaborated to study the DNA methylation contribution to the phenotypic variability of *A. thaliana* (Johannes, Porcher et al. 2009). The epiRILs permit a detailed assessment of the long-term molecular and phenotypic consequences of stable DNA methylation changes (Johannes, Porcher et al. 2009, Johannes and Colomé-Tatché 2011). The heritability of DNA methylation variation observed in a trait-specific manner in epiRILs of *A. thaliana* suggests that plants may have developed mechanisms for using epigenomic disturbance events that avoid the negative effects of inbreeding depression (Roux, Colomé-Tatché et al. 2011).

The epiRILs approach from plants could be borrowed to elaborate recombinant inbreed lines of the snail *B. glabrata* by reproducing the snail in self-fertilization and combined this with the use of DNMTi or knock out mutants with global DNA methylation changes to introduce epigenetic changes and evaluate if the epimutations induced are segregated in self-fertilization lines.

Nevertheless, gene editing *in vivo* is still under development in our biological model *B. glabrata* (Famakinde 2018, Coelho, Rodpai et al. 2020), and for the moment, the only suitable way to modify its DNA methylation level is the use of DNMT inhibitors. Consequently, I elaborated a multigenerational experiment, by crossing first two snails with divergent global methylation levels (hypomethylated and hypermethylated) that are offspring of the snails exposed to Flv1 and BA1 DNMT inhibitors, these couples were named epi-lines and four couple control snails were done as control lines. The offspring of this crossing was then reproduced by self-fertilization, the snails obtained by self-fertilization from the epi-lines were named epigenetic recombinant inbred lines (epiRILs) and the snails obtained by self-fertilization from control lines were named recombinant inbred lines (RILs). When the snails of the generation F2 were isolated for their reproduction by self-fertilization, some of them did not reproduce until 3 months later, this period is known as "delayed selfing" that consisted in a waiting time, due to the preference of the snail for outcrossing, but in the absence of a snail mate the snail will wait for a period of time and finally reproduce by selfing as a reproductive assurance strategy (Tsitrone, Duperron et al. 2003). Therefore, one of the difficulties with this approach is that to have recombinant inbred snail lines, it can take a long time in this species because in each generation the "delayed selfing period" will be present.

In the phenotypic results of this experiment, the fecundity mean of all outcrossed individuals was significantly higher than that of all selfed individuals, similar to the results found in *Biomphalaria camerunensis* (Kengne-Fokam, Nana-Djeunga et al. 2016); suggesting an inbreeding depression. Nevertheless, a high variability in fecundity was observed among epiRILs, some of them reproduced highly in self-fertilization and some other did not reproduce at all. These results suggested that the inbreeding depression effect was not present in all individuals and that some of them, on the contrary, presented a better fitness. Beneficial effects that resulted from crossing related individuals is a phenomenon that has been observed in mouse (Nebert, Gálvez-Peralta et al. 2010), where inbreeding of a knockout mouse line resulted in healthier individuals compared with the outcrossing generation which suggested that inbreeding can lead not only to depression but also to an improved phenotype (Nebert,

Gálvez-Peralta et al. 2010). It could be interesting to continue the self-fertilization of the snails to analyse for how many generations the improved fecundity is present, because it could be a temporary improvement such as that observed in *Physa acuta* inbred lines, where it has been found that phenotypic variance is higher under selfing and that it improves the response to selection at the beginning but then it becomes less responsive to selection (Noël, Jarne et al. 2017).

Another phenotype of interest in the snail is its compatibility with the parasite *Schistosoma mansoni*. Previous studies have suggested that compatibility is, in part, under epigenetic control (Perrin, Lepesant et al. 2013, Fneich, Théron et al. 2016) and I wondered whether or not the epiRILs of *B. glabrata* could displayed phenotypic plasticity when exposed to the parasite *S. mansoni*, for that I measured the prevalence and intensity of infection of control lines and epi-lines. The phenotypic infection results showed that the controls snails displayed homogeneous prevalence of infection (~80%) and similar intensities of infection (2.03±0.39), on the other hand, some epiRILs showed significant reduced prevalence of infection (until 20%) and more variability in the prevalence and intensity of infection, some epiRILs showed significant decreased of the intensity of infection compared to control RILs. These results suggested that probably the crossing of divergent epigenomes generate a gradient of DNA methylation in the offspring and that some DNA methylation changes could be segregated in the self-fertilization lines, allowing to display phenotypic plasticity in the prevalence and intensity of infection. This hypothesis remains speculative since the epi-sequencing (genome and epigenome) of the snails was not done but it is one of the main perspectives of this thesis. However, as these changes were observed after only two generations and that the phenotypic variability was only present in epiRILs and not in RILs, this suggested that these phenotypic changes were rather due to epigenetic alterations than genetic ones. This multigenerational experiment will allow to analyze if a biparental crossing and a subsequent self-fertilization decrease genetic variability in the snail, by analyzing the single nucleotide polymorphisms of the fourth generation (F3) and it will be also possible to analyze whether some epimutations were segregated and inherited from F1 to F3 generation by analyzing DNA methylation polymorphisms, both analysis will be done with epiGBS sequencing output.

DNA methylation enzymes and chromatin interactions

Histone modifications and DNA methylation are linked to influence gene expression in mammals, both marks modulate gene expression during the development (Cedar and Bergman 2009). In the case of invertebrates, evidence of a relationship between these two epigenetic marks has been found in the fruit fly *Drosophila melanogaster*, where DNA hypermethylation induce structural chromosome aberrations (Weissmann, Muyrers-Chen et al. 2003) and in the ant *Camponotus floridanus*, where DNA methylation was found to be highly associated with the histone post-transcriptional modification H3K4me3 (Glastad, Hunt et al. 2015). Further evidence was found in the honeybee *A. mellifera* and the ant *Solenopsis invicta*, where *"DNA methylation was targeted to genes marked by active histone modification"* (Hunt, Glastad et al. 2013).

DNMTi could be used to understand the interplay between these two epigenetic marks. There is an example in *Daphnia magna*, where the treatment with a 5-AzaC inhibitor induced changes in DNA methylation of the offspring which induced also changes in the histone modifications (H3K4me3 and H3K27me3) and in the gene expression (Lindeman, Thaulow et al. 2019), these results indicated that exposure of mature adults to 5-AzaC caused more pronounced effects on the early development of the offspring similar to the results we found with the hypomethylation global effect in the offspring of the snails *B. glabrata* treated with the inhibitor Flv1. Pharmacological modification of DNA methylation in *B. glabrata* could contribute to studying the interaction between gene body methylation and histone modification by treating snails with the DNMT inhibitor Flv1 and then measuring DNA methylation changes by sequencing the methylome with epiGBS or WGBS and histone modification by sequencing the chromatin with ATAQ-seq or CHIP-seq.

In animals and plants, intragenic DNA methylation is associated with euchromatin, the lightly condensed form of chromatin that contains regions of DNA that are transcriptionally active (Yang, Han et al. 2014, Bewick and Schmitz 2017) and, in contrast, promoter methylation is associated to heterochromatin, the condensed form of chromatin that is inactive for transcription (Klein and Costa 1997, Murakami 2013). In invertebrates, DNA methylation is predominantly found in an intragenic context, and the patterns of genomic DNA methylation are very different from the patterns found in vertebrates (Sarda, Zeng et al. 2012). Vertebrate genomes are heavily methylated in most developmental phases (Ehrlich, Gama-Sosa et al. 1982) while invertebrate genomes displayed low levels of DNA methylation (Suzuki and Bird 2008).These differences suggest that the DNA methylation machinery between both groups

could be also different; especially the enzyme readers that convey DNA methylation into chromatin structure and the enzyme writers that add the methyl group to the cytosine (C. Grunau personal communication).

The methylation readers MBD2 and MBD3 are the only vertebrate methyl-CpG binding proteins for which the homologue MBD2/3 can be identified in invertebrate genomes, suggesting that MBD2/3 represents the original methyl-CpG binding protein (Hendrich and Tweedie 2003). DNMTs and MBD2/3 have been found in the snail *B. glabrata* for sharing homology at the catalytic domains with the vertebrate enzymes (DNMT1, DNMT2, MBD2 and MBD3) (Fneich et al. 2013, Geyer et al. 2017). Probably, there is a specific DNA methylation machinery in mollusks, which have different functions to the homologues found in mammals, an additional characterization of these enzymes would allow us to better determine the DNA methylation function and its relationship with histone modifications.

Further work is needed to be able to purify and characterize the DNMTs and MBDs enzymes of mollusks. There exist techniques that allow the characterization of enzymes based in the use of DNMT inhibitors that bind to DNMTs and can be fused to chemical probes for visualization and purification (M. Lopez personal communication). DNMTs and MBD characterization in non-invertebrate models could reveal insights into the diversity and function of the epigenetic machinery across invertebrate species and to find key differences compared to the vertebrate's enzymes that could allow to understand better the evolution of this epigenetic mark from invertebrates to vertebrates.

Intragenic DNA methylation and gene expression in invertebrates

In invertebrates, gene body methylation (GBM) may regulate alternative splicing that increases protein diversity (Flores, Wolschin et al. 2012). GBM has been found to be associated with exon skipping and alternative splicing in the ants *Camponotus floridanus* and *Harpegnathos saltator* and methylated genes showed monoallelic expression (Bonasio, Li et al. 2012). In the honeybee *Apis mellifera*, methylated genes are enriched for alternative splicing, DNA methylation may regulate the production of splice variants by inducing exon insertion during transcription which results in longer genes (Flores, Wolschin et al. 2012), this was further analyze by inducing a knock down of the enzyme DNMT3 by RNA interference which showed that the global DNA methylation is reduced and produced changes in exon skipping and intron retention (Li-Byarlay, Li et al. 2013) and another work support the link

between GBM and alternative splicing due to the finding of differential methylation levels in genes belonging to the histone gene family (Lyko, Foret et al. 2010). In the ant *Solenopsis invicta* similar DNA methylation was found between individuals with the same ploidy (diploid and haploid) even when they were morphologically dissimilar due to caste differentiation suggesting that GBM may be involved in ploidy compensation. (Glastad, Hunt et al. 2014).

In the anemone *Nematostella vectensis* and the silkworm *Bombyx mori* GBM is positive correlated with gene expression (Xiang, Zhu et al. 2010, Zemach, McDaniel et al. 2010), in *Ciona intestinalis*, CpG methylation is principally located in transcription units of houskeeping genes perhaps to avoid transcriptional noise (Suzuki, Kerr et al. 2007). In the case of *A. mellifera* there is no correlation, instead, moderately transcribed genes are more methylated than poorly and highly expressed genes (Zemach, McDaniel et al. 2010). In the oyster *C. gigas*, a relationship was found between gene function and GBM, genes with housekeeping functions are highly methylated and genes involved in inducible functions are less methylated (Gavery & Roberts, 2010; Roberts and Gavery, 2012). It has been suggested that high methylation of housekeeping is a homeostatic mechanism which may prevent transcriptional plasticity in critical genes while low methylation in inducible genes facilitates environmentally responsive expression by increasing transcriptional plasticity (Gavery & Roberts, 2010; Roberts and Gavery, 2012).

In *Daphnia magna* and *D. pulex*, a negative correlation was found between GBM and gene family size, the very low level or absence of methylation in large gene families involved in response to environmental stress suggests that GBM could control gene family diversification stimulating phenotypic variation (Asselman, De Coninck et al. 2016). To further recognize if GBM can affect transcription and then phenotype, further studies are needed to identify the role of GBM in gene expression, which remains elusive.

Targeted gene body methylation changes in the snail *Biomphalaria glabrata*

The use of DNMT inhibitors is not suitable for identifying a causal relationship between gene body methylation and gene expression due to the inability to target the chemical inhibitory effect to a particular genomic region or locus. A better approach to study the causes of GBM in gene expression is to introduce targeted DNA methylation changes at the gene bodies with the use of epigenetic engineering tools, this approach could allow to directly measure the

impact of gene body methylation level in the gene expression of targeted genes. Recently, the use of dCas9, a mutant of the nuclease Cas9 with the nuclease activity deactivated was engineered to be able to target a sequence without causing a double stranded break and allowing the binding of writing or eraser enzymes involved in DNA methylation to a desired locus (Pulecio, Verma et al. 2017). There are epigenetic engineering tools that are capable of induce DNA methylation changes at a particular locus, these tools are available for human cell cultures and mice model (Oka, Rodić et al. 2006, Vojta, Dobrinić et al. 2016, Huang, Su et al. 2017, Lei, Zhang et al. 2017, Holtzman and Gersbach 2018). I tested a vector system called dCas9-SunTag-DNMT3A that showed efficiently targeted DNA methylation in the promoter of a *Hox* gene in a human cell line (Huang, Su et al. 2017) and I applied this tool to find whether it was also effective in eliciting a targeted DNA methylation *in vivo* in the snail *B. glabrata*, a non-model invertebrate species.

I found that the two plasmids coding for the system dCas9-SunTag-DNMT3A system were expressed in the embryos of the snail *B. glabrata*. This plasmids contain the promoter SV40, this promoter was previously shown to be expressed in other mollusc species, e.g. lipofection-mediated transfection of the oyster *C. gigas* heart primary cell cultures was achieved under transcriptional control of cytomegalovirus, CMV, and SV40 promoters (Buchanan, Nickens et al. 2001). I examined the plasmids expression during the embryo development by visualizing the fluorescence of the reporter genes BFP and GFP, this allowed me to identify that the plasmids were expressed transiently during 5 days, I recognized that this period was the time window to effectuate the target methylation.

Initially, after cotransfection with the two plasmids and the sgRNA, I did not achieve a targeted DNA methylation change. I reasoned that the co-transfection of two plasmids coding for the system dCas9-SunTag-DNMT3A and the sgRNA, did not achieve the methylation at the target loci due to the possibility that the sgRNA get degraded before the proteins encoded in the plasmids get expressed. The gRNA cannot be added to a vector since we ignore the RNA polymerase type in our model *B. glabrata*. Consequently, I decided to perform two microinjections, one with the plasmids coding for dCas9-SunTag-BFP and DNMT3A-GFP and another 72 hrs later (when plasmids get expressed) with the sgRNA. For this, screening of the embryos that showed the fluorescence of the reporter genes was necessary before the second microinjection with the sgRNA.

The double microinjection strategy allowed me to demonstrate that the system dCas9-Suntag-DNMT3A permitted the targeted methylation of a *homeobox* gene *in vivo* in the snail *B.*

glabrata. The transfected organism presented a mosaic integration of the plasmids which induced different effects in the targeted methylation, the four snails methylated showed a heterogeneous CpG methylation percentage (CpG%) with three snails showing one highly methylated CpG site (12-76%) and in one snail displaying a moderately methylated level (~4%) but in up to 5 CpG sites within 458 bp. The reasons of this high heterogeneity can be due to the dissimilar integration of vector constructions to the transfected cells, transfection is done at the embryo yolk and not in the embryo itself, and probably the penetration of the vector to the embryo cells occurs at different times.

In vivo transfection challenges

There are three options for the *in vivo* nucleic acid delivery in target tissues or cells: the viral vectors, the physical transfection methods, and the chemical-based nanoparticles (Yin, Kanasty et al. 2014). Viral vectors are highly efficient to target specific tissues or cells but the inconvenient is that it is essential to have a suitable biosafety environment to manipulate them and they are prohibitively expensive to produce for non-model species. An easier and cost-effective alternative is the use of chemical-based nanoparticles that encapsulate plasmid vectors to introduce them to target tissues or cells. These polymer-based reagents are used to transfect tissues *in vivo* in mice model, displaying high efficiency. I tested one of these reagents (*in vivo* jetPEI) in different embryo stages of the snail *B. glabrata*, and I found high mortalities on the embryos stages before gastrula stage probably due to a toxic effect of the transfection reagent , and is until gastrula stage microinjection that I observed that the vectors were expressed at the ectodermal layer, probably the cells before gastrula stage are more difficult to transfect, during embryo development, from one cell to blastula stages, the embryo has a continuous sheet of undifferentiated cells and it is until gastrulation that the cells begin to differentiate in cell lineages and this differentiated cells are probably more permissive to transfection.

The transfection at a multicellular stage induced the mosaic integration of the targeted DNA methylation modifications. To avoid this, it would be necessary to transfect at an earlier stage, at one-cell stage if possible, but instead of microinjecting the plasmid DNA and sgRNA, it would be necessary to find a non-invasive method to avoid the mortality of the embryos. One possible method that deserves further optimization is the *in vivo* electroporation since we only tested the parameters used in mice model. This technique could allow the transfection of

multiple embryos at the same time, which is suitable for high throughput screening, this technique has already led to successful results in other invertebrate models such as the oyster *C. gigas*, where the PiggyBac Transposon was transfected successfully through *in vivo* electroporation (Chen, Wu et al. 2018).

More optimization is required also in evaluate the on target and off-targeted effects of the epigenome editing tools, I did not evaluate the off-targeted effects, and this cannot be completely discarded. Nevertheless, sequence of the whole genome (WGBS) is very expensive and it could be better to use bioinformatics prediction tools to evaluate the sgRNA off-targets to then sequence the possible amplicons that could be also impacted by the targeted methylation changes by bisulfite amplicon sequencing, a technique that we showed is feasible in the snail *B. glabrata*.

The development of transfection and epigenome engineering tools can be used to investigate the causal relationship between gene body methylation (GBM) and gene expression. The snails with targeted CpG methylation changes can be used for a dual extraction of DNA and RNA to verify targeted DNA methylation and measure changes in transcription levels and splicing by RNA-Seq providing in this way insights about the function of GBM in a mollusk genome. Furthermore, elucidating the role of GBM in *B. glabrata* a non-model invertebrate, could help to understand its evolutionary divergence between vertebrates and invertebrates.

A multi-scale approach to study the role of DNA methylation in phenotypic variation and gene expression in a mollusk species

The three approaches presented in this thesis could be combined to offer a multi-scale approach to explore the role of DNA methylation in the snail *B. glabrata* or another mollusk or invertebrate species.

First, inbred lines should be generated by reproducing the snails in self-fertilization to decrease genetic diversity, then the snails with low genetic variability (verified by single nucleotide polymorphisme analysis), can be treated with the DNMT inhibitor Flv1 and continue in self-fertilization during at least 3 more generations. After that, comparisons between the phenotype of treated and control snails across generations could be done, and epigenotyping sequencing could allow us to compare also their genetic and DNA methylation polymorphisms, by considering genetic relatedness and epigenetic differences, we could use a

multiple-matrix approach (Thomson, Winney et al. 2018) to evaluate the contribution of genetic and epigenetic information to the observed phenotypic variability. Later, if differential methylated regions are identified as possible candidate regions altering a phenotypic trait, this could be further evaluated by an epigenetic engineering approach. Once the candidate genes altering a phenotype through their changes of DNA methylation are identified, a targeted DNA methylation or demethylation could be induced in the GBM of candidate genes to verify if DNA methylation changes have a direct impact in their gene expression, moreover, histone modifications could be also sequenced, to evaluate the relationship between GBM and chromatin structure. With the use of all these approaches it can now be anticipated to unambiguously answer the question if there is a causal relationship between DNA methylation, chromatin structure, gene expression and phenotypic plasticity.

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Résumé de thèse en français

Contexte de la recherche

I. La plasticité phénotypique

La variation phénotypique est la variation d'un trait dans une population quelle que soit la cause (génétique ou environnementale). La plasticité phénotypique est la capacité d'un génotype à exprimer différents phénotypes sous l'influence de l'environnement (Pigliucci 2001). Elle peut être adaptative ou non adaptative. Dans le cas d'une plasticité phénotypique adaptative, cette dernière peut influencer la valeur adaptative (ou « fitness ») des individus, contribuant ainsi à l'évolution des organismes (Grenier et al 2016). L'évaluation de la variabilité phénotypique est cruciale pour étudier les conséquences écologiques et évolutives du caractère adaptatif de la plasticité phénotypique (Coleman et al. 1994). De nouvelles preuves montrent que certains traits possèdent une plasticité phénotypique pouvant apporter un avantage face à la sélection naturelle (Robinson et Wilson, 1996, Svanbäck et Eklöv 2006). Il existe de nombreuses formes de plasticité phénotypique, elle peut être par exemple induite par les proies, les prédateurs, les parasite ou dépendre du polyphénisme des individus (Stearns 2006).

Sur le plan génétique, la plasticité phénotypique est attendue comme conséquence des différences dans l'expression des allèles dans différents environnements et par des fluctuations des interactions entre les loci (Scheiner, 1993). La présence d'une variation génétique pour un phénotype et la force de sélection agissant sur ce phénotype peuvent favoriser une réponse évolutive. La génétique quantitative étudie la manière dont les phénotypes sont affectés par l'action de nombreux gènes (Hill 2010). La détection de nombreux marqueurs moléculaires, l'accès au séquençage génomique et l'amélioration des approches statistiques telles que la cartographie des loci de caractères quantitatifs (LCQs) ont transformé le domaine de la génétique quantitative. Les LCQs peuvent être identifiés grâce à des marqueurs génétiques, par exemple le polymorphisme d'un seul nucléotide (PSN) ou encore les insertions ou délétions de quelques nucléotides et les microsatellites influençant un caractère quantitatif (Mackay 2009).

La génétique des populations comprend la composition génétique des individus et l'hérédité des gènes à travers les générations. La constitution génétique d'une population est désignée par le pourcentage d'individus appartenant à un génotype particulier. Ces proportions sont appelées *fréquences génotypiques* et peuvent changer par des processus tels que la migration, la mutation, la recombinaison et la sélection. Les mutations génétiques augmentent

progressivement la variabilité génétique dans des environnements fluctuants, les nouvelles mutations pouvant contribuer à l'adaptation (Carja et al 2014). Si l'étude de la génétique des populations a été utile pour comprendre la manière dont la diversité génétique peut être contrôlée par l'environnement, les mécanismes provoquant des réponses rapides à des environnements complexes sont encore inconnus. Si les réponses aux changements environnementaux sont contrôlées en partie au niveau moléculaire, d'autres mécanismes non génétiques peuvent néanmoins hypothétiquement être impliqués et induire des variantes phénotypiques. L'acquisition récente de nouvelles connaissances en biologie moléculaire ainsi que les progrès du séquençage massif ont généré des preuves qui suggèrent que la séquence d'ADN seule ne peut totalement expliquer la plasticité phénotypique adaptative observée dans des environnements fluctuants (Keller 2014).

II. Théorie étendue de l'évolution

La théorie synthétique de l'évolution est la théorie évolutionniste actuelle pour expliquer l'origine de la diversité biologique. Les découvertes récentes de nouveaux phénomènes suggèrent néanmoins que tout n'est pas expliqué par cette théorie. La théorie synthétique de l'évolution se concentre principalement sur la variation génétique issue de mutations aléatoires, l'hérédité par l'ADN et la sélection naturelle des gènes comme la seule cause d'adaptation (Orr 2005). Les sciences biologiques ont largement progressé entrainant l'apparition de nouveaux domaines de recherche tels que l'écologie moléculaire et la biologie des systèmes. De plus, notre compréhension de l'évolution s'est considérablement étendue, donnant naissance à la théorie étendue de l'évolution qui prend en compte les découvertes les plus récentes issues de l'héritabilité inclusive, de la plasticité développementale, de la génomique et d'autres domaines de recherche (Laland et al 2015). Une des différences entre la synthèse moderne et la théorie étendue de l'évolution est la prise en compte de l'héritabilité inclusive dans cette dernière. L'hérédité inclusive inclus l'hérédité non génétique ainsi que d'autres mécanismes par lesquels la descendance ressemble à ses parents. L'hérédité épigénétique, écologique et culturelle sont pris en compte dans l'hérédité, la fondation et la dispersion des phénotypes induits par l'environnement. L'hypothèse principale de l'hérédité inclusive est que les caractéristiques acquises peuvent être transmises aux générations suivantes et impliquées dans des mécanismes évolutifs.

Des preuves récentes démontrent que l'hérédité génétique et non génétique peuvent participer à l'hérédité des phénotypes. L'hérédité non génétique comprend les effets parentaux, l'hérédité écologique, l'hérédité culturelle et l'hérédité épigénétique (Danchin et al. 2011).

Les effets parentaux se réfèrent à l'impact que l'environnement parental induit sur le phénotype de leur descendance et qui sont totalement indépendants du propre génotype de la descendance. Ils peuvent survenir lorsque l'expression des gènes des progéniteurs se transforme en une composante environnementale perturbant le développement de la descendance. Par exemple, chez *Drosophila melanogaster*, l'ARN messager d'origine maternelle s'accumule dans l'œuf et régule l'ovogenèse et le développement précoce de l'embryon (Barckmann et Simonelig 2013).

L'hérédité écologique est la transmission de la construction de niche à la descendance. La construction de niche consiste à des altérations écologiques environnementales effectuées par les organismes. Les niches sont modifiées par les organismes à travers leur métabolisme, leur comportement et leurs actions. Il existe ainsi de nombreux exemples d'espèces animales qui construisent des nids, des trous, des ruches, des termitières, etc. (Laland et al 2009).

L'hérédité culturelle est la transmission d'une génération à l'autre d'informations qui incluent le comportement, les coutumes sociales, la langue, etc. L'hérédité culturelle est transmise, par exemple, par la communication, l'instruction, l'imitation, l'apprentissage social. L'information culturelle peut être héritée et peut évoluer au cours du temps. De plus, l'hérédité culturelle peut augmenter la valeur sélective d'un individu, permettant ainsi aux organismes de s'adapter et de transmettre un comportement adaptatif à la descendance et permettant une adaptation plus rapide aux changements environnementaux que l'hérédité génétique seule (Dachin et al 2011).

III. L'information épigénétique et l'hérédité épigénétique

L'information épigénétique consiste en des changements dans l'activité des gènes qui n'impliquent aucune modification de la séquence d'ADN. Les modifications épigénétiques sont réversibles et peuvent avoir lieu dans les cellules à la suite d'un changement environnemental. Elles sont transmises par voie mitotique et certaines d'entre elles par la voie méiotique (Nicoglou et Merlin 2017). Les modifications épigénétiques sont des différences de niveau de marques biochimiques présentes sur les histones, les protéines qui maintiennent l'ADN compact. Elles comprennent également les marques, par exemple un groupe méthyle qui se lie à certains nucléotides tels que la cytosine ou l'adénine. Ces changements sont appelés des épimutations, et il a été montré que le taux d'épimutations est significativement plus élevé que les mutations génétiques (Horsthemke 2006). Une classification des épimutations est fournie par Roquis et al. 2016 qui les divisent en trois catégories : épimutations induites, aléatoires et dépendantes du génotype. Les épimutations induites sont déclenchées par une condition

environnementale. Les épimutations aléatoires sont spontanées et indépendantes du génotype ou de l'environnement et les épimutations dépendantes du génotype sont totalement dépendantes du génotype et indépendantes des conditions environnementales (Roquis, Rognon et al.2016). La possible indépendance des épimutations par rapport à leur origine génotypique ainsi que la persistance et la stabilité des marques épigénétiques constituent un système d'hérédité qui opère à la frontière de l'information génétique et des mécanismes moléculaires (Eichten, Swanson-Wagner et al.2011, Aliaga, Bulla et al.2019).

L'hérédité épigénétique est la transmission de marques épigénétiques des parents à la descendance. Ces marques épigénétiques proviennent d'un stimulus environnemental initial et peuvent se stabiliser au fil du temps (Nicoglou et Merlin 2017). Il existe deux classes de transmission de marques épigénétiques entre différentes générations appelées effets épigénétiques intergénérationnels : effets multigénérationnels et transgénérationnels (Skinner 2008).

L'effet multigénérationnel se produit lorsque l'effet générationnel résulte d'une exposition directe des cellules germinales, des gamètes ou des embryons à un stress environnemental qui induit un changement épigénétique (Fallet et al. 2020). Par exemple, chez l'huître *Crassostrea gigas*, une exposition à un polluant chimique affecte le méthylome de la progéniture (Rondon et al. 2017).

Les effets transgénérationnels, sont les effets transmis sur plus de trois générations consécutives et qui ne sont pas liés à une exposition directe à un stress environnemental sur l'organisme affecté. Ainsi la longévité des descendants de la deuxième et troisième génération de *Caenorhabditis elegans* est, par exemple, stimulée par des changements dans l'histone modificateur de la chromatine H lysine 4 triméthylé (H3K4me3) de la génération parentale (Greer et al. 2011).

Les marques épigénétiques sont transmises des cellules mères aux cellules filles pendant la mitose mais le potentiel de transfert des marques épigénétiques d'une génération à une autre restent au cœur des débats en raison de l'existence d'une réinitialisation de l'épigénome pendant la gamétogenèse (Doerfler et Böhm 2006). L'hérédité des modifications épigénétiques via la lignée germinale implique de s'émanciper de la réinitialisation d'une génération à l'autre. En effet, si l'hérédité épigénétique trangénérationnelle a été démontrée dans des modèles invertébrés tels que *Drosophila melanogaster* (Wang et al. 2013) et *C. elegans* (Kishimoto et

al. 2017), elle n'a pas été suffisamment étudiée dans d'autres groupes d'invertébrés tels que les mollusques.

Les modifications épigénétiques peuvent potentiellement produire des variations phénotypiques qui sont transmises aux générations suivantes de cellules ou d'organismes bien que la variation génétique soit reconnue comme indispensable pour les variantes phénotypiques héréditaires. En effet, certains phénotypes adaptatifs hérités ne peuvent pas être expliqués par des mutations génétiques sporadiques, et nécessite un support héréditaire transmis sur de longues périodes. Certaines hypothèses suggèrent que les populations peuvent s'adapter à l'environnement grâce à des variation épigénétique qui apparaissent avant les mutations génétiques (Thorson, Smithson et al. 2017). Cette hypothèse suggère ainsi des phénotypes adaptatifs peuvent émerger rapidement sous l'influence de la sélection naturelle agissant aussi bien sur la variation épigénétique que génétique (Vogt 2018). Ainsi, l'épigénétique représente une extension de la théorie synthétique de l'évolution en reconnaissant la possibilité d'un nouveau système de variation phénotypique transgénérationnelle pouvant émerger rapidement et être héréditaire dans des situations particulières et pour certains traits acquis (Jablonka 2013). L'évolution basée sur la seule variation génétique est difficile à comprendre dans les scénarios où les espèces sont confrontées à des changements environnementaux rapides tels que le changement climatique (Hoffmann et Sgrò 2011). La variation épigénétique et génétique peut contribuer à la spéciation, phénomène s'exerçant lorsque deux populations sont déconnectées géographiquement, puisqu'elles collectent des marques épigénétiques différentes qui peuvent être suivies par la fixation de mutations d'ADN (Pál et Miklós 1999). Récemment, un nouveau cadre conceptuel utilisant des approches de la biologie des systèmes pour concilier l'hérédité génétique et épigénétique a été développé (Cosseau, Wolkenhauer et al.2017).

Il existe des preuves que la variation héréditaire des caractères écologiquement importants peut être façonnée par des mécanismes épigénétiques, même en l'absence de variation génétique, en plus des changements épigénétiques qui peuvent être hérités de manière stable au fil des générations (Johannes, Porcher et al.2009). En outre, les processus épigénétiques peuvent éventuellement fournir un système d'hérédité alternatif, la variation épigénétique, qui, contrairement à la variation génétique, est provoquée directement par les pressions environnementales et pourrait par conséquent offrir un moyen rapide et réversible de changement évolutif (Bossdorf, Richards et al. 2008, Cosseau, Wolkenhauer et al.2017).

IV. DNA methylation

La méthylation de l'ADN est un mécanisme par lequel le groupe méthyle (CH_3) peut se lier aux nucléotides de la cytosine ou de l'adénine, la méthylation de l'adénine est la liaison d'un groupe méthyle à la position N (azote) -6 de l'adénine et la méthylation de la cytosine consiste en l'ajout d'un groupe méthyle à la position C (carbone) -5 de la cytosine. Dans certains eucaryotes multicellulaires, la méthylation de l'ADN existe sur les cytosines dans un contexte CpG et varie considérablement entre les taxons. Chez les animaux, les niveaux de méthylation de l'ADN sont aussi très variables. Elle peut ainsi aussi bien être absente, comme chez le nématode *Caenorhabditis elegans* dont le génome ne code pas pour une ADN méthyltransférase, ou être présente à un niveau très bas, comme chez *Drosophila melanogaster*, qui présente un niveau très faible de méthylation de l'ADN (Zemach, McDaniel et al.2010)

De nombreux invertébrés présentent un modèle de méthylation de l'ADN de type mosaïque consistant en de larges domaines d'ADN méthylé séparés par de larges domaines d'ADN non méthylé (Hendrich et Tweedie 2003). La méthylation intragénique est un attribut général des génomes d'invertébrés pour lesquels la méthylation des transposons (TE) et des éléments répétitifs n'est que modérée. Chez les invertébrés, la méthylation intragénique de l'ADN pourrait être liée au niveau de transcription (le plus méthylé, le plus exprimé) mais son rôle reste une question ouverte. Une corrélation intéressante avec l'épissage alternatif et l'évolution du génome a été rapportée (Rivière 2014).

La méthylation de l'ADN chez les invertébrés se trouve principalement dans les corps géniques (introns et exons). Ce type de méthylation est appelé méthylation du corps génique (GBM) et est consensuellement considéré comme la forme ancestrale de l'ADN (Suzuki, Kerr et al.2007, Feng, Cokus et al.2010). Le GBM est observé chez les invertébrés, les vertébrés et les plantes. La fonction de la méthylation du corps du gène sur l'expression génique est différente de celle de la méthylation du promoteur qui est associée à la répression de l'expression génique. Le GBM est lié pour sa part à la transcription active chez les animaux (Zemach, McDaniel et al.2010, Sarda, Zeng et al. 2012). Chez les invertébrés, la méthylation de l'ADN est moins étudiée que chez les vertébrés bien que certaines études n'aient montré une association entre le niveau de transcription et le GBM (Rivière 2014), et suggéré un rôle de la méthylation intragénique dans l'épissage alternatif (Lyko, Foret et al.2010). Les effets fonctionnels de la méthylation intragénique de l'ADN ne sont néanmoins qu'à peine abordés dans de nombreux groupes d'invertébrés et notamment chez les mollusques.

V. Espèces de mollusques utilisés comme modèles biologiques

1. L'escargot d'eau douce *Biomphalaria glabrata*

Biomphalaria glabrata est un mollusque gastéropode de la famille Planorbidae qui est présent des Grandes Antilles au Brésil, aux Petites Antilles et jusqu'au Venezuela (Paraense 2001, Mavárez, Steiner et al. 2002). *B. glabrata* est l'hôte intermédiaire du parasite trématode *Schistosoma mansoni*, l'agent pathogène causal schistosomiase, une maladie tropicale négligée (Toledo et Fried 2010). En tant qu'hôte intermédiaire, *B. glabrata* a un rôle principal dans la transmission de la schistosomiase. Actuellement, la méthode la plus utilisée pour lutter contre l'escargot est l'utilisation de molluscicides, mais ils sont très toxiques pour les organismes aquatiques et n'ont qu'une efficacité limitée car les escargots deviennent rapidement résistants (Souza 1995). Une approche alternative pour lutter contre la maladie consisterait à contrôler le mollusque (Geyer et al.2017). Le parasite *S. mansoni* a un cycle de vie complexe avec deux hôtes obligatoires : un hôte intermédiaire où il se multiplie de manière asexuée (*B. glabrata),* et l'hôte définitif où il effectue la reproduction sexuée pouvant être aussi bien un humain ou un rongeur (Nelwan, 2019).

Les escargots *B. glabrata* présentent des différences dans leur compatibilité avec le parasite *S. mansoni*, certains sont résistants à l'infection tandis que d'autres sont sensibles. Ce phénomène est dû à un polymorphisme de compatibilité : les populations d'escargots et de parasites sont soit compatibles (l'infection est possible) soit incompatibles, avec différents degrés de compatibilité entre les deux (Theron, Rognon et al.2014, Mitta, Gourbal et al.2016). Chez un escargot compatible, la larve miracidium est capable de pénétrer dans le pied l'escargot et de déclencher l'infection, au cours de laquelle elle se développe en un sporocyste primaire. En revanche, un escargot résistant encapsulera le sporocyste par les hémocytes et détruira le parasite par une réaction cytotoxique impliquant des radicaux libres (Hahn, Bender et al. 2001).

L'étude des bases moléculaires des phénotypes de sensibilité et de résistance chez les escargots pourrait ouvrir des alternatives prometteuses aux mesures de contrôle actuelles de la schistosomiase. Traditionnellement, les mutations génétiques étaient considérées comme la seule base des variantes phénotypiques héréditaires. Les loci d'incompatibilité/résistance des escargots ont été cartographiés, mais ils semblent dépendre de la souche. On sait aujourd'hui que des phénotypes héréditaires peuvent être produits sans modification du génotype et que derrière ces variations se trouvent des mécanismes épigénétiques. La contribution relative des

composants génétiques et épigénétiques de la compatibilité de *B. glabrata* au parasite fait l'objet d'un actuel débat académique (Knight, Ittiprasert et al.2016, Sullivan 2018).

2. L'escargot d'eau douce *Physa acuta*

Physa acuta est un escargot pulmonaire aquatique originaire d'Amérique du Nord. C'est une espèce invasive qui se distribue sur divers continents, probablement due à la dispersion accidentelle médiée par le transport de plantes exotiques (Vinarski 2017). Cet escargot est un organisme de biosurveillance en raison de sa sensibilité aux contaminants métalliques (Spyra, Cieplok et al.2019).

L'un des aspects non génétiques qui a commencé à être étudié chez *P. acuta* est la réactivité environnementale de sa méthylation d'ADN. Il a été démontré que *P. acuta* présentait des changements de méthylation de l'ADN en réponse à l'exposition à des polluants chimiques environnementaux tels que les glucocorticoïdes (Bal, Kumar et al.2017) et le fongicide vinclozoline (Müller, Charaf et al.2016, Sánchez-Argüello, Aparicio et al.2016). De plus, ces polluants chimiques modifient non seulement leur méthylation de l'ADN, mais ils déclenchent également des effets phénotypiques, par exemple, des changements dans la structure de la coquille et la taille des escargots exposés. Des mesures de méthylation de l'ADN ont été rapportées chez *P. acuta* dans les trois études mentionnées précédemment (Muler et al.2016, Sanchez-Arguello et al.2016 et Bal et al.2017). Ces études permettent de déduire que la méthylation d'ADN dans un contexte CpG est présente dans son génome. Le méthylome n'a pas été séquencé, mais la présence de 5mC a été détectée par une méthode basée sur l'immunodosage quantitatif.

3. L'huître marine *Crassostrea gigas*

L'huître du Pacifique *C. gigas* est une espèce estuarienne et peut également habiter dans les zones marines intertidales. Elle a une large tolérance à la salinité de 20 à 25 ‰ et peut vivre à des températures comprises entre -1,8 et 35 ° C. En raison de son développement rapide et de sa tolérance aux fluctuations environnementales, *C. gigas* est devenue l'huître de choix pour l'ostréiculture dans diverses régions du monde. L'espèce est très féconde avec des femelles de grande longueur (15 cm) produisant 50 à 200 millions d'œufs en un seul frai. Néanmoins, *C. gigas* souffre de nombreuses maladies avec des taux de mortalité élevés. L'une de ces maladies est actuellement étudiée au laboratoire IHPE : le syndrome de mortalité des huîtres du Pacifique (POMS) qui affecte les juvéniles et qui a une forte incidence dans les régions littorales de nombreux pays, principalement la France. Elle est provoquée par le virus OsHV-1, qui induit

une immunodéficience chez l'huître conduisant à une dysbiose (désequilibre de la microbiote) et aboutissant finalement à une septicémie (de Lorgeril, Petton et al.2020).

La méthylation de l'ADN chez *C. gigas* est intragénique et présente des variations en fonction des contextes physiologiques. De plus, la méthylation de l'ADN influence l'expression des gènes et est essentielle au développement de l'huître (Riviere, Wu et al. 2013). Les trois enzymes impliquées dans l'établissement et le maintien de la méthylation de l'ADN (DNTM1, DNMT2 et DNMT3) sont présents dans l'huître *C. gigas* (Wang, Li et al. 2014).

Objectifs de la thèse

Il existe de nombreuses preuves que la plasticité phénotypique observée dans les organismes est due à l'interaction entre les informations génétiques et non génétiques. Néanmoins, pendant longtemps, seules les approches centrées sur les gènes ont été utilisées pour expliquer la plasticité phénotypique et l'adaptation des espèces. L'objectif de ma thèse est d'analyser si l'information non génétique a un impact sur la plasticité phénotypique et si celle-ci se transmet de génération en génération. La compréhension des mécanismes moléculaires permettant l'adaptation rapide des mollusques vecteurs de parasites à de nouveaux environnements est importante pour le contrôle des maladies. L'adaptation rapide est difficile à expliquer par la génétique mendélienne traditionnelle et il existe des preuves solides soutenant que les mécanismes épigénétiques sont à l'origine des adaptations rapides chez d'autres espèces.

Je me suis concentré sur une information non génétique particulière appelée l'épigénétique, qui consiste en une couche d'informations qui détermine la manière dont les gènes vont être exprimés par la cellule. Cette information consiste en des modifications biochimiques ne changeant pas les nucléotides de la séquence d'ADN et pouvant être transmis par mitose et parfois aussi par méiose. J'ai étudié une marque épigénétique nommée la méthylation de l'ADN qui est modulée par l'environnement et qui joue un rôle dans la plasticité phénotypique chez de nombreuses espèces, principalement les plantes et les vertébrés. Néanmoins, le rôle de la méthylation de l'ADN dans la génération de variations phénotypiques chez les invertébrés a été moins étudié.

Nous avons abordé la question du rôle de la méthylation de l'ADN dans la génération de la plasticité phénotypique et de son hérédité dans un modèle invertébré, l'escargot *Biomphalaria glabrata,* l'hôte intermédiaire du parasite *Schistosoma mansoni,* l'agent causal de la schistosomiase. La méthylation de l'ADN chez *B.glabrata* est régulée par l'infection du parasite *S.mansoni* (Knight, Ittiprasert et al.2016) et par le stress environnemental (Geyer, Niazi et al.2017). En outre, il a été démontré que la méthylation de l'ADN affecte son expression génique (Ittiprasert, Miller et al.2015), suggérant qu'elle peut ainsi affecter la variation phénotypique et possiblement l'adaptation de l'escargot à de nouveaux environnements.

Pour répondre à la question principale de cette thèse, une manipulation expérimentale de la méthylation de l'ADN chez l'escargot a été nécessaire afin d'induire des changements dans sa méthylation et d'en étudier les effets sur le phénotype.

Par conséquent, deux approches ont été proposées dans cette thèse pour introduire des épimutations chez l'escargot *B. glabrata* :

1) L'épimutagenèse aléatoire en utilisant des inhibiteurs chimiques des enzymes DNMTs et par ségrégation conséquente des épimutations dans des lignées d'autofécondation

2) Méthyler les cytosines d'un locus ciblé avec un outil d'ingénierie épigénétique ciblé consistant en la transfection des plasmides codant pour l'ADN méthyltranférase DNMT3 fusionnée au dCas9 (Cas9 avec l'activité nucléase désactivé).

Pour la première approche,

- Un inhibiteur efficace des ADN méthyltransférases (DNMTi) était nécessaire pour pouvoir modifier pharmacologiquement la méthylation de l'ADN des escargots. J'ai testé de nouveaux DNMTi basés sur ceux utilisés dans la recherche sur le cancer et qui ont été synthétisés par nos collaborateurs de l'Institut des Biomolécules Max Mousseron (IBMM) à Montpellier ; de l'Institut Pasteur à Paris, France et du Laboratoire ETaC CNRS-FRE3600 à Toulouse, France.

- Pour tester si le nouveau DNMTi, efficace chez des cultures de cellules humaines, était également efficace chez l'escargot *B. glabrata*, une méthode pour mesurer le niveau global de 5 méthylcytosine (5mC) dans nombreux échantillons et à un faible coût a été optimisée. Cette méthode a été comparé ensuite à un kit commercial de détection de 5mC du type ELISA.

- Une fois la méthode pour mesurer le niveau global de 5mC optimisée et un DNMTi efficace trouvé, une méthode rentable qui permet d'explorer les régions génomiques impactées par les traitements DNMTi était essentielle. J'ai testé la méthode de séquençage épigénotypique epiGBS pour la première fois chez une espèce de mollusque et l'ai comparée au WGBS pour évaluer la fiabilité de la méthode pour l'identification des régions différentiellement méthylées à la suite d'une manipulation pharmacologique de la méthylation de l'ADN.

Pour la deuxième approche,

- L'optimisation d'une technique de transfection a été réalisée pour pouvoir introduire des épimutations ciblées sur l'embryon de l'escargot *B. glabrata*.

• Un outil d'ingénierie épigénétique validé dans une lignée de cellules cancéreuses humaines a été testé *in vivo* sur l'embryon de l'escargot *B. glabrata*.

Résultats principaux et discussion du Chapitre I.

Inhibition pharmacologique de la méthylation de l'ADN pour étudier la contribution de cette marque épigénétique à la variation phénotypique d'une espèce de mollusque

La nécessité d'un inhibiteur de DNMT efficace et non toxique

Le rôle de la méthylation de l'ADN dans la plasticité phénotypique a été démontré chez de nombreuses espèces, principalement chez les plantes et les vertébrés (Johannes, Porcher et al. 2009, Jones 2012) et ce sujet est actuellement étudié chez certaines espèces d'invertébrés. Il existe par exemple des preuves du rôle de la méthylation de l'ADN dans la production de plasticité développementale chez l'abeille *Apis mellifera*, e. g. les changements de la méthylation de l'ADN sont impliqués dans la différenciation des castes (Elango, Hunt et al. 2009, Lyko, Foret et al. 2010) et dans la régulation de l'état de reproduction (Kucharski, Maleszka et al. 2008). De plus, chez la fourmi *Camponotus floridanus*, la métylation de l'ADN contribue à la variation de taille des fourmis ouvrières en régulant la transcription du récepteur du facteur de croissance épidermique (Alvarado, Rajakumar et al. 2015).

La méthylation de l'ADN est également impliquée dans la plasticité comportementale. Par exemple, chez le bourdon *Bombus terrestris*, il existe des différences dans l'état de méthylation de l'ADN entre les travailleurs reproducteurs et non reproducteurs. Un traitement avec l'inhibiteur de DNMT 5-aza-2'-désoxycytidine a d'ailleurs montré que le conflit reproductif parmi les travailleurs augmentait chez les abeilles juvéniles traitées avec le DNMTi (Amarasinghe, Clayton et al. 2014). Chez l'abeille Apis mellifera, la réversibilité de la transition de l'abeille nourrice à la butineuse est associée à des changements réversibles de méthylation de l'ADN (Herb, Wolschin et al.2012). Chez d'autres invertébrés, il existe des preuves du rôle de la méthylation de l'ADN dans le développement. Elle joue par exemple un rôle majeur dans le développement précoce du mollusque *Crassostrea gigas* (Riviere, Wu et al. 2013). De plus, des changements de méthylation de l'ADN ont été trouvés en réponse à des conditions environnementales changeantes comme c'est par exemple le cas chez *Daphnia magna* pour laquelle la méthylation de l'ADN est altérée par

la prédation, les changements de régime alimentaire et les altérations de la salinité (Asselman, De Coninck et al. 2015).

Une des limites de l'étude de la méthylation de l'ADN chez les invertébrés est la faible disponibilité des outils moléculaires qui permettent la manipulation expérimentale de la méthylation de l'ADN dans les organismes non modèles. L'un de ces outils sont les inhibiteurs de DNMT (DNMTi), largement utilisés dans les modèles de vertébrés en particulier les mammifères, chez certaines espèces végétales et chez un plus petit nombre d'espèces d'invertébrés (cf. introduction du chapitre I). Bien que l'utilisation de DNMTi puisse générer d'éventuels effets secondaires indésirables, il s'agit de l'outil existant le plus accessible pour la manipulation expérimentale de la méthylation de l'ADN pour les espèces non modèles en l'absence de mutants knock-out avec une méthylation de l'ADN modifiée (Verhoeven, von Holdt et al.2016).

Actuellement, l'utilisation du DNMTi chez les invertébrés repose sur trois composés disponibles dans le commerce qui sont très efficaces chez les vertébrés mais présentent une toxicité élevée chez les invertébrés et certaines espèces de vertébrés (Ueno, Katayama et al.2002). Ces composés sont les analogues de la cytidine 5 azacytidine, 2 '-désoxy-5-azacytidine et zébularine. Des progrès récents ont été réalisés dans la synthèse d'inhibiteurs de DNMTs de nouvelle génération qui sont moins toxiques, plus spécifiques et plus stables dans les solutions aqueuses. Ces nouveaux composés sont des analogues non cytidiniques qui ne s'incorporent pas à l'ADN pour être actifs, ce qui induit une moindre toxicité (Gros, Fahy et al.2012, Erdmann, Halby et al.2015). Malgré ces améliorations, l'efficacité inhibitrice in vivo de ces nouveaux composés dans un modèle d'invertébré et l'héritabilité des changements de méthylation de l'ADN induits n'avaient pas été testées avant ces travaux.

Validation de deux méthodes pour mesurer la méthylation de l'ADN chez une espèce de mollusque

Afin d'identifier des inhibiteurs de DNMT efficaces, j'ai validé deux méthodes rentables et à haut débit pour mesurer la méthylation de l'ADN, l'une au niveau global du génome et l'autre à une résolution nucléotidique, pour une utilisation dans une espèce de mollusque. La première est une méthode à base d'anticorps appelée dot blot qui est basée sur la reconnaissance de 5mC par un anticorps anti-5mC. Ce test a été utilisé pour mesurer les

changements globaux de 5 mC dans plusieurs échantillons à un prix inférieur par rapport au kit commercial du type ELISA utilisant le même principe. Ensuite, j'ai utilisé la méthode épigénotypage par séquençage (epiGBS), une méthode de séquençage de nouvelle génération, pour déterminer les changements de méthylation de l'ADN à la résolution nucléotidique dans un pourcentage du génome. Comparé au séquençage bisulfite du génome complet (WGBS), il permet de séquencer plusieurs échantillons à un prix beaucoup plus bas et est donc plus approprié pour des études de population. EpiGBS est une technique adaptée aux espèces non modèles qui a été largement utilisée dans les espèces végétales où elle a montré une haute fiabilité et faisabilité de séquencer la méthylation de l'ADN. Les plantes présentent un modèle de méthylation « mosaïque » comme de nombreuses espèces d'invertébrés. EpiGBS a déjà été utilisé pour séquencer le méthylome du crustacé *Daphnia magna*, montrant qu'il s'agit d'une méthode efficace pour détecter la méthylation de l'ADN chez une espèce d'invertébrés (van Gurp et al.2016). De plus, les avantages de l'epiGBS sont qu'il est rentable par rapport à RRBS et WGBS, il est plus adapté aux génomes méthylés en mosaïque en raison de la flexibilité du choix des enzymes de restriction, il permet d'obtenir des polymorphismes de méthylation de l'ADN et des polymorphismes génétiques à partir des mêmes échantillons. De plus, l'amélioration récente du pipeline rend accessible l'analyse de la méthylation de l'ADN et des polymorphismes mononucléotidiques (Gawehns, Postuma et al.2020). Pour ces raisons, nous avons décidé de tester si epiGBS pouvait être une méthode efficace pour séquencer l'épigénome et le génome des escargots *B. glabrata* traités avec le DNMTi.

Une comparaison de la profondeur de séquençage entre epiGBS et WGBS a été effectuée pour évaluer le pourcentage d'informations de méthylation d'ADN obtenues dans epiGBS et pour évaluer la corrélation entre les deux méthodes. J'ai trouvé que l'epiGBS permettait d'obtenir jusqu'à 4% de la profondeur de séquençage obtenue avec WGBS. De plus, les valeurs de méthylation du corps génique (GBM) obtenues par les deux méthodes étaient fortement corrélées et la distribution des quantiles de GBM était également similaire. D'autres optimisations de la technique sont possibles, telles que l'utilisation d'autres combinaisons d'enzymes de restriction, et des analyses de digestions *in silico* peuvent être effectuées pour anticiper quelles régions génomiques vont être séquencées, permettant l'amélioration de la détection des régions différentiellement méthylées qui en sont une des difficultés d'epiGBS (Paun, Verhoeven et al.2019). Néanmoins, cette méthode a permis la

détection de cytosines différentielment méthylées différentielles (DMCs) entre les contrôles et les escargots traités au DNMTi.

Modification pharmacologique de la méthylation de l'ADN chez *B. glabrata*

J'ai évalué s'il était possible de moduler la méthylation de l'ADN avec des inhibiteurs chimiques de DNMTs (DNMTi) de nouvelle génération. L'efficacité inhibitrice et la toxicité de 3 types de DNMTi ont été évaluées chez l'escargot d'eau douce *B. glabrata*, l'hôte intermédiaire de *Schistosoma mansoni*. Les trois types de DNMTi évalués étaient : la zébularine, les composés de nitroflavanone (Pechalrieu et al.2020) et les composés analogues de bisubstrate (Halby et al.2017). La toxicité a été évaluée en mesurant les effets sur la survie et la fécondité des escargots traités au DNMTi et de sa descendance.

J'ai trouvé que les trois types de DNMTi présentaient un effet différent dans la méthylation de l'ADN de l'escargot *B. glabrata*. La zébularine n'a montré une diminution significative de la méthylation globale dans aucune des deux générations, BA1 a diminué de manière significative la méthylation de l'ADN dans la génération F0 mais a montré une tendance à augmenter la méthylation de l'ADN dans la génération F1 et Flv1 a montré une diminution significative de la méthylation de l'ADN global dans les deux générations consécutives.

La zébularine n'a pas réduit de manière significative le niveau global de 5 mC mais a montré une tendance à diminuer dans la génération exposée, cet inhibiteur de DNMT a diminué le niveau global de 5mC dans le cerveau de l'abeille *Apis mellifera* (Biergans, Giovanni Galizia et al.2015) où il était associé à la régulation positive des gènes associés à la mémoire. De plus, la zébularine a induit la diminution de l'expression du DNMT3 dans le cerveau de cette abeille (Lockett, Helliwell et al.2010). Une explication possible de ces résultats contrastés sont les différences dans l'administration du traitement, *A. mellifera* a été traité par voie topique sur le thorax qui est une méthode efficace pour la libération au cerveau et avec une concentration élevée de zébularine (2 mM) et dans mes expériences, la zébularine a été administrée dans de l'eau à une concentration beaucoup plus faible (10 µM) et le composé pourrait être instable en solution aqueuse ainsi que la concentration pourrait être insuffisante pour permettre une hypométylation global. L'utilisation de la zébularine dans les cellules cancéreuses humaines est généralement effectuée à des concentrations élevées et avec des applications continues, par exemple, un traitement continu à la zébularine à une concentration de 500 µM a induit une déméthylation efficace dans les cellules de carcinome de la vessie T24 (Cheng, Weisenberger et al. 2004).

Le composé bisubstrat inactif (BA-neg) a provoqué une mortalité significative, une diminution significative de la fécondité et une réduction significative du niveau de 5mC chez les escargots traités avec ce composé. Ces résultats indiquent que les composés bisubstrates analogues (BA1 et BA-neg) ont des effets toxiques chez l'escargot. Il a été démontré que l'exposition à des substances toxiques induit des changements de méthylation de l'ADN (Rondon et al. 2017, Reamon-Buettner, Mutschler, and Borlak 2008, Baccarelli and Bollati 2009). BA1 a diminué le niveau de 5mC dans le F0 et augmenté le niveau de 5mC dans la génération F1, l'augmentation du taux de 5mC chez la descendance non traitée pourrait être un mécanisme compensatoire en raison de l'effet toxique induit sur la lignée germinale. Des résultats similaires ont été trouvés chez l'huître *Crassostrea gigas*, où une exposition à l'herbicide diuron augmente le niveau de méthylation de l'ADN dans le sperme des géniteurs exposés et chez la progéniture (Bachère, Barranger et al.2017), une hyperméthylation de l'ADN du sperme des géniteurs exposés a été transmise à la progéniture, le diuron est un pesticide toxique qui pourrait induire un mécanisme compensatoire dans la méthylation globale du sperme de l'huître.

Il a été largement démontré que la méthylation de l'ADN peut être modifiée par des composés toxiques tels que les métaux, les polluants et les perturbateurs endocriniens (Bacarelli et Bollati, 2009), c'est un sujet traité dans les études toxicologiques environnementales. Les polluants aquatiques peuvent influencer le niveau de méthylation de l'ADN chez des espèces d'invertébrés telles que le cladocère *Daphnia magna* et l'huître du Pacifique *C. gigas* et ces changements peuvent être transmises aux générations suivantes (Vandegehuchte, Lemière et al.2010, Rondon, Grunau et al. 2017). De plus, certaines espèces de mollusques comme l'escargot d'eau douce *Physa acuta* (Jeong, Patnaik et al.2015, Bal, Kumar et al.2017), sont utilisées comme espèces de biosurveillance pour étudier la pollution chimique, la composition des métaux lourds et les facteurs de stress microbiens en milieu aquatique et il a été suggéré que les changements de méthylation de l'ADN chez ce mollusque et d'autres espèces soient utilisés comme biomarqueur du stress environnemental (Rey, Eizaguirre et al.2020).

Parmi les trois types de composés testés, le Flv1 a montré la meilleure efficacité en tant qu'inhibiteur de DNMT chez l'escargot. C'est un bon candidat pour réduire pharmacologiquement la méthylation de l'ADN car il n'a pas montré de toxicité, il n’a pas affecté la survie ni la fécondité. En outre, un effet multigénérationnel a été provoqué par ce composé, une région différentiellement hypométhylée a été trouvée en commun entre les

générations F0 et F1. De plus, un plus grand nombre de régions différentiellement méthylées a été trouvé chez la progéniture de la génération exposée. L'explication la plus parcimonieuse de ce résultat est que la lignée germinale a été exposée à l'inhibiteur Flv1 lorsque des escargots matures ont été exposés, puisque l'enzyme DNMT1 est impliquée dans le maintien de la méthylation de l'ADN pendant la méiose et le développement embryonnaire chez d'autres mollusques (par exemple, *Patinopecten yessoensis*) (Li, Zhang et al.2019), le DNMTi inhibe la DNMT1 (prouvé par une inhibition *in vitro*) et induit des changements dans le développement de la progéniture, qui se traduisent par l'élargissement de la variance des traits morphométriques de la coquille observée dans la génération F1. Non seulement la variation morphométrique de la coquille était plus élevée chez la progéniture, mais l'effet global hypométhylé était également plus prononcé chez la progéniture, ce qui pouvait avoir un impact sur la gamétogenèse et le développement précoce des escargots. Ces résultats sont en accord avec l'hypothèse selon laquelle la méthylation de l'ADN joue un rôle important dans le développement des mollusques (Fallet, Luquet et al.2020), par exemple, la méthylation de l'ADN est essentielle dans le développement de l'huître *C. gigas*, la méthylation de l'ADN dans le promoteur pourrait contrôler l'expression des gènes homéotiques, ce groupe de gènes est exprimé spatialement et temporellement au cours du développement embryonnaire ce qui est cohérent avec leurs changements de niveau de méthylation de l'ADN observées à travers les stades du développement embryonnaire (Riviere, Wu et al.2013).

Flv1 comme outil pour étudier l'hérédité des changements de méthylation de l'ADN

Une analyse plus approfondie pourrait permettre de démontrer un effet direct du DNMTi Flv1 sur la lignée germinale, en mesurant directement le niveau de méthylation de l'ADN des cellules germinales (en prélevant l'ovotestis) d'un escargot témoin et d'un escargot traité au Flv1 comme précédemment fait dans l'huître *C. gigas*, où le niveau global de méthylation du sperme d'huîtres exposées au diuron a été mesuré (Bachère, Barranger et al.2017).

Il pourrait également être intéressant de vérifier si les changements épigénétiques sont hérités à une troisième génération pour étudier si les marques épigénétiques germinales sont héritées de manière transgénérationnelle. Comme je n'ai analysé que deux générations consécutives, je n'ai pas pu démontrer un effet transgénérationnel. Pour cela, il aurait fallu faire une expérience multigénérationnelle sur plus de trois générations, pour analyser si les épimutations induites par les traitements DNMTi pouvaient être transmises jusqu'à une génération qui ne serait pas directement exposée au traitement chimique.

L'étude de l'hérédité des marques environnementales épigénétiques à travers la lignée germinale a été très mal explorée chez les espèces d'invertébrés. Cela a été exploré chez les vertébrés (Crews, Gore et al. 2007) et les plantes (Paszkowski et Grossniklaus 2011). Les changements épigénétiques acquis sont observés avec un grand scepticisme en raison de l'existence d'une reprogrammation épigénétique dans la lignée germinale des embryons de mammifères (Hajkova 2011). Cependant, des preuves d'hérédité épigénétique a travers la lignée germinale chez les mammifères ont été fournies chez la souris, où il a été constaté que l'état de méthylation de l'ADN de certains gènes échappait à la reprogrammation épigénétique dans la lignée germinale, transférant un signal épigénétique à la progéniture (Rakyan, Chong et al.2003, Gapp et Bohacek 2018). Chez le poisson zèbre, il a été démontré que les marques épigénétiques ne sont pas complètement effacées pendant le développement et elles peuvent être héritées des parents à la descendance (Ortega-Recalde, Day et al.2019).

Dans le cas des invertébrés, des preuves d'effacement incomplet des marques épigénétiques parentales lors de la reprogrammation épigénétique ont été trouvées chez Drosophila, où une mutation entraînant une augmentation de la méthylation de l'ADN dans un promoteur de gène était héréditaire (Xing, Shi et al.2007). L'hérédité épigénétique transgénérationnelle de la longévité due à un rétablissement incomplet de la marque d'histone H3K4me3 lors de la reprogrammation épigénétique a été trouvée chez *Caenohabditis elegans* (Greer, Maures et al. 2011). Néanmoins, il manque plus de preuves de transmission de marques épigénétiques à travers la lignée germinale chez les invertébrés. Le composé Flv1 a permis d'induire une région différentiellement hypométhylée dans deux générations consécutives. Puisque Flv1 a démontré une efficacité inhibitrice du DNMT chez deux autres espèces de mollusques, l'huître *Crassostrea gigas* et l'escargot d'eau douce *Physa acuta*, il serait maintenant envisageable d'étudier si une épimutation induite par le DNMTi peut être héritée de manière transgénérationnelle. Flv1 peut être un outil pour étudier l'hérédité épigénétique chez les mollusques et probablement d'autres espèces d'invertébré.

Méthylation de l'ADN et variation phénotypique

Un autre aspect que j'ai évalué était si la modification de la méthylation de l'ADN pouvait induire une variabilité phénotypique, et pour cela, j'ai mesuré les traits morphométriques de la coquille des escargots traités et témoins et de leurs descendants respectifs. J'ai trouvé que

le composé Flv1 augmentait la variation des traits morphométriques de la coquille dans les deux générations analysées. L'effet d'hypométhylation induit par Flv1 a été vérifié par séquençage bisulfite à haut débit dans les deux générations, et l'activité d'inhibition du DNMT *in vitro* du composé Flv1 a été vérifiée dans un extrait nucléaire de cellules *Bge*, la lignée cellulaire embryonnaire de l'escargot *B. glabrata*. Ces résultats suggèrent que l'augmentation de la variabilité phénotypique pourrait être une conséquence des changements de méthylation de l'ADN déclenchés par Flv1. La variation des traits morphométriques chez les escargots traités a indiqué des différences de croissance, les épimutations causées par Flv1 pourraient fournir un mécanisme potentiel pour induire une plasticité phénotypique développementale qui a agi également dans la lignée germinale.

Le stress environnemental peut induire une modification aléatoire des schémas de méthylation de l'ADN, ce qui pourrait entraîner l'élargissement de la variance phénotypique (Angers, Castonguay et al.2010). Des modifications aléatoires de la méthylation de l'ADN ont été induites avec l'inhibiteur Flv1, mais de manière surprenante, une diminution de la variabilité de la méthylation de l'ADN chez les escargots traités a été observée par rapport aux témoins. La diminution significative de la méthylation globale de l'ADN pourrait être due à la répression, induite par la déméthylation sur le corps du gène, d'un gène codant pour une méthyltransférase SAM-dépendante qui utilise le donneur S-adénosyl-L-méthionine (SAM) comme cofacteur pour méthyler de nombreuses biomolécules (Struck, Thompson et al. 2012). La régulation négative de ce gène pourrait produire une boucle de rétroaction négative qui a diminué le niveau de 5mC à plusieurs gènes en influençant l'homéostasie de SAM, qui a modifié la méthylation globale dans la génération exposée et sa lignée germinale, ce qui a induit un effet de déméthylation plus important chez la progéniture. En outre, l'altération de l'homéostasie SAM pourrait avoir perturbé les processus cellulaires dépendants de SAM dans les deux générations.

Je ne peux pas confirmer que les changements de méthylation de l'ADN sont la seule cause des effets phénotypiques observés, et la variabilité génétique de l'escargot ne peut être complètement exclue. Les escargots utilisés dans nos traitements ont été sélectionnés au hasard dans le même élevage mais ils n'étaient pas des lignées consanguines, et il a été démontré que même lorsque les escargots ont été élevés en laboratoire pendant de nombreuses années, ils montrent des variations génétiques (Carvalho, Caldeira et al. 2001). Une approche pour démêler la contribution génétique et épigénétique à la plasticité phénotypique pourrait être de réduire la variabilité génétique en élaborant des lignées

consanguines et de combiner cela avec l'utilisation de DNMTi. Cette approche existe déjà chez les plantes, les lignées consanguines épigénétiques recombinantes (epiRILs) consistent à augmenter la similitude génétique par autofécondation et à introduire une variation épigénétique en croisant ces lignées consanguines avec un mutant pour le gène DDM1, codant pour une chromatine ATPase impliquée dans le maintien de la méthylation de l'ADN, les mutants DDM1 présentent une diminution de 70% dans leur méthylation globale de l'ADN (Kakutani, Jeddeloh et al. 1995).

Les EpiRILs ont été élaborés pour étudier la contribution de la méthylation de l'ADN à la variabilité phénotypique chez *A. thaliana* (Johannes, Porcher et al. 2009). Les epiRILs permettent une évaluation détaillée des conséquences moléculaires et phénotypiques à long terme des changements stables de la méthylation de l'ADN (Johannes, Porcher et al. 2009, Johannes et Colomé-Tatché 2011). L'héritabilité de la variation de la méthylation de l'ADN et leurs implications dans certains phénotypes chez les epiRILs de *A. thaliana* suggère que les plantes peuvent avoir développé des mécanismes pour utiliser les événements de perturbation épigénomique afin d'éviter les effets négatifs de la dépression de consanguinité (Roux, Colomé-Tatché et al.2011). En m'inspirant de cette approche, j'ai élaboré une expérience multigénérationnelle, en croisant des escargots avec des niveaux de méthylation globaux divergents (hypométhylés et hyperméthylés) qui sont la progéniture des escargots exposés aux inhibiteurs de Flv1 et BA1, ces couples ont été nommés épi-lignées et quatre couples d'escargots témoins ont été croisés et utilisés comme les lignes témoins. La progéniture de ce croisement a ensuite été reproduite par autofécondation, les escargots obtenus par autofécondation à partir des épi-lignées ont été nommés lignées consanguines épigénétiques recombinantes (épiRIL) et les escargots obtenus par autofécondation à partir de lignées témoins ont été nommés lignées consanguines recombinantes. (RIL).

Un phénotype d'intérêt chez l'escargot *B. glabrata* est sa compatibilité avec le parasite *S. mansoni*. Des études antérieures ont suggéré que la compatibilité est, en partie, sous contrôle épigénétique (Perrin, Lepesant et al.2013, Fneich, Théron et al.2016) et je me suis demandé si les epiRILs de *B. glabrata* pouvaient ou non présenter une plasticité phénotypique lorsqu'ils étaient exposés au parasite *S. mansoni*, pour cela j'ai mesuré la prévalence et l'intensité de l'infestation des lignées témoins et des épi-lignées. Les résultats ont montré que les escargots témoins présentaient une prévalence d'infestation constante (~ 80%) et des intensités d'infestation similaires (2,03 ± 0,39), d'autre part, les epiRILs ont montré une prévalence d'infestation significativement réduite (jusqu'à 20%) et plus de variabilité dans

la prévalence et l'intensité de l'infestation, certains epiRILs ont montré une diminution significative de l'intensité de l'infestation par rapport aux lignées consanguines témoins. Ces résultats suggèrent que probablement le croisement d'épigénomes divergents génère un gradient de méthylation de l'ADN chez la progéniture et que certains changements de méthylation de l'ADN pourraient être séparés dans les lignées d'autofécondation, permettant d'afficher une plasticité phénotypique dans la prévalence et l'intensité de l'infestation. Cette hypothèse reste spéculative puisque l'épi-séquençage (génome et épigénome) des escargots n'a pas été faite mais c'est l'une des principales perspectives de cette thèse.

Enzymes de méthylation de l'ADN et interactions avec la chromatine

Les modifications des histones et la méthylation de l'ADN sont liées pour influencer l'expression des gènes chez les mammifères, les deux marques modulent l'expression des gènes au cours du développement (Cedar et Bergman 2009). Dans le cas des espèces d'invertébrés, le lien entre la méthylation de l'ADN et les modifications des histones a été moins étudié, des preuves d'une relation entre ces deux marques épigénétiques ont été trouvées chez *Drosophila melanogaster*, où l'hyperméthylation de l'ADN induit des aberrations chromosomiques structurelles (Weissmann, Muyrers-Chen et al.2003) et chez la fourmi *Camponotus floridanus*, où la méthylation de l'ADN était fortement associée à la modification post-transcriptionnelle de l'histone H3K4me3 (Glastad, Hunt et al. 2015).

L'inhibiteur de DNMT Flv1 pourrait être utilisé pour comprendre l'interaction entre ces deux marques épigénétiques. Il y a un exemple chez *Daphnia magna*, où le traitement avec un inhibiteur de 5-AzaC a induit des changements dans la méthylation de l'ADN de la progéniture ainsi que des changements dans les modifications des histones (H3K4me3 et H3K27me3) dans l'expression génique (Lindeman, Thaulow et al. 2019), ces résultats ont indiqué que l'exposition d'adultes matures au 5-AzaC provoquait des effets plus prononcés sur le développement précoce de la progéniture similaires aux résultats que nous avons trouvés avec l'effet global d'hypométhylation chez la progéniture des escargots *B. glabrata* traités avec l'inhibiteur Flv1. La modification pharmacologique de la méthylation de l'ADN chez *B. glabrata* pourrait contribuer à étudier l'interaction entre la méthylation du corps du gène et la modification des histones en traitant les escargots avec l'inhibiteur Flv1 puis en mesurant les changements de méthylation de l'ADN en séquençant le méthylome avec epiGBS ou WGBS et les modifications des histones en séquençant la chromatine avec ATAQ-seq ou CHIP-seq.

Chez les animaux et les plantes, la méthylation intragénique de l'ADN est associée à l'euchromatine, la forme décondensée de la chromatine qui contient des régions de l'ADN qui sont transcriptionnellement actives (Yang, Han et al.2014, Bewick et Schmitz 2017) et, en revanche, la méthylation du promoteur est associée à l'hétérochromatine, la forme condensée de la chromatine et inactive pour la transcription (Klein et Costa 1997, Murakami 2013). Chez les invertébrés, la méthylation de l'ADN se trouve principalement dans un contexte intragénique, et les modèles de méthylation de l'ADN génomique sont très différents de ceux trouvés chez les vertébrés (Sarda, Zeng et al. 2012). Les génomes des vertébrés sont fortement méthylés dans la plupart des phases de développement (Ehrlich, Gama-Sosa et al.1982) tandis que les génomes des invertébrés affichent de faibles niveaux de méthylation de l'ADN (Suzuki et Bird 2008). Ces différences suggèrent que la machinerie de méthylation de l'ADN entre les deux groupes peut également être différente ; en particulier les lecteurs d'enzymes qui transmettent la méthylation de l'ADN dans la structure de la chromatine et les enzymes qui ajoutent le groupe méthyle à la cytosine.

Des enzymes DNMTs et MBD2/3 ont été trouvés chez *B. glabrata* pour partager l'homologie au niveau des domaines catalytiques avec les enzymes des vertébrés (DNMT1, DNMT2, MBD2 et MBD3) (Fneich et al.2013, Geyer et al.2017). Cependant, l'ensemble de la structure et de la fonction des DNMT et des MBD chez les espèces de mollusques n'a pas été bien étudiée. Il existe probablement une machinerie de méthylation de l'ADN spécifique chez les mollusques, qui ont des fonctions différentes de celles des homologues trouvés chez les mammifères, mais une caractérisation supplémentaire de ces enzymes nous permettrait de mieux déterminer la fonction de méthylation de l'ADN et sa relation avec les modifications des histones. Des travaux supplémentaires sont nécessaires pour être en mesure de purifier et de caractériser les enzymes DNMT et MBD des mollusques et d'autres espèces d'invertébrés. Il existe des techniques qui permettent la caractérisation des enzymes basées sur l'utilisation d'inhibiteurs des DNMTs qui se lient aux DNMTs et peuvent être fusionnées à des sondes chimiques pour la visualisation et la purification (communication personnelle de M. Lopez). La caractérisation des DNMTs et MBDs dans un mollusque pourrait révéler des informations sur la diversité et la fonction de la machinerie épigénétique dans les espèces invertébrées et trouver des différences clés par rapport aux enzymes des vertébrés qui pourraient permettre de mieux comprendre l'évolution de cette marque épigénétique.

Résultats principaux et discussion Chapitre II.

Épimutagenèse ciblée chez l'escargot *Biomphalaria glabrata*

Méthylation intragénique de l'ADN et expression génique chez les invertébrés

Chez les invertébrés, la méthylation intragénique fait actuellement l'objet d'études et il a été constaté qu'elle est peut-être impliquée dans la régulation de l'épissage alternatif qui augmente la diversité des protéines (Flores, Wolschin et al.2012). Chez l'abeille *Apis mellifera*, les gènes méthylés sont enrichis pour un épissage alternatif, la méthylation de l'ADN peut réguler la production de variantes d'épissage en induisant l'insertion des exons lors de la transcription, ce qui entraîne des gènes plus longs (Flores, Wolschin et al.2012), cela a été analysé plus en détail en induisant un renversement de l'enzyme DNMT3 par ARN d'interférence qui a montré que la méthylation globale de l'ADN est réduite et produit des changements dans le saut d'exon et la rétention d'intron (Li-Byarlay, Li et al.2013).

Chez l'anémone *Nematostella vectensis* et le ver à soie *Bombyx mori* la méthylation intragénique est positivement corrélée avec l'expression des gènes (Xiang, Zhu et al.2010, Zemach, McDaniel et al.2010), chez *Ciona intestinalis*, la méthylation des CpG est principalement localisée dans les unités de transcription des gènes de ménage peut-être pour éviter le bruit transcriptionnel (Suzuki, Kerr et al. 2007). Dans le cas de l'abeille *A. mellifera*, il n'y a pas de corrélation, mais les gènes modérément transcrits sont plus méthylés que les gènes faiblement et fortement exprimés (Zemach, McDaniel et al. 2010). Chez l'huître du Pacifique *C. gigas*, une relation a été trouvée entre la fonction génique et la méthylation intragénique, les gènes avec des fonctions de ménage sont fortement méthylés et les gènes impliqués dans les fonctions inductibles sont moins méthylés (Gavery et Roberts, 2010 ; Roberts et Gavery, 2012). Il a été suggéré qu'une méthylation élevée des gènes de ménage est un mécanisme homéostatique qui peut empêcher la plasticité transcriptionnelle dans les gènes critiques tandis qu'une faible méthylation dans les gènes inductibles facilite l'expression sensible à l'environnement en augmentant la plasticité transcriptionnelle.

Chez *Daphnia magna* et *D. pulex*, une corrélation négative a été trouvée entre la méthylation intragénique et la taille de la famille de gènes, le niveau très faible ou l'absence de méthylation dans les familles de longs gènes impliquées dans la réponse au stress environnemental, suggère que la méthylation intragénique pourrait contrôler la

diversification des familles de gènes stimulant la variation phénotypique (Asselman, De Coninck et al.2016). Pour mieux reconnaître si la méthylation intragénique peut affecter la transcription des gènes et puis le phénotype, d'autres études sont nécessaires pour identifier le rôle de la méthylation intragénique dans l'expression génique, qui reste inconnu.

Changements ciblés de la méthylation intragénique chez l'escargot *Biomphalaria glabrata*

L'utilisation d'inhibiteurs de DNMTs ne convient pas pour identifier une relation causale entre la méthylation intragénique et l'expression du gène en raison de l'incapacité de cibler l'effet inhibiteur chimique sur une région ou un locus génomique particulier. Une meilleure approche pour étudier les causes de la méthylation intragénique dans l'expression génique consiste à introduire des changements ciblés de la méthylation intragénique à l'aide d'outils d'ingénierie épigénétique. Récemment, l'utilisation de dCas9, un mutant de la nucléase Cas9 avec l'activité nucléase désactivée a été conçue pour pouvoir cibler une séquence sans provoquer une coupure de l'ADN et permettre la liaison d'enzymes impliquées dans la méthylation de l'ADN à un locus souhaité. (Pulecio, Verma et al.2017). Il existe des outils d'ingénierie épigénétique capables d'induire des changements de la méthylation de l'ADN à un locus particulier, ces outils sont disponibles pour les cultures de cellules humaines et pour les espèces modèles tels que la souris (Oka, Rodić et al.2006, Vojta, Dobrinić et al.2016, Huang, Su et al.2017, Lei, Zhang et al.2017, Holtzman et Gersbach 2018). J'ai testé un système vectoriel appelé dCas9-SunTag-DNMT3A qui a montré une efficacité à méthyler le promoteur d'un gène Hox dans une lignée cellulaire humaine (Huang, Su et al.2017) et j'ai appliqué cet outil pour déterminer s'il était également efficace pour provoquer une méthylation ciblée de l'ADN *in vivo* chez l'escargot *B. glabrata*, une espèce invertébrée non modèle.

J'ai trouvé que les deux plasmides codant pour le système dCas9-SunTag-DNMT3A ont été exprimés dans les embryons de l'escargot *B. glabrata*. Ces plasmides contiennent le promoteur viral SV40, il a été précédemment montré que ce promoteur était exprimé dans d'autres espèces de mollusques, par exemple, la transfection médiée par lipofection des cultures de cellules primaires du cœur de l'huître *C. gigas* a été réalisée sous contrôle transcriptionnel de promoteurs SV40 et CMV (Buchanan, Nickens et al. 2001). J'ai examiné l'expression des plasmides pendant le développement de l'embryon en visualisant la fluorescence des gènes rapporteurs BFP et GFP, cela m'a permis d'identifier que les

plasmides étaient exprimés de manière transitoire pendant 5 jours, j'ai reconnu que cette période était la fenêtre de temps pour effectuer la méthylation ciblée.

Après une première co-transfection avec les deux plasmides et le guide ARN (ARNg), la méthylation ciblée de l'ADN n'a pas été achevé. L'ARNg s'est possiblement dégradé avant que les protéines codées dans les plasmides ne se soient exprimées. L'ARNg ne peut pas être ajouté à un vecteur car nous ignorons le type d'ARN polymérase dans notre modèle *B. glabrata*. Par conséquent, j'ai décidé d'effectuer deux micro-injections, l'une avec les plasmides codant pour dCas9-SunTag-BFP et DNMT3A-GFP et 72 heures plus tard (lorsque les plasmides sont exprimés) avec le gARN.

La stratégie de double microinjection m'a permis de démontrer que le système dCas9-SunTag-DNMT3A permettait la méthylation ciblée d'un gène homéobox *in vivo* chez l'escargot *B. glabrata*. Les escargots transfectés présentaient une intégration mosaïque des plasmides qui induisait des effets différents dans la méthylation ciblée, les quatre escargots méthylés présentaient un pourcentage de méthylation CpG hétérogène (CpG%) avec trois escargots montrant deux ou trois sites CpG hautement méthylés (4-76%) et un escargot présentant un niveau modérément méthylé (4-15%) mais dans 6 sites CpG. Les raisons de cette forte hétérogénéité peuvent être dues à des différences dans le nombre de cellules transfectées parmi les embryons.

Les défis de la transfection *in vivo*

Il existe trois options pour la délivrance d'acide nucléique *in vivo* dans les tissus ou cellules cibles : les vecteurs viraux, les méthodes de transfection physique et les nanoparticules. Les vecteurs viraux sont très efficaces pour cibler des tissus ou des cellules spécifiques, mais l'inconvénient est qu'il est essentiel de disposer d'un environnement de biosécurité approprié pour les manipuler et ils ont un coût prohibitif pour les espèces non modèles.

Une alternative plus simple et rentable est l'utilisation de nanoparticules chimiques qui encapsulent des vecteurs plasmidiques pour les introduire dans les tissus ou cellules cibles. Ce réactif à base de polymère est appliqué dans les tissus chez la souris où il affiche une efficacité élevée. Dans mon cas, j'ai testé ce réactif à différents stades embryonnaires de l'escargot *B. glabrata*, j'ai trouvé des mortalités élevées sur les stades embryonnaires avant le stade gastrula (au stade deux cellules, 8 cellules, 16 cellules et blastula), et après le stade gastrula, j'ai observé que les vecteurs étaient exprimés au niveau de la couche ectodermique. Avant le stade gastrula les cellules ont été plus difficiles à transfecter et ont montré une

sensibilité élevée à la toxicité du réactif, ce qui a été révélé par les pourcentages élevés de mortalité.

La transfection à un stade multicellulaire a induit l'intégration mosaïque des modifications de méthylation de l'ADN ciblées. Pour éviter cela, il serait nécessaire de transfecter à un stade plus précoce, au stade une cellule si possible, mais au lieu de micro-injecter l'ADN plasmidique et le sgRNA, il serait nécessaire de trouver une méthode non invasive pour éviter la mortalité des embryons. Une méthode possible qui mérite une optimisation supplémentaire est l'électroporation *in vivo* puisque nous n'avons testé que les paramètres utilisés chez la souris. Cette technique pourrait permettre la transfection de plusieurs embryons en même temps, de plus, cette technique a déjà conduit à des résultats positifs dans d'autres modèles d'invertébrés tels que l'huître *C. gigas*, où le transposon PiggyBac a été transfecté avec succès grâce à électroporation *in vivo* (Chen, Wu et al.2018)

Le développement d'outils de transfection et d'ingénierie de l'épigénome peut être utilisé pour étudier la relation causale entre la méthylation intragénique et l'expression des gènes. Les escargots avec des changements de méthylation CpG ciblés peuvent être utilisés pour une double extraction d'ADN et d'ARN pour vérifier la méthylation ciblée de l'ADN et mesurer les changements dans les niveaux de transcription et l'épissage par RNA-Seq, fournissant ainsi des informations sur la fonction du GBM dans le génome d'un mollusque. De plus, élucider le rôle de la méthylation intragénique chez *B. glabrata*, un invertébré non modèle, pourrait aider à comprendre sa divergence évolutive entre les vertébrés et les invertébrés.

Une approche multi-méthodes pour étudier le rôle de la méthylation de l'ADN dans la variation phénotypique et l'expression génique chez une espèce de mollusque

Les trois approches présentées dans cette thèse pourraient être combinées pour offrir une approche multi-méthodes pour explorer le rôle de la méthylation de l'ADN chez l'escargot *B. glabrata* ou une autre espèce de mollusque ou d'invertébré.

Premièrement, des lignées consanguines pourraient être générées en reproduisant les escargots en autofécondation pour diminuer la variabilité génétique, puis les escargots à faible variabilité génétique (vérifiée par analyse SNP), peuvent être traités avec l'inhibiteur de DNMT Flv1 et continuer sa reproduction en autofécondation pendant au moins 3 générations. Après cela, des comparaisons entre le phénotype des escargots traités et témoins à travers les générations pourraient être faites, et l'épigénotypage pourrait nous

permettre de comparer également leurs polymorphismes génétiques et de méthylation de l'ADN, en considérant la parenté génétique et les différences épigénétiques, nous pourrions utiliser des matrices de covariance (Thomson, Winney et al.2018) pour évaluer la contribution des informations génétiques et épigénétiques à la variabilité phénotypique observée. Une fois que les gènes candidats altérant un phénotype par leurs changements de méthylation de l'ADN sont identifiés, la méthylation ou la déméthylation ciblée de l'ADN pourrait être induite dans les gènes candidats pour vérifier si les changements de méthylation de l'ADN ont un impact direct sur leur expression génique. De plus, nous pourrions analyser l'association entre les changements de méthylation de l'ADN et les structures de la chromatine. Avec l'utilisation de toutes ces approches, nous pourrions répondre à la relation causale entre la méthylation de l'ADN, la structure de la chromatine, l'expression des gènes et la plasticité phénotypique.