Aquatic eDNA: beyond species presence

Zhao, B.

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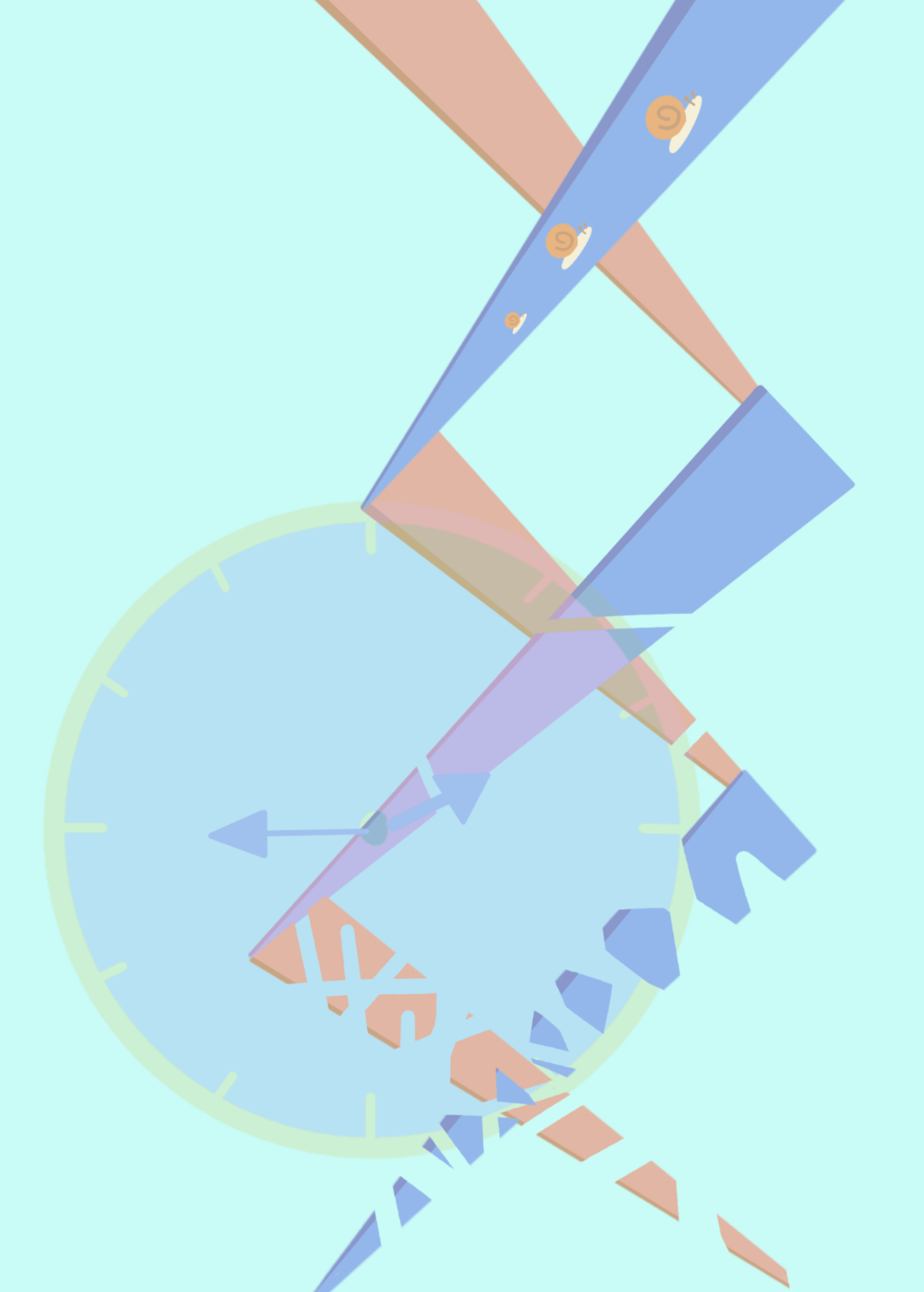

CHAPTER 4

Environmental DNA methylation of Lymnaea stagnalis varies with age and is hypermethylated compared to tissue DNA

*Beilun Zhao[a], Peter M. van Bodegom[a], Krijn Trimbos[a]

[a]Department of Environmental Biology, Institute of Environmental Sciences, Leiden University, Einsteinweg 2, 2333 CC Leiden, Netherlands

*Corresponding author: Beilun Zhao

E-mail: b.zhao@cml.leidenuniv.nl

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ABSTRACT

Environmental DNA (eDNA) approaches contributing to species identifications are quickly becoming the new norm in biomonitoring and ecosystem assessments. Yet, information such as age and health state of the population, which is vital to species biomonitoring, has not been accessible from eDNA. DNA methylation has the potential to provide such information on the state of a population. Here, we measured the methylation of eDNA along with tissue DNA (tDNA) of *Lymnaea stagnalis* at four life stages. We demonstrate that eDNA methylation varies with age and allows distinguishing among age classes. Moreover, eDNA was globally hypermethylated in comparison to tDNA. This difference was age-specific and connected to a limited number of eDNA sites. This differential methylation pattern suggests that eDNA release with age is partially regulated through DNA methylation. Our findings help to understand mechanisms involved in eDNA release and shows the potential of eDNA methylation analysis to assess age classes. Such age class assessments will encourage future eDNA studies to assess fundamental processes of population dynamics and functioning in ecology, biodiversity conservation and impact assessments.

KEYWORDS. Environmental DNA, Age Assessment, eDNA Methylation, Aquatic Biomonitoring, eDNA Release Mechanism

4.1 Introduction

Applications of environmental DNA (eDNA)-based methods have been rapidly increasing for surveying species and biodiversity over the last decade due to their proven non-invasiveness and cost-effectiveness [14,15,56,178]. However, to expand the applications of eDNA methods in biomonitoring, a deeper understanding of eDNA characteristics is crucial. For example, due to its characteristics such as the variation in decay rates [32,54,165], eDNA methods cannot distinguish between dead and living genetic material [100]. This complicates the assessment of the exact presence of the surveyed species at a specific location in space and time [127,179]. Additionally, by using eDNA methods, direct observations of the target organism are lost, and hence information on the state of the target species is not directly accessible. Many biomonitoring applications do, however, demand such information, for instance, to detect the health condition and age within the target population [127,179]. These limitations of the current eDNA methodology hamper the full deployment of eDNA applications in biomonitoring.

DNA methylation (DNAm) is the cell physiological process through which methyl groups are added to the DNA molecule. As one of the most important epigenetic modifications, DNAm plays an important role in a broad range of biological processes including but not limited to development, gene regulation and disease progression [115–118]. Methylation changes the activity of a DNA segment without changing the sequence [117]. Over the past decades, DNAm analysis has emerged as a promising approach in human medicine, to understand the mechanisms of tumorigenesis and provided biomarkers for early detection, diagnosis, and prognosis in cancer patients [116,118–120,180]. In addition, DNAm across a broad spectrum of tissues and cell types has been considered as an ageing clock due to its mechanistic role in ageing [181–183]. Measurement of changes across a few hundred specific CpG(5'-Cytosine-phosphate-Guanine-3') sites allowed predicting the accurate chronological age of individuals in a variety of species, such as humans, mice and bats [123–125].

While DNAm in multiple tissues and cells is increasingly understood, whether the same methylation pattern is also maintained in eDNA has remained unclear. Because eDNA originates from cellular material shed by organisms [32], from cells that are most likely not as active as those still in the body, eDNAm is possibly not consistent with that of tissue DNAm (tDNAm). However, considering the stability and slow change in methylation [115,128], such consistency in methylation patterns might very well exist. For example, the methylation pattern in cell-free DNA in the blood is consistent with their original cells or tissues [129]. Taking the strong functional connection between methylation and the state of the body into consideration, eDNAm has the potential to assess age, even though it differs from the tDNAm patterns.

eDNA from aquatic systems has been shown to represent a detailed picture of the community that lives in and around it, and has largely benefited biomonitoring, conservation and detection of rare and cryptic species over the last decade [56,70,184,185]. Information on the state of the population, such as its age, might be just as important for calculating population growth rates, and is essential to predict species survival as well as devising conservation strategies [127,179]. In this way, eDNAm analysis could potentially expand eDNA applications and hugely contribute to biomonitoring in a non-invasive and cost-effective way, through replacement of some of the harmful and laborious traditional methods (e.g., catching, observation) [51,160,161]. However, while eDNA methylation analysis could potentially open the door to investigate the states of populations, it has not been studied or utilized up till now.

Therefore, this study aimed to assess whether there are differences in aquatic eDNAm between age classes which is one of the most important pieces of information about the state of a population, and the differences between eDNAm and tDNAm. Specifically, we were interested in 1) how eDNAm and tDNAm vary between age classes, 2) whether eDNAm and tDNAm share the same pattern, and 3) how the differences between eDNAm and tDNAm vary with age. For this purpose, we studied methylation patterns from both eDNA and tDNA in four different age classes in separated tank experiments. *Lymnaea stagnalis* was used as model species mainly because it is hermaphrodite, thus excluding the impact of the potential sex-specific methylation differences on the analysis.

4.2 Methods

4.2.1 Tank experiments and sample collection

Long-term cultured *L. stagnalis* from Vrije University Amsterdam (originating from the Amsterdam mass culture) was used as a model species in the present study for the following reasons. First, one of our previous studies showed that *L. stagnalis* release a relatively large amount of eDNA [174]. Second, the assembled *L. stagnalis* genome (GenBank, GCA_900036025.1) published in 2016 can be used as a reference genome in bioinformatic analysis. Last, *L. stagnalis* is hermaphrodite. eDNA is a mixture of released DNA from multiple individuals. To make the age-methylation in eDNA clear, this species was utilized to eliminate the possibility of sex-specific age-associated alterations [186] in DNAm. This does not suggest that eDNAm analysis can only apply to hermaphrodite species. This does not suggest that eDNAm analysis can only apply to hermaphrodite species. There are no ethical issues associated with using this species [187].

All individual snails were from the same family. The snails in the oldest age class (200 days) were parents of the snails in other age classes. Each age class was cultured in a separate tank.

After culturing *L. stagnalis* for a sustained period, we transferred individual snails corresponding to the ages of 1, 30, 90 and 200 days, which corresponds to infant, child, teenager and adult life stages [188], to clean separate tanks (Figure 4.1). Three tanks of each age class were set up as replicates in a climate room at Leiden University, the Netherlands, in June 2020. The density in each tank of one age class from young to old was roughly 500, 150, 50 and 30 individuals per litre culturing water to attain a similar total biomass in each tank (given differences in body size at different age classes) with the aim of achieving similar amounts of released eDNA in each tank. The temperature of the climate room was set at 22 °C, with light between 7 am and 11 pm every day. The starting and sampling time of each tank was determined based on the birth date and age of the snails, within four months from the start of the experiment.

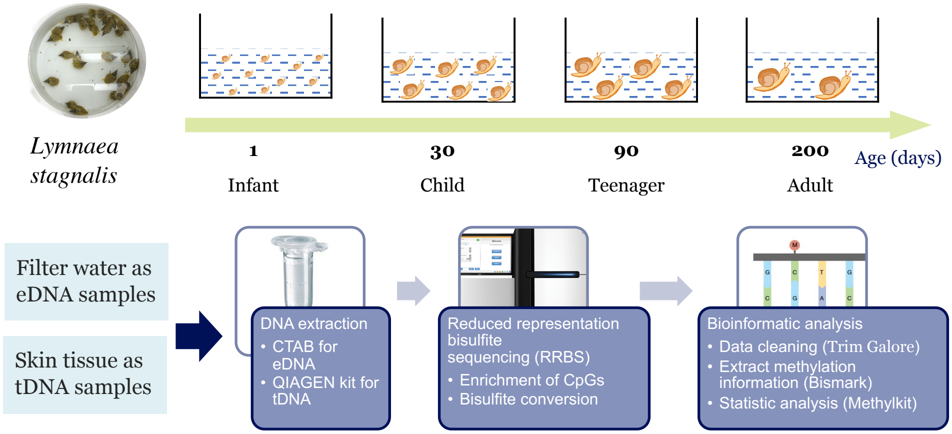

Figure 4.1 Workflow and methods. *Lymnaea stagnalis* was used as model species in the present study. Tank experiences were set up using *L. stagnalis* at age of 1, 30, 90 and 200 days, which correspond to infant, child, teenager and adult life stages. Three tanks were set up for each age group as replicates. Both environmental DNA (eDNA) and tissue DNA (tDNA) were collected for RRBS and subsequent bioinformatics analysis.

After two days without feeding (to reduce DNA from food), 120 mL culturing water in each tank was filtered by a plastic syringe (BD Plastipak™) using serial polyethersulfone (PES) membrane filters (Sterlitech) of pore sizes equal to 1.2 μm to collect eDNA. Our previous study [174] showed that eDNA particles of *L. stagnalis* were mostly over 1.2 μm. Moreover, at this size eDNA is at least still in chromosomes, and avoids potential uncertainty on methylation associated with eDNA degradation. After filtering, each filter was immediately put into a 2 mL tube together with 700 μL CTAB Lysis buffer (AppliChem GmbH, DE) before being stored at 4 °C. All plastic syringes and membrane containers were soaked in 10 % bleach over 10 minutes before being washed with deionized water, then air-dried on clean paper towels before use. Correspondingly, in each tank, the epidermis from 6 individuals was collected and mixed to obtain one tissue sample to represent the population and reduce genetic variability. Tissue samples were stored at -20 °C before the extraction within one week. Since global DNAm has been identified to be quite stable for months under various storage conditions and temperatures [189,190], we assume that the preservation method used in this study did not affect the results.

4.2.2 DNA extraction and library preparation

Total eDNA was extracted from each filter one day after collection, following a CTAB (Cetyltrimethyl Ammonium Bromide) protocol which has been described in previous studies [92,110], and eluted in 100 µL Tris-EDTA buffer solution (Sigma-Aldrich, US). Tissue DNA (tDNA) was extracted from each sample using the Qiagen Blood and Tissue kit following the manufacturer's protocol. All extracted DNA was stored at -20 °C until further processing. DNA concentrations of every obtained DNA sample were quantified using Thermo Scientific™ NanoDrop™ 2000.

Reduced Representation Bisulfite Sequencing (RRBS) libraries of each eDNA and tDNA sample were constructed using Zymo-Seq RRBS™ Library Kit following the manufacturer's protocol. Briefly, MspI digestion was firstly performed using around 200 ng input DNA to enrich CpG-dense sites, followed by an adapter ligation and gap-filling step. After that, bisulfite conversion was carried out before purifying the DNA. Last, an index primer amplification was carried out as follows: 30 s at 94 °C, 9 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 68 °C, then 5 min at 68 °C before conserving the product at 4 °C. Each sequencing library was quantified using the ddPCR™ Library Quantification Kit (1863040). High-throughput paired-end 150 bp sequencing was then done on the Illumina HiSeq X Ten Sequencing platform by Novogene Beijing (https://www.novogene.com). We performed RRBS in order to achieve low duplication rates (9 PCR cycles) and high genomic coverage methylation data through enriching CpG sites. The bisulfite conversion reaction and paired-end 150 bp sequencing also allowed us to analyse non-CpG methylation situation. DNA methylation level and context in invertebrate genomes have been found to be far more variable than in vertebrates [121,191,192]. Therefore, we argue that it is essential that also non-CpG (e.g., CHG and CHH, where H corresponds to Adenine, Thymine or Cytosine) methylation is included in invertebrate methylation studies.

4.2.3 Methylation analysis

Quality control and adapter trimming of the raw sequencing data was carried out using Trim Galore (v0.6.5, https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with its -rrbs option. DNAm at single-site resolution was extracted by Bismark software (v0.22.3, https://www.bioinformatics.babraham.ac.uk/projects/bismark/) using the *L. stagnalis* genome (GenBank, GCA_900036025.1) as the reference genome to generate BAM files containing methylation information of each sample using its default options (commands, software versions and options are shown in supporting information 2). Clean read numbers and mapping efficiency of each library are shown in Data S4.1. DNA methylations were mostly found in CpG, CHG and CHH sites. The global methylation levels of these sites were calculated in this step. A Kruskal-Wallis test was used to evaluate the difference in global methylation level between age classes of eDNA and tDNA separately. A Dunn's-test was performed to analyse the differences between each pair of age classes as the posthoc test of Kruskal-Wallis, using the PMCMRplus package in R (https://CRAN.R-project.org/package=PMCMRplus; v1.9.0). For each type of sites (CpG, CHG or CHH), a Wilcox Signed-Rank test was carried out to evaluate the differences in global methylation levels between eDNA and tDNA.

To further analyse the eDNAm differences with age at single-site resolution, the differentially methylated sites between each pair of two age classes in eDNA were extracted from the BAM files together with their methylation percentage in each sample by the MethylKit package [193] (v1.14.2) in R (v4.0.2). Sites were considered different if q-value < 0.01 and per cent methylation difference was larger than 25%. The q-value is a corrected p-value based on the sliding linear model (SLIM) method [194], suggested by the workflow, and has been used in most related studies [195,196]. In addition, methylated sites that differed from at least one age class to two or three other age classes were used to test whether these sites allowed clustering samples by age using the default clustering method in pheatmap package (https://CRAN. R-project. org/package=pheatmap; v1.0.12). These sites were also used in a Principal component analysis (PCA) using the FactoMineR (v2.4) package. The missMDA (v1.18) package was used to impute the missing values of the dataset. To analyse the differences of eDNAm compared to tDNAm, differentially methylated sites between eDNA and tDNA of each age class were extracted using the same method as above. Figures were generated by ggplot 2 (v3.3.2) [197], ggpubr (http://www.sthda.com/english/rpkgs/ggpubr; v0.4.0), VennDiagram [198], (v1.6.20), pheatmap and factoextra (v1.0.7) packages in R.

4.2.4 Data used in methylation analysis

The global methylation levels measured by RRBS might be different from those of a whole-genome bisulfite sequencing (WGBS) method, since the RRBS method specifically targets CpG sites but does not cover the whole genome [199]. To ensure good quality of the data and a high confidence methylation percentage, a minimum coverage of 10 reads per site was imposed in the single site differential methylation analysis [193]. On average, more than 225 K and 776 K sites were covered in the eDNA and tDNA samples respectively, including CpG, CHG and CHH sites. One possible explanation of the difference in the number of sites between eDNA and tDNA is that the eDNA samples include all DNA in the water not only DNA of *L.stagnalis* but also other organisms, leading to a lower percentage of *L.stagnalis* DNA in the total eDNA present in the sample compared to tDNA samples.

4.3 Results

4.3.1 eDNAm changes with age

The global methylation levels (percentages) of CpG, CHG and CHH sites of *L. stagnalis* eDNA were different between age classes for CHG and CHH sites ($P < 0.05$, Kruskal-Wallis Test) as shown in Figure 4.2a. Global methylation level increased from the age of 1 to 30 days, and then continuously decreased towards the age of 200 days. The Dunn's-test to evaluate the pairwise differences showed that differences were only significant between age classes of 30 and 200 days ($P < 0.05$, Table S4.1). Differences in methylation with age became more apparent ($P < 0.01$, Wilcox Signed-Rank Test) when grouping the age classes between young (from 1 to 90 days) and adult life stages (200 days; Figure S4.1). The global methylation level of CHH sites with a range from 12.9 to 59 % was much higher and broader than of CHG (5-17.5 %), and CpG (6.9-13.4 %). For CpG, no differences between age classes were observed in its global methylation level ($P = 1$, Table S4.1). The exact p-value of each Dunn's-test are provided in Table S4.1.

To better understand the differential methylation with age in eDNA in addition to the global methylation level, we compared the methylation level at a single-site resolution between each pair of two age classes. A total of 383 sites (Data S4.2) was found to be differentially methylated from at least one age class to two or three other age classes. These sites were defined as eDNAm age-associated sites in the present study. Interestingly, all these sites were CHH sites. Using the methylation data of these sites, eDNA samples were clustered completely according to age (Figure 4.3 and Figure S4.2) using the default clustering method in the pheatmap package, indicating the age-related changes in DNAm. Most of these sites were separated into different contigs across the genome with only a few sites in the same contig (Figure S4.3).

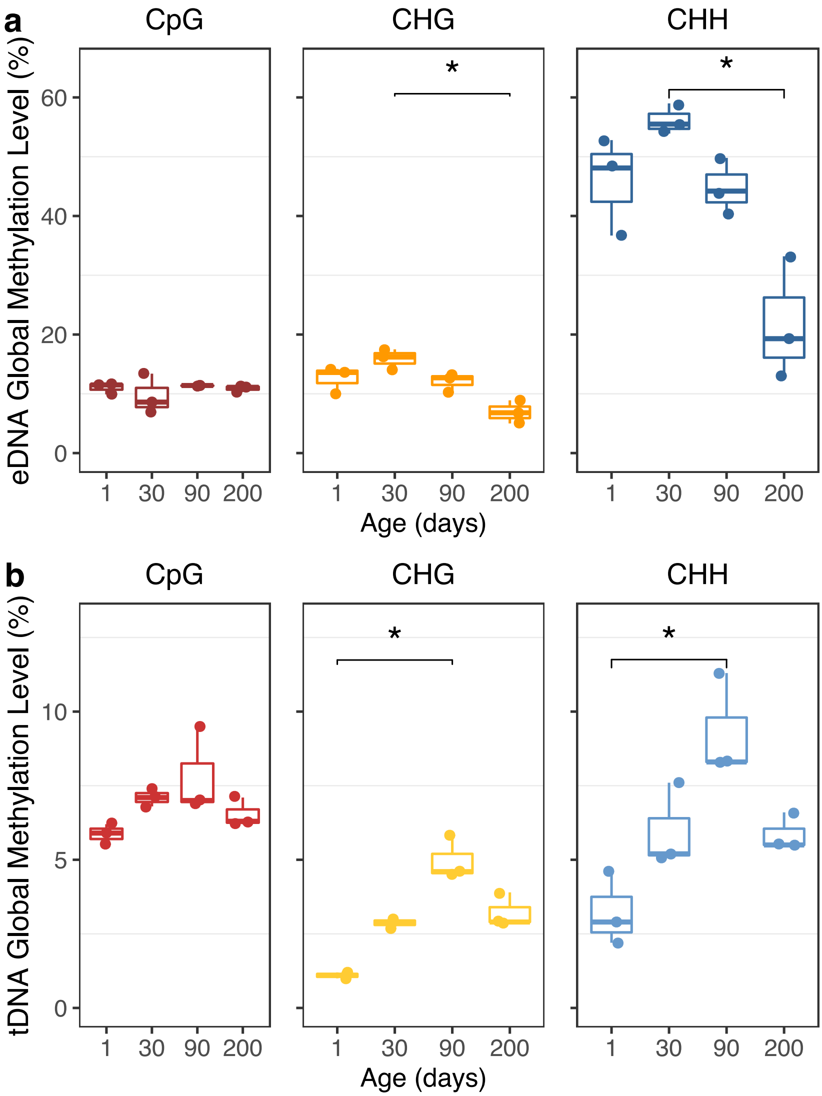

Figure 4.2 a) Environmental DNA (eDNA) global methylation level of CpG (red), CHG (orange) and CHH (blue) sites in four age groups of *L. stagnalis*. **b)** tDNA global methylation level of CpG (red), CHG (orange) and CHH (blue) sites in four age groups of *L. stagnalis*. Note that the ordinate ranges are different. Significant differences between age groups from Dunn's-test as a posthoc test of Kruskal-Wallis are shown by asterisks on the top. (*, $P < 0.05$).

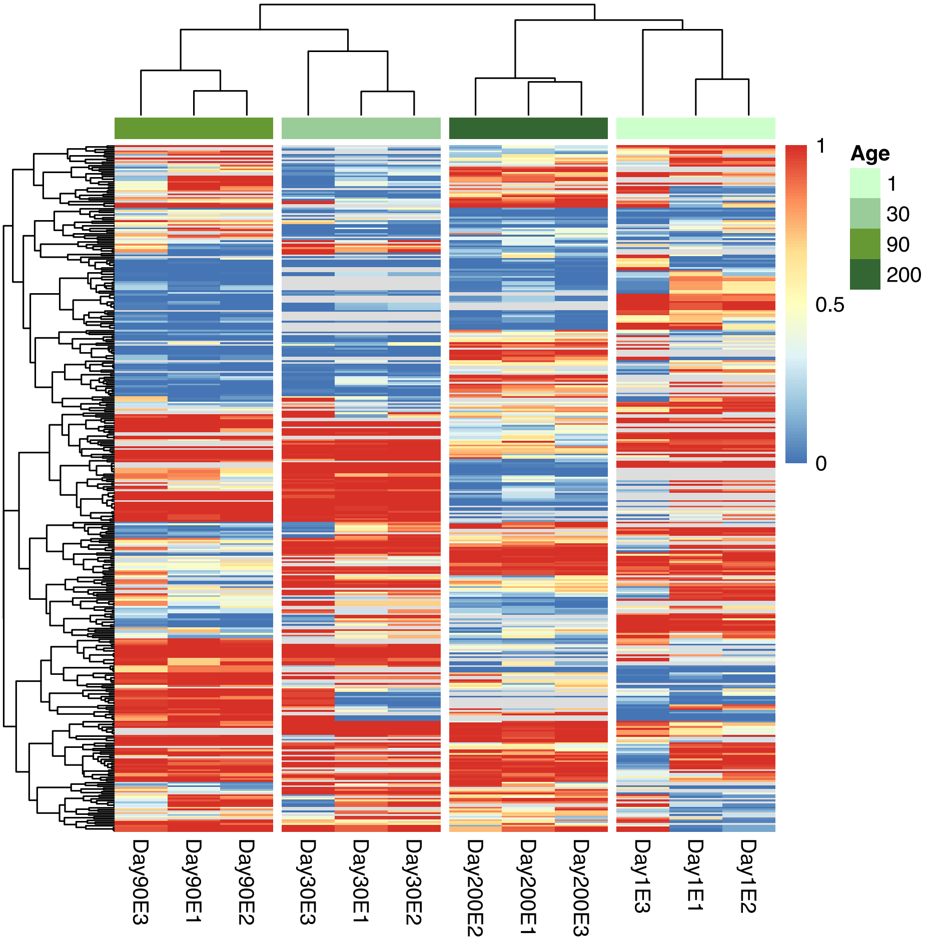

Figure 4.3 Heatmap of 383 age-associated methylation sites in *L. stagnalis* eDNA. Colours show the methylation levels (proportion, blue to red). Each site was detected in at least three age groups, grey indicates the absence of data. Each row represents one site, each column represents one sample. Columns are clustered as age (days) groups (green bar on the top) using the default clustering method in pheatmap package.

4.3.2 The eDNA is hypermethylated compared to tDNA

In order to explore how the methylation patterns of eDNA and tDNA differed across the age groups, we clustered our samples based on methylation data of CpG, CHG and CHH sites separately. The results indicated that methylation patterns between eDNA and tDNA were only similar for the CHG sites and only for the age class of 200 days (Figure S4.4). By CHG sites methylation, the three eDNA samples at the age of 200 days sorted with the tDNA samples, while eDNA and tDNA were differentiated by CpG and CHH methylation. Age is more important than DNA origin in CpG methylation. However, in eDNA, it can only separate young (1 and 30 days) and old (90 and 200 days). The eDNA global methylation levels were significantly dissimilar from tDNA on all CpG, CHG and CHH sites in the present study ($P < 0.01$, Wilcox Signed-Rank test), indicating whole-genome hypermethylation of eDNA (Figure 4.4). In addition, the age methylation trend was also different between eDNA and tDNA. While the highest eDNAm levels were present at the age class of 30 days (Figure 4.2a), in tDNA the highest levels were found at the age class of 90 days (Figure 4.2b). However, in one aspect, tDNA was consistent with eDNA, which was that the differences in the global methylation level among age classes were only shown on CHG and CHH sites ($P < 0.05$, Kruskal-Wallis test), but not on the CpG site ($P = 0.068$, Kruskal-Wallis test; Figures 4.2 and 4.4).

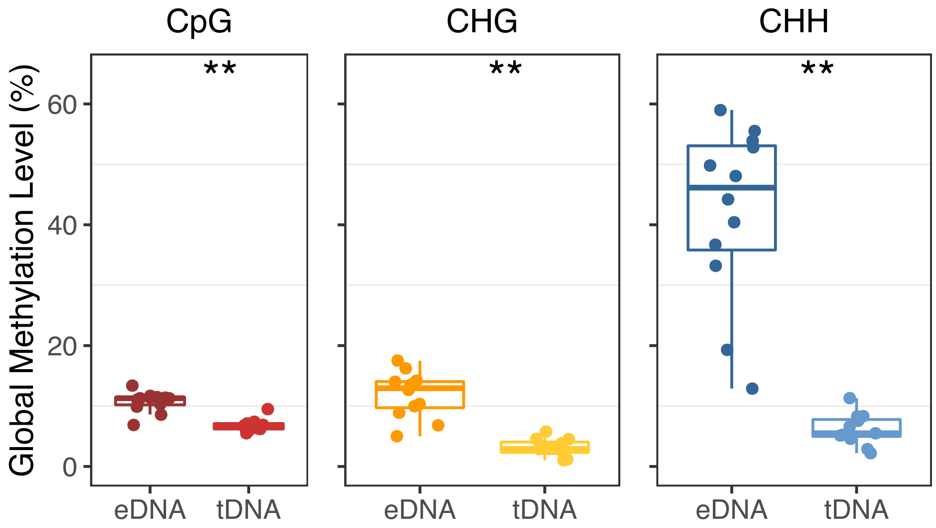

Figure 4.4 Comparison of global methylation level between environmental DNA (eDNA) and tissue DNA (tDNA) of CpG (red), CHG (orange) and CHH (blue) sites separately. The asterisks on the top indicate significant differences by a Wilcox Signed-Rank test. (*, $P < 0.05$; **, $P < 0.01$).

4.3.3 eDNA-tDNA differential methylation is age-specific

To further evaluate eDNA methylation, we extracted those eDNA sites that were methylated differentially from tDNA, for each age class separately (Data S4.3-4.6). In all age classes, more hypermethylated sites were apparent than hypomethylated sites in the eDNA (Figure 4.5a), which was in line with the global hypermethylation of eDNA. Both hyper- and hypo- methylated sites varied among age classes, while only a few sites were shared by more than one age class (Figures 4.5b and c). eDNA and tDNA samples were separated completely in all age classes (Figures 4.5d, e, f and g), supporting the significant differences between eDNA and tDNA methylation.

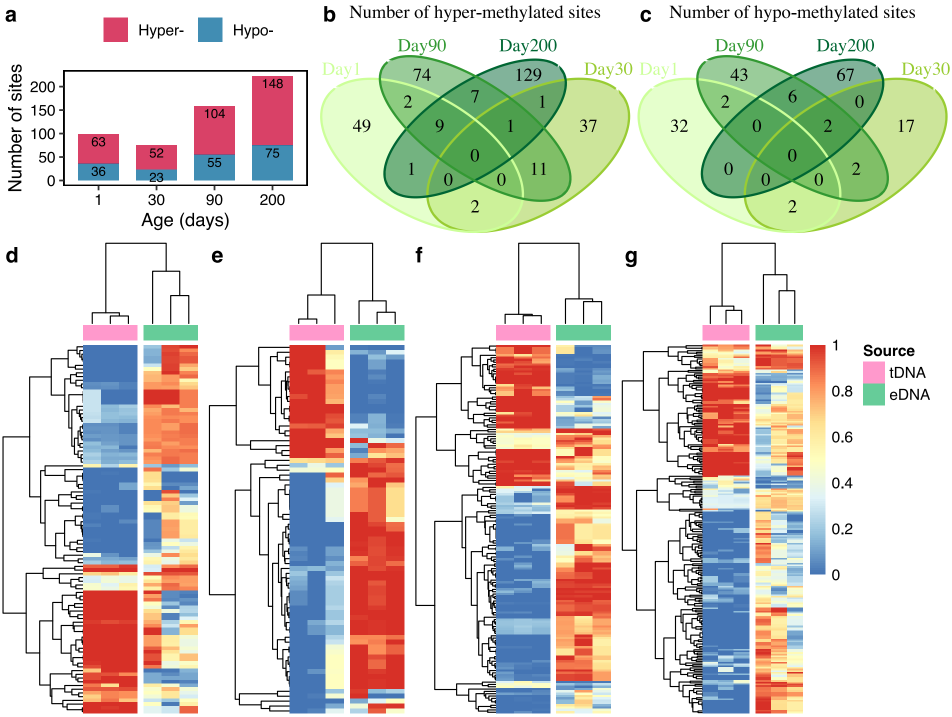

Figure 4.5 Differentially methylated sites of each age group in environmental DNA (eDNA) against tissue DNA (tDNA). **a)** The number of hyper- (red) and hypo- (blue) methylated sites. **b)** Venn diagram of the number of hyper-methylated sites in age groups. **c)** Venn diagram of the number of hypo-methylated sites in age groups. Heatmaps show the methylation level of differentially methylated sites in tDNA (pink) and eDNA (green), **d, e, f** and **g** represent age groups of 1, 30, 90 and 200 days respectively. Colours show the methylation levels (proportion, blue to red). Each row represents one site, each column represents one sample.

Interestingly, the number of sites differentially methylated between eDNA and tDNA had a pattern opposite to that of the eDNA global methylation levels with age (compare Figures 4.2 and 4.5a). The age class of 30 days held the highest global eDNAm level but contained fewer eDNA-tDNA differentially methylated sites (75 sites) than other age classes. By contrast, the age class of 200 days had the lowest global eDNAm level, and its eDNAm was most close to that of tDNAm, but contained the most eDNA-tDNA differentially methylated sites (223 sites). Consequently, the methylation difference between eDNA and tDNA of these sites showed more significance in the age class of 30 days (Figure 4.5e) than of 200 days (Figure 4.5g), while the other two age classes were in between (Figures 4.5d, 4.5f and Figure S4.5). Compared to tDNA, eDNA release by young *L. stagnalis* was differentially methylated in a limited cluster of sites, especially for the age class of 30 days, possibly the fastest body-growing life stage (child stage). Whereas when *L. stagnalis* were adult (200 days), the differential methylation of eDNA was less site-specific, indicating that the methylation differences between eDNA and tDNA are age-specific.

4.4 Discussion

While age-related differences in DNAm have been explored in tissue samples of several species [122–124,200,201], we show for the first time that these differences also exist in eDNAm. In the current study, we found 383 age-associated methylated sites in *L. stagnalis* eDNA. The methylation data of these sites was sufficient to cluster eDNA samples according to age. We additionally demonstrate here that eDNA methylation analysis has the potential to assess age. This would find broad applications in both the field of biomonitoring and conservation. In this way, eDNAm analysis will allow for more efficient and non-invasive age surveillance compared to current methods, such as demersal trawl surveys [51], or counting observations [160,161]. Considering the huge impact of age on population dynamics [127,179], eDNAm analysis could potentially expand the application of eDNA methods in environmental biology research.

We also found that eDNAm, both in terms of global methylation as well as in its methylation patterns, was highly different from tDNAm. First, eDNA was highly hypermethylated. Hypermethylation of DNA is known to inhibit gene transcriptions and expressions, and plays an important role in controlling gene activities thereby impacting many physiological processes like cell differentiation and body development [202]. eDNA may be expected to be hypermethylated given that it is from released genetic materials that is no longer as functional as those cells still in living bodies. Second, the eDNAm pattern with age did not fully correspond to tDNAm. The highest global methylation level showed up at the age of 30 days in eDNA, whereas it was at the age of 90 days in tDNA. Third, the methylation differences were age-specific. We found that the eDNA hypermethylation in young age groups targeted to a specific cluster of DNA sites, while a broader range of sites was involved in the differential methylation in the adult age group. Previous studies indicated that the eDNA release rate per body weight of young fish is higher than that of adults [53]. If this also applies to *L. stagnalis*, this notion, together with the higher global eDNAm levels of young *L. stagnalis* (Figure S4.1) in this study, will supports a positive connection between eDNA release rate and hypermethylation. This suggests eDNA release patterns may in part be a response to DNAm to get rid of dysfunctional, i.e., hypermethylated, cells (next to eDNA release due to damage, sloughed-off cells or other unintended reasons). This observation might help to understand the molecular mechanisms of eDNA release.

As alternative explanation for the methylation differences between eDNA and tDNA, one might argue that the differences originate from inherited methylation differences across tissues. However, while previous studies have reported DNAm to be different between different tissues in several species [183,203,204], the global methylation level differences among these tissues were not nearly as pronounced as the differences between eDNAm and tDNAm found in the present study (Figure 4.3a). For example, the genome-wide CpG methylation level of *Chlamys farreri* ranged from 20.9% to 21.7% across five tissues [183] and it ranged from 50.18 to 53.99% across seven tissues in the pig genome [203]. The CpG methylation percentage of eDNA and tDNA in *L. stagnalis* were 10.73% and 6.83% on average. The difference was more obvious for CHH sites (the most dominant methylation sites found in this study), which was 42.15% and 6.09% on average, respectively. Another alternative explanation for the differences between eDNA and tDNA methylation patterns might be the methylation discrepancy between pluripotent (e.g., germ cells) and somatic cells. Non-CpG methylations are predominately found in pluripotent cells but rarely in somatic cells of invertebrates due to their different functions and differentiations [205]. In the present study, eDNAm global level was higher than tDNAm in all three types of sites, which was not similar to the differences between pluripotent and somatic cells. Additionally, no egg bags were observed during any of the tank experiments, indicating that DNA from pluripotent cells were not the dominant eDNA source in our tank experiments. Therefore, we assume that the differences between eDNAm and tDNAm were not caused by the methylation differences between pluripotent and somatic cells. Therefore, we consider eDNA release as a mechanism to remove dysfunctional hypermethylated cells the most likely explanation for the large differences found in methylation levels of tDNA and eDNA.

A few issues need to be considered before using eDNAm to assess age classes in the field. Given that the genome of different species methylate differently, together with the genomic differences between species, we suggest that tank experiments of target species with different age classes can be run -similar to this study- to develop a species-specific assessment model of age by methylation for biomonitoring. Because of the differences in DNA methylation levels and in context between invertebrate and vertebrate genomes [121,191,192], as well as the discrepancies discovered in this study between eDNA and tDNA, non-CpGs methylation should be considered in addition to CpGs methylation. Because of the non-linear relationship between eDNAm and age, utilizing eDNAm to assess age may only be practicable for a few age classes, meaning that more age classes than the four utilized in the current proof-of-principle study would be preferable. This may aid biomonitoring of target species, assuming that methylation patterns as observed in tanks are similar to those in field conditions. In field conditions, methylation patterns may deviate to a certain extent, due to differences in e.g., temperature, circadian rhythm or diet. However, we expect those different conditions to impact a different cluster of DNA sites than those sites impacted by aging, because they relate to different physiological mechanisms, and therefore to different genes. While this remains to be tested, we suggest to not use global methylation levels as an indicator of age in target species in biomonitoring, but to target specifically those hundreds of sites that are directly related to age differences (as shown for our study organism in Figure 4.2a).

Another complication for biomonitoring applications is that individuals of different ages usually live together in the field resulting in eDNAm patterns representing a mix of age classes. It will thus be necessary to evaluate whether age-specific methylation sites exist in the target species for biomonitoring and subsequently select a set of sites that in combination contain the full suite of age classes expected in the field. The patterns in Figure 4.2a suggest that such age-specific methylation sites exist. Instead of determining the age of each individual, eDNAm pattern of these age-specific sites provides a proxy for different age classes within a population (within a single species). This information facilitates the prediction of the development of a population owing to the strong relationships between population age and productivity [127,206]. The extent to which such unique age-related sites methylation exists in eDNA, and how the eDNAm pattern is related to age require further studies. Next, by using methylation detection methods such as methylation arrays which enable quantitative assessments of selected methylation sites, quick methylation detection of those age-related sites from eDNA samples is possible [207]. These techniques can be custom designed to detect methylation of a cluster of target sites, which are suitable for eDNA in which the target DNA only takes up a very low percentage of the total DNA. In combination, this might allow for age assessment by eDNAm analysis. Recent studies have found specific cytosines in highly-conserved stretches of DNA, whose methylation levels change with age across mammalian species [182] or across odontocetes [208], which indicates that age assessment based on DNAm across species should be theoretically possible.

eDNAm analysis might also open up the possibility to investigate population abundances. eDNA concentration has been used to estimate the abundance or density of a population [70,185,209]. However, the variation in eDNA release rate [53] with age can cause major uncertainty in this assessment. Since eDNAm analysis can provide an age profile, it will be possible to calculate abundances more accurately if combined with the knowledge of eDNA release rate of different age classes. Moreover, if the direct connection between eDNA methylation and release rate is validated (see above), eDNAm data may be used to estimate the eDNA release rate without the need for specific knowledge on eDNA release rate of age classes.

Apart from age, DNAm has been used as a biomarker for disease [119], such as cancer [118,120,180] and psychiatric disorders [116]. The strong connection between DNAm and health conditions provides an untapped potential for investigating the health conditions of target species using eDNAm analysis. Additionally, DNAm is directly related to environmental factors [210,211], including but not limited to temperature [212], high-altitude [213], noise and pollution [214–216], making it potentially possible to use eDNAm analysis to evaluate environmental change (once the relationship between these drivers and eDNAm of the target species has been quantified). Complementary to real-time functional gene analysis [61] and metatranscriptome analysis using RNA [63,65,217], methylation can help to get insight into the long-term activity of genes as it provides a blueprint of functional information over a longer period due to its slow change and stability [115,128]. In combination, eDNAm analysis could provide a comprehensive understanding of a population of a target species.

Our study shows -for the first time- that eDNA methylation is different between age classes, which indicates that eDNAm might have the potential to assess age. This study further shows that the difference in methylation between eDNA and tDNA is age-specific, suggesting a connection between eDNA release and methylation. Benefiting from the very well-developed methylation detection techniques that suit multiple demands, eDNA methylation analysis has the full potential to become a time and labour-saving method in environmental science. Therefore, we believe age assessment is probably just the first step of eDNAm analysis application in biomonitoring and environmental research.

Data Accessibility Statement

All data used in this study are freely available. BAM files for each sample containing all alignments plus methylation call strings are available from NCBI SRA database with BioProject accession number of PRJNA730179 (https://www.ncbi.nlm.nih.gov/sra/PRJNA730179). The methylation values of 383 age-associated sites in eDNA, as well as the eDNA-tDNA differentially methylated sites are available as supporting data. Commends and scripts used in the analysis are available in supporting information 2.

Benefit-Sharing Statement

Benefits Generated: This research provides the first view of eDNA methylation, supports the possibility of using eDNA methylation in assessing age, and contributes to understanding the features of eDNA by the methylation differences in eDNA and tDNA. Further, the result will help improve eDNA method in biomonitoring.

Author Contributions

Peter van Bodegom, Krijn Trimbos and Beilun Zhao designed the experiments and wrote the draft of the manuscript. Beilun Zhao performed the experiments and analyzed the data. The authors declare no competing financial interest.

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APPENDIX

Supplemental Table and Figures

Table S4.1 *P*-values of pairwise comparisons using Dunn's-test for evaluating differences in global methylation level of each type of sites (CpG, CHG and CHH respectively; C, cytosine; p, phosphate; G, guanine; H, adenine, thymine or cytosine) as a function of age groups as a posthoc test of the Kruskal-Wallis Test. The P value adjustment method was holm. eDNAm refers to environmental DNA methylation, tDNAm refers to tissue DNA methylation.

		eDNAm			tDNAm		
	Age (days)	1	30	90	1	30	90
CpG	30	1	-	-	0.12	-	-
	90	1	1	-	0.12	0.95	-
	200	1	1	1	0.77	0.92	0.92
CHG	30	0.52	-	-	0.51	-	-
	90	0.65	0.45	-	0.01*	0.44	-
	200	0.35	0.02*	0.52	0.44	0.73	0.51
CHH	30	0.57	-	-	0.52	-	-
	90	0.91	0.57	-	0.01*	0.44	-
	200	0.57	0.01*	0.57	0.44	0.73	0.52

* indicates *P* < 0.05.

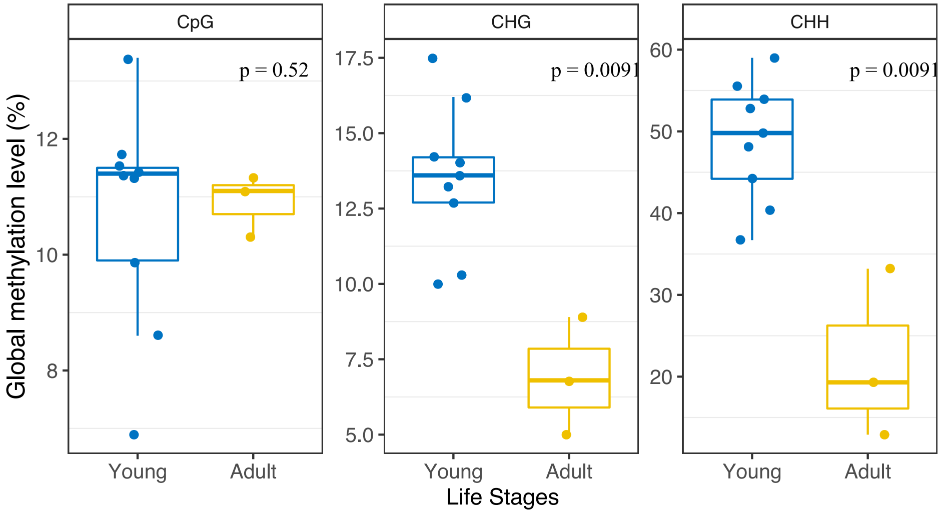

Figure S4.1 Environmental DNA (eDNA) global methylation level of young (1-90 days, blue) and adult (200 days, yellow) groups. *P* values on the top indicating significant differences according to the Wilcox Signed-Rank test.

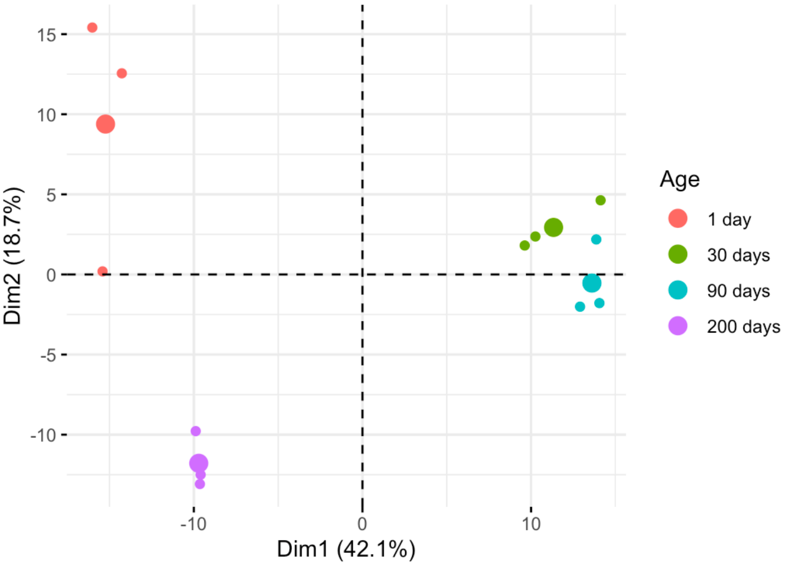

Figure S4.2 PCA of eDNA samples based on 383 age-associated differential methylation sites. Different replicates for each age class are shown separately.

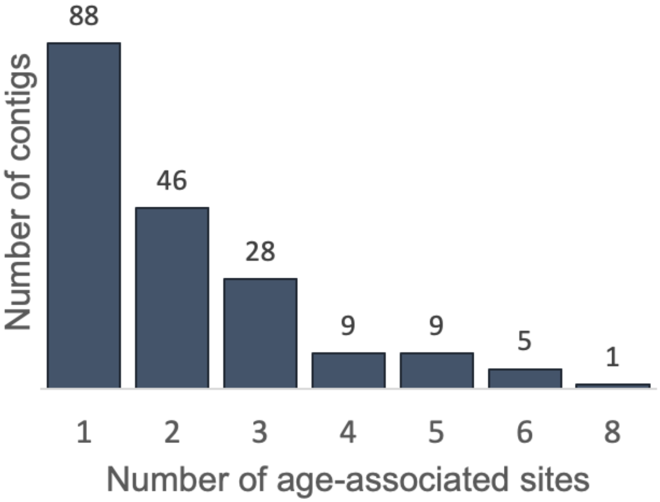

Figure S4.3 Number of contigs with its corresponding number of age-associated sites in eDNA methylation.

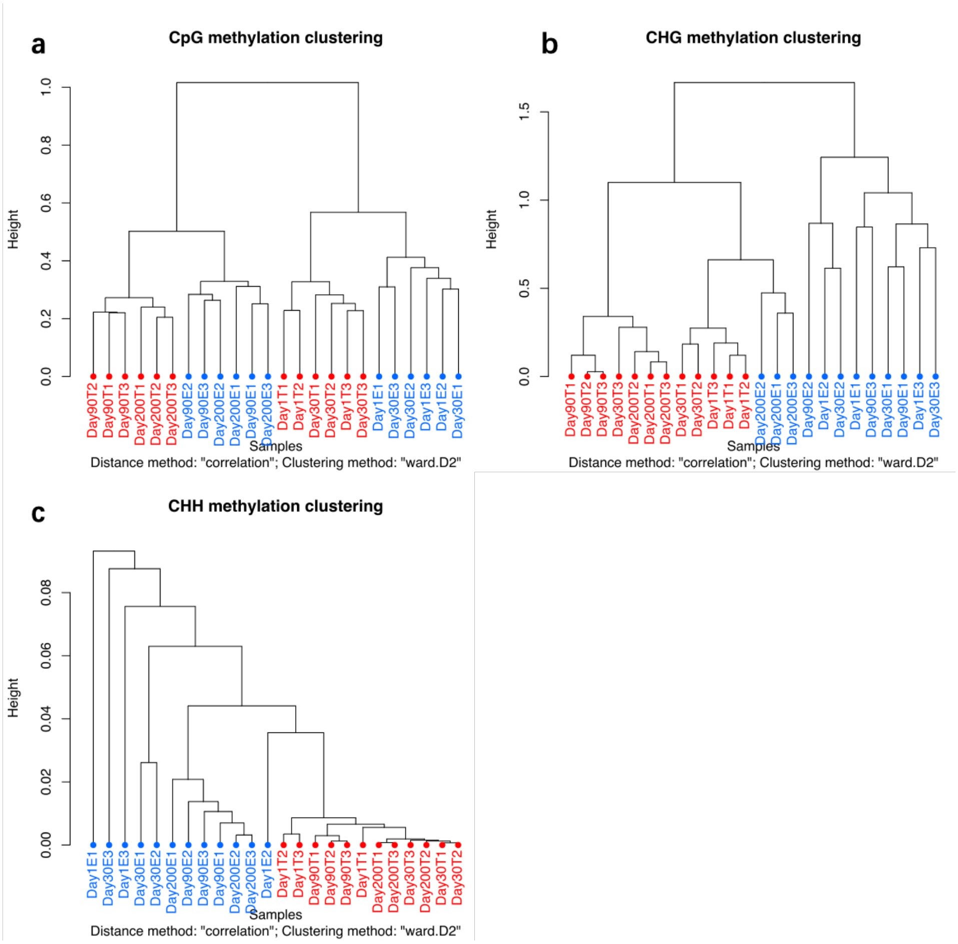

Figure S4.4 Clustering of samples based on methylation of CpG **(a)**, CHG **(b)** and CHH **(c)** sites. Age is more important than DNA origin in CpG methylation as shown in **(a)**, eDNA samples are separated into young (1 and 30 days) and old (90 and 200 days). By CHG sites methylation, the three eDNA samples at the age of 200 days sorted with the tDNA samples **(b)**. eDNA and tDNA were differentiated by CHH sites methylation **(c)**.

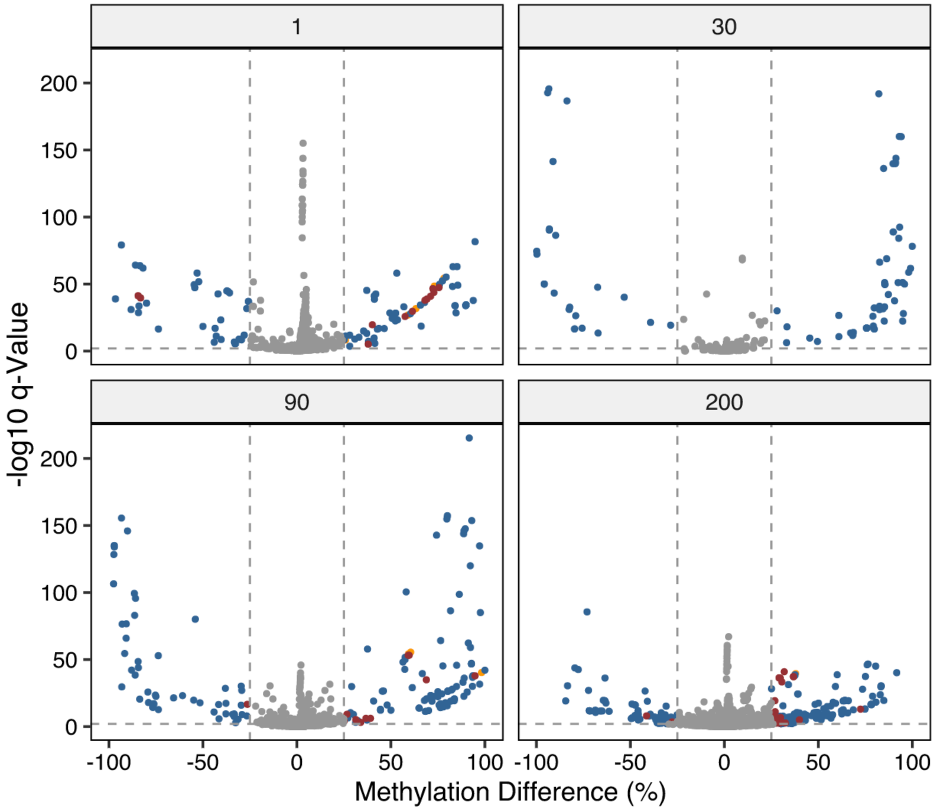

Figure S4.5 Volcano figure showing the methylation difference (%) of each site between tissue DNA and eDNA and the corresponding adjusted q-value in each age class. Colours indicate the type of sites, CpG (red), CHG (yellow), CHH (blue).

Supplemental Data

Data S4.1 Clean read numbers and mapping efficiency of each library.

Name	Read	Clean Reads	Bismark_Sequence pairs analysed	Unique best hit	Mapping efficiency (%)
Day1E1	Forward	36.509.125	60946164	1060821	1,7
Day1E1	Reverse	40.626.610			
Day1T1	Forward	3.781.614	6952814	1235049	17,8
Day1T1	Reverse	4.241.030			
Day1E2	Forward	28.720.745	48965303	865270	1,8
Day1E2	Reverse	32.055.838			
Day1T2	Forward	5.257.513	7296701	1258079	17,2
Day1T2	Reverse	5.586.045			
Day1E3	Forward	27.820.922	48112853	979496	2
Day1E3	Reverse	31.116.975			
Day1T3	Forward	2.725.173	4461088	709935	15,9
Day1T3	Reverse	2.975.068			
Day30E1	Forward	30.852.967	48488405	899013	1,9
Day30E1	Reverse	34.133.933			
Day30T1	Forward	5.845.248	9060113	1460712	16,1
Day30T1	Reverse	6.355.156			
Day30E2	Forward	2.947.644	46889938	844453	1,8
Day30E2	Reverse	32.529.967			
Day30T2	Forward	6.375.632	10097115	1677161	16,6
Day30T2	Reverse	6.945.449			
Day30E3	Forward	27.976.103	45658190	751170	1,6
Day30E3	Reverse	30.915.423			
Day30T3	Forward	5.764.021	9369697	890597	9,5
Day30T3	Reverse	637.025			
Day90E1	Forward	31.555.842	50237217	1009578	2
Day90E1	Reverse	34.582.197			
Day90T1	Forward	6.284.934	8071193	1220056	15,1
Day90T1	Reverse	6.637.088			
Day90E2	Forward	25.825.673	44004497	724404	1,6
Day90E2	Reverse	28.593.305			
Day90T2	Forward	7.592.453	10348100	1572242	15,2
Day90T2	Reverse	8.073.551			
Day90E3	Forward	35.371.174	49053333	1098186	2,2
Day90E3	Reverse	37.997.043			
Day90T3	Forward	6.431.322	8661035	1294540	14,9
Day90T3	Reverse	6.845.629			
Day200E1	Forward	26.749.953	46560520	883412	1,9
Day200E1	Reverse	29.801.259			
Day200T1	Forward	7.695.244	12303609	1702889	13,8
Day200T1	Reverse	8.359.145			
Day200E2	Forward	26.484.849	45153372	1381174	3,1
Day200E2	Reverse	29.400.607			
Day200T2	Forward	7.217.800	11329765	1560993	13,8
Day200T2	Reverse	7.846.203			
Day200E3	Forward	24.242.996	41724363	1896610	4,5
Day200E3	Reverse	26.803.897			
Day200T3	Forward	6.006.143	9441526	1284974	13,6
Day200T3	Reverse	6.547.340			

Data S4.2 Methylation percentage of a total of 383 sites in *Lymnaea stagnalis* genome that was differentially methylated from at least one age class to two or three other age classes. These sites were defined as eDNAm age-associated sites in the present study.

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01000342.1	4096	0,07	0,09	1,00	0,83	0,88	0,07	0,99	0,98	0,64	0,65	0,64	0,88
FCFB01000342.1	4108	0,93	0,97	0,17	0,97	0,94	0,93	0,98	0,98	0,98	0,58	0,45	0,38
FCFB01000988.1	20049	0,30	0,91	0,36	0,90	0,95	0,83	0,87	0,81	0,80	0,87	0,96	0,98
FCFB01000988.1	20050	0,70	0,09	0,64	0,05	0,00	0,00	0,06	0,22	0,20	0,11	0,02	0,02
FCFB01000988.1	20056	0,30	0,91	0,36	0,95	0,95	1,00	0,94	0,81	0,80	0,86	0,98	0,99
FCFB01004269.1	4606	1,00	0,98	0,46	1,00	1,00	0,92	1,00	1,00	1,00	0,85	0,30	0,63
FCFB01009015.1	4696	0,99	0,97	0,15	0,77	0,92	0,98	0,45	0,67	0,96	0,16	0,26	0,28
FCFB01016529.1	613	1,00	0,97	0,08	0,98	0,98	0,99	1,00	1,00	1,00	0,96	0,99	0,98
FCFB01016529.1	622	1,00	1,00	0,82	1,00	1,00	1,00	0,24	0,41	0,90	0,95	0,97	0,98
FCFB01016529.1	625	1,00	0,98	0,08	0,96	0,98	0,99	0,25	0,41	0,90	0,98	0,99	1,00
FCFB01018690.1	21029	1,00	1,00	0,99	1,00	0,90	1,00	0,09	0,02	0,03	1,00	1,00	1,00
FCFB01023009.1	8808	0,29	0,73	0,00	0,13	0,05	0,64	0,83	0,80	1,00	0,18	0,05	0,11
FCFB01030999.1	10899	0,96	0,95	0,67	0,96	0,97	0,85	0,57	0,86	0,71	0,50	0,44	0,12
FCFB01030999.1	10903	0,34	0,51	0,00	0,35	0,08	0,71	0,57	0,57	0,50	0,28	0,14	0,07
FCFB01039333.1	165	0,06	0,06	0,55	0,00	0,00	0,00	0,03	0,00	0,00	0,04	0,00	0,00
FCFB01040148.1	1505	0,50	0,82	0,00	0,35	0,01	0,00	1,00	0,80	0,05	0,39	0,16	0,38
FCFB01041107.1	8790	0,07	0,03	1,00	0,67	0,67	0,02	0,93	1,00	0,33	1,00	0,95	0,96
FCFB01042712.1	4179	0,56	0,72	0,94	1,00	1,00	1,00	0,97	0,86	0,96	1,00	1,00	1,00
FCFB01042712.1	4180	0,56	0,72	0,94	1,00	1,00	1,00	0,97	0,80	0,96	1,00	1,00	1,00
FCFB01046721.1	663	0,82	0,90	0,31	1,00	1,00	0,80	1,00	0,89	1,00	1,00	0,98	0,92
FCFB01046721.1	664	0,00	0,00	0,67	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
FCFB01046721.1	666	1,00	1,00	0,33	1,00	1,00	1,00	1,00	1,00	1,00	0,78	1,00	0,98

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01046721.1	667	0,00	0,00	0,67	0,00	0,00	0,00	0,00	0,00	0,00	0,22	0,00	0,03
FCFB01046721.1	672	0,00	0,00	0,66	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02
FCFB01050668.1	1580	0,90	0,81	0,97	0,94	0,96	0,92	0,19	0,28	0,80	0,93	0,96	0,96
FCFB01050668.1	1586	1,00	0,98	0,84	0,88	0,89	0,95	0,26	0,27	0,88	0,69	0,65	0,69
FCFB01050668.1	1592	0,47	0,24	0,05	0,80	0,84	0,82	0,13	0,07	0,62	0,13	0,10	0,08
FCFB01058186.1	390	0,62	0,85	0,57	1,00	1,00	1,00	1,00	1,00	1,00	0,96	1,00	0,94
FCFB01058186.1	394	1,00	1,00	1,00	1,00	1,00	1,00	1,00	0,99	1,00	0,71	0,74	0,65
FCFB01058186.1	403	0,25	0,43	0,00	1,00	1,00	1,00	1,00	1,00	1,00	0,67	0,74	0,71
FCFB01061499.1	8907	0,00	0,00	0,00	0,33	0,50	0,13	0,00	0,00	0,07	0,12	0,00	0,00
FCFB01064855.1	1244	0,03	0,11	1,00	0,92	1,00	1,00	1,00	1,00	0,94	0,91	0,73	1,00
FCFB01064855.1	1248	1,00	0,89	0,00	0,09	0,00	0,00	0,00	0,00	0,06	0,64	0,33	0,27
FCFB01064855.1	1250	0,00	0,11	1,00	0,92	1,00	1,00	1,00	1,00	0,94	0,91	0,73	1,00
FCFB01064855.1	1251	0,03	0,11	0,43	0,92	1,00	1,00	1,00	1,00	0,91	0,71	0,13	0,73
FCFB01064855.1	1253	0,00	0,11	1,00	0,92	1,00	1,00	1,00	1,00	0,94	0,91	0,73	1,00
FCFB01064855.1	1261	0,98	0,89	0,57	0,08	0,00	0,00	0,00	0,00	0,09	0,83	0,93	0,55
FCFB01076149.1	19955	0,84	1,00	1,00	1,00	0,90	0,17	0,97	1,00	0,95	0,96	0,99	1,00
FCFB01076897.1	2056	0,00	0,08	0,29	0,02	0,07	0,02	0,94	0,94	0,76	0,00	0,00	0,50
FCFB01078356.1	296	1,00	1,00	1,00	1,00	1,00	1,00	0,74	0,13	0,24	0,64	0,12	0,78
FCFB01078356.1	301	0,00	0,00	0,09	0,15	0,00	0,00	0,26	0,87	0,76	0,50	0,88	0,22
FCFB01078356.1	302	1,00	1,00	0,05	1,00	1,00	1,00	0,89	0,89	1,00	1,00	1,00	1,00
FCFB01078356.1	304	0,75	1,00	0,00	0,86	0,78	1,00	0,89	0,89	1,00	0,85	1,00	1,00
FCFB01078356.1	311	1,00	1,00	0,05	1,00	1,00	1,00	0,63	0,04	0,25	0,74	0,12	0,78
FCFB01078356.1	316	0,89	0,91	1,00	0,58	0,67	1,00	0,74	0,14	0,22	0,45	0,00	0,67
FCFB01080856.1	642	1,00	0,95	0,90	0,96	1,00	1,00	0,48	0,54	0,33	0,99	0,99	1,00

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01080856.1	643	0,96	1,00	0,13	0,24	0,77	0,02	0,53	0,47	0,71	0,00	0,02	0,01
FCFB01080856.1	648	0,10	0,06	0,13	0,04	0,16	0,00	0,52	0,48	0,69	0,01	0,01	0,01
FCFB01080856.1	651	0,09	0,00	0,89	0,25	0,31	0,99	0,47	0,49	0,28	0,91	0,94	0,98
FCFB01081796.1	3492	0,01	0,03	0,96	0,58	0,70	0,72	0,98	1,00	0,99	0,57	0,67	0,17
FCFB01087352.1	3450	0,94	0,60	1,00	0,64	0,72	0,12	0,13	0,02	0,07	0,24	0,13	0,22
FCFB01087352.1	3452	0,29	0,80	0,83	0,38	0,31	1,00	0,97	0,91	0,98	0,97	0,98	0,98
FCFB01093018.1	1413	0,88	0,88	0,75	0,95	0,97	1,00	0,01	0,00	0,85	0,57	0,79	0,71
FCFB01093018.1	1425	0,23	0,06	0,50	0,01	0,00	0,00	0,00	0,00	0,01	0,50	0,76	0,41
FCFB01093018.1	1428	0,19	0,06	0,69	0,91	0,97	1,00	0,01	0,06	0,85	0,33	0,11	0,38
FCFB01097445.1	632	0,23	0,54	0,76	0,14	0,03	0,33	0,93	0,88	0,84	0,74	0,90	0,93
FCFB01097445.1	643	0,89	0,79	0,56	0,92	0,98	0,86	0,63	0,59	0,65	0,63	0,72	0,60
FCFB01103780.1	1843	0,71	1,00	0,60	0,78	1,00	0,94	0,00	0,00	0,00	1,00	1,00	1,00
FCFB01106002.1	6135	0,58	0,57	0,02	0,12	0,03	0,02	0,00	0,00	0,02	0,14	0,03	0,27
FCFB01106002.1	6139	0,94	0,80	0,13	0,95	0,98	1,00	0,99	0,98	1,00	0,75	0,69	0,88
FCFB01124373.1	355	0,93	0,92	0,00	0,47	0,17	0,62	0,00	0,09	0,72	1,00	0,83	1,00
FCFB01124477.1	960	0,65	0,17	0,22	0,28	0,15	0,00	0,01	0,02	0,02	0,05	0,06	0,00
FCFB01124477.1	962	0,65	0,17	0,22	0,28	0,15	0,00	0,01	0,00	0,02	0,05	0,06	0,00
FCFB01142548.1	1074	0,77	0,94	1,00	0,54	0,75	0,93	0,11	0,10	0,50	0,59	0,24	0,14
FCFB01142548.1	1076	0,07	0,03	1,00	0,46	0,40	0,77	0,90	1,00	0,71	0,60	0,76	0,86
FCFB01142548.1	1079	0,77	0,94	1,00	0,55	0,75	0,27	0,10	0,10	0,36	0,60	0,21	0,14
FCFB01151726.1	5818	0,92	0,08	1,00	0,84	0,85	0,05	0,00	0,00	0,05	0,92	0,17	1,00
FCFB01151726.1	5821	0,00	0,00	0,00	0,16	0,15	0,95	1,00	0,86	0,95	0,04	0,00	0,00
FCFB01151726.1	5822	1,00	1,00	1,00	1,00	1,00	0,98	1,00	1,00	1,00	0,16	0,83	0,04
FCFB01151726.1	5830	0,92	0,07	1,00	1,00	1,00	1,00	1,00	0,86	1,00	0,96	0,38	1,00

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01154087.1	1805	1,00	1,00	1,00	0,58	0,88	0,00	0,03	0,05	0,05	1,00	1,00	0,83
FCFB01168257.1	1681	1,00	0,99	0,10	0,81	0,33	0,99	0,90	0,95	0,98	0,64	0,58	0,48
FCFB01168257.1	1696	0,98	0,99	0,11	0,98	1,00	0,99	0,89	0,93	0,97	1,00	1,00	0,99
FCFB01208026.1	1613	0,62	0,75	0,99	0,05	0,02	0,01	1,00	1,00	0,16	0,90	0,91	0,74
FCFB01208026.1	1617	1,00	1,00	0,98	1,00	1,00	1,00	1,00	1,00	0,99	0,64	0,61	0,87
FCFB01210245.1	2852	0,00	0,00	0,00	0,11	0,05	0,00	0,33	0,35	0,33	0,00	0,02	0,00
FCFB01210245.1	2854	0,88	0,46	0,76	1,00	1,00	1,00	1,00	1,00	1,00	0,98	0,98	1,00
FCFB01210245.1	2863	1,00	1,00	0,52	1,00	1,00	1,00	1,00	1,00	0,99	0,52	0,73	0,42
FCFB01210245.1	2869	1,00	1,00	0,90	0,98	1,00	1,00	0,40	0,54	0,47	0,98	0,98	0,99
FCFB01275188.1	290	0,64	0,77	0,20	0,83	1,00	0,98	0,88	0,20	0,91	0,72	0,62	0,71
FCFB01019059.1	37208	1,00	1,00	0,78	1,00	1,00	0,00	0,13	0,18	0,11			
FCFB01019059.1	37213	0,00	0,00	0,22	0,00	0,01	1,00	0,88	0,83	0,90			
FCFB01024767.1	13400	0,00	0,10	1,00	0,06	0,00	0,00	1,00	0,83	0,41			
FCFB01028736.1	4381	0,97	0,76	1,00	0,39	0,22	0,00	1,00	1,00	0,41			
FCFB01028736.1	4384	0,97	0,77	0,42	0,40	0,23	0,01	0,00	0,00	0,01			
FCFB01029853.1	8882	1,00	1,00	0,88	0,57	0,82	0,06	0,00	0,00	0,11			
FCFB01029853.1	8892	0,04	0,66	0,38	0,44	0,18	0,94	1,00	1,00	0,89			
FCFB01073020.1	4748	1,00	1,00	0,38	0,03	0,04	0,00	0,59	0,92	0,20			
FCFB01073020.1	4756	0,00	0,00	0,63	0,97	0,96	1,00	0,41	0,08	0,79			
FCFB01081797.1	8369	0,98	0,96	0,97	0,34	0,06	0,80	0,12	0,14	0,29			
FCFB01081797.1	8370	0,05	0,04	0,03	0,69	0,95	0,20	0,88	0,86	0,71			
FCFB01081797.1	8371	0,93	0,89	0,06	0,97	1,00	1,00	1,00	1,00	1,00			
FCFB01081797.1	8373	0,91	0,85	0,03	0,32	0,06	0,80	1,00	1,00	1,00			
FCFB01081797.1	8379	0,05	0,04	0,94	0,00	0,00	0,00	0,88	0,88	0,71			

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01084658.1	31696	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00			
FCFB01087771.1	2128	0,67	0,60	0,00	0,03	0,12	0,09	0,30	0,24	0,02			
FCFB01087771.1	2132	0,33	0,40	1,00	0,97	0,88	0,91	0,70	0,82	0,98			
FCFB01087771.1	2147	0,33	0,40	1,00	0,97	0,88	0,91	0,69	0,82	0,98			
FCFB01099912.1	455	0,70	0,47	0,17	0,03	0,00	0,02	0,00	0,00	0,02			
FCFB01099912.1	456	0,71	0,47	0,17	0,03	0,00	0,01	0,00	0,00	0,02			
FCFB01107026.1	12144	0,81	0,13	1,00	1,00	1,00	1,00	1,00	1,00	1,00			
FCFB01107026.1	12148	0,36	0,90	0,00	0,96	1,00	1,00	0,95	1,00	1,00			
FCFB01107026.1	12163	0,36	0,90	0,00	0,90	1,00	1,00	0,95	1,00	1,00			
FCFB01114063.1	5202	0,76	0,96	0,00	0,40	0,10	1,00	0,03	0,09	0,70			
FCFB01114063.1	5208	0,72	0,95	0,00	0,10	0,00	1,00	0,01	0,02	0,68			
FCFB01114063.1	5209	0,28	0,05	1,00	0,90	1,00	0,01	0,99	0,98	0,32			
FCFB01114063.1	5211	0,73	0,95	0,00	0,10	0,00	1,00	1,00	1,00	0,98			
FCFB01114063.1	5212	0,28	0,06	1,00	0,90	0,99	0,00	0,99	0,98	0,32			
FCFB01118056.1	1163	0,04	0,50	0,43	0,02	0,04	0,96	1,00	1,00	0,97			
FCFB01118056.1	1171	0,04	0,40	0,35	0,00	0,01	0,96	1,00	1,00	0,97			
FCFB01118056.1	1174	0,03	0,10	0,25	0,02	0,03	0,96	1,00	1,00	0,97			
FCFB01118056.1	1179	0,00	0,00	0,13	0,00	0,01	0,96	1,00	1,00	0,97			
FCFB01118056.1	1184	0,00	0,00	0,13	0,00	0,00	0,96	1,00	1,00	0,97			
FCFB01126140.1	5555	0,30	0,00	0,54	1,00	1,00	1,00	1,00	1,00	1,00			
FCFB01126140.1	5558	0,60	0,54	0,46	1,00	0,95	1,00	1,00	1,00	0,98			
FCFB01126140.1	5561	0,40	0,54	0,00	0,00	0,05	0,00	0,08	0,00	0,02			
FCFB01146771.1	2284	0,02	0,11	0,00	0,47	0,15	1,00	1,00	1,00	0,98			
FCFB01146771.1	2290	0,98	0,94	1,00	0,52	0,85	0,00	0,00	0,00	0,02			

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01150471.1	389	0,00	0,00	0,00	0,00	0,00	1,00	1,00	1,00	0,82			
FCFB01150471.1	391	1,00	1,00	1,00	1,00	1,00	0,00	0,00	0,00	0,24			
FCFB01150471.1	400	1,00	1,00	1,00	1,00	1,00	0,10	0,00	0,00	0,24			
FCFB01150471.1	401	0,00	0,00	0,00	0,00	0,00	1,00	1,00	1,00	0,76			
FCFB01150471.1	406	0,00	0,00	0,00	0,02	0,01	1,00	1,00	1,00	0,76			
FCFB01151726.1	5809	1,00	1,00	0,22	1,00	1,00	1,00	1,00	1,00	1,00			
FCFB01151726.1	5811	1,00	1,00	1,00	0,84	0,85	0,05	0,00	0,00	0,05			
FCFB01198073.1	1199	0,21	0,42	1,00	0,11	0,00	0,02	0,01	0,00	0,00			
FCFB01198073.1	1202	0,86	0,92	1,00	0,10	0,00	0,04	0,01	0,00	0,09			
FCFB01198073.1	1205	0,21	0,58	1,00	0,10	0,00	0,00	0,01	0,00	0,00			
FCFB01225179.1	1891	0,98	1,00	1,00	0,71	0,91	0,00	0,08	0,20	0,10			
FCFB01254115.1	722	1,00	1,00	0,09	0,08	0,00	0,02	0,00	0,00	0,06			
FCFB01254115.1	733	0,00	0,00	0,91	0,93	1,00	1,00	1,00	1,00	0,94			
FCFB01321990.1	290	0,07	0,00	0,00	0,85	0,87	1,00	1,00	1,00	0,98			
FCFB01321990.1	292	0,93	1,00	1,00	0,15	0,23	0,00	0,00	0,00	0,02			
FCFB01321990.1	298	0,93	1,00	1,00	0,17	0,22	0,00	0,09	0,00	0,04			
FCFB01321990.1	300	0,07	0,00	0,00	0,85	0,87	1,00	1,00	1,00	0,98			
FCFB01321990.1	303	0,93	1,00	1,00	0,15	0,22	0,00	0,00	0,00	0,02			
FCFB01321990.1	304	0,93	1,00	1,00	0,15	0,22	0,00	0,00	0,00	0,02			
FCFB01002288.1	1807				0,49	0,78	0,05	0,00	0,20	0,02	0,80	1,00	1,00
FCFB01002288.1	1808				0,56	0,25	0,99	1,00	1,00	1,00	0,20	0,13	0,67
FCFB01004285.1	1506				1,00	1,00	1,00	0,32	0,10	0,43	0,80	0,83	1,00
FCFB01004809.1	3521				0,11	0,75	1,00	1,00	1,00	1,00	0,32	0,67	0,45
FCFB01011554.1	3901				1,00	1,00	1,00	1,00	1,00	1,00	0,50	0,33	0,90

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01011779.1	8292				0,99	1,00	1,00	0,28	0,47	0,84	0,78	0,81	0,85
FCFB01011779.1	8295				0,99	1,00	1,00	0,72	0,55	0,60	0,84	0,43	0,62
FCFB01011779.1	8297				0,95	1,00	1,00	1,00	0,98	1,00	0,54	0,44	0,38
FCFB01014883.1	1349				0,74	0,84	0,99	0,83	0,53	0,86	0,00	0,01	0,00
FCFB01014883.1	1352				0,00	0,06	0,00	0,00	0,00	0,02	0,83	0,95	0,75
FCFB01014883.1	1354				0,74	0,87	0,99	0,85	0,50	0,87	0,27	0,10	0,00
FCFB01016107.1	10345				0,75	0,93	0,06	0,52	0,27	0,31	0,90	1,00	1,00
FCFB01017702.1	34994				0,00	0,00	0,00	0,14	0,00	0,00	0,95	1,00	1,00
FCFB01017702.1	35001				1,00	1,00	1,00	1,00	1,00	1,00	0,05	0,00	0,00
FCFB01019801.1	16500				0,33	0,15	0,02	0,11	0,29	0,08	1,00	1,00	1,00
FCFB01023604.1	6614				0,00	0,00	0,02	0,00	0,00	0,00	0,59	0,54	0,57
FCFB01023604.1	6615				1,00	1,00	1,00	1,00	1,00	1,00	0,12	0,43	0,14
FCFB01023604.1	6626				0,06	0,00	0,00	0,00	0,00	0,02	0,56	0,50	0,57
FCFB01028921.1	20477				0,34	0,27	0,07	0,62	0,88	0,38	1,00	1,00	1,00
FCFB01031202.1	7756				1,00	1,00	1,00	1,00	1,00	1,00	0,29	0,00	0,00
FCFB01038320.1	759				0,02	0,00	0,00	1,00	1,00	0,40	0,84	0,57	0,86
FCFB01042182.1	20791				0,47	0,73	1,00	0,33	0,09	0,10	0,80	1,00	0,43
FCFB01043744.1	5223				0,38	0,33	0,02	0,05	0,22	0,10	0,95	0,90	0,86
FCFB01045921.1	1228				0,42	0,33	0,00	0,92	0,99	0,49	0,94	0,81	0,60
FCFB01045921.1	1233				0,62	0,67	1,00	0,08	0,01	0,49	0,00	0,00	0,20
FCFB01045921.1	1236				0,62	0,67	1,00	0,08	0,04	0,49	0,06	0,18	0,60
FCFB01045921.1	1239				0,00	0,00	0,00	0,92	0,99	0,52	0,17	0,24	0,44
FCFB01046615.1	9719				0,91	0,97	0,49	1,00	0,86	0,37	1,00	0,92	1,00
FCFB01051497.1	1271				1,00	1,00	1,00	0,44	0,60	1,00	0,20	0,00	0,14

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01051497.1	1273				1,00	1,00	1,00	1,00	1,00	1,00	0,20	0,00	0,29
FCFB01052781.1	1954				0,00	0,01	0,00	1,00	1,00	0,13	0,62	0,20	0,50
FCFB01052781.1	1963				1,00	1,00	1,00	0,00	0,00	0,87	0,43	0,83	0,52
FCFB01058186.1	391				1,00	1,00	1,00	1,00	0,99	1,00	0,74	0,76	0,71
FCFB01064292.1	610				1,00	1,00	1,00	1,00	1,00	1,00	0,40	0,44	0,08
FCFB01064403.1	5520				0,00	0,00	0,03	0,00	0,00	0,00	0,76	0,88	0,78
FCFB01064403.1	5526				0,00	0,00	0,05	0,00	0,00	0,00	0,71	0,88	0,78
FCFB01069167.1	93				0,69	0,70	1,00	0,31	0,43	0,83	0,00	0,08	0,00
FCFB01069167.1	96				0,70	0,70	1,00	0,33	0,45	0,83	0,13	0,17	0,00
FCFB01069167.1	102				0,70	0,70	1,00	0,33	0,43	0,83	0,00	0,09	0,00
FCFB01069167.1	105				0,30	0,30	0,00	0,67	0,55	0,17	1,00	0,91	1,00
FCFB01069605.1	667				1,00	1,00	1,00	0,86	0,67	0,76	0,01	0,00	0,00
FCFB01069605.1	673				1,00	1,00	1,00	0,87	0,67	0,76	0,02	0,00	0,00
FCFB01069605.1	680				1,00	1,00	1,00	0,80	0,67	0,76	0,00	0,01	0,00
FCFB01069755.1	8155				0,98	1,00	1,00	1,00	1,00	1,00	0,10	0,30	0,20
FCFB01069755.1	8165				1,00	1,00	1,00	1,00	1,00	1,00	0,10	0,30	0,53
FCFB01073332.1	763				0,09	0,60	1,00	1,00	0,90	0,92	0,67	0,81	0,18
FCFB01073332.1	769				0,91	1,00	0,06	1,00	1,00	1,00	1,00	1,00	1,00
FCFB01073906.1	9236				0,00	0,00	0,01	0,00	0,00	0,00	1,00	1,00	1,00
FCFB01080090.1	124				0,88	0,99	0,07	1,00	1,00	0,19	0,85	0,78	0,87
FCFB01082287.1	21726				1,00	1,00	1,00	1,00	1,00	1,00	0,17	0,00	0,12
FCFB01083052.1	1182				1,00	1,00	0,43	0,59	0,67	0,43	0,83	0,27	0,17
FCFB01084658.1	15657				1,00	1,00	0,97	0,67	0,45	0,96	0,00	0,00	0,10
FCFB01084658.1	15668				1,00	1,00	0,97	0,67	0,45	0,96	0,32	0,00	0,11

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01084658.1	15672				0,00	0,00	0,05	0,00	0,00	0,00	0,88	1,00	0,90
FCFB01084668.1	2097				0,38	0,51	0,96	0,89	1,00	1,00	1,00	0,91	1,00
FCFB01084668.1	2100				1,00	1,00	0,93	1,00	1,00	1,00	0,28	0,13	0,08
FCFB01084668.1	2106				1,00	1,00	0,93	1,00	1,00	1,00	0,28	0,09	0,08
FCFB01089343.1	143				1,00	1,00	1,00	1,00	0,78	1,00	0,17	0,26	0,38
FCFB01089343.1	151				1,00	1,00	1,00	1,00	0,78	1,00	0,42	0,29	0,50
FCFB01090299.1	9445				0,00	0,00	0,00	0,00	0,00	0,00	0,63	0,57	0,85
FCFB01091372.1	381				0,98	1,00	1,00	1,00	1,00	1,00	0,07	0,00	0,00
FCFB01091372.1	383				0,02	0,00	0,01	0,00	0,00	0,00	0,93	1,00	1,00
FCFB01091372.1	384				0,98	1,00	1,00	1,00	1,00	1,00	0,07	0,00	0,00
FCFB01093162.1	36				0,00	0,00	0,00	0,00	0,00	0,00	0,92	1,00	1,00
FCFB01093162.1	40				1,00	1,00	0,99	0,95	1,00	1,00	0,08	0,00	0,01
FCFB01093162.1	52				1,00	1,00	1,00	1,00	1,00	1,00	0,08	0,00	0,01
FCFB01093596.1	1884				0,40	1,00	1,00	0,33	0,00	0,42	0,33	0,00	0,00
FCFB01096446.1	7397				0,80	0,98	0,00	0,00	0,06	0,04	0,78	0,59	0,81
FCFB01101657.1	4979				0,25	0,16	0,09	0,22	0,63	0,05	0,89	0,95	0,96
FCFB01107500.1	6690				1,00	1,00	1,00	0,09	0,02	0,27	0,50	1,00	0,14
FCFB01107500.1	6692				1,00	1,00	1,00	1,00	1,00	1,00	0,61	1,00	0,29
FCFB01108805.1	1802				0,24	0,02	0,01	1,00	1,00	0,18	0,90	0,79	0,97
FCFB01108805.1	1806				0,78	0,99	0,99	0,00	0,04	0,82	0,10	0,21	0,03
FCFB01108805.1	1807				0,78	0,98	0,99	0,00	0,00	0,82	0,15	0,21	0,03
FCFB01108805.1	1813				0,22	0,02	0,01	1,00	1,00	0,18	0,90	0,79	0,97
FCFB01112633.1	6817				1,00	0,96	0,20	1,00	1,00	1,00	0,13	0,56	0,67

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01118339.1	2388				0,00	0,00	0,00	0,04	0,07	0,17	1,00	0,98	0,92
FCFB01118442.1	1482				0,00	0,00	1,00	0,00	0,00	0,20	0,47	0,12	0,21
FCFB01118661.1	237				0,84	0,95	0,98	0,35	0,17	0,61	0,64	0,62	0,96
FCFB01126784.1	19552				0,00	0,00	0,00	0,92	0,87	0,59	0,00	0,00	0,00
FCFB01126784.1	19553				1,00	1,00	0,82	0,96	0,97	0,95	0,00	0,09	0,17
FCFB01128139.1	3328				1,00	1,00	0,96	0,80	0,28	0,71	0,30	0,08	0,20
FCFB01137816.1	3539				0,28	0,83	0,95	0,33	0,29	0,21	0,17	0,22	0,08
FCFB01137816.1	3554				0,28	0,83	0,91	0,33	0,29	0,25	0,17	0,22	0,09
FCFB01144716.1	2171				1,00	1,00	1,00	0,82	0,25	0,87	0,50	0,14	0,40
FCFB01155803.1	1616				0,96	1,00	1,00	0,69	1,00	1,00	0,60	0,00	0,13
FCFB01155803.1	1619				0,96	1,00	0,99	0,69	1,00	1,00	0,60	0,00	0,13
FCFB01162114.1	1167				0,39	0,05	0,00	0,00	0,00	0,00	0,90	1,00	1,00
FCFB01188496.1	657				1,00	1,00	1,00	1,00	1,00	1,00	0,00	0,00	0,00
FCFB01188496.1	665				1,00	1,00	1,00	1,00	1,00	1,00	0,00	0,00	0,00
FCFB01214434.1	1978				0,14	0,50	0,16	0,55	0,00	0,10	1,00	1,00	1,00
FCFB01215204.1	2036				0,42	0,26	0,01	1,00	1,00	0,53	0,63	0,88	0,86
FCFB01215204.1	2042				1,00	1,00	0,99	0,41	0,58	0,87	1,00	0,95	1,00
FCFB01225617.1	1842				0,91	1,00	0,38	0,44	0,65	0,74	0,72	0,63	0,70
FCFB01232303.1	596				0,48	0,36	0,00	0,70	0,65	0,76	0,83	1,00	1,00
FCFB01255054.1	264				0,71	0,61	0,60	1,00	0,88	1,00	0,94	0,68	0,44
FCFB01294911.1	558				0,29	0,30	0,00	0,11	0,00	0,00	0,94	1,00	1,00
FCFB01300892.1	551				0,00	0,00	0,00	0,01	0,03	0,02	0,62	0,90	0,80
FCFB01003472.1	36475	1,00	0,94	1,00				0,17	0,08	0,00	0,85	0,75	0,67
FCFB01009985.1	16720	0,19	0,35	0,00				0,88	0,72	0,48	0,42	0,33	0,30

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01009985.1	16722	0,69	0,65	0,94				0,00	0,06	0,00	0,00	0,00	0,00
FCFB01009985.1	16728	0,79	0,75	0,96				0,00	0,00	0,05	0,10	0,00	0,02
FCFB01010669.1	6280	0,92	1,00	1,00				0,41	0,35	0,85	0,94	0,98	1,00
FCFB01010669.1	6281	0,00	0,00	0,00				0,62	0,67	0,15	0,00	0,00	0,00
FCFB01014988.1	5268	0,29	0,15	1,00				0,00	0,00	0,00	0,00	0,00	0,00
FCFB01014988.1	5274	1,00	0,23	1,00				0,00	0,00	0,00	0,00	0,02	0,00
FCFB01015742.1	1735	0,89	0,87	1,00				1,00	1,00	1,00	0,63	0,35	0,73
FCFB01022486.1	3703	1,00	1,00	1,00				0,00	0,00	0,08	0,16	0,27	0,31
FCFB01024389.1	6916	0,15	0,00	0,03				1,00	0,99	0,97	0,45	0,95	0,60
FCFB01041278.1	3751	0,00	0,00	0,00				0,34	0,35	0,44	0,42	0,05	0,08
FCFB01041352.1	13132	1,00	1,00	1,00				0,29	0,14	0,29	0,75	0,56	0,40
FCFB01041453.1	1383	0,75	0,57	0,13				0,10	0,00	0,20	0,56	0,67	0,53
FCFB01047729.1	862	0,00	0,00	0,00				0,86	0,96	0,90	1,00	0,75	1,00
FCFB01048258.1	15155	0,06	0,03	1,00				1,00	0,80	0,50	0,80	0,88	0,97
FCFB01052260.1	1816	1,00	0,73	0,47				0,83	1,00	0,93	0,19	0,40	0,13
FCFB01052260.1	1831	0,86	0,73	0,27				0,00	0,00	0,00	0,25	0,14	0,00
FCFB01053501.1	383	1,00	1,00	0,39				1,00	1,00	1,00	1,00	0,98	1,00
FCFB01054657.1	1700	0,83	0,67	0,39				0,15	0,30	0,17	0,15	0,23	0,15
FCFB01062652.1	4087	0,92	0,88	1,00				0,47	0,63	0,49	0,94	0,86	0,97
FCFB01062652.1	4093	0,29	0,36	0,76				1,00	0,95	0,84	0,08	0,14	0,11
FCFB01062652.1	4095	0,76	0,75	0,24				1,00	1,00	1,00	0,99	1,00	0,97
FCFB01062652.1	4098	0,97	0,88	1,00				0,47	0,65	0,54	0,94	0,86	0,94
FCFB01062652.1	4100	1,00	1,00	1,00				0,53	0,38	0,54	0,10	0,14	0,03
FCFB01062652.1	4104	0,34	0,36	0,82				1,00	0,98	0,88	0,13	0,16	0,05

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01062652.1	4107	0,93	1,00	1,00				0,53	0,38	0,60	0,96	1,00	0,98
FCFB01062652.1	4110	0,10	0,12	0,06				0,53	0,36	0,48	0,12	0,16	0,08
FCFB01066866.1	15527	0,00	0,00	0,00				1,00	1,00	0,95	0,64	1,00	0,60
FCFB01066866.1	15533	0,00	0,00	0,00				1,00	1,00	0,95	0,64	0,92	0,60
FCFB01066866.1	15540	1,00	1,00	1,00				0,00	0,00	0,05	0,36	0,08	0,40
FCFB01070975.1	3467	1,00	0,92	0,83				0,00	0,00	0,33	0,05	0,36	0,13
FCFB01077018.1	484	0,72	0,41	0,53				0,00	0,11	0,05	0,10	0,04	0,01
FCFB01077018.1	494	0,72	0,41	0,51				0,00	0,10	0,05	0,10	0,05	0,00
FCFB01079503.1	626	1,00	1,00	1,00				1,00	1,00	1,00	0,67	0,29	0,30
FCFB01079503.1	629	1,00	1,00	1,00				1,00	1,00	1,00	0,47	0,14	0,30
FCFB01079503.1	640	1,00	1,00	1,00				1,00	1,00	1,00	0,65	0,29	0,30
FCFB01079880.1	3398	0,50	0,00	1,00				0,00	0,06	0,00	0,63	0,25	0,20
FCFB01089594.1	1898	0,00	0,00	1,00				0,00	0,07	0,19	0,88	1,00	0,79
FCFB01089594.1	1904	0,00	0,00	0,00				0,00	0,07	0,19	0,81	0,97	0,55
FCFB01089594.1	1906	0,00	0,00	1,00				0,00	0,07	0,19	0,87	1,00	0,80
FCFB01089594.1	1915	1,00	1,00	0,00				1,00	0,94	1,00	0,94	0,97	0,79
FCFB01093755.1	1959	1,00	0,71	0,00				0,93	1,00	1,00	0,18	1,00	0,73
FCFB01093755.1	1964	0,00	0,29	1,00				0,00	0,00	0,00	0,09	0,10	0,00
FCFB01095113.1	156	1,00	0,60	1,00				0,00	0,00	0,00	0,00	0,00	0,00
FCFB01095113.1	159	1,00	0,60	1,00				0,00	0,00	0,00	0,00	0,00	0,00
FCFB01105402.1	1817	0,96	0,63	0,65				0,10	0,60	0,33	0,14	0,06	0,00
FCFB01111984.1	637	0,14	0,67	1,00				1,00	1,00	1,00	1,00	1,00	0,98
FCFB01111984.1	639	0,14	0,67	1,00				1,00	1,00	1,00	1,00	1,00	0,98

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01111984.1	651	0,86	0,33	0,00				0,00	0,00	0,00	0,00	0,00	0,02
FCFB01113245.1	3514	0,92	1,00	1,00				1,00	0,83	1,00	0,38	0,21	0,80
FCFB01113245.1	3521	0,92	1,00	1,00				1,00	0,83	1,00	0,00	0,14	0,20
FCFB01113245.1	3523	0,25	0,67	0,80				1,00	0,83	0,93	0,00	0,00	0,00
FCFB01113331.1	1794	0,00	0,00	0,04				0,87	0,86	0,65	0,00	0,00	0,00
FCFB01113331.1	1806	0,00	0,00	0,00				0,87	0,86	0,65	0,00	0,00	0,01
FCFB01113331.1	1811	0,00	0,00	0,00				0,87	0,88	0,65	0,00	0,00	0,02
FCFB01130734.1	1894	0,00	0,11	0,66				0,00	0,50	0,17	0,00	0,00	0,00
FCFB01134328.1	1518	0,06	0,24	0,43				0,12	0,10	0,20	0,21	0,89	0,96
FCFB01134328.1	1521	0,96	0,71	0,50				0,88	0,91	0,80	0,18	0,21	0,07
FCFB01142893.1	318	0,80	0,60	0,04				0,05	0,00	0,12	0,21	0,01	0,00
FCFB01142893.1	325	0,80	0,60	0,04				0,05	0,22	0,24	0,22	0,01	0,03
FCFB01142893.1	333	0,80	0,61	0,04				0,05	0,00	0,18	0,22	0,01	0,00
FCFB01142893.1	337	0,80	0,60	0,04				0,05	0,22	0,24	0,29	0,03	0,04
FCFB01142893.1	339	0,79	0,59	0,04				0,00	0,18	0,24	0,07	0,01	0,03
FCFB01144512.1	697	0,00	0,29	0,36				0,20	0,80	0,31	0,69	0,38	0,73
FCFB01145528.1	358	0,09	0,10	0,22				1,00	0,82	0,70	0,43	0,67	1,00
FCFB01145528.1	364	0,01	0,08	0,00				0,94	0,82	0,52	0,43	0,67	0,75
FCFB01145528.1	374	0,01	0,08	0,78				0,94	0,82	0,49	0,43	0,67	0,75
FCFB01147219.1	232	0,00	0,05	0,14				0,12	0,18	0,05	0,51	0,37	0,71
FCFB01168968.1	1622	0,97	0,86	0,29				1,00	1,00	0,82	0,45	0,20	0,80
FCFB01168968.1	1632	0,01	0,00	0,47				0,73	0,44	0,36	0,36	0,90	0,90
FCFB01168968.1	1633	0,99	1,00	0,59				0,36	0,54	0,73	0,64	0,30	0,80

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01174673.1	2148	0,27	0,29	0,56				1,00	1,00	1,00	0,92	1,00	1,00
FCFB01174673.1	2151	0,73	0,71	0,56				0,00	0,00	0,09	0,25	0,00	0,00
FCFB01174673.1	2153	1,00	1,00	0,67				1,00	1,00	1,00	0,50	0,56	0,75
FCFB01198322.1	4478	0,88	0,86	1,00				0,00	0,00	0,00	0,21	0,07	0,02
FCFB01198322.1	4485	0,88	0,86	1,00				0,00	0,00	0,00	0,21	0,07	0,02
FCFB01198322.1	4488	0,88	0,86	1,00				0,00	0,00	0,00	0,21	0,07	0,02
FCFB01198322.1	4493	0,88	0,57	1,00				0,00	0,00	0,00	0,00	0,00	0,00
FCFB01198322.1	4495	0,13	0,14	0,00				1,00	1,00	1,00	0,79	0,93	0,98
FCFB01199498.1	615	1,00	1,00	0,04				0,99	0,95	0,68	1,00	1,00	1,00
FCFB01204157.1	1204	1,00	1,00	0,17				0,75	0,75	0,96	0,94	1,00	0,50
FCFB01213984.1	238	1,00	0,93	0,12				1,00	1,00	0,97	1,00	1,00	1,00
FCFB01213984.1	241	0,33	0,87	0,76				0,25	0,47	0,38	0,42	0,46	0,27
FCFB01223955.1	2073	0,14	0,30	0,71				1,00	1,00	1,00	1,00	1,00	1,00
FCFB01225398.1	396	1,00	1,00	1,00				0,14	0,01	0,01	0,25	0,16	0,30
FCFB01225398.1	403	1,00	1,00	0,92				0,37	0,30	0,24	0,46	0,41	0,50
FCFB01247832.1	313	1,00	1,00	1,00				1,00	1,00	1,00	0,20	0,16	0,39
FCFB01260649.1	83	1,00	1,00	1,00				0,57	0,23	0,77	0,40	0,00	0,44
FCFB01260649.1	85	0,96	0,93	0,20				0,59	0,70	0,92	0,48	0,53	0,67
FCFB01260649.1	87	0,96	0,94	0,00				0,17	0,38	0,82	0,19	0,33	0,42
FCFB01263184.1	677	0,86	1,00	1,00				0,49	0,68	0,28	1,00	1,00	1,00
FCFB01280708.1	508	0,86	0,83	0,08				0,00	0,00	0,00	0,00	0,06	0,00
FCFB01280708.1	524	0,86	0,84	0,08				0,00	0,00	0,00	0,00	0,06	0,00
FCFB01002326.1	9331	0,92	0,91	1,00	0,96	1,00	0,91				0,61	0,60	0,29
FCFB01002326.1	9350	0,00	0,00	0,00	0,35	0,39	0,00				0,00	0,00	0,00

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01007362.1	333	1,00	1,00	1,00	0,75	0,45	1,00				1,00	1,00	1,00
FCFB01007574.1	5165	0,01	0,04	0,98	0,03	0,09	0,11				0,58	0,75	0,43
FCFB01007574.1	5174	0,02	0,04	0,03	0,74	0,73	0,56				0,32	0,50	0,57
FCFB01009009.1	1668	0,48	0,40	1,00	0,18	0,50	0,50				0,83	0,88	0,94
FCFB01011377.1	14289	0,58	0,75	0,94	0,00	0,01	0,00				0,07	0,00	0,00
FCFB01015988.1	12562	0,97	0,90	0,14	0,98	1,00	1,00				0,44	0,16	0,16
FCFB01016038.1	1206	1,00	1,00	1,00	1,00	1,00	1,00				0,56	0,33	0,20
FCFB01016038.1	1215	0,20	0,00	0,00	0,03	0,03	0,07				0,33	0,33	0,20
FCFB01020785.1	13917	0,90	0,94	0,29	0,94	1,00	1,00				0,59	0,63	0,38
FCFB01040148.1	1502	0,50	0,82	0,00	0,36	0,01	0,01				0,94	0,58	0,63
FCFB01044932.1	4651	1,00	0,30	1,00	0,50	1,00	1,00				0,41	0,67	0,43
FCFB01050786.1	23662	0,95	1,00	0,00	1,00	1,00	1,00				0,12	0,00	0,11
FCFB01052098.1	21815	1,00	1,00	1,00	1,00	1,00	1,00				0,05	0,05	0,00
FCFB01060683.1	653	1,00	1,00	1,00	1,00	1,00	1,00				0,03	0,00	0,06
FCFB01060683.1	654	0,67	0,92	0,96	0,50	0,44	1,00				1,00	1,00	0,94
FCFB01060683.1	662	1,00	0,92	0,00	1,00	1,00	1,00				0,03	0,00	0,06
FCFB01060683.1	663	1,00	0,92	0,00	1,00	1,00	1,00				1,00	1,00	1,00
FCFB01060683.1	665	1,00	1,00	1,00	1,00	1,00	1,00				0,03	0,00	0,06
FCFB01063552.1	2463	0,05	0,63	0,11	0,00	0,00	0,00				0,33	0,21	0,42
FCFB01063552.1	2469	0,07	0,58	0,89	0,02	0,00	0,01				0,50	0,64	0,74
FCFB01063552.1	2472	0,85	0,88	0,17	0,19	0,02	0,05				0,45	0,26	0,43
FCFB01063869.1	12883	0,93	0,93	0,86	1,00	1,00	1,00				1,00	0,67	0,38
FCFB01063869.1	12889	1,00	1,00	1,00	1,00	1,00	1,00				0,80	0,83	0,13
FCFB01065451.1	461	0,03	0,11	0,33	0,00	0,00	0,13				1,00	0,63	1,00

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01072910.1	9249	0,79	0,95	0,67	0,16	0,03	0,95				0,80	0,78	0,83
FCFB01075461.1	539	1,00	1,00	0,03	0,85	1,00	1,00				1,00	0,92	0,93
FCFB01081058.1	194	0,66	0,05	0,88	0,97	0,99	0,43				0,40	0,39	0,21
FCFB01081058.1	195	0,99	0,97	0,14	0,99	0,99	1,00				0,92	0,81	0,95
FCFB01081058.1	198	0,98	0,91	0,97	0,98	1,00	1,00				0,72	0,70	0,63
FCFB01088939.1	1453	0,95	0,83	0,14	0,95	0,89	0,91				0,33	0,09	0,10
FCFB01099809.1	1504	0,92	1,00	0,41	0,50	0,80	0,99				0,23	0,19	0,54
FCFB01108160.1	4732	0,00	0,00	1,00	0,14	0,00	0,00				0,64	0,13	0,08
FCFB01114965.1	9126	0,98	1,00	0,71	0,14	0,01	0,01				0,58	0,61	0,62
FCFB01114965.1	9133	0,85	0,87	0,79	0,99	1,00	1,00				0,52	0,61	0,65
FCFB01124854.1	2461	0,98	0,94	0,60	0,11	0,20	1,00				0,78	0,17	0,86
FCFB01124854.1	2468	0,05	0,16	0,20	0,89	0,70	0,00				0,56	0,83	0,71
FCFB01135735.1	839	0,92	0,63	0,97	0,67	0,60	1,00				0,26	0,29	0,56
FCFB01143881.1	337	0,99	0,99	0,89	0,34	0,16	0,17				0,36	0,10	0,29
FCFB01143881.1	343	1,00	0,99	0,94	0,97	1,00	1,00				0,36	0,36	0,43
FCFB01175538.1	4903	0,11	0,38	0,00	0,99	1,00	1,00				1,00	0,98	0,89
FCFB01217469.1	23	0,60	0,43	0,80	1,00	1,00	1,00				1,00	1,00	1,00
FCFB01217469.1	26	0,60	0,40	0,80	1,00	1,00	1,00				0,95	1,00	1,00
FCFB01217469.1	32	0,40	0,61	0,20	0,00	0,00	0,00				0,00	0,00	0,00
FCFB01217469.1	34	0,65	0,40	1,00	1,00	1,00	1,00				0,95	1,00	0,98
FCFB01217469.1	35	0,59	0,40	0,80	1,00	1,00	1,00				0,95	1,00	1,00
FCFB01217469.1	43	0,80	0,80	0,64	0,29	0,00	0,00				0,20	0,19	0,38
FCFB01250400.1	476	0,88	0,88	0,24	0,17	0,38	0,07				0,38	0,40	0,92
FCFB01250400.1	477	0,95	0,75	1,00	0,09	0,38	0,06				0,57	1,00	0,69

Chr	start	Day1E1	Day1E2	Day1E3	Day30E1	Day30E2	Day30E3	Day90E1	Day90E2	Day90E3	Day200E1	Day200E2	Day200E3
FCFB01250400.1	479	0,95	0,88	1,00	0,23	0,38	0,06				0,86	1,00	0,69
FCFB01250400.1	480	0,82	0,25	0,76	0,20	0,08	0,07				0,62	0,20	0,43
FCFB01250400.1	485	1,00	1,00	0,24	0,96	0,92	1,00				0,46	0,40	0,73
FCFB01279345.1	492	0,15	0,00	0,00	0,64	0,40	0,00				0,00	0,00	0,00

Data S4.3 Methylation percentage of the differently methylated DNA sites in *Lymnaea stagnalis* genome between eDNA and tissue DNA for age class of 1 day.

Chr	Start	Day1T1	Day1E1	Day1T2	Day1E2	Day1T3	Day1E3	Type
FCFB01067681.1	14	0,17	0,83	0,27	0,82	0,16	0,82	CpG
FCFB01067681.1	20	0,16	0,86	0,29	0,75	0,18	0,78	CpG
FCFB01067681.1	30	0,13	0,90	0,20	0,86	0,13	0,82	CpG
FCFB01067681.1	33	0,11	0,86	0,17	0,89	0,07	0,75	CpG
FCFB01067681.1	35	0,11	0,80	0,17	0,85	0,07	0,85	CpG
FCFB01067681.1	40	0,09	0,86	0,09	0,88	0,07	0,82	CpG
FCFB01067681.1	55	0,07	0,83	0,06	0,68	0,04	0,81	CpG
FCFB01067681.1	67	0,16	0,83	0,14	0,82	0,02	0,87	CpG
FCFB01089978.1	5781	0,93	0,06	0,95	0,11	0,83	0,12	CpG
FCFB01089978.1	5790	1,00	0,12	0,95	0,12	1,00	0,12	CpG
FCFB01209936.1	550	0,12	0,41	0,13	0,62	0,10	0,54	CpG
FCFB01209936.1	565	0,34	0,90	0,46	0,79	0,56	0,78	CpG
FCFB01067681.1	9	0,72	0,93	0,68	0,93	0,60	0,94	CHG
FCFB01067681.1	15	0,17	0,73	0,29	0,86	0,22	0,84	CHG
FCFB01067681.1	23	0,16	0,87	0,28	0,82	0,15	0,84	CHG
FCFB01067681.1	47	0,06	0,80	0,06	0,89	0,04	0,84	CHG
FCFB01067681.1	56	0,06	0,80	0,05	0,71	0,04	0,81	CHG
FCFB01000988.1	20049	0,98	0,30	0,99	0,91	1,00	0,36	CHH
FCFB01000988.1	20050	0,00	0,70	0,00	0,09	0,00	0,64	CHH
FCFB01000988.1	20056	1,00	0,30	1,00	0,91	1,00	0,36	CHH
FCFB01001312.1	1912	0,00	0,95	0,01	0,00	0,00	0,00	CHH
FCFB01008219.1	12788	0,00	1,00	0,00	0,85	0,00	0,72	CHH
FCFB01008219.1	12801	1,00	0,01	1,00	0,57	1,00	0,50	CHH
FCFB01008432.1	7155	0,00	0,60	0,02	0,52	0,00	0,00	CHH
FCFB01008432.1	7159	1,00	0,40	0,98	0,48	1,00	1,00	CHH
FCFB01008432.1	7170	0,00	0,40	0,02	0,29	0,00	0,00	CHH
FCFB01008432.1	7171	0,00	0,60	0,02	0,51	0,00	0,00	CHH
FCFB01015135.1	9974	0,00	0,50	0,00	0,40	0,00	0,07	CHH
FCFB01015988.1	12562	0,00	0,97	0,00	0,90	0,00	0,14	CHH
FCFB01018271.1	36534	1,00	0,73	1,00	0,44	1,00	0,92	CHH
FCFB01018271.1	36540	0,00	0,27	0,00	0,56	0,00	0,08	CHH
FCFB01035591.1	1991	1,00	0,02	0,71	0,14	1,00	0,00	CHH
FCFB01035591.1	1995	0,00	1,00	0,29	0,86	0,00	1,00	CHH
FCFB01035591.1	1999	0,00	1,00	0,29	0,86	0,00	1,00	CHH
FCFB01035591.1	2004	0,00	1,00	0,29	0,86	0,00	1,00	CHH
FCFB01035591.1	2011	0,00	1,00	0,29	0,86	0,00	1,00	CHH
FCFB01039333.1	165	0,00	0,06	0,00	0,06	0,11	0,55	CHH

Chr	Start	Day1T1	Day1E1	Day1T2	Day1E2	Day1T3	Day1E3	Type
FCFB01042712.1	4179	1,00	0,56	1,00	0,72	1,00	0,94	CHH
FCFB01042712.1	4180	1,00	0,56	1,00	0,72	1,00	0,94	CHH
FCFB01046721.1	663	0,91	0,82	0,90	0,90	0,98	0,31	CHH
FCFB01046721.1	664	0,00	0,00	0,00	0,00	0,00	0,67	CHH
FCFB01046721.1	666	1,00	1,00	1,00	1,00	1,00	0,33	CHH
FCFB01046721.1	667	0,02	0,00	0,00	0,00	0,00	0,67	CHH
FCFB01046721.1	672	0,00	0,00	0,00	0,00	0,00	0,66	CHH
FCFB01048258.1	15155	1,00	0,06	1,00	0,03	1,00	1,00	CHH
FCFB01051395.1	3480	1,00	0,20	1,00	0,04	1,00	0,50	CHH
FCFB01054657.1	1700	0,07	0,83	0,40	0,67	0,00	0,39	CHH
FCFB01055290.1	14611	1,00	0,03	1,00	0,18	1,00	0,71	CHH
FCFB01055290.1	14612	0,00	0,97	0,00	0,78	0,00	0,29	CHH
FCFB01055290.1	14614	1,00	0,03	1,00	0,33	1,00	0,71	CHH
FCFB01061499.1	8897	1,00	0,43	0,95	0,55	1,00	0,86	CHH
FCFB01067681.1	11	0,17	0,86	0,28	0,86	0,20	0,80	CHH
FCFB01067681.1	26	0,14	0,90	0,24	0,86	0,17	0,81	CHH
FCFB01067681.1	27	0,14	0,86	0,22	0,86	0,15	0,85	CHH
FCFB01067681.1	42	0,07	0,80	0,07	0,89	0,04	0,84	CHH
FCFB01067681.1	44	0,09	0,86	0,06	0,86	0,07	0,81	CHH
FCFB01067681.1	48	0,07	0,86	0,06	0,89	0,04	0,84	CHH
FCFB01075100.1	13376	0,88	0,20	0,74	0,50	0,71	0,29	CHH
FCFB01077018.1	484	0,06	0,72	0,00	0,41	0,00	0,53	CHH
FCFB01077018.1	494	0,06	0,72	0,00	0,41	0,00	0,51	CHH
FCFB01080580.1	2196	1,00	0,00	1,00	0,03	1,00	0,13	CHH
FCFB01080580.1	2199	0,00	0,81	0,00	0,90	0,00	0,75	CHH
FCFB01080580.1	2202	0,00	0,20	0,00	0,43	0,00	0,88	CHH
FCFB01080580.1	2205	0,00	0,81	0,00	0,53	0,00	0,75	CHH
FCFB01083675.1	346	1,00	0,05	1,00	0,02	1,00	0,80	CHH
FCFB01083675.1	355	0,00	0,96	0,00	1,00	0,00	0,20	CHH
FCFB01085219.1	1456	0,85	0,63	0,79	0,38	0,82	0,50	CHH
FCFB01085219.1	1457	0,15	0,38	0,22	0,69	0,18	0,57	CHH
FCFB01085219.1	1465	0,92	0,75	0,93	0,44	0,94	0,47	CHH
FCFB01085219.1	1468	0,85	0,63	0,69	0,31	0,82	0,43	CHH
FCFB01087352.1	3452	1,00	0,29	1,00	0,80	1,00	0,83	CHH
FCFB01087352.1	3461	0,00	0,71	0,00	0,20	0,00	0,00	CHH
FCFB01088939.1	1453	0,00	0,95	0,00	0,83	0,00	0,14	CHH
FCFB01088979.1	2936	0,66	0,44	0,87	0,13	1,00	0,59	CHH
FCFB01097445.1	632	0,94	0,23	0,98	0,54	0,96	0,76	CHH

Chr	Start	Day1T1	Day1E1	Day1T2	Day1E2	Day1T3	Day1E3	Type
FCFB01111984.1	637	1,00	0,14	1,00	0,67	1,00	1,00	CHH
FCFB01111984.1	639	1,00	0,14	1,00	0,67	1,00	1,00	CHH
FCFB01111984.1	651	0,00	0,86	0,00	0,33	0,00	0,00	CHH
FCFB01130734.1	1894	0,00	0,00	0,00	0,11	0,12	0,66	CHH
FCFB01130979.1	88	0,00	0,95	0,00	0,93	0,00	0,13	CHH
FCFB01130979.1	90	0,00	0,95	0,00	0,93	0,00	0,13	CHH
FCFB01130979.1	91	1,00	0,05	1,00	0,07	1,00	0,88	CHH
FCFB01132183.1	2114	0,23	0,08	0,55	0,17	0,56	0,19	CHH
FCFB01134328.1	1518	1,00	0,06	1,00	0,24	1,00	0,43	CHH
FCFB01134328.1	1521	0,00	0,96	0,00	0,71	0,00	0,50	CHH
FCFB01142893.1	318	0,00	0,80	0,04	0,60	0,00	0,04	CHH
FCFB01142893.1	325	0,00	0,80	0,00	0,60	0,00	0,04	CHH
FCFB01142893.1	333	0,00	0,80	0,00	0,61	0,00	0,04	CHH
FCFB01142893.1	337	0,00	0,80	0,00	0,60	0,05	0,04	CHH
FCFB01142893.1	339	0,00	0,79	0,00	0,59	0,00	0,04	CHH
FCFB01144512.1	700	0,39	0,88	0,46	0,85	0,53	0,58	CHH
FCFB01217469.1	23	1,00	0,60	1,00	0,43	1,00	0,80	CHH
FCFB01217469.1	26	1,00	0,60	1,00	0,40	1,00	0,80	CHH
FCFB01217469.1	32	0,00	0,40	0,00	0,61	0,00	0,20	CHH
FCFB01217469.1	34	1,00	0,65	1,00	0,40	1,00	1,00	CHH
FCFB01217469.1	35	1,00	0,59	1,00	0,40	1,00	0,80	CHH
FCFB01217469.1	38	1,00	0,41	1,00	0,25	1,00	0,67	CHH
FCFB01217469.1	40	0,41	0,82	0,32	0,80	0,39	0,52	CHH
FCFB01217469.1	43	0,44	0,80	0,31	0,80	0,39	0,64	CHH

Data S4.4 Methylation percentage of the differently methylated DNA sites in *Lymnaea stagnalis* genome between eDNA and tissue DNA for age class of 30 day.

Chr	Start	Day30T1	Day30E1	Day30T2	Day30E2	Day30T3	Day30E3	Type
FCFB01002763.1	71	0,00	0,92	0,00	0,67	0,40	0,86	CHH
FCFB01002763.1	73	0,00	0,92	0,00	0,67	0,40	0,86	CHH
FCFB01002763.1	77	0,00	0,92	0,00	0,67	0,40	0,86	CHH
FCFB01002763.1	80	0,00	0,92	0,00	0,67	0,40	0,86	CHH
FCFB01002763.1	89	0,00	0,92	0,00	0,67	0,40	1,00	CHH
FCFB01002763.1	90	1,00	0,08	1,00	0,33	0,60	0,14	CHH
FCFB01002763.1	97	0,00	0,92	0,00	0,67	0,40	0,86	CHH
FCFB01003142.1	1634	1,00	0,05	1,00	0,00	0,81	0,00	CHH
FCFB01006232.1	4942	1,00	0,11	1,00	0,00	0,50	0,00	CHH
FCFB01006232.1	4949	1,00	0,11	1,00	0,00	0,50	0,00	CHH
FCFB01006232.1	4952	0,00	0,89	0,00	1,00	0,50	1,00	CHH
FCFB01006232.1	4958	0,00	0,89	0,00	1,00	0,50	1,00	CHH
FCFB01010492.1	1165	1,00	0,00	1,00	0,00	0,80	0,00	CHH
FCFB01010492.1	1167	0,00	1,00	0,00	1,00	0,20	1,00	CHH
FCFB01010492.1	1172	1,00	0,00	1,00	0,00	0,80	0,00	CHH
FCFB01010492.1	1173	0,20	1,00	0,00	1,00	0,20	1,00	CHH
FCFB01015988.1	12555	1,00	0,15	1,00	0,01	0,95	0,04	CHH
FCFB01015988.1	12562	0,00	0,98	0,07	1,00	0,48	1,00	CHH
FCFB01015988.1	12564	1,00	0,15	1,00	0,02	0,93	0,04	CHH
FCFB01019441.1	49779	0,00	1,00	0,00	1,00	0,50	1,00	CHH
FCFB01020365.1	1392	0,14	1,00	0,00	1,00	0,29	1,00	CHH
FCFB01023281.1	672	0,20	0,96	0,40	0,89	0,20	1,00	CHH
FCFB01023281.1	674	0,00	0,96	0,00	0,89	0,00	1,00	CHH
FCFB01030999.1	10899	0,05	0,96	0,00	0,97	0,06	0,85	CHH
FCFB01049326.1	16796	0,00	0,17	0,00	0,22	0,20	0,98	CHH
FCFB01049326.1	16802	0,00	1,00	0,00	1,00	0,20	0,98	CHH
FCFB01049326.1	16809	1,00	0,82	1,00	0,78	0,80	0,03	CHH
FCFB01050299.1	659	0,13	0,93	0,00	1,00	0,23	1,00	CHH
FCFB01050668.1	1578	1,00	0,00	1,00	0,00	0,09	0,01	CHH
FCFB01050668.1	1585	0,00	0,89	0,00	0,93	0,91	1,00	CHH
FCFB01056745.1	9505	0,00	0,82	0,00	0,91	0,44	1,00	CHH
FCFB01056745.1	9514	1,00	0,18	1,00	0,09	0,54	0,00	CHH
FCFB01056745.1	9515	0,00	0,82	0,00	0,91	0,46	1,00	CHH
FCFB01061499.1	8907	0,00	0,33	0,00	0,50	0,00	0,13	CHH
FCFB01064671.1	2549	1,00	0,00	1,00	0,00	1,00	0,00	CHH
FCFB01064671.1	2557	1,00	0,00	1,00	0,00	1,00	0,00	CHH
FCFB01064671.1	2560	0,00	1,00	0,00	1,00	0,00	1,00	CHH

Chr	Start	Day30T1	Day30E1	Day30T2	Day30E2	Day30T3	Day30E3	Type
FCFB01072226.1	6218	0,95	0,04	1,00	0,01	0,78	0,00	CHH
FCFB01072226.1	6221	0,11	0,96	0,00	0,99	0,21	1,00	CHH
FCFB01072226.1	6228	0,05	0,95	0,00	0,99	0,22	1,00	CHH
FCFB01072226.1	6232	0,05	0,96	0,00	0,99	0,21	1,00	CHH
FCFB01072226.1	6235	0,05	0,95	0,00	0,99	0,21	1,00	CHH
FCFB01076149.1	19955	1,00	1,00	1,00	0,90	1,00	0,17	CHH
FCFB01081058.1	194	0,00	0,97	0,00	0,99	0,13	0,43	CHH
FCFB01081058.1	198	0,00	0,98	0,13	1,00	0,25	1,00	CHH
FCFB01086732.1	5935	0,00	0,92	0,00	0,46	0,35	1,00	CHH
FCFB01086732.1	5950	0,00	0,92	0,04	0,46	0,35	0,23	CHH
FCFB01087352.1	3450	1,00	0,64	1,00	0,72	0,91	0,12	CHH
FCFB01087352.1	3452	1,00	0,38	1,00	0,31	1,00	1,00	CHH
FCFB01088939.1	1453	0,00	0,95	0,00	0,89	0,14	0,91	CHH
FCFB01096978.1	3282	0,00	0,99	0,08	1,00	0,15	1,00	CHH
FCFB01096978.1	3284	1,00	0,01	1,00	0,00	0,85	0,00	CHH
FCFB01096978.1	3287	1,00	0,02	0,92	0,00	0,85	0,00	CHH
FCFB01096978.1	3295	0,00	0,99	0,08	1,00	0,15	1,00	CHH
FCFB01097413.1	46	0,00	0,84	0,00	0,88	0,24	0,83	CHH
FCFB01097413.1	49	1,00	0,16	1,00	0,13	0,78	0,17	CHH
FCFB01097445.1	632	0,91	0,14	0,92	0,03	0,96	0,33	CHH
FCFB01097445.1	643	0,65	0,92	0,68	0,98	0,70	0,86	CHH
FCFB01107048.1	1798	0,00	0,94	0,00	1,00	0,50	1,00	CHH
FCFB01107082.1	651	0,07	0,95	0,00	0,99	0,11	0,99	CHH
FCFB01107082.1	659	0,03	0,95	0,00	0,99	0,11	0,99	CHH
FCFB01107082.1	666	0,13	0,95	0,17	0,99	0,11	0,99	CHH
FCFB01118442.1	1482	0,00	0,00	0,02	0,00	0,44	1,00	CHH
FCFB01118938.1	3692	1,00	0,14	1,00	0,00	0,71	0,18	CHH
FCFB01118938.1	3695	0,00	0,85	0,00	1,00	0,29	0,82	CHH
FCFB01126784.1	19553	0,00	1,00	0,00	1,00	0,70	0,82	CHH
FCFB01138844.1	558	0,00	1,00	0,00	0,71	0,33	0,78	CHH
FCFB01142186.1	2463	1,00	0,02	1,00	0,00	0,75	0,00	CHH
FCFB01142186.1	2474	0,00	0,98	0,00	1,00	0,25	1,00	CHH
FCFB01155803.1	1616	0,00	0,96	0,00	1,00	0,00	1,00	CHH
FCFB01155803.1	1619	0,00	0,96	0,00	1,00	0,00	0,99	CHH
FCFB01163516.1	513	0,00	0,99	0,00	1,00	0,50	1,00	CHH
FCFB01163516.1	515	0,00	0,99	0,00	1,00	0,50	1,00	CHH
FCFB01281800.1	491	0,00	1,00	0,00	0,90	0,00	1,00	CHH
FCFB01281800.1	494	0,43	1,00	0,44	0,90	0,50	1,00	CHH

Data S4.5 Methylation percentage of the differently methylated DNA sites in *Lymnaea stagnalis* genome between eDNA and tissue DNA for age class of 90 day.

Chr	Start	Day90T1	Day90E1	Day90T2	Day90E2	Day90T3	Day90E3	Type
FCFB01015015.1	412	0,41	0,60	0,37	0,69	0,43	1,00	CpG
FCFB01026221.1	30997	0,09	0,25	0,05	0,50	0,10	0,29	CpG
FCFB01067681.1	40	0,61	0,82	0,51	0,72	0,49	0,81	CpG
FCFB01067681.1	55	0,20	0,80	0,15	0,69	0,16	0,75	CpG
FCFB01067681.1	67	0,22	0,78	0,18	0,71	0,17	0,83	CpG
FCFB01090249.1	1131	0,49	0,19	0,47	0,13	0,45	0,27	CpG
FCFB01122052.1	7354	0,51	0,95	0,48	0,93	0,45	0,67	CpG
FCFB01141377.1	3586	0,03	0,75	0,01	0,80	0,02	0,63	CpG
FCFB01167088.1	6	0,40	0,85	0,45	0,86	0,47	0,76	CpG
FCFB01268192.1	166	0,12	1,00	0,00	1,00	0,08	1,00	CpG
FCFB01281122.1	678	0,22	0,56	0,16	0,50	0,22	0,48	CpG
FCFB01067681.1	47	0,20	0,78	0,15	0,68	0,16	0,79	CHG
FCFB01067681.1	56	0,19	0,80	0,16	0,72	0,16	0,77	CHG
FCFB01122052.1	7348	0,52	0,86	0,50	1,00	0,45	0,61	CHG
FCFB01268192.1	165	0,00	1,00	0,00	1,00	0,08	1,00	CHG
FCFB01002763.1	71	0,00	0,88	0,00	0,60	0,00	0,64	CHH
FCFB01002763.1	73	0,00	0,88	0,00	0,60	0,00	0,64	CHH
FCFB01002763.1	77	0,00	0,88	0,00	0,60	0,00	0,64	CHH
FCFB01002763.1	80	0,00	0,88	0,00	0,60	0,00	0,64	CHH
FCFB01002763.1	89	0,00	0,88	0,00	0,60	0,00	0,64	CHH
FCFB01002763.1	90	1,00	0,13	1,00	0,40	1,00	0,36	CHH
FCFB01002763.1	94	0,00	0,88	0,00	0,60	0,00	0,64	CHH
FCFB01002763.1	97	0,08	0,88	0,00	0,60	0,00	0,62	CHH
FCFB01007975.1	1501	1,00	0,02	0,80	0,03	1,00	0,00	CHH
FCFB01007975.1	1506	0,00	0,98	0,00	0,97	0,00	1,00	CHH
FCFB01009136.1	1304	0,94	0,15	0,72	0,00	0,82	0,05	CHH
FCFB01010669.1	6280	0,96	0,41	0,99	0,35	0,99	0,85	CHH
FCFB01010669.1	6281	0,00	0,62	0,00	0,67	0,00	0,15	CHH
FCFB01011758.1	1553	0,38	0,99	0,00	0,99	0,33	1,00	CHH
FCFB01013160.1	2861	1,00	0,73	1,00	0,60	1,00	0,75	CHH
FCFB01013160.1	2867	0,00	0,36	0,05	0,80	0,00	0,38	CHH
FCFB01015135.1	9965	0,02	0,17	0,00	0,71	0,00	0,29	CHH
FCFB01015135.1	9974	0,00	0,17	0,01	0,71	0,00	0,30	CHH
FCFB01018540.1	1929	1,00	0,63	0,89	0,00	0,86	0,07	CHH
FCFB01018540.1	1933	0,00	0,38	0,12	1,00	0,14	0,93	CHH
FCFB01018540.1	1942	1,00	0,63	0,89	0,00	0,86	0,07	CHH
FCFB01018540.1	1947	1,00	0,63	0,89	0,00	0,86	0,07	CHH

Chr	Start	Day90T1	Day90E1	Day90T2	Day90E2	Day90T3	Day90E3	Type
FCFB01018540.1	1953	0,00	0,38	0,11	1,00	0,14	0,93	CHH
FCFB01020571.1	6648	0,28	0,56	0,32	0,63	0,21	0,55	CHH
FCFB01020911.1	25602	1,00	0,53	1,00	1,00	1,00	0,65	CHH
FCFB01020911.1	25612	1,00	0,53	1,00	1,00	1,00	0,65	CHH
FCFB01020911.1	25617	1,00	0,53	1,00	1,00	1,00	0,65	CHH
FCFB01021203.1	21811	0,00	0,78	0,00	1,00	0,00	0,71	CHH
FCFB01021203.1	21813	0,00	0,80	0,00	1,00	0,00	0,57	CHH
FCFB01021203.1	21819	1,00	0,22	1,00	0,00	1,00	0,43	CHH
FCFB01021203.1	21820	1,00	0,22	1,00	0,00	1,00	0,43	CHH
FCFB01021203.1	21823	0,00	0,77	0,00	1,00	0,00	0,57	CHH
FCFB01021203.1	21825	0,00	0,78	0,00	1,00	0,00	0,57	CHH
FCFB01023313.1	1612	0,00	0,33	0,20	1,00	0,00	0,78	CHH
FCFB01029098.1	1070	0,00	0,97	0,16	1,00	0,06	1,00	CHH
FCFB01030999.1	10899	0,07	0,57	0,00	0,86	0,00	0,71	CHH
FCFB01030999.1	10903	0,07	0,57	0,00	0,57	0,00	0,50	CHH
FCFB01032104.1	8712	1,00	0,78	1,00	0,57	1,00	0,73	CHH
FCFB01032240.1	3260	0,94	0,03	1,00	0,03	1,00	0,08	CHH
FCFB01037227.1	6728	1,00	0,57	1,00	0,57	1,00	0,60	CHH
FCFB01040994.1	13595	0,00	0,50	0,00	0,91	0,06	0,76	CHH
FCFB01040994.1	13598	0,00	0,50	0,00	0,91	0,00	0,73	CHH
FCFB01040994.1	13601	1,00	0,50	1,00	0,09	1,00	0,47	CHH
FCFB01040994.1	13616	0,56	0,67	0,47	1,00	0,44	0,83	CHH
FCFB01041278.1	3751	0,00	0,34	0,01	0,35	0,00	0,44	CHH
FCFB01041563.1	19049	0,00	0,17	0,00	0,67	0,00	0,50	CHH
FCFB01041563.1	19050	1,00	0,83	1,00	0,67	1,00	0,50	CHH
FCFB01042360.1	19650	0,00	0,80	0,00	0,60	0,00	0,89	CHH
FCFB01042360.1	19653	1,00	0,20	1,00	0,40	1,00	0,11	CHH
FCFB01042401.1	11370	0,64	0,02	0,83	0,01	0,78	0,01	CHH
FCFB01048258.1	15155	1,00	1,00	1,00	0,80	1,00	0,50	CHH
FCFB01048974.1	1893	0,00	0,89	0,00	0,93	0,00	1,00	CHH
FCFB01048974.1	1908	0,00	0,90	0,00	0,93	0,00	1,00	CHH
FCFB01048974.1	1913	0,00	0,89	0,00	0,93	0,00	1,00	CHH
FCFB01049052.1	1627	0,27	0,97	0,42	1,00	0,22	0,96	CHH
FCFB01050668.1	1578	0,96	0,01	1,00	0,02	0,67	0,01	CHH
FCFB01050668.1	1580	1,00	0,19	1,00	0,28	1,00	0,80	CHH
FCFB01050668.1	1585	0,07	0,99	0,00	0,97	0,17	0,96	CHH
FCFB01050668.1	1586	1,00	0,26	1,00	0,27	1,00	0,88	CHH
FCFB01051483.1	4519	0,92	0,02	0,93	0,00	0,89	0,00	CHH

Chr	**Start**	**Day90T1**	**Day90E1**	**Day90T2**	**Day90E2**	**Day90T3**	**Day90E3**	**Type**
FCFB01052260.1	1816	0,07	0,83	0,00	1,00	0,00	0,93	CHH
FCFB01052260.1	1825	0,00	0,83	0,00	1,00	0,00	1,00	CHH
FCFB01053598.1	209	0,00	0,40	0,00	0,67	0,00	0,78	CHH
FCFB01053598.1	219	0,00	0,40	0,00	0,67	0,00	0,78	CHH
FCFB01058820.1	4646	0,00	0,82	0,11	1,00	0,20	1,00	CHH
FCFB01058820.1	4648	1,00	0,18	0,89	0,00	0,80	0,00	CHH
FCFB01058820.1	4650	0,00	0,81	0,12	1,00	0,20	1,00	CHH
FCFB01058820.1	4656	0,00	0,82	0,11	1,00	0,20	1,00	CHH
FCFB01067681.1	42	0,19	0,80	0,20	0,66	0,18	0,77	CHH
FCFB01067681.1	44	0,19	0,80	0,18	0,68	0,18	0,77	CHH
FCFB01067681.1	48	0,19	0,78	0,16	0,63	0,16	0,77	CHH
FCFB01070185.1	3844	0,00	1,00	0,00	1,00	0,00	0,71	CHH
FCFB01071064.1	11618	0,03	0,95	0,02	0,96	0,07	0,80	CHH
FCFB01071064.1	11626	0,03	0,95	0,05	0,97	0,07	0,80	CHH
FCFB01071064.1	11634	0,03	0,95	0,02	0,96	0,09	0,80	CHH
FCFB01080856.1	639	0,24	1,00	0,13	1,00	0,33	0,98	CHH
FCFB01080856.1	642	0,94	0,48	1,00	0,54	0,64	0,33	CHH
FCFB01080856.1	643	0,82	0,53	0,87	0,47	1,00	0,71	CHH
FCFB01087352.1	3450	0,96	0,13	0,98	0,02	0,98	0,07	CHH
FCFB01087352.1	3461	0,00	0,08	0,00	0,13	0,00	0,45	CHH
FCFB01088237.1	3475	0,00	0,85	0,07	0,65	0,00	0,50	CHH
FCFB01088237.1	3478	0,09	0,85	0,07	0,67	0,00	0,50	CHH
FCFB01089643.1	1400	0,14	1,00	0,21	0,98	0,17	0,99	CHH
FCFB01103833.1	714	1,00	0,57	1,00	0,06	1,00	0,06	CHH
FCFB01105402.1	1817	0,02	0,10	0,00	0,60	0,00	0,33	CHH
FCFB01111650.1	1907	0,94	0,02	0,78	0,01	1,00	0,02	CHH
FCFB01111650.1	1910	0,53	0,00	0,28	0,02	0,33	0,01	CHH
FCFB01113331.1	1794	0,02	0,87	0,01	0,86	0,05	0,65	CHH
FCFB01113331.1	1806	0,02	0,87	0,00	0,86	0,07	0,65	CHH
FCFB01113331.1	1811	0,04	0,87	0,01	0,88	0,05	0,65	CHH
FCFB01117601.1	5283	0,06	0,96	0,00	0,99	0,04	0,91	CHH
FCFB01118938.1	3692	0,97	0,43	1,00	0,67	0,96	0,40	CHH
FCFB01118938.1	3695	0,06	0,57	0,00	0,33	0,00	0,53	CHH
FCFB01120444.1	1581	0,40	0,08	0,50	0,00	0,51	0,15	CHH
FCFB01122052.1	7352	0,51	0,90	0,48	0,86	0,45	0,67	CHH
FCFB01122717.1	2890	1,00	0,05	0,84	0,00	0,71	0,00	CHH
FCFB01122717.1	2898	1,00	0,06	0,84	0,00	0,71	0,01	CHH
FCFB01122717.1	2900	0,00	0,95	0,16	1,00	0,29	1,00	CHH

Chr	Start	Day90T1	Day90E1	Day90T2	Day90E2	Day90T3	Day90E3	Type
FCFB01126784.1	19552	0,00	0,92	0,06	0,87	0,00	0,59	CHH
FCFB01126784.1	19553	0,00	0,96	0,13	0,97	0,00	0,95	CHH
FCFB01126784.1	19562	0,00	0,96	0,13	0,97	0,00	0,93	CHH
FCFB01126784.1	19568	0,83	1,00	0,75	1,00	0,50	0,93	CHH
FCFB01127938.1	6619	1,00	0,01	1,00	0,00	0,92	0,00	CHH
FCFB01129450.1	1462	0,00	0,75	0,00	0,94	0,00	0,63	CHH
FCFB01129450.1	1465	0,00	0,63	0,00	0,94	0,00	0,63	CHH
FCFB01129450.1	1471	0,00	0,63	0,00	0,94	0,00	0,63	CHH
FCFB01129450.1	1472	0,00	0,63	0,00	0,94	0,00	0,63	CHH
FCFB01129450.1	1477	1,00	0,38	1,00	0,09	1,00	0,35	CHH
FCFB01133142.1	183	0,00	1,00	0,00	0,78	0,00	0,71	CHH
FCFB01133142.1	198	0,00	1,00	0,00	0,82	0,00	0,71	CHH
FCFB01134328.1	1509	1,00	0,12	1,00	0,17	1,00	0,22	CHH
FCFB01134328.1	1518	1,00	0,12	1,00	0,10	0,90	0,20	CHH
FCFB01134328.1	1521	0,00	0,88	0,00	0,91	0,10	0,80	CHH
FCFB01155803.1	1616	0,10	0,69	0,00	1,00	0,00	1,00	CHH
FCFB01155803.1	1619	0,14	0,69	0,00	1,00	0,00	1,00	CHH
FCFB01158699.1	1699	0,99	0,95	1,00	0,30	1,00	0,68	CHH
FCFB01158699.1	1707	1,00	0,95	1,00	0,30	1,00	0,68	CHH
FCFB01168968.1	1632	0,89	0,73	0,70	0,44	1,00	0,36	CHH
FCFB01168968.1	1633	1,00	0,36	0,90	0,54	1,00	0,73	CHH
FCFB01171622.1	57	0,27	0,94	0,17	0,98	0,19	0,94	CHH
FCFB01171622.1	61	0,02	0,93	0,01	0,96	0,03	0,92	CHH
FCFB01191329.1	1438	0,00	0,78	0,00	0,80	0,00	0,40	CHH
FCFB01191329.1	1443	0,00	0,78	0,00	0,80	0,00	0,40	CHH
FCFB01191329.1	1447	1,00	0,22	1,00	0,20	1,00	0,40	CHH
FCFB01212038.1	3052	0,13	1,00	0,11	1,00	0,00	1,00	CHH
FCFB01212038.1	3055	0,88	0,00	0,88	0,01	1,00	0,00	CHH
FCFB01249223.1	496	1,00	0,09	1,00	0,00	1,00	0,10	CHH
FCFB01249223.1	497	0,00	0,91	0,00	1,00	0,00	0,91	CHH
FCFB01268192.1	157	0,00	1,00	0,00	1,00	0,00	1,00	CHH
FCFB01276698.1	161	0,00	0,90	0,00	0,93	0,00	0,94	CHH
FCFB01276698.1	164	0,70	1,00	0,71	1,00	0,69	1,00	CHH
FCFB01276698.1	167	0,00	0,90	0,00	0,93	0,00	0,94	CHH
FCFB01276698.1	169	1,00	0,10	1,00	0,21	1,00	0,06	CHH
FCFB01276698.1	170	0,00	0,90	0,00	0,93	0,00	0,94	CHH
FCFB01282215.1	581	0,00	0,51	0,00	0,58	0,00	0,07	CHH
FCFB01282215.1	584	1,00	0,50	1,00	0,50	1,00	0,93	CHH

Chr	Start	Day90T1	Day90E1	Day90T2	Day90E2	Day90T3	Day90E3	Type
FCFB01282215.1	589	0,00	0,60	0,00	0,71	0,04	0,57	CHH
FCFB01282215.1	599	1,00	0,80	0,96	0,71	0,96	0,46	CHH
FCFB01306716.1	480	1,00	0,03	1,00	0,01	1,00	0,13	CHH
FCFB01306716.1	481	0,00	0,97	0,00	0,99	0,00	0,87	CHH
FCFB01306716.1	484	1,00	0,03	1,00	0,01	1,00	0,13	CHH
FCFB01306716.1	497	1,00	0,03	1,00	0,01	1,00	0,12	CHH
FCFB01306716.1	498	1,00	0,03	1,00	0,01	1,00	0,12	CHH
FCFB01327802.1	51	0,33	0,96	0,31	0,99	0,57	0,98	CHH

Data S4.6 Methylation percentage of the differently methylated DNA sites in *Lymnaea stagnalis* genome between eDNA and tissue DNA for age class of 200 day.

Chr	Start	Day200	Day200E1	Day200T2	Day200E2	Day200T3	Day200E3	Type
FCFB01015468.1	2309	0,36	0,43	0,56	0,82	0,10	0,61	CpG
FCFB01025914.1	2092	0,53	0,80	0,45	0,88	0,33	0,67	CpG
FCFB01028332.1	1497	0,00	0,95	0,20	0,76	0,00	0,62	CpG
FCFB01060608.1	1311	0,79	0,38	0,47	0,41	0,66	0,09	CpG
FCFB01063832.1	985	0,10	0,27	0,81	0,53	0,11	0,69	CpG
FCFB01067681.1	40	0,47	0,78	0,53	0,79	0,42	0,72	CpG
FCFB01067681.1	55	0,33	0,66	0,33	0,72	0,32	0,68	CpG
FCFB01067681.1	67	0,35	0,66	0,34	0,75	0,36	0,71	CpG
FCFB01077342.1	8346	0,27	0,57	0,38	0,69	0,09	0,73	CpG
FCFB01077342.1	8353	0,36	0,43	0,38	0,57	0,09	0,69	CpG
FCFB01097619.1	61	0,20	0,00	0,31	0,68	0,00	0,54	CpG
FCFB01118955.1	708	0,08	0,75	0,09	0,33	0,00	0,13	CpG
FCFB01122847.1	1484	0,57	1,00	0,57	0,95	0,71	0,86	CpG
FCFB01153747.1	269	0,08	0,35	0,02	0,11	0,12	0,46	CpG
FCFB01153747.1	271	0,09	0,36	0,02	0,11	0,11	0,43	CpG
FCFB01153747.1	286	0,04	0,35	0,04	0,05	0,13	0,43	CpG
FCFB01153747.1	290	0,12	0,47	0,04	0,07	0,16	0,49	CpG
FCFB01186689.1	732	0,45	0,82	0,94	0,88	0,59	0,93	CpG
FCFB01197776.1	1271	0,33	0,80	0,43	0,60	0,38	0,63	CpG
FCFB01262921.1	616	0,26	0,52	0,37	0,73	0,31	0,52	CpG
FCFB01324012.1	266	0,71	0,40	0,50	0,68	0,83	0,30	CpG
FCFB01067681.1	47	0,33	0,63	0,33	0,75	0,31	0,68	CHG
FCFB01067681.1	56	0,32	0,66	0,33	0,75	0,32	0,68	CHG
FCFB01000603.1	1617	0,00	1,00	0,00	0,67	0,00	0,58	CHH
FCFB01000603.1	1620	0,00	1,00	0,00	0,67	0,00	0,58	CHH
FCFB01000603.1	1622	0,00	1,00	0,00	0,67	0,00	0,58	CHH
FCFB01000603.1	1627	0,00	1,00	0,00	0,66	0,00	0,58	CHH
FCFB01000603.1	1630	0,00	1,00	0,00	0,67	0,00	0,58	CHH
FCFB01001047.1	20213	0,00	0,63	0,00	0,27	0,00	0,07	CHH
FCFB01001495.1	11862	0,00	1,00	0,08	0,69	0,13	0,53	CHH
FCFB01001976.1	47236	0,00	0,97	0,00	0,48	0,00	0,65	CHH
FCFB01001976.1	47251	0,00	0,97	0,00	0,48	0,00	0,65	CHH
FCFB01003142.1	1611	0,00	0,80	0,00	0,40	0,00	0,30	CHH
FCFB01005129.1	4136	0,00	0,93	0,00	0,06	0,00	0,10	CHH
FCFB01005234.1	198	0,00	0,89	0,17	0,11	0,13	0,50	CHH
FCFB01006224.1	524	1,00	0,40	1,00	0,82	1,00	0,67	CHH
FCFB01008219.1	12788	0,00	0,96	0,15	0,76	0,09	0,44	CHH

Chr	Start	Day200	Day200E1	Day200T2	Day200E2	Day200T3	Day200E3	Type
FCFB01008219.1	12793	0,00	0,96	0,00	0,78	0,00	0,40	CHH
FCFB01008219.1	12796	1,00	0,04	1,00	0,24	1,00	0,61	CHH
FCFB01009669.1	832	0,14	0,89	0,18	0,71	0,45	0,65	CHH
FCFB01011855.1	2286	0,00	0,94	0,00	0,89	0,00	0,63	CHH
FCFB01011855.1	2294	1,00	0,06	1,00	0,13	1,00	0,38	CHH
FCFB01013186.1	6100	0,53	1,00	0,83	0,91	0,45	0,94	CHH
FCFB01013186.1	6109	0,50	1,00	0,83	0,91	0,40	0,93	CHH
FCFB01013198.1	9894	0,00	0,82	0,40	0,88	0,00	0,49	CHH
FCFB01013198.1	9901	1,00	0,32	1,00	0,41	1,00	0,73	CHH
FCFB01013864.1	1503	0,17	0,78	0,31	0,60	0,29	0,43	CHH
FCFB01013864.1	1518	1,00	0,43	0,77	0,50	0,86	0,67	CHH
FCFB01015135.1	9965	0,00	0,55	0,03	0,30	0,03	0,48	CHH
FCFB01015135.1	9974	0,00	0,55	0,03	0,31	0,03	0,48	CHH
FCFB01017217.1	335	0,00	0,80	0,00	0,63	0,00	0,07	CHH
FCFB01017217.1	336	0,00	0,80	0,00	0,64	0,00	0,07	CHH
FCFB01018540.1	1929	0,94	0,22	0,83	0,63	0,80	0,60	CHH
FCFB01018540.1	1933	0,11	0,89	0,17	0,39	0,20	0,40	CHH
FCFB01018540.1	1942	0,89	0,22	0,83	0,69	0,80	0,60	CHH
FCFB01018540.1	1947	0,89	0,22	0,83	0,63	0,80	0,60	CHH
FCFB01018540.1	1953	0,11	0,88	0,17	0,38	0,20	0,40	CHH
FCFB01024634.1	6943	1,00	0,46	1,00	0,68	1,00	0,90	CHH
FCFB01024634.1	6946	0,00	0,79	0,07	0,85	0,00	0,23	CHH
FCFB01024634.1	6955	0,00	0,71	0,00	0,43	0,00	0,14	CHH
FCFB01024634.1	6959	0,00	0,79	0,00	0,81	0,00	0,23	CHH
FCFB01026912.1	10666	0,17	0,85	0,11	0,93	0,08	0,96	CHH
FCFB01027245.1	371	0,00	0,97	0,00	0,67	0,00	0,69	CHH
FCFB01027458.1	847	0,00	0,56	0,00	0,36	0,00	0,15	CHH
FCFB01027458.1	857	1,00	0,44	1,00	0,62	1,00	0,85	CHH
FCFB01028882.1	2659	0,43	0,19	1,00	0,12	0,22	0,05	CHH
FCFB01031845.1	904	0,58	0,00	0,30	0,17	0,23	0,03	CHH
FCFB01032036.1	147	1,00	0,37	1,00	0,67	1,00	0,63	CHH
FCFB01032036.1	149	1,00	0,07	1,00	0,67	1,00	0,52	CHH
FCFB01032503.1	14215	0,00	0,57	0,00	0,40	0,09	0,19	CHH
FCFB01032503.1	14229	1,00	0,43	1,00	0,60	0,95	0,86	CHH
FCFB01032503.1	14232	0,00	0,57	0,00	0,40	0,09	0,14	CHH
FCFB01034552.1	11656	0,00	0,63	0,00	0,21	0,06	0,26	CHH
FCFB01034552.1	11666	1,00	0,38	1,00	0,79	0,94	0,74	CHH
FCFB01034705.1	538	0,57	0,17	0,26	0,31	0,42	0,12	CHH

Chr	Start	Day200	Day200E1	Day200T2	Day200E2	Day200T3	Day200E3	Type
FCFB01035311.1	4312	0,00	0,50	0,00	0,60	0,00	0,20	CHH
FCFB01038657.1	4955	0,00	0,67	0,00	0,65	0,00	0,37	CHH
FCFB01041210.1	372	0,83	0,00	0,92	0,00	1,00	0,46	CHH
FCFB01041210.1	375	0,17	0,71	0,00	0,67	0,00	0,54	CHH
FCFB01044129.1	2633	0,00	0,67	0,00	0,17	0,00	0,38	CHH
FCFB01044597.1	2626	0,38	0,60	0,00	0,65	0,00	0,60	CHH
FCFB01044597.1	2632	0,38	0,77	0,00	0,54	0,00	0,74	CHH
FCFB01044597.1	2644	0,40	0,74	0,00	0,34	0,00	0,64	CHH
FCFB01047459.1	10178	1,00	0,17	1,00	0,67	1,00	0,67	CHH
FCFB01047459.1	10180	1,00	0,17	1,00	0,67	1,00	0,67	CHH
FCFB01048617.1	3080	0,00	0,68	0,00	0,67	0,00	0,33	CHH
FCFB01048617.1	3095	0,00	0,80	0,00	0,67	0,00	0,33	CHH
FCFB01050668.1	1578	0,50	0,01	0,20	0,06	0,44	0,07	CHH
FCFB01050668.1	1580	0,60	0,93	0,60	0,96	0,89	0,96	CHH
FCFB01050668.1	1585	0,50	0,91	0,80	0,92	0,56	0,88	CHH
FCFB01050668.1	1586	0,90	0,69	1,00	0,65	0,89	0,69	CHH
FCFB01051761.1	5240	0,00	0,80	0,00	0,27	0,00	0,21	CHH
FCFB01051761.1	5243	1,00	0,20	1,00	0,73	1,00	0,79	CHH
FCFB01052260.1	1816	0,00	0,19	0,00	0,40	0,00	0,13	CHH
FCFB01052327.1	19694	1,00	0,43	0,90	0,75	1,00	0,60	CHH
FCFB01056445.1	43	0,00	0,86	0,00	1,00	0,00	0,05	CHH
FCFB01056445.1	47	0,00	0,86	0,20	1,00	0,00	0,05	CHH
FCFB01058651.1	22507	0,00	0,46	0,25	0,30	0,00	0,57	CHH
FCFB01058651.1	22514	0,00	0,50	0,25	0,63	0,00	0,64	CHH
FCFB01059131.1	27479	0,00	0,57	0,00	0,83	0,00	0,38	CHH
FCFB01059131.1	27501	0,00	0,57	0,00	0,86	0,00	0,50	CHH
FCFB01059139.1	734	0,85	0,54	1,00	0,71	1,00	0,70	CHH
FCFB01059459.1	16477	0,00	0,88	0,00	0,50	0,00	0,29	CHH
FCFB01059459.1	16488	0,00	0,88	0,00	0,48	0,00	0,29	CHH
FCFB01059754.1	3042	0,88	0,63	1,00	0,58	0,82	0,60	CHH
FCFB01061839.1	5200	0,00	0,67	0,00	0,18	0,08	0,38	CHH
FCFB01061839.1	5207	1,00	0,50	1,00	0,82	0,92	0,63	CHH
FCFB01064133.1	3422	1,00	0,36	1,00	0,62	1,00	0,84	CHH
FCFB01067161.1	26724	0,86	0,11	0,92	0,15	0,90	0,07	CHH
FCFB01067681.1	42	0,35	0,68	0,37	0,74	0,35	0,67	CHH
FCFB01067681.1	44	0,34	0,68	0,33	0,74	0,33	0,69	CHH
FCFB01067681.1	48	0,32	0,66	0,34	0,72	0,31	0,69	CHH
FCFB01072118.1	1121	0,88	0,10	0,88	0,44	0,67	0,60	CHH

Chr	Start	Day200	Day200E1	Day200T2	Day200E2	Day200T3	Day200E3	Type
FCFB01072118.1	1125	0,89	0,10	0,88	0,44	0,64	0,60	CHH
FCFB01075764.1	1204	0,14	0,80	0,09	0,56	0,00	0,50	CHH
FCFB01078276.1	2671	1,00	0,33	1,00	0,44	1,00	1,00	CHH
FCFB01078276.1	2680	1,00	0,29	1,00	0,44	1,00	1,00	CHH
FCFB01082372.1	511	0,00	1,00	0,00	0,43	0,00	0,15	CHH
FCFB01082372.1	523	1,00	0,10	1,00	0,71	1,00	0,92	CHH
FCFB01082401.1	10516	0,00	0,67	0,00	0,33	0,20	0,00	CHH
FCFB01082401.1	10518	0,00	0,67	0,00	0,33	0,20	0,00	CHH
FCFB01083253.1	23202	0,91	0,18	1,00	0,60	1,00	0,79	CHH
FCFB01083253.1	23203	0,91	0,18	1,00	0,60	1,00	0,77	CHH
FCFB01083253.1	23206	0,09	0,82	0,00	0,40	0,00	0,22	CHH
FCFB01085074.1	3093	0,09	0,96	0,00	0,98	0,00	0,92	CHH
FCFB01085272.1	155	1,00	0,29	1,00	0,30	1,00	0,35	CHH
FCFB01086480.1	1070	0,57	0,00	0,40	0,00	0,10	0,07	CHH
FCFB01087352.1	3450	0,93	0,24	0,92	0,13	0,92	0,22	CHH
FCFB01088939.1	1429	1,00	0,56	0,79	0,63	0,96	0,75	CHH
FCFB01088939.1	1434	0,00	0,44	0,21	0,38	0,04	0,25	CHH
FCFB01088939.1	1435	0,00	0,44	0,21	0,43	0,04	0,25	CHH
FCFB01088939.1	1438	1,00	0,50	0,79	0,63	0,96	0,75	CHH
FCFB01088939.1	1439	0,00	0,40	0,21	0,38	0,04	0,25	CHH
FCFB01088939.1	1443	1,00	0,60	0,79	0,63	0,96	0,75	CHH
FCFB01088939.1	1444	0,00	0,50	0,21	0,44	0,05	0,25	CHH
FCFB01096572.1	22767	0,00	0,73	0,00	0,40	0,00	0,53	CHH
FCFB01100499.1	9611	1,00	0,17	1,00	0,40	1,00	0,88	CHH
FCFB01100499.1	9612	0,00	0,83	0,00	0,60	0,00	0,12	CHH
FCFB01100499.1	9622	1,00	0,17	1,00	0,40	1,00	0,88	CHH
FCFB01100499.1	9625	1,00	0,17	1,00	0,50	1,00	0,88	CHH
FCFB01100499.1	9626	0,00	0,83	0,00	0,60	0,00	0,12	CHH
FCFB01100499.1	9633	0,00	0,83	0,00	0,60	0,00	0,12	CHH
FCFB01101766.1	2323	0,15	0,90	0,04	0,42	0,16	0,71	CHH
FCFB01101766.1	2325	0,85	0,10	1,00	0,55	0,94	0,29	CHH
FCFB01102646.1	10587	1,00	0,33	1,00	0,13	1,00	0,89	CHH
FCFB01102646.1	10595	0,00	0,67	0,00	0,88	0,00	0,12	CHH
FCFB01106470.1	4846	0,00	0,84	0,00	0,60	0,00	0,38	CHH
FCFB01107148.1	609	0,08	0,50	0,00	0,63	0,00	0,13	CHH
FCFB01109288.1	5109	0,00	0,25	0,00	0,27	0,00	0,36	CHH
FCFB01110016.1	8055	0,30	0,89	0,00	0,57	0,17	0,50	CHH
FCFB01110016.1	8057	0,30	0,89	0,00	0,71	0,14	0,50	CHH

Chr	Start	Day200	Day200E1	Day200T2	Day200E2	Day200T3	Day200E3	Type
FCFB01110018.1	760	0,14	1,00	0,18	0,76	0,00	0,56	CHH
FCFB01110018.1	770	0,14	0,67	0,18	0,39	0,00	0,33	CHH
FCFB01110018.1	773	1,00	0,67	1,00	0,64	1,00	0,76	CHH
FCFB01110285.1	486	1,00	0,16	0,95	0,59	1,00	0,72	CHH
FCFB01110285.1	490	0,00	0,84	0,05	0,40	0,00	0,27	CHH
FCFB01110285.1	494	0,00	0,84	0,05	0,40	0,00	0,32	CHH
FCFB01110285.1	497	1,00	0,16	0,95	0,60	1,00	0,74	CHH
FCFB01110285.1	503	1,00	0,16	0,95	0,60	1,00	0,74	CHH
FCFB01114875.1	589	0,00	0,88	0,00	0,78	0,00	0,14	CHH
FCFB01114875.1	591	0,00	0,88	0,00	0,78	0,00	0,14	CHH
FCFB01114912.1	3250	1,00	0,20	1,00	0,60	1,00	0,93	CHH
FCFB01115117.1	2317	0,00	1,00	0,00	0,87	0,00	0,52	CHH
FCFB01116934.1	9374	0,80	0,08	1,00	0,35	0,86	0,27	CHH
FCFB01120444.1	1581	0,43	0,00	0,57	0,05	0,49	0,20	CHH
FCFB01128171.1	97	0,82	1,00	0,92	0,44	0,92	0,66	CHH
FCFB01128711.1	893	1,00	0,08	1,00	0,50	1,00	0,29	CHH
FCFB01128711.1	895	0,00	0,92	0,00	0,33	0,00	0,71	CHH
FCFB01128711.1	896	1,00	0,08	1,00	0,50	1,00	0,29	CHH
FCFB01128711.1	901	1,00	0,08	1,00	0,50	1,00	0,29	CHH
FCFB01129450.1	1462	0,00	0,64	0,00	0,21	0,00	0,00	CHH
FCFB01129450.1	1471	0,00	0,64	0,00	0,21	0,00	0,00	CHH
FCFB01129450.1	1472	0,00	0,64	0,00	0,22	0,00	0,00	CHH
FCFB01129450.1	1477	1,00	0,36	1,00	0,79	1,00	1,00	CHH
FCFB01133490.1	6079	0,80	0,33	1,00	0,71	0,81	0,79	CHH
FCFB01134128.1	1066	1,00	0,13	1,00	0,42	1,00	0,63	CHH
FCFB01134128.1	1067	1,00	0,13	1,00	0,39	1,00	0,63	CHH
FCFB01134128.1	1068	0,45	0,06	0,54	0,05	0,71	0,44	CHH
FCFB01134128.1	1071	0,00	0,88	0,00	0,58	0,00	0,36	CHH
FCFB01134128.1	1074	1,00	0,13	1,00	0,41	1,00	0,64	CHH
FCFB01134128.1	1075	0,00	0,88	0,00	0,59	0,00	0,36	CHH
FCFB01134128.1	1079	0,00	0,90	0,00	0,58	0,00	0,36	CHH
FCFB01134128.1	1082	0,00	0,90	0,00	0,58	0,00	0,36	CHH
FCFB01136884.1	463	0,98	0,29	1,00	0,58	0,95	0,70	CHH
FCFB01139722.1	296	0,91	0,13	0,89	0,13	1,00	0,15	CHH
FCFB01140380.1	310	0,03	0,80	0,00	0,59	0,00	1,00	CHH
FCFB01142584.1	1828	0,00	0,67	0,00	0,10	0,00	0,16	CHH
FCFB01142663.1	771	0,79	0,78	0,71	0,56	0,85	0,36	CHH
FCFB01142663.1	783	0,79	0,78	0,73	0,56	0,85	0,36	CHH

Chr	Start	Day200	Day200E1	Day200T2	Day200E2	Day200T3	Day200E3	Type
FCFB01143881.1	351	0,10	0,64	0,29	0,36	0,00	0,29	CHH
FCFB01147059.1	4974	0,00	0,70	0,15	0,63	0,21	0,33	CHH
FCFB01159118.1	234	0,00	0,80	0,00	0,36	0,00	0,00	CHH
FCFB01159118.1	246	0,00	0,70	0,00	0,33	0,00	0,17	CHH
FCFB01159118.1	249	0,00	0,65	0,00	0,42	0,00	0,14	CHH
FCFB01159118.1	255	0,00	0,78	0,00	0,42	0,00	0,24	CHH
FCFB01165212.1	1270	0,89	0,00	1,00	0,30	0,80	0,60	CHH
FCFB01165212.1	1274	0,11	1,00	0,00	0,74	0,20	0,40	CHH
FCFB01169754.1	2692	0,00	0,67	0,00	0,60	0,00	0,20	CHH
FCFB01169754.1	2693	0,13	0,29	0,13	0,50	0,00	0,30	CHH
FCFB01169754.1	2700	0,13	0,64	0,13	0,46	0,00	0,36	CHH
FCFB01169754.1	2701	0,13	0,36	0,13	0,62	0,00	0,29	CHH
FCFB01170491.1	517	0,00	0,88	0,06	0,86	0,00	0,82	CHH
FCFB01170491.1	518	0,00	0,88	0,06	0,86	0,00	0,82	CHH
FCFB01170491.1	523	0,00	0,88	0,06	0,86	0,00	0,82	CHH
FCFB01170491.1	529	1,00	0,12	0,94	0,14	1,00	0,18	CHH
FCFB01170702.1	247	0,00	0,80	0,00	0,56	0,00	0,30	CHH
FCFB01174828.1	2427	0,25	0,60	0,00	0,17	0,00	0,72	CHH
FCFB01174828.1	2441	0,75	0,40	1,00	0,83	1,00	0,29	CHH
FCFB01196316.1	134	0,00	0,92	0,00	1,00	0,17	0,11	CHH
FCFB01205325.1	434	0,20	1,00	0,20	1,00	0,43	0,75	CHH
FCFB01226071.1	1541	0,00	0,86	0,00	0,80	0,00	0,68	CHH
FCFB01235648.1	1323	0,00	0,82	0,00	0,14	0,00	0,18	CHH
FCFB01235648.1	1325	0,11	0,83	0,00	0,29	0,20	0,18	CHH
FCFB01245310.1	568	0,17	0,88	0,00	0,80	0,00	0,67	CHH
FCFB01245310.1	581	0,17	0,88	0,00	0,80	0,00	0,67	CHH
FCFB01249518.1	632	0,63	0,22	1,00	0,50	0,89	0,59	CHH
FCFB01250948.1	94	0,57	1,00	0,80	0,92	0,40	0,90	CHH
FCFB01250948.1	102	0,43	0,00	0,20	0,08	0,60	0,10	CHH
FCFB01250948.1	106	0,43	1,00	0,80	0,91	0,40	0,90	CHH
FCFB01250948.1	109	0,57	1,00	0,80	0,92	0,40	0,90	CHH
FCFB01253946.1	871	0,00	0,44	0,00	0,40	0,00	0,13	CHH
FCFB01281800.1	489	0,00	0,67	0,00	0,81	0,00	0,21	CHH
FCFB01281800.1	494	0,75	1,00	0,54	1,00	0,41	0,74	CHH

Commands

Bismark command

```
# Prepare reference genome

/User/Bismark_0.22.3

perl bismark_genome_preparation  --verbose /User/snailGenome

# Align sequencing data of each sample to the reference genome

perl bismark --genome /Users/snailGenome/  --samtools_path /Users/samtools-1.10/ --
path_to_bowtie2 /Users/miniconda3/bin/ -1 /Users/1-ATCACG_FKDL202588528-1a-
1_H7W2MCCX2_L1_1_val_1.fq.gz -2

# The version of bowtie2 is V. 2.4.1.

# Extract context-dependent (CpG/CHG/CHH) methylation

perl bismark_methylation_extractor --samtools_path /Users/samtools-1.10/ --bedGraph --CX --
scaffolds --comprehensive /Users/Bismark-0.22.3/Day1_tDNA_P1.bam

# Sort data sequence of each bam file

/Users/samtools-1.10/samtools sort -o /Users/Desktop/Bismark_bam/ Day1_tDNA_P1.bam
/Users/Bismark-0.22.3/Day1_tDNA_P1.bam
```

R script

```
# Kruskal-Wallis test evaluating the difference in global methylation level of each type of sites
(CpG, CHG and CHH) between age groups of eDNA and tDNA separately,

Kruskal.test(Percentage ~ Age, data = MethPerT[which(MethPerT$Type == 'CpG'),
c('Percentage','Age')])

# Dunn's-test analysing the differences between each pair of age groups as the posthoc test of
Kruskal-Wallis test

library(PMCMRplus)

posthoc.kruskal.dunn.test(Percentage~Age,data= MethPerT[which(MethPerT$Type == 'CpG'),
c('Percentage','Age')])

# Generate Fig.4.2

library(ggplot2)
```

```
ibrary(ggpubr)

ggboxplot(MethPerE,x="Age",y="Percentage",color = "Type",palette = "jco",add =
"jitter")+facet_wrap(~Type,scales = "
fixed",nrow=1)+theme_bw()+theme(panel.grid.major=element_line(colour=NA),
strip.background = element_rect(fill = "white"),legend.position="none", axis.title.y =
element_text(size=12),axis.title.x = element_text(size = 11), title =
element_text(size=10),axis.text = element_text(size=10)) +scale_color_manual(values =
c("#993333","#ff9900","#336699"))+labs(y="eDNA Global methylation level (%)",x="Age
(days)")

# Analyse the eDNAm changes with age at single-site resolution

library(parallel)

library(stats4)

library(BiocGenerics)

library(GenomeInfoDb)

library(GenomicRanges)

library(methylKit)

# Comparison between age classes of 1 and 30 days, other pairs applied the same commend.
BAM files are available from NCBI SRA database with BioProject accession number of
PRJNA730179

E1_30.bam.list=list("Day1_eDNA_P1.bam", " Day1_eDNA_P2.bam", " Day1_eDNA_P3.bam",
" Day30_eDNA_P1.bam", " Day30_eDNA_P2.bam", " Day30_eDNA_P3.bam")

E1_30.myobjbamCpG=processBismarkAln(E1_30.bam.list,sample.id=list("Day1E1","Day1E2",
"Day1E3","Day30E1","Day30E2","Day30E3"),assembly="hg18",treatment=c(0,0,0,1,1,1),nolap
=TRUE,read.context="CpG",save.folder=NULL)

E1_30.CpGmeth=unite(E1_30.myobjbamCpG, destrand=FALSE)

E1_30.CpGDiff=calculateDiffMeth(E1_30.CpGmeth)

E1_30.CpGDiff25p=getMethylDiff(E1_30.CpGDiff,difference=25,qvalue=0.01,type="all")

E1_30.CpGDiff25p

E1_30.myobjbamCHG=processBismarkAln(E1_30.bam.list,sample.id=list("Day1E1","Day1E2",
"Day1E3","Day30E1","Day30E2","Day30E3"),assembly="hg18",treatment=c(0,0,0,1,1,1),nolap
```

```
=TRUE,read.context="CHG",save.folder=NULL)

E1_30.CHGmeth=unite(E1_30.myobjbamCpG, destrand=FALSE)

E1_30.CHGDiff=calculateDiffMeth(E1_30.CpGmeth)

E1_30.CHGDiff25p=getMethylDiff(E1_30.CpGDiff,difference=25,qvalue=0.01,type="all")

E1_30.CHGDiff25p

E1_30.myobjbamCHH=processBismarkAln(E1_30.bam.list,sample.id=list("Day1E1","Day1E2",
"Day1E3","Day30E1","Day30E2","Day30E3"),assembly="hg18",treatment=c(0,0,0,1,1,1),nolap
=TRUE,read.context="CHH",save.folder=NULL)

E1_30.CHHmeth=unite(E1_30.myobjbamCpG, destrand=FALSE)

E1_30.CHHDiff=calculateDiffMeth(E1_30.CpGmeth)

E1_30.CHHDiff25p=getMethylDiff(E1_30.CpGDiff,difference=25,qvalue=0.01,type="all")

E1_30.CHHDiff25p

# Generate Fig.4.3 using the 383 methylated sites that differed from at least one age group to
two or three other age groups (Data S2).

library(RColorBrewer)

library(pheatmap)

Age_Sites_annotation_col = data.frame(Age =
c("1","1","1","30","30","30","90","90","90","200","200","200"))

rownames(Age_Sites_annotation_col) = colnames(Age_Sites_matrix)

Age_Sites_ann_colors = list(Age = c("1"= "#ccffcc", "30"="#99cc99",
"90"="#669933","200"="#336633"))

pheatmap(Age_Sites_matrix, color = colorRampPalette(rev(brewer.pal(n = 7, name
="RdYlBu")))(100), annotation_col = Age_Sites_annotation_col, annotation_colors =
Age_Sites_ann_colors,legend_breaks = c(0,0.5,1),legend_labels = c("0","0.5","1") ,cutree_cols
=4, cellwidth = 25, ,show_colnames = FALSE )

# Analyse the differences of eDNAm compared to tDNAm

# Generate Fig.4.4

Fig4a<-ggarrange(ggboxplot(MethPer[which(MethPer$Type == 'CpG'),
c('EorT','Percentage')],x="EorT",y="Percentage",color = "EorT",palette =
```

```
c("#993333","#cc3333"),add =
"jitter")+theme_bw()+theme(panel.grid.major=element_line(colour=NA),strip.background =
element_rect(fill = "white"),legend.position="none", axis.title.y = element_text(size=12),title =
element_text(size=10),axis.text = element_text(size=10),axis.title.x = element_blank(),plot.title
= element_text(hjust = 0.5))+ stat_compare_means(label = "p.signif",label.x.npc =
0.6,label.y.npc = 0.95,size=6,symnum.args = list(cutpoints = c(0,0.01), symbols =
c("**")))+labs(y="Global methylation level (%)", title="CpG")+ylim(0,65),
ggboxplot(Study2_MethPer[which(Study2_MethPer$Type == 'CHG'),
c('EorT','Percentage')],x="EorT",y="Percentage",color = "EorT",palette =
c("#ff9900","#ffcc33"),add =
"jitter")+theme_bw()+theme(panel.grid.major=element_line(colour=NA),strip.background =
element_rect(fill = "white"),legend.position="none",title = element_text(size=10),axis.text =
element_text(size=10),axis.title = element_blank(),plot.title = element_text(hjust =
0.5),axis.text.y = element_blank())+stat_compare_means(label = "p.signif",label.x.npc =
0.6,label.y.npc = 0.95,size=6,symnum.args = list(cutpoints = c(0,0.01), symbols =
c("**")))+labs(title="CHG")+ylim(0,65),
ggboxplot(Study2_MethPer[which(Study2_MethPer$Type == 'CHH'),
c('EorT','Percentage')],x="EorT",y="Percentage",color = "EorT",palette =
c("#336699","#6699cc"),add =
"jitter")+theme_bw()+theme(panel.grid.major=element_line(colour=NA),strip.background =
element_rect(fill = "white"),legend.position="none",title = element_text(size=10),axis.text =
element_text(size=10),axis.title = element_blank(),plot.title = element_text(hjust =
0.5),axis.text.y = element_blank())+stat_compare_means(label = "p.signif",label.x.npc =
0.6,label.y.npc = 0.95,size=6,symnum.args = list(cutpoints = c(0,0.01), symbols =
c("**")))+labs(title="CHH")+ylim(0,65),nrow = 1,widths = c(1,0.95,0.95))

Fig4b<-ggboxplot(MethPerT,x="Age",y="Percentage",color = "Type",palette = "jco",add =
"jitter")+facet_wrap(~Type,scales = "
fixed",nrow=1)+theme_bw()+theme(panel.grid.major=element_line(colour=NA),
strip.background = element_rect(fill = "white"),legend.position="none", axis.title.y =
element_text(size=12),axis.title.x = element_text(size = 11), title =
element_text(size=10),axis.text = element_text(size=10)) +scale_color_manual(values =
c("#cc3333","#ffcc33","#6699cc"))+labs(y="tDNA Global methylation level (%)",x="Age (days)")

ggarrange(Fig4a,Fig4b,nrow = 2,labels = c("a","b"))

#Comparison between eDNAm and tDNAm of the age classes of 1 day, other age classes
applied the same commend. BAM files are available from NCBI SRA database with BioProject
```

```
accession number of PRJNA730179

Age1.bam.list=list("Day1_tDNA_P1.bam", " Day1_eDNA_P1.bam", " Day1_tDNA_P2.bam", " Day1_eDNA_P2.bam", " Day1_tDNA_P3.bam", "Day1_eDNA_P3.bam")

Age1.myobjbamCpG=processBismarkAln(Age1.bam.list,sample.id=list("Day1T1","Day1E1","Day1T2","Day1E2","Day1T3","Day1E3"),assembly="hg18",treatment=c(0,1,0,1,0,1),nolap=TRUE,read.context="CpG",save.folder=NULL)

Age1.CpGmeth=unite(Age1.myobjbamCpG, destrand=FALSE)

Age1.CpGDiff=calculateDiffMeth(Age1.CpGmeth)

Age1.CpGDiff25p=getMethylDiff(Age1.CpGDiff,difference=25,qvalue=0.01,type="all")

Age1.CpGDiff25p

Age1.myobjbamCHG=processBismarkAln(Age1.bam.list,sample.id=list("Day1T1","Day1E1","Day1T2","Day1E2","Day1T3","Day1E3"),assembly="hg18",treatment=c(0,1,0,1,0,1),nolap=TRUE,read.context="CHG",save.folder=NULL)

Age1.CHGmeth=unite(Age1.myobjbamCpG, destrand=FALSE)

Age1.CHGDiff=calculateDiffMeth(Age1.CpGmeth)

Age1.CHGDiff25p=getMethylDiff(Age1.CpGDiff,difference=25,qvalue=0.01,type="all")

Age1.CHGDiff25p

Age1.myobjbamCHH=processBismarkAln(Age1.bam.list,sample.id=list("Day1T1","Day1E1","Day1T2","Day1E2","Day1T3","Day1E3"),assembly="hg18",treatment=c(0,1,0,1,0,1),nolap=TRUE,read.context="CHH",save.folder=NULL)

Age1.CHHmeth=unite(Age1.myobjbamCpG, destrand=FALSE)

Age1.CHHDiff=calculateDiffMeth(Age1.CpGmeth)

Age1.CHHDiff25p=getMethylDiff(Age1.CpGDiff,difference=25,qvalue=0.01,type="all")

Age1.CHHDiff25p

# Generate Fig.4.5 using the different methylated sites between eDNA and tDNA (Data S3-6).

library(ggplot2)

library(ggpubr)

library(ggplotify)
```

```
Fig5a<-ggplot(data= E_T_diffSiteAmount,aes(x=Age, y=Site,fill=hy),short.panel.labs =
FALSE)+geom_bar(stat="identity",width = 0.7,alpha=0.75)+theme_light()+labs(y="Number of
sites",x="Age (days)") +theme_pubr(border = TRUE,base_size =
9)+theme(panel.grid.major=element_line(colour=NA),strip.background =
element_blank(),axis.title.y = element_text(size=9),axis.text = element_text(size=8),legend.text
= element_text(size =
8),legend.title=element_blank(),legend.position="top")+scale_fill_manual(values =
c("#cc0033","#006699"))+geom_text(aes(label = Site), size = 2.5, hjust = 0.5, vjust = 1.3,
position = "stack")

Fig5b<-venn.diagram(HyperE_T_venn_list, NULL, fill = c('#ccff99', '#99cc33', '#339933',
'#006633'), alpha = 0.50, cat.col = c('#ccff99', '#99cc33', '#339933', '#006633'), cat.cex = 0.7,
cat.fontfamily = 'serif', col = c('#ccff99', '#99cc33', '#339933', '#006633'), cex = 0.7, fontfamily =
'serif',main="Number of hyper-methylated sites",main.cex = 0.8)

Fig5c<-venn.diagram(HypoE_T_venn_list, NULL,   fill = c('#ccff99', '#99cc33', '#339933',
'#006633'), alpha = 0.50, cat.col = c('#ccff99', '#99cc33', '#339933', '#006633'), cat.cex = 0.7,
cat.fontfamily = 'serif', col = c('#ccff99', '#99cc33', '#339933', '#006633'), cex = 0.7, fontfamily =
'serif',main="Number of hypo-methylated sites",main.cex = 0.8)

Annotation_col = data.frame(Source = factor(rep(c("tDNA","eDNA"), 3)))

rownames(Annotation_col) = colnames(Age1_matrix)

Ann_colors = list(Source = c(tDNA = "#ff99cc", eDNA = "#66cc99"))

heat1<-pheatmap(Age1_matrix, annotation_col = Annotation_col,annotation_colors =
Ann_colors,gaps_col = 3,cutree_col = 2,border=FALSE,show_colnames=F,legend =
FALSE,annotation_legend = FALSE, annotation_names_col=F)

Fig5d = as.ggplot(heat1)

heat30<-pheatmap(Age30_matrix, annotation_col = Annotation_col,annotation_colors =
Ann_colors,gaps_col = 3,cutree_col = 2,border=FALSE,show_colnames=F,legend =
FALSE,annotation_legend = FALSE)

Fig5e = as.ggplot(heat30)

heat90<-pheatmap(Age90_matrix, annotation_col = Annotation_col,annotation_colors =
Ann_colors,gaps_col = 3,cutree_col = 2, border=FALSE, show_colnames=F,legend =
FALSE,annotation_legend = FALSE)

Fig5f = as.ggplot(heat90)
```

```
heat200<-pheatmap(Age200_matrix, annotation_col = Annotation_col,annotation_colors = Ann_colors,gaps_col = 3,cutree_col = 2, border=FALSE, show_colnames=F,legend = FALSE,annotation_legend = FALSE)

Fig5g = as.ggplot(heat200)

fig5up<-ggarrange(Fig5a,Fig5b,Fig5c,nrow = 1,labels = c("a", "b","c"),widths = c(1,1,1))

fig5down<-ggarrange(Fig5d,Fig5e,Fig5f,Fig5g, ncol=4,widths = c(0.55,0.55,0.55,1),labels = c("d","e","f","g"))

>ggarrange(fig5up,fig5down,nrow = 2,heights = c(0.5,1))
```