Epigenetic changes associated with reproductive investment and life-history trade-offs in lekking male black grouse (*Lyrurus tetrix*)

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Ethics approval statement

All field work was ethically approved by the Central Finland Environmental Centre (permissions KSU-2003-L-25/254 and KSU-2002-L4/254) with additional permissions for specific sites (KSU-2004-L-72/254).

Data and code availability statement

Multiplexed EpiGBS sequencing data can be found under NCBI BioProject PRJNA1085187 with SRA BioAccession Numbers SAMN51757821 – SAMN51757844. Whole genome resequencing data can be found under the same NCBI BioProject with SRA BioAccession Number SAMN51785344. All original code, metadata and phenotypic files have been deposited on Figshare with DOI 10.6084/m9.figshare.30186223 (Chen et al., 2025). A large intermediate datafile containing the raw, unfiltered, methylKit object has been deposited on Figshare with DOI 10.6084/m9.figshare.30186571.v1 (Chen, 2025) Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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Conflict of interest disclosure

The authors declare no conflicts of interest.

Abstract

Life-history trade-offs are a central concept in evolutionary biology, yet their underlying molecular mechanisms are not yet fully understood. Whilst much research has focused on genetic variation, epigenetic mechanisms, which regulate gene regulation, may be equally important. To investigate this, we collected blood samples from 50 male black grouse (*Lyrurus tetrix*) before and after the breeding (lekking) period and quantified genome-wide DNA methylation changes using reduced representation bisulphite sequencing. We identified 1,026 CpG sites that changed significantly in methylation across the breeding period, many residing within genes involved in the regulation of RNA biosynthesis. We tested whether these DNA methylation changes were associated with reproductive investment and future fitness-relevant traits: survival and the expression of post-breeding sexual ornaments, which reflect body condition after the strenuous lekking period. Dozens of CpG sites showed significant associations, often clustering within the same genes, suggesting that epigenetic changes associated with reproduction and survival are localized rather than widespread. Moreover, changes at three CpG sites exhibited opposite relationships between current reproductive investment and future fitness-relevant traits, suggesting that epigenetic mechanisms might contribute to shaping life-history trade-offs. Our study demonstrates that DNA methylation changes are associated with the expression of costly reproductive traits, highlighting the importance of epigenetic mechanisms in shaping life-history traits and fitness.

Keywords: DNA methylation, lekking, sexual ornaments, epigenetics, trade-off, black grouse.

Introduction

Reproductive investment entails a life-history trade-off, balancing current reproduction against future long-term fitness, a concept central to sexual selection theory (Roff, 1993; Stearns, 1998; Williams, 1966). Reproductive investment is physiologically and energetically costly (Speakman, 2008), and when resources are limited, individuals must trade this investment off against survival or future reproduction to maximize their fitness (Jennions et al., 2001). Beyond resource limitation, trade-offs can also emerge because life-history traits are not expressed in isolation across an individual's lifetime but as parts of integrated systems that generate organismal complexity (Mauro & Ghalambor, 2020), where multiple traits can share underlying biological pathways, such as the melano-corticosterone system (Roulin & Ducrest, 2011). However, the molecular mechanisms underlying life-history trade-offs remain poorly understood. Changes in gene regulatory processes, such as epigenetic mechanisms (Jaenisch & Bird, 2003; Richards, 2006), play a key role in shaping life-history traits (Lindner, Laine, et al., 2021; Lindner, Verhagen, Viitaniemi, Laine, Visser, Husby, & Van Oers, 2021) and might therefore provide a molecular mechanism underlying life-history trade-offs (McCaw et al., 2024; Zhou & Liu, 2025).

Epigenetic mechanisms affect gene expression without changing the primary nucleotide sequence (Richards, 2006) and include DNA methylation and histone modifications. The epigenome integrates genetic and environmental information, including the early-life environment and stress (Berbel-Filho et al., 2019; Feil & Fraga, 2012; Sepers et al., 2021) to modulate gene expression (Jaenisch & Bird, 2003). DNA methylation is one of the most studied epigenetic mechanisms (Laine et al., 2022) and involves the attachment of a methyl group to DNA. In vertebrates, DNA methylation at CpG sites – dinucleotides composing a cytosine base pair followed by a guanine – in promoter regions (Jones, 2012) prevents transcription factors from binding to the DNA and is therefore typically associated with reduced gene expression (Jones, 2012). The functional effects of CpG methylation in other genomic regions are less well known, although intragenic methylation may be associated with alternative-splicing (Maunakea et al., 2013; Shayevitch et al., 2018).

Temporal changes in promoter DNA methylation can alter gene expression (Lindner, et al., 2021) and result in phenotypic changes in wild animals, such as the timing of the expression of phenological traits (Fishman & Tauber, 2024). In wild birds, seasonal variation in DNA methylation and gene expression has been linked to the initiation of reproduction (Lindner, Laine, et al., 2021; Viitaniemi et al., 2019), thermoregulation (Swanson et al., 2009) and song control (Thompson et al., 2012), highlighting the role of epigenetic changes in regulating key fitness traits. However, it is unknown whether individuals who invest more in reproduction show the largest or smallest temporal epigenetic changes. Moreover, if temporal epigenetic changes influence trait expression later in life, they may represent a molecular mechanism underlying trade-offs between current and future reproduction.

The black grouse (*Lyrurus tetrix*) provides a unique opportunity to investigate how temporal epigenetic changes are related to reproductive investment, the expression of future traits and life-history trade-offs. Black grouse males undergo a short but intense 4–6 week lekking period in spring, where they compete for non-resource holding territories to secure copulations (Alatalo et al., 1991). This risky, time- and energy-consuming period (Boyko et al., 2004) is characterized by significant body mass losses and increased parasite loads (Lebigre et al., 2013), which involve immune system and metabolic changes that have been linked to DNA

methylation and gene regulation changes in other species (Bollepalli et al., 2018; He et al., 2018; Morales-Nebreda et al., 2019).

Male quality in black grouse is signaled through lekking performance as well as the expression of multiple sexual ornaments (Kervinen et al., 2016; Rintamäki et al., 2001). The most successful males fight most intensively and defend the most central territories on the lek (Hämäläinen et al., 2012). Whereas lek attendance is a measure of current investment, lek centrality also captures variation in both short- and long-term reproductive investment (Alatalo et al., 1991; Kokko et al., 1998, 1999), and mating success is the outcome of the investment in all behavioral and ornamental traits integrated over multiple time scales (Kervinen et al., 2016). If epigenetic changes that occur during the lekking season reflect the costs of increased reproductive investment, this may have functional consequences beyond the current reproductive season. Epigenetic changes may reflect reproductive costs if they are indicative of the resources allocated to reproduction at the expense of another trait, or if these changes trigger molecular responses, such as transcriptional or hormonal fluctuations, that negatively affect another trait (now or in the future). These consequences may include reduced survival or the diminished expression of sexual traits in the following season, which potentially constrain future reproductive potential. Black grouse molt immediately after the lekking period (de Vos, 1983; Siitari et al., 2007; Soulsbury et al., 2016), and therefore their post-reproductive body condition becomes imprinted in their sexual ornaments, such as lyre length and blue chroma, which are displayed during the subsequent reproductive period, around 10–11 months later (Siitari et al., 2007).

Here, we quantified erythrocyte DNA methylation changes in male black grouse sampled before and after the lekking season. To test whether CpG site methylation changes are associated with individual investment in reproduction, we linked three indicators of reproductive investment – lek attendance, centrality and mating success –to temporal methylation changes. To test whether CpG site methylation changes during the lekking season are costly for the expression of future traits, we linked methylation changes to survival until the next autumn season and plumage traits displayed in the subsequent lekking season. We found dozens of CpG sites associated with reproductive investment and the future expression of fitness-relevant traits, which were localized to a few genes rather than widespread. To test whether methylation changes can underly life-history trade-offs, we evaluated whether methylation changes at single genes can have effects on different traits in opposite directions by searching for CpG sites significantly associated with reproductive investment and future traits. Methylation at three CpG sites were positively associated with reproductive investment and negatively with future traits, consistent with this pattern. Our results indicate that epigenetic mechanisms are involved in the molecular mechanisms underlying life-history trade-offs.

Materials and Methods

Data and sample collection

Black grouse males were captured using baited walk-in traps across five lekking sites in Central Finland between 2005 and 2007 inclusive. A total of 50 birds were captured both before (January – March) and after the lekking season (April – May) as described in Lebigre *et al.* (Lebigre et al., 2013) (mean sampling interval = 100 days, range = 55 – 133 days) from

five different leks (Table S1). During capture, blood samples were taken from the branchial vein (1–2 ml) with a heparinized syringe and the birds were weighed to the nearest 10 g using a Pesola Spring balance. Blood was centrifuged for 5 minutes at 12,000 rpm to separate red blood cells and plasma. Red blood cells were stored in 70% ethanol at 4ºC and blood plasma at -80 °C until further analysis. During pre-lekking capture, the birds were aged according to plumage characteristics (Helminen, 1963) and if not present already, birds were ringed with both an aluminum tarsus ring containing a unique serial number as well as three color rings allowing identification from a distance for recording behavior. Five individuals were captured before and after the lekking season in two consecutive years, and one individual was caught at both time points in three consecutive years, totaling 116 captures that allow the comparison between pre- and post-lekking periods (Table S1).

Fitness measures

During the lekking season (March–April), males were observed using scan-sampling with a 5–20-minute interval. Lek attendance was quantified as the percentage of scans in which the individual was observed performing this behavior in that year. Lek centrality was measured as the average distance of a male to the lek center in that year, calculated using a 10 x 10-metre grid system per lek. During each scan, the position of a male was mapped to the closest 1 m on the grid and the median of all points was taken as its distance to the lek center. The center of individual male territories was determined as the median of all coordinates recorded per male during a given mating season, and the overall lek center was determined as the median of all the coordinates recorded during that mating season. Thus, lower lek centrality values are indicative of more centrally-displaying males (Höglund et al., 1997).

We quantified male mating success as the number of observed copulations with female black grouse on leks. Most black grouse matings take place on leks (Lebigre et al., 2007) and in general, females mate once with a single male. Previous work has validated that the observed copulations are highly concordant with true parentage inferred from genetic data (Alatalo, Burke, et al., 1996). Male survival was determined based on future observations. As black grouse males have high site fidelity (Caizergues & Ellison, 2002; Höglund et al., 1999; Warren & Baines, 2002), males were assumed to not have survived when they were never caught in the next winter (trapping generally started in January) or sighted in subsequent years.

EpiGBS3 library preparation and sequencing

We assessed genome-wide DNA methylation changes across the lekking period using pairs of blood samples that were collected in pre- (n = 58) and post-lekking (n = 58) time periods. We collected epigenetic data using EpiGBS3 (Barcelo-Serra et al., 2025), a cost-effective reduced-representation bisulphite sequencing method, adjusted from the EpiGBS2 protocol (Gawehns et al., 2022; Van Gurp et al., 2016) to work on Illumina Novaseq sequencing machines. Before conducting reduced-representation bisulphite sequencing, we first characterized genome-wide DNA methylation patterns in black grouse by whole-genome bisulphite sequencing a single sample as detailed in the Supplementary Information.

As in other vertebrates (Derks et al., 2016; Laine et al., 2016), CpG methylation in black grouse showed a pronounced decline around transcription start sites (TSS; Figure S1), a patterns typically linked to increased transcription (Keller et al., 2016). Because the genome-wide methylation landscape in black grouse matches that described for other vertebrate systems

(Jones, 2012; Siegfried & Simon, 2010), we are confident the same regulatory relationships apply in black grouse and therefore used EpiGBS3, which specifically targets CpG sites, for all further samples. (Baduel et al., 2024)

We prepared EpiGBS3 libraries at the Netherlands Institute of Ecology following the EpiGBS3 protocol (Gawehns et al., 2022). In brief, DNA was isolated from red blood cells using the Qiagen Blood and Tissue Extraction Kit. DNA concentrations were standardized and subsequently 800 ng of DNA per sample was digested with the restriction enzyme MspI. MspI identifies and cleaves genomic DNA at 5'-C^CGG fragments. Large fragments were removed using beads (0.8X AMPure XP beads) and remaining fragments were ligated to a barcoded adapter combination that was unique within the library. After ligation, the fragments of 22 samples were pooled into one sequencing library and exposed to sodium bisulphite to convert unmethylated cytosines to uracils. We amplified the fragments using 15 PCR cycles and the KAPA HIFI Uracil + hotstart ready mix. All resulting libraries were paired end sequenced on an Illumina NovaSeq X sequencing platform by Novogene (Cambridge, UK).

DNA methylation calling

The raw reads were processed using the bioinformatic pipeline included in the EpiGBS3 protocol (Gawehns et al., 2022). In brief, raw reads were demultiplexed, quality checked, filtered for adapter contamination and merged. The Illumina sequence and the custom adapters were trimmed, and remaining short reads were removed using cutadapt v2.10 (Martin, 2011). The quality of raw and cleaned reads were checked using fastqc v0.11.8 (Andrews et al., 2010) and multiqc v1.8 (Ewels et al., 2016). The cleaned reads were aligned to the black grouse reference genome (GCA_043882375.1) using paired-end and nondirectional mode using Bismark v0.22.3 (Krueger & Andrews, 2011) with Bowtie v.2.3.5.1 (Langmead et al., 2009). The mapped reads were merged using SAMtools v1.9 (Danecek et al., 2021) and CpG methylation was called while ignoring the first four base pairs in both reads using Bismark.

Principal component analysis

We merged the two strands and filtered for a minimum of 10X coverage per CpG site per sample using a custom bash script. Next, we conducted a principal component analysis (PCA) on these partially filtered reads to assess clustering between sequencing libraries, time periods, sampling years, and leks. The PCA was conducted using the stats R package v4.4.0 (Team, 2021) and included only CpG sites that were covered in all individuals (n = 215). Additionally, we tested for library effects on genome-wide mean CpG methylation percentage. We fitted two linear models with the R package lme4 v1.1.35.5 (Bates et al., 2015): the null model fitted to the mean CpG methylation percentage which includes male ID as a random effect, and the alternative model that is identical to the null model but additionally includes library as a fixed effect. To test for significant differences in average CpG methylation percentage between libraries, we conducted a one-way analysis of variance (ANOVA) on these two models.

Filtering for high-quality variant CpG sites

We excluded CpG sites with a coverage higher than the 99.9 percentile from further analysis to remove potential PCR duplicates, and united the reads using the R package methylKit v1.16.1 (Akalin et al., 2012), resulting in a total of 1,559,800 partially filtered CpG sites. Next,

we removed CpG sites that were nonvariant (either 0% or 100% methylated in all samples, removed 129,274 sites) and those with little variation among samples (removed 615,066 sites). The latter filter step excluded CpG sites that are 0% or 100% methylated in more than 70% of the samples. We additionally excluded CpG sites that were present in fewer than 29 samples (50%) at each time point (removed 460,811 sites). The filtered data set consisted of 354,649 CpG sites which were used for subsequent analysis.

Testing for temporal changes in DNA methylation

We identified CpG sites that significantly changed in DNA methylation across the lekking period using binomial generalized linear mixed effect models (GLMMs) that were constructed for each CpG site separately. The models predicted the number of methylated and unmethylated cytosines using the *cbind* function and included a two-level fixed effect of time period (pre-lekking versus post-lekking). To control for the non-independence of repeated samples across years, we included a random effect of male ID. The models were implemented using lme4 v1.1.35.5 (Bates et al., 2015) and were run in parallel for every CpG site that passed the filtering steps described above (n = 354,649) using the R package parallel v4.3.1 (Team, 2021). We excluded CpG site models if they did not converge or if its overdispersion ratio was higher than the 95th percentile. We corrected for multiple testing using the false discovery rate (FDR, (Benjamini & Hochberg, 1995)). A dynamic CpG site was defined as a CpG with a significant effect of the time period (FDR-corrected q < 0.05, thus allowing for 5% false discoveries) and an absolute mean methylation difference across individuals of at least 10%.

Methylation changes associated with reproductive investment

However, not all methylation changes that occur during the lekking season are associated with investment in reproduction, but could alternatively be caused by changes in the photoperiod, increasing temperatures, or other seasonal factors (Visser et al., 2010). Such factors could be involved in gearing up for the reproductive season, but are unrelated to the reproductive investment made in the season. To isolate those methylation changes associated with reproductive investment only, we next tested whether variation in reproductive investment was associated with variation in methylation changes at dynamic CpG sites. Reproductive investment was quantified using three traits associated with high energetic demands: lek attendance, lek centrality and mating success.

We built three sets of LMMs, one per investment trait, where each set included a LMM per dynamic CpG site. The models were fitted to ∆methylation and included the respective z-transformed behavioral trait as a fixed effect, as well as a two-level fixed effect of age (yearling versus adult) and a random effect of lek. The potential for DNA methylation changes intrinsically depends on the starting level of DNA methylation. For example, a CpG site with 100% DNA methylation prior to lekking can only decrease in methylation or remain stable, whereas a CpG site with 50% DNA methylation prior to lekking can increase or decrease by 50% or remain stable. We tested for this relationship by fitting a linear mixed effect model to ∆methylation and included pre-lekking methylation% as a fixed effect, as well as a random effect of CpG site and ID. We indeed observed the expected relationship, where higher pre-lekking values are associated with methylation decreases over time (Figure S2a) and higher absolute changes when pre-lekking methylations are low or high (Figure S2b). We therefore controlled for this inherent statistical relationship between pre-lekking DNA methylation and

methylation change potential by including the percentage of cytosines that were methylated pre-lekking as a fixed effect.

Similar to above, for each model set we took one random data point of the individuals with repeated samples to avoid issues with model convergence. Because the behavioral data are inherently incomplete (males that do not attend leks do not have centrality records), and because not every CpG site is covered in each sample, we only included CpG sites with sufficient data: for each set, we excluded CpG sites that contained less than 20 data points for the respective behavioral trait (see Table S2 for the number of CpG sites analyzed for each trait). CpG site models that did not converge were excluded from further analysis, overdispersion was evaluated by constructing *Q-Q* plots and p-values were corrected for multiple testing per model set using the FDR, allowing 5% false discoveries.

Methylation changes associated with future traits

To identify whether epigenetic changes are related to future traits and might therefore be indicative of physiological and energetic costs, we next tested whether ∆methylation was associated with survival to the next winter and the expression of ornamental traits. For each trait (survival, blue chroma in the next season, lyre size in the next season), we built a set of mixed effect models, one model per CpG site. Each model predicted the respective trait and included ∆methylation, %methylation pre-lekking, and age category as fixed effects and lek as a random effect. For survival, we used generalized mixed effects models with a binomial distribution, whereas LMMs were built for blue chroma and lyre size in the next season. As only males that survived until the next winter have records of ornamental traits, the sample size was smaller in these models. For each model set, we excluded CpG sites that contained less than 12 data points for the respective trait. Again, we took one random data point of the individuals with repeated samples.

Gene annotation

All filtered CpG sites were annotated using the black grouse genome annotation (https://github.com/rshuhuachen/ms_load_grouse/blob/main/data/genomic/annotation/PO2979_Lyrurus_tetrix_black_grouse.annotation.gff.gz, retrieved on September 20th, 2024). We annotated whether each CpG site was located in the region around a transcription start site (TSS), promoter, intron, exon, upstream or downstream region using the R packages GenomicFeatures v1.42.3 (Lawrence et al. 2013) and rtracklayer v1.50.0 (Lawrence et al. 2009). The TSS was defined as the region 300-bp upstream to 50-bp downstream of each gene's annotated starting position (Laine et al., 2016). Promoters were defined as the region 2,000-bp upstream to 200-bp downstream of the genes' annotated starting position, which therefore overlap with the TSS (Lindner, Laine, et al., 2021). Upstream and downstream regions were limited to 10,000-bp up- and down-stream of the gene body respectively (Laine et al., 2016; Lindner, Verhagen, Viitaniemi, Laine, Visser, Husby, & van Oers, 2021).

If a CpG site was found within a TSS, it was also inherently located within the promoter region; therefore, we annotated it solely as being located in the TSS in order to avoid redundancy. If a CpG site was found in regions of different genes (for example, downstream of gene A and in the promoter of gene B), we annotated it according to the gene body it was most closely located to calculated in base pairs. We tested whether dynamic CpG sites were over- or underrepresented in certain genomic regions by comparing the percentage of significant sites

in a certain region to the percentage of total filtered CpG sites in that same region using a binomial test.

GO enrichment analyses

We conducted three gene ontology (GO) enrichment analyses using GOrilla (Eden et al., 2009), where a target and a background list were provided to identify significant GO terms using GOrilla's default settings. The first analysis tested whether dynamic CpG sites were overrepresented in certain GO terms by using a target list containing genes in which we found a dynamic CpG site, whereas the background list contained genes that contained a CpG site that was included in our filtered data set, regardless of its significance. The second analysis tested whether CpG sites associated with reproductive effort were overrepresented in certain GO terms by using a target list containing genes in which we found a CpG site associated with one of the three reproductive investment traits before multiple testing correction. The background list consisted of genes that contained a dynamic CpG site. The last analysis was similar, where we tested whether CpG sites associated with survival or ornament expression were overrepresented in certain GO terms, where the target list contained genes in which we found a CpG site associated with survival, blue chroma expression or lyre size. An FDR-correction was applied for each analysis separately to correct for multiple testing, allowing for 5% false discoveries.

All statistical analyses were implemented in R v4.4.0 (Team, 2021) using Rstudio v2024.09.0 (Posit team, 2024) and the results were visualized using the R packages ggplot2 v3.5.1 (Wickham et al., 2019) and cowplot v1.1.3 (Wilke et al., 2021)

Results

Using EpiGBS3, we sequenced 1,559,800 raw CpG sites at a general mean coverage of 24X to produce a dataset of 354,649 high quality, variable CpG sites in 50 individual male black grouse. Principle component analysis revealed that the samples did not cluster according to the library they were sequenced in (Figure S2a), lek (Figure S2b), time-period (Figure S2c) or year (Figure S2d). We also found no effect of sequencing library on genome-wide mean CpG methylation (ANOVA: $F = 11.13$, $df = 9$, $p = 0.27$).

As described above, we applied FDR correction to minimize the likelihood of identifying Type I errors at the risk of excluding true positives. The models testing for temporal changes in DNA methylation per CpG site were implemented on the full dataset ($n = 116$), provided that the CpG site in question was sequenced in a given individual. By contrast, the models testing for associations with Δmethylation were implemented on a smaller dataset ($n > 12$) as not all individuals were observed on the leks or survived to the next year, increasing the likelihood that true positives were excluded after correction for multiple tests. Therefore, for the models testing for associations with Δmethylation, we reported both the results both without correction (using a significance threshold of $p < 0.05$), under the assumption that these may still capture biologically meaningful patterns, and with FDR correction (using a significance threshold of $q < 0.05$).

Temporal changes in DNA methylation

We identified 1,026 CpG sites (0.3%) that changed significantly (after false discovery rate correction) in methylation across the lekking period on a population level (hereafter referred

to as “dynamic CpG sites”; Figure 1a). The majority of dynamic CpG sites increased (n = 807) rather than decreased (n = 219) in methylation over time. We found an underrepresentation of dynamic CpG sites in promoter regions (exact binomial test $p < 0.001$; Figure 1b) and transcription start sites (exact binomial test $p < 0.001$; Figure 1b) and an overrepresentation in upstream (exact binomial test $p = 0.02$; Figure 1b) and intronic regions (exact binomial test $p < 0.001$; Figure 1b).

GO enrichment analysis identified several GO terms associated with the regulation of transcription, although only the term “regulation of RNA biosynthetic process” term passed FDR correction (Tables S3; S4). Although dynamic CpG sites were distributed across the entire genome (Figure 1c), we identified three genomic regions where multiple dynamic CpG sites clustered together within single genes. Gene *MAB21L2* on scaffold five contained six dynamic CpG sites in its promoter, gene *BEST1* on scaffold seven contained four dynamic CpG sites in its promoter, and gene *HES1-B* on scaffold ten contained five dynamic CpG sites in its down- and upstream regions. The number of dynamic CpG sites in these genes exceeded expectations based on the total number of analyzed CpG sites in those genes (exact binomial test $p < 0.001$). While these methylation changes were identified on the population level, there was also notable among-individual variation in both the extent and direction of methylation change (Figure 1d).

Epigenetic changes associated with reproductive investment

We identified 64 CpG sites where lek attendance was significantly associated with ∆methylation, although only a single CpG site in the promoter region of the uncharacterized gene *ANN00004* remained significant after FDR correction (Figure 2a). Males with higher lek attendance showed smaller methylation changes at this CpG site (*beta* estimate = -0.08, standard error (SE) = 0.02, degrees of freedom (df) = 26.3, FDR-corrected q = 0.01, Figure 3a). We also identified 62 CpG sites where lek centrality was associated with ∆methylation, but none of these passed FDR correction (Figure 2b). Mating success was associated with ∆methylation at 32 CpG sites (Figure 2c), with one CpG site located in the downstream region of the gene *FKBP8* passing FDR correction. Males with higher mating success showed smaller decreases in methylation at this CpG site (*beta* estimate = 0.20, SE = 0.02, df = 29.5, FDR-corrected q = 0.04, Figure 3b).

We identified multiple CpG sites that showed associations between different reproductive investment traits and changes in methylation. Twelve CpG sites were associated with both attendance and lek centrality and one CpG site was associated with both lek centrality and mating success (Table S5). GO analysis found that CpG sites associated with reproductive investment are enriched in genes involved in positive regulation of transcription and RNA biosynthesis, although none of the GO terms passed multiple-testing correction (Table S6).

Epigenetic changes associated with future fitness-relevant traits

If methylation changes during the lekking season incur costs, or indirectly by signaling that costly investments were made into reproduction, these changes might be associated with the expression of future traits including survival, a key life-history trait, as well as with the expression of ornamental traits. We identified 25 CpG sites where ∆methylation was significantly associated with survival (Figure 2d) and 13 CpG sites where ∆methylation was associated with the blue chroma expressed in the subsequent year (Figure 2e), but none of

these passed FDR correction. 16 CpG sites were identified where ∆methylation was associated with lyre size in the subsequent year, two of which passed FDR correction (Figure 2f). Males with higher ∆methylation levels had smaller lyres in the subsequent season for both CpG sites (*beta* estimate = -9.61 and -8.62, SE = 1.65 and 1.42, df = 13 and 9, FDR-corrected q = 0.007 and 0.01, respectively, Figure 3c,d). Because neither CpG site could be annotated, it is unknown in what gene region or gene these CpG sites were located in. No GO terms were significantly enriched for CpG sites associated with any of the future traits.

Trait-associated CpG sites cluster in the same genes

Several genes contained multiple CpG sites where methylation was associated with one or multiple traits before FDR correction (Table S5). For example, the *BEST1* gene contains four significant CpG sites in its promoter associated with lek attendance and/or centrality, where higher ∆methylation is associated with higher reproductive investment. Moreover, the *NFIC* gene contains three CpG sites located in its upstream region associated with survival or lek attendance, the intronic regions of *STARD3* contain three CpG sites associated with mating success or lek centrality, and the downstream region of gene *UBTF* contains two CpG sites associated with lek attendance, centrality and/or blue chroma coloration in the next season. The number of CpG sites associated with one or multiple traits was higher than expected based on the total number of dynamic CpG sites in these four genes (exact binomial test p < 0.01).

Identifying CpG sites that may mediate life-history trade-offs

If methylation changes are positively associated with reproductive investment and negatively associated with a fitness-relevant trait expressed in the future or *vice versa*, this might provide a molecular mechanism by which life-history trade-offs may arise. We identified three CpG sites that showed significant associations that were consistent with this pattern (Table 1), although they did not pass FDR correction. First, methylation changes at a CpG site in the downstream region of gene *ANKRD40* were positively associated with lek attendance and negatively associated with survival. Second, methylation changes at a CpG site in the exon of *JAM3* were positively associated with mating success and negatively associated with survival (Table 1). *JAM3* also contains another CpG site in its promoter region where DNA methylation changes were associated with the lyre size in the next year (*beta* estimate = -4.65, p = 0.04; Table S5), pointing to different functions of methylation within one gene. Third, methylation changes at a CpG site in the upstream region of *PLEKHA6* were positively associated with lek attendance and negatively associated with lyre size in the next year.

Discussion

Identifying the molecular mechanisms associated with reproductive investment is essential for understanding life-history trade-offs. Our results show that a small proportion of CpG sites in male black grouse change in methylation during the lekking season, which are related to the regulation of RNA biosynthesis. We further show that changes in DNA methylation that are associated with reproductive investment and the expression of future traits, including key life-history traits, are targeted in specific genes. Our findings indicate that epigenetic changes and consequently, gene regulatory changes, link investment in reproduction to survival and the subsequent expression of condition-dependent sexual traits, thereby contributing to our understanding of the molecular mechanisms responsible for life-history trade-offs.

Temporal methylation changes

Epigenetic factors are known to contribute to the regulation of circadian and seasonal cycles, including migration and the onset of reproduction. Here, we show that epigenetic changes also occur in male black grouse during the 4–6 week lekking season, a period of intense reproductive investment. Specifically, a small proportion of CpG sites (1,026, 0.3%) changed significantly in DNA methylation between the pre- and post-lekking stage, a proportion that is comparable to a previous study on erythrocyte methylation changes during the onset of reproduction in great tits (*Parus major*) (Lindner, Laine, et al., 2021). This indicates that targeted gene regulatory changes occur in black grouse during this critical reproductive period, with these changes predominantly occurring at genes involved in the regulation of RNA biosynthesis. Such changes may have widespread functional effects by altering the rate, frequency and extent of RNA production. Notably, the decreased expression of genes linked to RNA biosynthesis has been associated with processes such as oocyte maturation (Celichowski et al., 2018), supporting the idea that they could be important for reproduction.

We identified multiple dynamic CpG sites in three genes: *MAB21L2*, *BEST1* and *HES1-B*. *MAB21L2* is important for neural (Baldessari et al., 2004), heart and liver development (Saito et al., 2012) and the importance of its appropriate temporal expression has previously been emphasized in the context of chick eye development (Sghari & Gunhaga, 2018). *BEST1* is important for forming and regulating chloride ion channels. Genetic variants in this genes have been linked to variation in sperm motility and fertilization success (Milenkovic et al., 2019), as well as with various diseases including retinal dystrophy and other ocular abnormalities (Elbagoury et al., 2025; Huckfeldt & Sobrin, 2020). *HES1-B* is a transcriptional repressor that is critical for cell differentiation (Kageyama et al., 2000) and the inhibition of adipogenesis in birds (Wang et al., 2024). It is part of the cyclic Notch signaling pathway, a highly conserved system of cell-cell communication and gene regulation that is important for cell differentiation across life stages (Fongang & Kudlicki, 2016). Although the precise functional consequences of CpG methylation in these genes for black grouse reproduction remain unknown, they represent promising candidates for future studies.

Methylation changes associated with reproductive investment

We identified over a hundred CpG sites that were significantly associated with variation in reproductive investment. A dozen of these CpG sites were associated with more than one indicator of reproductive investment. This overlap may reflect the inherent collinearity among lek attendance, centrality and mating success, as males occupying the most central territories secure the most matings, and males cannot defend central territories without attending leks (Rintamäki et al., 2001). Alternatively, this overlap could point towards shared molecular processes underpinning these traits, such as metabolic changes, elevated heightened responses in response to increased parasite loads, coping with physiological and oxidative stress (Lebigre et al., 2013) or hormonal changes (Alatalo, Höglund, et al., 1996).

Genes containing CpG sites significantly associated with reproductive investment traits were predominantly involved in regulating transcription, although no GO terms passed FDR correction. This may indicate a possible link between DNA methylation changes and transcriptional activity related to lekking and sexual trait expression, or vice versa, as the direction of causality remains elusive. The CpG site where ∆methylation was significantly

associated with mating success was located in the *FKBP8* gene, which belongs to a protein family that is important for immunoregulation (Kang et al., 2005), apoptosis (Wong et al., 2008), starvation-induced autophagy and basic cellular processes involving protein folding and trafficking (Aguilera et al., 2022). We therefore tentatively conclude that decreased *FKBP8* expression might reduce mating success by compromising cellular homeostasis.

Methylation changes associated with future fitness-relevant traits

We tested whether DNA methylation changes during the reproductive season have consequences for the expression of future life-history traits. We identified a small number of CpG sites where methylation changes were indeed associated with survival and the condition-dependent expression sexual ornaments after the lekking season. Our results indicate that changes in DNA methylation at certain genes might be important for somatic maintenance, highlighting the importance of precise gene regulation for fitness. This reinforces the idea that disrupted gene regulation could have detrimental effects if genes are activated or silenced at inappropriate times or in the wrong context (Chen et al., 2025; Morton et al., 1956).

Overlapping CpG sites

We identified several genes that contained three or more CpG sites that were significantly associated with one or multiple traits. All four dynamic CpG sites identified in the *BEST1* gene were associated with reproductive investment. Additionally, *NFIC*, *STARD3*, *UBTF* contained multiple significant CpG sites. *NFIC* is a DNA binding protein that acts as a transcription and replication factor (Robinson et al., 2014), is important for growth and organ development (Zenker et al., 2019) and is a key epigenetic regulator during development (Fane et al., 2017). *STARD3* (also known as *MLN64*) is involved in cholesterol transport and homeostasis (Voilquin et al., 2019), is associated with fatty acid composition (Kigoshi et al., 2019) and is a homolog of the carotenoid binding protein of the silkworm (Sakudoh et al., 2007). However, it does not appear to be differentially expressed in carotenoid-colored tissues in either birds (Walsh et al., 2012) or cichlids (Judan Cruz et al., 2021). *UBTF* is a transcription factor that mediates the recruitment of RNA polymerase to promoter regions, maintains genomic stability, and mediates DNA accessibility (Sanij et al., 2015). Although the functional effects of these genes in the black grouse remain speculative, their known roles in development, transcription and energy metabolism are suggestive of roles in balancing reproductive investment and somatic maintenance.

A molecular mechanism underlying life-history trade-offs

If changes in DNA methylation are beneficial for one life-history trait but come at a cost for another, they could provide a molecular mechanism by which life-history trade-offs are mediated. We identified two genes where DNA methylation was associated in opposite directions with reproductive investment and survival, and an additional gene where DNA methylation was associated in opposite directions with both reproductive investment and lyre size in the next lekking season, although these CpG sites did not pass FDR correction. This finding suggests that epigenetic mechanisms may contribute to life-history trade-offs through antagonistic pleiotropic effects. To date, the genomic evidence for life-history trade-offs has largely focused on genetic correlations and demonstrations of antagonistic pleiotropy for specific genetic variants (Chang et al., 2024). For example, a transcription factor gene showing antagonistic pleiotropy has previously been identified in algae, indicating that such genes could induce up- and down-regulation of other molecular pathways (Saggere et al.,

2022). Our results hint towards a possible role of epigenetic and hence, gene regulatory mechanisms that may additionally or alternatively constrain life-history evolution, highlighting how antagonistic pleiotropy may act on different levels of cellular organization.

Expected transcriptional consequences

The significant CpG sites that we identified, including dynamic CpG sites associated with reproductive investment and future traits, were distributed across different genomic regions with diverse functional roles. Consequently, predicting their transcriptional consequences is not straightforward. Methylation changes in promoter CpG sites might lead to the up- or downregulation of genes by affecting their transcription, methylation changes in exonic CpG sites might be associated with alternative splicing patterns (Lev Maor et al., 2015), and methylation of intronic CpG sites might play a role in the silencing of harmful repetitive regions such as transposable elements (Derks et al., 2016; To et al., 2015). Importantly, we found that single genes sometimes contained multiple significant CpG sites in different regions, for example both in an exon and in a promoter, making it difficult to infer the net effects on transcript abundance and / or splicing. Linking methylation changes to gene expression changes is therefore essential for understanding the functional effects of temporal epigenetic changes, but unfortunately this was not possible in the current study because RNA sequencing data are not available.

Caveats

Although we identified many CpG sites associated with reproductive investment and/or future fitness-relevant traits, most did not pass FDR correction. Correcting for multiple testing is a conservative approach that minimizes Type I errors but also increases the risk of discarding true positives (i.e. Type II errors), particularly when sample sizes are limited. In such cases, only very strong effects can be reliably detected. Although we cannot say whether the identified CpG sites that did not pass FDR correction were false or true positives, the overlap of CpG sites across traits suggests that at least some of them are biologically meaningful. Further studies could address this limitation by increasing sample sizes to improve statistical power, and by sampling more timepoints in order to produce a more detailed picture of temporal changes in DNA methylation and their role in the expression of current and future fitness-relevant traits in the short- and long-term.

Another possible caveat is that the identified genes and/or CpG sites are not necessarily independent from each other; they may be inherited together due to linkage disequilibrium, which, which cannot be resolved using our current dataset of mainly unrelated males (Chen et al., 2025). Moreover, we analyzed the effects of single CpG sites in isolation, while it is also possible that epigenetic changes could interact across loci and biological pathways. While our data set did not allow to test for interacting and cumulative effects due to statistical limitations caused by sample size, future studies could take a more integrative approach to test this idea.

Conclusions

Understanding how organisms balance competing demands such as growth, reproduction and survival remains a central question in evolutionary biology. Life-history trade-offs emerge because finite energetic resources must be allocated among different traits, and additionally or alternatively, because traits often share underlying biological pathways. Our results suggest that epigenetic regulation provides a dynamic mechanism through which resource allocation

decisions can be implemented at the molecular level. More specifically, we show that changes in DNA methylation are associated with reproductive investment and future fitness-relevant traits at a handful of genes. We further show that temporal methylation changes at a few specific CpG sites are positively associated with reproduction and negatively associated with future fitness-relevant traits, which provides empirical support for the notion that epigenetic mechanisms may mediate fundamental life-history trade-offs. Methylation changes at key regulatory loci, such as those located in epigenetic regulator and transcription genes, may also have pleiotropic effects: while they can facilitate short-term reproductive investment, they may simultaneously compromise longevity and future reproductive potential. Thus, epigenetic regulation emerges as a plausible integrative mechanism linking gene expression to physiological, metabolic and hormonal processes that underpin the cost of reproduction.

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Author contributions

Conceptualization, all authors; data curation, R.S.C. and C.L.S.; methodology, R.S.C, C.L.S. and K.O.; investigation, R.S.C. and C.L.S.; formal analysis, R.S.C.; funding acquisition, C.L.S, K.O. and J.I.H.; resources, C.L.S. and K.O.; supervision, C.L.S., K.O. and J.I.H.; writing – original draft, R.S.C; writing – review & editing, all authors.

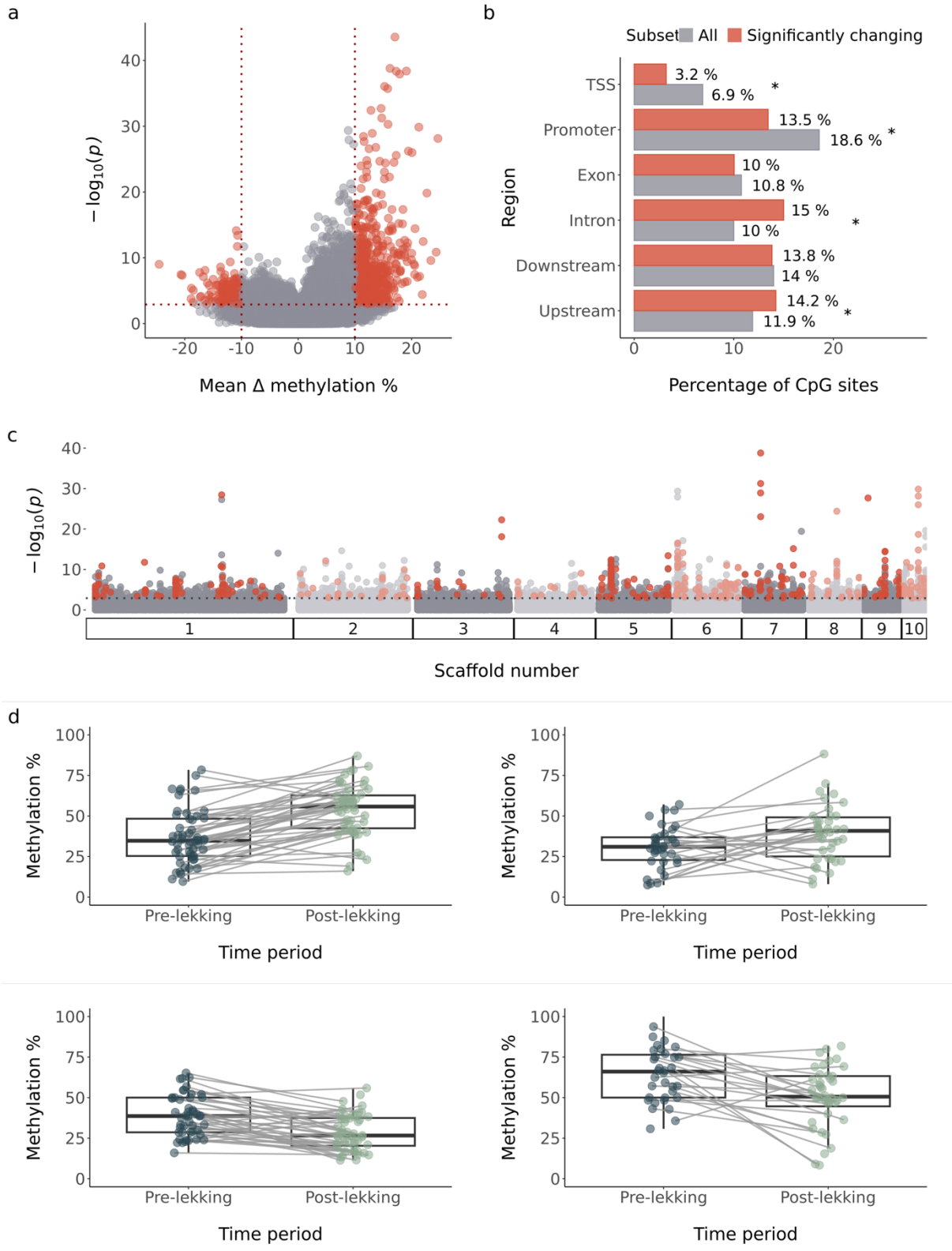

Figure 1. DNA methylation changes across the lekking season. (a) Volcano plot showing per filtered CpG site the relationship between mean Δmethylation percentage across individuals and their *p*-values, indicating whether the CpG significantly changed in DNA methylation across the lekking period after correction for the genome-wide false discovery rate (FDR). Dynamic CpG sites were defined as having an FDR-corrected $q < 0.05$ and an absolute mean Δmethylation > 10% (see the Methods for details) and are highlighted in red. The vertical

red dotted lines indicate Δmethylation values of -10% and 10%, respectively, and the horizontal line shows the significance threshold ($q < 0.05$); (b) Bar plot showing the percentage of all CpG sites and the percentage of dynamic CpG sites located in each of the six genomic regions. Asterisks indicate a significant over- or underrepresentation of CpG sites in the respective genomic region that significantly changed compared to all CpG sites (binomial test $p < 0.05$); (c) Manhattan plot showing the distribution of CpG sites across the ten largest scaffolds. Dynamic CpG sites, which passed the FDR-corrected significance threshold and had a minimum absolute mean Δmethylation > 10%, are highlighted in red, whereas non-dynamic CpG sites are shown in grey. Individual scaffolds are indicated by alternating shades of grey and red. The dotted horizontal line indicates the FDR-corrected significance threshold; (d) Raw data showing DNA methylation pre- and post-lekking for the two CpG sites that most significantly increased in DNA methylation (top) and the two CpG sites that most significantly decreased in DNA methylation (bottom). Grey lines connect pre- with post-lekking sample pairs (i.e. the same individual consecutively sampled during the same season). Thick horizontal lines indicate mean methylation %, while the lower and upper hinges correspond to the first and third quartiles, respectively, and the whiskers represent 1.5 times the interquartile range.

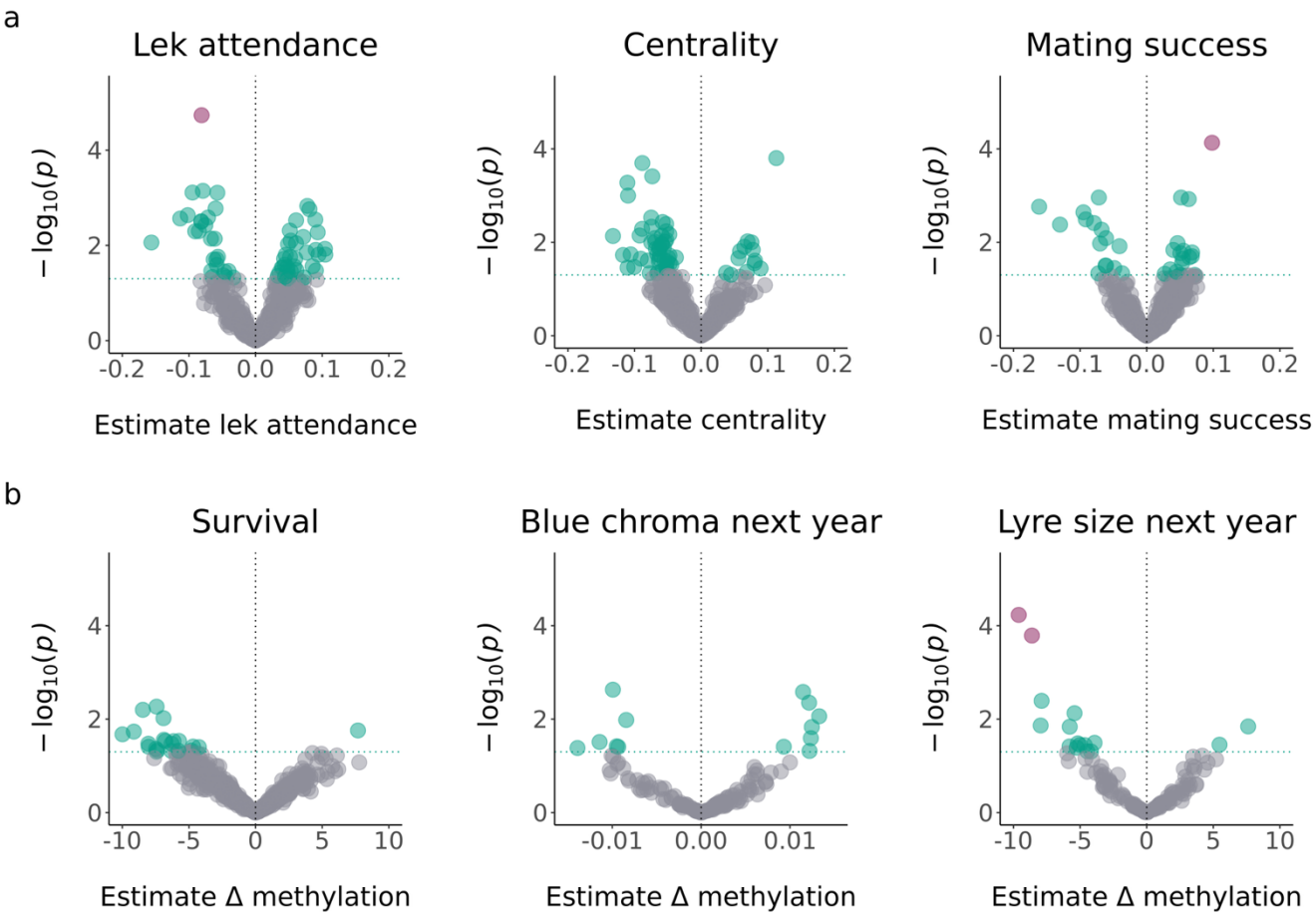

Figure 2. **Output of the models liking methylation changes to the investment and cost traits.** Volcano plots showing, per dynamic CpG site, the relationship between the beta estimate and p-value. For the investment traits (a), the effects of lek attendance, centrality and mating success on Δmethylation are shown. For the cost traits (b), the effects of Δmethylation on survival, blue chroma in the next year and lyre size in the next year are shown. The vertical dotted line indicates a *beta* estimate of 0 and the horizontal dotted line indicates the significance threshold prior to multiple testing correction. The blue points indicate CpG sites showing a significant association and the purple points indicate those that remain significant after applying FDR correction.

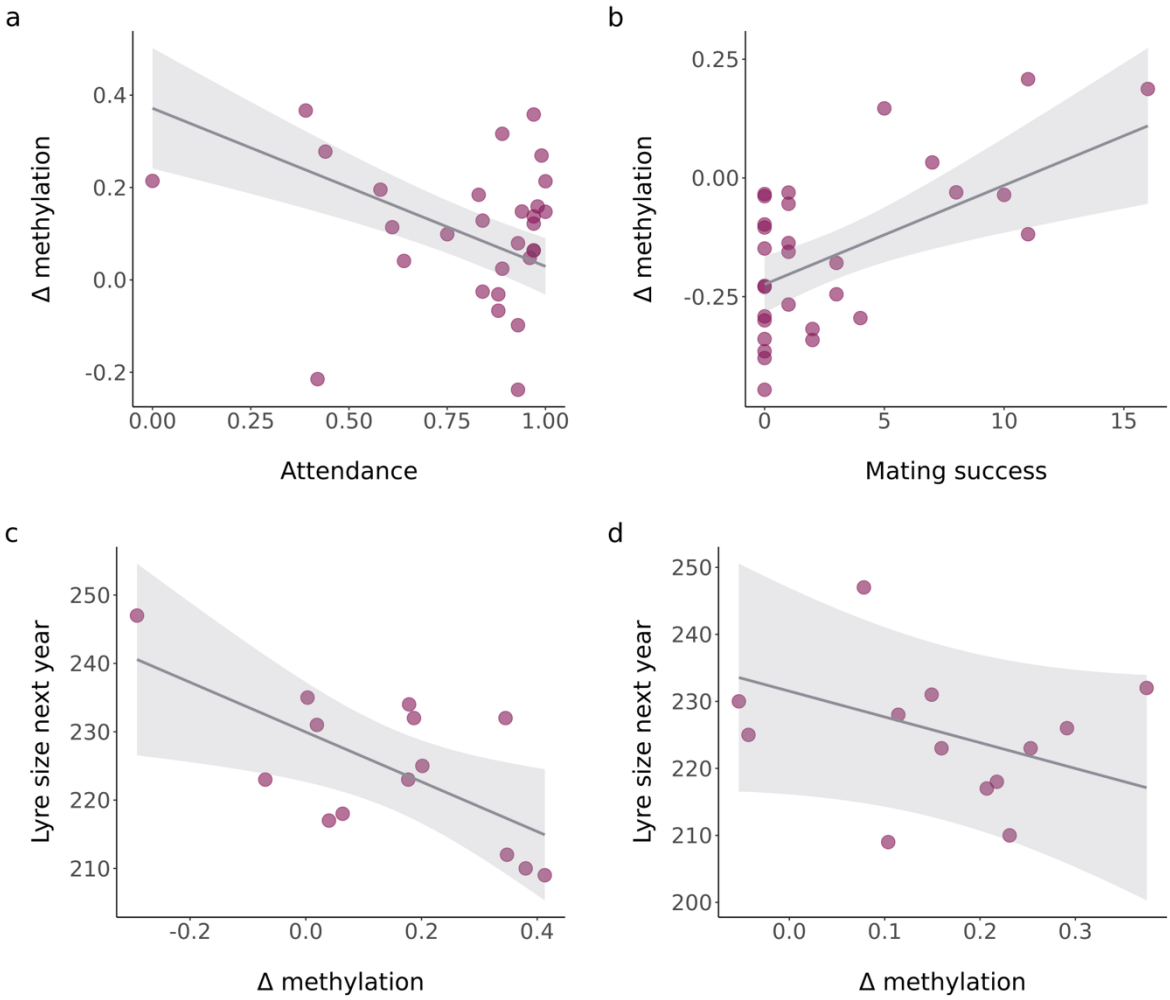

Figure 3. Scatterplots of Δmethylation at four CpG sites and the phenotypic traits Δmethylation was significantly associated with. a) The relationship between lek attendance and Δmethylation at the significant CpG site in the promoter of uncharacterised gene *ANN00004*; b) the relationship between mating success and Δmethylation at the significant CpG site in the downstream region of gene *FKBP8;* c) the relationship between Δmethylation and the lyre size expressed in the next year at the first unannotated significant CpG sites; and d) the relationship between Δmethylation and the lyre size expressed in the next year at the second unannotated significant CpG sites. The raw data are depicted as purple points and the grey lines show the predicted regression based on the linear mixed effects model described in the Methods. The shaded areas indicate the 95% confidence limits of the estimated marginal means for each data point.

Table 1. The three CpG sites where a pattern consistent with antagonistic pleiotropy was identified. We modelled how reproductive investment (attendance, mating success) was associated with ∆methylation and how ∆methylation was associated with cost traits (survival, lyre size next year; see Methods for details). Accordingly, attendance and mating success were predictors in their respective models, and ∆methylation was the response variable. Survival and lyre size in the next year were response variables in their respective models, where ∆methylation was the predictor. *Beta* estimates of the relevant fixed effects are shown together with their associated *p*-values prior to FDR correction.

Scaffold	**Position**	**Associated trait**	***Beta* estimate**	***p***	**Gene region**	**Gene**
19	9,737,771	Attendance	0.05	0.02	downstream	*ANKRD40*
		Survival	-8.03	0.04		
25	2,512,626	Mating success	0.06	0.02	exon	*JAM3*
		Survival	-6.78	0.03		
22	3,652,515	Attendance	0.05	0.05	upstream	*PLEKHA6*
		Lyre size next year	-7.96	0.01		

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Supplemental Information for:

Epigenetic changes associated with reproductive investment and life-history trade-offs in lekking male black grouse (*Lyrurus tetrix*)

Rebecca S. Chen, Carl D. Soulsbury, Joseph I. Hoffman and Kees van Oers

Supplementary Materials and Methods

Prior to our reduced-representation epigenetic analysis, we explored genome-wide patterns of DNA methylation in black grouse by bisulphite sequencing the whole genome of a single individual. CpG sites cluster in CpG islands, which are overrepresented in promoter regions (Vinson & Chatterjee, 2012). CpG methylation in vertebrates is typically reduced in promoter regions, allowing the binding of transcription factors which can consequently increase gene expression (Siegfried & Simon, 2010). However, DNA methylation patterns and features vary across taxa and within lineages (Schmitz et al., 2019). Because this is the first genome-wide epigenetic study on black grouse to our knowledge, we characterized CpG site methylation patterns across the genome and assessed whether the patterns are comparable to avian model species (e.g. the great tit).

WGBS library preparation and sequencing

First, we extracted DNA from red blood cells with the Qiagen Blood and Tissue Extraction kit of a single sample. The library preparation and sequencing were performed by BMK Gene. BMK Gene used 1 μg of genomic DNA to prepare the WGBS library using the EZ DNA Methylation-Gold™ Kit DNA methylation reagent kit method and protocol. In brief, DNA was fragmented, the ends were repaired, and adapters were ligated, an adenosine base pair was added to the 3' end of the strands and fragments were size-selected. The bisulphite treatment was applied, and the sequences were duplicated in a polymerase chain reaction. The library was sequenced on one lane from both ends of the PE150 bp fragments using an Illumina NovaSeq 6000 sequencing platform which generated 45.14 G of raw reads with a target coverage of 45X.

DNA methylation calling

Raw sequencing reads were trimmed to ensure high quality (≥ 20) and to remove adaptor sequences with a stringency of 2 overlapping bp using Trim Galore v0.6.10 (Krueger et al., 2023). Next, we prepared the black grouse reference genome (NCBI assembly GCA_043882375.1) using the bismark-genome-preparation command to bisulphite-convert and index the genome using bismark v0.24 (Krueger & Andrews, 2011), allowing the alignment of bisulphite-treated reads. We aligned the cleaned sequences to the bismark-prepared reference genome using bismark and extracted methylation calls using the bismark_methylation_extractor command from bismark. Only CpG site methylation was analyzed.

Methylation patterns across genomic regions

We filtered for reads with a minimum coverage of 10X. To characterize methylation levels across different genomic regions, we calculated the mean methylation level across genes for four regions: the transcription start site (TSS; 300 bp upstream to 50 bp downstream of the annotated starting position of each gene (Laine et al., 2016)), the gene body, upstream regions (10 kb upstream from the gene body) and downstream regions (10 kb downstream from the gene body). The mean methylation of TSS regions was calculated across the entire 350 bp window. We used a sliding window approach to calculate mean methylation of gene bodies following (Laine et al., 2016) with slight adaptations. First, we subdivided the gene body into

40 bins (where the bin length therefore differs depending on the gene size), and then calculated the mean methylation for each bin, with an overlap between neighboring bins of 50% of the bin size. A similar sliding window approach was used to calculate mean methylation of up- and downstream regions. Here, the regions were divided into 40 bins of 250 bp each, and the mean methylation was calculated for each bin with an overlap between neighboring bins of 125 bp. For this analysis, custom R scripts were generated using the R packages genomation v1.32.0 (Akalin et al., 2015), GenomicFeatures v1.52.2 (M. Carlson, 2017), rtracklayer 1.60.1 (Lawrence et al., 2009), windowscanr v0.1 (Tavares, 2024), and parallel v4.3.1 (Team, 2021).

Supplementary Results

We characterized genome-wide CpG methylation patterns in black grouse using whole genome bisulphite sequencing of a single sample. The filtered dataset consisted of 9,290,585 CpG sites, of which 4,941,240 were annotated. CpG sites in regions nearby the TSS had lower levels of DNA methylation compared to those in gene bodies and up- and downstream regions of the genome (Fig. S1a). We found large variation in TSS methylation, where the mode of CpG methylation was 0% but a second peak was identified around 70% (Fig. S1b). In general, these DNA methylation patterns are consistent with other avian study systems (Derks et al., 2016; Laine et al., 2016).

Supplementary Figures

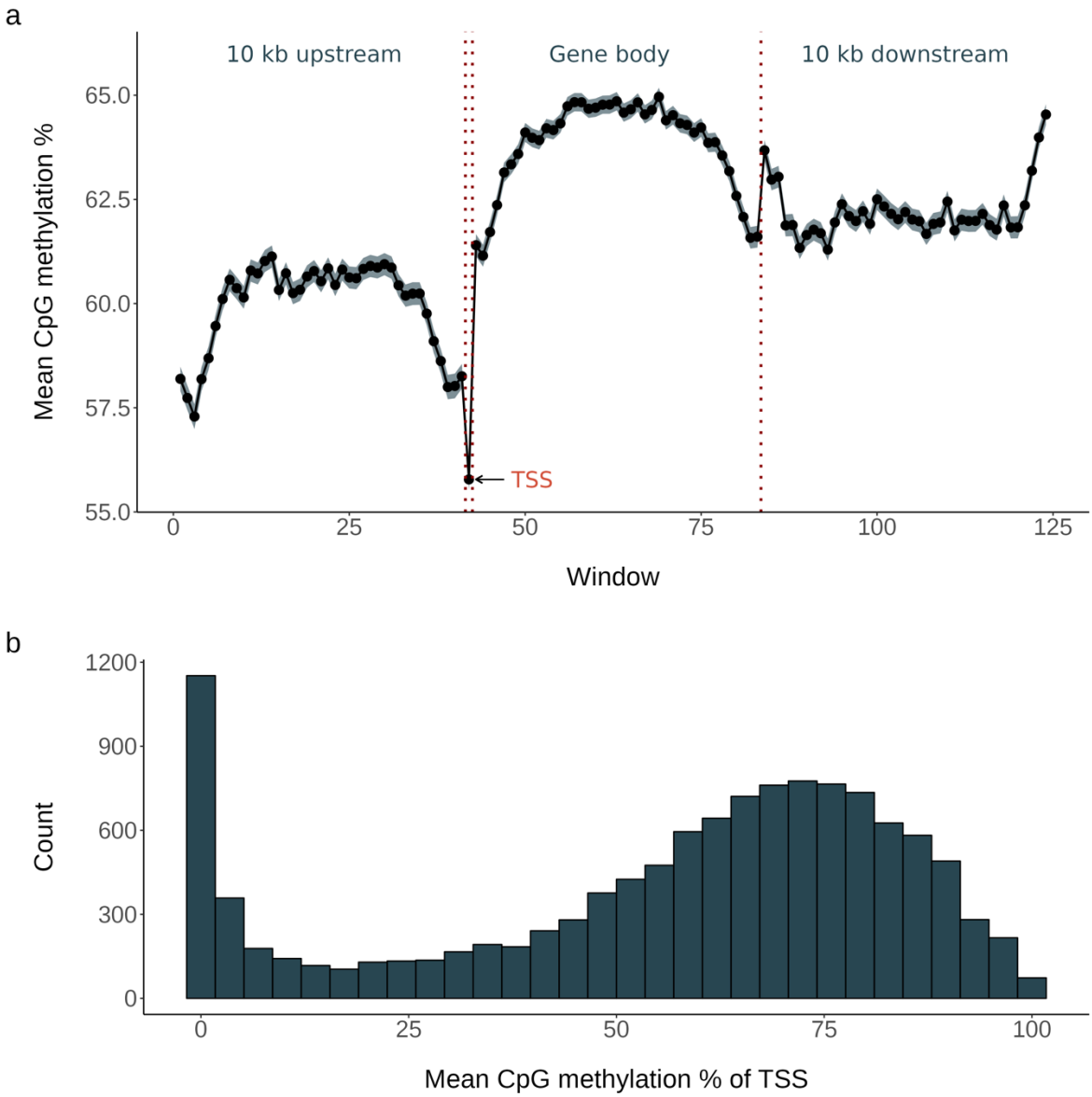

Figure S1. CpG site methylation across genomic regions quantified with whole genome bisulphite sequencing of one sample. (a) The mean CpG methylation % across genes for each window, separated by genomic region. Points represent the mean and shaded areas the standard error; (b) histogram of the mean CpG methylation % of transcription start sites (TSS) across genes.

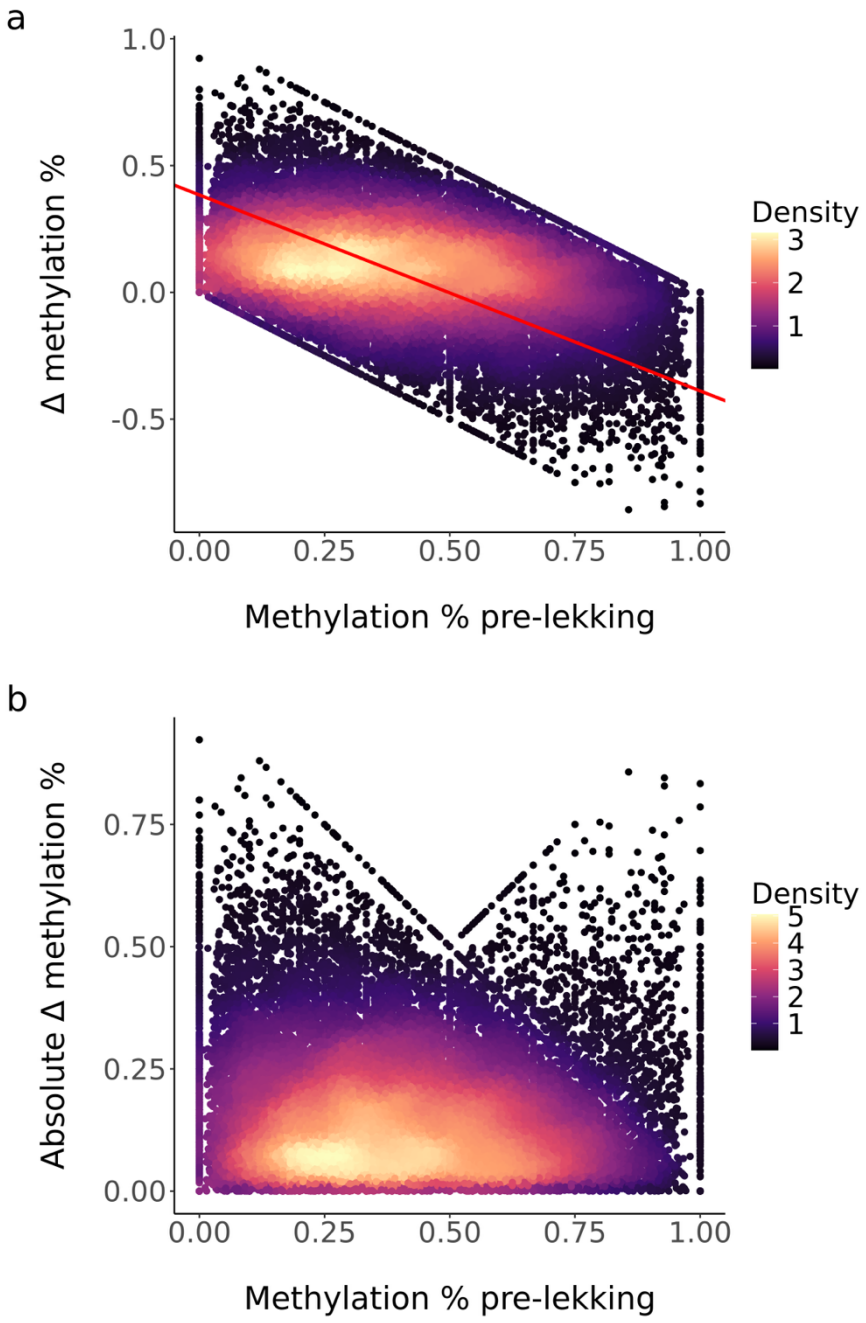

Figure S2. Scatterplots showing the relationship between pre-lekking methylation and Δmethylation. Each point represents a single CpG site in a single individual, and the color indicates the density of neighboring points to better visualize overlapping datapoints. In a), pre-lekking methylation % is plotted against Δmethylation and the red diagonal shows the regression that accounts for the non-independence of CpG sites among individuals and individual variation at the same CpG site. In b), pre-lekking methylation % is plotted against the absolute value of Δmethylation.

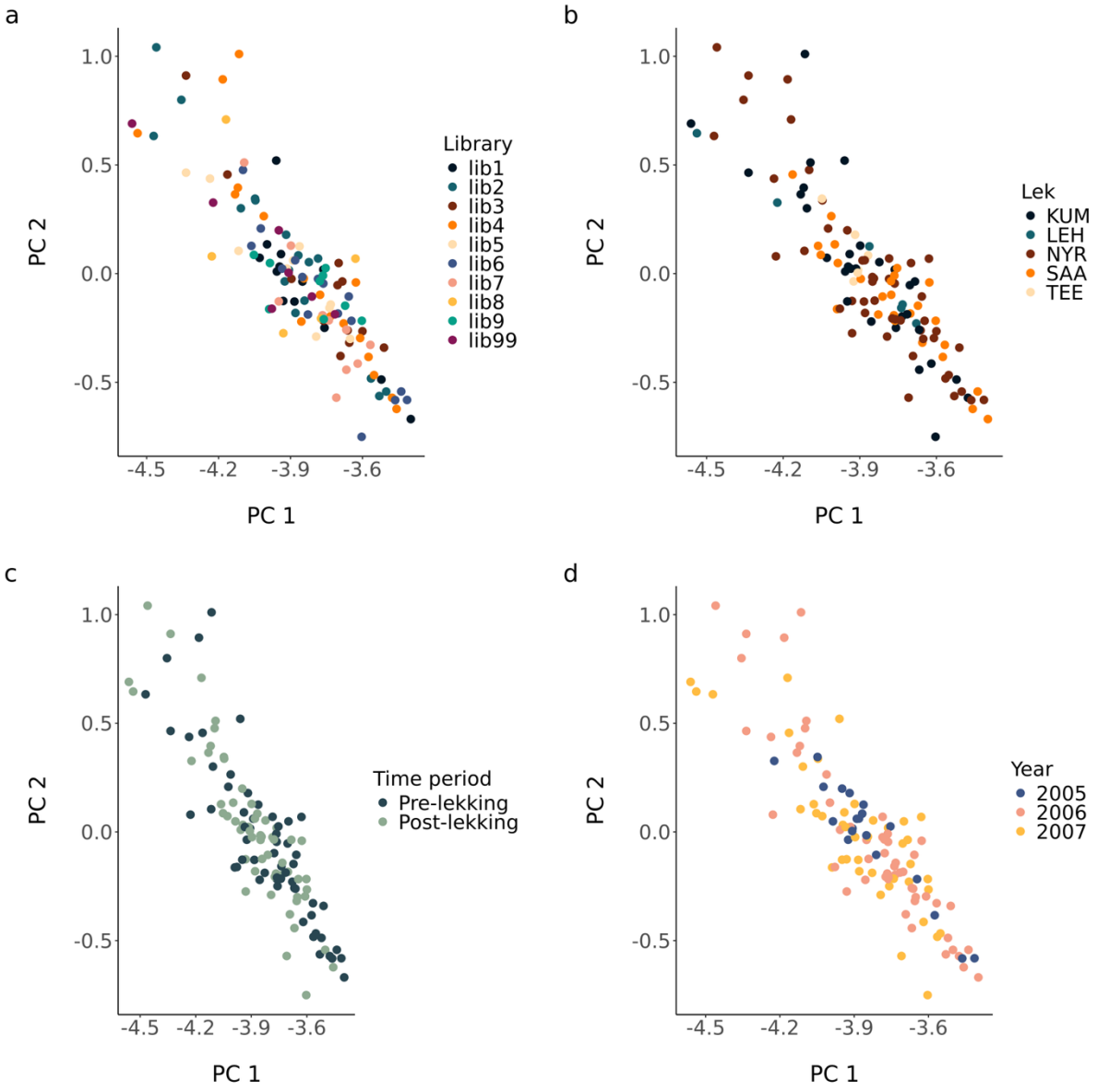

Figure S3. Principal component analysis of EpiGBS3 data. Scatterplots of the two first principal components (PC1 and PC2, respectively) quantified with a principal component analysis conducted on unfiltered CpG sites with complete data, colored by (a) EpiGBS library; (b) lek; (c) time period; and (d) sampling year. PC1 explained 97.5% of variance explained and PC2 0.86%.

Supplementary Tables

Supplementary Table S1. Structure of the data, including the exact dates when morphological measures and blood samples were taken at pre- and post-lekking time points. Lek abbreviations stand for the following: NYR = Nyrölä, KUM = Kummunsuo, LEH = Lehtosuo, SAA = Saarisuo, TEE = Teerrisuo. In the survival column, 1 stands for survived to the next autumn season, 0 stands for no survival.

ID	Lek	Age	Time period	Sampling date	Survived
D154259	TEE	adult	pre	25/02/2005	0
			post	24/05/2005	
D154299	TEE	adult	pre	13/01/2005	0
			post	26/05/2005	
D211312	NYR	adult	pre	31/01/2005	0
			post	14/05/2005	
D211334	LEH	adult	pre	18/02/2005	1
			post	19/05/2005	
D211334	LEH	adult	pre	17/01/2006	0
			post	12/05/2006	
D211431	NYR	adult	pre	07/03/2005	0
			post	21/05/2005	
D211445	TEE	adult	pre	25/02/2005	1
			post	21/05/2005	
D217821	NYR	adult	pre	20/01/2006	0
			post	15/05/2006	

D217896	KUM	adult	pre	30/01/2006	1
			post	10/05/2006	
D217910	NYR	adult	pre	15/02/2005	1
			post	23/05/2005	
D217910	NYR	adult	pre	13/01/2006	0
			post	10/05/2006	
D217911	NYR	adult	pre	21/01/2005	1
			post	17/05/2005	
D217949	NYR	adult	pre	04/02/2006	1
			post	17/05/2006	
D217949	NYR	adult	pre	24/01/2007	0
			post	09/05/2007	
D217961	NYR	adult	pre	20/01/2006	1
			post	13/05/2006	
D217964	NYR	adult	pre	04/02/2006	0
			post	15/05/2006	
D217965	NYR	adult	pre	20/01/2006	1
			post	10/05/2006	
D217977	KUM	adult	pre	30/01/2006	0
			post	13/05/2006	
D229025	KUM	adult	pre	24/01/2007	0
			post	07/05/2007	
D229082	NYR	adult	pre	20/01/2006	1

			post	12/05/2006	
D229093	NYR	adult	pre	13/01/2006	1
			post	15/05/2006	
D229096	NYR	adult	pre	02/02/2007	1
			post	08/05/2007	
D229097	SAA	adult	pre	24/01/2006	0
			post	07/05/2006	
D229103	SAA	adult	pre	10/01/2006	0
			post	06/05/2006	
D229160	SAA	adult	pre	10/02/2005	1
			post	13/05/2005	
D229160	SAA	adult	pre	13/02/2006	1
			post	11/05/2006	
D229160	SAA	adult	pre	30/01/2007	0
			post	05/05/2007	
D229161	SAA	adult	pre	10/02/2005	0
			post	13/05/2005	
D229162	SAA	adult	pre	23/01/2007	0
			post	15/05/2007	
D229165	KUM	adult	pre	12/03/2007	1
			post	06/05/2007	
D229167	KUM	adult	pre	16/02/2006	1
			post	10/05/2006	

D229167	KUM	adult	pre	16/01/2007	1
			post	04/05/2007	
D229170	SAA	adult	pre	13/02/2006	1
			post	10/05/2006	
D229170	SAA	adult	pre	23/01/2007	0
			post	05/05/2007	
D229171	SAA	adult	pre	28/02/2006	0
			post	11/05/2006	
D229186	SAA	adult	pre	10/01/2006	1
			post	11/05/2006	
D229192	KUM	adult	pre	23/01/2006	0
			post	11/05/2006	
D229193	KUM	adult	pre	09/02/2007	1
			post	04/05/2007	
D229509	KUM	adult	pre	30/01/2006	1
			post	16/05/2006	
D229516	LEH	yearling	pre	31/01/2006	1
			post	13/05/2006	
D229633	SAA	adult	pre	06/02/2006	1
			post	11/05/2006	
D229658	KUM	adult	pre	09/02/2007	1
			post	07/05/2007	
D229679	KUM	adult	pre	27/02/2006	1

			post	12/05/2006	
D229679	KUM	adult	pre	16/01/2007	1
			post	06/05/2007	
D229714	NYR	yearling	pre	13/01/2006	0
			post	15/05/2006	
D229718	NYR	yearling	pre	13/01/2006	1
			post	16/05/2006	
D229734	KUM	adult	pre	23/01/2006	1
			post	11/05/2006	
D229735	KUM	adult	pre	09/02/2007	0
			post	06/05/2007	
D229771	NYR	adult	pre	20/01/2006	1
			post	11/05/2006	
D229772	NYR	adult	pre	24/01/2007	1
			post	09/05/2007	
D229775	SAA	adult	pre	30/01/2007	0
			post	10/05/2007	
D229776	LEH	adult	pre	23/01/2007	1
			post	09/05/2007	
D229783	NYR	adult	pre	26/01/2006	1
			post	17/05/2006	
D237277	NYR	adult	pre	23/02/2007	0
			post	08/05/2007	

D237278	NYR	adult	pre	23/02/2007	0
			post	15/05/2007	
D237361	NYR	yearling	pre	24/01/2007	1
			post	15/05/2007	
D237378	NYR	yearling	pre	02/02/2007	0
			post	15/05/2007	
D237389	KUM	yearling	pre	20/02/2007	1
			post	09/05/2007	
D237394	NYR	yearling	pre	15/02/2007	1
			post	09/05/2007	

Supplementary Table S2. The number of CpG sites remaining after each filtering step for each statistical model. There are four filtering steps: CpG sites were only included if there was enough data for analysis (see Methods for details), if the CpG site model converged, if the CpG site did not show signs of dispersion issues, and if the CpG site showed statistical significance (FDR-corrected $q < 0.05$).

Model	Enough data	Converged	Dispersion	Significant	Significant after FDR
Changing CpG sites	354,649	345,937	328,640	1,482	1,026
Lek attendance	607	607	607	64	1
Lek centrality	534	534	534	62	0
Mating success	564	564	564	32	1
Survival	607	597	597	25	0
Blue chroma next year	125	125	125	13	0
Lyre size next year	125	125	125	16	2

Supplementary Table S3. **Biological functions identified using gene ontology (GO) enrichment analysis of dynamic CpG sites.** E is the enrichment score, B is the number of genes in the input list associated with the GO term and b is the number of genes in the input list associated with the term.

GO Term	Description	*p*	FDR *q*	E	B	b
GO:0000987	proximal promoter sequence-specific DNA binding	5.48 e^{-5}	1.98 e^{-1}	1.88	438	41
GO:0000981	DNA-binding transcription factor activity, RNA polymerase II-specific	5.54 e^{-5}	1.00 e^{-1}	1.85	468	43
GO:0003700	DNA-binding transcription factor activity	5.82 e^{-5}	7.03 e^{-2}	1.83	484	44
GO:0001012	RNA polymerase II regulatory region DNA binding	1.04 e^{-4}	9.39 e^{-2}	1.78	496	44
GO:0000977	RNA polymerase II regulatory region sequence-specific DNA binding	1.04 e^{-6}	7.51 e^{-2}	1.78	496	44
GO:1990837	sequence-specific double-stranded DNA binding	1.58 e^{-4}	9.52 e^{-2}	1.69	583	49
GO:0000978	RNA polymerase II proximal promoter sequence-specific DNA binding	1.82 e^{-4}	9.43 e^{-2}	1.82	432	39
GO:0000976	transcription regulatory region sequence-specific DNA binding	1.95 e^{-4}	8.84 e^{-2}	1.71	541	46
GO:0001067	regulatory region nucleic acid binding	2.13 e^{-4}	8.55 e^{-2}	1.70	543	46
GO:0044212	transcription regulatory region DNA binding	2.13 e^{-4}	7.70 e^{-2}	1.70	543	46
GO:0043565	sequence-specific DNA binding	2.79 e^{-4}	9.19 e^{-2}	1.63	629	51
GO:0003690	double-stranded DNA binding	3.39 e^{-4}	1.02 e^{-1}	1.63	618	50
GO:0140110	transcription regulator activity	4.01 e^{-4}	1.12 e^{-1}	1.55	753	58

Supplementary Table S4. **Biological processes identified using gene ontology (GO) enrichment analysis of dynamic CpG sites.** E is the enrichment score, B is the number of genes in the input list associated with the GO term and b is the number of genes in the input list associated with the term. The *q* that passed multiple testing correction (allowing for 5% false discoveries) is highlighted with an asterisk.

GO Term	Description	*p*	FDR *q*	E	B	b
GO:1903506	regulation of nucleic acid-templated transcription	9.99 e^{-6}	0.13	1.47	1439	105
GO:0006355	regulation of transcription, DNA-templated	9.99 e^{-6}	0.07	1.47	1439	105
GO:2001141	regulation of RNA biosynthetic process	1.10 e^{-5}	0.05*	1.47	1442	105
GO:0051252	regulation of RNA metabolic process	4.84 e^{-5}	0.16	1.40	1580	110
GO:2000026	regulation of multicellular organismal development	7.55 e^{-5}	0.20	1.55	937	72
GO:0006357	regulation of transcription by RNA polymerase II	8.39 e^{-5}	0.18	1.49	1092	81
GO:0001708	cell fate specification	1.43 e^{-4}	0.27	4.42	41	9
GO:0050793	regulation of developmental process	1.68 e^{-4}	0.28	1.45	1166	84
GO:1903508	positive regulation of nucleic acid-templated transcription	2.38 e^{-4}	0.35	1.57	771	60
GO:0045893	positive regulation of transcription, DNA-templated	2.38 e^{-4}	0.31	1.57	771	60
GO:1902680	positive regulation of RNA biosynthetic process	2.46 e^{-4}	0.29	1.56	772	60
GO:0007399	nervous system development	2.48 e^{-4}	0.27	2.45	156	19
GO:0051094	positive regulation of developmental process	4.42 e^{-4}	0.45	1.61	625	50
GO:0022603	regulation of atomical structure morphogenesis	4.53 e^{-4}	0.43	1.68	514	43

GO:0019219	regulation of nucleobase-containing compound metabolic process	4.67 e^{-4}	0.41	1.32	1741	114
GO:0045664	regulation of neuron differentiation	5.60 e^{-4}	0.46	1.86	335	31
GO:0009953	dorsal/ventral pattern formation	6.60 e^{-4}	0.51	4.54	31	7
GO:0045944	positive regulation of transcription by RNA polymerase II	6.80 e^{-4}	0.50	1.62	572	46
GO:0030154	cell differentiation	7.72 e^{-4}	0.53	1.47	891	65
GO:0051241	negative regulation of multicellular organismal process	8.23 e^{-4}	0.54	1.65	513	42
GO:0016477	cell migration	9.06 e^{-4}	0.57	1.77	375	33

Supplementary Table S5. All annotated CpG sites significantly associated with an investment or cost trait before applying a multiple-testing correction. For the investment traits (attendance, centrality and mating success), the respective trait was the predictor and ∆methylation was the response. For the cost traits (survival, lyre size in the next year, and blue chroma in the next year), ∆methylation was the predictor, and the respective trait was the response. *Beta* estimates of the relevant fixed effect are shown as well as their associated *p* and FDR-corrected *q*.

Scaffold number	**Position**	**Trait**	**Beta**	***p***	**FDR *q***	**Region**	**Gene**
9	36,006,955	Centrality	-0.05	0.006	0.24	upstream	*ACVR1*
6	25,173,106	Mating success	-0.07	0.046	0.70	downstream	*ADAMTSL2*
6	56,292,083	Mating success	-0.16	0.002	0.20	exon	*ADRB1*
27	3,851,345	Centrality	-0.07	0.003	0.24	exon	*ANK1*
13	18,332,312	Attendance	0.08	0.002	0.13	intron	*ANKRD11*
13	18,316,851	Survival	-6.31	0.048	0.79	upstream	*ANKRD11*
19	9,737,771	Attendance	0.05	0.017	0.30	downstream	*ANKRD40*
19	9,737,771	Survival	-8.03	0.039	0.79	downstream	*ANKRD40*
7	49,082,068	Mating success	-0.07	0.001	0.17	exon	*ANKRD9*
7	49,082,068	Survival	-7.43	0.05	0.79	exon	*ANKRD9*
25	5,152,922	Attendance	0.08	0.001	0.13	promoter	*APOA1*
7	16,104,769	Attendance	0.04	0.019	0.30	promoter	*BEST1*
7	16,104,769	Centrality	-0.05	0.007	0.24	promoter	*BEST1*
7	16,104,759	Centrality	-0.04	0.021	0.29	promoter	*BEST1*
7	16,104,775	Centrality	-0.05	0.004	0.24	promoter	*BEST1*

19	9,844,172	Mating success	0.05	0.028	0.70	intron	*CAC1G*
23	358,619	Survival	-11.24	0.010	0.79	promoter	*CAMTA1*
19	4,947,243	Blue chroma (next year)	0.01	0.009	0.26	promoter	*CANT1*
13	18,429,542	Centrality	-0.05	0.044	0.40	promoter	*CBFA2T3*
24	2,545,576	Centrality	0.04	0.045	0.40	TSS	*CILP2*
3	46,046,806	Centrality	-0.07	0.008	0.24	upstream	*COLEC12*
7	190,378	Survival	-4.83	0.047	0.79	downstream	*CPSF7*
28	355,968	Centrality	-0.07	0.005	0.24	upstream	*CRABP2*
13	18,012,675	Attendance	-0.06	0.046	0.46	intron	*CYB5B*
13	18,012,675	Centrality	0.08	0.010	0.24	intron	*CYB5B*
6	6,229,580	Mating success	-0.07	0.005	0.30	downstream	*DMRTA2*
4	56,198,050	Blue chroma (next year)	0.01	0.026	0.43	upstream	*DJB5*
23	506,065	Mating success	0.03	0.047	0.70	promoter	*DJC11*
26	3,774,540	Centrality	-0.10	0.034	0.35	exon	*DLI1*
24	462,215	Centrality	0.07	0.010	0.24	promoter	*DOT1L*
2	3,176,115	Centrality	-0.07	0.010	0.24	downstream	*ELP3*
19	9,920,788	Centrality	-0.11	0.001	0.11	promoter	*EPN3*
15	9,512,366	Mating success	0.05	0.01	0.46	downstream	*FAF2*
24	2,986,739	Mating success	0.10	< 0.001	0.04	downstream	*FKBP8*

11	111,251	Attendance	-0.06	0.017	0.30	intron	*FURIN*
7	15,878,140	Centrality	-0.06	0.013	0.26	promoter	*GAL*
12	8,736,017	Centrality	-0.05	0.036	0.36	upstream	*GATA2*
12	8,736,370	Survival	-9.99	0.021	0.79	upstream	*GATA2*
24	6,241,473	Mating success	0.06	0.001	0.17	exon	*GLI1*
24	1,324,651	Attendance	-0.06	0.02	0.30	intron	*GRAMD2B*
13	19,314,324	Attendance	-0.07	0.007	0.19	intron	*GSE1*
10	15,663,765	Centrality	0.04	0.05	0.42	downstream	*HES1-B*
10	15,663,816	Lyre size (next year)	5.47	0.035	0.37	downstream	*HES1-B*
1	85,356,738	Attendance	-0.10	0.002	0.13	promoter	*HMG14*
26	5,691,986	Centrality	-0.13	0.007	0.24	exon	*HMGCL*
22	6,616,045	Attendance	-0.08	0.004	0.14	promoter	*HOXB2*
24	1,105,654	Attendance	-0.09	0.005	0.16	intron	*HSD11B1L*
24	1,105,654	Centrality	0.08	0.028	0.32	intron	*HSD11B1L*
17	3,956,821	Attendance	0.06	0.033	0.40	downstream	*ID1*
25	2,512,503	Lyre size (next year)	-4.66	0.036	0.37	promoter	*JAM3*
25	2,512,626	Mating success	0.07	0.016	0.53	exon	*JAM3*
25	2,512,626	Survival	-6.78	0.029	0.79	exon	*JAM3*
22	5,532,734	Attendance	-0.09	0.005	0.16	intron	*JUP*
14	16,305,631	Centrality	-0.06	0.026	0.30	downstream	*LAMP2*
16	3,179,120	Centrality	-0.11	0.001	0.07	upstream	*LFNG*

6	10,440,648	Attendance	-0.16	0.009	0.21	upstream	*LRRC19*
26	5,443,524	Attendance	0.09	0.012	0.26	upstream	*MACF1*
26	5,443,524	Centrality	-0.08	0.046	0.40	upstream	*MACF1*
26	5,467,315	Centrality	-0.05	0.029	0.32	exon	*MACF1*
24	2,702,734	Attendance	-0.05	0.036	0.40	downstream	*MEF2B*
5	28,610,764	Attendance	0.09	0.016	0.29	exon	*MFHAS1*
5	28,610,764	Centrality	-0.09	0.022	0.29	exon	*MFHAS1*
6	2,881,316	Attendance	-0.03	0.047	0.46	downstream	*NFIA*
6	2,881,497	Attendance	0.04	0.029	0.38	downstream	*NFIA*
24	1,284,876	Survival	-10.5	0.037	0.79	upstream	*NFIC*
24	1,284,881	Survival	-9.14	0.018	0.79	upstream	*NFIC*
24	1,284,955	Attendance	-0.06	0.007	0.19	upstream	*NFIC*
13	14,932,523	Centrality	0.08	0.025	0.30	exon	*NLRC5*
19	5,502,407	Blue chroma (next year)	-0.01	0.031	0.43	exon	*NPLOC4*
23	1,421,756	Attendance	-0.06	0.002	0.13	promoter	*PANK4*
23	1,421,880	Attendance	-0.06	0.001	0.09	promoter	*PANK4*
22	3,652,515	Attendance	0.05	0.05	0.46	upstream	*PLEKHA6*
22	3,652,515	Lyre size (next year)	-7.96	0.014	0.26	upstream	*PLEKHA6*
1	146,148,448	Mating success	0.04	0.015	0.53	intron	*PLXNC1*
12	19,141,977	Centrality	-0.06	0.011	0.25	promoter	*PLXND1*
6	51,134,063	Mating success	-0.08	0.004	0.26	upstream	*PROM1A*

22	9,519,053	Survival	-4.26	0.04	0.79	exon	*PSMD11*
22	9,519,121	Centrality	-0.06	0.011	0.25	exon	*PSMD11*
6	9,950,458	Centrality	0.11	< 0.001	0.05	exon	*PTPRF*
9	21,606,343	Attendance	0.05	0.029	0.38	promoter	*RACGAP1*
9	21,606,328	Blue chroma (next year)	0.01	0.003	0.16	promoter	*RACGAP1*
18	6,910,579	Centrality	-0.06	0.041	0.40	promoter	*RCJMB04_21 H11*
7	25,450,447	Mating success	-0.09	0.003	0.26	exon	*RIOX1*
12	2,799,757	Centrality	0.07	0.043	0.40	promoter	*SEMA3B*
20	5,712,463	Survival	-6.91	0.01	0.79	intron	*SLC25A25*
20	5,712,468	Blue chroma (next year)	0.01	0.048	0.46	intron	*SLC25A25*
22	7,658,401	Attendance	0.09	0.005	0.16	upstream	*SLC25A39*
18	9,922,763	Blue chroma (next year)	0.01	0.039	0.43	promoter	*SMTN*
18	9,922,763	Centrality	-0.06	0.004	0.24	promoter	*SMTN*
19	4,859,312	Attendance	-0.11	0.003	0.13	downstream	*SOCS3*
19	5,590,706	Mating success	0.05	0.05	0.70	downstream	*SOX9*
22	994,621	Centrality	-0.07	0.018	0.28	exon	*SPDEF*
21	8,792,897	Lyre size (next year)	-5.42	0.007	0.23	downstream	*SRSF1*
21	8,793,064	Mating success	-0.13	0.004	0.26	downstream	*SRSF1*

8	50,726,315	Mating success	0.06	0.021	0.59	intron	*SSPO*
2	106,053,468	Lyre size (next year)	-4.19	0.049	0.38	upstream	*SSTR4*
6	9,779,970	Attendance	-0.04	0.047	0.46	downstream	*ST3GAL3*
22	6,261,487	Mating success	0.05	0.017	0.53	intron	*STARD3*
22	6,261,490	Centrality	-0.05	0.01	0.24	intron	*STARD3*
22	6,261,517	Centrality	-0.07	0.046	0.40	intron	*STARD3*
6	8,114,650	Mating success	0.04	0.038	0.70	intron	*TAL1*
6	8,114,811	Survival	-5.75	0.029	0.79	exon	*TAL1*
9	20,852,739	Attendance	0.21	< 0.001	0.06	intron	*TBR1*
9	20,852,739	Centrality	-0.12	0.019	0.28	intron	*TBR1*
11	13,037,331	Centrality	-0.11	0.035	0.36	promoter	*TCF12*
24	161,329	Attendance	0.05	0.035	0.40	exon	*TCF3*
24	161,513	Mating success	0.04	0.043	0.70	intron	*TCF3*
16	4,916,072	Attendance	0.04	0.045	0.46	intron	*TOM1L2*
17	3,936,611	Blue chroma (next year)	-0.01	0.038	0.43	promoter	*TPX2-B*
6	29,617,149	Attendance	0.09	0.003	0.13	promoter	*TUBGCP2*
6	29,617,149	Centrality	-0.08	0.025	0.30	promoter	*TUBGCP2*
22	7,638,774	Attendance	0.03	0.044	0.46	downstream	*UBTF*
22	7,638,774	Centrality	-0.05	0.009	0.24	downstream	*UBTF*

22	7,638,786	Blue chroma (next year)	-0.01	0.010	0.26	downstream	*UBTF*
22	5,929,242	Attendance	0.04	0.025	0.38	intron	*WNK4*
22	5,929,242	Centrality	-0.05	0.021	0.29	intron	*WNK4*
27	3,240,038	Attendance	-0.05	0.043	0.46	intron	*XPO7*
27	3,239,941	Blue chroma (next year)	-0.01	0.039	0.43	intron	*XPO7*
27	3,239,995	Lyre size (next year)	-5.78	0.015	0.26	intron	*XPO7*
24	1,067,454	Attendance	0.08	0.014	0.28	intron	*ZBTB7A*
24	1,067,454	Centrality	-0.09	0.005	0.24	intron	*ZBTB7A*
26	3,802,592	Attendance	-0.09	0.001	0.09	exon	*ZC3H12A*
13	10,836,664	Attendance	0.05	0.044	0.46	downstream	*ZNF423*
13	10,836,664	Centrality	-0.06	0.013	0.26	downstream	*ZNF423*
13	18,640,882	Centrality	-0.07	0.010	0.24	promoter	*ZNF469*
4	37,107,500	Attendance	0.10	0.016	0.29	downstream	Unnotated
5	12,092,564	Attendance	0.06	0.003	0.13	promoter	Unnotated
5	12,092,564	Centrality	-0.04	0.030	0.33	promoter	Unnotated
5	50,969,477	Attendance	-0.06	0.026	0.38	TSS	Unnotated
11	18,733,797	Mating success	0.05	0.022	0.60	intron	Unnotated
11	18,733,819	Mating success	0.07	0.020	0.59	intron	Unnotated
8	2,890,848	Mating success	0.07	0.050	0.70	exon	Unnotated

9	4,520,683	Survival	-7.47	0.043	0.79	exon	Unnotated
10	9,236,234	Mating success	-0.07	0.011	0.46	exon	Unnotated
5	34,123,180	Centrality	0.09	0.036	0.36	downstream	Unnotated
24	1,606,325	Mating success	-0.06	0.031	0.70	promoter	Unnotated
24	1,606,325	Centrality	0.07	0.012	0.26	promoter	Unnotated
24	1,606,364	Attendance	0.07	0.047	0.46	promoter	Unnotated
16	5,045,219	Attendance	-0.07	0.035	0.40	TSS	Unnotated

Supplementary Table S6. **Biological processes identified using gene ontology (GO) enrichment analysis of CpG sites associated with reproductive investment.** E is the enrichment score, B is the number of genes in the input list associated with the GO term and b is the number of genes in the input list associated with the term.

GO term	Description	*p*	FDR *q*	E	B	b
GO:0048522	positive regulation of cellular process	2.18 e^{-4}	1.00	1.47	145	43
GO:0048518	positive regulation of biological process	2.38 e^{-4}	0.56	1.44	155	45
GO:0045944	positive regulation of transcription by R polymerase II	3.36 e^{-4}	0.53	2.05	46	19
GO:0031328	positive regulation of cellular biosynthetic process	4.70 e^{-4}	0.55	1.84	62	23
GO:0009891	positive regulation of biosynthetic process	4.70 e^{-4}	0.44	1.84	62	23
GO:0051239	regulation of multicellular organismal process	5.51 e^{-4}	0.43	1.64	91	30
GO:0051254	positive regulation of R metabolic process	8.02 e^{-4}	0.54	1.82	60	22
GO:1903508	positive regulation of nucleic acid-templated transcription	8.02 e^{-4}	0.47	1.82	60	22
GO:0045893	positive regulation of transcription, D-templated	8.02 e^{-4}	0.42	1.82	60	22
GO:1902680	positive regulation of R biosynthetic process	8.02 e^{-4}	0.38	1.82	60	22

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