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DNA Methylation Dynamics Reflect Sex and Status Differences in Mortality Rates in a Polygynous Bat

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ABSTRACT

Males of polygynous mammals often do not live as long as females and, in some cases, exhibit evidence of earlier senescence. Patterns of DNA methylation (DNAm) have recently been used to predict chronological age in mammals. Whether DNAm also changes as a consequence of survival and senescence is largely untested in wild animals. In this study, we estimate mortality rates using recaptures of 2700 greater spear-nosed bats, *Phyllostomus hastatus*, over 34 years and DNAm profiled for over 300 adult bats. In this species, one male typically controls mating access to a group of unrelated females. Bayesian analysis reveals that mortality risk in males is 1.8 times that of females, and comparison of age-associated differences in DNAm indicates that DNAm changes 1.4 times faster in males than females. Therefore, even though the age of either sex is predicted by a common set of sites, the methylome of males is more dynamic than that of females. Sites associated with sex differences in the rate of DNAm change are sensitive to androgens and enriched on the X chromosome. Sites that exhibit hypermethylation are enriched in promoters of genes involved in the regulation of metabolic processes. Unexpectedly, subordinate males have higher mortality rates than reproductively dominant males and exhibit faster DNAm change than dominants at dozens of sites. Our results reveal that differences in mortality associated with sex and social status are reflected by changes in DNA methylation, providing novel insights into mechanisms of aging and mortality in this and likely other wild animal populations.

1 | Introduction

Recent studies have revealed that patterns of DNA methylation (DNAm) in mammalian cells change predictably with age (Horvath and Raj 2018; Lu et al. 2023). Typically, over 75% of cytosines in cytosine-phosphate-guanine (i.e., CpG) sites are methylated in an individual, but DNAm tends to decline with age across the genome (Unnikrishnan et al. 2018). However, at some sites, especially those in CpG-dense promoter regions, i.e., CpG islands (Day et al. 2013), DNAm increases with age in a clocklike manner. A series of studies, first conducted in humans (Hannum et al. 2013; Horvath 2013), but then in many other mammals (e.g., Lemaitre et al. 2022; Lu et al. 2023; Nakamura et al. 2023; Polanowski et al. 2014; Stubbs et al. 2017; Tangili et al. 2023; Thompson et al. 2017; Wilkinson et al. 2021), have shown that chronological age can be accurately predicted from the proportion of DNA that is methylated across a set of conserved sites. While different tissues show different DNAm aging patterns, e.g., those with proliferative versus differentiated cells (Lu et al. 2023; Moqri et al. 2024), age can be predicted with a single or "universal" epigenetic clock using DNA from any mammalian tissue (Lu et al. 2023). This result indicates that

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mammals undergo a common aging process that can be detected within their cells (Horvath and Raj 2018).

Environmental factors can, however, influence the rate of DNAm change and either accelerate or decelerate epigenetic aging. For example, calorie restriction, which increases lifespan in mice and primates, decelerates epigenetic aging (Cole et al. 2017; Maegawa et al. 2017). Similarly, hibernation in yellow-bellied marmots (Pinho et al. 2022) and bats (Sullivan et al. 2022) also slows epigenetic aging and is associated with extended lifespan (Al-Attar and Storey 2020; Turbill et al. 2011). Sex differences in lifespan are also commonly observed in mammals (Lemaitre et al. 2020). In humans, sex also influences epigenetic aging in some groups (Horvath et al. 2016; Yusipov et al. 2020). Determining if sex, social dominance, or other ecological factors predictably alter the methylome in wild animals could provide insight into mechanisms underlying life history differences within and between species. For example, studies in olive baboons (*Papio cynocephalus*) have demonstrated that dominant males show accelerated aging compared to subordinate males (Anderson et al. 2021) and that methylation profiles can be altered by diet (Lea et al. 2016) and early life adversity (Anderson et al. 2024). Sex differences in aging have also been reported for roe deer (Lemaitre et al. 2022) and sheep, where castration delays epigenetic aging in males (Sugrue et al. 2021) as a consequence of reduced androgen exposure (Sugrue et al. 2025).

Bats are of particular interest for studies of epigenetic aging because some species can live longer, relative to their body size, than any other mammal (Austad and Fischer 1991). How they survive so long is actively under investigation. Many, although not all, of the most long-lived species undergo hibernation (Wilkinson and Adams 2019). Several studies have also found evidence of adaptive changes in bat immune systems that may contribute to their unusual longevity (Gorbunova et al. 2020). These range from gene losses and gains that likely influence inflammasome responses (Moreno Santillan et al. 2021; Scheben et al. 2023) to differences in the rate of DNAm change between long-lived and short-lived species, in which short-lived species tend to exhibit faster rates of change (Wilkinson et al. 2021). Bat mating systems vary in the degree to which males compete for access to females, either before mating or via sperm competition (Wilkinson and McCracken 2003), and in many species social groups occur. The social environment has been linked to lifespan in humans and other mammals (Ellis et al. 2019; Snyder-Mackler et al. 2020), but not yet in bats.

Greater spear-nosed bats, *Phyllostomus hastatus*, provide an attractive system for investigating if DNA methylation change is influenced by mortality rates because they exhibit extreme polygyny. Males compete for control of stable groups of 15–25 females, and one dominant individual, referred to as the harem male, defends and mates with most or all of the females in a group each year (Adams and Wilkinson 2020; McCracken and Bradbury 1981). Both sexes disperse from their natal group, and females within a group are typically unrelated, with no evidence of inbreeding (Bohn et al. 2009; McCracken and Bradbury 1981; Wilkinson et al. 2019). Prior to attaining harem status, males reside in bachelor groups with little or no opportunity for mating (McCracken and Bradbury 1981). Harem males aggressively defend groups year-round and may retain control for up to four years, resulting in extreme reproductive skew (Wilkinson et al. 2016) not unlike elephant seals, *Mirounga angustirostris* (Ross et al. 2023). Harem males also have high levels of androgens and glucocorticoids year-round, but as in many other polygynous mammals (Beirne et al. 2015; Clutton-Brock and Isvaran 2007), males do not live as long as females (Wilkinson et al. 2024). Thus, males can be expected to experience accelerated patterns of physiological aging, which could influence actuarial senescence sooner than females.

Given the behavioural, hormonal, and survival differences between male and female greater spear-nosed bats, here we aimed to determine if the rate of aging estimated from mark-recapture data predicts change in DNAm with respect to sex and male reproductive status. We hypothesised that males would have higher mortality rates and show faster change in DNAm than females, based on evidence of female-biased longevity. In addition, given that harem males are typically older (Wilkinson et al. 2024) and expend more energy than bachelor males (Kunz et al. 1998), we expected them to exhibit more DNAm change than bachelor males. Rather than rely on deviations from an epigenetic clock, which require individuals of known chronological age to detect accelerated or decelerated epigenetic aging (e.g., Anderson et al. 2021), we first compared several epigenetic clocks to identify the most reliable unbiased estimator of chronological age, and then used it to compare rates of change in DNAm assayed from wing tissue between the sexes and male reproductive statuses. We then investigated the genomic locations and genes adjacent to significant sites to infer putative functional consequences of the methylation changes. We found that differences in DNA methylation reflect differences in mortality risk between the sexes and male reproductive status in both expected and unexpected ways.

2 | Materials and Methods

2.1 | Study Population and Sample Collection

Between 1990 and 2025 a total of 9428 greater spear-nosed bats, *Phyllostomus hastatus*, were captured or recaptured in four cave or building roosts (see Supporting Information) using a bucket trap or hand net attached to an extension pole in Trinidad, Lesser Antilles (Adams and Wilkinson 2020; Wilkinson et al. 2016) [some of these animals had been captured and banded in a previous study (McCracken and Bradbury 1981), and we obtained initial banding dates for them from G. McCracken]. Upon capture, bats were sexed, measured for size (forearm length), weight, and tooth wear on a 10-point scale. Additionally, individuals not previously captured were marked with a unique number stamped on a metal alloy band. One or two 4 mm biopsy punches were taken from the wing and stored at −20°C prior to processing or −80°C for long-term storage. DNA was extracted using a commercial kit (see Supporting Information for details). We used DNA samples extracted from wing tissue of 184 females and 153 males that were taken in 14 different years, with most (113 females, 81 males) collected in January or May 2023.

Animal handling methods follow guidelines by the American Society of Mammalogists and were approved by the University of Maryland Institutional Animal Care and Use Committee

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under licences from the Forestry Division of the Ministry of Agriculture, Land and Fisheries, Trinidad and Tobago (protocols FR-JUL-24-25, FR-JUL-21-46, FR-APR-18-16).

2.2 | Measuring DNA Methylation and Estimating Age

Methylation was measured from DNA samples by hybridization to a custom Illumina microarray (Arneson et al. 2022) containing 37,492 probes. Alignment of the 50 bp probe sequences to either the *Phyllostomus hastatus* (v1.1) or the *P. discolor* (v.1.0) genome identified unique positions for 29,799 CpG sites (Wilkinson et al. 2021). We included the *Phyllostomus discolor* chromosome-level assembly to infer X and autosomal linkage of mapped sites by comparison to the human genome annotation (Haghani et al. 2023). Because unmapped sites may exhibit non-specific binding, we limited analyses to sites that map to these two genomes. Sites were categorised as being intergenic, 3′ UTR, 5′ UTR, promoter (within 1 kb of the transcription start site), exon, or intron regions of annotated genes. The proportion of DNA molecules methylated at each CpG site (i.e., Beta values) was obtained after normalisation using the SeSAMe procedure (Zhou et al. 2018).

We compared age estimates derived from four epigenetic clocks available in the R package MammalMethylClock (Zoller and Horvath 2024). Each of these clocks was created by applying elastic-net regression to normalised methylation values measured in DNA from a tissue taken from a known-aged animal. It is important to recognise that not all of the sites in each clock correlate with age because the procedure is designed to minimise unexplained variation (Moqri et al. 2024). The "AllBat" clock used DNAm data derived from wing tissue of nearly 800 known-age individuals representing 26 bat species. This clock predicts age using 162 CpG sites (Wilkinson et al. 2021). The "Phastatus" clock was trained using 75 *P. hastatus* samples and uses 56 CpG sites. We also estimated age using two universal clocks, U2 and U3 (Lu et al. 2023). The U2 clock measures age relative to maximum lifespan and gestation length and uses 817 CpG sites. This clock was developed using DNAm array data from 11,754 DNA samples representing 185 mammal species (including the bat data mentioned above) and uses 817 CpG sites. The U3 clock also measures relative age but does so with respect to age at sexual maturity and gestation time. It is based on a log-linear model using the same data set as the U2 clock and uses 761 CpG sites. We evaluated the performance of these clocks using pairs of samples taken from 29 animals (13 females, 16 males) that were captured and sampled on two or three occasions 1 to 10 years apart. We used the proportion of variance explained, the median absolute error, the difference between the slope of the regression between the observed and estimated intervals and a slope of one, and the difference between male and female residuals to determine which clock provides the best and least-biased age estimate.

We estimated ages using DNAm for all bats with worn teeth, which indicate advanced age (Adams and Wilkinson 2020), so the distribution of older-age animals assayed for DNAm should be representative of the bats in the population. Young animals are under-represented in the DNAm data relative to their number in the population, but are comparable to the number of older animals to ensure that rate estimates were not influenced by outliers.

2.3 | Mortality Rate Estimates

We constructed a capture-mark-recapture (CMR) dataset using banding and recapture records for 2783 greater spear-nosed bats captured in three caves. Because we were interested in estimating differences in mortality among adults, all records of individuals <1 year of age were removed. Exact year of birth was known for 470 individuals in our sample. For the remaining individuals, we estimated birth dates using one of two methods: (1) using age estimates derived from DNAm by applying the AllBat clock (Wilkinson et al. 2021) ($N=246$ individuals, Figure S1); or (2) using age estimates derived from tooth wear ($N=2067$ individuals) (Wilkinson et al. 2024).

CMR analysis was conducted using the 'Bayesian Survival Trajectory Analysis' (BaSTA) package (Colchero et al. 2012) in R 4.3.1., which corrects for recapture probability (Colchero and Clark 2012). BaSTA estimates unknown birth and death dates using population averages, allowing for entire datasets to be retained even in the presence of unknown values.

We fit Gompertz two-parameter mortality models (Finch and Pike 1996; Gompertz 1825; Pletcher 1999) using a Markov Chain Monte Carlo (MCMC) procedure. The Gompertz model assumes that the mortality rate at age x, $\mu(x)=b_0\exp(b_1(x))$. We used b_1 to measure the age-associated increase in mortality, which is often called actuarial senescence or rate of aging (Lemaitre et al. 2020), and b_0 to measure non-age-dependent mortality risk, i.e. baseline mortality. We compared two models, one which included sex as a categorical covariate, and another which included male reproductive status (harem male or bachelor) as a categorical covariate. The model to detect an effect of sex included 2196 females and 587 males, while the male status model included data on 140 harem and 330 bachelor males. For males which changed status ($N=9$), we used the status for the oldest record, as this reflects the final fate of the male. We recorded eight transitions from bachelor to harem male and one transition from harem to bachelor male. We report means and 95% credibility intervals for each coefficient.

2.4 | DNA Methylation Dynamics

To identify CpG sites that exhibit sex differences in how DNAm changes with age, i.e. differentially methylated positions (DMPs), we fitted models for each site with age, sex, and their interaction as explanatory variables. A significant interaction indicates that males and females show different rates of age-associated change in DNAm at that site. Then, to evaluate sex-specific change in methylation, we fitted linear models with age as a predictor for each site in each sex independently and used the age coefficient to quantify the rate of change in DNAm at each site for each sex. We used all samples to test for a sex effect except those from seven individuals (five males, two females) that were less than 1 year of age when captured,

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two females with estimated ages less than 0.5, and one female whose sample did not pass quality control criteria. Given that we estimated age of both sexes using a common epigenetic clock, this approach should provide a conservative estimate of the number of sites which exhibit differences in DNA methylation rates between the sexes. Unless stated otherwise, we controlled for multiple testing by using a Benjamini–Hochberg False Discovery Rate (FDR) and identified significant sites if FDR $p < 0.05$. We tested if males and females show different absolute rates of change across sites using a paired Wilcoxon signed-rank test.

We also tested for differences in age-related change in DNAm from bachelor and harem males. For this analysis, we fitted linear models with age, male status, and their interaction as predictors for each site and used the age coefficient to quantify the change in DNAm at each site for status type. To ensure independence, we omitted 15 samples (8 harem and 7 bachelor) that were duplicates from the same animal and selected the sample that would maximise the overlap in ages for each status type. To validate this exclusion criterion, we also fitted two models to datasets in which the oldest or the youngest of the duplicates was excluded and confirmed that selecting to maximise age overlap provides a conservative estimate of the number of age-by-status interaction sites, and 85% or more of the interaction sites were detected in all analyses. This resulted in a dataset with 47 bachelor and 86 harem males.

Models of DNAm were fit in R 4.3.1. Other analyses were conducted in JMP Pro 17.2.

2.5 | DMP and Gene Set Enrichment Analyses

We used contingency tests to determine if age, sex, or interaction DMPs were independent of gene region or chromosome type (X or autosome). We inferred sites were on the X chromosome if they were X-linked in humans and on the same chromosome (or scaffold) in either the *P. discolor* or *P. hastatus* genome based on previous annotations of the microarray (Wilkinson et al. 2021). To facilitate interpretation, we report $\log_2$ odds ratios (OR) for 2×2 contingency tables.

We conducted enrichment analyses on DMPs using Experimentally derived Functional element Overlap analysis of ReGions from EWAS, eFORGE v.2.0 (Breeze et al. 2019). This program tests for enrichment (or depletion) of regulatory elements previously identified in 111 reference human epigenomes (Roadmap Epigenomics Consortium et al. 2015) by identifying CpG probe sequences associated with five different histone marks or 15 different (8 active and 7 repressed) predicted chromatin states. We conducted separate analyses for either all significant DMPs or the most significant 1000 sites if there were more than 1000 significant sites, for the four possible directions associated with an age-by-sex interaction, i.e. (1) male and female positive, i.e. hypermethylating, (2) male negative, female negative, i.e. hypomethylating, (3) male negative, female positive, or (4) male positive and female negative. As bat wing tissue is comprised of skin, muscle, and blood (Cheney et al. 2017), we only report results for cell lines derived from these tissues (14 blood cell lines, 6 fetal muscle lines, and 8 foreskin lines) and use a BY FDR (Benjamini and Yekutieli 2001) of 1% to identify enriched regulatory elements. In a prior study using the same array platform, a total of 3954 CpG sites exhibited significant differences in the rate of DNAm change between castrated and non-castrated sheep (Sugrue et al. 2021). Subsequent work using transgenic mice which lack androgen receptors indicates that androgen exposure can influence methylation (Sugrue et al. 2025). We used a Fisher's Exact test (FET, FDR $p < 0.05$) to compare DMPs to this list of sites to determine if androgen sensitivity influences DNAm dynamics in these bats.

We conducted several association tests to determine the function of genes near significant DMPs. As multiple probes on the array often are located nearest the same gene, we used the most significant DMP to identify the nearest gene and effect direction. We considered all 5631 genes closest to mapped CpG sites in the *P. hastatus* genome as background and then used PANTHER v.18.0 (Thomas et al. 2022) to test for enrichment of age-by-sex interaction genes with respect to protein class or biological process using a Fisher's Exact test (FET, FDR $p < 0.05$). Given that some eFORGE results indicated enrichment for regulatory element binding sites, we used Enrichr (Xie et al. 2021) CHEA to identify transcription factors that target genes associated with age-by-sex interaction DMPs. This program uses genes that have been identified as targets of 199 transcription factors in prior ChIP-seq, ChIP-ChIP or other transcription factor binding site profiling studies.

We carried out the same set of enrichment analyses for DMPs and genes near DMPs associated with male status.

3 | Results

3.1 | The AllBat Clock Provides the Best Estimate of Chronological Age

All four epigenetic clocks predicted the interval in time between successive captures of 29 animals, with median absolute error, MAE, ranging from 0.45 to 0.81, R^2 ranging from 0.786 to 0.911, and slopes ranging from 0.758 to 1.063. However, the "AllBat" clock had the lowest MAE, the highest R^2, and a slope (1.063) closest to 1, with no apparent sex bias (Figure 1). We therefore used the AllBat clock to estimate age in analyses involving DNA methylation described below.

3.2 | Rate of Aging in Males Is Greater Than in Females

After estimating birthdates for 2783 animals using juvenile captures, the AllBat DNAm clock or toothwear, Bayesian analysis of recapture data revealed that the aging rate, i.e., the exponent associated with how the mortality rate increases with age, for males was 1.81 times that of females (Figure 2) with no overlap in the 95% credibility intervals (CI) for each estimate (mean posterior $b1_{\text{males}} = 0.543$; CI = 0.491, 0.600; $b1_{\text{females}} = 0.305$; CI = 0.290, 0.321; KLDC = 1). Estimates for the mortality rate intercept, i.e., the baseline mortality, did not differ between the sexes (mean posterior $b0_{\text{males}} = -4.782$;

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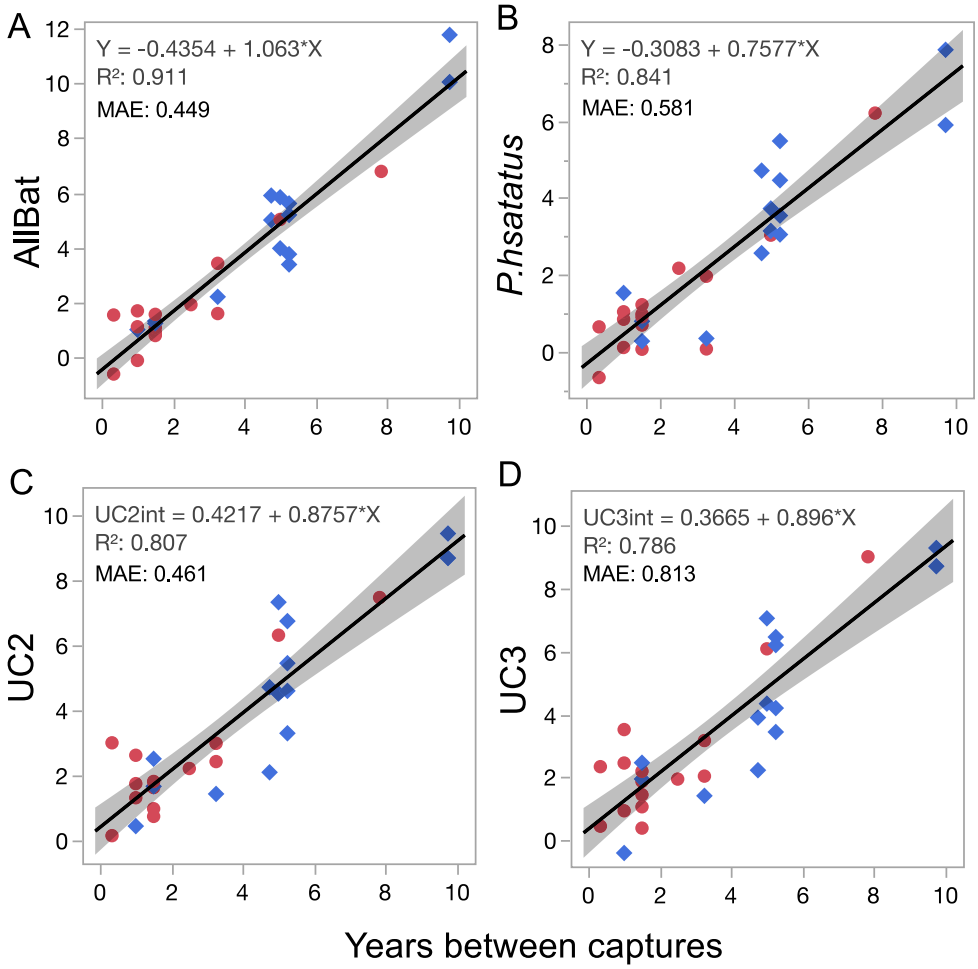

FIGURE 1 | Epigenetic clock comparisons. Predicted intervals in time for (A) AllBat epigenetic clock, (B) an epigenetic clock estimated for *P. hastatus*, (C) U2 and (D) U3 universal clocks (Lu et al. 2023). Females are indicated in blue triangles, males are in red filled circles. Comparison of four epigenetic clocks reveals that the AllBat epigenetic clock predicts the interval in time between captures more accurately as indicated by a smaller median absolute error (MAE), larger coefficient of determination, and an age coefficient closer to 1. The AllBat clock also had the smallest difference in MAE between the sexes (0.16) than any other epigenetic clock (0.28–0.38).

CI = −5.290, −4.410; $b0_{\text{females}} = -4.942$; CI = −5.128, −4.771; KLDC = 0.856).

3.3 | Male Reproductive Status Influences Mortality Rate

The aging rate of subordinate bachelor males was 1.61 times that of reproductively dominant harem males (Figure 2H) with no overlap in the 95% credibility intervals for each estimate (mean posterior $b1_{\text{bachelor}} = 0.897$; CI = 0. 795, 1.000; $b1_{\text{harem}} = 0.564$; CI = 0.449, 0.683; KLDC = 1). Baseline mortality between male status types did not differ (mean posterior $b0_{\text{bachelors}} = -5.443$; CI = −6.078, −4.842; $b0_{\text{harem}} = -5.704$; CI = −6.731, −4.776; KLDC = 0.684). To verify that differences in aging rates between male types are not due to mortality prior to when harem status could be attained, we removed the 114 bachelor males that never reached 3 years of age, which is the age of the youngest harem male (Figure S1). Reanalysis using the remaining 216 bachelor males indicates that the mortality rate of bachelor males is 1.72 times larger than that of harem males with no overlap in the 95% credibility intervals (mean posterior $b1_{\text{bachelor}} = 0.984$; CI = 0.854, 1.126; $b1_{\text{harem}} = 0.572$; CI = 0.452, 0.696; KLDC = 1).

3.4 | Age-Related Change in DNAm Is Greater for Males Than Females

Two lines of evidence indicate that age-associated change in DNAm for males is greater than in females. First, analysis of linear models for DNAm using age, sex, and their interaction reveals that 4275 sites had significant age-by-sex interactions (Figure 3). Among interaction sites, the absolute rate of change in males was greater than it was in females for 89.5% of sites (Figure 3C, $\chi^2 = 579.6$, $p = 2.27\text{e-}128$). A total of 13,441 sites were age-associated, with more sites exhibiting a decrease than an increase in DNAm with age (Figure S2A, $\chi^2 = 266.1$, $p = 4.13\text{e-}60$, $\log_2[\text{OR}] = 0.56$). DNAm differed by sex at 6551 sites, with more sites having higher DNAm in males than females than in the reverse comparison (Figure S2B, $\chi^2 = 1063.8$, $p = 1.23\text{e-}233$, $\log_2[\text{OR}] = 1.33$).

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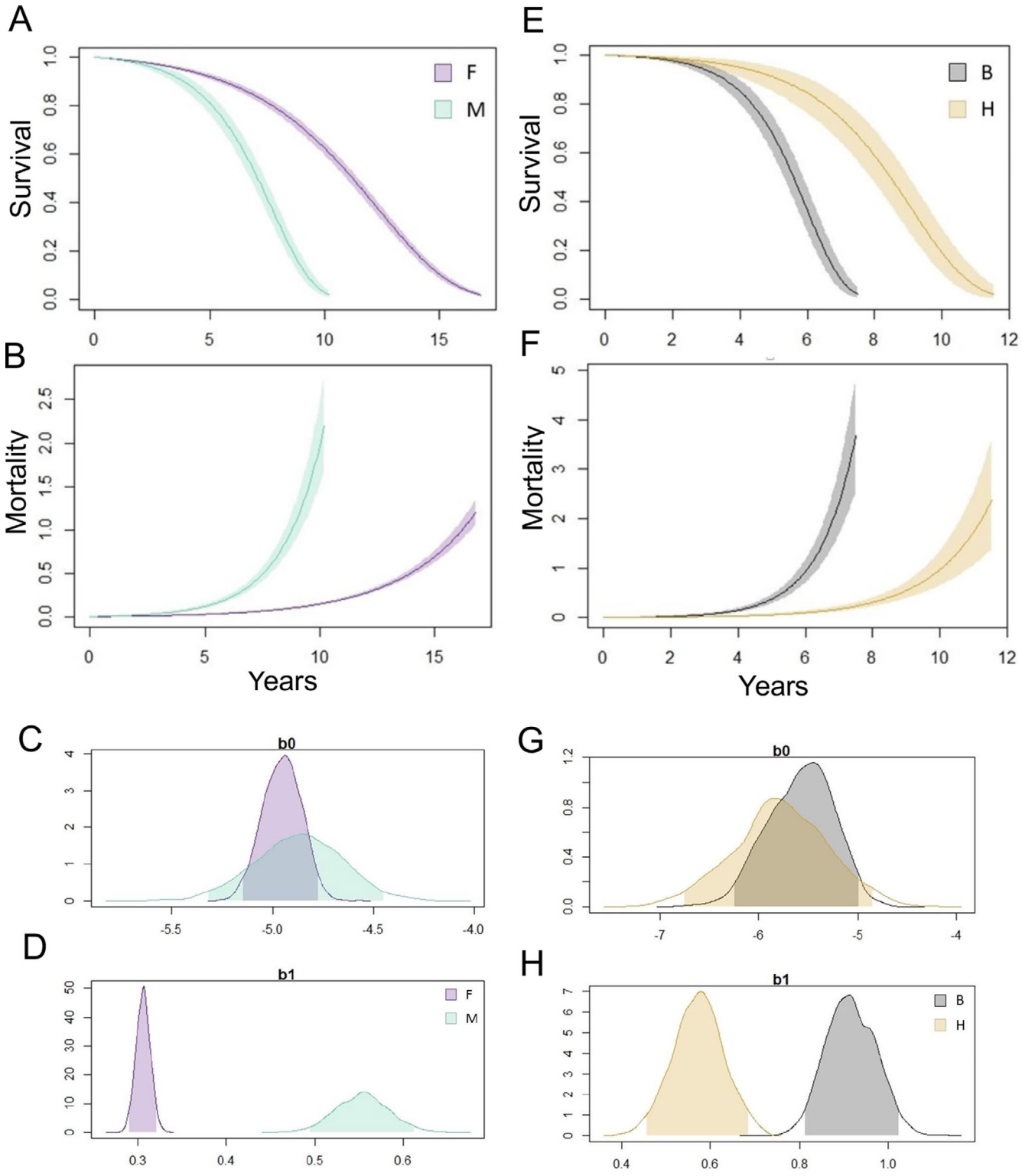

FIGURE 2 | Effect of sex and status on survival and mortality of greater spear-nosed bats. Bayesian estimates of age-specific (A) survival probability and (B) mortality rates for males and females. Probability density plots of posterior distributions of (C) the age independent (b_0) and (D) age dependent coefficient (b_1) for a 2-parameter Gompertz model demonstrate that the demographic aging rates (b_1) of males are greater than females. Estimation of the age-specific effect of reproductive status on (E) survival probability and (F) mortality rate with (G) b_0 and (H) b_1 coefficients reveals that subordinate bachelor males have higher demographic aging rates than reproductively dominant harem males. Shaded regions represent 95% credible intervals.

Second, linear models that fit DNAm for age separately by sex reveal that the number of sites with significant age-related change only in males (4716) is greater than the number of sites with age-related change only in females (2967) despite the greater age range in females, while 12,206 sites show evidence of age-related change in both sexes (Figure 3D). Furthermore,

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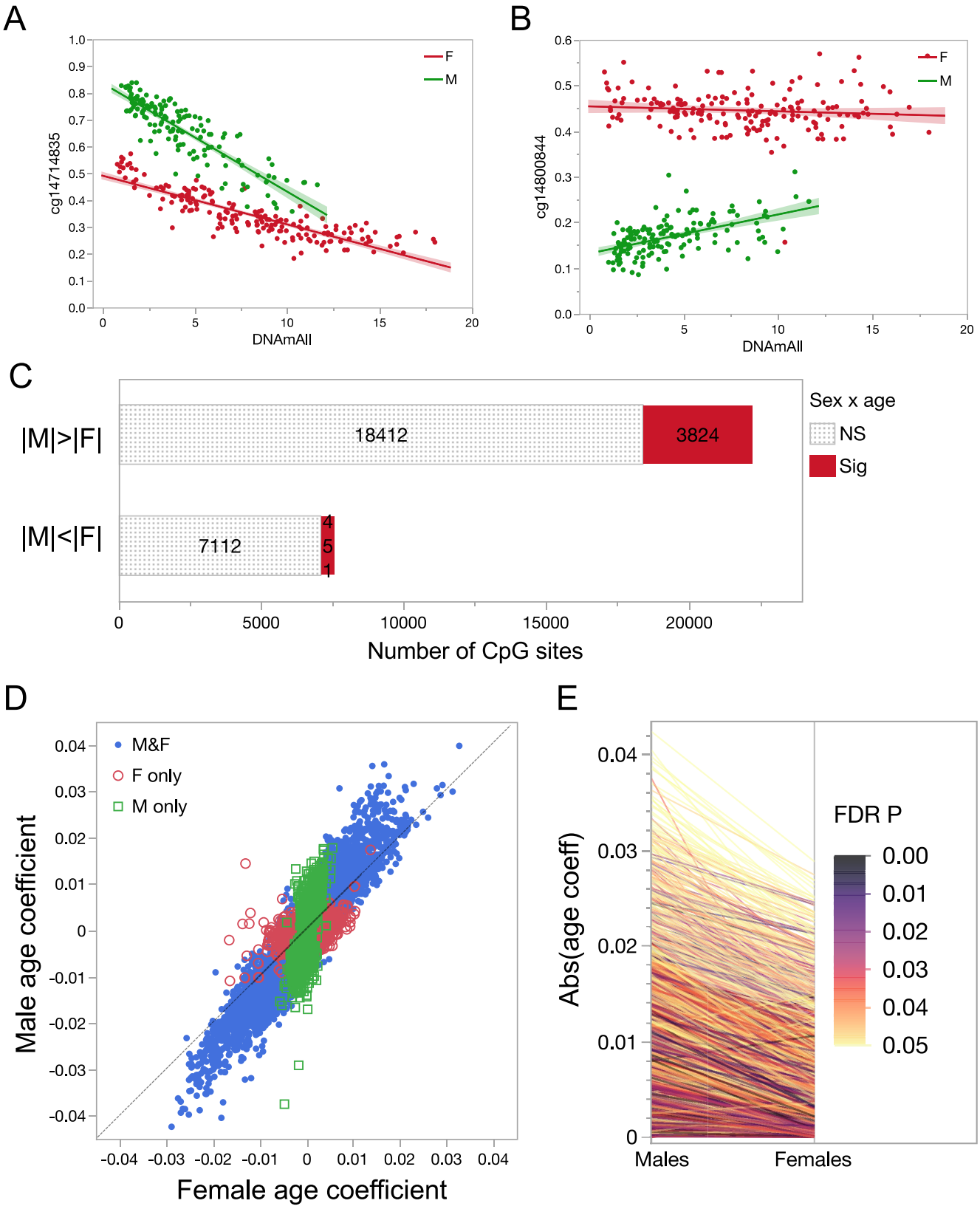

FIGURE 3 | Legend on next page.

DNAm at all 29,799 mapped sites also changes more rapidly in males than in females (abs[male slope] = 0.0038, abs[female slope] = 0.0025, Wilcoxon $S = 1.4e8$; $p < 0.0001$, Figure 3E) despite the intersex correlation ($r = 0.894$). Principal component analysis using all sites reveals that male and female rates would be equivalent if male slopes were reduced by 0.707, consistent with DNAm in males changing, on average, at a rate 1.41 times faster than in females.

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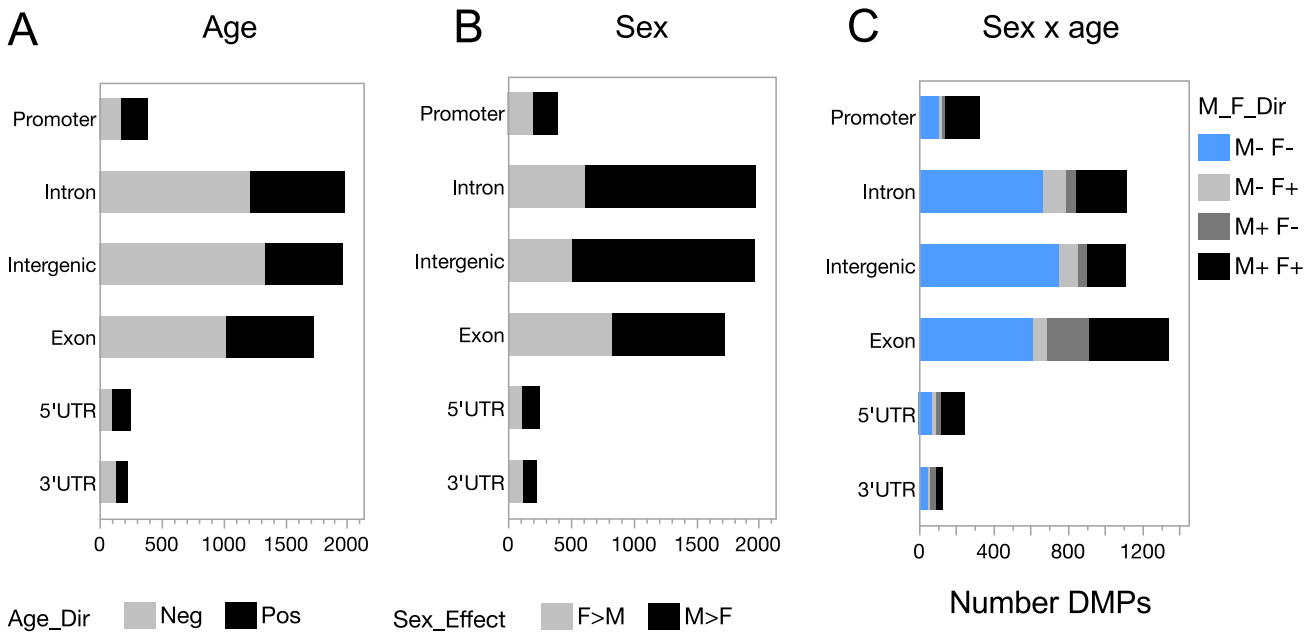
FIGURE 3 | Differential methylation with respect to age in males is greater than in females. Plot of DNAm *beta* against predicted age illustrate significant age-by-sex interactions for (A) cg14714835 in the exon of *CNKSR2*, an X-linked gene involved in Ras signalling, and (B) cg14800844 in the intron of *BCOR*, another X-linked gene which interacts with androgen receptors. Lines indicate least squares regressions with 95% confidence regions indicated by shading. (C) Among significant age-by-sex DMPs the absolute value of the DNAm on age coefficient for males is greater than that for females than the reverse. (D) DNAm on age coefficients for males plotted against age coefficients for females. Open red circles are sites that are only significant in females, open green squares are only significant in males, and blue dots are significant in both sexes. The dashed line indicates equality. (E) Absolute value of DNAm on age coefficients by sex for age-by-sex DMPs indicates that change in DNAm for males is greater than females. FDR *p* is indicated by colour.



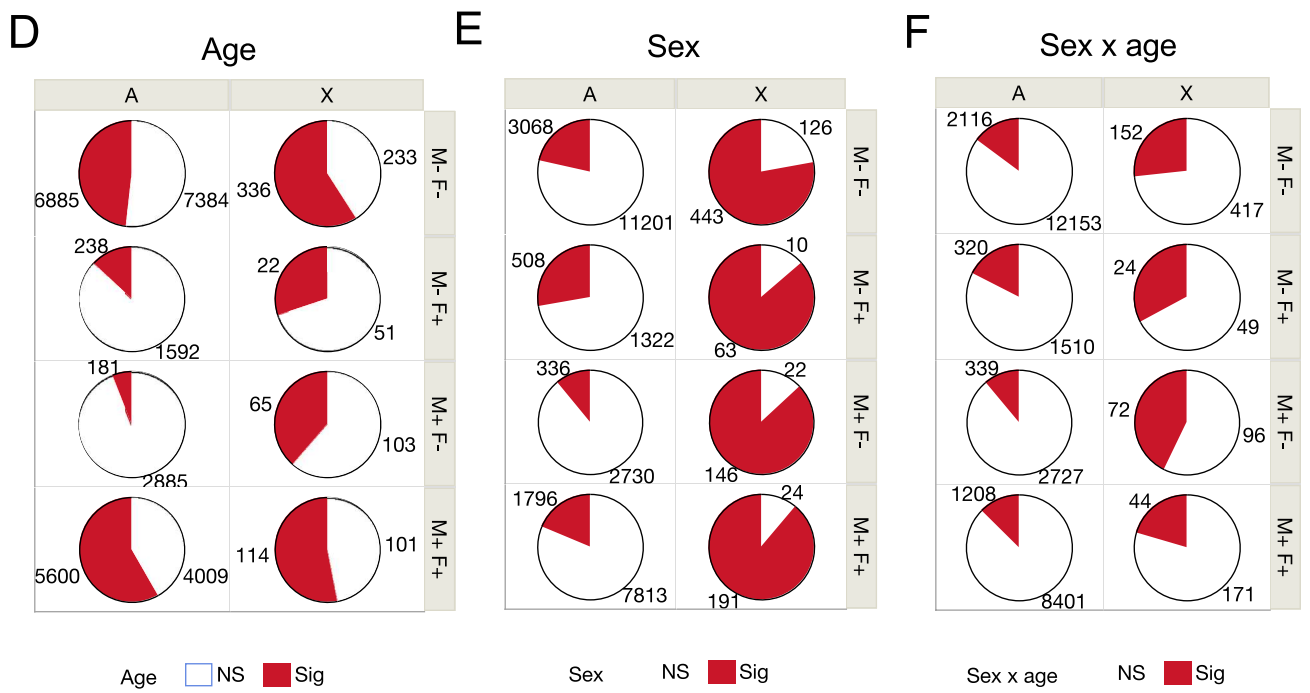


FIGURE 4 | Differentially methylated positions (DMPs) are nonrandomly distributed with respect to gene proximity and chromosome location. Distribution and direction of effect for (A) CpG sites with age effects, (B) sites with sex effects, and (C) sites with age-by-sex interactions. Negative age effects are hypomethylating while positive age effects are hypermethylating. Distribution of significant (FDR $p < 0.05$) and nonsignificant sites on autosomes (A) or the X chromosome that exhibited associations with (D) age, (E) sex, or (F) the interaction between age and sex. The sign of the age coefficient for males and females is indicated below or on the right of each panel.

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As noted in previous studies (Lu et al. 2023; Wilkinson et al. 2021), age-associated sites that gain methylation with age are enriched in promoter and 5′UTR regions (Figure 4A; Figure S3). In contrast to age-associated sites, sex-associated sites are under-represented in promoters, exons, and 5′UTR regions and over-represented in introns and intergenic regions (Figure 4B; Figure S3). Similarly, sites with significant age-by-sex interactions are over-represented in intergenic regions and under-represented in exons (Figure 4C; Figure S3). However, the direction of age-associated change for each sex among the significant interactions was nonrandomly distributed across genomic regions, as indicated by a contingency test ($\chi^2 = 1826$, df = 15, $p < 0.0001$). Interaction sites that were hypermethylating in both sexes were enriched in promoter and 5′UTR regions, while sites that were hypermethylating in males but hypomethylating in females were enriched in exons (Figure 4C).

In addition, age-by-sex interaction sites were enriched for androgen sensitivity, mirroring findings previously detected by comparing methylation between castrated and intact sheep (Sugrue et al. 2021) or androgen receptor knockout mice (Sugrue et al. 2025). A total of 759 of 3954 (19.2%) asDMPs had significant age-by-sex interactions, in comparison to 3516 of 25,845 (13.6%) non-androgen-sensitive sites ($\chi^2 = 87.3$, $p = 4.82\text{e-}21$, $\log_2[\text{OR}] = 0.59$).

3.5 | The X Chromosome Is Enriched for Age-by-Sex Interaction DMPs

A total of 1025 sites mapped to the X chromosome, and 954 (93.07%) of those sites exhibited differential methylation with respect to age, sex, or their interaction. A contingency test between chromosome type and age association was significant ($\chi^2 = 22.8$, $p = 9.60\text{e-}7$, $\log_2[\text{OR}] = 0.43$) due to age-associated sites being more abundant (537) on the X than expected (462.3) (Figure 4D). Consistent with the presence of X-inactivation in female mammals, sex-associated sites are also much more common on the X (843 observed, 225.3 expected; $\chi^2 = 2247.5$, $p < 1\text{e-}300$, $\log_2[\text{OR}] = 4.23$), although more than 5000 sex-associated sites were also located on an autosome (Figure 4E). Finally, sites with significant age-by-sex interactions were also found more often on the X (Figure 4F, 292 observed, 147.0 expected; $\chi^2 = 172.8$, $p = 9.28\text{e-}40$, $\log_2[\text{OR}] = 1.31$) and were distributed across the chromosome (Figure S4). A contingency test between the directionality of the male and female age relationships, i.e. hypermethylating versus hypomethylating, and type of chromosome using just age-by-sex interaction sites was also significant ($\chi^2 = 95.5$, $p = 7.56\text{e-}23$). Post hoc analysis reveals that this effect is due to 2.5 times more significant interaction sites with male positive, female negative slopes on the X than expected and 0.5 times fewer male positive, female positive slope sites on the X than expected. Taken together, there were nearly twice as many male-biased (116) as female-biased (68) hypermethylating sites with significant sex-by-age interactions on the X chromosome ($\chi^2 = 23.1$, $p < 0.001$).

3.6 | Age-by-Sex Interaction DMPs Are Associated With Transcription Regulators

Epigenomic enrichment analysis (Breeze et al. 2019) of age-by-sex DMPs reveals that tissue-specific associations with histone marks and predicted chromatin states found in human cell lines (Roadmap Epigenomics Consortium et al. 2015) depend on the direction of the interaction (Figure 5A,B). Interaction sites that had positive age relationships in both sexes were enriched for activating H3K4me1 and repressive H3K27me3 marks in cell lines from blood and muscle. Those tissues were enriched for enhancer (Enh), enhancer bivalent (EnhBiv), bivariate flanking (BivFlnk), and repressive polycomb (ReprPC) chromatin states. In contrast, interaction sites with a positive age relationship in males, but a negative age relationship in females, were enriched for activating H3K36me3 marks and the transcription (Tx) chromatin state in blood, muscle, and skin tissues. The only other evidence for enrichment occurred for sites that had a negative age relationship in both sexes, which were enriched for quiescent states in blood and enhancer states in muscle and skin cell lines.

Gene ontology over-representation analyses using the gene nearest significant age-by-sex interaction DMPs detected enrichment for biological processes involved in the regulation of transcription and the regulation of metabolism, especially for interaction sites associated with methylation that increases with age in both sexes, indicated as M+F+ (Figure 5C). Those genes are highly enriched for DNA-binding transcription factors and other transcriptional regulators (Figure 5D).

Enrichment analysis for target genes of 199 transcription factors identified in prior binding site profiling studies (Xie et al. 2021) using the genes located nearest significant age-by-sex interaction DMPs identified transcription factors that influence growth and development (Figure 5E). The top 10 (adjusted $p < 0.01$) transcription factors associated with genes that had positive male and female age relationships were two components of the polycomb repressive complex 2, *SUZ12* (Suppressor of Zeste 12) and *EZH2* (Enhancer of Zeste Homologue 2)—which have often been found associated with age, especially in regions with low methylation (Moqri et al. 2024)—*AR* (Androgen Receptor), *NANOG*, which helps embryonic stem cells (ESCs) maintain pluripotency, and three Yamanaka factors, *KLF4* (Kruppel-like factor 4), *SOX2* (SRY-box 2), and *POU5F1* (POU domain, class 5, transcription factor 1, also known as *OCT4*). Yamanaka factors enable reprogramming of cells into pluripotent stem cells (Ocampo et al. 2016). The gene list associated with negative male and positive female age slopes was also enriched for targets of *SUZ12* and *EZH2*, while the gene list for negative male and female age slopes was enriched primarily for *REST* (Restrictive element-1 silencing transcription factor), which acts as a repressor of genes that promote cell death during human aging (Lu et al. 2014).

3.7 | Male Reproductive Status Influences DNAm

Linear models on male DNAm using age, status, and their interaction reveal that 4933 sites differ with respect to status but only 27 sites had significant (FDR < 0.05) age-by-status interactions (Figure 6A,B; Figure S5). Status-associated sites are more likely to have higher mean DNAm in harem (H) males than in bachelor (B) males than expected (3600 observed, 2544.6 expected; $\chi^2 = 1083.6$, $p < 0.0001$, $\log_2[\text{OR}] = 1.58$) and the distribution for the H>B sites is skewed towards 1, or complete methylation (Figure 6C). In contrast, the H<B sites are skewed towards 0,

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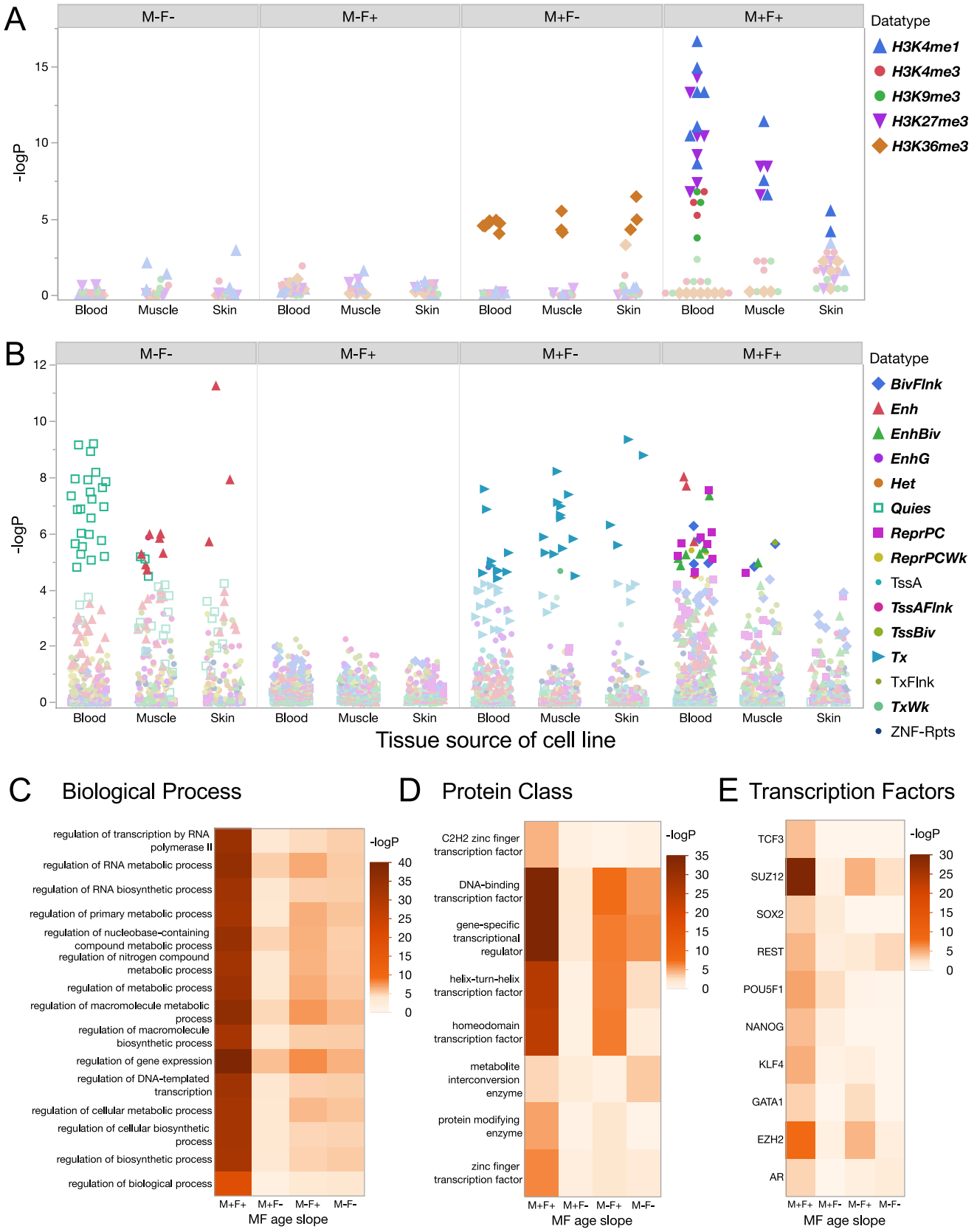

FIGURE 5 | Enrichment analyses using significant age-by-sex interaction DMPs implicate transcriptional regulation of metabolic processes. Epigenomic associations for age-by-sex interaction DMPs with (A) five histone marks and (B) 15 predicted chromatin states for blood, muscle or skin tissue sources from human cell lines. Bold symbols are significant (BY FDR $p < 0.01$). Gene ontology enrichment analyses for genes closest to a DMP with a significant age-by-sex interaction term for (C) the top 15 biological processes, (D) protein classes, and (E) transcription factors known to target those genes. Directionality of the male and female age associations with DNAm are indicated by +/− for each analysis.

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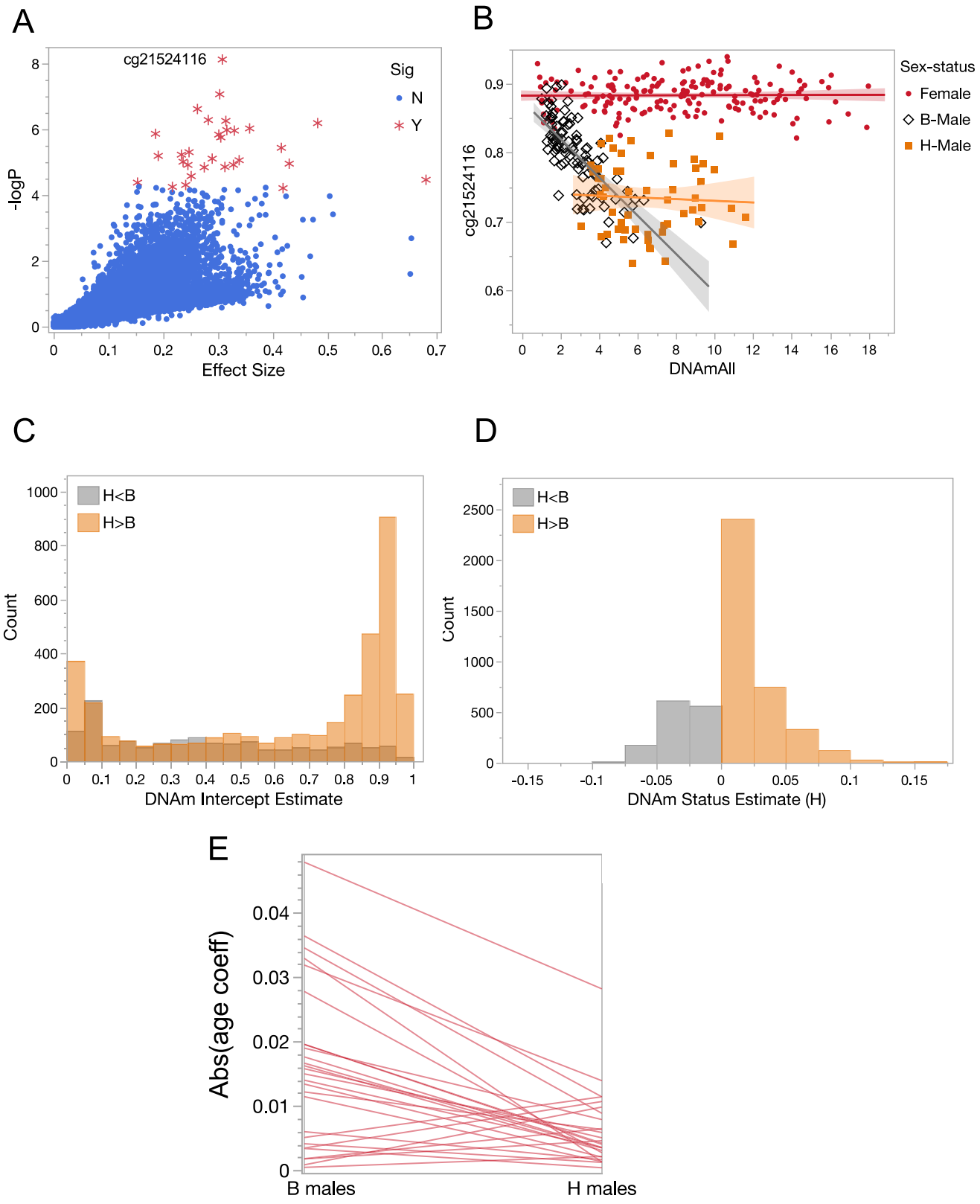

FIGURE 6 | Bachelor males differ from harem males in DNAm intercept and age relationships. (A) Plot of negative log P for age-by-status interactions against effect size, i.e. the standardised coefficient. Sites with FDR $p < 0.05$ are indicated with red asterisks. (B) The most significant age-by-status DMP, cg21524116, is in the intron of MKLN1 (muskelin 1) and exhibits more age-related change in bachelor than in harem males. Females in red dots differ from both types of males and do not change with age. Bachelor males in black triangles decline with age while Harem males in orange squares do not change with age. (C) Distribution of intercept estimates for additive models with significant (FDR $p < 0.05$) status effects and (D) distribution of the status effect. Coefficients in which the harem male estimate was greater or less than the bachelor male estimate are coloured. (E) Absolute value of DNAm on age coefficients for age-by-status DMPs shows that bachelor males change more rapidly than harem males.

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indicating regions of low methylation, with the effect size of status ranging from −0.08 to 0.15 (Figure 6D). Status-associated DMPs were also significantly more likely to involve sites with age-by-sex interactions ($\chi^2 = 551.8$, $p < 0.0001$, $\log_2[\text{OR}] = 1.26$). Among the 27 sites with significant age-by-status interactions, absolute age-associated change in DNAm for bachelor males is greater than for harem males (Figure 6E, Wilcoxon $S = 153$, $p < 0.0001$).

In contrast to the effects of sex, sites that differ with respect to male status are less likely to be on the X than expected (126 observed, 169.7 expected; $\chi^2 = 13.96$, $p = 0.0002$, $\log_2[\text{OR}] = -0.51$) and only 1 of 27 age-by-status DMPs is on the X. Significant status DMPs are not distributed at random among genomic regions ($\chi^2 = 68.0$, $p < 0.0001$), but instead are more likely to be in exons, promoter, or 5′UTR regions than in intergenic or 3′UTR regions, and are more likely to be androgen sensitive ($\chi^2 = 7.03$, $p = 0.008$, $\log_2[\text{OR}] = 1.26$).

3.8 | Status DMPS Are Also Associated With Transcription Regulators

Using the top 1000 significant status sites, which correspond to FDR $p < 0.0403$ if B>H and FDR $p < 0.0166$ if H>B, we found evidence of enrichment for histone marks and predicted chromatin states, but only for sites in which methylation was higher in bachelor than in harem males (Figure 7A,B). Those sites were enriched for activating H3K4me1 in muscle and skin, with the most significant association found for cell lines derived from leg muscle. Similarly, with regard to chromatin states, only sites where DNAm was higher in bachelor than harem males showed evidence of enrichment for enhancer and flanking transcription start sites (TssAFlnk) with the most significant associations found for epithelial and leg muscle cells.

Gene ontology over-representation analyses using genes nearest status DMPs reveal enrichment for several biological processes associated with the regulation of metabolism (Figure 7C) Those genes are enriched for transcription factors or other gene-specific transcriptional regulators (Figure 7D). Enrichment analysis for target genes of transcription factors using the genes nearest status DMPs identifies transcription factors that also influence growth and development (Figure 7E). The transcription factors with the strongest association among genes where the age slope for B>H are two components of the polycomb repressive complex 2, *SUZ12* and *EZH2*, as well as two repressors, *REST and SMAD4* (mothers against decapentaplegic homologue 4), a tumour suppressor that acts to mediate *TGF-b* (transforming growth factor beta) signal transduction. Associations among transcription factor targets are less strong for the genes where H>B, but include *SUZ12*, *SOX2*, *SMAD4*, *POU5F1* and *TCF3* (transcription factor 3), which is necessary for B and T lymphocyte development.

4 | Discussion

Greater spear-nosed bats exhibit strong sexual dimorphism for body size and canine length consistent with strong precopulatory sexual selection (Adams et al. 2020). One expected consequence of such selection is that male mortality would be expected to be higher than female mortality. In line with this prediction, our rate of aging estimates indicate that the male mortality rate is 1.8 times greater than the female mortality rate, consistent with maximum lifespans derived from prior banding studies (Wilkinson et al. 2021, 2016), which reported a 22-year-old female among 1375 recaptured females and a 9-year-old male among 470 recaptured males. However, despite not living as long as females, older male greater spear-nosed bats do not show obvious overt evidence of senescence, as might be expected for males from species with polygynous mating systems (Clutton-Brock and Isvaran 2007). Unlike some other polygynous mammals, such as red deer (Carranza et al. 2004) or badgers (Beirne et al. 2015), where male body mass declines after peak reproductive age, the oldest male bats we captured were harem males. Prior studies have found that harem males are in better body condition, with higher body mass for their skeletal size, than bachelor males (Adams and Wilkinson 2020). However, harem males also have higher levels of biologically active glucocorticoids than bachelor males, consistent with elevated stress (Wilkinson et al. 2024). Nevertheless, mortality rate estimates indicate that bachelor males have higher mortality than harem males.

We can suggest a couple of hypotheses for why harem and bachelor males differ. One possibility is that harem males are in better condition because they are intrinsically different and can tolerate higher levels of glucocorticoids than bachelor males (Wilkinson et al. 2024). Such variation in male quality has been described for at least one other extremely polygynous mammal, the southern elephant seal, *Mirounga leonina*. Male elephant seals that become reproductively dominant have higher survival as juveniles and as adults (Lloyd, Oosthuizen, Bester, et al. 2020; Lloyd, Oosthuizen, Fay, et al. 2020) and show no evidence of accelerated senescence as a consequence of being dominant. One factor that apparently influences whether a male seal is frail or robust is whether it was born with many conspecifics or not (Lloyd, Oosthuizen, Fay, et al. 2020). Whether such density-dependent effects on growth and development occur in the bats has not been investigated. Another possibility is that bachelor males experience higher extrinsic mortality due to predation, pathogen infection, or starvation. If so, how that occurs is not obvious. Bachelor and harem males roost in social groups in the same caves that can contain several hundred individuals (McCracken and Bradbury 1981). Harem males roost with groups of females that are stable over time. Bachelor males roost together in groups whose composition is less stable, but often not spatially far from female groups (Wilkinson et al. 2019). Females from the same social group benefit from foraging together and assisting with the protection of offspring (Wilkinson and Boughman 1998; Wilkinson et al. 2016). While less data is available for harem males, we know that they do not forage with females and spend more energy than bachelor males (Kunz et al. 1998). Thus, males and females are likely exposed to similar risks of predation and infection. Whether competition for food influences male growth and development and contributes to individual differences in condition warrants study.

Given the striking differences in mortality between males and females and the previous observation that short-lived bat species exhibit faster rates of DNAm change than long-lived bat

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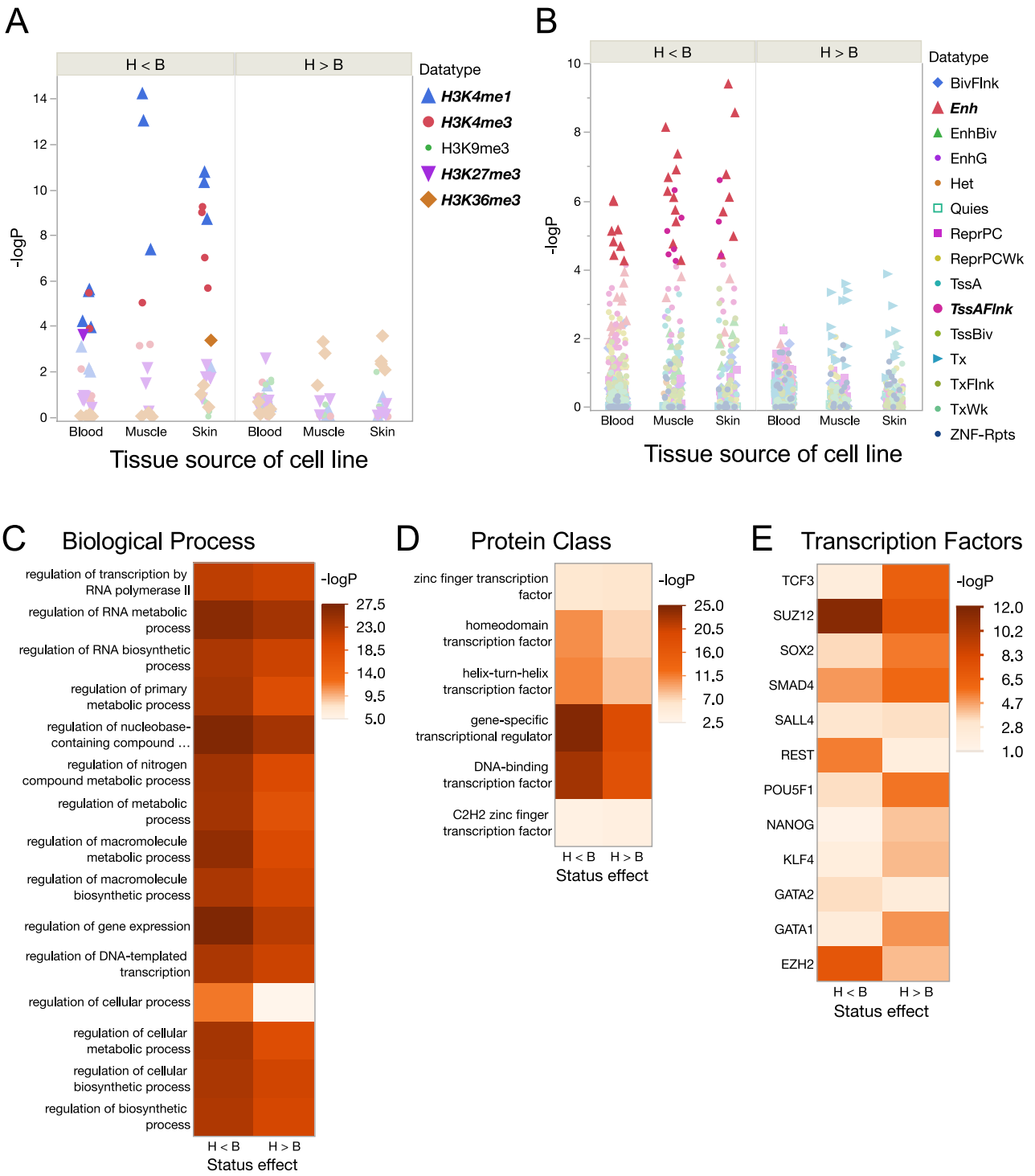

FIGURE 7 | Enrichment analyses using status DMPs reveal harem (H) males had lower DNAm than bachelor (B) males and indicate elevated metabolic processes. (A) The tissue with the highest enrichment for activating histone marks in blood was derived from primary natural killer cells, in muscle it was fetal muscle, and skin was fibroblast primary cells. (B) Predicted chromatin states show enrichment for enhancers in blood, muscle or skin tissue sources. Gene ontology enrichment analysis for genes closest to a DMP with a significant (FDR $p < 0.05$) status term for (C) the top 15 biological processes, (D) protein classes, and (E) transcription factors known to target those genes.

species (Wilkinson et al. 2021), we expected that shorter-lived greater spear-nosed males would exhibit faster rates of change in DNAm than longer-lived females of this species. Indeed, on average, DNAm is changing 1.4 times faster in males than in females, which is consistent with the prediction that males from species with polygynous mating systems should undergo senescence more rapidly than females (Clutton-Brock and Isvaran 2007). These findings are also consistent with studies on roe deer (*Capreolus capreolus*) which found that females exhibit a lower rate of epigenetic aging than males, as indicated by differences from sex-specific epigenetic clocks (Lemaitre et al. 2022).

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Analysis of the chromosomal locations of age-by-sex interaction DMPs reveals that the X chromosome is disproportionately affected by DNA methylation dynamics. Given that dosage compensation in female mammals is accomplished by random inactivation of one of their two X chromosomes by DNA methylation (Morey and Avner 2011), it may not seem surprising that the X is enriched for sites that exhibit different rates of change between the sexes. However, over 60% of X-linked sites on the array exhibit change associated with age in female bats. If those sites are involved in X-inactivation, then the active X must be exhibiting age-associated change in DNA methylation. Alternatively, those sites may escape or exhibit variable X-inactivation, which is known to occur in 23% of X-linked genes in humans (Tukiainen et al. 2017), but only 3% of X-linked genes in mice (Yang et al. 2010). Variation in X chromosome inactivation has been postulated as an underlying mechanism for sex differences in gene expression (Deng et al. 2014) with most genes that escape inactivation exhibiting female-biased expression (Liu et al. 2023) unless they are in a pseudo-autosomal region and show male-biased expression (Tukiainen et al. 2017). While neo-X chromosomes due to X-autosome fusions have been reported for other species in the family Phyllostomidae, there is no evidence of such chromosomal rearrangements in this species (Noronha et al. 2010) and we found that X-linked DMPs are not limited to a few clusters, but instead are distributed across the X chromosome (see Figure S4). It is unlikely, therefore, that many DMPs are in pseudo-autosomal regions.

Given that males and females share the same genome, how might males experience deleterious effects at an earlier age than females? One possibility is that age-dependent gene expression is regulated by steroid sex hormones. Consistent with this hypothesis, we found evidence that the X-linked transcription factor, androgen receptor (AR), targets genes near age-by-sex DMPs. For example, 19 significant age-by-sex DMPs are located in BCL 6 corepressor (BCOR), which is an androgen-dependent interaction partner of AR that is often mutated in cancers (Lempiainen et al. 2020). Humans have over 120,000 Androgen Response Elements capable of binding activated AR proteins (Wilson et al. 2016), which provides some indication of how widespread these effects might be. In addition, three sites on the array—cg11392697, cg13893024, and cg15635095—are located in an exon of AR, and all three undergo a decline in methylation with age and exhibit significant sex differences, suggesting that changes in the expression of this key transcription factor could also contribute to sex differences that influence mortality in the bats. The study on roe deer (Lemaitre et al. 2022) identified two sites that exhibited sex differences associated with aging rates. One of those sites, cg16767916, exhibited hypermethylation with age in both sexes, with males consistently higher than females in deer and in bats, and also differed in methylation between castrated and intact sheep. While more comparisons are clearly needed, these results are consistent with sex differences in DNAm dynamics being associated with androgen exposure.

While we originally suspected harem males would show evidence of faster aging relative to bachelor males, we instead found that rates of change in methylation for bachelor males were 2.8 times greater than those for harem males at sites that differed in rate between them. Moreover, the enrichment analyses indicate that there are functional differences in the patterns of methylation between bachelor and harem males, such that sites where methylation is higher in bachelor than harem males are associated with enhancer chromatin states. The nearest genes to those sites are transcription factors that influence metabolic processes. The most likely transcription factors include two components of the polycomb repressive complex 2 that regulate homeotic genes and are essential for growth and development (Piunti and Shilatifard 2021). Enrichment in functionally relevant genomic contexts suggests that some of these DNAm differences have downstream consequences on the animals' physiology. Differences in male status are known to influence DNAm profiles in baboons (Anderson et al. 2021), but, in contrast to the bats, dominant individuals experience accelerated aging. It is tempting to speculate that differences in how dominance is attained and is associated with stress hormones (Creel et al. 2013; Sapolsky 2005; Wilkinson et al. 2024) could be responsible for these DNAm differences. DNAm in baboons also can be influenced by diet (Lea et al. 2016) and early life adversity (Anderson et al. 2024), both of which could also be relevant for bats.

In contrast to sex differences, differences in DNAm between harem and bachelor males are concentrated on the autosomes. Sites that show differences in DNAm between castrated and non-castrated sheep (Sugrue et al. 2021) are also more likely to differ between males and females, as well as between harem and bachelor males. One of the sites emphasised in the sheep study was in MKLN1, muskelin 1, which became progressively hypomethylated with age in intact males but remained stable in castrated male and female sheep. Recent work has shown that this site can be used as an epigenetic predictor of long-term androgen exposure in mice (Sugrue et al. 2025). Intriguingly, in the bats, this site also decreases in methylation with age, but only in bachelor males. There is no effect of age on DNAm in harem males and females, but baseline DNAm levels are higher and more stable in females (cf. Figure 6B). Urinary androgen levels increase with age in both bachelor and harem males; however, androstenedione is higher in bachelor males than harem males matched for age (Wilkinson et al. 2024). Harem males do, though, maintain high levels of androgens year-round. One of the sites in the AR gene, cg13893024, also shows a significant effect of status, with harem males having higher DNAm than bachelor males at that site. Thus, while the role of androgens in mediating hypomethylation at some sites, such as in MKLN1, between the sexes may be conserved across species, their effects on male social status may be more complex and not simply a consequence of long-term exposure to androgens.

One potential limitation of our study is that we use a DNAm clock to estimate age. It is possible, therefore, that some of the 13,341 age-associated DMPs are spurious because they are correlated with one or more of the 162 sites used in the AllBat clock. However, it is worth noting that the AllBat clock was developed with data from 26 species, including known-age greater spear-nosed bats that had been banded as pups and recaptured as adults (Wilkinson et al. 2021). Linear models of DNAm on age from those known-age bats identified 17,820 age-associated DMPs (FDR $p < 0.05$), which exhibit over 87% overlap with the age-associated DMPs in this study. Consequently, we think few, if any, of the age-associated DMPs reported here are spurious. A second potential limitation is that we infer change in DNAm over time primarily by sampling males and females at different

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ages. While we have repeated samples from some individuals (cf. Figure 1), in most cases they are not from males before and after they transition from reproductively subordinate bachelors to dominant harem males. Such repeated sampling could determine if dominance is acquired by males experiencing progressive changes over time or if individuals have inherently different DNA methylation and gene expression trajectories. Future longitudinal studies are needed to distinguish between these alternatives and to determine if these patterns are shared by other highly polygynous mammals. Nonetheless, the results of the present study provide valuable insights into the relationship between DNAm and differences in survival between the sexes, as well as revealing unexpected effects of social status on aging.

Author Contributions

D.M.A. and G.S.W. designed research; D.M.A., G.S.W., and J.G.R. performed research; D.M.A., G.S.W., J.G.R., and S.B.S.W.H. analysed data; G.S.W. provided funding and supervision; D.M.A. and G.S.W. wrote the paper. All authors edited and provided feedback on the manuscript.

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Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Normalised methylation values for each sample, along with sample metadata, are available from NCBI GEO as series GSE164127 and GSE273259. The design of the Illumina microarray (HorvathMammalMethylChip40) is available from the Gene Expression Omnibus (GEO) at NCBI as platform GPL28271. Microarray probe annotations for the *P. hastatus* and *P. discolor* genomes as well as the results of linear models, gene ontology over-representation tests, and epigenomic association tests are available at https://doi.org/10.6084/m9.figshare.c.7582406.v1. *Code availability*: R code for implementing the analyses described in this paper is also available at https://doi.org/10.6084/m9.figshare.c.7582406.v1.

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