Sex-chromosome-dependent aging in female heterogametic methylomes

Authors:

Marianthi Tangili [1] * †, Joanna Sudyka [1,2] †, Fabricio Furni[1], Per J. Palsbøll[1,3], Simon Verhulst* [1]

Affiliations:

[1] Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, The Netherlands

[2] Institute of Environmental Sciences, Jagiellonian University, Kraków, Poland

[3]Center for Coastal Studies, Provincetown, Massachusetts, USA

† These authors contributed equally to this work.

* Corresponding authors: Marianthi Tangili, Email: tangilim@gmail.com and Simon Verhulst, Email: s.verhulst@rug.nl

Abstract:

DNA methylation-based indices predict mortality and provide insights into aging mechanisms. Even though sex chromosomes are thought to play an important role in sex-specific aging, they have been largely ignored in epigenetic aging studies. We identified age-related CpG (AR-CpG) sites in the full methylomes of two avian species (zebra finch and jackdaw), including for the first time the avian sex chromosomes (Z and the female-specific, haploid W). In both species, we find a high concentration of AR-CpG sites on the W chromosome, with DNA methylation levels predominantly decreasing with age. Our results highlight distinct age-related changes in sex chromosome DNA methylation compared to the rest of the genome, suggesting that this feature of sex chromosomes may contribute to sex-dependent aging.

Main

The underlying causes of aging, the decline in biological functioning with time, remain an enigma. Phenotypic and molecular biomarkers that serve as proxies of biological age (*1*) are instrumental in unraveling aging mechanisms. In this context, indices based on age-dependent epigenetic modifications have shown an unrivaled capacity to predict chronological and biological age (*2*). These indices are mostly based on age-related changes in DNA methylation (DNAm) at CpG sites; sites in the genome where a 5' cytosine is followed by a guanine (*3*). Indices of chronological age based upon the degree of DNAm in certain CpG loci ('epigenetic clocks') have opened novel, pivotal windows into the complex landscape of age-associated phenotypes. Numerous epigenetic clocks have been developed in recent years for humans (*4–6*), model (*7*) and non-model animals (*8*, *9*), and even plants (*10*). Because DNAm contributes to the regulation of gene expression (*3*) and is known to change with age (*4*), studying DNAm in relation to aging across a broad range of species holds the potential to identify fundamental sites, sequences, and processes related to aging conserved across diverse taxa. Casting the net widely will enable identifying aging pathways shared among species, or classes, that likely are fundamental to aging.(*11*).

Sex differences in age-related pathology and lifespan are ubiquitous in the animal kingdom, with the homogametic sex in female heterogametic species living on average 7.1% longer (*12*), suggesting a link between sex chromosomes and aging. However, epigenetic aging studies routinely ignore sex chromosomes, with a few notable exceptions only in humans (*13*, *14*). Employing a novel, robust pipeline we identified age-related CpG (AR-CpG) sites where the degree of DNAm correlates with age among blood samples from captive zebra finches (*Taeniopygia guttata*) and wild jackdaws (*Corvus monedula*). We employed longitudinal data, i.e. multiple samples collected from an individual during its lifespan, to identify AR-CpG sites across the whole genome. We included, for the first time, the avian sex chromosomes (Z and the female-specific haploid W) and tested for deviations of the observed distribution of AR-CpG sites across chromosomes from null expectations. We hypothesized that an important role of sex chromosomes in aging will be revealed in AR-CpG sites being more numerous on sex chromosomes.

We provide details of the pipeline we developed to identify AR-CpG sites from longitudinal methylomes in the supplementary material (Text 1 and Fig. S1, S2). In both species, 0.006% of CpG sites were identified to show age-related changes in DNAm (zebra finch: 1,085/18,906,252 CpG sites; jackdaw: 1,299/21,835,349; Fig. S1). We did not detect AR-CpG sites in the mitochondrial genome, but AR-CpG sites were present on most nuclear chromosomes (Fig. 1). AR-CpG sites were more numerous than expected by chance on the W chromosome in both species (Fig.2; zebra finch: 17% of all AR-CpG sites, Table S2, $p<0.0001$; jackdaw: 14% of all AR-CpG sites, Table S3, $p<0.0001$, Fig.2). The AR-CpG site density on the Z chromosome was elevated in the zebra finch (13% of all age-related sites; $\chi^2= 2622$, df=36, $p<0.001$, Fig.2) and depressed in the jackdaw (3% of all AR-CpG sites; $\chi^2=2243$, df=35, $p<0.001$, Fig.2). The density of AR-CpG sites also varied among the autosomal chromosomes. In the zebra finch, AR-CpG sites were overrepresented on six chromosomes, and underrepresented on 22 chromosomes (Table S2). The corresponding numbers in the jackdaw were six and nine respectively (Table S3). In the zebra finch, DNAm level increased with age on 56% of AR-CpG sites (n= 604; Fig.

1C), which contrasts with the jackdaw, where DNAm increased with age on 31% of AR-CpG sites (n = 407; Fig. 1D).

AR-CpG sites were predominantly located in intergenic regions in both species, followed by introns, promoters, and exons in the zebra finch and introns, exons, and promoters in the jackdaw (Fig. 3A, D). The observed distribution deviated significantly from null expectations (zebra finch: χ^2= 1040.3, df=3, p<0.001, jackdaw: χ^2=321.49, df=3, p<0.001), also when considered separately for each annotation category (Table S4). AR-CpG sites were absent from exons in the W chromosome in both species (Fig. 3B, F, Fig. S3).

Functional consequences of DNAm mostly arise when a promoter is methylated, suppressing gene expression, making promoter DNAm of special interest. On the sex chromosomes promoters, DNAm increased with age on 12 sites and decreased on 21 sites (Fig, 3D). In the jackdaw, all AR-CpG sites in promoters increased with age (n=4; Fig. 3H). In the zebra finch autosomal chromosomes, 60 AR-CpG sites were detected in promoter regions, with DNAm increasing with age on 29 sites, and decreasing with age on 31 sites (Fig. 3D). In the jackdaw autosomal chromosomes, 46 AR-CpG sites were found in promoters among which 15 and 31 increased and decreased in DNAm with age, respectively (Fig3H). For a small number of AR-CpG sites in promoters the specific genes could be identified (Table S5). The GO enrichment analysis of the genes whose promoters contained AR-CpG sites were not involved in shared biological processes or molecular functions for either species. In both species, DNAm decreased with age on the majority of intergenic W chromosome AR-CpG sites (Fig.S3). Details of the direction of change in the degree of DNAm at AR-CpG sites per annotation category across all chromosomes in each species are depicted in Fig. S3.

The number of AR-CpG sites identified by our pipeline depends on the stringency of the selection criteria, raising the question whether this biases our findings. We therefore repeated the analysis with less stringent criteria, resulting in a six-fold increase in AR-CpG sites (Fig.S4,S5). However, the overall trends regarding the distribution of AR-CpG sites across the chromosomes remained the same in both species (Fig. S4, S5), leading us to assume that our results were robust with respect to pipeline criteria.

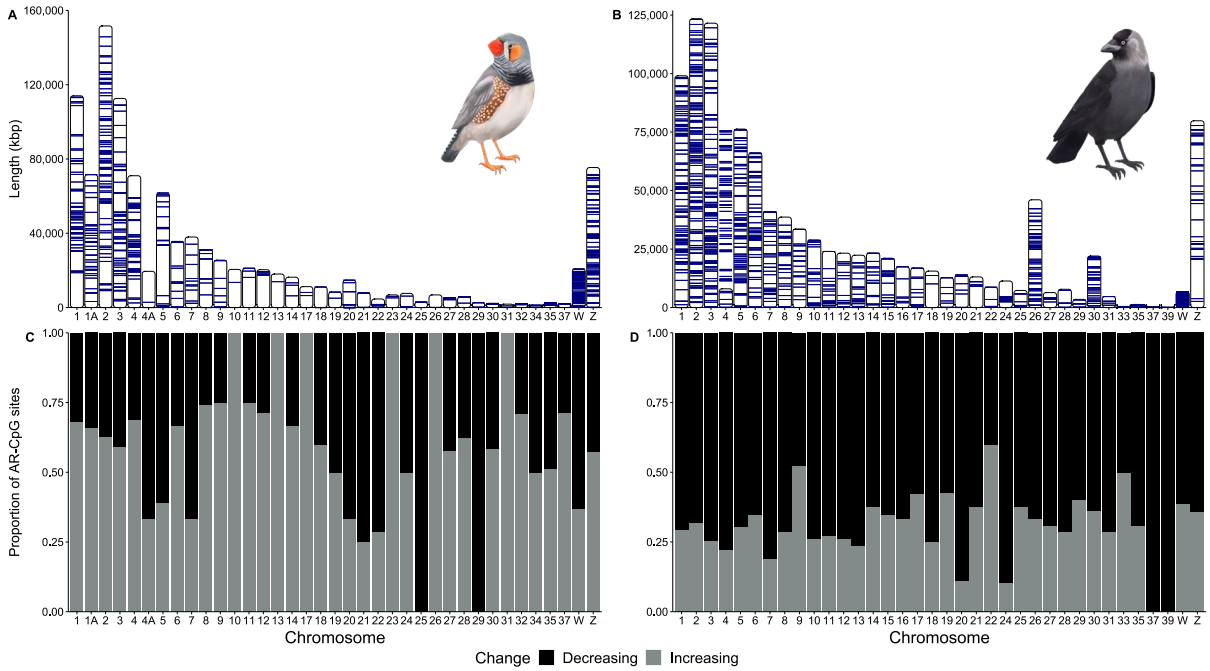

Figure 1. Location of AR-CpG sites across chromosomes and direction of change in DNAm. *Position of each AR-CpG site per chromosome for (**A**) the zebra finch and (**B**) the jackdaw. Proportion of AR-CpG sites on each chromosome whose level of DNAm increased (grey) or decreased (black) with age in the zebra finch (**C**) and the jackdaw (**D**). No AR-CpG sites were detected on zebra finch chromosomes 15, 16, 33, 36 and jackdaw chromosomes 23, 32, 34, 36, 38. The actual AR-CpG site positions for both species can be found in* (*15*).

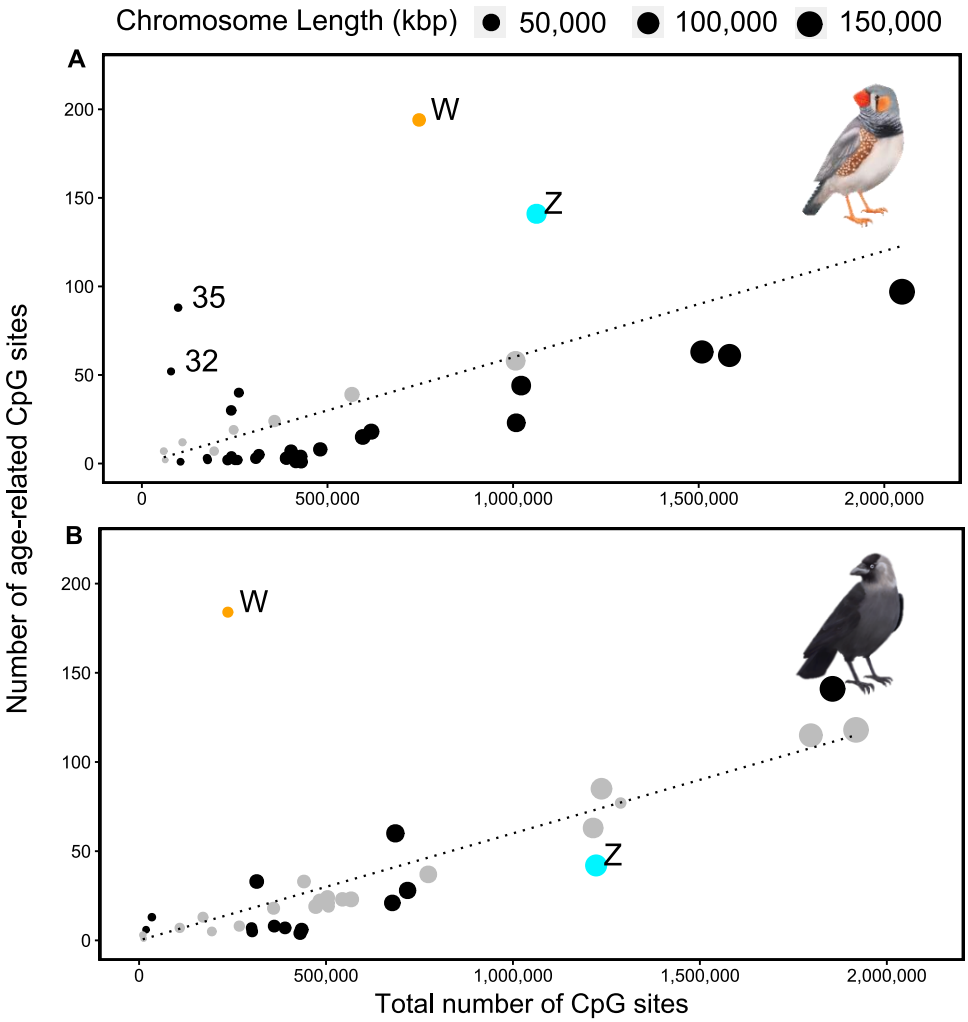

Figure 2. Number of AR-CpG sites in relation to total CpG sites per chromosome. *(**A**) Zebra finch and (**B**) jackdaw. The diameter of each data point is proportional to chromosome length in kilobase pairs (kbp). Black data points denote autosomal chromosomes with an AR-CpG density that deviated significantly from the null expectations (dotted line).*

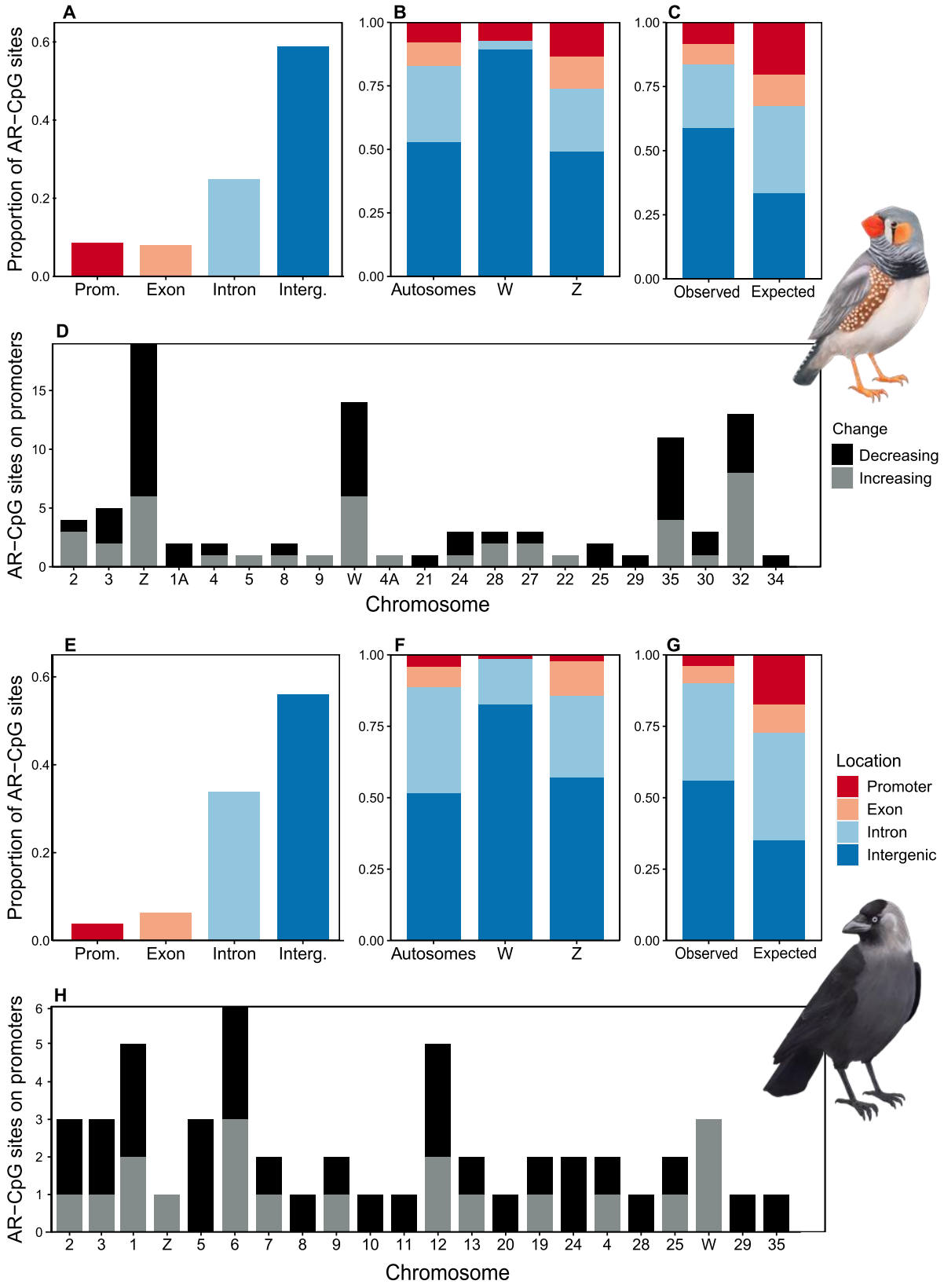
A
B
C
D
E
F
G
H
Proportion of AR−CpG sites
AR−CpG sites on promoters
Prom.
Exon
Intron
Interg.
Autosomes
W
Z
Observed
Expected
Chromosome
Change
Decreasing
Increasing
Location
Promoter
Exon
Intron
Intergenic

Figure 3. Functional annotation of AR-CpG sites. *Proportion of identified AR-CpG sites in each annotation category (***A & E***), by chromosome type (***B & F***) and observed vs. expected in the whole genome (***C & G***) for the zebra finch and jackdaw respectively. Count of AR-CpG sites increasing or decreasing in the degree of DNAm in promoters per chromosome (ordered by decreasing length) for the zebra finch and jackdaw, respectively (***D & H***).*

Our finding of a high density of AR-CpG sites on the W chromosome in two avian species highlights the potential of sex chromosome DNAm to drive sex-specific aging (*13*, *14*). A unique strength of our study was the longitudinal character of the data, combined with whole-genome methylome data, which provided us with a high-resolution view of the entire methylome. Consequently, we detected previously unrecognized sex-chromosome-specific patterns of epigenetic aging, unlike earlier attempts that were restricted to targeting specific genomic regions (*16*) or relied on cross-sectional analyses (*17*). The similarity of our findings between jackdaws and zebra finches, such as the comparable overall number of AR-CpG sites, their increase and decrease in DNAm over time as well as their distribution within each annotation category on the W chromosome suggest our findings may apply generally to passerine birds.

Whether DNAm increases (hypermethylation) or decreases (hypomethylation) with age typically varies among CpG sites (*18*). On the W chromosome in both species, DNAm decreased with age in the majority of AR-CpG sites. The avian W chromosome contains a high density of transposable elements (*19*), whose expression has negative fitness consequences (*20*), which may explain why DNAm is substantially higher on W compared to the autosomal chromosomes (*21*, also in our study species, data not shown). Decreasing DNAm with age likely leads to declining suppression of transposable element expression, and thereby increased genomic instability and deregulation of gene expression, both drivers of senescence (*23*, *24*). Across all chromosomes, but on W chromosomes in particular, AR-CpG sites were disproportionally located in intergenic regions. Besides transposons, intergenic regions also harbor microRNAs (*18*) that modulate longevity and the aging processes (*26*) while playing a significant role in cell proliferation, cell death, and hematopoiesis (*27*). Dysregulation of microRNA gene expression can lead to malignancy (*28*) and disrupted immune cell homeostasis (*29*). Decreased levels of DNAm with age in intergenic regions of the W chromosome suggests loss of the ability to inhibit detrimental genetic elements such as deleterious mutations leading to genomic instability, which is one of the hallmarks of aging (*24*). Our findings therefore indicate that epigenetic aging of the W chromosome via DNAm contributes to the observed shorter lifespans of female birds compared to males (*30*).

The density of AR-CpG sites was higher than expected by chance on the zebra finch Z chromosome, whereas there were slightly fewer AR-CpG sites than expected on the jackdaw Z chromosome. This difference matches the sex difference in lifespan in the two species: in zebra finches, females have a shorter lifespan (*31*), whereas there is no difference in lifespan between the sexes in jackdaws (*32*). The density of AR-CpG sites deviated significantly from the null expectations on most autosomes, but most deviations were small. The notable exceptions were chromosomes 32 and 35 in the zebra finch, where the density of AR-CpG sites was higher than expected. Whether variation in the AR-CpG density among chromosomes (other than the W chromosome) reflects the frequency of transposable elements or other functional components among chromosomes remains to be investigated.

Our finding that AR-CpG sites on the W chromosome mostly decreased in DNAm with age and that most such sites were located in intergenic regions contrasts research on the human haploid, heterogametic Y chromosome where the majority of AR-CpG sites were found to become hypermethylated with age and were located in genes (*13*). On the other hand, a single early study found the mammalian X chromosome to harbor relatively few AR-CpG sites (*33*), as we found for the jackdaw Z chromosome. The avian sex chromosomes ZW are not homologous to the mammalian XY (*34*) and it is therefore unsurprising that DNAm patterns differ from male heterogametic (XY) species. Our findings suggest that DNAm on sex chromosomes likely plays different roles in male and female heterogametic systems. Furthermore, our results underscore the notion that some of the key mechanisms driving sex-specific differences in aging might be fundamental and thus conserved among closely related species, but not across taxa. Consequently, efforts should be directed towards deciphering the role of sex chromosomes in aging across a broader range of species as well as understanding the mechanisms underlying this process in species with various sex determination systems.

Ethics approval

All methods and experiments detailed in this manuscript were performed under the approval of the Central Committee for Animal Experiments (Centrale Commissie Dierproeven) of the Netherlands, under licenses AVD1050020174344 and AVD1050020184967.

Acknowledgments

We thank the Center for Information Technology at the University of Groningen for support and access to the Peregrine and Hábrók high-performance computing clusters. We express our gratitude to Ellis Mulder for her help in the laboratory, Berber Maarsingh for illustrations, and Raphaël Scherrer for assistance with the statistical analyses. We are also indebted to the animal caretakers at the University of Groningen as well as numerous students whose invaluable help made this project possible.

Funding: MT was supported by an Adaptive Life Scholarship awarded by the University of Groningen. JS received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement no. 101025890. Contributions by FB and PJP were supported by the European Union’s Horizon 2020 Research and Innovation Programme under the Marie Skłodowska-Curie grant agreement no. 813383, and the University of Groningen.

Author contributions: MT, JS, and SV conceived and designed the study. MT and JS performed the bioinformatics and data analysis under the supervision of SV and PJP. FF performed the genomic annotation. MT and SV wrote the manuscript with input from all authors. All authors approved the final version of the manuscript. **Competing interests:** The authors declare no competing interests**.**

Data and materials availability

Raw reads for whole methylomes of both species are available at the National Center for Biotechnology Information under BioProject ID PRJNA1108628. The bioinformatics code and R code used in this study can be found at https://datadryad.org/stash/share/6L2TYwRWmgzi4c35v1pf987XyECT1wIdJBoqcwwEXeA.

References

1. X. Xia, W. Chen, J. McDermott, J. D. J. Han, Molecular and phenotypic biomarkers of aging. *F1000Research* **6** (2017).
2. S. Pal, J. K. Tyler, Epigenetics and aging. *Sci. Adv.* **2**, 1–19 (2016).
3. L. D. Moore, T. Le, G. Fan, DNA methylation and its basic function. *Neuropsychopharmacology* **38**, 23–38 (2013).
4. S. Horvath, DNA methylation age of human tissues and cell types. *Genome Biol.* **16** (2013).
5. M. E. Levine, A. T. Lu, A. Quach, B. H. Chen, T. L. Assimes, L. Hou, A. A. Baccarelli, J. D. Stewart, Y. Li, E. A. Whitsel, G. Wilson, A. P. Reiner, A. Aviv, K. Lohman, Y. Liu, L. Ferrucci, An epigenetic biomarker of aging for lifespan and healthspan. *Aging (Albany. NY).* **10**, 573–591 (2018).
6. R. E. Marioni, S. Shah, A. F. Mcrae, B. H. Chen, E. Colicino, S. E. Harris, J. Gibson, A. K. Henders, P. Redmond, S. R. Cox, A. Pattie, J. Corley, L. Murphy, N. G. Martin, G. W. Montgomery, A. P. Feinberg, M. D. Fallin, M. L. Multhaup, A. E. Jaffe, R. Joehanes, J. Schwartz, A. C. Just, K. L. Lunetta, J. M. Murabito, J. M. Starr, S. Horvath, A. A. Baccarelli, D. Levy, P. M. Visscher, N. R. Wray, I. J. Deary, DNA methylation age of blood predicts all-cause mortality in later life. *Genome Biol.* **16**, 1–12 (2015).
7. Y. Han, M. Nikolić, M. Gobs, J. Franzen, G. de Haan, H. Geiger, W. Wagner, Targeted methods for epigenetic age predictions in mice. *Sci. Rep.* **10** (2020).
8. M. Tangili, A. J. Slettenhaar, J. Sudyka, H. L. Dugdale, I. Pen, P. J. Palsbøll, S. Verhulst, DNA methylation markers of age(ing) in non-model animals. *Mol. Ecol.* **32**, 4725–4741 (2023).
9. A. Lu, Z. Fei, A. Haghani, T. R. Robeck, J. A. Zoller, C. Li, Universal DNA methylation age across mammalian tissues. *Nat. Aging* **3**, 1144–1166 (2023).
10. S. T. Gardner, E. M. Bertucci, R. Sutton, A. Horcher, D. Aubrey, B. B. Parrott, Development of DNA methylation-based epigenetic age predictors in loblolly pine (Pinus taeda). *Mol. Ecol. Resour.* **23**, 131–144 (2023).
11. M. Moqri, C. Herzog, J. R. Poganik, K. Ying, J. N. Justice, D. W. Belsky, A. T. Higgins-chen, B. H. Chen, A. A. Cohen, G. Fuellen, S. Hägg, R. E. Marioni, M. Widschwendter, K. Fortney, P. E. Slagboom, E. Verdin, A. B. Maier, V. Sebastiano, Validation of biomarkers of aging. *Nat. Med.*, doi: 10.1038/s41591-023-02784-9 (2024).
12. Z. A. Xirocostas, S. E. Everingham, A. T. Moles, The sex with the reduced sex chromosome dies earlier: A comparison across the tree of life. *Biol. Lett.* **16**, 1–6 (2020).
13. J. B. Lund, S. Li, K. Christensen, J. Mengel-From, M. Soerensen, R. E. Marioni, J. Starr, A. Pattie, I. J. Deary, J. Baumbach, Q. Tan, Age-dependent DNA methylation patterns on the Y chromosome in elderly males. *Aging Cell* **19**, 1–8 (2020).
14. G. Li, C. Wang, X. Guan, Y. Bai, Y. Feng, W. Wei, H. Meng, M. Fu, M. He, X. Zhang, Y. Lu, Y. Lin, H. Guo, Age-related DNA methylation on Y chromosome and their associations with total mortality among Chinese males. *Aging Cell* **21**, 1–12 (2022).
15. M. Tangili, J. Sudyka, F. Furni, P. J. Palsbøll, S. Verhulst, Dataset- Identification of age-related CpG sites from longitudinal avian methylomes. Dryad (2024). https://doi.org/doi:10.5061/dryad.wm37pvmw8.
16. B. S. Meyer, M. Moiron, C. Caswara, W. Chow, O. Fedrigo, G. Formenti, B. Haase, K. Howe, J. Mountcastle, M. Uliano-Silva, J. Wood, E. D. Jarvis, M. Liedvogel, S. Bouwhuis, Sex-specific changes in autosomal methylation rate in ageing common terns. *Front. Ecol. Evol.* **11**, 1–9 (2023).
17. D. Sun, T. S. Layman, H. Jeong, P. Chatterjee, K. Grogan, J. R. Merritt, D. L. Maney, S.

V. Yi, Genome-wide variation in DNA methylation linked to developmental stage and chromosomal suppression of recombination in white-throated sparrows. *Mol. Ecol.* **30**, 3453–3467 (2021).

18. S. Li, L. Christiansen, K. Christensen, T. A. Kruse, P. Redmond, R. E. Marioni, I. J. Deary, Q. Tan, Identification, replication and characterization of epigenetic remodelling in the aging genome: A cross population analysis. *Sci. Rep.* **7**, 1–8 (2017).
19. V. Peona, O. M. Palacios-Gimenez, J. Blommaert, J. Liu, T. Haryoko, K. A. Jønsson, M. Irestedt, Q. Zhou, P. Jern, A. Suh, The avian W chromosome is a refugium for endogenous retroviruses with likely effects on female-biased mutational load and genetic incompatibilities. *Philos. Trans. R. Soc. B Biol. Sci.* **376** (2021).
20. V. M. Warmuth, M. H. Weissensteiner, J. B. W. Wolf, Accumulation and ineffective silencing of transposable elements on an avian W Chromosome. *Genome Res.* **32**, 671–681 (2022).
21. M. Tone, Y. Sakaki, T. Hashiguchi, S. Mizuno, Genus specificity and extensive methylation of the W chromosome-specific repetitive DNA sequences from the domestic fowl, Gallus gallus domesticus. *Chromosoma* **89**, 228–237 (1984).
22. M. Tangili, J. Sudyka, F. Furni, P. J. Palsbøll, S. Verhulst, Absence of DNA methylation-driven dosage compensation on female heterogametic sex chromosomes. *in prep.* (2024).
23. F. Gaudet, J. G. Hodgson, A. Eden, L. Jackson-grusby, J. Dausman, J. W. Gray, H. Leonhardt, R. Jaenisch, Induction of Tumors in Mice by Genomic Hypomethylation. *Science (80-.).* **300**, 489–492 (2003).
24. L. López-Gil, A. Pascual-Ahuir, M. Proft, Genomic Instability and Epigenetic Changes during Aging. *Int. J. Mol. Sci.* **24**, 1–28 (2023).
25. C. Pheiffer, R. T. Erasmus, A. P. Kengne, T. E. Matsha, Differential DNA methylation of microRNAs within promoters , intergenic and intragenic regions of type 2 diabetic , pre-diabetic and non-diabetic individuals. *Clin. Biochem.* **49**, 433–438 (2016).
26. H. E. Kinser, Z. Pincus, MicroRNAs as modulators of longevity and the aging process. *Hum. Genet.* **139**, 291–308 (2020).
27. V. R. Ambros, The function of animal MicroRNAs. *Nature* **431**, 350–355 (2004).
28. S. Costinean, N. Zanesi, Y. Pekarsky, E. Tili, S. Volinia, N. Heerema, C. M. Croce, Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in Eμ-miR155 transgenic mice. *Proc. Natl. Acad. Sci.* **103**, 7024–7029 (2006).
29. S. A. Rose, A. Wroblewska, M. Dhainaut, H. Yoshida, J. M. Shaffer, A. Bektesevic, B. Ben-zvi, A. Rhoads, E. Y. Kim, B. Yu, Y. Lavin, M. Merad, J. D. Buenrostro, B. D. Brown, A microRNA expression and regulatory element activity atlas of the mouse immune system,. *Nat. Immunolgy* **22**, 914–927 (2021).
30. J. Tower, M. Arbeitman, The genetics of gender and life span. *J. Biol.* **8**, 8–10 (2009).
31. M. Briga, E. Koetsier, J. J. Boonekamp, B. Jimeno, S. Verhulst, Food availability affects adult survival trajectories depending on early developmental conditions. *Proc. R. Soc. B Biol. Sci.* **284**, 20162287 (2017).
32. J. J. Boonekamp, M. Salomons, S. Bouwhuis, C. Dijkstra, S. Verhulst, Reproductive effort accelerates actuarial senescence in wild birds: An experimental study. *Ecol. Lett.* **17**, 599–605 (2014).
33. N. Acevedo, L. E. Reinius, M. Vitezic, V. Fortino, C. Söderhäll, H. Honkanen, R. Veijola, O. Simell, J. Toppari, J. Ilonen, M. Knip, A. Scheynius, H. Hyöty, D. Greco, J. Kere, Age-associated DNA methylation changes in immune genes , histone modifiers and chromatin remodeling factors within 5 years after birth in human blood leukocytes. *Clin. Epigenetics* **7**, 1–20 (2015).

34. I. Nada, Z. Shan, M. Schartl, D. W. Burt, M. Koehler, H.-G. Northwang, F. Grutzner, I. R. Paton, D. Windsor, I. Dunn, W. Engel, P. Staeheli, S. Mizuno, T. Haaf, M. Schmid, 300 million years of conserved synteny between chicken Z and human chromosome 9. *Nat. Genet.* **21**, 258–259 (1999).

List of Supplementary Materials

Materials and Methods
Supplementary Text 1
Figs. S1 to S4
Tables S1 to S5
References (35-45)