Sex-specific changes in autosomal methylation rate in ageing common terns

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Abstract

Senescence, an age-related decline in survival and/or reproductive performance, occurs in species across the tree of life. Molecular mechanisms underlying this within-individual phenomenon are still largely unknown, but DNA methylation changes with age are among the candidates. Using a longitudinal approach, we investigated age-specific changes in autosomal methylation of common terns, relatively long-lived migratory seabirds known to show senescence. We collected blood at 1-, 3- and/or 4-year intervals, extracted DNA from the erythrocytes and estimated autosomal DNA methylation by mapping Reduced Representative Bisulfite Sequencing reads to a new reference genome. We found autosomal methylation levels to decrease with age within females, but not males, and no evidence for selective (dis)appearance of birds of either sex in relation to their methylation level. Moreover, although we found positions in the genome to consistently differ in their methylation levels, individuals did not show such strong consistent differences. These results pave the way for studies at the level of genome features or specific positions, which should elucidate the functional consequences of the patterns we observe, and how they translate to the ageing phenotype.

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Abstract

Senescence, an age-related decline in survival and/or reproductive performance, occurs in species across the tree of life. Molecular mechanisms underlying this within-individual phenomenon are still largely unknown, but DNA methylation changes with age are among the candidates. Using a longitudinal approach, we investigated age-specific changes in autosomal methylation of common terns, relatively long-lived migratory seabirds known to show senescence. We collected blood at 1-, 3- and/or 4-year intervals, extracted DNA from the erythrocytes and estimated autosomal DNA methylation by mapping Reduced Representative Bisulfite Sequencing reads to a new reference genome. We found autosomal methylation levels to decrease with age within females, but not males, and no evidence for selective (dis)appearance of birds of either sex in relation to their methylation level. Moreover, although we found positions in the genome to consistently differ in their methylation levels, individuals did not show such strong consistent differences. These results pave the way for studies at the level of genome features or specific positions, which should elucidate the functional consequences of the patterns we observe, and how they translate to the ageing phenotype.

Keywords: aging, avian senescence, epigenetics, ontogeny, RRBS,

Sterna hirundo

Introduction

Senescence is a within-individual decline in survival probability (actuarial senescence) and/or reproductive performance (reproductive senescence) with age. Although the rate and shape of the decline vary both among and within species, this detrimental process occurs in species across the tree of life (Shefferson *et al.* 2017). It is hypothesized to have evolved because unavoidable extrinsic mortality reduces the strength of selection against poor performance with age (Fisher 1930; Medawar 1952; Williams 1957; Hamilton 1966). From a genetic point of view, this so-called 'selective shadow' could allow for the accumulation of late-acting deleterious mutations over evolutionary time (mutation accumulation, Medawar 1952), or for selection favouring alleles with beneficial effects early, but detrimental effects late in life (antagonistic pleiotropy, Williams 1957). Moreover, early-life investment of limited resources in reproduction over that in perfect somatic maintenance and repair would also be evolutionarily beneficial, such that senescence could be a 'best-of-a-bad-job' consequence of accumulated, unrepaired damage (disposable soma, Kirkwood 1977).

Although the classical theories of mutation accumulation and antagonistic pleiotropy assume a purely sequence-level genetic basis to senescence, the disposable soma theory does not. Given its firm foundation in life history theory, resource acquisition and allocation (*sensu* van Noordwijk & de Jong 1986) are important facets to consider. Both are known to show phenotypic plasticity in response to environmental variation (e.g.

Erikstad *et al.* 1998; Descamps *et al.* 2016), and experimental manipulation of one of these facets without adjustment of the other is known to affect rates of senescence (e.g. Boonekamp*et al.* 2014). As such, part of the senescence process is also expected to be underpinned by more flexible, regulatory processes (Wilson *et al.* 2008). In line with this, numerous studies across taxa have shown that senescence is underpinned by both genetic and epigenetic processes (Sen *et al.* 2016; Melzer *et al.*2020).

Epigenetic processes are those that affect the regulation of gene expression (Holliday 2006). This regulation is complex and encompasses several mechanisms, a major one of which is DNA methylation, the addition of a methyl-group to the fifth carbon site of a cytosine in a CpG (5'-C-phosphate-G-3') dinucleotide context (Jaenisch & Bird 2003; Miranda & Jones 2007; Suzuki & Bird 2008). Age-specific DNA methylation has been described for many model species (e.g. Maegawa*et al.* 2010; Hannum *et al.* 2013; Tharakan *et al.*2020), and

the methylation status of specific CpGs has been shown to be a powerful predictor of both chronological and biological age, i.e. to function as an epigenetic clock (Bocklandt *et al.* 2011). Using a cross-sectional analytical approach, epigenetic clocks have meanwhile been characterized for a variety of organisms, including humans (Weidner*et al.* 2014), dogs and wolves (Thompson *et al.* 2017) and cetaceans (Robeck *et al.* 2021). Importantly and additionally, a rare longitudinal study following the same set of human twins across 10 years, revealed (i) the methylation of CpG sites to change with age*within subjects* , (ii) this change to mostly occur in gene-sets involved in ageing-related degenerative disorders, and (iii) most of the change to be explained by individual-specific environmental factors rather than inherited (genetic) factors (Tan *et al.* 2016). Although age-specific methylation seems the norm at least in model organisms, the direction of methylation changes with age is harder to predict: both passive non-directional changes (representing epigenetic drift, Fraga *et al.* 2005; Tan *et al.* 2016), and active directional changes (hyper- and hypomethylation, Zampieri *et al.*2015; Ciccarone *et al.* 2018; but see Unnikrishnan *et al.*2019) have been reported.

Research on senescence, including its epigenetic underpinning, has so far mostly focused on humans or model organisms kept under controlled laboratory conditions. Extending the taxonomic range and incorporating field studies is crucial to understand the evolutionary ecology of senescence (Monaghan *et al.* 2008). For this extension, birds are an especially interesting taxon. They have longer life spans relative to their body size than mammals (e.g. Lindstedt & Calder 1976), and various populations of birds with vastly different life histories have been studied over several decades, such that many basic insights into their senescence patterns can now be obtained (Bouwhuis & Vedder 2017). Bird genomes are rather compact, show high levels of synteny across species (Zhang *et al.* 2014), and, similar to those of other vertebrates, are globally methylated (Suzuki & Bird 2008; Li *et al.* 2011, Sepers *et al.* 2019). With respect to age-specific DNA methylation in birds, we are aware of the existence of an epigenetic clock for short-tailed shearwaters (*Ardenna tenuirostris* ; De Paoli Iseppi *et al.* 2019), and of findings of early-life within-individual increases in global methylation in both great tit (*Parus major* ; Watson *et al.* 2019) and zebra finch (*Taeniopygia guttata* ; Sheldon *et al.* 2020) nestlings. However, we are not aware of any study reporting age-specific DNA methylation patterns in avian adulthood, or in avian late-life specifically.

When studying age-specific trait expression, many studies, including most of those producing epigenetic clocks or otherwise studying age-specific differences in DNA methylation patterns, use cross-sectional samples and analysis tools. Patterns revealed by cross-sectional approaches, however, are the result of a combination of within- and among-individual processes. If we aim for understanding the within-individual process of senescence, we need to account for the effect of compositional changes of a population, for example when birds with a certain level of DNA methylation are more likely to die and selectively disappear from the study population (e.g. Vaupel & Yashin 1985; Forslund & Part 1995). Mixed-effect models applied to (partly) longitudinal data to specifically test whether within-individual patterns and population-level patterns are the same, or differ, are a powerful analytical tool to do so (van de Pol & Wright 2009). Across taxa, efforts have increasingly been made to validate and complement findings from cross-sectional analyses of senescence through longitudinal studies (Nussey *et al.* 2008; Gaillard *et al.*2017), and this methodological turn is also reflected in studies of human DNA methylation (Bollati *et al.* 2009; Tan *et al.*2016). Longitudinal studies of DNA methylation and ageing in model species and natural populations are, however, missing and needed (Bell*et al.* 2019).

Here, we report on a longitudinal study of autosomal methylation levels in the common tern. Common terns are long-lived migratory seabirds whose patterns of senescence have been the topic of various studies. Although breeders of both sexes show little sign of reproductive senescence - they breed earlier in the year and fledge more offspring as they grow older (Nisbet *et al.* 2002; Zhang *et al.* 2015c; Nisbet*et al.* 2020) - breeding and survival probabilities are known to decline with age (Zhang *et al.* 2015b; Vedder *et al.*2021b). In addition, there is evidence for sex-specific transgenerational senescence, with daughters of older mothers and sons of older fathers suffering from reduced lifetime reproductive success (Bouwhuis *et al.* 2015). Studies aiming to identify a molecular basis for these within- and transgenerational effects have so far focused on telomere dynamics, and found that: (i) telomeres shorten with age (Bichet *et al.* 2020); (ii) telomere length is genetically correlated with lifespan (Vedder *et al.* 2021a); and (iii) paternal age is negatively correlated

with offspring telomere length (Bouwhuis *et al.* 2018). The explanatory power of telomere length, however, is very low for all of these patterns (e.g. 1.1% of phenotypic variation in lifespan is explained by additive genetic variation in telomere length, Vedder *et al.* 2021a), such that additional mechanisms are expected. To evaluate whether age-specific changes in global DNA methylation could be such a mechanism, we sequenced and*de novo* assembled a chromosome-scale high-quality reference genome and used it to compare within-individual age-specific changes in DNA methylation at shared sites across the genome. Although rates of within-generational senescence do not differ between the sexes (Zhang*et al.* 2015a), transgenerational effects are known to be sex-specific (Bouwhuis *et al.* 2015), such that we also considered sex-specificity of any patterns in autosomal methylation status.

Materials and Methods

Study population

The data we present were collected as part of a long-term individual-based study of a mono-specific common tern colony located on six artificial concrete islands at the Banter See (53°36'N, 08deg06'E) in Wilhelmshaven, Germany. Fledglings from this colony have been marked with metal rings since 1984, and subcutaneously injected with transponders since 1992 (Becker & Wendeln 1997). This transponder-marking combined with placement of antennae on elevated platforms on the edges of the colony site and around each nest during incubation (shared between both parents), allows for a well-described family structure of all philopatric birds (e.g. Moiron *et al.*2020).

Reference genome

To create the chromosome-scale reference genome, blood of one adult female common tern was collected in 100% EtOH and stored at -80degC. Four sequence datasets were generated following the VGP 1.5 pipeline (Rhie*et al.* 2021): 67.91x Pacific Biosciences (Pacbio) continuous long reads (CLR); 698.35x Bionano Genomics optical maps; 169.30x 10X Genomics linked-reads; and 79.62x Arima Hi-C Illumina reads.

Briefly, 30µg of High Molecular Weight DNA (HMW DNA) was isolated from the whole blood sample using a modified (for avian nucleated erythrocytes) agarose plug protocol of the Bionano Prep Blood and Cell Culture DNA Isolation Kit (cat no. RE-130-10). Lysates were embedded into agarose plugs, followed by Proteinase K and RNase A treatments and 1X TE drop dialysis purification. To create the Pacbio data, DNA was sheared using a 26G blunt end needle (Pacbio protocol PN 101-181-000 Version 05) to approximately ˜40kb fragment length. We used 10µg of this fragmented DNA to generate a large-insert Pacbio library using the Pacific Biosciences Express Template Prep Kit v1.0 (#101-357-000). The library was then size selected (>15kb) using the BluePippin system (Sage Science). The resulting PacBio Library was sequenced on 10 PacBio 1M v3 smrtcells (#101-531-000) on a Sequel instrument with the sequencing kit 3.0 (#101-427-500) and a 10 hours movie with 2 hours pre-extension time. Unfragmented HMW DNA was used to generate a linked-read library on the 10X Genomics Chromium (Genome Library Kit & Gel Bead Kit v2 PN-120258, Genome Chip Kit v2 PN-120257, i7 Multiplex Kit PN-120262). We sequenced this 10X library on an Illumina Novaseq S4 150bp PE lane. uHMW DNA was labeled for Bionano Genomics optical mapping using the Bionano Prep Direct Label and Stain (DLS) Protocol (30206E) and 1 flow cell was run on the Saphyr instrument. Hi-C libraries were generated with the Arima Genomics v1.0 2-enzyme protocol (P/N: A510008), according to the manufacturer's protocol and sequenced on Illumina HiSeq X.

The resulting four data types were processed using the VGP v1.5 pipeline (Rhie *et al.* 2021), which includes: assembling Pacbio contigs using *FALCON v2018.31.08-03.06* ; *FALCON-Unzip v6.0.0.47841* ; purging false haplotype duplications with *purge_haplotigs v1.0.3+ 1.Nov. 2018* ; scaffolding with 10X with *scaff10x v4.1.0;*scaffolding with *Bionano Solve DLS v3.2.1* ; scaffolding with Hi-C data with *Salsa HiC v2.2* ; filling in gaps and polishing for base call accuracy with CLR and *Arrow smrtanalysis v6.0.0.47841* ; and polishing with Illumina short reads with longranger *align v2.2.2* ; and *freebayes v1.3.1* . The resulting assembly was then manually curated to fix any errors, using *gEVAL* and Hi-C short read linked-read mapping profiles as described in Howe et al. (2021).*BUSCO v4.1.4* with the bird lineage dataset (*aves_odb10*) was used to assess

assembly completeness. The reference genome was submitted to NCBI with the following accession number: GCA_009819605.1, as part of the Vertebrate Genome Project (VGP) (https://vgp.github.io/genomeark). Our reference genome has GC-rich promoter regions due to the use of Pacbio long reads that get through them (Rhie *et al.* 2021; Kim *et al.* 2021). Reference genomes consisting of short-read assemblies (e.g., using Illumina reads) exhibit GC bias, where GC-rich regions such as promoters could be incorrectly assembled or even missing (Kim *et al.* 2021).

Methylation sequencing

Blood of 34 adults of 17 breeding pairs was sampled in the years 2013 (N=16), 2014 (N=32) and 2017 (N=26) using larval stages of the bloodsucking bug *Dipetalogaster maximus.* Bugs were placed into dummy eggs with holes through which they could stick out their mouth pieces and placed in the nests of incubating focal birds (Becker*et al.* 2006; Arnold *et al.* 2008; Bichet *et al.*2019). After 20-30 minutes, "bug eggs" were collected and the focal birds' blood, sucked by the bug, was removed from the bugs' abdomen using a syringe. Upon collection, whole blood was stored in EDTA buffer (2%) in a fridge (3-7°C) for up to 3 weeks, before red blood cells were transferred to glycerol buffer (40%) and frozen at -80° C. Each bug was used only once to prevent contamination of blood samples.

Genomic DNA was extracted and libraries for RRBS were generated as described in Klughammer *et al.* (2015), following standard steps such as MspI digestion, end-fill-in, A-tailing and size selection. This included the enrichment of the libraries with Pfu-Turbo Cx Hotstart DNA polymerase to allow the assessment of the bisulfite conversion rate. After clean-up and quality control, libraries were sequenced with an Illumina HiSeq 4000 (50bp SE).

Methylation data selection

Across all 74 samples, individuals were sequenced with an average of 51,482,011 (range: 24,942,071 - 82,760,425) reads. After conversion of unmapped bam files to fastq with *SamToFastq.jar* from*picard v1.118* , the quality of the sequencing reads was checked using *Fastqc v0.11.5* (Andrews 2010) and *Multiqc v1.8* (Ewels *et al.* 2016). Spiked-in sequences were used to estimate over- and underconversion using *RefFreeDMA* (Klughammer*et al.* 2015). *Trimgalore* v0.3.3 (implemented in*RefFreeDMA;* Krueger *et al.* , 2021) was used to remove adaptors to guarantee good mapping and remove low quality bases ([?]20) as well as short read fragments ([?] 16 bp).

We used *Bismark v0.22.3* (Krueger & Andrews 2011) to prepare and index the reference genome for subsequent mapping of the bisulfite-converted reads using *Bowtie 2 v2.4.1* (Langmead & Salzberg 2012), allowing for one mismatch (–score_min L,0,-0.20). Methylation extraction was conducted with *Bismark Extractor v0.22.3* (Krueger & Andrews 2011) with the 'ignore' option to remove unmethylated cytosines introduced during the end-repair step. The R-package *Methylkit v.1.16.1* in R *v.4.0* (Akalin *et al.* 2012; R Core Team, 2021) and its function *methRead* were used to load and analyse the methylation calls.

Methylation calls were filtered to those with a minimum coverage of 10 reads per CpG position. CpGs with a coverage >99.9th percentile most likely result from PCR bias and were removed. Across all 74 samples, there were on average 6,361,176 CpG positions before, and 539,538 CpG positions after, filtering for >10x coverage. Methylation call distributions between samples were normalized using the*normalizeCoverage* function implemented in *Methylkit* to reduce the bias of systematic oversampling in some samples. We then merged CpG positions of the different samples using the *unite*function to make sure positions were sequenced in at least 70% (N=52) of the samples to facilitate a longitudinal analysis approach. This also meant we only covered CpG positions on autosomes, as CpGs from the sex chromosomes were excluded. Following Meng *et al.* (2010) and Sziraki *et al.* (2018), sites that showed little or no variation were removed as well, applying a threshold of a standard deviation <0.1. This resulted in 1,365,573 observations of 23,647 sites, used to determine methylation levels as the fractional methylation rate per CpG position per individual.

Statistical analyses

To identify and partition sources of variation in autosomal methylation levels, we used the R package

glmmTMB (Brooks *et al.* 2017) to run a generalized linear mixed model (GLMM) with the BFGS algorithm as an optimizer, using the number of methylated and unmethylated Cs (represented by the sequenced Cs and Ts) for each position as our dependent variable, assuming a binomial error distribution (Lea *et al.* 2017). As fixed effects we added sex (as a two-level class variable using males as the reference category) and age (as a covariate). With respect to the latter, each individual's age was partitioned into an 'average age' and 'delta age' component following van de Pol & Wright (2009). Average age was calculated as the average of all ages at which we assessed a bird's autosomal methylation level, while delta age was calculated as the difference between the bird's actual age and its average age (i.e. delta age = age – average age). When adding both age variables as covariates, average age represents the among-individual, and delta age the within-individual age effect (van de Pol & Wright 2009). If the among- and within-individual age effects were to differ, this would indicate that the effect of age among individuals cannot be explained by changes within individuals, revealing age-specific selective (dis)appearance of individuals with certain levels of methylation (van de Pol & Wright 2009). We additionally included the interaction between sex and delta age in our model to test for sex-differences in the within-individual age trajectory of methylation. Random effects included were 'bird identity', genomic position ('position identity') and an observation-level random effect ('observation'). The latter was added to account for overdispersion, which was detected using the R package *performance* (Ludecke*et al.* 2021), whereas the first two were included to account for pseudoreplication caused by repeated sampling of individuals and genomic positions, respectively. Model evaluation and summary of parameter estimates and statistics were conducted using the R package*parameters* (Ludecke *et al.* 2020). The marginal effects of the interaction were plotted using the R-package *sjplot* test (Ludecke 2018).

Results

Reference genome

The reference genome was generated using 68x PacBio sequencing reads, Bionano Genomics optical maps, 10X Genomics linked-reads and Arima Hi-C reads. This allowed us to scaffold the assembly to chromosome-level (Rhie *et al.* 2021) and we successfully assigned 99.3% of the assembled sequence to 25 identified autosomes, two sex chromosomes and the mitochondrial genome, leaving only 95 scaffolds unlocalised. The total length of the primary haplotype assembly was 1.23 Gbp with a contig N50 of 22.0 Mb and a scaffold N50 of 85.5 Mb. It included 96% complete assembled single copy genes according to BUSCO analysis, with only 1.3% fragmented, 0.4% falsely duplicated, and 2.3% missing (n = 8,338 genes). This represents a high-quality assembly, surpassing the aspired VGP contiguity metrics ˜20-fold (Rhie*et al* 2021).

Age-specific global methylation

Across the 74 samples, 29,044,662 RRBS reads (˜ 56% mapping efficiency) could be uniquely mapped to the reference genome. The mean bisulfite conversion rate was 99.2%. When analyzing sources of variation in methylation rates across the 23,647 CpG positions occurring in at least 70% of samples, we found a significant interaction between sex and delta age (Table 1). This interaction showed that methylation rates declined with age within individual females, but did not change with age within individual males (Figure 1). The effect of average age was non-significant and the credible intervals of the average and delta age components strongly overlapped (Table 1), suggesting no selective (dis)appearance of individuals based on their autosomal methylation rates. When assessing the random effects, most variance was explained by position identity (Table 1), showing that genomic positions differ in their average level of methylation. Bird identity explained variation in methylation rates to a much lesser extent (Table 1), such that there is little evidence for consistent differences in methylation among individuals.

Discussion

DNA methylation patterns at CpG sites are increasingly used as biomarkers, so-called epigenetic clocks, to predict both chronological and biological age across species and taxa (e.g. Lu *et al.* 2021). How DNA methylation changes within individuals and whether it can explain phenotypic senescence patterns, however, is still largely unknown (Bell *et al.* 2019). Here, we used blood samples from common terns collected at 1-, 3-

and/or 4-year intervals and a longitudinal analysis approach to investigate whether autosomal methylation levels changed with age within individual birds, and whether any change differed between males and females sharing environments and broods. We found female genomes to become less methylated as these females aged, whereas there was no such age-specific decline of autosomal methylation in males. Moreover, we found the estimates for the within- and among-individual components of age to be similar, such that there was no indication for selective (dis)appearance of individuals based on their methylation pattern. Finally, we provide evidence for positions in the genome to consistently differ in their methylation levels, whereas evidence for consistent differences among individuals was considerably less.

Our finding of female common terns showing a decrease in methylation as they aged fits with findings of global hypomethylation in older compared to younger mammals (Zampieri *et al.* 2015; Ciccarone *et al.*2018; Sziraki et al. 2018; but see Unnikrishnan *et al.* 2019). Such methylation loss is thought to partly originate from demethylation of large regions of repetitive sequences, CpG-poor promoters or large hypomethylated blocks of "open sea" regions outside the CpG islands (Bollati *et al.* 2009; Heyn *et al.* 2012; Yuan *et al.*2015). Whether these changes affect chromatin configuration and thus genome (in)stability, and whether changes in promoter methylation interact with histone modifications and transcription factors to alter expression remains to be investigated (Zampieri *et al.* 2015; Ciccarone *et al.* 2018).

Male terns, in contrast, showed no signs of decreased methylation as they grew older. Although many studies developing epigenetic clocks have assumed age-related changes to be similar across the sexes and used mixed-sex datasets to obtain them (e.g. Raj *et al.* 2021; Horvath*et al.* 2021), others have found sex-differences in these clocks (e.g. in some human ethnicities (Horvath *et al.* 2016), baboons (Anderson *et al.* 2021) or elephants (Prado *et al.* 2021)). Moreover, a rare longitudinal study in a wild population of roe deer also showed sex-specific epigenetic clock regions, with an accelerated ageing signal in males, which are known to undergo stronger survival senescence in this species (Lemaitre *et al.* 2021). Combined with our findings, this suggests that methylation tests for sex-specificity should best be the norm.

Interestingly, male and female terns from our study population do not differ in the onset or rate of senescence in survival or breeding probabilities (Zhang *et al.* 2015b; Vedder *et al.* 2021b), such that sex-specificity in the ageing process is only found in how parental age affects the quality of the offspring that recruit back into the population (with maternal age negatively affecting the reproductive performance of daughters and paternal age negatively affecting survival of sons (Bouwhuis *et al.* 2015)). As such, we did not necessarily expect sex differences in the age-specificity of the birds' autosomal methylation level. The fact that we were able to observe them, raises the question of which site-specific methylation patterns drive the pattern observed on the global level. As mentioned above, global loss is thought to originate from the demethylation of specific regions: repetitive sequences, CpG-poor promoters or large hypomethylated blocks of "open sea" regions outside the CpG islands (Bollati *et al.*2009; Heyn *et al.* 2012; Yuan *et al.* 2015). CpG island promoters, on the other hand, have been found to show age-specific increases in methylation (Heyn *et al.* 2012; Day *et al.*2013). As such, global demethylation may perhaps be compensated for by such increases in males, but not females. In combination with the fact that we found genomic positions to consistently differ in methylation levels, this stresses the need for moving from the level of global autosomal methylation assessment that we and others (e.g. Watson*et al.* 2019; Sheldon *et al.* 2020) have started with, to fully annotating the (common tern) genome and studying patterns across genomic features and focal sites.

Besides finding a sex-specific within-individual change in autosomal methylation level with age, and significant among-position consistency in methylation level, we found little evidence for consistent among-individual levels of autosomal methylation across years, or for selective (dis)appearance of birds in relation to their methylation level. This suggests that birds differentially change their autosomal methylation from year to year, with these changes perhaps reflecting their individual-specific condition or environment, but not relating to their recruitment as a breeder in the study population or their local survival. Implementing a random regression analytical framework is data-hungry and not possible with our current dataset, but linking among-year changes in e.g. body mass or other measures of physiology seems a promising research avenue, especially when taking the analyses to a site-specific level, such that distinct genotype-methylation-phenotype

correlations can be identified.

The strength of our study lies in its longitudinal sampling and analytical approach, which allows us to characterise within-individual changes, rather than infer them from cross-sectional data. At the same time, however, this sampling approach may come with some limitations. Non-destructive, longitudinal sampling in natural populations often relies on using blood as the focal tissue, but how DNA methylation of (in our case) erythrocytes translates to phenotypes mostly remains an open question that needs addressing (Husby 2020), ideally in experimental study systems. Moreover, because we used RRBS, a high-throughput and low-cost method, to assess methylation, it is important to realise that this method introduces biases towards high density CpG regions (Smith *et al.* 2009; Gu *et al.* 2011); we have focally answered the question of what happens at CpG islands during ageing (Beck *et al.* 2021). Keeping this in mind, however, our study has provided evidence for a sex-specific within-individual change in autosomal methylation with age and shown that different positions in the genome are consistently differentially methylated, such that future work, changeing the perspective from genome-wide average estimates to the specific genome feature or base pair level, can elucidate whether, where and how much methylation might affect ageing males and females, as such establishing a longitudinal epigenetic clock.

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Data accessibility

The common tern reference genome we developed (bSteHir1) is accessible in NCBI (https://www.ncbi.nlm.nih.gov/bioproject/560234), as well in Genome Ark (https://vgp.github.io/genomeark/Sterna_hirundo/). All RRBS data will be deposited into the public database at ENA https://www.ebi.ac.uk/ena. The final dataset and our R code will be uploaded to Dryad upon acceptance of the manuscript.

Author Contributions

SB collected the blood samples and life history data on the terns, ML and SB designed the study and ML organized sequencing of the samples. BSM analyzed the methylation data and performed the statistical analyses with the help of MM. The reference genome was generated as part of the Vertebrate Genome Project, coordinated by EDJ. OF coordinated the genome assembly; BH and JM were responsible for sample processing and generated the genome sequence data; CC, GF and MU-S for the genome assembly; WC, JW and KH curated the final reference genome. BSM and SB wrote the manuscript with input from ML, MM and EDJ. All authors commented on drafts of the manuscript.

Tables and Figures

Table 1. Result of a Generalised Linear Mixed Model with binomial error distribution testing whether variation in autosomal methylation level is explained by sex (males as a reference category) and the between- (average age) and within-individual (delta age) components of age. Provided are parameter estimates and associated 95% credible intervals (95% CI). Significant effects (p-value < 0.05 and 95% CI which do not overlap with zero) are presented in bold.

parameter	estimate	95% CI	z	p
fixed effects				
intercept	-0.131	**-0.226, -0.035**	-2.677	**0.007**
sex	-0.036	-0.100, 0.029	-1.086	0.277
average age	0.000	-0.007, 0.007	0.093	0.926
delta age	0.001	-0.002, 0.003	0.514	0.607
sex:delta age	-0.004	**-0.008, -0.001**	-2.399	**0.016**
random effects				
bird identity	0.095	0.074, 0.121		
position identity	2.739	2.714, 2.764		

parameter	estimate	95% CI	z	p
observation	1.263	1.26, 1.265		

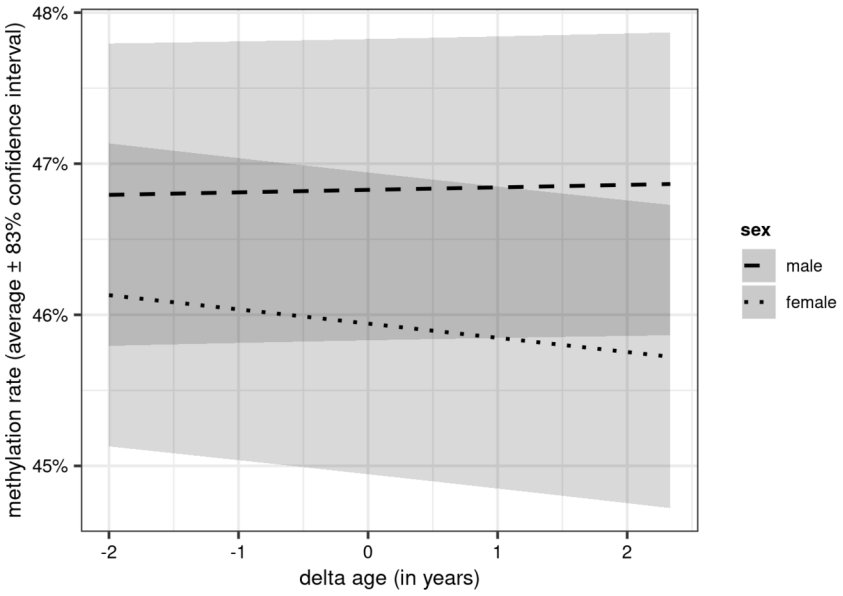
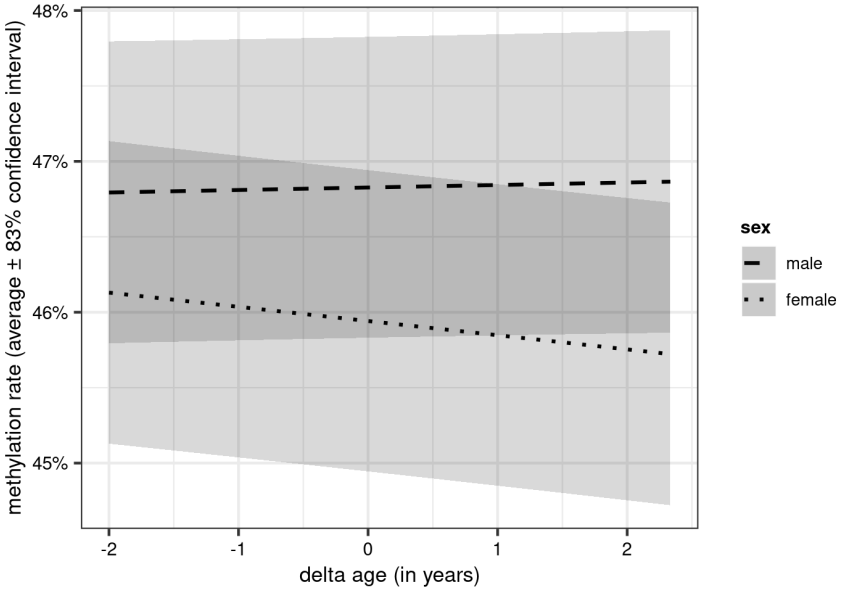


Figure 1. Autosomal DNA methylation rate declines with age within individual female, but not male, common terns. Lines represent model predictions, grey areas the 83% credible interval.



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`Table1.xlsx` available at https://authorea.com/users/462472/articles/557893-sex-specific-changes-in-autosomal-methylation-rate-in-ageing-common-terns