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Epigenetic age estimation of wild mice using faecal samples

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Abstract

Age is a key parameter in population ecology, with a myriad of biological processes changing with age as organisms develop in early life then later senesce. As age is often hard to accurately measure with non-lethal methods, epigenetic methods of age estimation (epigenetic clocks) have become a popular tool in animal ecology and are often developed or calibrated using captive animals of known age. However, studies typically rely on invasive blood or tissue samples, which limit their application in more sensitive or elusive species. Moreover, few studies have directly assessed how methylation patterns and epigenetic age estimates compare across environmental contexts (e.g. captive or laboratory-based vs. wild animals). Here, we built a targeted epigenetic clock from laboratory house mice (strain C57BL/6, *Mus musculus*) using DNA from non-invasive faecal samples, and then used it to estimate age in a population of wild mice (*Mus musculus domesticus*) of unknown age. This laboratory mouse-derived epigenetic clock accurately predicted adult wild mice to be older than juveniles and showed that wild mice typically increased in epigenetic age over time, but with wide variation in epigenetic ageing rate among individuals. Our results also suggested that, for a given body mass, wild mice had higher methylation across targeted CpG sites than laboratory mice (and consistently higher epigenetic age estimates as a result), even among the smallest, juvenile mice. This suggests wild and laboratory mice may display different CpG methylation levels from very early in life and indicates caution is needed when developing epigenetic clocks on laboratory animals and applying them in the wild.

KEYWORDS
ageing, DNA methylation, epigenetic clock, *Mus musculus*

1 | INTRODUCTION

Age is a key characteristic for any organism, with numerous biological processes from immune maturation to reproduction being age related (Georgountzou & Papadopoulos, 2017). Yet, measuring age in wild individuals can be challenging as date of birth is often unknown. Classic methods for assessing age in wild animals are often inaccurate (e.g. body size) or destructive (e.g. eye lens weight),

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raising ethical concerns and preventing longitudinal studies. An alternative approach for measuring the age of wild individuals relies on the measurement of epigenetic marks in particular genomic regions. Specifically, at some CpG sites (cytosines followed by a guanine; Moore et al., 2013), the proportion of methylated cytosines appears to change linearly with age. DNA methylation can influence biological ageing through various molecular mechanisms, including repression of chromatin state and promotor silencing among others (Moore et al., 2013). Together these CpG sites with age-related methylation patterns can be used to derive an 'epigenetic clock'. An epigenetic clock, trained using samples from individuals of known age, can then be used to predict age in individuals of unknown age. Such epigenetic clocks can provide a more accurate estimate of chronological age among wild animals than visible characteristics (Larison et al., 2021; Mayne et al., 2022). Epigenetic clocks have now been developed for a wide range of animal species including baboons, chimpanzees, humpback whales, wolves, green turtles and zebras (Anderson et al., 2021; Bors et al., 2021; De Paoli-Iseppi et al., 2017; Fairfield et al., 2021; Ito et al., 2018; Jarman et al., 2015; Larison et al., 2021; Mayne et al., 2022; Pinho et al., 2022; Polanowski et al., 2014; Sullivan et al., 2022; Tangili et al., 2023; Thompson et al., 2017; Wilkinson et al., 2021; Wright et al., 2018), as well as plants (Gardner et al., 2023).

Alongside measuring chronological age, epigenetic clocks also appear to capture signals of biological age, typically considered to reflect the accumulated damage and functional decline in cells, tissues and organs (Yousefzadeh et al., 2021). Accelerated epigenetic ageing has been linked to various communicable and non-communicable diseases in both humans and laboratory mice (Ambatipudi et al., 2017; Cao et al., 2022; Harvanek et al., 2021; Joyce et al., 2021; Morales Berstein et al., 2022; Peng et al., 2019). Insights have also come from the wild: high social rank is associated with accelerated epigenetic ageing in wild baboons (Anderson et al., 2021), and hibernation slows down ageing in marmots and bats (Pinho et al., 2022; Sullivan et al., 2022). Thus, the use of epigenetic clocks may provide a means of estimating chronological age among wild animals while simultaneously providing insight into biological ageing in natural settings.

Previous studies using epigenetic clocks have focused on humans (Ambatipudi et al., 2017; Cao et al., 2022; Harvanek et al., 2021; Joyce et al., 2021; Morales Berstein et al., 2022; Peng et al., 2019), laboratory (Han et al., 2018; Kerepesi et al., 2022) or wild animals (Anderson et al., 2021; De Paoli-Iseppi et al., 2017; Lemaître et al., 2022; Polanowski et al., 2014; Prado et al., 2021). A number of studies have also included both wild and captive (e.g. from a zoo) individuals (Fairfield et al., 2021; Ito et al., 2018; Mayne et al., 2022; Robeck et al., 2021; Wilkinson et al., 2021). However, a comparison of wild and laboratory individuals has not been previously conducted. Comparing epigenetic age and ageing between laboratory and wild individuals of the same species could help us understand drivers of biological ageing and its variability in individuals from contrasting genetic and environmental backgrounds.

Here, we build an epigenetic clock using samples from laboratory mice (*Mus musculus*) and use this laboratory-based clock to predict age in house mice (*M. musculus domesticus*) from a wild population. Our aims were threefold: (1) to see whether we could develop an epigenetic clock from laboratory-based animals capable of accurately capturing differences in chronological age within a wild population, (2) to compare estimates of epigenetic age in laboratory compared to wild mice of a given size and to gain insight into biological ageing in laboratory versus wild settings and (3) to assess the extent of variability in biological (epigenetic) ageing rate among wild mice. We used faecal samples as a source of DNA, to develop a non-invasive method that allows longitudinal sampling without ethical or logistical limitations on sampling frequency and allows epigenetic age estimates to be made in contexts where animal capture or handling are impossible. To our knowledge, this is the first epigenetic clock built with faecal samples. Our results not only show the potential of such an approach, but also indicate substantial differences in DNA methylation levels between an inbred laboratory population and an outbred wild population, even from very early in life.

2 | MATERIALS AND METHODS

2.1 | Sample collection

A total of 137 faecal samples were collected from 65 individual *M. musculus* C57BL/6 laboratory mice (30 females, 35 males) from two animal facilities. The samples were collected in May–November 2021 at the Biomedical Services Building, Oxford, UK (animal facility A) and King's College, London, UK (animal facility B). The chronological age of the mice varied from 7 to 339 days, covering approximately the first third of expected C57BL/6 life span (30–32 months; Schultz et al., 2020). The mice were kept in standard housing and were not subject to any interventions before or during sampling. During sample collection, body mass was recorded for mice from animal facility B but not for mice from animal facility A. However, body mass is tightly correlated with age among juvenile house mice (JAX, 2022a, 2022b; Spangenberg et al., 2014), allowing accurate estimation of mass from age. As such, we estimated body mass for 25 mice under 7 weeks of age from animal facility A [for older mice from this facility ($n = 8$) body mass was not estimated and consequently samples from those mice were not included in analyses involving body mass]. Body mass estimation was done based on Spangenberg et al. (2014) for 7- to 20-day-old pups and The Jackson Laboratory C57BL/6 body mass references for 3- to 7-week-old pups (JAX, 2022a, 2022b). For the latter age group, estimation was done separately for females and males using the Jackson Laboratory sex-specific data (JAX, 2022a, 2022b). To collect faecal samples, mice were briefly placed on a sterile surface until defecation. Faecal pellets were collected in a sterile manner, immediately preserved in DNA/

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RNA Shield (Zymo Research, Irvine, CA, USA) and stored frozen at −80°C until further processing (up to ≤12 months).

Wild house mouse (*M. musculus domesticus*) sampling was conducted in April–May 2019, July 2019, September–October 2019, August–September 2020 and April–May 2021 on Skokholm Island, Wales, UK. Mice were trapped overnight using small galvanized metal Sherman live traps baited with peanuts and non-absorbent cotton wool for bedding and with a spray of sesame oil outside the trap as a lure. Across each of two broad sampling areas (one near the coast and one in the island interior, named 'Quarry' and 'Observatory' respectively), on each trapping night 150 traps were set at dusk and checked at dawn. To prevent cross-contamination, any traps showing signs of mouse presence were washed and sterilized before being reset using bleach solution (including at least a 60-min soak in 20% bleach solution) to destroy bacterial cells and DNA. All newly captured mice were permanently identified by subcutaneous injection of a passive integrated transponder (PIT) tag. Upon each capture, each mouse was either tagged or identified (if a recapture), aged, sexed and measured before being released within 3 m of its trapping point. Sex was determined using anogenital distance and reproductive state. Reproductive state was recorded as either non-perforate, perforate, suspected pregnant or lactating for *females*, and testes abdominal, small or large for *males*. At each capture, body mass was recorded. Mice were placed in a small cotton bag for weighing to the nearest 0.1 g, and in a transparent plastic bag for measurement of body length (measured as snout-vent length, SVL, from the tip of the nose to the base of the tail, with mice gently straightened before measuring). Reproductive state was classified as active or inactive, with females being reproductively active when either pregnant, lactating or perforate, and males being reproductively active when testes were visibly descended (classed as small or large). Age was roughly classified to one of three age groups using body mass and reproductive state. Reproductively inactive mice weighing ≤15.0 g were classified as *juveniles*, mice >20.0 g of body mass were classified as *adults* regardless of reproductive state and mice falling between these two categories (reproductively active mice ≤15.0 g of body mass, as well as all mice weighing 15.1–20.0 g) were classed as *sub-adults*.

Faecal samples were collected from traps in a sterile manner (shortly after mice were removed from traps the following day), preserved in DNA/RNA Shield and stored in a −20°C freezer until the end of fieldwork (maximum 6 weeks after sample collection). At this point they were returned to the laboratory frozen and stored at −80°C until DNA extraction (up to 17 months later). A total of 215 samples were selected from all collected samples (>900) for further processing, by first selecting a longitudinal dataset (mice sampled ≥2 times; total of 54 individuals) with as much variation as possible in morphometric variables (age, body mass, sex and reproductive status), as well as environmental variables (sampling season and sampling area) and then supplementing this with additional (equally variable) cross-sectional samples (one sample per animal) to increase the number of individuals for cross-sectional analyses up to 130. Variation in variables was achieved by randomly selecting approximately equal numbers of samples across categories, e.g. across juvenile, sub-adult and adult mice.

2.2 | DNA extraction, bisulphite conversion and PCR amplification

DNA was extracted from faecal samples using the ZymoBIOMICS DNA MiniPrep Kit according to the manufacturer's protocol (Zymo Research, Irvine, CA, USA). DNA was then bisulphite-converted using the Zymo EZ DNA Methylation-Gold Kit to convert unmethylated cytosines to uracil and then thymine (Zymo Research, Irvine, CA, USA). PCR amplification was conducted for five genes previously reported to correlate with chronological age in *M. musculus*: *Prima1*, *Hsf4*, *Kcns1*, *Gm9312* and *Gm7325* (Han et al., 2018). Amplification was conducted using the PyroMark PCR Kit according to manufacturer's instructions and primers for the five genes (Qiagen, Hilden, Germany; Table S2; Han et al., 2018). PCR conditions were as follows: 15 min initial denaturation at 95°C, 50 cycles of 30 sec denaturation at 58°C, 30 sec primer annealing at 58°C and 30 sec extension at 72°C, followed by a 10-minute final extension at 72°C. Amplification success was confirmed using gel electrophoresis. PCR was repeated with the same conditions for any reaction that did not produce a band on the gel. The five amplicons (PCR products) were pooled for each sample by combining all PCR products per sample. DNA was quantified with Qubit Fluorometer High Sensitivity dsDNA kit and normalized to 6.25 ng/μL (Thermo Fisher Scientific, Waltham, MA, USA).

2.3 | Sequencing, basecalling and demultiplexing

The Oxford Nanopore Technology (ONT) platform was used for library sequencing, and all ONT procedures were conducted according to manufacturer's instructions and ONT protocol NBA_9102_v109_revl_09Jul2020 (Oxford Nanopore Technologies, Oxford, UK). Samples were processed in six batches. For each batch, we first used the ONT Ligation Sequencing Kit (SQK-LSK109) to repair and dA-tail the DNA ends, followed by ligation of sequencing adaptors to the prepared ends. We then barcoded pooled amplicons using the ONT Native Barcoding Expansion kit (EXP-NBD104 or EXP-NBD196) such that each sample had a unique barcode. Subsequently barcoded amplicons were combined into a single library (a total of six libraries were prepared as samples were processed in six batches). Libraries were sequenced individually. Approximately 15 ng of the prepared library was loaded onto a prepared ONT MinION Mk1B R9.4.1 flow cell and sequenced using the ONT MinKNOW software v21.10.4, resulting in a mean of 49,969 reads per sample. A negative control (where DNAse-free H_2O was used instead of pooled amplicons at the start of Nanopore pipeline) was included in three sequencing runs and these generated a mean of 200 (range 17–519) reads. One flow cell was used twice and washed between runs with the ONT Flow Cell Wash kit (EXP-WSH003). Different barcodes were used

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for negative controls across the two sequencing runs where the same flow cell was used to enable testing for carry-over of reads (only 17 potential carry-over reads were detected). Raw sequencing data were basecalled and demultiplexed using High Accuracy basecalling on the ONT Guppy software v5.0.11. The basecalled FASTQ files were then run through the Apollo pipeline v0.1 (https://github.com/WildANimalClocks/apollo, https://doi.org/10.5281/zenodo.8426692) to acquire methylation rates for each CpG site within the five genes (73 CpG sites in total, 4–27 CpG sites per gene). Apollo requires a minimum read count of 50 for reporting on a particular site. Using the alignment with the reference genes, target sites (cytosines within CpGs) were identified in each read and determined as either methylated (cytosine) or unmethylated (uracil). The process was continued for each read, resulting in a proportion of methylated cytosines at each CpG site.

2.4 | Analyses

The data were analysed and visualized in R v4.1.2 (R Core Team, 2023). The epigenetic clock was built using the cross-sectional data of 50 samples from C57BL/6 mice housed in two facilities (Table S1). Using CpG site-specific methylation rates, we used the package glmnet v4.1-3 (Friedman et al., 2010) to perform a cross-validated elastic net regularization using the cv.glmnet function with a LASSO model (mixing parameter alpha = 1) and a leave-one-out cross-validation (nfolds = nrow). From inspecting relationships between methylation levels in CpG sites and body mass (Figures S1–S4), we expected only a subset of CpG sites to be relevant for predicting age and thus the alpha parameter was set to 1 (LASSO penalty). We further investigated the effect of different alpha values on model fit by varying alpha between 0 (ridge penalty) and 1 (LASSO penalty) at increments of 0.05 and selecting a value that maximized model fit (minimized the mean squared error). This analysis was run 10 times. This analysis did not suggest a strong lead candidate for alpha value; however, our initial alpha value of 1 was among the strongest candidates and as such we proceeded with alpha = 1. We then fitted a final glmnet model using an optimal lambda value determined by the cross-validation (lambda = 0.913). Epigenetic age was then predicted based on the glmnet model using the predict() function. This clock was validated on an additional 15 C57BL/6 mice from facility B. Using one sample per animal, we first measured the correlation between epigenetic age and chronological age and assessed clock performance for estimating chronological age of laboratory mice using mean absolute error (MAE; Tangili et al., 2023). We then ran a linear mixed effects model using lmer function from lme4 R package (Bates et al., 2015) to measure the influence of sex and sequencing batch on epigenetic age predictions. For this model we used two samples from each of 15 mice (30 samples in total) and included animal ID as a random factor.

The above-described epigenetic clock (Clock 1) was developed to test and showcase how accurate a faeces-based targeted epigenetic clock can be; however, as our aim was to develop a laboratory-based epigenetic clock that could be used to predict age in wild mice, we then built a second epigenetic clock (Clock 2) as described earlier but using only those CpG sites that (1) showed parallel trends in their methylation rates against body mass in laboratory and wild mice (i.e. ones that increased/decreased in methylation with body mass in both systems based on linear regression slope estimates quantifying the change in DNA methylation levels per unit change in body mass; Figures S1–S4) and (2) had methylation rates for the majority of wild mouse samples (we failed to acquire sufficient read counts for methylation rate measurement for all 11 CpG sites from the gene *Gm7325* in 9% of all wild mouse samples). Following these principles, we included a total of 53 CpG sites in Clock 2. The lambda value used in this clock (determined with cross-validation, as for Clock 1) was 16.950. Clock 2 was similarly validated with the independent laboratory dataset and then used to estimate epigenetic age for all wild house mouse samples for which methylation rates at the CpG sites included in the clock were successfully measured ($n = 201$; 93% of all wild mouse samples; Table S1) using linear modelling. Intercepts and coefficients for both epigenetic clocks developed are presented in Table S5.

To examine method repeatability, 11 out of the 201 wild mouse DNA samples were processed twice through BS treatment, PCR and sequencing. First and repeat DNA aliquots were processed in two distinct batches. Repeatability was estimated for (1) methylation levels of CpG sites included in Clock 2 ($n = 11$; Table S4) and (2) epigenetic age estimates, using R package rptR (Stoffel et al., 2017) with 1000 parametric bootstraps. Sequencing batch and sample ID were used as predictors (random effects). After assessing repeatability, duplicates were removed from the data by randomly selecting one observation per sample ID.

To test for the effect of covariates on predicted epigenetic age in the laboratory mouse validation dataset, we fit a linear model with epigenetic age as the dependent variable and chronological age, sex, cage and sequencing run ID as predictor variables. ANOVA was used to test whether predicted epigenetic age varied significantly by wild mouse age categories (juvenile/sub-adult/adult) and post hoc Wilcoxon rank sum tests were used to test whether the predicted epigenetic age of wild mice varied significantly between specific age category pairs (juvenile vs. sub-adult, juvenile vs. adult, sub-adult vs. adult). The ability of the clock to detect an increase in age among wild mice sampled on two consecutive occasions was tested using a one-tailed binomial test. The null hypothesis for this test was that the probability of mice increasing in epigenetic age between consecutive time points (p) = .5 (i.e. mice are just as likely to increase or decrease in epigenetic age over time), while the alternative hypothesis was that $p > .5$. We also used linear models to test (1) whether among wild mice, time between sampling points predicted absolute change in epigenetic age as well as (2) whether sex, trapping area, season (*spring*: April/May; *summer*: July/August; *autumn*: September/October), reproductive activity or body mass at first capture predicted the rate of epigenetic ageing $\left(\frac{\text{absolute change in epigenetic age}}{\text{days elapsed}}\right)$. Mice with less than 27 days between time points were excluded from this longitudinal analysis

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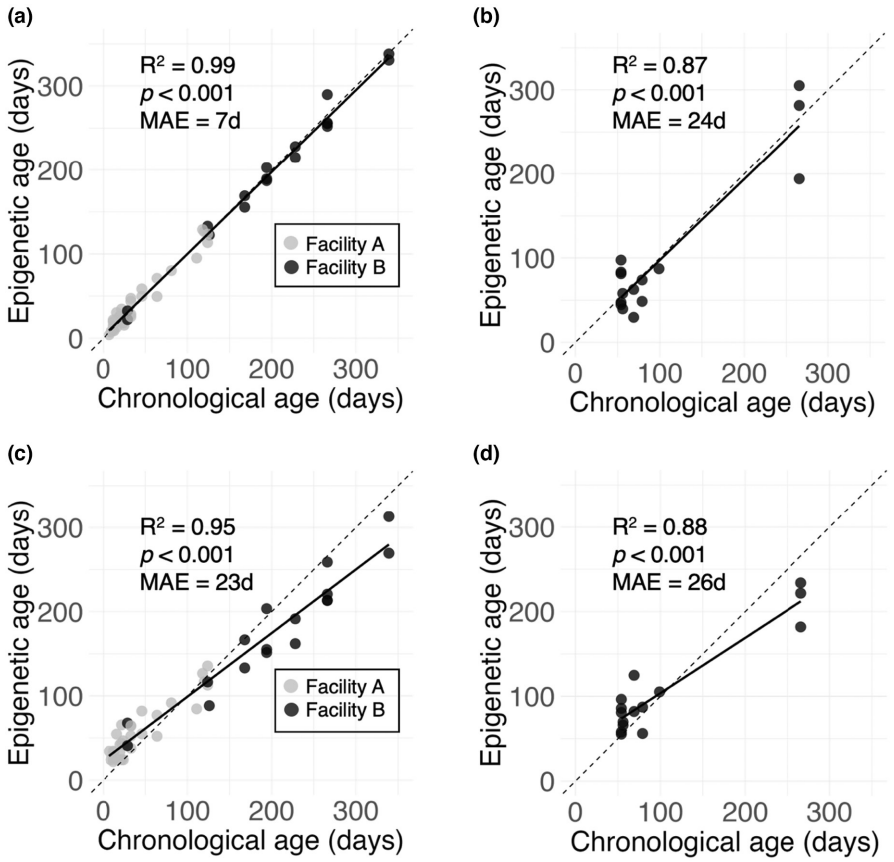

FIGURE 1 The relationship between DNA methylation based (epigenetic) age and chronological age in C57BL/6 mice (a, c) used to train the epigenetic clock model or (b, d) an independent set of mice (a validation set) not used in building the epigenetic clock model. Two epigenetic clocks were built: one with 22 CpG sites from five genes (Clock 1) (a, b) and one with 11 CpG sites from two genes (Clock 2) (c, d). Circles represent individual mice, circle colour in (a) indicates animal facility (*grey* = facility A, *black* = facility B). Solid lines are linear regression lines, while dashed lines are a reference line of $y = x$ (the hypothetical relationship if chronological age and epigenetic age estimates were exactly equivalent).

as the mean absolute error (MAE) of the clock in the validation dataset was 26 days (Figure 1b).

To explore whether laboratory and wild mice might differ in methylation levels across CpG sites from targeted genes that showed parallel trends in the laboratory and the wild (i.e. genes that decreased/increased in methylation with body mass in both systems: *Hsp4*, *Gm9312*, *Kcns1*, *Gm7325*; gene *Prima1* was excluded since it increased in methylation in wild mice but decreased in laboratory mice; Figure S1), we used body mass as a proxy of age. While the reliability of body mass as an indicator of age declines after initial growth during the first few weeks of life, it continues to increase with chronological age in both C57BL/6 laboratory and wild mice beyond this time and thus can be used as a rough estimate of age in adults as well (Figure S5; Gerber et al., 2021; Gray et al., 2015; JAX, 2022a, 2022b). We used a Bayesian regression model run with function brm in R package brms (Bürkner, 2017) with methylation level as the response variable, and source (laboratory/wild) and body mass as predictors. The model also included an animal ID random effect since the data contained ≥1 sample per individual, as well as a nested random effect Gene/Position to account for measurement of methylation at multiple positions within targeted genes. Further, we used a generalized additive model to test whether source (laboratory/wild) predicted epigenetic age. The following models were compared to explore potential non-linearities in the relationship between epigenetic age and body mass, and whether this relationship varied by source: (1) gam(Epigenetic age ~ Body mass), (2) gam(Epigenetic age ~ s(Body mass)), (3) gam(Epigenetic age ~ Body mass + Source), (4) gam(Epigenetic age ~ s(Body mass) + Source), (5) gam(Epigenetic age ~ Body mass * Source) and (6) gam(Epigenetic age + s(Body mass, by = Source)). Model 5, with a linear body mass efect that varied by source, had the best model fit (assessed from GCV, AIC and adjusted *R*-squared values) and was then used to test whether source predicted epigenetic age. Female wild mice with signs of ongoing or recent pregnancy (those recorded as suspected pregnant, $n = 9$), were excluded from these models including body mass, as body mass will be a less accurate age proxy in these individuals. Who were significantly heavier than other females (one sample per animal ID, $n = 48$; linear model, $F_{1,54} = 29.28$, $p < .001$).

Finally, to explore whether the *rate* of epigenetic ageing $\left(\frac{\text{absolute change in epigenetic age}}{\text{days elapsed}}\right)$ differed between laboratory and wild mice we used two methods. First, we tested whether the mean rate of epigenetic ageing differed between laboratory and wild mice. To do this we constructed a linear model using data from repeat-sampled mice, where rate of epigenetic ageing was the response and source (laboratory/wild) was the predictor, while including body mass and sex as covariates. All repeat-sampled laboratory mice were adults, whereas repeat sampled wild mice included animals classed as both adult and juvenile at the first sampling point. As such, we also repeated this analysis excluding all juveniles for comparability. Second, we used Levene's test to ask whether the rate of epigenetic ageing differed according to (1) age category in wild mice (binary assessment; juvenile vs. sub-adult/adult) or (2) source (laboratory vs. wild).

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2.5 | Ethics statement

Wild mouse work was conducted under Home Office licence PPL PB0178858 held at the University of Oxford and with research permits from the Islands Conservation Advisory Committee (ICAC) and Natural Resources Wales.

3 | RESULTS

3.1 | Measurement of CpG site methylation levels from faecal samples

We used non-invasively collected faecal samples from laboratory and wild house mice (*M. musculus* and *M. musculus domesticus* respectively) as a source of host DNA for the measurement of methylation levels at specific CpG sites. A sufficient amount of host DNA was extracted and subsequently sequenced despite use of a microbial DNA purification kit: methylation levels were successfully measured across all 73 CpG sites from the targeted house mouse genes (*Hsf4*, *Gm9312*, *Kcns1*, *Gm7325* and *Prima1*; Table S2) in all samples from laboratory mice ($n=80$) and in 81% (183 out of 226) samples from wild mice (mean read depth per gene ranged 5,703–16,849 across samples). Methylation levels were successfully measured in 96% (217 out of 226) wild mouse samples when considering only those CpG sites included in the clock (Clock 2) subsequently used to predict age in wild mice (11 CpG sites from genes *Hsf4* and *Kcns1*, see below). Repeatability of the method was assessed with 11 wild mouse DNA extractions processed twice (see Section 2). Repeatability of methylation levels was 0.674 [standard error (SE)=0.052, $p<.001$], while repeatability of epigenetic age estimates was 0.929 (SE=0.072, $p<.001$).

3.2 | Construction of a non-invasive epigenetic clock

We first built an epigenetic clock using samples from C57BL/6 laboratory mice ($n=50$, one sample per animal) to generate a targeted epigenetic clock using methylation levels from 73 CpG sites across five genes that were previously associated with age in laboratory mice (*Hsf4*, *Gm9312*, *Kcns1*, *Gm7325* and *Prima1*; Table S1; Han et al., 2018). Elastic net regression identified 22 CpG sites from the five targeted genes that exhibited variability in methylation patterns with age; three from *Hsf4*, six from *Gm9312*, six from *Kcns1*, one from *Gm7325* and one from *Prima1* (Table S3). This epigenetic clock (Clock 1) had a mean absolute error (MAE) of 7 days (~0.7% of expected C57BL/6 life span; Schultz et al., 2020) in the training set (Pearson's $r=.996$, $p<.001$; Figure 1a). We validated the clock by applying it to an independent set of C57BL/6 mice that were not used in training the clock ($n=15$). Among these laboratory mice (one sample per animal), epigenetic age was also strongly correlated with chronological age (Pearson's $r=.935$, $p<.001$, MAE=24 days; Figure 1b). Neither sex nor sequencing run had a significant effect on epigenetic age (linear mixed effects model with two samples per animal: chronological age $F=111.526$, $p<.001$; sex $F=0.046$, $p=.833$; sequencing run $F=1.602$, $p=.217$; animal ID included as a random effect). This demonstrates that non-invasive faecal samples can be used to generate an epigenetic clock in laboratory mice with equivalent or higher accuracy in estimating chronological age compared to a clock previously derived using blood samples (Han et al., 2018; MAE=35–41 days in two validation datasets).

As our aim was to develop a laboratory-based epigenetic clock that could be applied to wild mice, we built a second clock that did not include CpG sites that either showed non-parallel methylation level patterns across the two systems or for which we failed to acquire methylation levels in a substantial number of wild mouse samples (see Methods for more detail). For this clock (Clock 2), elastic net regression identified 11 CpG sites from genes *Hsp4* and *Kcns1* (Table S4), 7 of which were also included in the first clock (Table S3). Here, the slope deviated more from 1 (where 1 would indicate perfect positive linear relationship between chronological and epigenetic age) than did the first clock (slope estimate 0.756 ± 0.024 standard error vs. 0.976 ± 0.013 standard error in training set; Figure 1a,c). However, the clock still had a high accuracy with a MAE of 23 days in the training set (Pearson's $r=.977$, $p<.001$; Figure 1c) and 26 days in the validation set (Pearson's $r=.938$, $p<.001$; Figure 1d). This second clock was then used for further analyses in this study.

3.3 | Chronological age prediction in wild mice

We next applied Clock 2 (Figure 1c,d) to 201 faecal samples from 118 wild house mice to test if it could be used to estimate chronological age in wild individuals of unknown age. Mice of all available body sizes were included with the aim of capturing as much age variation as possible (body mass range 5.9–43.0 g, mean 18.8, median 19.2). The epigenetic age of wild mice varied from −18 to 426 days [mean 213, median 193; 2 out of 201 samples (1%) had a negative epigenetic age].

Epigenetic age varied significantly between mice from different age categories that were assigned in the field using external characteristics (juvenile/sub-adult/adult; ANOVA, $F_{2,191}=131$, $p<.001$; Figure 2a). Moreover, among 35 wild mice sampled twice between 30 and 340 days apart (mean 127, median 79), majority ($n=30$; 86%) were epigenetically older at the latter time point (one-tailed binomial test for H_0 $p=.5$, $p<.001$). In general, the number of days between sampling time points positively predicted change in epigenetic age (linear model, $F_{1,28}=7.814$, $p=.009$), but there was wide variation in the slope observed among individuals (Figure 2b). Rate of epigenetic ageing $\left(\frac{\text{absolute change in epigenetic age}}{\text{days elapsed}}\right)$ was not predicted by any investigated variables (linear model with log-transformed response variable; trapping area $F_{1,23}=1.878$, $p=.184$; season $F_{2,23}=2.307$, $p=.122$; sex $F_{1,23}=0.237$, $p=.631$; reproductive activity $F_{1,23}=1.790$, $p=.194$ and body mass $F_{1,23}=0.013$, $p=.909$ at first sampling point). Together these results indicate that an epigenetic clock trained with samples from inbred laboratory mice can be used

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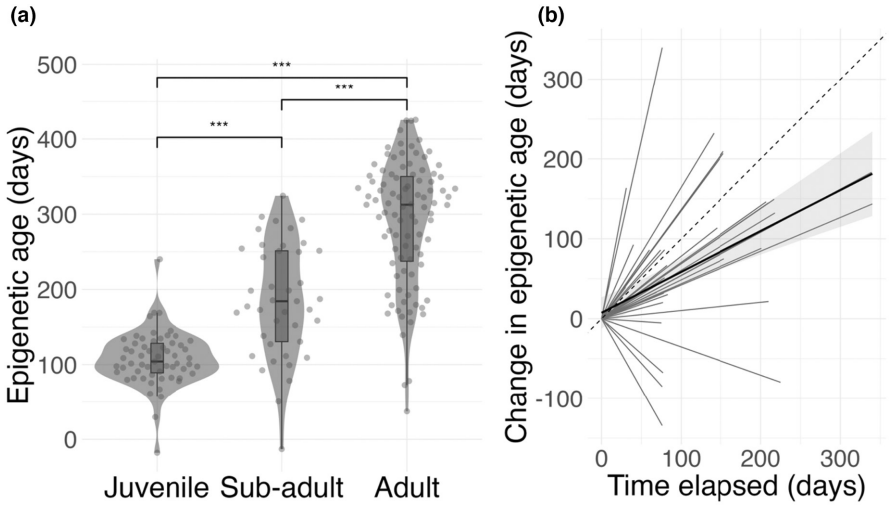

FIGURE 2 (a) Epigenetic age of wild mice phenotypically characterized as juvenile ($n = 62$), sub-adult ($n = 37$) or adult ($n = 95$) predicted with an epigenetic clock built using C57BL/6 laboratory mice. Age category was assigned in the field using body size and appearance (see Section 2). Median epigenetic age was 109 days in juveniles, 179 days in sub-adults and 296 days in adults. Statistical differences between different age categories were tested with Wilcoxon rank sum tests (***$p < .001$). (b) Change in epigenetic age between two time points in wild mice sampled twice between 30 and 340 days apart ($n = 35$). Grey lines represent individual mice, black line is a linear regression line with 95% confidence interval bands and the dashed line is a reference line of $y = x$ (the hypothetical relationship if chronological age and epigenetic age estimates were exactly equivalent). Epigenetic age increased with time for 30 (86%) of 35 mice.

to provide an estimate of chronological age in outbred wild mice, though not one that is highly accurate.

3.4 | Multiple times higher methylation levels in wild compared to laboratory mice

To investigate whether wild mice had different methylation levels to laboratory mice in early life and beyond, we explored the relationship between methylation levels and body mass across mice of all sizes. Methylation levels were strongly predicted by source [brm model; source, posterior mean 1.10, 95% credible interval (CIs) 0.90–1.31; body mass, posterior mean 0.05, 95% CI 0.05–0.06; model includes animal ID random effect as well as a nested random effect Gene/Position], such that wild mice had higher levels of methylation in the genes showing parallel methylation trends across laboratory and wild mice (Figures S1–S4).

We next assessed whether this higher methylation level in wild mice resulted in higher epigenetic age for a given chronological age compared to laboratory mice. In the absence of known chronological age for wild mice, we used body mass to provide an upper limit age estimate for individuals classed as juveniles. Others have reported that 12- to 13-day-old wild house mice from mainland Europe weigh around 7 g (range 3.6–10.5 g, mean 6.8; Gerber et al., 2021) and another study showed that 14-day-old wild-derived but captive house mice from Gough Island (home to the largest wild house mice recorded) weigh around 8.5 g (range ~7–10.5 g, raw data not available; Gray et al., 2015; Figure S5). Thus, irrespective of context, house mice between 12 and 14 days typically are expected to weigh 7–10.5 g (Gerber et al., 2021; Gray et al., 2015). We therefore examined epigenetic age from a random cross-sectional set of juvenile wild Skokholm Island mice that fall within this body mass range and thus which we expect to be no more than 25 days old ($n = 19$, body mass 6.1–10.4 g with a mean of 8.5 g; Figure S5). Among these individuals, the mean epigenetic age estimate was 106 days [range 18–169, median 104; one sample had a negative epigenetic age; for comparison, the epigenetic age in laboratory mice <14 days of age ($n = 7$) ranged 22–34 with a mean of 29 days], which is more than four times their expected chronological age (Figure S5). Looking across wild mice of all body masses, the same pattern was maintained, with wild mice having higher epigenetic age estimates than laboratory mice (generalized additive model; body mass, $F = 38.10$, $p < .001$, body mass * source, $F = 13.40$, $p < .001$; Figure 3a).

To further examine whether older epigenetic age profiles among wild mice might be due to accelerated ageing through exposure to environmental stressors, such as food shortage or climatic variation, we studied the rate of epigenetic ageing across laboratory and wild mice for which two time points were available (laboratory $n = 15$; wild $n = 35$). While the rate of epigenetic ageing appeared slightly shallower in wild mice (Figure 3b), source did not predict rate of epigenetic ageing (linear model: source, $F_{1,39} = 0.010$, $p = .923$; controlling for sex, $F_{1,39} = 0.408$, $p = .527$ and body mass at first time point, $F_{1,39} = 0.105$, $p = .747$). Laboratory mice included in this analysis were all adults, while the longitudinal data of wild mice included adult and juvenile mice; however, exclusion of juvenile wild mice (≤15 g of body mass and reproductively inactive, $n = 8$) did not appear to have a strong effect on these trends (linear model: source, $F_{1,31} = 0.004$, $p = .948$; controlling for sex, $F_{1,31} = 0.213$, $p = .648$ and body mass at first time point, $F_{1,31} = 0.226$, $p = .638$). Further, although the variance in estimates of epigenetic ageing rates was lower for juvenile

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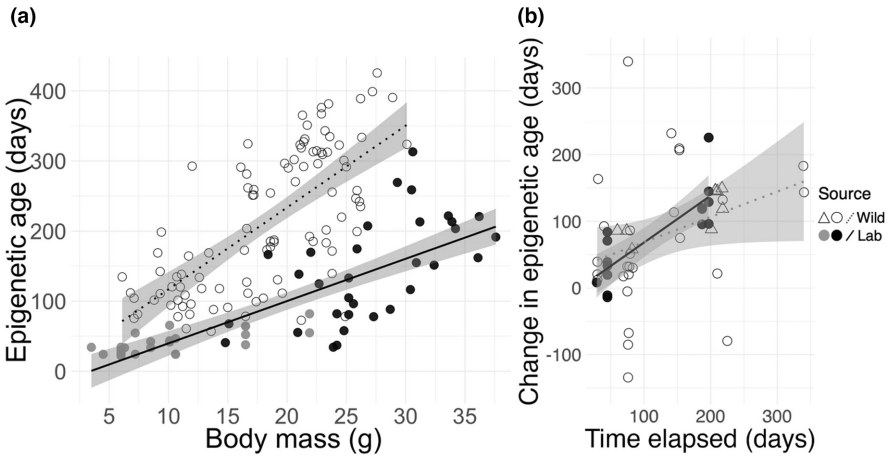

FIGURE 3 Wild mice have elevated epigenetic age compared to laboratory mice for a given body mass, but do not show a different rate of epigenetic ageing in adulthood. (a) Epigenetic age of wild ($n = 99$) and laboratory mice ($n = 57$) in relation to body mass. Empty circles are wild mice and filled circles are laboratory mice; black circles are laboratory mice for which body mass was recorded during sample collection and grey circles are laboratory mice for which body mass was estimated post hoc (see Section 2). (b) Change in epigenetic age in relation to time elapsed in laboratory ($n = 15$) and wild mice ($n = 35$) sampled twice a minimum of 26 days apart. Body mass varied 19.2–26.3 g for laboratory mice and 7.0–37.7 g for wild mice. Empty triangles ($n = 8$) are wild mice classed as 'juvenile' at the first time point, empty circles ($n = 27$) are wild mice classed as 'sub-adult' or 'adult' at the first time point and filled circles are laboratory mice. Lines are linear regression lines (*dashed* = wild mice, *solid* = laboratory mice) and shading indicates 95% confidence intervals.

than non-juvenile (sub-adult/adult) wild mice, this variance difference was not statistically significant (Levene's test, $F_{1,33} = 1.770$, $p = .193$; Pearson's correlation between change in epigenetic age and days elapsed: juvenile wild mice, $r = .79$, $p = .021$; non-juvenile wild mice, $r = .32$, $p = .102$; Figure 3b). Similarly, higher variation in epigenetic ageing rates observed in wild compared to laboratory mice was not statistically significant (Levene's test, $F_{1,48} = 3.428$, $p = .070$; Figure 3b).

4 | DISCUSSION

Here, we tested an approach for estimating age in wild house mice by building an epigenetic clock using samples from inbred C57BL/6 laboratory mice and using it to estimate age in outbred wild mice of unknown chronological age. Faecal samples were used as a source of host DNA and proved suitable for measuring of DNA methylation and epigenetic age, indicating their potential as a non-invasive alternative to the blood or tissue samples more commonly used in epigenetic clocks (Han et al., 2018). The clock effectively distinguished wild juveniles from adults and typically showed increases in predicted age over time among repeat-captured individuals. The success rate of the latter (86% individuals predicted older at a later time point) was similar to what has been previously been detected in a wild baboon study (Anderson et al., 2021).

However, while the clock accurately predicted age in an independent set of laboratory mice (with error of ±26 days; ~3% of expected C57BL/6 life span), we observed high variation among wild mice in how their epigenetic age changed over chronological time, suggesting our clock had far less accuracy in predicting chronological age in this different ecological context. Others have had better success in applying clocks built with captive individuals to wild individuals (e.g. Mayne et al., 2022 in green turtles; Robeck et al., 2021 in cetaceans; correlation between chronological and epigenetic age in these studies 0.67–0.98 vs. 0.36 between change in time and change in epigenetic age in our study). However, these studies have built epigenetic clocks using samples from captive individuals where there is genetic and environmental variation, such as animals from zoos or outdoor enclosures. Our clock was built with samples from inbred laboratory mice housed under very stable environmental conditions, but applied to wild mice that are outbred and exposed to a highly variable temperate climate. Studies of different laboratory strains have confirmed that epigenetic clocks may behave differently in different genetic backgrounds. For instance, Han et al. showed that DBA/2 mice were up to twice as old epigenetically as C57BL/6 mice (Han et al., 2018). Moreover, DNA methylation may be influenced by inbreeding (Han et al., 2021; Venney et al., 2016) and environmental factors (Parrott et al., 2014; Viitaniemi et al., 2019; Zocher et al., 2021) and wild animals are generally exposed to more variable environments than their inbred laboratory counterparts. As such, the contrasting genetic and environmental backgrounds in our mouse systems may partly explain why age estimates in wild mice based on a clock from laboratory mice had low accuracy.

Technical factors may have also contributed to the low accuracy of a laboratory animal-based clock when used to estimate age in wild animals. The accuracy of chronological age prediction particularly for older mice might have been affected by the relatively lower number of laboratory mice aged over 3 months in the training set. This is because the clock could be more inclined to capture patterns prevalent in younger mice, potentially resulting in an incomplete representation of the diverse epigenetic changes associated with ageing later in life. Further, while all samples from laboratory mice

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were preserved immediately after defecation, the time between defecation and sample preservation varied in wild mice (where samples were collected from traps which animals had been in overnight, up to 13 h). It is possible some DNA degradation occurred before the samples were preserved in a stabilizing buffer, affecting the methylation profiles. It is also possible that host cell profiles vary to some extent between faecal samples from laboratory and wild mice. Since methylation levels vary between tissue types (Han et al., 2018), differences in faecal cell profiles could have contributed to our findings on methylation levels in laboratory versus wild mice.

As our study is the first to develop a laboratory-based epigenetic clock based on faecal samples and apply it to a wild setting, we cannot assess whether the relatively low accuracy in chronological ageing we achieved is species or sample type specific. It is also possible that epigenetic changes with age are comparable across settings (laboratory and wild), but that biological ageing varies more in the wild than it does in the laboratory, obscuring any chronological signal in epigenetic markers. Further studies would be needed to understand whether this is the case and whether laboratory-based clocks might still hold value for estimating either chronological or biological ageing in wild animals. An alternative clock to ours could be trained using samples from captive individuals with greater genetic diversity, such as more genetically diverse laboratory mice rather than an inbred strain or using samples from free-living populations where chronological age can be accurately estimated, e.g. outbred semi-natural populations where individuals can be tracked from birth (Gerber et al., 2021).

The epigenetic age of wild mice from Skokholm Island varied from −18 to 426 days [mean 213, median 193; 2 (1% of all 201) samples had negative epigenetic ages, −18 and −13 days]. The presence of negative predicted ages in wild individuals may be due to measurement error, but it may also be due to the inherent biological differences in age-related epigenetic changes in wild versus laboratory populations.

Despite our epigenetic clock very accurately predicting the chronological age of laboratory mice, several lines of evidence suggest that in wild mice, our epigenetic age estimates were overestimates of chronological age. First, among juveniles, for which body mass is an accurate predictor of age across both wild and laboratory mice (Gerber et al., 2021; Gray et al., 2015; JAX, 2022a, 2022b), epigenetic age estimates were several times higher than their expected chronological age from body mass (Gerber et al., 2021; Gray et al., 2015). Second, we found that wild mice exhibited higher levels of CpG site methylation (and subsequently several times higher estimates of epigenetic age) across all body masses, compared to laboratory mice. While accelerated weight gain in ad libitum-fed laboratory mice may contribute to lower methylation levels among adult laboratory mice compared to wild mice, it may also be that a more challenging environment experienced by wild mice increases methylation and consequently accelerates epigenetic clocks. Our comparison of methylation levels and epigenetic age of wild versus laboratory mice is specific to a comparison with the C57BL/6 strain. However, it is perhaps noteworthy that the approximately 5- to 10-fold difference in epigenetic age between C57BL/6 laboratory mice and wild mice found here is larger than the previously reported twofold difference in epigenetic age between C57BL/6 mice and another inbred laboratory strain, DBA/2 (Han et al., 2018).

To test whether the older epigenetic age profile of wild mice could be explained by accelerated ageing post-weaning (i.e. from when they are trappable), we investigated the rate of epigenetic ageing using individuals captured and sampled twice over time. If anything, the rate of epigenetic ageing appeared slightly slower and more variable in wild mice, though this observation relied on a small sample size and was not statistically significant. Various factors can influence methylation levels and these factors could differ between the laboratory and wild settings here, such as abiotic factors (e.g. temperature), inbreeding (Han et al., 2021; Venney et al., 2016) and food shortage (as caloric restriction may slow epigenetic ageing, Maegawa et al., 2017; Hahn et al., 2017). Moreover, as we observed heightened epigenetic age in wild compared to laboratory mice even during the first ~2 weeks of life, we speculate that peri- and early postnatal effects on offspring DNA methylation may vary between laboratory and wild mice. Various human, mouse and other animal studies have demonstrated an association between prenatal maternal experience (such as food shortage, diet, infection, substance exposure and stress) and offspring DNA methylation patterns, with differences from the prenatal (foetal) phase still detectable in later life (Camerota et al., 2021; Heijmans et al., 2008; Joubert et al., 2016; Kertes et al., 2016; Lan et al., 2013; Richetto et al., 2017; Tobi et al., 2009; Vangeel et al., 2017).

In our present study we used targeted sequencing to measure methylation levels in genes of interest. As such, while methylation levels are higher in wild than laboratory mice in these targeted genes, this may not be the case for genome-wide methylation. Further, when comparing methylation levels and epigenetic age estimates across laboratory and wild mice, we have used body mass as a proxy for age across laboratory and wild mice. Body mass is, however, only a rough proxy and mass–age relationships are likely to vary somewhat between laboratory and wild settings. To more definitively explore whether methylation levels and biological age for a given chronological age vary across these contexts, comparisons of epigenetic ageing patterns in (semi-)wild mice of known chronological age with these laboratory and wild populations would be very valuable.

While our approach of training an epigenetic clock with laboratory individuals and using it to estimate age in wild individuals did not allow accurate estimation of chronological age, our results demonstrate such an approach can still be effective in distinguishing between juvenile and adult individuals. Such information may be useful in contexts where a faecal deposit is found but the individual is not observed, such as in field-based projects of animals that are hard or impossible to capture. At the same time, this method can provide interesting insights into biological ageing when applied to wild animals of known chronological age or to individuals sampled longitudinally such that changes in epigenetic age can be estimated (Brivio et al., 2015; De Paoli-Iseppi et al., 2017; Powell & Proulx, 2003). Considering the much greater variability in epigenetic

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ageing rates we observed in wild compared to laboratory animals, our results suggest wild systems may provide an informative environment in which to study drivers of epigenetic age acceleration. Our current study did not have sufficient power to ask why wild individuals might vary so widely in epigenetic ageing rates and most longitudinally sampled individuals were adults. Further work using a larger sample size and greater coverage of different life stages would be valuable to systematically explore potential drivers of this fascinating variation.

In summary, our data indicate the potential to use a non-invasive, DNA methylation-based epigenetic clock built with samples from laboratory mice to estimate the age of wild mice. While this approach did not provide highly accurate estimates of chronological age, it can be used to measure variation in biological ageing in future longitudinal studies, making it a promising tool for studies of ontogeny and senescence in wild settings.

AUTHOR CONTRIBUTIONS

EH and SCLK set up the wild mouse study system. EH, SJ, AR and KW collected the samples. AO and TJL developed the software Apollo. EH conducted the laboratory work and analysed the data with support from TJL, SCLK and AR. EH wrote the manuscript with contributions from all authors.

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CONFLICT OF INTEREST STATEMENT

Authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Data and R scripts are available at https://github.com/eveliinahanski/musmus_epiage

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REFERENCES

Ambatipudi, S., Horvath, S., Perrier, F., Cuenin, C., Hernandez-Vargas, H., le Calvez-Kelm, F., Durand, G., Byrnes, G., Ferrari, P., Bouaoun, L., Sklias, A., Chajes, V., Overvad, K., Severi, G., Baglietto, L., Clavel-Chapelon, F., Kaaks, R., Barrdahl, M., Boeing, H., … Herceg, Z. (2017). DNA methylome analysis identifies accelerated epigenetic ageing associated with postmenopausal breast cancer susceptibility. *European Journal of Cancer*, *75*, 299–307. https://doi.org/10.1016/j.ejca.2017.01.014

Anderson, J. A., Johnston, R. A., Lea, A. J., Campos, F. A., Voyles, T. N., Akinyi, M. Y., Alberts, S. C., Archie, E. A., & Tung, J. (2021). High social status males experience accelerated epigenetic ageing in wild baboons. *eLife*, *10*, e66128. https://doi.org/10.7554/eLife.66128

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Bors, E. K., Baker, C. S., Wade, P. R., O'Neill, K. B., Shelden, K. E. W., Thompson, M. J., Fei, Z., Jarman, S., & Horvath, S. (2021). An epigenetic clock to estimate the age of living beluga whales. *Evolutionary Applications*, *14*(5), 1263–1273. https://doi.org/10.1111/eva.13195

Brivio, F., Grignolio, S., Sica, N., Cerise, S., & Bassano, B. (2015). Assessing the impact of capture on wild animals: The case study of chemical immobilisation on alpine ibex. *PLoS One*, *10*(6), e0130957. https://doi.org/10.1371/journal.pone.0130957

Bürkner, P.-C. (2017). brms: An R package for Bayesian multilevel models using Stan. *Journal of Statistical Software*, *80*(1), 1–28. https://doi.org/10.18637/jss.v080.i01

Camerota, M., Graw, S., Everson, T. M., McGowan, E. C., Hofheimer, J. A., O'Shea, T. M., Carter, B. S., Helderman, J. B., Check, J., Neal, C. R., Pastyrnak, S. L., Smith, L. M., Dansereau, L. M., DellaGrotta, S. A., Marsit, C. J., & Lester, B. M. (2021). Prenatal risk factors and neonatal DNA methylation in very preterm infants. *Clinical Epigenetics*, *13*(1), 171. https://doi.org/10.1186/s13148-021-01164-9

Cao, X., Li, W., Wang, T., Ran, D., Davalos, V., Planas-Serra, L., Pujol, A., Esteller, M., Wang, X., & Yu, H. (2022). Accelerated biological ageing in COVID-19 patients. *Nature Communications*, *13*(1), 2135. https://doi.org/10.1038/s41467-022-29801-8

De Paoli-Iseppi, R., Deagle, B. E., McMahon, C. R., Hindell, M. A., Dickinson, J. L., & Jarman, S. N. (2017). Measuring animal age with DNA methylation: From humans to wild animals. *Frontiers in Genetics*, *8*, 106. https://doi.org/10.3389/fgene.2017.00106

Fairfield, E. A., Richardson, D. S., Daniels, C. L., Butler, C. L., Bell, E., & Taylor, M. I. (2021). Ageing European lobsters (*Homarus gammarus*) using DNA methylation of evolutionarily conserved ribosomal DNA. *Evolutionary Applications*, *14*(9), 2305–2318. https://doi.org/10.1111/eva.13296

Friedman, J., Hastie, T., & Tibshirani, R. (2010). Regularization paths for generalized linear models via coordinate descent. *Journal of Statistical Software*, *33*(1), 1–22.

Gardner, S. T., Bertucci, E. M., Sutton, R., Horcher, A., Aubrey, D., & Parrott, B. B. (2023). Development of DNA methylation-based epigenetic age predictors in loblolly pine (*Pinus taeda*). *Molecular Ecology Resources*, *23*(1), 131–144. https://doi.org/10.1111/1755-0998.13698

Georgountzou, A., & Papadopoulos, N. G. (2017). Postnatal innate immune development: From birth to adulthood. *Frontiers in Immunology*, *8*, 957. https://doi.org/10.3389/fimmu.2017.00957

Gerber, N., Auclair, Y., König, B., & Lindholm, A. K. (2021). Population density and temperature influence the return on maternal investment in wild house mice. *Frontiers in Ecology and Evolution*, *8*, 602359. https://doi.org/10.3389/fevo.2020.602359

Gray, M. M., Parmenter, M. D., Hogan, C. A., Ford, I., Cuthbert, R. J., Ryan, P. G., Broman, K. W., & Payseur, B. A. (2015). Genetics of rapid and extreme size evolution in Island mice. *Genetics*, *201*(1), 213–228. https://doi.org/10.1534/genetics.115.177790

Hahn, O., Grönke, S., Stubbs, T. M., Ficz, G., Hendrich, O., Krueger, F., Andrews, S., Zhang, Q., Wakelam, M. J., Beyer, A., Reik, W., & Partridge, L. (2017). Dietary restriction protects from age-associated DNA methylation and induces epigenetic reprogramming

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of lipid metabolism. *Genome Biology*, *18*(1), 56. https://doi.org/10.1186/s13059-017-1187-1

Han, T., Wang, F., Song, Q., Ye, W., Liu, T., Wang, L., & Chen, Z. J. (2021). An epigenetic basis of inbreeding depression in maize. *Science Advances*, *7*(35), eabg5442. https://doi.org/10.1126/sciadv.abg5442

Han, Y., Eipel, M., Franzen, J., Sakk, V., Dethmers-Ausema, B., Yndriago, L., Izeta, A., de Haan, G., Geiger, H., & Wagner, W. (2018). Epigenetic age-predictor for mice based on three CpG sites. *eLife*, *7*, e37462. https://doi.org/10.7554/eLife.37462

Harvanek, Z. M., Fogelman, N., Xu, K., & Sinha, R. (2021). Psychological and biological resilience modulates the effects of stress on epigenetic ageing. *Translational Psychiatry*, *11*(1), 601. https://doi.org/10.1038/s41398-021-01735-7

Heijmans, B. T., Tobi, E. W., Stein, A. D., Putter, H., Blauw, G. J., Susser, E. S., Slagboom, P. E., & Lumey, L. H. (2008). Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(44), 17046–17049. https://doi.org/10.1073/pnas.0806560105

Ito, H., Udono, T., Hirata, S., & Inoue-Murayama, M. (2018). Estimation of chimpanzee age based on DNA methylation. *Scientific Reports*, *8*(1), 9998. https://doi.org/10.1038/s41598-018-28318-9

Jarman, S. N., Polanowski, A. M., Faux, C. E., Robbins, J., de Paoli-Iseppi, R., Bravington, M., & Deagle, B. E. (2015). Molecular biomarkers for chronological age in animal ecology. *Molecular Ecology*, *24*(19), 4826–4847. https://doi.org/10.1111/mec.13357

JAX®. (2022a). *Body weight information for aged C57BL/6J*. https://www.jax.org/jax-mice-and-services/strain-data-sheet-pages/body-weight-chart-aged-b6

JAX®. (2022b). *Body weight information for C57BL/6J*. https://www.jax.org/jax-mice-and-services/strain-data-sheet-pages/body-weight-chart-000664

Joubert, B. R., Felix, J. F., Yousefi, P., Bakulski, K. M., Just, A. C., Breton, C., Reese, S. E., Markunas, C. A., Richmond, R. C., Xu, C. J., Küpers, L. K., Oh, S. S., Hoyo, C., Gruzieva, O., Söderhäll, C., Salas, L. A., Baïz, N., Zhang, H., Lepeule, J., … London, S. J. (2016). DNA methylation in newborns and maternal smoking in pregnancy: Genome-wide consortium meta-analysis. *American Journal of Human Genetics*, *98*(4), 680–696. https://doi.org/10.1016/j.ajhg.2016.02.019

Joyce, B. T., Gao, T., Zheng, Y., Ma, J., Hwang, S. J., Liu, L., Nannini, D., Horvath, S., Lu, A. T., Bai Allen, N., Jacobs, D. R., Jr., Gross, M., Krefman, A., Ning, H., Liu, K., Lewis, C. E., Schreiner, P. J., Sidney, S., Shikany, J. M., … Lloyd-Jones, D. (2021). Epigenetic age acceleration reflects long-term cardiovascular health. *Circulation Research*, *129*(8), 770–781. https://doi.org/10.1161/CIRCRESAHA.121.318965

Kerepesi, C., Meer, M. V., Ablaeva, J., Amoroso, V. G., Lee, S. G., Zhang, B., Gerashchenko, M. V., Trapp, A., Yim, S. H., Lu, A. T., Levine, M. E., Seluanov, A., Horvath, S., Park, T. J., Gorbunova, V., & Gladyshev, V. N. (2022). Epigenetic ageing of the demographically non-ageing naked mole-rat. *Nature Communications*, *13*(1), 355. https://doi.org/10.1038/s41467-022-27959-9

Kertes, D. A., Kamin, H. S., Hughes, D. A., Rodney, N. C., Bhatt, S., & Mulligan, C. J. (2016). Prenatal maternal stress predicts methylation of genes regulating the hypothalamic-pituitary-adrenocortical system in mothers and newborns in the Democratic Republic of Congo. *Child Development*, *87*(1), 61–72. https://doi.org/10.1111/cdev.12487

Lan, X., Cretney, E. C., Kropp, J., Khateeb, K., Berg, M. A., Peñagaricano, F., Magness, R., Radunz, A. E., & Khatib, H. (2013). Maternal diet during pregnancy induces gene expression and DNA methylation changes in fetal tissues in sheep. *Frontiers in Genetics*, *4*, 49. https://doi.org/10.3389/fgene.2013.00049

Larison, B., Pinho, G. M., Haghani, A., Zoller, J. A., Li, C. Z., Finno, C. J., Farrell, C., Kaelin, C. B., Barsh, G. S., Wooding, B., Robeck, T. R., Maddox, D., Pellegrini, M., & Horvath, S. (2021). Epigenetic models developed for plains zebras predict age in domestic horses and endangered equids. *Communications Biology*, *4*(1), 1412. https://doi.org/10.1038/s42003-021-02935-z

Lemaître, J. F., Rey, B., Gaillard, J. M., Régis, C., Gilot-Fromont, E., Débias, F., Duhayer, J., Pardonnet, S., Pellerin, M., Haghani, A., Zoller, J. A., Li, C. Z., & Horvath, S. (2022). DNA methylation as a tool to explore ageing in wild roe deer populations. *Molecular Ecology Resources*, *22*(3), 1002–1015. https://doi.org/10.1111/1755-0998.13533

Maegawa, S., Lu, Y., Tahara, T., Lee, J. T., Madzo, J., Liang, S., Jelinek, J., Colman, R. J., & Issa, J. J. (2017). Caloric restriction delays age-related methylation drift. *Nature Communications*, *8*(1), 539. https://doi.org/10.1038/s41467-017-00607-3

Mayne, B., Mustin, W., Baboolal, V., Casella, F., Ballorain, K., Barret, M., Vanderklift, M. A., Tucker, A. D., Korbie, D., Jarman, S., & Berry, O. (2022). Age prediction of green turtles with an epigenetic clock. *Molecular Ecology Resources*, *22*(6), 2275–2284. https://doi.org/10.1111/1755-0998.13621

Moore, L. D., Le, T., & Fan, G. (2013). DNA methylation and its basic function. *Neuropsychopharmacology*, *38*(1), 23–38. https://doi.org/10.1038/npp.2012.112

Morales Berstein, F., McCartney, D. L., Lu, A. T., Tsilidis, K. K., Bouras, E., Haycock, P., Burrows, K., Phipps, A. I., Buchanan, D. D., Cheng, I., the PRACTICAL consortium, Martin, R. M., Smith, G. D., Relton, C. L., Horvath, S., Marioni, R. E., Richardson, T. G., & Richmond, R. C. (2022). Assessing the causal role of epigenetic clocks in the development of multiple cancers: A Mendelian randomization study. *eLife*, *11*, e75374. https://doi.org/10.7554/eLife.75374

Parrott, B. B., Bowden, J. A., Kohno, S., Cloy-McCoy, J. A., Hale, M. D., Bangma, J. T., Rainwater, T. R., Wilkinson, P. M., Kucklick, J. R., & Guillette, L. J. (2014). Influence of tissue, age, and environmental quality on DNA methylation in *Alligator mississippiensis*. *Reproduction*, *147*(4), 503–513. https://doi.org/10.1530/REP-13-0498

Peng, C., Cardenas, A., Rifas-Shiman, S. L., Hivert, M. F., Gold, D. R., Platts-Mills, T. A., Lin, X., Oken, E., Avila, L., Celedón, J. C., Weiss, S. T., Baccarelli, A. A., Litonjua, A. A., & DeMeo, D. L. (2019). Epigenetic age acceleration is associated with allergy and asthma in children in Project Viva. *The Journal of Allergy and Clinical Immunology*, *143*(6), 2263–2270.e14. https://doi.org/10.1016/j.jaci.2019.01.034

Pinho, G. M., Martin, J. G. A., Farrell, C., Haghani, A., Zoller, J. A., Zhang, J., Snir, S., Pellegrini, M., Wayne, R. K., Blumstein, D. T., & Horvath, S. (2022). Hibernation slows epigenetic ageing in yellow-bellied marmots. *Nature Ecology & Evolution*, *6*(4), 418–426. https://doi.org/10.1038/s41559-022-01679-1

Polanowski, A. M., Robbins, J., Chandler, D., & Jarman, S. N. (2014). Epigenetic estimation of age in humpback whales. *Molecular Ecology Resources*, *14*(5), 976–987. https://doi.org/10.1111/1755-0998.12247

Powell, R. A., & Proulx, G. (2003). Trapping and marking terrestrial mammals for research: Integrating ethics, performance criteria, techniques, and common sense. *ILAR Journal*, *44*(4), 259–276. https://doi.org/10.1093/ilar.44.4.259

Prado, N. A., Brown, J. L., Zoller, J. A., Haghani, A., Yao, M., Bagryanova, L. R., Campana, M. G., E Maldonado, J., Raj, K., Schmitt, D., Robeck, T. R., & Horvath, S. (2021). Epigenetic clock and methylation studies in elephants. *Ageing Cell*, *20*(7), e13414. https://doi.org/10.1111/acel.13414

R Core Team. (2023). *R: A language and environment for statistical Computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Richetto, J., Massart, R., Weber-Stadlbauer, U., Szyf, M., Riva, M. A., & Meyer, U. (2017). Genome-wide DNA methylation changes in a mouse model of infection-mediated neurodevelopmental disorders. *Biological Psychiatry*, *81*(3), 265–276. https://doi.org/10.1016/j.biopsych.2016.08.010

13652394x, 2024, 8, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/mec.17330 by University of Turku, Wiley Online Library on [09/04/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Robeck, T. R., Fei, Z., Lu, A. T., Haghani, A., Jourdain, E., Zoller, J. A., Li, C. Z., Steinman, K. J., DiRocco, S., Schmitt, T., Osborn, S., Van Bonn, B., Katsumata, E., Mergl, J., Almunia, J., Rodriguez, M., Haulena, M., Dold, C., & Horvath, S. (2021). Multi-species and multi-tissue methylation clocks for age estimation in toothed whales and dolphins. *Communications Biology*, *4*(1), 642. https://doi.org/10.1038/s42003-021-02179-x

Schultz, M. B., Kane, A. E., Mitchell, S. J., MacArthur, M. R., Warner, E., Vogel, D. S., Mitchell, J. R., Howlett, S. E., Bonkowski, M. S., & Sinclair, D. A. (2020). Age and life expectancy clocks based on machine learning analysis of mouse frailty. *Nature Communications*, *11*(1), 4618. https://doi.org/10.1038/s41467-020-18446-0

Spangenberg, E., Wallenbeck, A., Eklöf, A. C., Carlstedt-Duke, J., & Tjäder, S. (2014). Housing breeding mice in three different IVC systems: Maternal performance and pup development. *Laboratory Animals*, *48*(3), 193–206. https://doi.org/10.1177/0023677214531569

Stoffel, M. A., Nakagawa, S., & Schielzeth, H. (2017). rptR: Repeatability estimation and variance decomposition by generalized linear mixed-effects models. *Methods in Ecology and Evolution*, *8*, 1639–1644. https://doi.org/10.1111/2041-210X.12797

Sullivan, I. R., Adams, D. M., Greville, L. J. S., Faure, P. A., & Wilkinson, G. S. (2022). Big brown bats experience slower epigenetic ageing during hibernation. *Proceedings of the Biological Sciences*, *289*(1980), 20220635. https://doi.org/10.1098/rspb.2022.0635

Tangili, M., Slettenhaar, A. J., Sudyka, J., Dugdale, H. L., Pen, I., Palsbøll, P. J., & Verhulst, S. (2023). DNA methylation markers of age(ing) in non-model animals. *Molecular Ecology*, *32*(17), 4725–4741. https://doi.org/10.1111/mec.17065

Thompson, M. J., VonHoldt, B., Horvath, S., & Pellegrini, M. (2017). An epigenetic ageing clock for dogs and wolves. *Ageing*, *9*(3), 1055–1068. https://doi.org/10.18632/ageing.101211

Tobi, E. W., Lumey, L. H., Talens, R. P., Kremer, D., Putter, H., Stein, A. D., Slagboom, P. E., & Heijmans, B. T. (2009). DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. *Human Molecular Genetics*, *18*(21), 4046–4053. https://doi.org/10.1093/hmg/ddp353

Vangeel, E. B., Pishva, E., Hompes, T., van den Hove, D., Lambrechts, D., Allegaert, K., Freson, K., Izzi, B., & Claes, S. (2017). Newborn genome-wide DNA methylation in association with pregnancy anxiety reveals a potential role for *GABBR1*. *Clinical Epigenetics*, *9*, 107. https://doi.org/10.1186/s13148-017-0408-5

Venney, C. J., Johansson, M. L., & Heath, D. D. (2016). Inbreeding effects on gene-specific DNA methylation among tissues of Chinook salmon. *Molecular Ecology*, *25*(18), 4521–4533. https://doi.org/10.1111/mec.13777

Viitaniemi, H. M., Verhagen, I., Visser, M. E., Honkela, A., van Oers, K., & Husby, A. (2019). Seasonal variation in genome-wide DNA methylation patterns and the onset of seasonal timing of reproduction in great tits. *Genome Biology and Evolution*, *11*(3), 970–983. https://doi.org/10.1093/gbe/evz044

Wilkinson, G. S., Adams, D. M., Haghani, A., Lu, A. T., Zoller, J., Breeze, C. E., Arnold, B. D., Ball, H. C., Carter, G. G., Cooper, L. N., Dechmann, D. K. N., Devanna, P., Fasel, N. J., Galazyuk, A. V., Günther, L., Hurme, E., Jones, G., Knörnschild, M., Lattenkamp, E. Z., … Horvath, S. (2021). DNA methylation predicts age and provides insight into exceptional longevity of bats [published correction appears in Nat Commun. 2021 May 5;12(1):2652] [published correction appears in Nat Commun. 2022 Sep 7;13(1):5266]. *Nature Communications*, *12*(1), 1615. https://doi.org/10.1038/s41467-021-21900-2

Wright, P. G. R., Mathews, F., Schofield, H., Morris, C., Burrage, J., Smith, A., Dempster, E. L., & Hamilton, P. B. (2018). Application of a novel molecular method to age free-living wild Bechstein's bats. *Molecular Ecology Resources*, *18*(6), 1374–1380. https://doi.org/10.1111/1755-0998.12925

Yousefzadeh, M., Henpita, C., Vyas, R., Soto-Palma, C., Robbins, P., & Niedernhofer, L. (2021). DNA damage-how and why we age? *eLife*, *10*, e62852. https://doi.org/10.7554/eLife.62852

Zocher, S., Overall, R. W., Lesche, M., Dahl, A., & Kempermann, G. (2021). Environmental enrichment preserves a young DNA methylation landscape in the aged mouse hippocampus. *Nature Communications*, *12*(1), 3892. https://doi.org/10.1038/s41467-021-23993-1

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