Title: Inferences from epigenetic information in an ecological context: a case study of early-life environmental effects on DNA methylation in zebra finches

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Abstract

DNA methylation (DNAm) plays a key role in mediating phenotypic responses to environmental variation. Various approaches exist to link DNAm variation to phenotypes, ranging from single nucleotide resolution to the development of composite indexes. We here discuss the conceptual differences between these approaches through a case study using whole genome, longitudinal DNAm data from adult zebra finches raised in experimentally created small or large broods. Specifically, we (i) identified CpG sites where DNAm was affected by brood size, and (ii) developed two DNAm-based indexes of the early-life environment (DMSi: based on differentially methylated sites with respect to rearing brood size; SMLmi: supervised machine learning-derived index optimized to predict brood size). We also compared results obtained by either merging or analyzing longitudinal DNAm samples separately and discussed how the permanence or transience of DNAm changes shapes responses to environmental variation through life. Our findings confirm that early-life environment leaves lasting DNAm signatures detectible in adulthood, and this effect is stronger later in life. Importantly, both indexes predicted early-life growth rates, demonstrating that DNAm-based indexes can be leveraged to retroactively quantify aspects of early-life conditions, providing a powerful novel tool in the study of wild populations.

Keywords: DNA methylation, brood size, early-life stress, epigenetic data, case study

1. Introduction

Evolutionary theory has long considered genetic variation to be the main driver of phenotypic plasticity. However, mounting evidence reveals the insufficiency of genetic variation in explaining the rapid phenotypic responses of wild animals to environmental changes (Charmantier et al., 2008; Réale et al., 2003). Epigenetic modifications are known to be sensitive to the environment (Angers et al., 2010; Li et al., 2021), modulate gene expression (Gibney & Nolan, 2010), and generate phenotypic variation without altering the primary nucleotide sequence with direct effects on evolution and adaptation (Kronholm & Collins, 2016). Emerging research also recognizes epigenetic mechanisms such as chromatin modifications and DNA methylation (DNAm) as dynamic regulators that modulate phenotypic plasticity and thereby link environmental variation to phenotypic outcomes. Epigenetic modifications are especially relevant in contexts when genetic variation is limited, potentially generating heritable phenotypic diversity that can be subject to natural selection (Lind & Spagopoulou, 2018).

Since methylation of different parts of the genome has different effects on gene expression (Moore et al., 2013), τhe design and resolution of DNAm studies can greatly impact the insights that can be drawn. All levels of analysis complement each other, providing a comprehensive understanding of the DNAm landscape and how that influences gene expression, cellular function and ultimately individual phenotype across different biological contexts. Global DNAm, which provides a single overall measure of DNAm across the entire genome of an individual (e.g. with gel-based techniques or ELISA kits) is a simple, fast and cost-effective way of studying DNAm. It can provide a broad overview of the epigenetic state of individuals, cells or tissues, which can be indicative of overall genomic stability and health (reviewed by Li & Tollefsbol, 2021). However, effects of age and environmental factors can result in increased DNAm at some CpG sites, while simultaneously resulting in DNAm decreases at other sites. As a consequence, whole genome DNAm levels are much coarser and less informative as they are skewed by repetitive regions and cannot be directly connected to regulatory pathways as evidenced by previous research in mammals and birds (Laubach et al., 2021; Meyer et al., 2023).

Higher-resolution approaches assess DNAm at the level of individual CpG sites (positions in the genome where a 5' cytosine is followed by a guanine, Moore et al., 2013) providing valuable insights, especially when directly linked to gene expression of a gene with known effects on the phenotype or life history of the organism. In both biomedical and ecological contexts, DNAm patterns of specific CpG sites have been associated with numerous traits. In clinical research, CpG-specific DNAm patterns have been linked to specific diseases (Hedman et al., 2017) and neuropsychiatric disorders (Zheleznyakova et al., 2016). In evolutionary ecology, the methylation of specific CpG sites, or entire genes has been studied in relation to a plethora of ecologically relevant traits across a wide variety of species. In birds, methylation has been associated with brood size of rearing (Jimeno et al., 2019; Sepers et al., 2021), food deprivation (Xiao et al., 2020), and urbanization (Caizergues et al., 2022). In wild baboons, methylation was linked to resource availability (Lea et al., 2016), in lizards to habitat quality (Hu et al., 2019), and in crustaceans to the exposure to pollution (Harney et al., 2022). Collectively, these findings confirm that DNAm is sensitive to environmental perturbations and a putative mechanism for generating phenotypic variation.

A fundamentally different approach to make inferences based on DNAm, compared to the focus on individual CpG sites, is the development of methylation-based indexes. Utilizing the methylation of selected positions in the genome, an algorithm can be trained and later used as a

predictive tool using e.g. supervised machine learning. Such indexes have proven to be valuable for diagnostic and therapeutic purposes, for example for non-invasive cancer detection and identifying cancer types (Draškovič & Hauptman, 2024; Wei et al., 2021), prediction of patient response to chemotherapy (Wick et al., 2014), and assessment of the impact of lifestyle and therapeutic interventions (Keller et al., 2020; Lu et al., 2023). This approach has in particular been applied to develop epigenetic 'clocks' of chronological age, algorithms that use DNAm patterns to predict chronological age for humans, model, and non-model animals (Horvath, 2013; Petkovich et al., 2017; Tangili et al., 2023).

Despite the growing use of DNAm based indexes, it is still debated whether the CpG sites underlying such indexes are functionally involved in the targeted process, or if the level of methylation at the identified sites correlates with those processes in absence of any biological function. The functional role of the DNAm of CpG sites outside of regulatory regions remains poorly understood (Moore et al., 2013) and confounded by the limited overlap between the CpG sites identified across different studies aimed at the same biological process within the same species (Galkin et al., 2020; Liu et al., 2020). Part of the explanation might be that phenotypic variation rarely correlates strongly with single genes and hence methylation-based indexes likely capture CpG sites across gene networks scattered across the genome. In this sense, an index comprising DNAm at many CpG sites may better match the causes of the phenotypic variation under study, compared to the DNAm of single sites. Nevertheless, whether these sites are causally linked to the observed phenotypes remains an open question, demanding more research across diverse species to elucidate the underlying mechanisms.

We here present a case study to explore different approaches to utilize DNAm data to gain information on the role of DNAm in mediating the effects of the environment on the phenotype. DNAm, via its ability to regulate gene expression, has emerged as a potential mediator of the effects that specifically early-life environment can exert on adult life-history traits and fitness outcomes by becoming genomically embedded (Szyf & Bick, 2012). To this end, we have utilized longitudinal whole genome methylation data from fifty adult zebra finches (*Taeniopygia castanotis*) of both sexes that were reared in experimentally created small or large broods. This manipulation is of interest, firstly because it is reasonable to assume that parents and offspring have evolved responses to variation in brood size, and hence we can be certain that phenotypic effects are not artefacts. Secondly, previous studies from the present study population and others, have documented multiple long-term effects of this manipulation; for example, individuals reared in large broods have shorter lifespans (Briga et al., 2017) and lower sexual attractiveness (Holveck & Riebel, 2010).

In our case study, we first identified CpG sites with differential methylation between treatments. Secondly, to translate methylation information into interpretable metrics, we developed two methylation-based indexes: an epigenetic early life quality index (DMSi) calculated from methylation data of differentially methylated sites between individuals raised in small and large broods and a predicted brood size index (SMLmi) calculated using a supervised machine learning algorithm with the goal to predict brood size of rearing. We hypothesized that these indexes could serve to quantify effects of early life conditions on the methylome within the treatment categories, and as such provide a more accurate reflection of an individual's phenotypic state than the coarse, binary trait of manipulated brood size. A second aim was to test whether in this way an estimate could be obtained of the early life conditions experienced by individuals first encountered in adulthood, as frequently happens in studies in the wild.

Longitudinal data, collected from the same individuals at multiple time points, are essential when studying processes such as development, aging and adaptation and allow for the continuous monitoring of the molecular state of an individual. However, such data is scarce and most epigenetic studies are cross-sectional. Knowing how to analyze and interpret longitudinal epigenetic data is crucial for understanding complex phenotypic traits, but there is as still little experience with utilizing longitudinal samples in epigenetic studies. In our case study we sampled all individuals twice, once in early adulthood, and a second time closer to the end of their lives, and explored different ways to combine the information of longitudinal samples.

2. Case study

2.1 Case study: methods

2.1.1 Subjects and samples

Details on subjects used in the present study are presented in Tangili et al. (2025). In brief, adult zebra finches were randomly matched and provided with nest boxes and nesting material. Fortified canary food was supplied to the parents in addition to tropical seed mixture and water until the hatching of the first chick in the nest but not thereafter. Nestboxes were checked daily for the presence of eggs or chicks. Chicks were cross-fostered at age five days or before to broods of either two or six chicks, which is the range of brood sizes observed in the wild (Zann, 1996). On the day of cross-fostering, all chicks were weighed and individually marked, and weighed again when 15 days old (or as near to that as practically possible). Siblings were assigned to different broods, and parents never reared their own offspring. When reaching adulthood (≥3 months), birds were moved to outdoor aviaries each containing single sex groups of 18–24 adults (Briga et al. 2017). We here used blood samples collected from the brachial vein and stored in -80°C in glycerol buffer from 50 individuals distributed equally over the small and large broods (n=25 x 2) and almost equally over the two sexes (26 males, 24 females). Each bird was sampled twice with an average sampling interval of 995 (S.D.=742) days - see Fig.S1 for the age distribution at sampling.

2.1.2 Enzymatic Methyl-seq data

We extracted DNA according to the manufacturer's protocol using innuPREP™ DNA Mini Kit (Analytik Jena GmBH) from 3 uL of nucleated red blood cells. Next-generation sequencing was conducted by the Hospital for Sick Children (Toronto, Canada). The amount of DNA was quantified using a Qubit High Sensitivity Assay (Thermo Fisher Scientific). Two hundred ng of DNA was used as input material for library preparation using the Next Enzymatic Methyl-seq™ Kit (New England Biolabs Inc.) following the manufacturer's instructions. Briefly, DNA was fragmented by sonication to an average length of ~500 base pairs (bp) using a Covaris LE220 (Covaris Inc.). Fragmented DNA was end-repaired and ligated to Illumina sequencing adapters. The 5-methylcytosines and 5-hydroxymethylcytosines were oxidized by TET2 and cytosines were deaminated by APOBEC. Methyl-seq libraries were subjected to five PCR amplification cycles. Libraries assessed on a Bioanalyzer™ DNA High Sensitivity chip (Agilent Inc.). The amount of DNA was quantified by quantitative PCR using the Kapa Library Quantification Illumina/ABI Prism™ kit according to the manufacturer's instructions (La Roche Ltd.). Libraries

were pooled in equimolar amounts and sequenced to a minimum of ~100M paired-end reads (2x150bp) per sample on a 10B flow cell using a NovaSeqX™ (Illumina Inc.) platform.

Sequences were trimmed using Trim Galore! v0.6.10 (Krueger et al., 2023) in paired-end mode. The data were assessed before and after trimming using FastQC v. 011.9 (Andrews, 2010) and MultiQC v. 11.14 (Ewels et al., 2016). Alignments were performed using. Trimmed reads were aligned against the *in silico* bisulfite converted zebra finch (*Taeniopygia guttata*) reference genome (GCA_003957565.4, Rhie et al., 2021) using Bismark v. 0.14.433 (Krueger & Andrews, 2011) and the Bowtie 2 v. 2.4.5 alignment algorithm (Langmead & Salzberg, 2012). The average mapping efficiency was at 65.14% (SD: 2.79).

Mean autosomal DNAm did not appear to be affected by the storage time (range 8-16 years) of the samples (estimate= 0.03 ± 0.04, t = 0.85, p = 0.39).

2.1.3 Differentially methylated site (DMS) identification

We performed an epigenome wide association study (EWAS) in order to identify differentially methylated sites (DMSs) between individuals raised in small and large broods using *Methylkit* v. 1.18.0 (Akalin et al., 2015). To increase precision of the DNAm estimates, and because we were interested in long-term effects of brood size on DNAm, data of the two observations per individual were merged by adding the amount of methylated counts and dividing by the coverage of both observations. The average initial number of sites per individual was 13,956,712. We filtered for a minimum coverage of 12 and maximum coverage of 99.9 to avoid PCR bias (Wreczycka et al., 2017), resulting in an average of 7,406,171 sites per individual. Read coverage distributions were normalized between samples using the *normalizeCoverage* function of the *Methylkit* package. We used the *unite* function in the *Methylkit* package to only retain CpG sites shared by all samples, which yielded 2,064,943 sites. Finally, CpG sites with methylation of 0% or 100% DNAm in all samples were also excluded, resulting to a final number data set 2,052,610 CpG sites. We calculated DNAm difference per CpG site using the *calculateDiffMeth* function of the *Methylkit* package using Fisher's exact test (Fisher, 1922) with Bonferroni correction (Dunn, 1961). Following Wreczycka et al., (2017) we defined DMSs as CpG sites where the difference in percent methylation between individuals in the two treatments was at least 25%. The results of this analysis are reported in Tangili et al. (2025), and we here extend this analysis to include EWASs of the effect of brood size on the first and second sample of all individuals separately. For these analyses, we filtered for a minimum coverage of five and maximum coverage of 99.9 and we used an absolute difference in methylation of 15% between the two brood sizes. After filtering, 37,169 and 56,884 CpG sites were retained for samples collected at a young and old age respectively.

2.1.4 Epigenetic early life quality index (DMSi) calculation

We calculated an epigenetic early life quality index, which we termed DMSi, as follows: DNAm of all identified DMS was mean centered, and for sites where DNAm was on average higher in birds from enlarged broods, mean centered DNAm was multiplied by -1. DNAm was then averaged per individual over all DMS. To facilitate comparisons with future studies we z-scored the index prior to analyses.

2.1.5 Supervised Machine Learning methylation index (SMLmi) calculation

As a second method to develop a DNAm index of early life quality, we performed an elastic net regression with a leave-one-out cross-validation (LOOCV) framework using DNAm at variant sites data (i.e. DNAm not of zero or one hundred in all samples) with a coverage between ten and 99.9. We used DNAm per CpG site per sample as the predictor variable and brood size of rearing as continuous dependent variable (coded as -0.5 and +0.5 for small and large broods respectively). The elastic net mixing parameter was set to $\alpha = 0.1$, as a titration procedure showed this value to yield the most accurate model. A cross-validated elastic net model using function *cv.glmnet* from package *glmnet* v. 4.1-8 (Friedman et al., 2010) was used to identify the optimal regularization parameter λ (lambda.min). A 10-fold LOOCV approach was used to estimate how well the methylation of CpG sites predicted rearing brood size.

2.1.6 Statistical analyses

Data were analyzed using linear mixed effects models using package *lme4* v. 1.1.35.1 (Bates et al., 2015) in R v. 4.1.2 (R Core Team, 2023). We employed Akaike Information Criterion (AIC) values (Akaike, 1974) to compare the predictive performance of models. Partial correlations were calculated using the *pcor* function from the *ppcor* v. 1.1 package (Kim, 2015) in R.

2.2 Case study: differentially methylated sites (DMSs)

We previously reported the effects of rearing brood size on the methylome, combining the methylation data from the two samples collected at different timepoints for each individual (Tangili et al., 2025). This approach yielded a total of 149 CpG sites with an absolute methylation difference of ≥25% between individuals raised in small and large broods (Fig.1a). Those DMSs were distributed among 19 autosomal chromosomes. DNAm was found to be higher in birds raised in large broods at 79 DMSs and lower in 70 DMSs. We here extend this analysis, by identifying DMSs between birds raised in small and large broods separately for the two samples from each individual (young and old). In this way, we can evaluate the effect of pooling samples taken at different ages on the detection of DMSs, on the consistency of DMSs over life, and on the age dependence of frequency and magnitude of DMSs.

We identified 25 and 106 brood size-related DMSs when individuals were respectively young or old (Fig.1b and c), with three DMSs shared between the two age cohorts (in chromosomes 2, 10, and 12). Comparison of these numbers is complicated by the different number of CpG sites included in the analysis of samples at different ages, but also expressed as a proportion of the number of CpG sites there were more DMS among the samples collected late in life (0.19%) compared to samples collected early in life (0.069%; χ^2=21.65, d.f.=1, p<0.001). Moreover, when comparing the absolute difference in methylation of all CpG sites in the two cohorts using a Wilcoxon rank-sum test, this revealed a small but highly significant difference between groups (Fig.2; W = 946,481,574, p<0.001). Thus, the magnitude of the effects of early life conditions on DNAm differed significantly within individuals over life, with the changes being more pronounced later in life.

The identification of CpG sites across the genome whose methylation is significantly different between birds raised in small and large broods reinforces the idea that environmental factors during the crucial developmental stages can become biologically embedded in the genome with direct and possibly long-term effects on life-history and fitness-related traits, with DNAm being a

potential mediator of such effects (Szyf & Bick, 2012). These findings are in line with previous work on nestling zebra finches (Jimeno et al., 2019; Sheldon et al., 2018) and great tits (Sepers et al., 2021) that showed consistent effects of brood size on DNAm. These changes in DNAm between individuals raised in different brood sizes might underlie the phenotypic differences observed between treatment groups such as the significantly shorter lifespan of zebra finches raised in large broods we have previously identified (Briga et al., 2017). Using longitudinal samples, we here extend these findings, by showing that early life environment effects on DNAm are stronger later in life. This raises the question whether early life environmental effects on the phenotypic level also increased with age, but this remains to be investigated. In any case, our findings highlight the potential role of DNAm in mediating the enduring impact of early-life environmental conditions on adult phenotypes (Dettmer & Chusyd, 2023).

2.3. Case study: Differentially Methylated Site index (DMSi)

Methylation-based, predictive indexes are widely used in clinical research and diagnostics (Xu et al., 2024; Yousefi et al., 2022; Zhang et al., 2025), but have so far only been used to predict chronological age in an ecological context (Tangili et al., 2023). Developing methylation-based biomarkers could allow retroactive quantification of early-life stress and other significant life events. The development of such biomarkers holds promise especially when studying wild populations, where detailed, individual life-history information might be labor-intensive to obtain and therefore scarce. The development of targeted, cost-effective methods to measure DNAm of selected CpG sites (e.g. Morselli et al., 2021), makes feasible the collection of population data with unprecedented accuracy.

We constructed an epigenetic index of early developmental conditions (i.e. brood size) using the methylation of the DMSs identified between zebra finches raised in small and large broods (DMSi). Because birds raised in large broods achieved a shorter lifespan (Briga et al 2017), we hypothesized that this index would serve as a quantitative measure of the overall phenotypic quality of each individual. The cutoff to identify DMS of 25% is arbitrary, and we therefore first examined what extent results depended on this cutoff level. To this end, we additionally calculated the DMSi from the sites whose absolute methylation difference was ≥20% (n=1045), instead of ≥25%. We find the two DMSi's to be highly correlated (r=0.98, p<0.001; Fig.S2), despite the inclusion of 896 additional DMSs at the lower methylation difference threshold. This is likely due to the index being primarily driven by the sites with the largest differences in methylation, suggesting that the index is robust against the inclusion of CpG sites with smaller differences in methylation.

Given actuarial senescence in zebra finches, in combination with the shorter lifespan of birds reared in large broods, and the shorter lifespan of females (Briga et al., 2017), we anticipated the DMSi to lower in females and decrease with age. With respect to sex, this prediction was confirmed, in that female DMSi was lower than male DMSi, independent of rearing brood size (Table 1; Fig.3a). In contrast, DMSi changed very little with age within individuals (Fig.4). However, we believe this apparent stability to be an artefact of the chosen method. Specifically, the DMSs whose methylation was used to calculate the DMSi were identified based on methylation data merged across both timepoints. Thus, only CpG sites where DNAm differed between brood sizes regardless of age will have been selected. We therefore also calculated the DMSi separately based on samples collected early or late in life, i.e. two DMSi values per individual (Fig.3b,c). Using age category specific DMSi estimates, there were significant effects of

brood size and age, with individuals raised in large broods and young individuals exhibiting significantly lower DMSi. However, there were no significant effects of sex or interactions between age cohort and sex or brood size (Table 2a), indicating that brood size and age independently affect the DMSi. Moreover, individual identity accounted for a significant portion of the variation in DMSi values (repeatability 69% after controlling for fixed effects; Table 2), highlighting that DMSi levels differ consistently between individuals.

2.4. Case study: Supervised Machine Learning methylation index (SMLmi)

When predicting brood size using the merged samples per individual, a ridge regression (α=0, using the methylation of all CpG sites to predict brood size with optimized weights) yielded the best result. However, the predictive accuracy was only 42%, i.e. slightly worse than random assignment. This indicates that no individual CpG or small subset of CpGs in the pooled samples was strongly predictive of rearing brood size, which contrasts with the identification of DMSs in this data set (Fig. 1a). A predictive modeling approach on all individual samples using elastic net regression with leave-one-out cross-validation yielded a different result, predicting rearing brood size with an accuracy of 89% (Fig.5). When inspecting the 11 wrongly assigned samples, it became apparent that eight of these samples were from four individuals of which both samples were wrongly assigned (Table S1). The elastic net model SMLmi utilized the methylation of 455 CpG sites spread over 30 autosomal chromosomes and the Z chromosome (Fig.S3), and 25 CpG sites were shared between the DMS and the CpGs included in SMLmi (Fig.S4). While the SMLmi differed strongly between birds reared in small and large broods (as expected), age and sex did not explain significant variation in the SMLmi (Table 2b).

2.5. Case study: Comparing indexes

We identified a strong negative correlation between DMSi and SMLmi (Fig.6; r=-0.80, $p < 0.001$). This pattern suggests that both indices are capturing biologically meaningful epigenetic information about the early-life environment. However, for individuals raised in small broods the correlation between the two metrics was weaker (r=-0.44, $p < 0.001$) compared to individuals raised in large broods (r=-0.62, $p < 0.001$). The steeper negative slope, and stronger correlation, among individuals reared in large broods can be taken to imply that the impact of brood size on methylation is more pronounced in individuals facing higher amounts of early-life stress. This suggests phenotypic variation may also be larger among individuals reared in large broods, but this remains to be investigated. The finding that the relationship between the indexes differs between birds reared in small and large broods indicates that one or both indexes do not perfectly reflect rearing brood size.

When examining the DNAm differences between individuals raised in small and large broods we found these to be minimal across all CpG sites that remained after the initial filtering (Fig.7). Not surprisingly, the methylation difference between individuals raised in small and large broods for DMS was large, because this is how these were defined. Of more interest therefore is the distribution of DNAm differences between birds reared in small and large broods of the CpGs included in the SMLmi as selected in the elastic net regression procedure. We find these differences to be intermediate to the distributions of all CpG sites and the selected DMS. This distinction reflects the different goals of the two analyses; identifying the CpG sites with the largest methylation differences versus accurately predicting brood size. The CpGs selected to increase SMLmi performance do not necessarily represent the largest differences in methylation

between treatments, with machine learning algorithms selecting features even with subtle differences that, when combined, are highly predictive.

2.6. Case study: Indexes and early-life growth rate

To assess to what extent the two methylation-based indexes relate to individual variation in early-life conditions, we correlated DMSi and SMLmi to pre-fledging growth rates. Early-life growth rate is a robust predictor of fitness prospects across a wide range of species (Douhard et al., 2014; Drake et al., 2025), including birds (Brown et al., 2022). Both indices significantly predicted individual variation in growth rate (Fig.8a,b; Table S2,3). The partial correlation coefficients for mass gain per day were 0.45 for the DMSi and -0.44 for the SMLmi. Comparison of the AIC values of the predictive models revealed that the DMSi was a substantially better predictor of early-life growth than SMLmi (AIC = −212.01 and −45.22, respectively). However, when adding rearing brood size to the models in tables S2 and S3, this caused the index effects to be no longer significant (DMSi: $\beta = 0.02 \pm 0.05$, $t = 0.36$, $p = 0.72$; SMLmi: $\beta = 0.03 \pm 0.05$, $t = 0.53$, $p = 0.6$). Thus, both indexes can be predictive of early-life growth, but are not an improvement over knowing the actual early-life conditions.

3. Discussion

Epigenetic information can be utilized in different ways to understand how environmental factors modulate phenotypic traits in the short and long-term affecting organismal sensitivity, resilience and adaptability. Studying DNAm at the level of each individual CpG site offers the highest resolution for understanding how DNAm directly affects phenotypes depending on its location, because the methylation of different parts of the genome has different effects on gene expression (Moore et al., 2013). However, even CpG site-level analyses can differ in resolution. Depending on the approach employed to quantify methylation, and the resulting resolution of the data, studies may examine methylation in CpG sites across the entire genome, focus on CpG islands, or target specific genomic regions or functionally relevant loci. However, focusing exclusively on genes or genomic regions predicted to be affected by a treatment introduces significant limitations. Novel effects outside of the predefined regions may be overlooked, leading to a possibly misleading understanding of the treatment's effects on the epigenome. Furthermore, this approach limits the opportunity to use the data in ways not envisaged when designing the study. Taking into consideration all the above, comprehensive, high resolution, genome-wide analyses are essential to capture the full spectrum of epigenetic differences between phenotypes that can be leveraged to get insights into the mechanistic underpinnings of phenotypic differences between individuals.

Leveraging whole-genome methylation data from adult zebra finches raised in either small or large broods, we presented a case study to compare different ways to quantify the long-term consequences of early-life developmental conditions using longitudinal samples. When identifying DMSs, longitudinal samples, taken at different times from an individual, can either be merged, increasing precision, or analyzed separately. We previously presented an analysis of the merged samples (Tangili et al. 2025) and here compared those results with separate analysis of the samples taken when early and later in life. On the one hand, judging by the number of DMSs identified, merging samples was the more powerful approach, and we consider these findings the best estimate of the long-term effect of rearing brood size on DNAm. On the other hand, merging the samples concealed the interesting finding that DNAm differences due to rearing

brood size were larger later in life, raising the question whether phenotypic differences due to rearing brood size were also larger later in life. Moreover, there was limited overlap between DMSs identified in early and late life samples, perhaps reflecting how birds reared in small and large broods age differently (Briga et al. 2017). Thus, we conclude (not surprisingly), that whether it is recommended to merge longitudinal samples depends on the details of the question that is being addressed, with merging being useful to increase precision when estimating long-term effects, while addressing age-related variation requires samples taken at different times to be analyzed separately.

Whether longitudinal samples were merged or not also affected the development of indexes of early-life conditions. For example, we observed a strong reduction in power of the SMLmi to predict brood size from the merged samples compared to a similar analysis based on the individual samples. This effect may stem directly from merging two samples taken at different phases in life. The finding of small overlap of DMS with respect to rearing brood size between the samples taken at young and old ages is in line with this explanation. This finding indicates that using aggregated longitudinal data might not be suitable for trait prediction and offers further support to the idea that the difference in methylation between individuals raised in small versus large broods varies across different life stages. On the other hand, the DMSi based on merged samples showed the expected difference between the sexes, while it was not suitable to investigate age effects due to the inherent bias in DMS selection towards CpG sites that change relatively little with age. We therefore tend towards recommending the calculation of indexes using individual samples, but acknowledge that more comparisons between indexes from merged versus single samples will be necessary before general conclusions can be reached with confidence.

When studying natural populations, developmental conditions of an often large proportion of individuals will not be known. The indexes we calculated based on the effects of manipulated brood size on DNAm can in theory offer the opportunity to obtain information on the developmental conditions of individuals first encountered in adulthood. The high predictive accuracy of brood size by SMLmi is encouraging in this respect, as was the strong association between the indexes and early-life growth rate. The actual brood size of rearing was still a better predictor of growth rate than either index, but when direct information about developmental conditions is absent, indexes as developed in this study are possibly the best available alternative, besides traits that can be measured on adults (e.g. morphological traits that are fixed early in life). We recognize, however, that such indexes will have to be developed and validated for each population or study system, and also within populations it remains to be investigated as how stable such indexes are in space and time. Nonetheless, our findings suggest that at least within the context of an ongoing study where developmental conditions are known for part of the population, DNAm based indexes provide a unique tool to assess the developmental conditions of individuals first encountered as adults.

Indexes integrating DNAm information from many CpG sites are likely to have greater explanatory power with respect to phenotypic variation in most cases, when compared to individual CpG sites. Nevertheless, we acknowledge that in order to fully understand the mechanisms through which DNAm mediates phenotypic plasticity, it is ultimately necessary to investigate the functional roles of individual CpG sites. While both indexes we calculated (DMSi & SMLmi) were highly correlated with each other, the two indexes draw information from sets of CpG sites that showed limited overlap. Instead of considering this a weak point, we see it as

broadening of the opportunity to explore the mechanisms underlying phenotypic variation. This divergence suggests that the two indexes capture multiple and possibly partially independent epigenetic signatures that can contribute to the same phenotypic outcomes. Thus, the development of DNAm based indexes of phenotypic traits can be seen as a stepping stone towards the identification of (networks of) CpG sites that together play an important role in shaping phenotypic variation.

As illustrated by our findings when comparing results based on either single samples or merged longitudinal samples per individual, DNAm is a dynamic and modifiable process, not only during early life and development but throughout an individual's entire lifespan. Information on the dynamics of DNAm is limited, in particular in non-model systems, but whether DNAm responses to environmental changes are transient or permanent has large consequences for the evolution of such responses (Fig.9). Environmental changes that induce a phenotypic response through DNAm can impact fitness in different ways, both before and during adulthood, depending on whether the change in DNAm is transient or persistent. In either case, it can be assumed that an environmentally-induced change in methylation provides fitness benefits in the short term when it is an evolved response. However, different scenarios can be envisaged for long-term consequences. In one scenario, the induced DNAm causes a permanent (favorable) change in the phenotype, in which case the fitness benefits are permanent, regardless of whether the change in DNAm is maintained, making this scenario less relevant in the context of this discussion. Alternatively, methylation patterns can be stably maintained when the different DNAm state is also beneficial in adulthood, maintaining also associated fitness benefits. When the effect on DNAm is transient, and no longer beneficial, the DNAm change may diminish as the individual matures, becoming neutral with respect to fitness. Lastly, an antagonistic pleiotropic effect can arise, where due to a constraint on phenotypic flexibility a change in DNAm that is beneficial early in life is permanent and becomes deleterious in later life. The above examples showcase how early-life methylation modifications resulting from early-life environmental exposures can be reinforced, attenuated or altered by later-life exposures, which can lead to complex, lifelong phenotypic effects. A better understanding of the extent to which DNAm dynamics are transient or permanent will be essential for our understanding of the impact of DNAm on the relationship between environment, aging, phenotype and fitness.

Acknowledgements

We thank the Center for Information Technology at the University of Groningen for support and access to Hábrók high-performance computing cluster. We express our gratitude to Ellis Mulder for her help in the laboratory, to Michael Briga and Blanca Jimeno for performing the developmental manipulation and maintaining the long-term experiment and to the animal caretakers at the University of Groningen as well as numerous students whose invaluable help made this project possible.

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Ethics

All methods and experiments detailed in this manuscript were performed under the approval of the Central Committee for Animal Experiments (Centrale Commissie Dierproeven) of the Netherlands, under license AVD1050020174344.

Data accessibility

Raw reads of the whole methylomes of all samples are available at the National Center for Biotechnology Information under BioProject ID PRJNA1108628. The bioinformatics code is available in https://datadryad.org/dataset/doi:10.5061/dryad.wm37pvmw8, the R code and intermediate data files used in this study are available in http://datadryad.org/share/ZxtGRgc_KmEIC-S2ZKYyAj0cLw_CdjcNT1UmfT4XeT8 (reviewer URL).

Author's contributions

MT and SV designed the study. MT performed the analyses. MT and SV wrote the manuscript with input from all authors. All authors approved the final version of the manuscript.

Competing interests

We have no competing interests.

Funding

Contributions by PJP were supported by the European Union's Horizon 2020 Research and Innovation Programme under the Marie Skłodowska-Curie grant agreement no. 813383, and the University of Groningen.

Figures and Tables

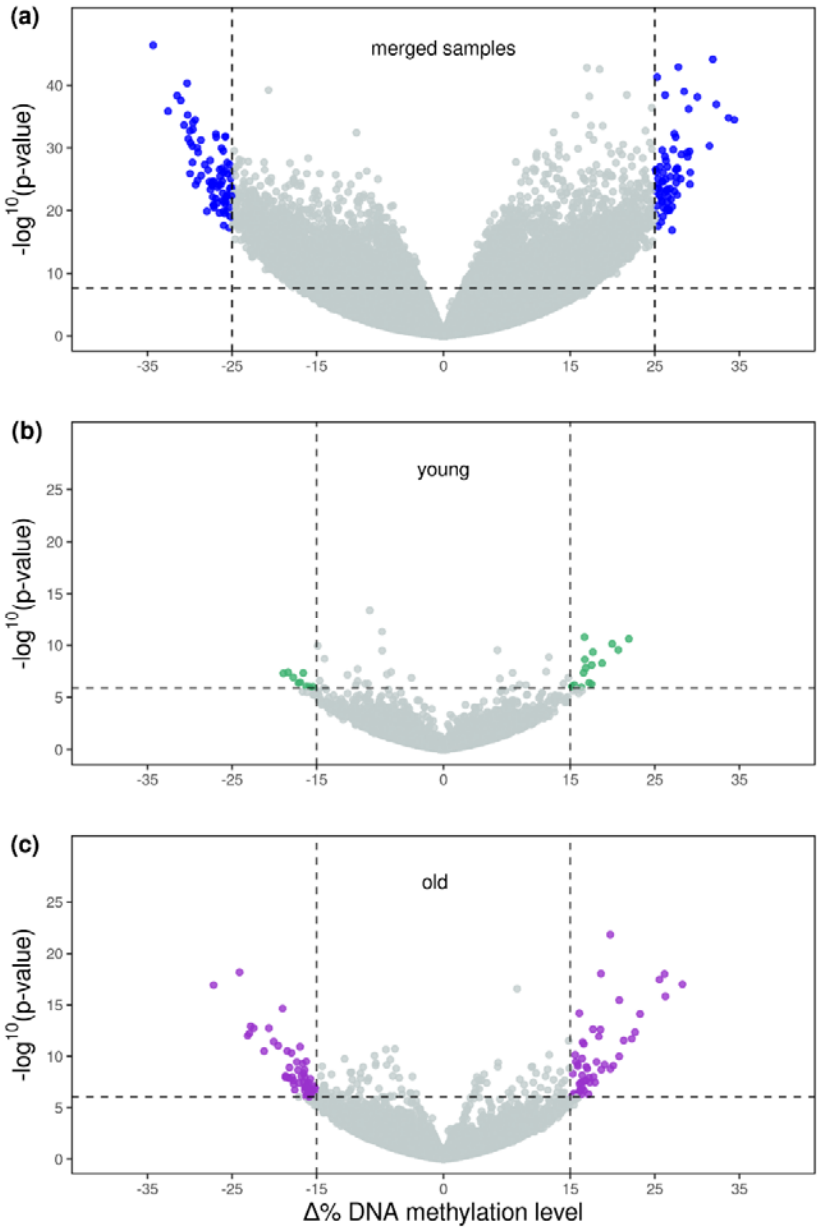

Figure 1. Volcano plot of Bonferroni-corrected p-values as a function of % DNAm differences between individuals raised in small and large broods for individuals merged together (a), young cohort (b), old cohort (c). Each point represents a CpG site, with colored points indicating biologically and statistically significant differences in % DNAm between individuals raised in small

and large broods. The dashed horizontal lines mark the genome wide significance thresholds while the dashed vertical lines represent absolute difference in DNAm.

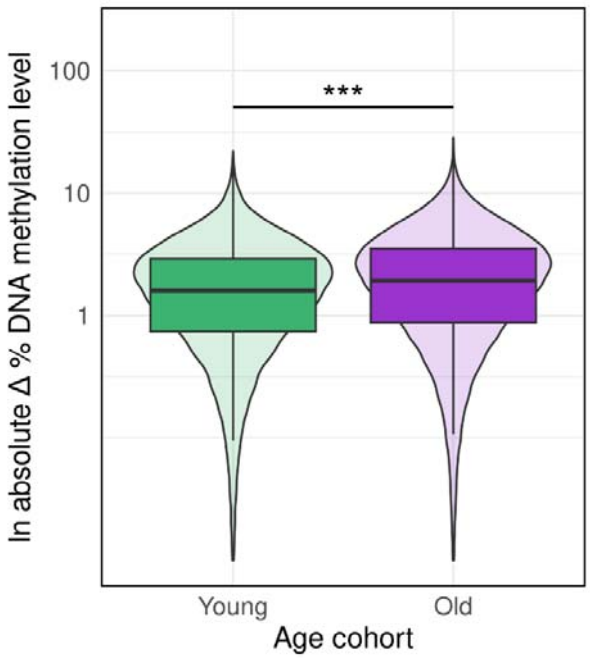

Figure 2. Distribution of CpG-site specific absolute methylation differences between zebra finches raised in small and large broods for the two age cohorts. The y-axis values were transformed using the natural logarithm.

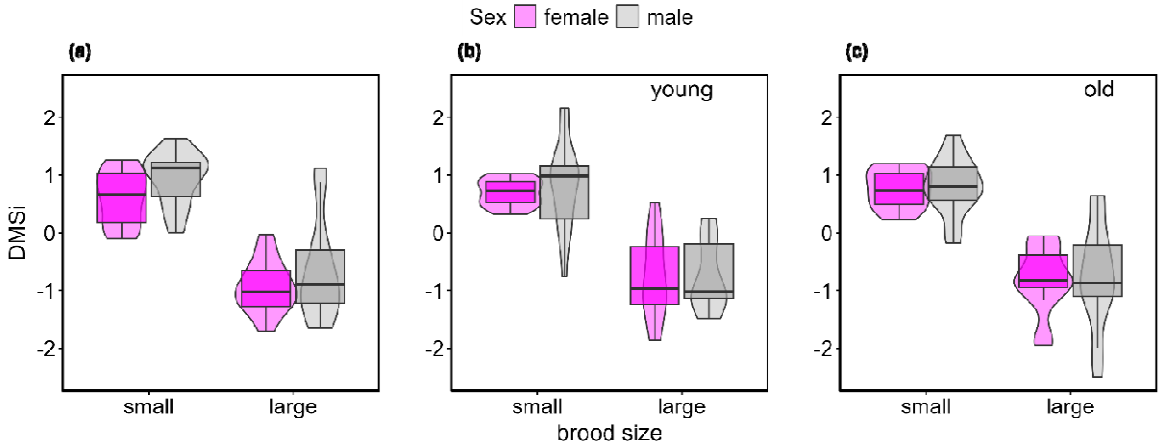

Figure 3. Levels of DMSi (z-scores) in zebra finches raised in small and large broods divided by sex in the merged samples (a), and samples in the young (b) and old (c) age cohort. In (b), DMSi was calculated based on methylation of DMS identified between young individuals raised in small versus large broods; in (c), DMSi was calculated from the methylation of DMS identified between old individuals raised in small versus large broods.

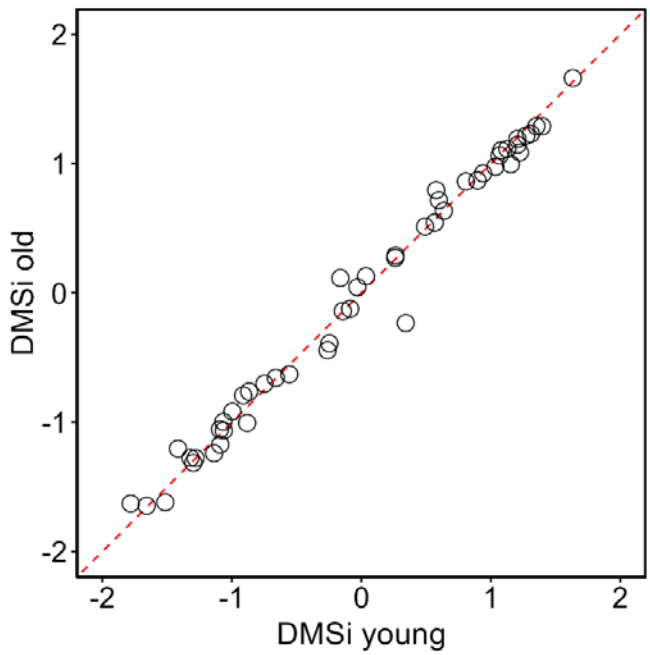

Figure 4. Within individual changes in DMSi (r = 0.992, p < 0.001). The dashed line indicates equal values (y=x), so no within-individual change in DMSi with age.

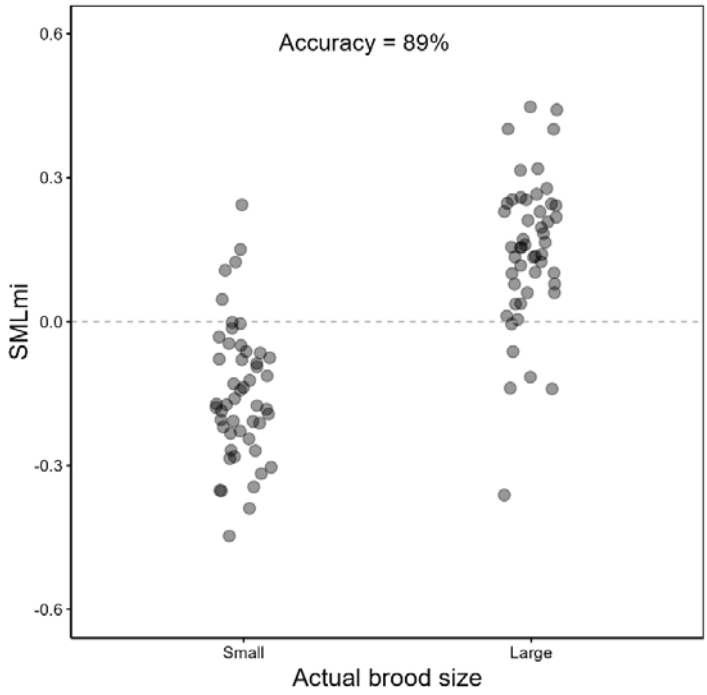

Figure 5. Predicted brood size (small brood: -0.5, large brood: 0.5) from the DNA methylation of 456 selected CpG sites. The model has a predictive accuracy of 89% (89/100 samples correctly assigned to their brood size). The points above the dashed line on the left and under the dashed line on the right were wrongly assigned.

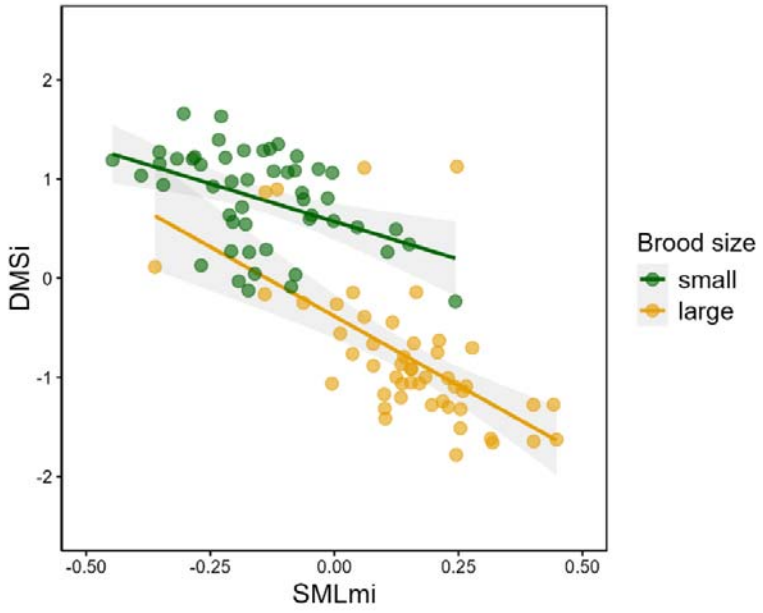

Figure 6. Correlation between DMSi and predicted brood size values (r = -0.80, p < 0.001). For individuals raised in small broods r=-0.44 (p < 0.001) and for individuals raised in large broods r=-0.62 (p < 0.001). Colors denote the brood size of rearing. Solid line represents the best-fit regression through the data and the colored area is 95% CI.

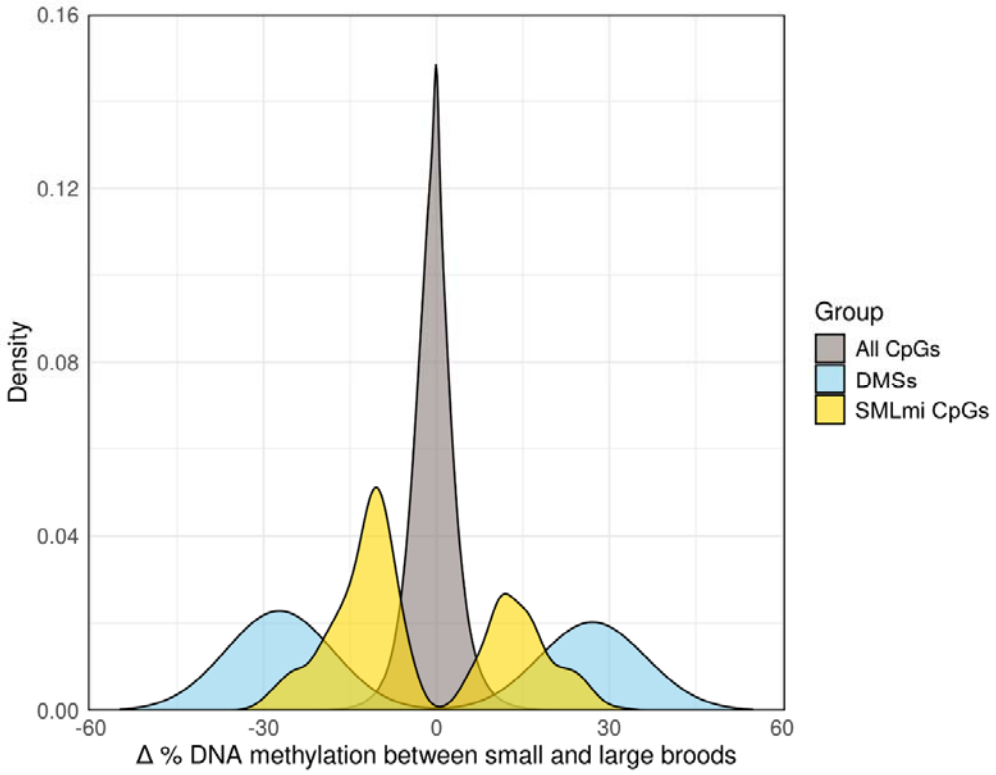

Figure 7. Density plots of methylation differences between individuals raised in small and large broods. Grey plot: CpG sites in the whole dataset of merged samples after filtering (n= 2,052,610). Blue plot: the DMSs in the merged samples (absolute 25% difference; n=149). Yellow plot: CpGs selected for the SMLmi (n=455).

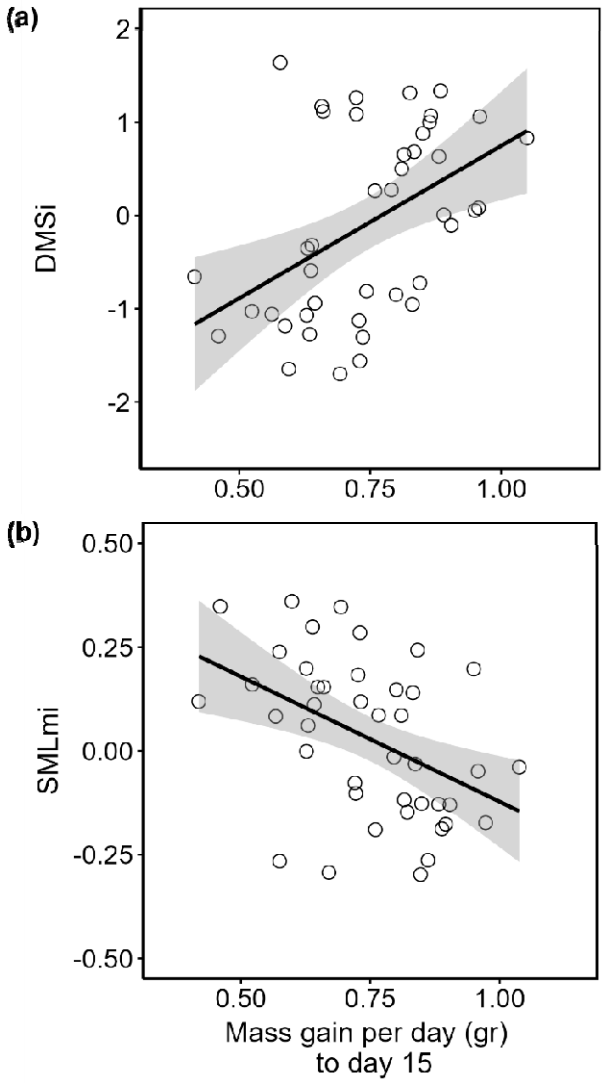

Figure 8. Correlations of DMSi (a) and SMLmi (b) with mass gain per day (gr) from day five to day 15 (n=42). Solid line represents the best-fit regression through the data and the colored area is 95% CI. Growth rate data were corrected for age at measurement using the estimates in Tables S2 and S3.

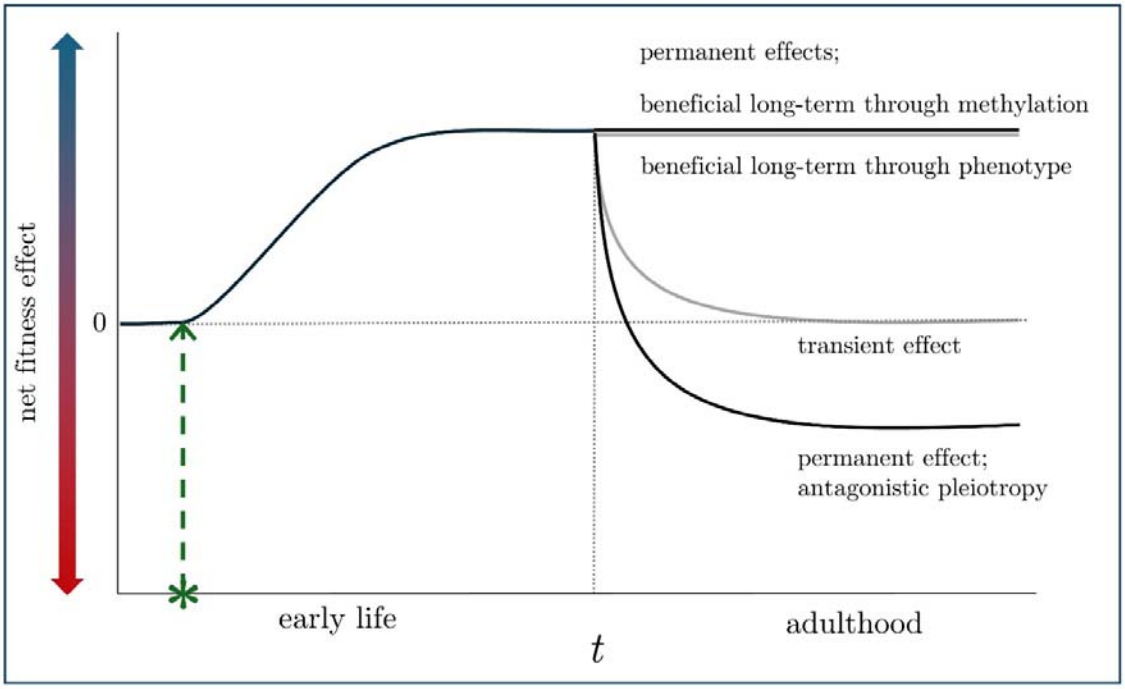

Figure 9. Short and long-term effects of early-life environmental change on fitness through methylation. Solid lines show the net fitness effects of methylation changes caused either by environmental change (green star and vertical arrow) or the onset of adulthood (vertical dotted line). Black solid lines represent the presence and grey solid lines the absence of methylation changes. For simplicity, the effects of methylation change are here considered either fully permanent or fully transient and the net fitness changes are only due to changes in methylation.

Tables

Table 1. Results of linear model exploring the effects of brood size and sex per sample on the DMSi index. Variables were mean centered with females, and small brood coded as -0.5 and males, and large brood coded as +0.5.

	DMSi		
Predictor	*Estimate (±SE)*	*t-value*	*Pr(>|t|)*
Intercept	-0.006 (±0.06)	-0.11	0.92
brood size	-1.57 (±0.12)	-13.48	<0.001
sex	0.31 (±0.12)	0.12	0.01

Table 2. The DMSi index calculated from the methylation of DMS identified comparing young and old zebra finches raised and small vs. large broods in relation to brood size and sex. Variables were mean centered with females, small broods and young individuals coded as -0.5 and males, large broods and old individuals coded as +0.5. Bird identity was included as a random effect.

(a)	**DMSi**			
Predictors	*Estimates (±SE)*	*df*	*t-value*	*Pr(>|t|)*
Intercept	-0.001 (±0.78)	47	-0.017	0.99
brood size	-1.49 (±0.16)	47	-9.5	<0.001
age cohort	0.60 (±0.07)	47	8.92	<0.001
sex	0.07 (±0.16)	47	0.42	0.67
brood size* age cohort	-0.08 (±0.13)	47	-0.58	0.56
sex * age cohort	-0.02 (±0.13)	47	-0.14	0.89
Random Effects				
BirdID	0.24			
residual	0.11			
(b)	**SMLmi**			
Predictors	*Estimates (±SE)*	*df*	*t-value*	*Pr(>|t|)*
Intercept	6.13e-04 (±0.019)	47	0.03	0.98
brood size	0.295 (±0.038)	47	7.72	<0.001
age cohort	0.017 (±0.018)	47	0.94	0.35
sex	-0.032 (±0.038)	47	-0.83	0.41
brood size* age cohort	0.011 (±0.037)	47	0.32	0.76
sex * age cohort	1.34e-03 (±0.037)	47	0.04	0.97
Random Effects				
BirdID	0.01			
residual	0.008			

Supplementary Information

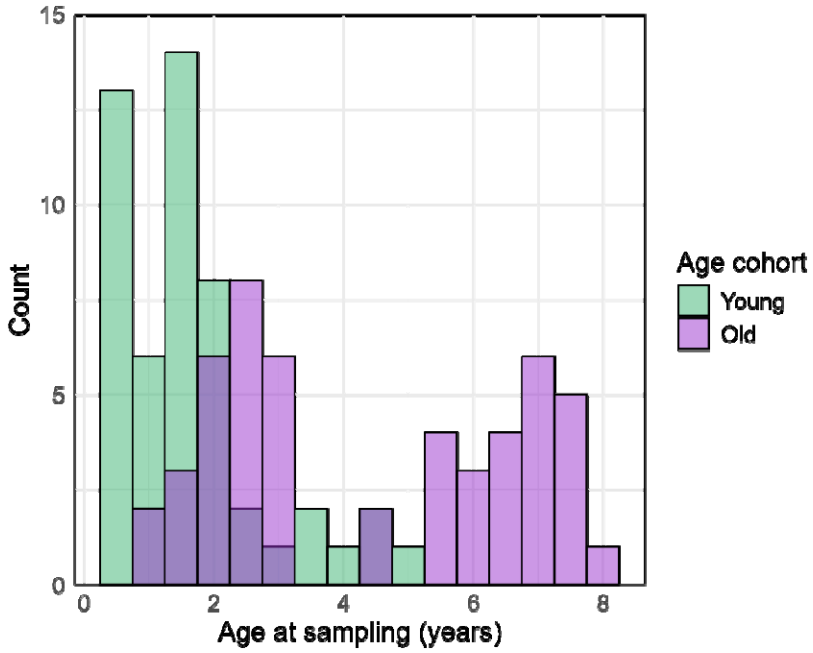

Figure S1. Age at sampling (years) distribution of samples included in the analyses. Color denotes relative time of sampling (young=first sample from an individual, old= second sample of an individual).

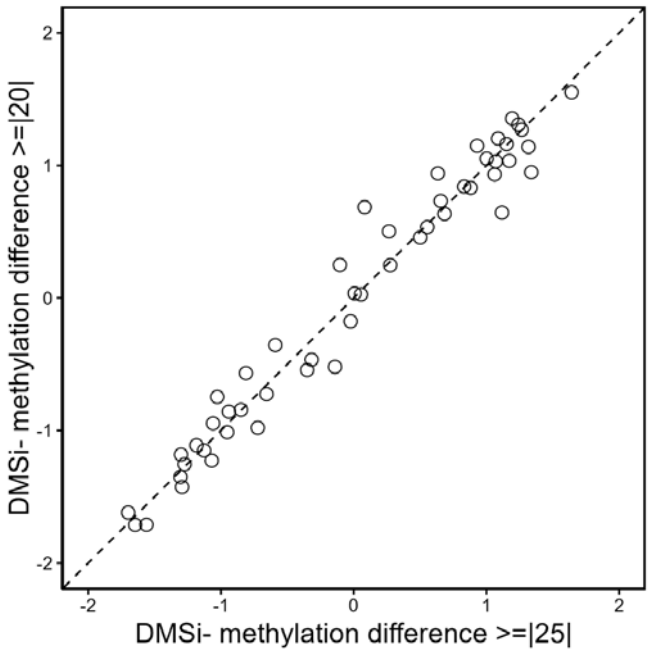

Figure S2. Comparison of the epigenetic quality index (DMSi) calculated from the methylation of differentially methylated sites identified for absolute methylation difference of 20% and 25%.

Table S1. Sample information and predicted brood size. Small brood size is coded as -0.5 and large brood size as +0.5. Accuracy denotes whether or not the sample was assigned to the correct brood size. Predictions were considered correct if they matched the direction of the actual brood size deviation (i.e., both positive or both negative).

Sample	BirdID	Brood size	Predicted brood size	Accuracy
1	1810	-0.5	0.190	Incorrect
2	1825	-0.5	-0.030	Correct
3	1904	0.5	-0.106	Incorrect
4	1907	-0.5	-0.046	Correct
5	1920	0.5	0.068	Correct
6	1944	-0.5	-0.114	Correct
7	1953	-0.5	-0.085	Correct
8	1983	0.5	0.103	Correct
9	2045	-0.5	0.003	Incorrect
10	2053	-0.5	-0.059	Correct
11	2058	-0.5	-0.078	Correct
12	2069	0.5	0.010	Correct
13	2087	-0.5	-0.078	Correct
14	2108	0.5	-0.030	Incorrect
15	2124	-0.5	-0.060	Correct
16	2142	-0.5	-0.086	Correct
17	2153	0.5	-0.093	Incorrect
18	2155	-0.5	-0.190	Correct
19	2163	0.5	0.141	Correct
20	2175	-0.5	-0.055	Correct
21	2229	0.5	0.086	Correct
22	2260	0.5	0.125	Correct
23	2291	0.5	0.084	Correct
24	2236	0.5	0.195	Correct
25	2259	0.5	0.086	Correct
26	1983	0.5	0.028	Correct
27	2429	0.5	0.111	Correct
28	2473	-0.5	-0.342	Correct
29	1923	0.5	0.115	Correct
30	1942	0.5	0.001	Correct
31	2058	-0.5	-0.175	Correct
32	2269	-0.5	-0.078	Correct
33	2087	-0.5	-0.179	Correct
34	2172	-0.5	-0.079	Correct
35	2288	-0.5	-0.028	Correct
36	2423	0.5	0.268	Correct
37	2487	-0.5	-0.024	Correct
38	2045	-0.5	-0.108	Correct
39	2257	-0.5	-0.049	Correct

40	2277	0.5	-0.060	Incorrect
41	2291	0.5	0.050	Correct
42	2418	0.5	0.065	Correct
43	2390	-0.5	-0.055	Correct
44	2511	-0.5	-0.205	Correct
45	1953	-0.5	-0.019	Correct
46	2163	0.5	0.092	Correct
47	2247	0.5	0.141	Correct
48	2260	0.5	0.200	Correct
49	2463	0.5	-0.028	Incorrect
50	1920	0.5	0.097	Correct
51	2155	-0.5	-0.043	Correct
52	1810	-0.5	0.148	Incorrect
53	1825	-0.5	-0.060	Correct
54	2053	-0.5	0.089	Incorrect
55	2069	0.5	0.079	Correct
56	2153	0.5	-0.116	Incorrect
57	2508	0.5	0.024	Correct
58	2508	0.5	0.102	Correct
59	2116	0.5	-0.003	Incorrect
60	2259	0.5	0.147	Correct
61	2423	0.5	0.293	Correct
62	2257	-0.5	-0.023	Correct
63	1897	0.5	0.091	Correct
64	2463	0.5	-0.035	Incorrect
65	2038	0.5	-0.046	Incorrect
66	2229	0.5	0.044	Correct
67	2277	0.5	0.055	Correct
68	2354	-0.5	-0.127	Correct
69	2124	-0.5	-0.109	Correct
70	1907	-0.5	0.012	Incorrect
71	2511	-0.5	-0.296	Correct
72	2429	0.5	0.280	Correct
73	2473	-0.5	-0.081	Correct
74	2035	0.5	0.069	Correct
75	1944	-0.5	0.071	Incorrect
76	1897	0.5	0.084	Correct
77	2108	0.5	0.014	Correct
78	1923	0.5	0.166	Correct
79	2172	-0.5	-0.057	Correct
80	2415	-0.5	-0.141	Correct
81	2392	-0.5	-0.195	Correct
82	2038	0.5	0.144	Correct
83	1904	0.5	-0.096	Incorrect
84	1942	0.5	0.094	Correct
85	2470	-0.5	-0.120	Correct

86	2142	-0.5	-0.044	Correct
87	2487	-0.5	0.034	Incorrect
88	2035	0.5	0.110	Correct
89	2116	0.5	0.129	Correct
90	2175	-0.5	-0.148	Correct
91	2470	-0.5	-0.235	Correct
92	2247	0.5	0.076	Correct
93	2415	-0.5	0.047	Incorrect
94	2269	-0.5	-0.045	Correct
95	2390	-0.5	-0.184	Correct
96	2288	-0.5	-0.042	Correct
97	2236	0.5	0.192	Correct
98	2354	-0.5	-0.138	Correct
99	2418	0.5	0.097	Correct
100	2392	-0.5	-0.263	Correct

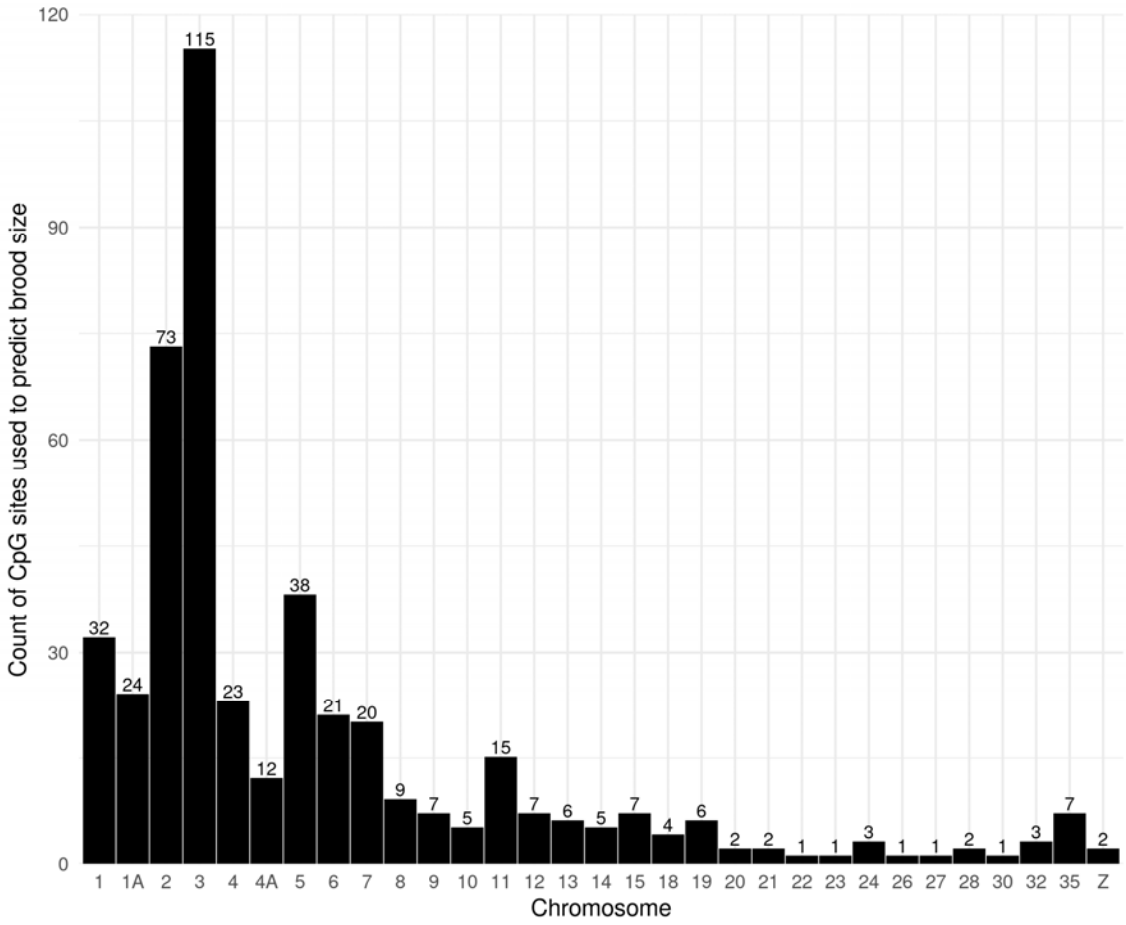

Figure S3. CpG sites in the final brood size prediction model per chromosome.

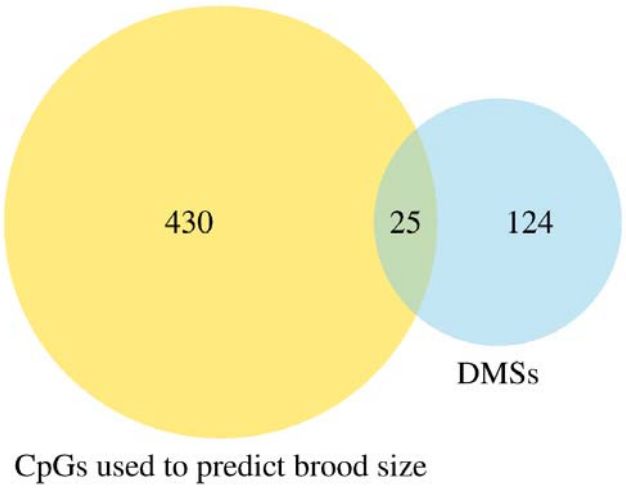

Figure S4. Shared CpG sites between DMSs when pooling samples per individual, collected at young and old age, and the CpG sites used to predict brood size (i.e. included in the SMLmi).

Table S2. Results of linear model exploring the effects of DMSi and age at measurement on mass gain per day.

	Mass gain per day (gr)		
Predictor	*Estimate (±SE)*	*t-value*	*Pr(>|t|)*
Intercept	1.38 (±0.16)	8.45	<0.001
DMSi	0.07 (±0.02)	3.14	0.003
age at first mass measurement	0.04 (±0.02)	2.7	0.01
age at day 15 measurement	-0.06 (±0.01)	-4.86	<0.001

Table S3. Results of linear model exploring the effects of DMSi and age at measurement on mass gain per day.

	Mass gain per day (gr)		
Predictor	*Estimate (±SE)*	*t-value*	*Pr(>|t|)*
Intercept	1.43 (±0.16)	8.82	<0.001
SMLmi	-0.34 (±0.11)	-3.09	0.004
age at first mass measurement	0.04 (±0.02)	2.63	0.01
age at day 15 measurement	-0.06 (±0.01)	-5.11	<0.001