The effect of experimental lead pollution on DNA methylation in a wild bird population

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Abstract

Anthropogenic pollution is known to negatively influence an organism's physiology, behavior and fitness. Epigenetic regulation, such as DNA methylation, has been hypothesized as a potential mechanism to mediate such effects, yet studies in wild species are lacking. We first investigated the effects of early-life exposure to the heavy metal lead (Pb) on DNA methylation levels in a wild population of great tits (*Parus major*), by experimentally exposing nestlings to Pb at environmentally relevant levels. Secondly, we compared nestling DNA methylation from a population exposed to long-term heavy metal pollution (close to a copper smelter), where birds suffer from pollution-related decrease in food quality, and a control population. For both comparisons, the analysis of about one million CpGs covering most of the annotated genes revealed that pollution-related changes in DNA methylation were not genome wide, but enriched for genes underlying developmental processes. However, the results were not consistent when using binomial or beta binomial regression highlighting the difficulty of modeling variance in CpGs. Our study indicates that post-natal anthropogenic heavy metal exposure can affect methylation levels of development related genes in a wild bird population.

Introduction

Epigenetic control of gene expression, such as DNA methylation, is increasingly recognized as playing a major role in many different cellular processes. DNA methylation is the addition of a methyl (-CH_3) group to the 5' carbon site of cytosines catalyzed by DNA-methyltransferases that occurs mainly at CpG sites in animals. Especially in CpG islands within promotor regions, DNA methylation is found to be negatively associated with gene expression. Epigenetic changes are linked to variation in phenotype and behavior and are associated with prevalence for various diseases (Angers et al. 2010, Rosenfeld 2010, Skinner et al. 2010).

Methylation patterns can be affected by various environmental factors such as maternal nutrition and maternal care (e.g. Weaver et al. 2004, Heijmans et al. 2008, Faulk and Dolinoy 2011, Feil and Fraga 2012), but also by various pollutants and other early-life stressors, both pre- and postnatally, as discovered in humans and mouse models (reviewed by Cheng et al. 2012, Head et al. 2012, Head 2014, Ray et al. 2014, Ruiz-Hernandez et al. 2015). The potential effects of environmental factors on epigenetic regulation are highly important for ecological and ecotoxicological fields, but research in wild vertebrate populations is only emerging (Bossdorf et al. 2008, Head et al. 2012, Liebl et al. 2013, Wenzel and Piertney 2014, Riyahi et al. 2015, Rubenstein et al. 2016, Verhoeven et al. 2016, Sepers et al. 2019). However, epigenetics can significantly improve our understanding of the mechanisms underlying natural phenotypic variation and the responses of organisms to environmental change (Verhoeven et al. 2016). Furthermore, potential transgenerational epigenetic effects could explain why populations are slow to recover even after pollution removal (Head 2014).

Heavy metals, such as Pb, are global, persistent human-induced pollutants that are among the potential contaminants affecting DNA methylation status (reviewed in Bihaqi 2019). For example in human epidemiological studies, developing fetuses show a decrease in

global methylation levels as a result of historical maternal Pb exposure and accumulation (Pilsner et al. 2009, Wright et al. 2010). Furthermore, in rat, mouse and monkey models, experimental peri- and post-natal Pb exposure decreases DNA methyltransferase activity and affects DNA methylation, which are subsequently related to behavioral alternations (Wu et al. 2008, Faulk et al. 2013, Faulk et al. 2014, Luo et al. 2014, Sanchez-Martin et al. 2015, Singh et al. 2018, Nakayama et al. 2019). In birds, metal exposure has been found to affect offspring growth (Burger and Gochfeld 2000) and multiple aspects of physiology, including stress hormone and stress protein levels (Eeva et al. 2014), oxidative stress levels (Koivula and Eeva 2010) and immune function (reviewed in Boyd 2010). However, the potential epigenetic alterations by early-life exposure to metal pollution, potentially underlying such effects in birds, have not been studied.

In addition to the direct effect of metals, large-scale metal pollution can decrease resource availability and quality in wild populations (Eeva and Lehikoinen 2004, Eeva et al. 2005), which could subsequently also influence methylation patterns: Along with toxicants, altered nutrition and diet, especially diet poor in methyl donors, are well-known epigenetic modifiers in animal models (reviewed in Choi and Friso 2010, Rosenfeld 2010, Konycheva et al. 2011). Also protein or lipid-altered diets can cause major changes in the epigenome (Burdge et al. 2007, Aagaard-Tillery et al. 2008, Choi and Friso 2010). All in all, we expect populations inhabiting polluted environments to have altered DNA methylation patterns, either due to direct or indirect pollution effects.

Here we investigated whether experimental and anthropogenic early-life exposure to the heavy metal Pb alters genome-wide DNA methylation status in a wild population of great tits (*Parus major*). First, we experimentally exposed nestlings to dietary Pb at levels found close to active pollution sources in Europe and compared to respective controls. The exposure covered the whole postnatal pre-fledging period. Second, we compared methylation

patterns from nestlings in a population close to an anthropogenic pollution source, copper smelter (Eeva et al. 1997) to nestlings from an unpolluted population. Around the smelter, nestlings are exposed to multiple metals (in low concentrations) pre-and postnatally and experience an altered nutritional quality and quantity compared to controls. In our recent work using the same experimental protocol we found that Pb exposure and altered nutrition during nestling development lead to changes in e.g. growth, oxidative stress markers, stress protein levels and vitamin metabolism, but the mechanisms, potentially epigenetic regulation are not understood (Eeva et al. 2014, Rainio et al. 2015b, Ruiz et al. 2016).

Our objectives are two-fold. First, by comparing nestlings exposed to the experimental Pb treatment to the control group we aim to detect methylation changes directly induced by Pb during post-natal stage. Second, by comparing nestling methylation patterns from an environment with a long history of metal pollution, to an unpolluted population we aim to detect long term effects of pollutants on great tit methylation levels, that could be mediated via prenatal or postnatal metal exposure or via altered nutrition (latter coined as indirect effect of metal pollution). By these two comparisons we expect to find differentially methylated regions associated with genes that can potentially explain how wild bird populations cope with anthropogenic pollution. Given that there are not yet established golden standards for analyzing methylation data in an ecological context, we used and compared two frequently used analytical tools to detect differentially methylated sites (Wreczycka et al. 2017, Zhang et al. 2018).

Methods

Study species

The great tit is a small passerine bird and a model species in ecological and evolutionary research, with ample ecological and genetic background information available. It is an

insectivorous non-migratory bird that commonly breeds in nest boxes, making it an ideal species for experimental manipulations. Importantly, as one of the only non-domesticated bird species, both the genome and methylome are available (Derks et al. 2016, Laine et al. 2016, Verhulst et al. 2016).

(1) Experimental Pb treatment and control population

The Pb exposure, dosages and sampling are described in detail in (Eeva et al. 2014, Rainio et al. 2015b, Ruuskanen et al. 2015). Briefly, breeding was monitored to record hatching dates of great tit chicks in a population with low pollution levels in southwestern Finland (Turku, 60°26'N, 22°10'E). From day 3 after hatching (hatch date = 0) until day 14 (i.e. in total 12 days) whole broods were subjected to Pb with daily oral dosing with the following treatments: HIGH dose (4 µg Pb/g body mass, N = 15 broods) or CONTROL (distilled water, N = 15 broods). Based on calculations of fecal Pb levels in passerines at several polluted and reference sites across Europe (Nyholm 1994, Belskii et al. 1995b Eeva and Lehikoinen 1996; Eeva et al. 2005b; Dauwe et al. 2000; Dauwe et al. 2004, Eeva et al. 2009a, Berglund et al. 2010) the estimated Pb intake averages 2.2–8.5 µg/g body mass daily in polluted and 0.2–0.5 µg/g in control areas. Therefore, the dose represented environmentally relevant exposure levels occurring in polluted areas in Europe. The exposure period covered most of the post-hatching nestling period, i.e. most important developmental period in altricial birds.

(2) Polluted environment: Copper smelter population

The Harjavalta copper smelter (61°20'N, 22°10'E) was built in 1945 and great tits have been monitored since 1991 in the vicinity (<2 km) of the pollution source. Thus, this study site is suitable for investigating long-term exposure of several metals (e.g., Pb, arsenic, cadmium, copper, nickel) as well as lower food availability and quality (Eeva and Lehikoinen 2004).

The Mean fecal Pb concentrations have varied between 8-10 µg/g (dry mass, geometric mean) (Berglund et al. 2015) in nestlings in SMELTER. Assuming two year generation time interval, great tits have been exposed to environmental pollution for about 12 generations after the study population was established by placing nest boxes. These nests were monitored in the same way as in HIGH and CONTROL and nestlings were dosed with distilled water. The sample in the SMELTER consisted of 19 nests. The smelter site is ca 95 km from the experimental site. The breeding habitat was similar in both CONTROL and SMELTER, representing pine dominated forests with mixed spruce and birch. However, in the CONTROL area some oaks were scattered in the forest. The same control group was used for HIGH and SMELTER comparisons.

Blood sampling protocol, DNA isolation and RRBS library preparation

Blood samples were collected from 7-day old nestlings for sex-determination (following Griffiths et al. 1998) and only females were chosen for CONTROL, HIGH and SMELTER groups. Measurements were taken of multiple physiological indices (Eeva et al. 2014, Rainio et al. 2015a). Fresh fecal samples were collected for measuring metal concentrations (see below). Whole blood samples were collected directly in liquid nitrogen from nestlings at age of 14 days (i.e. after 12 days of treatment) for analyses of DNA methylation status and physiological indices. Samples were stored at −80°C until analysis. Ten unrelated (one nestling/brood) samples from female nestlings in HIGH and CONTROL groups, and eight samples from SMELTER group were selected randomly (total N = 28 samples). The experiment was conducted under licenses from the Animal Experiment Committee of the State Provincial Office of Southern Finland (license number ESAVI/846/ 04.10.03/2011) and the Centre for Economic Development, Transport and the Environment, ELY Centre

Southwest Finland (license number VARELY/149/07.01/2011). The details of the DNA isolation and RRBS library preparation are given in the supplementary material.

Metal analyses

For detailed analyses, see Eeva et al. (2014). Briefly, two fecal samples (one male and one female) from the same brood were combined to assess brood level metal exposure (total N = 35 broods). The determination of metal concentrations (As, Cd, Cu, Ni, Pb) was conducted with ICP-MS with detection limit of 1 ppt (ng/l) and below. The calibration of the instrument was done with a commercial multi-standard from Ultra Scientific, IMS–102, ICP-MS calibration standard 2 and certified reference materials were used for method validation. Data was analyzed with GLMs (SAS 9.4) with Tukey post-hoc tests.

Bisulfite sequencing analysis

The initial quality check with Fastqc (Andrews 2010) indicated presence of Illumina universal adapter contamination and low quality (Q < 20) bases in the 3' end of the raw reads. The adapter sequences were removed with Cutadapt (Martin 2014) and the low quality bases were filtered using Condentri (Smeds and Kunstner 2011) with default settings. The quality filtered reads were then mapped against the Great tit reference genome (Assembly Parus_major1.0.3; NCBI Bioproject PRJNA208335, Laine et al. 2016) using Bismark aligner with default parameters (L, 0, -0.2) allowing 2−3 mismatches or a comparable number of indels per 100 bp read (Krueger and Andrews 2011). Methylation information was extracted from alignment files using the bismark_methylation_extractor tool (Krueger and Andrews 2011). The resulting methylation levels per base pair were inspected to detect potential methylation bias in the beginning and in the end of read 1 and 2 (Hansen et al. 2012). There

was lower methylation in the beginning and higher in the end of read 2. Therefore, the first four bases and the last base were removed from the read 2 for subsequent analyses (supplementary figure 1). On average, we recovered 16.08 million raw reads (range 13.55-21.15) from each RRBS library and after quality filtering 11.72 million reads remained (range 10.12-15.06). On average 6.36 million (54%) of the quality filtered reads were uniquely mapped against the Great tit reference genome. This translates to an average 322.45 million cytosine bases analyzed, of which 179.95 million cytocines (56%) were methylated. We estimated the bisulfite conversion rate by aligning the reads against great tit mitochondrial DNA, which is mostly un-methylated (Mechta et al. 2017) and calculated the conversion rate as 1- methylation% in CpG context. The bisulfite conversion rate was 97.7-99.1%.

In order to call methylated CpG sites from the Bismark methylation extractor files, the function *readBismarkCoverage* in the R package Methylkit (Akalin et al. 2012) was used. Using a minimum coverage threshold of 10, on average 1 309 860 (range 1 062 814-1 545 820) methylated CpG sites were obtained for the CONTROL-HIGH comparison and 1344110 (range 1062814-1774992) CpG sites for the CONTROL-SMELTER comparison. The CpGs were then filtered by extreme coverage to remove e.g. potential PCR duplicates using 99.9% percentile upper threshold as implemented in the function *filterByCoverage* in R package Methylkit. Methylated CpG sites were also median normalized to take into account differing library sizes using *normalizeCoverage* function in R package Methylkit. Finally, CpG sites were united such that the data set contained only CpG sites covered by a minimum of seven individuals per group. The subsequent data sets comprised 1023725 and 903449 CpG sites for CONTROL-HIGH and CONTROL-SMELTER comparisons, respectively. 879056 CpG sites were shared between these two comparisons .The range of mean and

median coverage was 25.94-33.31 and 17-27 in the CONTROL- HIGH comparison. The respective statistics were 33.73-41.50 and 27-37 in the CONTROL- SMELTER comparison.

Statistical analyses

Two commonly used methods were used for the identification of differentially methylated CpG sites. First, a generalized linear model was used as implemented in the R package Methylkit. This method assumes that the methylated and un-methylated counts follow a binomial distribution and the effect of group/treatment can be estimated with a log-likelihood test (Akalin et al. 2012). Second, a generalized linear model assuming beta binomial distribution was used taking into account potential overdispersion by estimating a gene-specific shrinkage operator as implemented in R package dss (Feng et al. 2014). Currently, potential overdispersion has not thoroughly been tested in MethylKit and thus was not applied. In both methods, the model was fitted for each CpG site separately and we compared the pairwise methylation differences between the CONTROL and HIGH and CONTROL and SMELTER groups. For beta binomial regression the original p-values were recalculated based on the test statistics as implemented in the R-package fdrtool (Strimmer 2008b). Since the binomial regression method implemented in the R package Methylkit does not report test statistics and the method is not recommended to be used with U-shaped p-value distributions (Strimmer 2008b), the re-estimation of p-values were conducted only for the beta binomial regression. The model fits were evaluated by inspecting the resulting p-value histograms. Under a proper null model one would expect that the p-value histogram follows approximately uniform distribution, but if there is an effect of treatment then a surplus of small p-values is expected (Fodor et al. 2007, Barton et al. 2013, Garamszegi and de Villemereuil 2017). Deviations from the uniform distribution may provide information about the misspecification of the model or problems in the data.

The test statistics of goodness-of-fit test (Chi-square) of the p-value histograms and visual inspection indicated deviations from the uniform distribution in both methods (Fig 1, Table 1). However, the deviation in the beta binomial regression was smaller than the deviation in the binomial regression and the test statistics were lower when the p-values were re-calculated with fdrtool (Table 2). The deviations from the uniform distribution possibly indicate that our data do not fit to model assumptions or problems with the raw data (Strimmer 2008a, b). Also, methods for multiple testing assume uniform distribution (Strimmer 2008a). Therefore, we further investigated the deviation from uniform distribution by filtering the potentially uninformative CpGs. We used independent filtering approach where those p-values deviating from the uniform are filtered out based on appropriate filter statistics. In gene expression data the mean count for each transcript across all samples has been successfully used as a filter statistics (Bourgon et al. 2010). Using similar approach, we calculated the mean of methylated counts (i.e. Cs) for each CpG across all individuals. Note that the count of C determines relative to the count of T (un-methylated) the methylation level of a given CpG. We applied a threshold for the rank of mean methylated C counts and filtered out those CpGs that were causing the deviation from the uniform distribution (Supplementary figures 6 and 7) by keeping most of the significant CpGs. By removing 30% of the lowest C counts we recovered p-value distribution closer to the uniform distribution and a surplus for small ($p<0.05$) p-values (Figure 2, Table 2). The filtering was carried out using R package genefilter (Gentleman et al. 2018). The filtering approach also increased the number significant CpG sites after controlling for multiple testing (fdr <0.05) in all comparisons (Table 2).

Identification of DMRs

Differentially methylated regions (DMRs) or clusters of differentially methylated CpG sites were identified based on the results of both binomial and beta binomial models. The

following criteria were used to identify DMRs: (i) minimum size of a DMR = 50 bp, (ii) minimum number of CpGs in the DMR = 3 and (iii) the percentage of CpGs with fdr <0.05 = 50 % , (v) and more liberally by including the percentage of CpGs with p-value < 0.01 in the cluster = 50%. The identification of DMRs was conducted in the R package dss.

Annotation of differentially methylated regions

The location and association of the all CpGs with a given genomic feature was determined using the Great tit genome assembly and annotation 1.1 (Laine et al. 2016). More specifically, each CpG was annotated with respect to location in genes and promoter regions, which were defined as 3 kb upstream from the gene start (Viitaniemi et al. 2019). We excluded alternative transcripts in defining promoters. Some CpGs annotated to both genes and promoters. The annotation was conducted using the *IntersectBed* option in the BedTools package to identify the only the overlapping genomic features (Quinlan and Hall 2010). Altogether, CpGs present in the CONTROL-HIGH and CONTROL-SMELTER comparisons were annotated to 13364 and 12972 genes, respectively. These data sets cover 72% and 70% of the total number of the annotated genes (18550) in the Great tit genome. Of all the 1023725 CpGs analyzed in the CONTROL-HIGH comparison, 683392 CpGs were found within genes (66.8%) and 451843 (44.1%) within promoters. Of the 903449 CpGs in CONTROL-SMELTER comparison 602997 (66.7%) were located within genes, and 398381 (44.1%) CpGs within promoters. The annotation includes also the overlapping parts of gene bodies and promoters.

STRING database (Szklarczyk et al. 2015) was used to identify gene ontology categories associated with the DMRs. Annotated genes without generic names i.e. genes with LOC identifier were excluded from the analyses. A hierarchical clustering with a user-specified cutoff value C (0.5) was used as implemented in REVIGO database for merging of semantically similar GO categories corresponding to 1% chance of merging two randomly

generated categories (Supek et al. 2011). The lowest fdr corrected p-values of the initial enrichment analyses were used to select a representative GO term for each merged category.

Results

Metal exposure

Our dietary Pb treatment (HIGH Pb) significantly increased fecal Pb concentrations compared to the CONTROL (Table 1). At the SMELTER site, we found intermediate fecal Pb levels, not significantly different from either HIGH or CONTROL (Table 1). In the SMELTER area, concentrations of other measured heavy metals (As, Cu, Cd, Ni) were higher than in CONTROL or HIGH treatment (Table 1).

DNA methylation

Descriptive methylation patterns among the treatment groups

The average methylation percentages across all CpGs were 27.97 and 28.01 in CONTROL-HIGH Pb comparison, and 26.82 and 26.67 in CONTROL-SMELTER comparison. The mean difference in methylation in CONTROL-HIGH and CONTROL-SMELTER comparisons were −0.0013% and 0.0015%, respectively. There were no marked differences in the methylation percentage using 2% cutoff between the major chromosomes in either comparison (Supplementary figure 2). Also, there were no clear patterns in sample clustering in either of the comparisons based on hierarchical clustering or principal components analysis (Supplementary figure 3, Supplementary figure 4).

Differentially methylated CpG sites and DMRs in CONTROL HIGH comparison

We identified 96 377 (9.4%) differentially methylated CpGs in the CONTROL-HIGH comparison using binomial GLM (Table 2) at p-level 0.05, and after fdr correction (<0.05) 9555 CpGs remained. Almost equal proportion of the significant CpGs showed hypomethylation (45.9%) in CONTROL and hypermethylation (54.1%) in HIGH (supplementary figure 5). Beta binomial GLM identified 16852 (1.6%) differentially methylated CpGs in CONTROL-HIGH comparison at p-level 0.05 (Table 2) and seven CpGs after fdr correction. Altogether, 336 DMRs were detected in binomial regression and 72 DMRs in beta binomial regression in CONTROL-HIGH comparison.

Differentially methylated CpG sites and DMRs in CONTROL SMELTER comparison

We identified 129 830 (14.4%) CpGs in CONTROL-SMELTER comparison using binomial GLM at p-level 0.05 and 25222 CpGs remained after fdr correction. 52.5% of the significant CpGs were hypomethylated and 47.5% were hypermethylated (supplementary figure 5). Beta binomial GLM identified 22669 (2.5%) CpGs in CONTROL-SMELTER comparison at p-level <0.05 and 33 remained after fdr correction. Altogether, 781 DMRs were detected in binomial regression and 159 DMRs in beta binomial regression in CONTROL-SMELTER comparison.

The overlap between differentially methylated CpG sites and DMRs between the two comparisons

2789 (2.9%) of the significant CpGs showing hypomethylation, and 3022 (3.1%) showing hypermethylation were shared between CONTROL-HIGH and CONTROL-SMELTER, respectively (supplementary figure 5). 946 (1.5%) of the significant CpGs showing hypomethylation, and 947 (1.5%) showing hypermethylation were shared between CONTROL-HIGH and CONTROL-SMELTER comparisons (Supplementary figure 5). Using binomial regression, 30 DMRs that had exactly the same starting position were shared

between CONTROL-HIGH and CONTROL-SMELTER comparisons. Using beta binomial regression, three DMRs were shared between these two comparisons. Of the DMRs identified in binomial regression, 54% and 40% of were hypomethylated in CONTROL compared to HIGH and SMELTER, respectively. In beta binomial regression 48% and 38% of the DMRs were hypomethylated in CONTROL compared to HIGH and SMELTER, respectively. This suggests that there was no clear pattern of hypo- or hypermethylation in respect to pollution.

Annotation of the DMRs

The mean methylation in DMRs (identified by binomial regression) was higher for gene bodies than promoters in CONTROL (50.4% vs. 25.7%) in HIGH (49.7% vs. 24.9%). Similar pattern was found in DMRs between CONTROL (48.4% vs. 30.1%) and SMELTER (46.7% vs. 26.6%). These differences were also statistically significant (permutation test, 1000 replicates, $p = 0.001$). The DMRs identified in binomial regression in CONTROL-HIGH were annotated to 123 unique genes and 53 promoter regions excluding predicted genes. CONTROL-SMELTER DMRs were annotated to 281 genes, and 115 promoter regions. The DMRs from the beta binomial regression were annotated to 33 unique genes and, 8 promoter regions in. CONTROL-HIGH and to 66 genes and 34 promoter regions in CONTROL-SMELTER. In CONTROL HIGH comparison, the number of DMRs showing hyper or hypo methylation in gene bodies and promoters were similar. In SMELTER site, gene bodies and promoters shows tendency for hypermethylation (57% of the DMRs in gene bodies and 67% in promoters, respectively).

Gene enrichment analyses indicated 15 statistically significant ($fdr < 0.05$) gene ontologies in CONTROL-HIGH comparison and 62 gene ontologies in CONTROL-SMELTER comparison, when using DMRs from the binomial regression. No statistically significant gene ontologies were found in either comparison among the DMRs identified in beta binomial regression. After merging semantically similar gene ontologies using REVIGO

355 database, 5 and 11 enrichments remained in CONTROL-HIGH and CONTROL-SMELTER
356 comparisons, respectively (Figure 3, Figure 4). Most of the gene ontologies were associated
357 with developmental processes and were described under GO terms such as “system
358 development” or “nervous system development” (Figure 3, Figure 4). Other categories
359 involved cell-cell signaling or categories involving in transmitting information between cell
360 and its surroundings (Figures 3, 4). Finally, we also report 10 DMRs with the largest
361 differences in methylation levels (Supplementary Table 1). These included 12 genes (*POMC,*
362 *ITGA11, LEKR1, USH2A, ZPR1, JMJD1C, ADAMTS3, PDE1C, TBP, PAPD4, GCC1* and
363 *UTRN)* that may serve as potential candidates for further studies on the effects of pollution on
364 organisms via DNA methylation.

365

366 **Discussion**

367 We studied whether early-life exposure to pollution affects DNA methylation patterns in wild
368 great tit populations. We found evidence that both direct Pb exposure during post-hatching
369 stage and long-term anthropogenic pollution affect methylation levels of a small number
370 (0.25-2.1%) genes from which we were able collect data. The number of CpGs and DMRs
371 varied between binomial and beta binomial regression to a large extent such that binomial
372 regression was more liberal than beta binomial regression. We found that genes associated
373 with early developmental traits were enriched among the DMRs in binomial regression
374 potentially linking methylation differences to biologically meaningful traits in birds living in
375 polluted environments. However, this result was not consistent between the two statistical
376 methods highlighting the difficulty of modeling the variance in the CpGs. Nevertheless, our
377 results suggest that post-hatching, not only prenatal, environment modifies DNA methylation
378 patterns in wild vertebrates.

Causal and direct effects of Pb pollution on DNA methylation: CONTROL-HIGH comparison

Our data on fecal metal levels presented here, as well as data on bone Pb levels (Pb accumulates in bone) from the same broods (Eeva et al. 2014, Ruuskanen et al. 2015) shows that the HIGH group was indeed exposed to higher levels of Pb than CONTROL during the post-hatching period. The measurements correspond to observed Pb levels in polluted environments across Europe (Belskii et al. 1995a, Belskii et al. 1995b, Eeva and Lehikoinen 1996, Belskii et al. 2005, Berglund and Nyholm 2011), thus validating the effectiveness and environmental relevance of the Pb exposure treatment.

The HIGH-CONTROL comparison represents direct effects of Pb exposure post-hatching. There were no differences in the general methylation levels (hypo- or hypermethylation) between the two groups in either at the CpG or DMRs, in contrast for example to previous epidemiological studies in humans (Pilsner et al. 2009, Wright et al. 2010). The identified GO terms that were found to be enriched using the binomial regression analysis suggest that high Pb exposure may affect methylation of genes associated with biological processes such as system development and developmental processes. In previous studies, similar developmental pathways have been identified in rodents, but also sex-specific differences reported (Singh et al. 2018). These results makes sense in the light of what is known from previous studies in the same study system. For example, in the HIGH Pb treatment, vitamin A, retinol and stress protein levels were higher than in the CONTROL (Eeva et al. 2014, Ruiz et al. 2016). However, we acknowledge that the patterns that we found in blood tissue can be different in other tissues, but that the majority of the findings are likely to be similar for other tissues, as found previously in the study species (Derks et al. 2016, Lindner et al. 2021 Verhulst et al. 2016, Husby 2020). Also, relative little is known about the temporal stability of CpG methylation in great tits or other birds (Sepers et al. 2019). Viitaniemi et al. (2019) found that the majority of the CpGs showed stabile

methylation during the breeding season of an experimental great tit population. We cannot however, rule out the possibility that these changes are transient, and different methylation patterns may emerge in another life stage than 14 day-old offspring. Furthermore, If the observed methylation differences Pb to altered gene expression at the target genes, our results imply that the effects of pollution on such a variable set of genes may alter various developmental and cellular processes and ultimately health and phenotype.

Effects of long-term environmental pollution on DNA methylation: CONTROL-SMELTER comparison

At the SMELTER site, a population residing in an environment with long-term pollution exposure, birds were exposed to various pollutants, such as copper, nickel, cadmium and arsenic, originating from the nearby copper smelter (Eeva et al. 2014), with levels higher compared to CONTROL population. However, importantly, food quality and availability likely differed between CONTROL and SMELTER, as pollution reduces some important food sources such as caterpillars, and other insects in the area (Eeva and Lehikoinen 1996, Eeva et al. 2003). *Detailed studies on invertebrate abundance at polluted and control sites have shown that especially the amount of nutritious (e.g. rich in carotenoids) caterpillar larvae of moths have decreased in the polluted area (Eeva and Lehikoinen 1997; Sillanpää et al. 2009). This difference in abundance can be seen in the diet of great tit nestlings: the diet of great tit nestlings contained ca. 20 % less (based on biomass) moths and caterpillars as compared to the control area (Eeva et al. 2005). Decreased caterpillar availability manifest in inferior growth and less yellow plumage of great tit nestlings (Eeva et al. 2009).* The metal concentrations observed at the SMELTER area are generally below the critical levels associated with sub-clinical effects (Berglund et al. 2012), suggesting that indirect pollution effects via lower quality food is more likely (Eeva et al. 2005). The CONTROL and SMELTER population are not likely to differ genetically (given the low genetic

differentiation in this species even at European scale, e.g. Lemoine et al. 2016) and the habitats are relatively similar. However, at the SMELTER site, individuals are be exposed to pollutants already pre-hatching (e.g. Ruuskanen et al. 2015) contrary to experimental birds, but the importance of timing of the exposure is not well understood.

Contrary to predictions we did not find general differences in hypo/hypermethylation in CpGs or DMRs. Only when the DMRs annotated to genes were inspected, a tendency for hypermethylation was detected especially in the promoters of 155 genes (binomial) and 34 genes (beta-binomial) was detected. We detected a signal on differential methylation for genes related to nervous system in CONTROL-SMELTER comparison, which could potentially point to cognitive or behavioral changes. Parallel to our results, both prenatal Pb and malnutrition have recently been found to influence methylation of genes in pathways associated with neuronal proliferation and differentiation in mice and embryonic cell models (Senut et al. 2012, Senut et al. 2014, Weng et al. 2014, Sanchez-Martin et al. 2015, Singh et al. 2018, Dou et al. 2019). In humans, captive animal models and wildlife, both early nutrition and metal exposure, particularly Pb, have well-documented detrimental effects on cognitive abilities and behavior that persist into adulthood (e.g., impaired learning, memory, increased aggression, hyperactivity Brown et al. 1971, Morgan et al. 2000, Burger and Gochfeld 2005, Carere et al. 2005, Arnold et al. 2007, Chen et al. 2012, Ruuskanen et al. 2015). Until now, the role of epigenetic mechanisms underlying such effects has not been thoroughly characterized. Our results can thus stimulate further research on the potential epigenetic mechanisms explaining the long-lasting influences of early-life adverse environment on behavioral and cognitive traits. If the observed methylation differences Pb to altered gene expression at the target genes (see below), they could contribute to the potential developmental problems associated with poor nutrition. For example, we found that SMELTER group showed lower growth rates, higher antioxidant enzyme and stress hormone

454 levels, lower hematocrit and survival probability than CONTROL (Eeva et al. 2014, Rainio et
455 al. 2015b, Ruiz et al. 2016).
456
457 *Overlaps between the two comparisons*
458
459 We found very little overlap (~1-3%) in methylation of individual CpG sites between the
460 CONTROL-HIGH and CONTROL-SMELTER comparisons. However, on the DMR level
461 and their annotations showed some overlap indicating that the exposure to the Pb treatment
462 and metal-polluted at the smelter site can induce similar methylation changes. This suggests
463 some direct effects of metals also at the smelter site. The gene ontology enrichments also
464 mainly pointed that developmental processes were similar in these two comparisons
465 suggesting that the overall effect of pollution is in the same direction. However, the majority
466 of methylation differences in CONTROL and SMELTER are thus likely to be explained by
467 (i) other elements than Pb, (ii) and/or indirect effect of food, (iii) or their combination, or by
468 (iv) SMELTER site birds exposed to metals prenatally, compared to only postnatal exposure
469 in the HIGH group. Currently, we cannot distinguish between these alternatives. In general,
470 the number of DMRs between CONTROL and SMELTER were considerably higher than in
471 CONTROL-HIGH comparison probably reflecting exposure to a more stressful environment,
472 both nutritional stress and direct exposure to pollutants of various types pre and post-natally.
473
474 *Functional consequences of varying DNA methylation levels?*
475 Importantly, when interpreting the potential functional consequences of the observed
476 methylation differences, one needs to note that not all these genes with DMRs have been
477 characterized in birds (and annotation has been done using mainly chicken and zebra finch

gene models). Thus, the function of these genes is not well understood. Secondly, the link between DNA methylation and gene expression is not always straightforward depending on the genomic feature where the methylation changes occur (Jones 2012). Promoter methylation has been found to be inversely correlated with gene expression (Lou et al. 2014), but gene body methylation can have similar effects (Dixon et al. 2018). However, we hypothesize that differential methylation at the observed sites affects gene activity and ultimately multiple cellular, developmental and physiological processes. Indirect evidence for a functional interpretation is provided by a recent great tit study using whole-genome bisulfite and RNA-seq data. This study showed that across all genes, higher CG methylation at transcription start sites and within gene bodies was associated with lower gene expression (Laine et al. 2016). Finally, without detailed knowledge on gene function or differences in expression, it is difficult to judge whether the observed changes in methylation cause differences in phenotype and physiology we previously observed between HIGH Pb exposure and CONTROL groups (Eeva et al. 2014, Rainio et al. 2015b, Ruiz et al. 2016). Therefore, follow-up studies are needed to investigate how the observed parameters are affected by differential methylation in one or more of the regions.

The performance of the binomial and beta-binomial models

We employed two commonly used methods to detect CpGs and evaluated their performance using p-value histograms. Either one of these methods did not recover uniform p-value histograms in our data when applied to overall coverage threshold of 10x, pointing out that the p-values are not reliable as such. When we applied a filtering approach, developed for gene expression count data, we were able to recover uniform distribution for both methods. Thus, it appears that uninformative counts i.e. low counts for methylated state can induce a clear deviation from the uniform distribution. In other words, the small methylation differences between treatment groups are potentially difficult to model using the two

503 statistical approaches. While we applied an overall coverage threshold of 10x to our data, it
504 seems that another filtering step is needed for C counts to recover uniform p-value
505 distribution at least in our data set.

506 Overall, the performance of the binomial and beta binomial regression reflects the
507 outcome of previous studies on simulated and empirical data sets: binomial regression has
508 been found to be more liberal in finding CpGs as compared to beta binomial regression
509 (Dolzhenko and Smith 2014, Park and Wu 2016, Wreczycka et al. 2017), and we also found
510 considerably more CpGs and DMRs in both comparisons using binomial than beta binomial
511 regression. The likelihood of false negatives is higher when stringency of accounting for the
512 number of tests is lower. Also, this is more apparent in the individual CpG site analysis then
513 in the DMR analysis. Yet, in this novel and explorative study, identifying a set of potentially
514 affected CpGs and DMRs, very strict correction for the number of tests would lead to a large
515 type II error and thus failure to recognize differentially methylated sites or regions as
516 significant. Overall, the comparison between the two methods is challenging in empirical
517 data sets but both methods seem to recover uniform p-value distribution when uninformative
518 CpGs are filtered out. We view our analysis as a starting point for further functional
519 validation of our findings.

520 *Conclusions*

521 In this study, we explored the environmental causes of epigenetic variation in an ecological
522 model organism, which is a novel and emerging research field. We found evidence that
523 differentially methylated regions contain genes enriched for biologically meaningful
524 processes and suggest potential targets for future research. Although we used a method that
525 does not cover the whole genome, we were able to analyze methylation patterns covering
526 most of the annotated genes in great tit genome. However, the results were not consistent
527 between binomial and beta binomial regression, which warrants caution when selecting

analysis methods and interpreting results using different methods. Finally, the functional consequences of variable methylation patterns found in this study are yet to be discovered and a more comprehensive approach combining other molecular levels as well functional studies is needed.

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Disclosure of interest

We have no conflict of interest to declare.

Data accessibility

The sequence data are deposited in the SRA database under accession number PRJNA589705.

Author contributions

SR, TE and HM designed the study. SR and TE collected the data. HM, SR and KvO designed the sequencing. HM conducted statistical and bioinformatic analyses. KvO provided the genome resources. SR, HM, and KvO interpreted the data. All authors contributed to manuscript preparation.

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883

884 **Tables**

885 **Table 1.** Metal concentrations (µg/g, dry weight) in feces of seven day old *Parus major*
886 nestlings in the three treatment groups. The values are geometric means with 95% CIs. GLM
887 and Tukey's test: means with the same letter are not significantly different. N indicates
888 number of broods.

Metal	HIGH n = 12	SMELTER n = 11	CONTROL n = 12	$F_{ndf, ddf}$	p
Pb	8.0 (4.8-13.3) a	4.4 (2.6-7.6) ab	2.2 (1.3-3.6) b	$3.70_{2,32}$	0.03
As	0.40 (0.25-0.62) a	4.3 (2.7-6.9) b	0.60 (0.38-0.95) a	$24.4_{2,32}$	<0.0001
Cd	0.73 (0.51-1.04) a	1.93 (1.34-2.78) b	0.54 (0.38-0.76) a	$10.2_{2,32}$	0.0004
Cu	34.6 (27.1-44.1) a	111 (86-144) b	29.6 (23.2-29.6) a	$12.9_{2,32}$	<0.0001
Ni	2.24 (1.6-3.1) a	20.6 (14.5-29.2) b	1.99 (1.4-2.8) a	$34.5_{2,32}$	<0.0001

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890

891 **Table 2.** The number and percentage (in parentheses) of p-values for CpG sites less than 0.05
892 in different comparisons. Fdr refers to multiple testing correction using Benjamini &
893 Hochberg (1995) method. The test statistics of goodness-of-fit test (χ^2) of the p-value
894 histograms indicated deviations uniform p-value distribution in unfiltered data, which
895 improved (i.e. higher χ^2 values)with filtering ('filter').

Comparison	p-value < 0.05	fdr < 0.05	χ^2 -statistics
Binomial CONTROL HIGH	96377 (9.4)	9555	793200
Binomial CONTROL SMELTER	129830 (14.4)	25222	851790
Beta-binomial CONTROL HIGH	16852 (1.6)	7	358240
Beta-binomial CONTROL SMELTER	22669 (2.5)	33	236840
fdrtool CONTROL HIGH (beta-binomial)	62789 (6.1)	520	192880
fdrtool CONTROL SMELTER (beta-binomial)	67418 (7.5)	1811	149650
Binomial CONTROL HIGH - filter	89486 (12.5)	11334	122810
Binomial CONTROL SMELTER -filter	122463 (19.4)	30782	420650
Beta-binomial CONTROL HIGH - filter	61867 (8.6)	759	31295
Beta-binomial CONTROL SMELTER - filter	66714 (10.5)	2492	67606

896

897

Figures

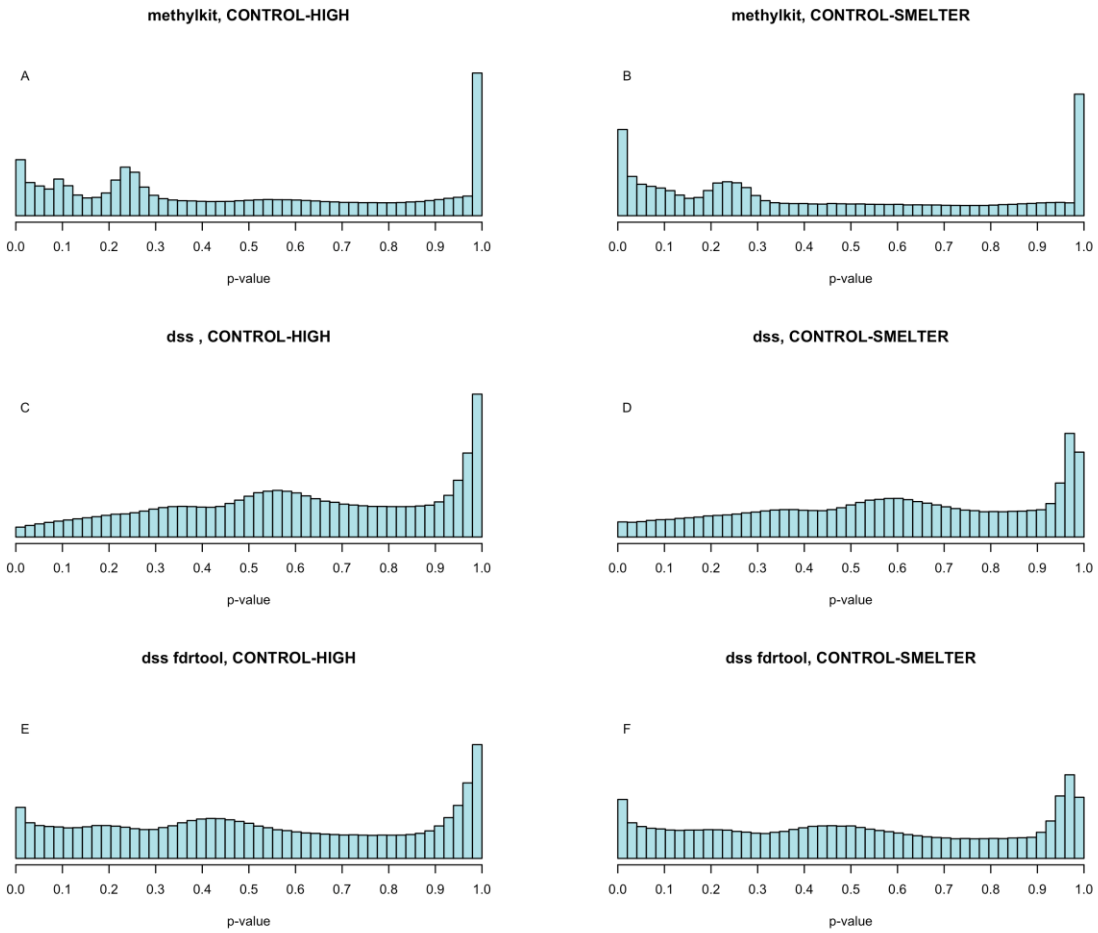


Figure 1

P-value histograms of the binomial (methylkit, A, B) and beta-binomial regression (dss, C, D) in CONTROL-HIGH and CONTROL-SMELTER comparisons. The p-values of the beta binomial regression were re-calculated based on the test statistics as implemented in the fdrtool R-package (E, F).

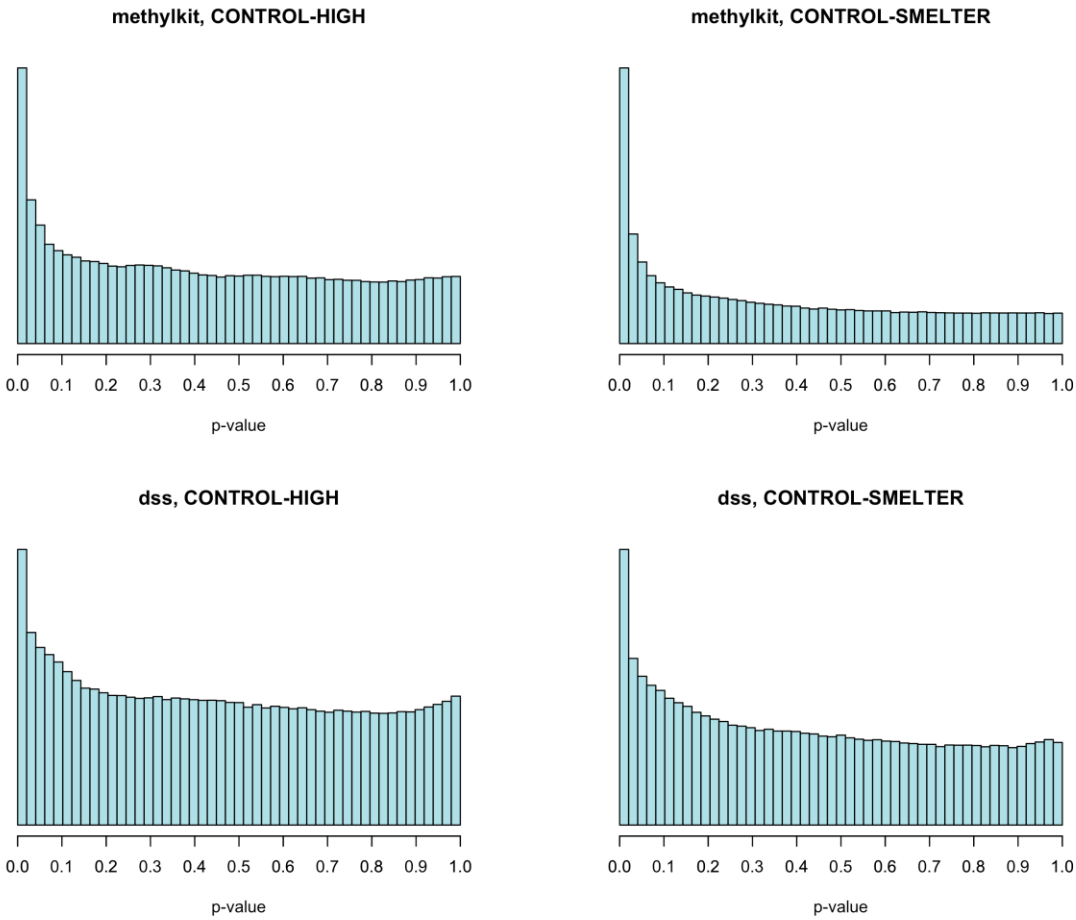
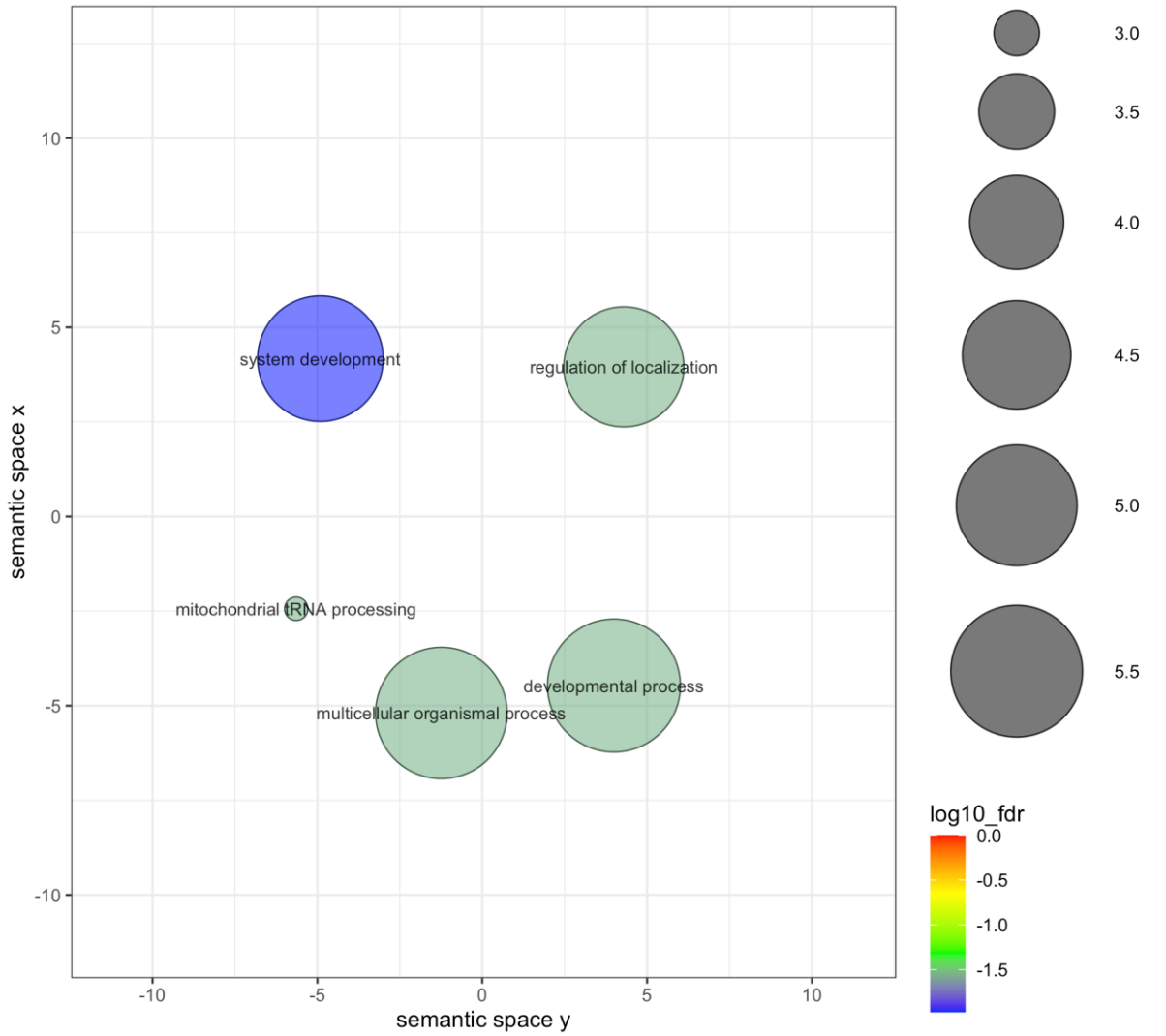


Figure 2. The p-value histograms after low coverage filtering in binomial regression (methylkit) and in beta binomial regression (dss). The p-values in beta binomial regression are based on the re-calculated p-values in fdrtool.



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915 Figure 3. Results of the gene enrichment test for DMRs identified in the binomial regression
916 in the CONTROL-HIGH comparison after merging semantically similar gene ontology
917 categories. Circle size indicates the frequency of the GO term in the underlying GO database
918 (bubbles of more general terms are larger; http://revigo.irb.hr/) and color scale shows the fdr
919 (log10 scale) of the representative GO term for each merged category.

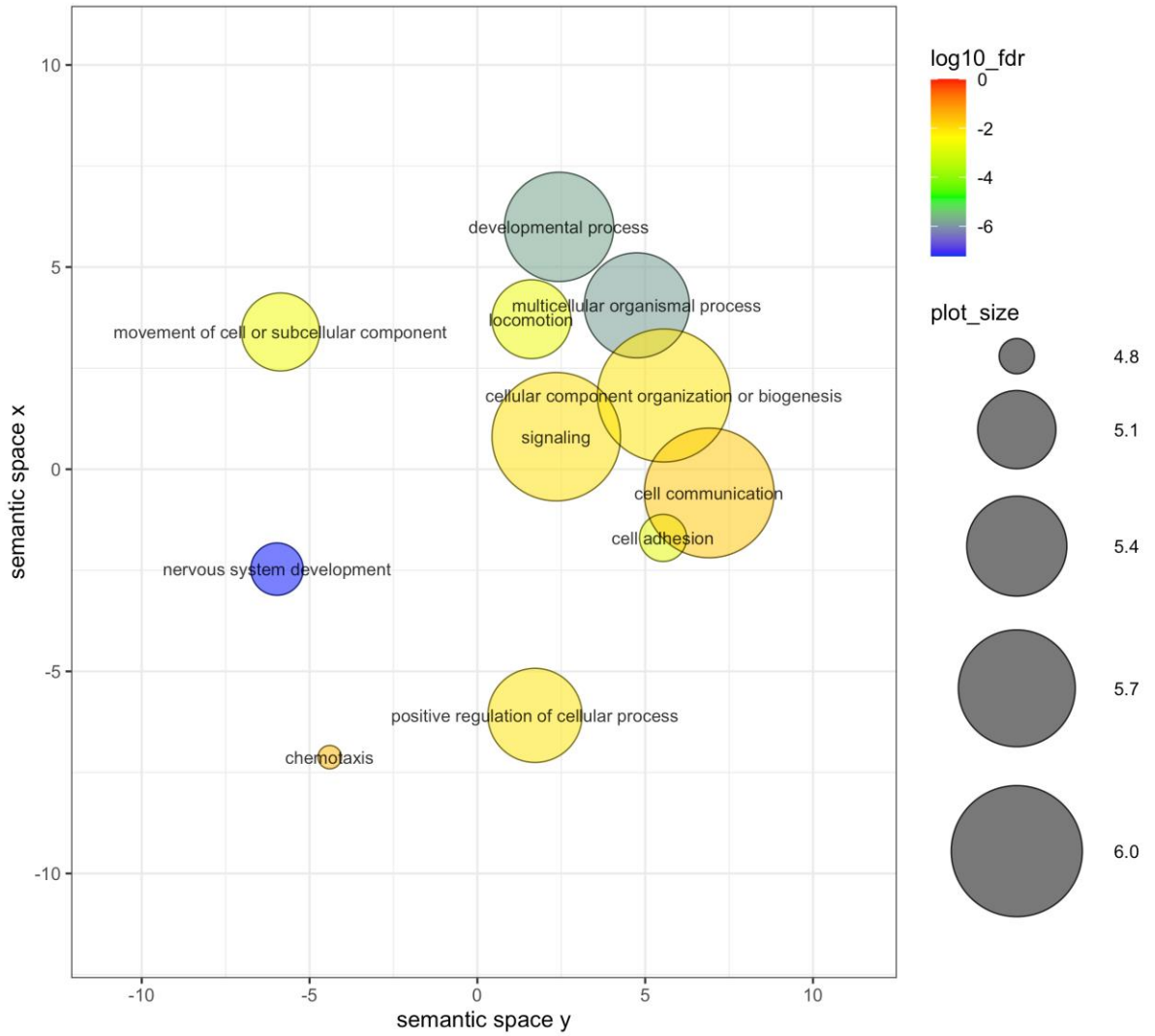
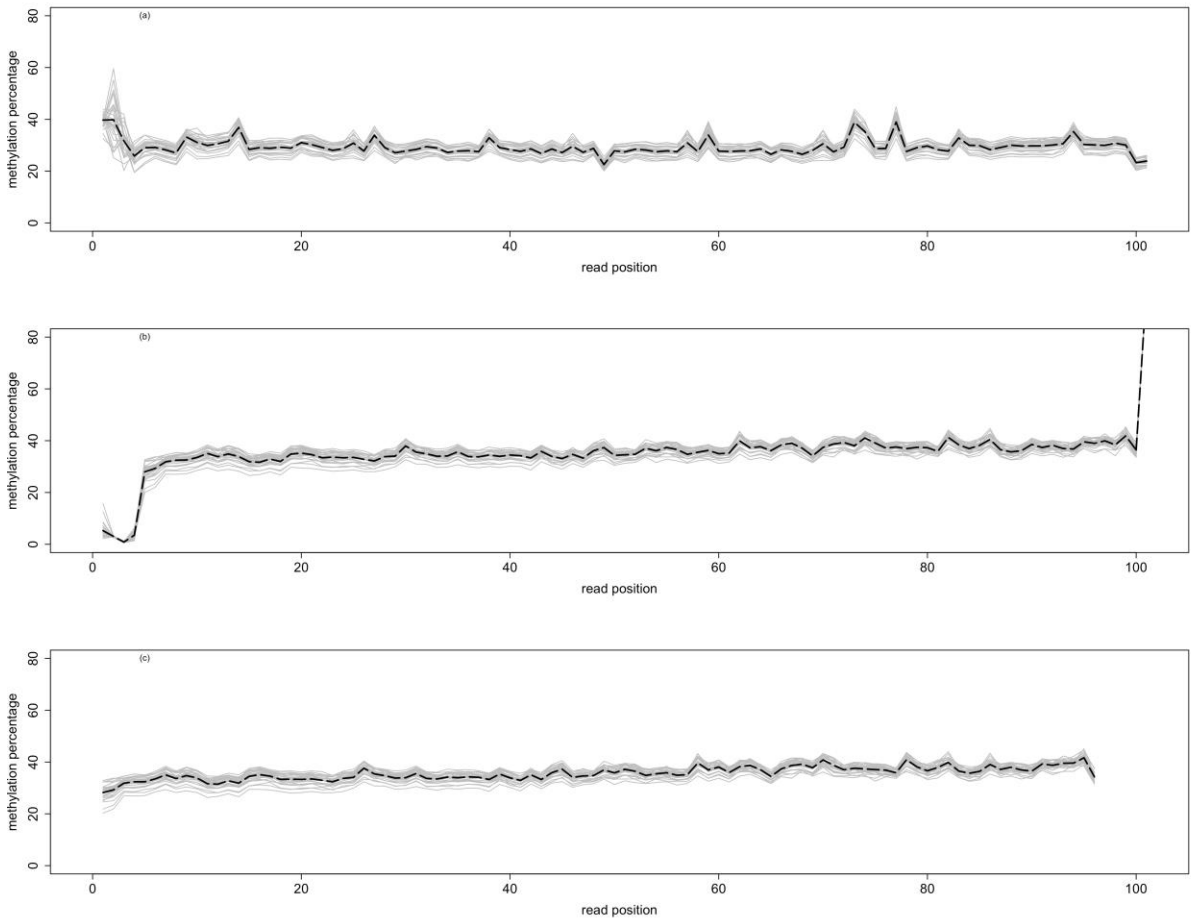


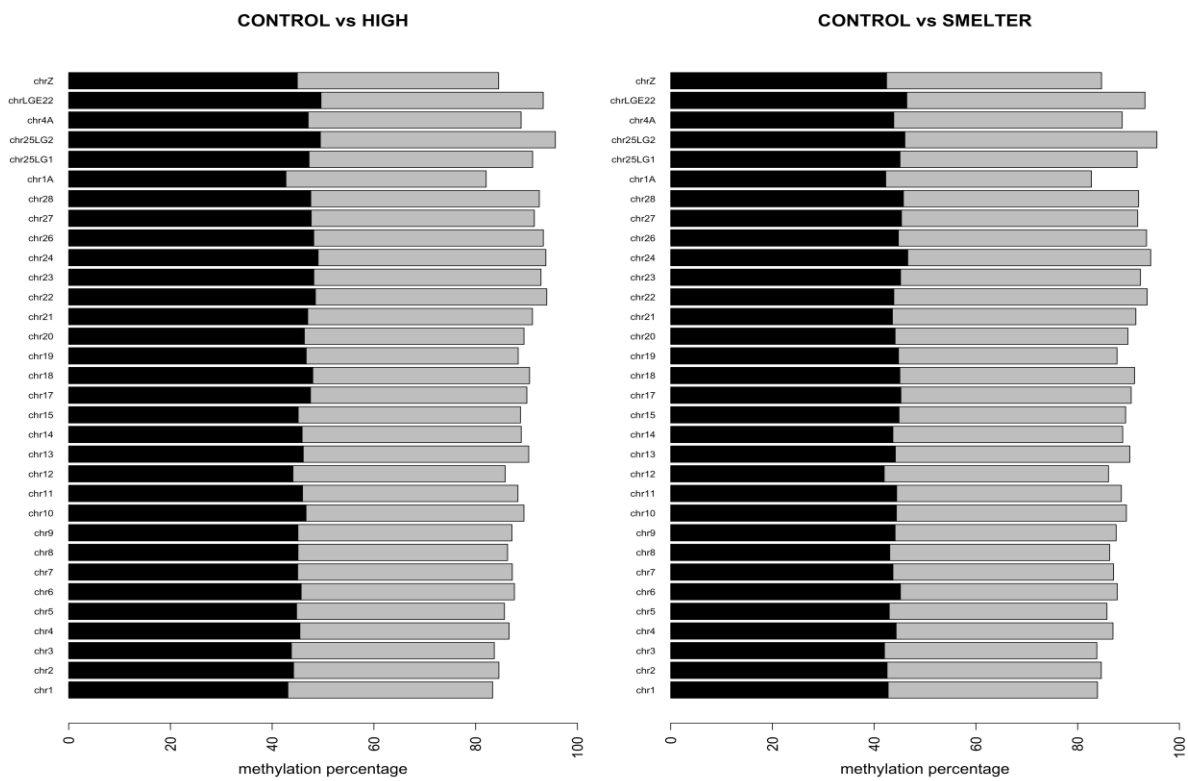
Figure 4. Results of the gene enrichment test for DMRs identified in the binomial regression in the CONTROL-SMELTER comparison after merging semantically similar gene ontology categories. Circle size indicates the frequency of the GO term in the underlying GO database (bubbles of more general terms are larger; http://revigo.irb.hr/) and color scale shows the fdr (log10 scale) of the representative GO term for each merged category.

Supplementary material



Supplementary figure 1

Methylation bias (M-bias) plots for read 1 (a) and for read 2 before (b) and after (c) cutting the bases showing lower or higher methylation than the other bases in the read. Four bases were cut from the beginning of the read 2 and one from the end of the read 2. The grey lines show the methylation percentage in all libraries along the position in the reads and the black line shows the mean methylation level across all libraries. On the x-axis is the position in the read and on the y-axis the methylation percentage.



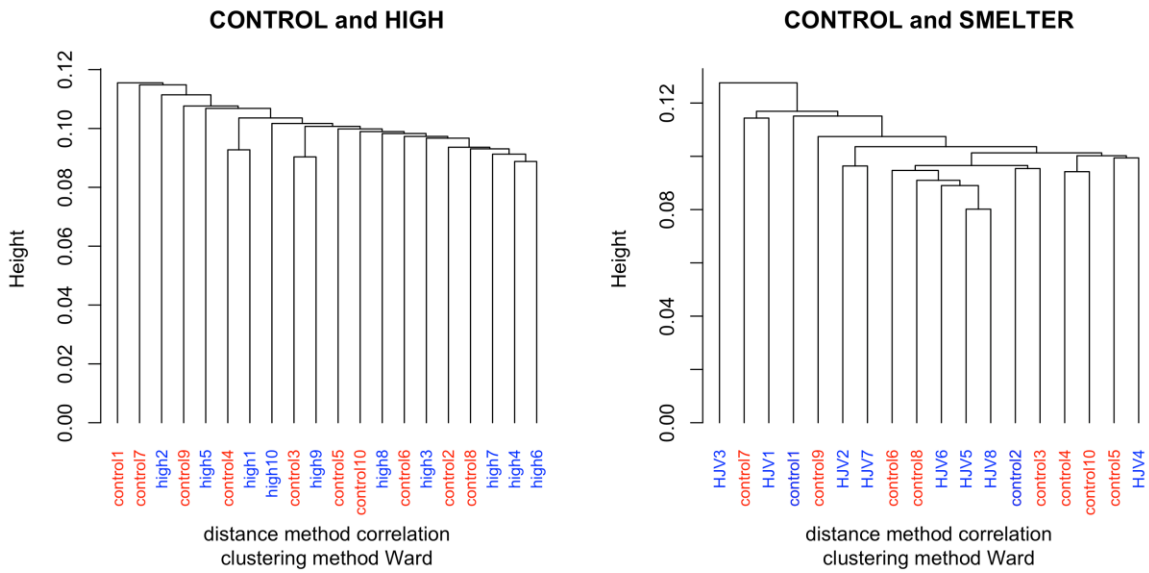
939

940 Supplementary figure 2

941 The percentage of hypo (black) and hypermethylated (grey) CpGs in Great tit major

942 chromosomes. In (a) CONTROL and HIGH and in (b) CONTROL SMELTER comparisons.

943



944

945 Supplementary figure 3

946 Hierarchical clustering of all individuals in (a) CONTROL HIGH and (b) CONTROL
947 SMELTER comparisons.

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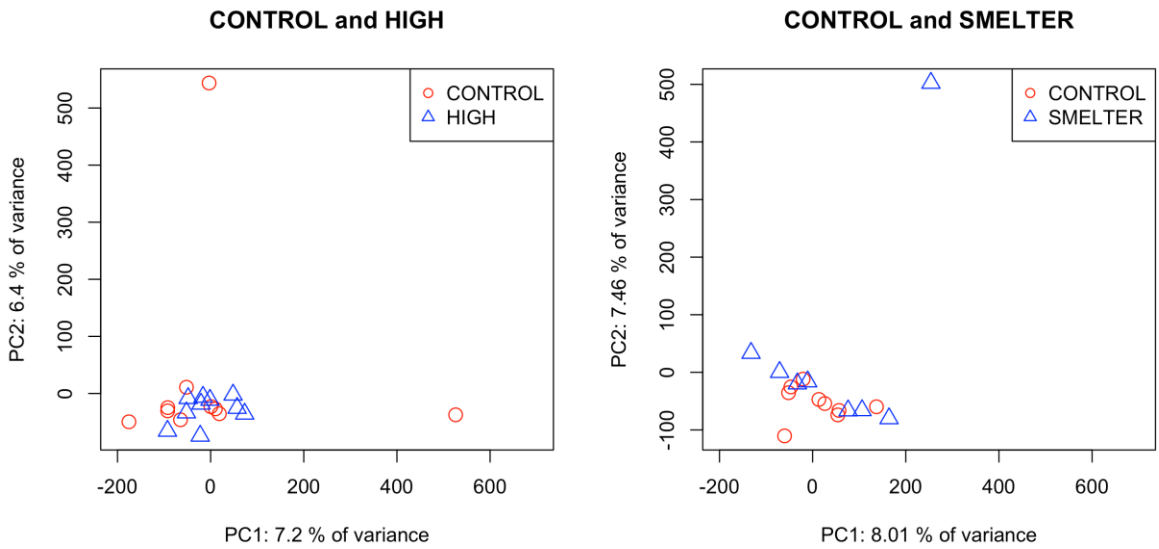
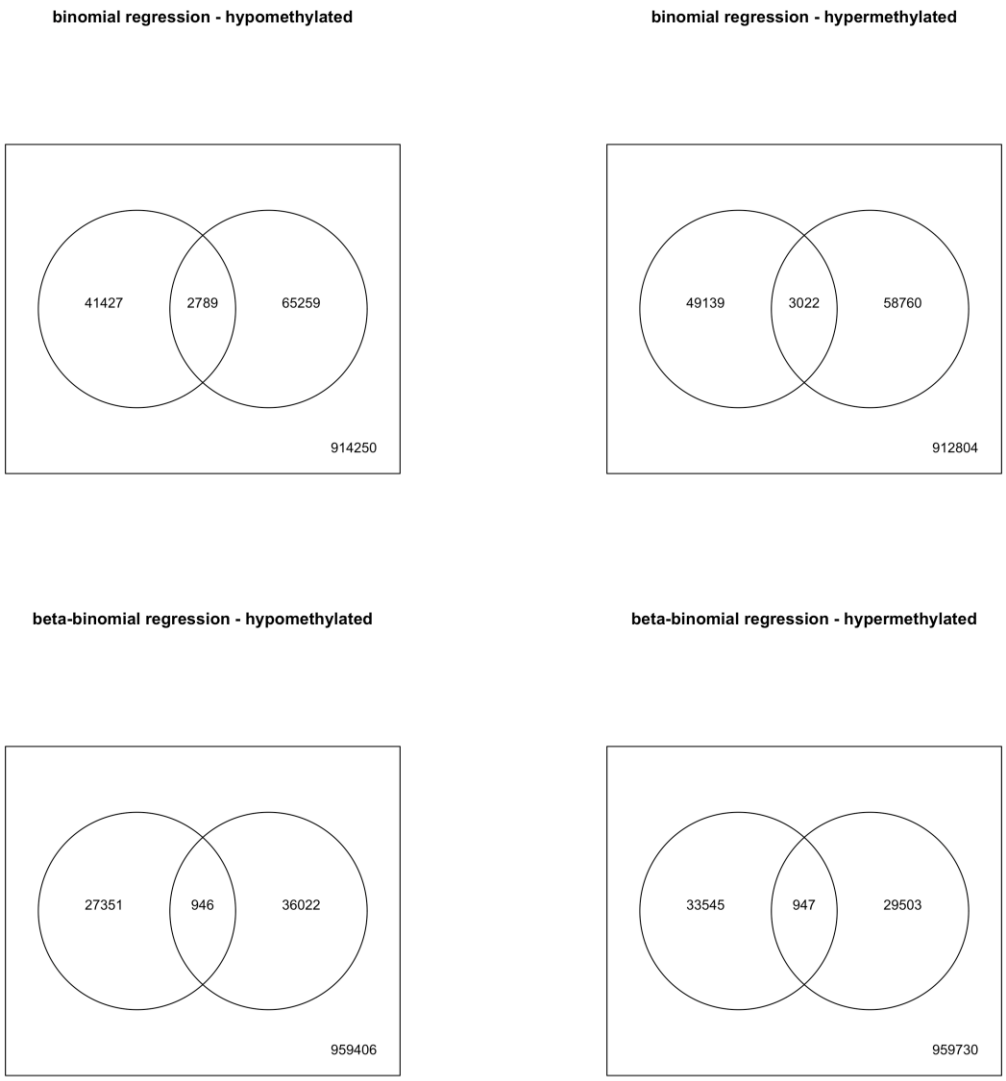


949

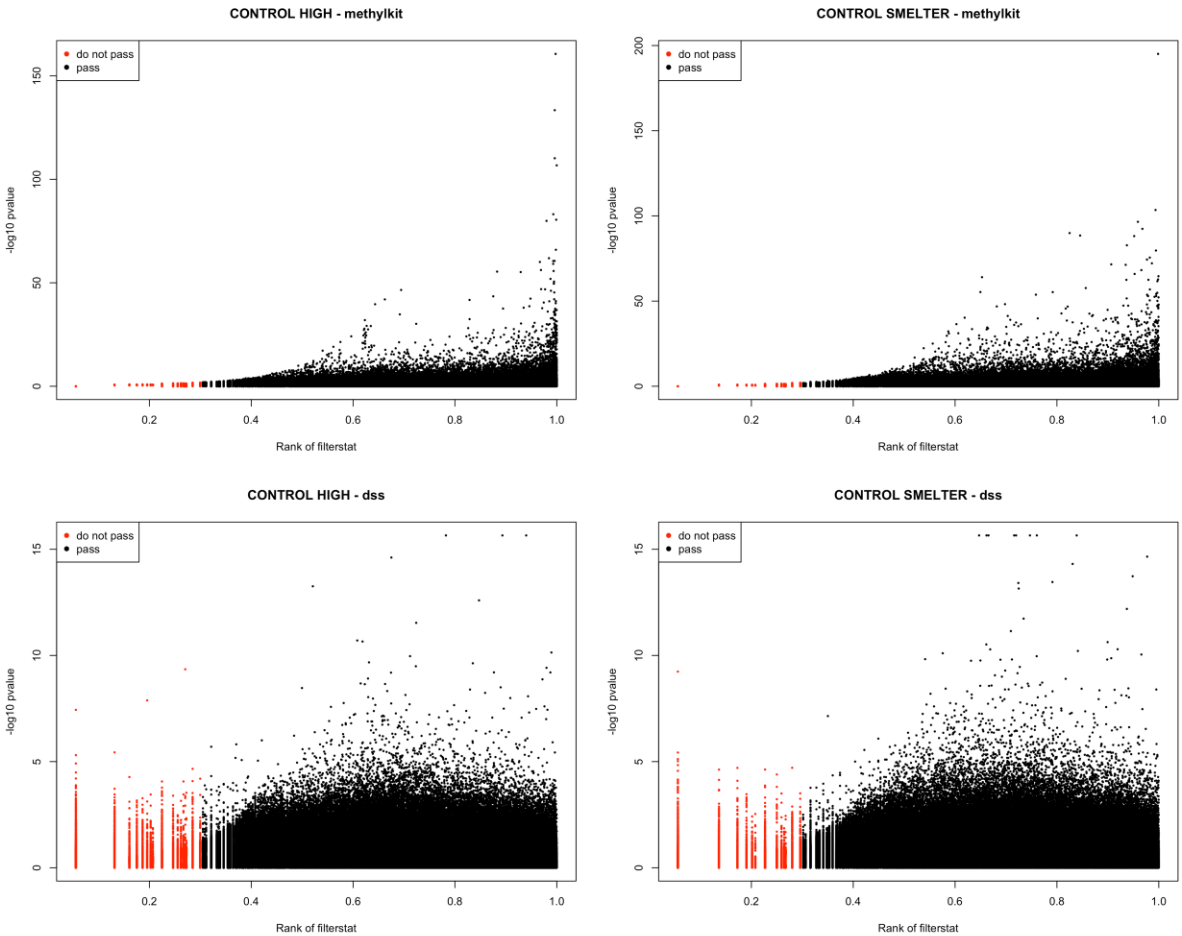
950 Supplementary figure 4

951 Principal component analysis of all individuals and CpGs in CONTROL HIGH (a) and in
952 CONTROL SMELTER (b) comparisons. The x-axis shows the variance explained by PC1
953 and y-axis variance explained by PC2.

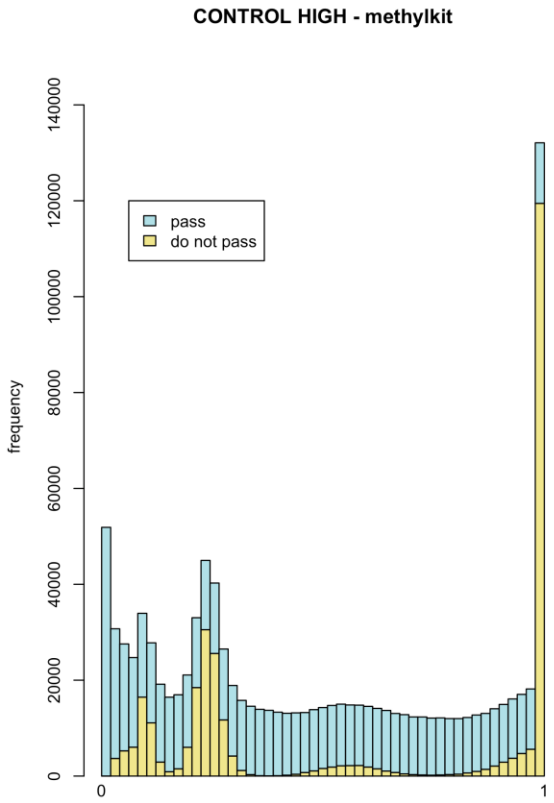


Supplementary figure 5.

Venn diagrams showing the overlap of the significant CpGs between CONTROL-HIGH (left side of each diagram) and CONTROL-SMELTER (right side of each diagram) comparisons. In the upper panel is the overlap in binomial regression and in the lower panel the overlap in beta-binomial regression. Numbers at the lower right in each box show total number of sites.



Supplementary figure 6. The principle of filtering uninformative CpGs. On the x-axis is the rank of filter statistics i.e. the mean coverage of Cs in each analyzed CpG. On the y-axis is the -log10 p-value obtained either from binomial regression (methylkit) or from beta binomial regression (dss). The red filled circles are CpGs not passing the filtering threshold (30%) while the filled black circles are CpGs passing the filtering threshold.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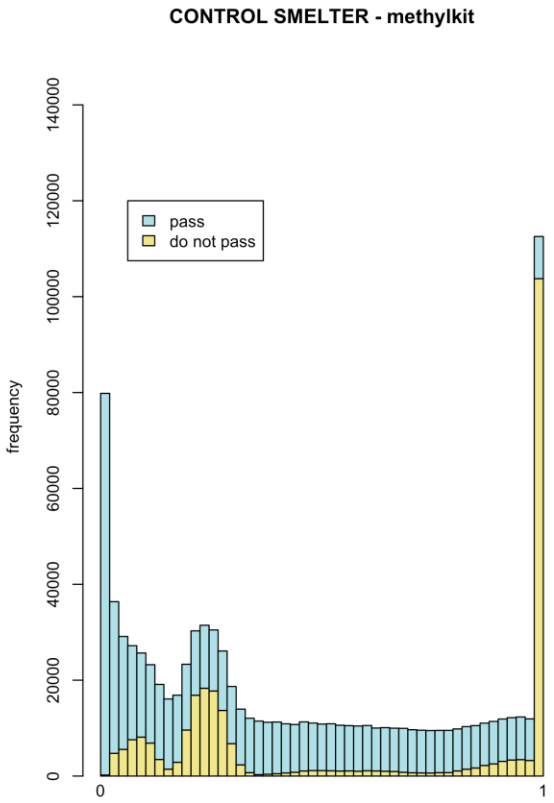
pass
do not pass
frequency
0
20000
40000
60000
80000
100000
120000
140000
0
1


CONTROL SMELTER - methylkit
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do not pass
frequency
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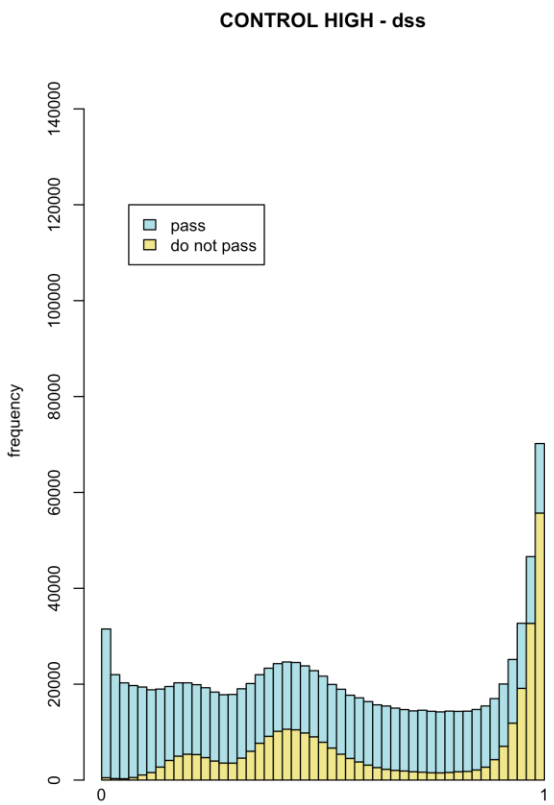
60000
80000
100000
120000
140000
0
1


CONTROL HIGH - dss
pass
do not pass
frequency
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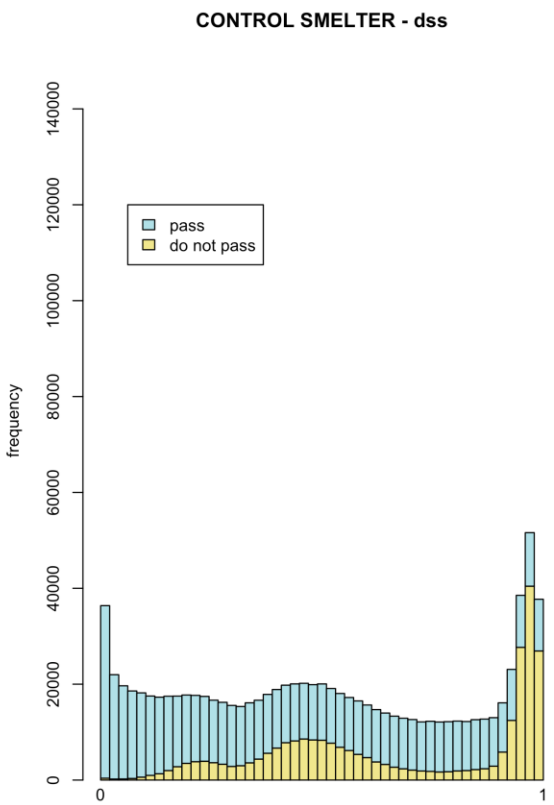
20000
40000
60000
80000
100000
120000
140000
0
1


CONTROL SMELTER - dss
pass
do not pass
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120000
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967

Supplementary figure 7. The filtering approach shown as p-value histogram. The yellow color shows the effect of removing low coverage CpG sites along the p-value distribution. The blue colour shows the p-values remaining after the low coverage filtering.

DNA isolation

DNA isolation was performed at the Center of Evolutionary Applications (University of Turku, Finland). We used whole blood samples, which can be acquired without sacrificing the individuals. In birds erythrocytes have nuclei and therefore >95% of the gained DNA is from erythrocytes. DNA was extracted from c. 10 − 20 µl whole blood using the salt extraction method modified from Aljnabi & Martinez (1997). Extracted DNA was treated with RNase-I according to the manufacturer's protocol. DNA concentrations were measured fluorometrically with a Qubit High Sensitivity kit (ThermoFisher Scientific) and we assessed DNA integrity by running each DNA sample on an agarose gel.

RRBS library preparation

We used a reduced representation bisulfite sequencing (RRBS) approach, which enriches the regions of the genome that have a high CpG content (Meissner et al. 2005). It was previously shown in the study species that the vast majority of the methylated cytocines (97%) were derived from CpG context in blood (Derks et al. 2016). Sequencing was conducted at the Finnish Microarray and Sequencing Center in Turku, Finland. The library preparation was started from 200 ng of genomic DNA and was carried out according to a protocol adapted from Boyle et al. (2012). The first step in the workflow involved the fragmentation of genomic DNA with MspI where the cutting pattern of the enzyme (C^CGG) was used to systematically digest DNA to enrich for CpG dinucleotides. After a fragmentation step, a

single reaction was carried out to end repair and A-tail (required for the adapter ligation) the MspI digested fragments using Klenow fragment (3’ => 5’ exo), following the purification of A-tailed DNA with bead SPRI clean-up method (AMPure magnetic beads). A unique Illumina TruSeq indexing adapter was then ligated to each sample during adapter ligation step to be able to identify pooled samples of one flow cell lane. To reduce the occurrence of adapter dimers, a lower concentration of adapters (1:10 dilution) was used than recommended by the manufacturer. These ligated DNA fragments were purified with the bead SPRI clean-up method before putting samples through bisulfite conversion to achieve C-to-U conversion of unmethylated cytosines, whereas methylated cytosines remain intact. Bisulfite conversion and sample purification were done according to the Invitrogen MethylCode Bisulfite Conversion Kit. Aliquots of converted DNA were amplified by PCR (16 cycles) with Taq/Pfu Turbo Cx Polymerase, a proofreading PCR enzyme that does not stall when it encounters uracil, the product of the bisulfite reaction, in the template. PCR-amplified RRBS libraries were purified using two subsequent rounds of SPRI bead clean-ups to minimize primer dimers in the final libraries. The high quality of the libraries was confirmed with Advanced Analytical Fragment Analyzer and the concentrations of the libraries were quantified with Qubit® Fluorometric Quantitation, Life Technologies. We selected fragment sizes ranging between 150 − 1000 bp (average sizes were 250-350 bp) for sequencing.

Sequencing

The samples were normalized and pooled for the automated cluster preparation, which was carried out with an Illumina cBot station. The 28 libraries were randomly combined in three pools, 10 or 8 samples in each pool and sequenced in 3 lanes. The samples were sequenced with an Illumina HiSeq 2500 instrument using TruSeq v3 sequencing chemistry. Paired-end sequencing with 2 x 100 bp read length was used with 6 bp index run.

Supplementary Table 1. Top 10 DMRs for CONTROL-HIGH and CONTROL-SMELTER comparisons, separately for binomial and betabinomial models.Mean Met% = average methylation percentage. Met% difference = difference, in percentages, in methylation between two groups. CO = control, HI = HIGH, SME = SMELTER

CHROMOSOME	POSITION (bp)		ANNOTATION	DMR length	No. CpGs	Mean met% CO	Mean met% HI/SME	Met% difference
A) CO-HIGH binomial								
chr10	19104012	19104107	ITGA11	96	4	80.08	49.96	30.12
chr9	24013175	24013233	LEKR1	59	7	45.12	69.51	-24.38
chr13	12002300	12002394	.	95	8	31.02	54.51	-23.49
chr28	1285543	1285681	.	139	4	60.02	83.44	-23.42
chr3	20274597	20274654	USH2A	58	6	51.39	73.61	-22.22
chr24	788116	788169	ZPR1	54	4	40.76	61.20	-20.44
chr22	703067	703161	.	95	8	37.47	18.45	19.02
chr4	1948465	1948599	ADAMTS3	135	11	33.67	52.10	-18.43
chr2	81838048	81838308	.	261	18	36.30	18.74	17.56
chrZ	65661754	65661805	.	52	4	40.04	22.79	17.25
B) CO-SMELTER binomial								
chrZ	19688875	19689005	.	131	14	63.56	23.61	39.95
chr2	53829014	53829116	PDE1C	103	6	94.64	61.62	33.02
Scaffold1294	1027	1153	.	127	6	42.69	67.69	-24.99
chrZ	21902415	21902582	PAPD4	168	13	8.46	32.57	-24.11
chr7	9525141	9525265	.	125	7	66.83	44.56	22.26
chr3	48718277	48718390	UTRN	114	8	74.32	52.12	22.20
chr28	1285543	1285688	.	146	5	67.49	89.66	-22.17
chr3	41373464	41375606	TBP	2143	10	38.19	16.11	22.08
chr6	1317263	1317448	LOC107207020	186	5	85.72	63.69	22.03
chr15	13816009	13816974	POMC	966	40	52.83	31.41	21.42

CHROMOSOME	POSITION (bp)		ANNOTATION	DMR lenght	No. CpGs	Mean met% CO	Mean met% HI/SME	Met% difference
C) CO-HIGH betabinomial								
chr10	19104012	19104107	ITGA11	96	4	80.08	49.96	30.12
chr9	24013175	24013233	LEKR1	59	7	45.12	69.51	-24.38
chr13	12002300	12002394	.	95	8	31.02	54.51	-23.49
chr3	20274597	20274654	USH2A	58	6	51.39	73.61	-22.22
chr24	788116	788169	ZPR1	54	4	40.76	61.20	-20.44
chr15	13816420	13816728	POMC	309	25	51.26	30.95	20.31
Scaffold306	178895	179055	.	161	16	45.08	65.33	-20.25
chr6	4145270	4145351	JMJD1C	82	5	71.25	51.89	19.36
chr22	703067	703161	.	95	8	37.47	18.45	19.02
chr4	1948465	1948599	ADAMTS3	135	11	33.67	52.10	-18.43
D) CO-SMELTER betabinomial								
chrZ	19688875	19689005	.	131	14	63.56	23.61	39.95
chr2	53829014	53829116	PDE1C	103	6	94.64	61.62	33.02
chr3	41373476	41375606	TBP	2131	8	32.04	5.65	26.39
chr6	1317265	1317448	LOC107207020	184	4	83.96	59.20	24.76
chrZ	21902415	21902570	PAPD4	156	10	6.63	31.20	-24.56
chr15	13816009	13816877	POMC	869	36	51.97	29.03	22.94
Scaffold525	2758	2857	GCC1	100	28	53.35	30.49	22.86
chr3	161130	161201	.	72	10	61.32	83.76	-22.44
chr7	9525141	9525265	.	125	7	66.83	44.56	22.26
chr3	48718277	48718390	UTRN	114	8	74.32	52.12	22.20

Genes at differentially methylated regions

Among the set of 10 most differentially methylated regions across the treatment groups, we identified 12 genes (*POMC, ITGA11, LEKR1, USH2A, ZPR1, JMJD1C, ADAMTS3*, *PDE1C, TBP, PAPD4, GCC1* and *UTRN).* Of these 12, *POMC*, showed lower methylation (ca. 20%, thus theoretically higher expression) in methylation *both* in SMELTER and HIGH treatments compared to CONTROL. *POMC* (pro-opiomelanocortin) is a neuronal hormone, which is cleaved to multiple key by-products, including (i) corticotropin (ACHT), controlling the stress response, (ii) appetite control and (iii) b-endorphin (Marco et al. 2016). Methylation of *POMC* has been associated with nutritional state (in rats, Ramamoorthy et al. 2018), maternal under nutrition (in ovine Stevens et al. 2010) and offspring early-life stress (in mice, Wu et al. 2014). Here, we report for the first time that *POMC* methylation may mediate early-life stress (nutritional and/or metal exposure) also in a wild vertebrate population. Furthermore, methylation of another stress related gene, PDEC1 (phosphodiesterase 1C) was also decreased in SMELTER compared to control. Expression of PDEC1 gene has been found to be associated with aldosterone stress hormone in chicken (e.g. Fallahsharoudi et al. 2017).

The other differentially methylated genes in relation to metal exposure were related to (i) DNA damage: *JMJD1C* is a candidate histone demethylase and also plays a role in the pathway DNA-damage response (e.g. Watanabe et al. 2013). Our data suggests that its methylation was decreased (theoretical expression increased) in high Pb exposure compared to control, which is logical given that Pb exposure is likely to cause more oxidative stress and DNA damage (e.g. Wu et al. 2008, Rainio et al. 2015a). Other studies on Pb exposure also found differences in methylation in detoxification pathways (Sen et al. 2015); (ii) growth and development: *LEKR1* (Freathy et al. 2010), *ADAMTS3* (Janssen et al. 2016), *UTNR* (in mammals, e.g. Schofield et al. 1993). Furthermore, Pb has specifically been found to impair

neurodevelopment (e.g. Morgan et al. 2000, Burger and Gochfeld 2005). Our data shows that methylation of *ZPR1* (zinc finger protein gene), an important protein in neural development (e.g. Doran et al. 2006) was increased by ca. 20% (theoretical expression decreased) in Pb exposure compared to controls, which may warrant further studies on *ZPR1*, Pb exposure and neurodevelopment. Previous studies have reported alternation in methylation of other genes related to neurodevelopment, such as another zinc finger protein gene, *Zfp974* and *Zfp787, ARTN, C5aR1* (Dou et al. 2019), *Syt2, Prkg1, Pcdhb20, Slc2a3, Klhl1 and Snap29* (Singh et al. 2018) and *PAX1* and *MSI1* (Senut et al. 2014) (iii) transcription and intracellular processes: *TBP* is universal transcription factor required for all of the eukaryotic RNA polymerases (Shimada et al. 2003), *PAPD4* is a poly(A) RNA polymerase (Burroughs et al. 2010), while *GCC1* is associated with Golgi apparatus structure (Gosavi et al. 2018). Thus, these genes may serve as potential candidates for further studies on the effects of pollution on organisms via DNA methylation.