Title

Elevated epigenetic age in wild compared to lab-raised house mice

Authors

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Competing interests

Authors declare no competing interests.

Abstract

Age is a key parameter in population ecology, with myriad biological processes changing with age as organisms develop early in life and 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, few studies have directly compared epigenetic age estimates between wild and captive or lab-reared animals of the same species, even though age-related epigenetic changes can be influenced by environmental conditions. Here, we built an epigenetic clock from standard laboratory house mice (C57BL/6, *Mus musculus)* and then used it to estimate age in a population of wild mice (*Mus musculus domesticus*) of unknown age. We show that this clock accurately predicts adult wild mice to be older than juveniles and that wild mice typically increase in epigenetic age over time, but with wide variation in epigenetic ageing rate among wild individuals. We further show that, for a given body mass, wild mice are epigenetically older than lab mice, and that this difference is not explained by accelerated ageing post-capture but is observed even among the smallest juvenile mice. This suggests different epigenetic age profiles in mice with contrasting genetic and environmental backgrounds arise very early in life and may be driven by peri- and postnatal effects on offspring DNA methylation.

Keywords

Epigenetic clock, DNA methylation, ageing, *Mus musculus*

Introduction

42 Age is a key characteristic for any organism, with numerous biological processes from immune
43 maturation to reproduction being age-related. Yet, measuring age in wild individuals can be
44 challenging as date of birth is often unknown. Classic methods for assessing age in wild animals
45 are often imprecise (e.g., body size) or destructive (e.g., eye lens weight), raising ethical concerns
46 and preventing longitudinal studies. An alternative approach for measuring the age of wild
47 individuals relies on the measurement of epigenetic marks in particular genomic regions.
48 Specifically, at some CpG sites (those where a cytosine is followed by a guanine; Moore et al.,
49 2013) the proportion of methylated cytosines appears to change linearly with age, and together
50 these sites can be used to derive an 'epigenetic clock'. An epigenetic clock trained using samples
51 from individuals of known age can then be used to predict age in individuals of unknown age.
52 Such epigenetic clocks can provide a more accurate estimate of chronological age among wild
53 animals than visible characteristics (Mayne et al., 2022; Larison et al., 2021). Epigenetic clocks
54 have now been developed for a wide range of animal species including baboons, chimpanzees,
55 humpback whales, wolves, green turtles, and zebras (Jarman et al., 2015; Anderson et al., 2021;
56 Pinho et al., 2022; De Paoli-Iseppi et al., 2017; Polanowski et al., 2014; Thompson et al., 2017;
57 Wright et al., 2018; Mayne et al., 2022; Larison et al., 2021; Bors et al., 2021; Sullivan et al., 2022;
58 Tangili et al., 2023; Ito et al., 2018; Fairfield et al., 2021; Wilkinson et al., 2021), as well as plants
59 (Gardner et al., 2023).
60
61 Alongside measuring chronological age, epigenetic clocks also appear to capture signals of
62 biological age, typically considered to reflect the accumulated damage and functional decline in
63 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 (Joyce et al., 2021; Morales Berstein et al., 2022; Ambatipudi et al., 2017; Harvanek et al., 2021; Cao 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 (Joyce et al., 2021; Morales Berstein et al., 2022; Ambatipudi et al., 2017; Harvanek et al., 2021; Cao et al., 2022; Peng et al., 2019), laboratory (Han et al., 2018; Kerepesi et al., 2022) or wild animals (Prado et al., 2021; Polanowski et al., 2014; Anderson et al., 2021; De Paoli-Iseppi et al., 2018; Lemaître et al., 2022). A number of studies have also included both wild and captive (e.g., from a zoo) individuals (Mayne et al., 2022; Robeck et al., 2021; Ito et al., 2018; Fairfield 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 lab 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 lab-based clock to predict age in house mice (*Mus musculus domesticus*) from a wild population. Our aims were threefold: (1) to see whether we could develop an epigenetic clock from

86 lab-based animals capable of accurately capturing differences in chronological age within a wild
87 population (2) to compare estimates of epigenetic age in lab compared to wild mice of a given size,
88 to gain insight into biological ageing in lab vs wild settings and (3) to assess the extent of variability
89 in biological (epigenetic) ageing rate among wild mice. We used faecal samples as a source of
90 DNA, to develop a non-invasive method that allows longitudinal sampling without ethical or
91 logistical limitations on sampling frequency, and allows applications of epigenetic age estimates
92 in contexts where animal capture or handling are not possible. To our knowledge, this is the first
93 epigenetic clock built with faecal samples. Our results show the potential of such an approach, but
94 also indicate substantial differences in the estimated epigenetic age of an inbred laboratory
95 population and an outbred wild population, even from very early in life.
96

97 **Materials and Methods**

98 *Sample collection*

99 A total of 137 faecal samples were collected from 65 individual *Mus musculus* C57BL/6 laboratory
100 mice (30 females, 35 males) from two animal facilities. The samples were collected in May–
101 November 2021 at the Biomedical Services Building, Oxford, UK (Animal facility A), and King's
102 College, London, UK (Animal facility B). The chronological age of the mice varied from 7 to 339
103 days. The mice were kept in standard housing and were not subject to any interventions before or
104 during sampling. During sample collection, body mass was recorded for mice from Animal facility
105 B but not for mice from Animal facility A. However, body mass is tightly correlated with age
106 among juvenile house mice (Spangenberg et al., 2014; Jax, 2022a; Jax, 2022b) allowing accurate

107 estimation of mass from age. As such, we estimated body mass for 25 mice under seven weeks of
108 age from Animal Facility A (for older mice from this facility (*n*=8) body mass was not estimated
109 and consequently samples from those mice were not included in analyses including body mass).
110 Body mass estimation was done based on Spangenberg et al. (2014) for 7–20-day old pups and
111 The Jackson Laboratory C57BL/6 body mass references for 3–7-week-old pups (Jax, 2022a; Jax,
112 2022b). For the latter age group, estimation was done separately for females and males using the
113 Jackson Laboratory sex-specific data (Jax, 2022a; Jax, 2022b). To collect faecal samples, mice
114 were briefly placed on a sterile surface until defecation. Faecal pellets were collected in a sterile
115 manner, immediately preserved in DNA/RNA Shield, and stored frozen at -80°C until further
116 processing.
117
118 Wild house mouse (*Mus musculus domesticus*) sampling was conducted in April–May 2019, July
119 2019, September–October 2019, August–September 2020, and April–May 2021 on Skokholm
120 Island, Wales, UK. Mice were trapped overnight using small Sherman live traps baited with
121 peanuts and non-absorbent cotton wool for bedding, and with a spray of sesame oil outside the
122 trap as a lure. Across each of two broad sampling areas (one near the coast and one in the island
123 interior, named "Quarry" and "Observatory" respectively), on each trapping night 150 traps were
124 set at dusk and checked at dawn. To prevent cross-contamination, any traps showing signs of
125 mouse presence were washed and sterilised before being reset using bleach solution (including a
126 ≥60 min soak in 20% bleach) to destroy bacterial cells and DNA.
127

128 All newly captured mice were permanently identified by subcutaneous injection of a passive
129 integrated transponder (PIT) tag. Upon each capture, each mouse was either tagged or identified
130 (if a recapture), aged, sexed, and measured before being released within 3m of its trapping point.
131 Age category was assigned based on size and pelage characteristics: small (typically <15g of mass
132 and <80mm of length) mice were classified as juveniles and full-sized mice (typically >20g and
133 >80mm of length) were classified as adults. Mice falling between these two size categories were
134 ranked as sub-adults. Sex was determined using anogenital distance and reproductive state.
135 Reproductive state was recorded as either non-perforate, perforate, suspected pregnant or lactating
136 for females, and testes abdominal, small or large for males. At each capture, body mass and body
137 condition were recorded. Body condition was scored from 1 to 4 by palpating the lower spine and
138 hips to estimate the amount of subcutaneous fat.
139
140 Faecal samples were collected from traps in a sterile manner (shortly after mice were collected
141 from traps the following day), preserved in DNA/RNA Shield and stored in a -20°C freezer until
142 the end of fieldwork (maximum 6 weeks after sample collection). At this point they were returned
143 to the laboratory frozen and stored at -80°C until DNA extraction. A total of 215 samples were
144 selected from all collected samples (>900) for further processing, by first selecting a longitudinal
145 dataset (mice sampled ≥2 times over time; total of 54 individuals) with as much variation as
146 possible in morphometric variables (age, body mass, sex, and reproductive status), as well as
147 environmental variables (sampling season and sampling area) and then supplementing this with
148 additional (equally variable) cross-sectional samples (one sample per animal) to increase number
149 of individuals for cross-sectional analyses up to 130. Variation in variables was achieved by

150 randomly selecting approximately equal numbers of samples across categories e.g., across
151 juvenile, sub-adult and adult mice.
152

153 *DNA extraction, bisulfite conversion and PCR amplification*

154 DNA was extracted from faecal samples using the ZymoBIOMICS DNA MiniPrep Kit according
155 to the manufacturer's protocol (Zymo Research, Irvine, California, USA). DNA was then bisulfite-
156 converted using the Zymo EZ DNA Methylation-Gold Kit to convert unmethylated cytosines to
157 uracil and then thymine (Zymo Research, Irvine, California, USA). PCR amplification was
158 conducted for five genes previously reported to correlate with chronological age in *Mus musculus*;
159 *Prima1, Hsf4, Kcns1, Gm9312*, and *Gm7325* (Han et al., 2018). Amplification was conducted
160 using the PyroMark PCR Kit according to manufacturer's instructions and primers for the five
161 genes (QIAGEN, Hilden, Germany; Table S2; Han et al., 2018). PCR conditions were as follows:
162 15 min initial denaturation at 95°C, 50 cycles of 30 sec denaturation at 58°C, 30 sec primer
163 annealing at 58°C and 30 sec extension at 72°C, followed by a 10 minute final extension at 72°C.
164 Amplification success was confirmed using gel electrophoresis. PCR was repeated with the same
165 conditions for any reaction that did not produce a band on the gel. The five amplicons (PCR
166 products) were pooled for each sample into 5-gene libraries by combining all PCR products per
167 sample. DNA was quantified with Qubit Fluorometer High Sensitivity dsDNA kit and normalised
168 to 6.25 ng/μl (Thermo Fisher Scientific, Waltham, Massachusetts, USA).
169

170 *Sequencing, basecalling, and demultiplexing*

171 The Oxford Nanopore Technology (ONT) platform was used for library sequencing, and all ONT
172 procedures were conducted according to manufacturer's instructions and ONT protocol
173 NBA_9102_v109_revl_09Jul2020 (Oxford Nanopore Technologies, Oxford, UK). We first used
174 the ONT Ligation Sequencing Kit (SQK-LSK109) to repair and dA-tail the DNA ends, followed
175 by ligation of sequencing adaptors to the prepared ends. We then barcoded libraries using the ONT
176 Native Barcoding Expansion kit (EXP-NBD104 or EXP-NBD196). Approximately 15 ng of the
177 prepared library was loaded onto a prepared ONT MinION Mk1B R9.4.1 flow cell and sequenced
178 using the ONT MinKNOW software v21.10.4. Libraries were sequenced across a total of six runs
179 resulting in a mean of 49,969 reads per sample. A negative control (where DNAse free H_2O was
180 used instead of pooled amplicons at the start of Nanopore pipeline) was included in three
181 sequencing runs, and these generated a mean of 200 (range 17–519) reads. One flow cell was used
182 twice, and washed between runs with the ONT Flow Cell Wash kit (EXP-WSH003). Different
183 barcodes were used for negative controls across the two sequencing runs where the same flow cell
184 was used to enable testing for carry-over of reads (only 17 potential carry-over reads were
185 detected). Raw sequencing data was basecalled and demultiplexed using High Accuracy
186 basecalling on the ONT Guppy software v5.0.11. The basecalled FASTQ files were then run
187 through the Apollo pipeline v0.1 (https://github.com/WildANimalClocks/apollo, DOI:
188 10.5281/zenodo.8426692) to acquire methylation rates for each CpG site within the five genes.
189 Using the alignment with the reference genes, target sites (cytosines within CpGs) were identified
190 in each read and determined as either methylated (cytosine) or unmethylated (uracil). The process
191 was continued for each read, resulting in a proportion of methylated cytosines at each CpG site.
192

193 *Analyses*

194 The data was analysed and visualised in R v4.1.2 (R Core Team, 2023). The epigenetic clock was
195 built using the cross-sectional data of 50 samples from C57BL/6 mice housed in two facilities.
196 Using CpG site specific methylation rates, we used the package glmnet v4.1–3 (Friedman et al.,
197 2010) to perform a cross-validated elastic net regulatisation using the cv.glmnet function using a
198 LASSO model (mixing parameter alpha=1) and a leave-one-out cross validation (nfolds=nrow).
199 We then fitted a final glmnet model using optimal lambda value determined by the cross-
200 validation. Epigenetic age was then predicted based on the glmnet model using the predict()
201 function. This clock was validated on an additional 30 samples from C57BL/6 mice (*n*=15 mice,
202 two samples per animal). We assessed clock performance for estimating chronological age of lab
203 mice using mean absolute error (MAE; Tangili et al., 2023). We then built another epigenetic clock
204 as described above using only those CpG sites that (1) showed parallel trends in their methylation
205 rates against body mass in lab and wild mice (i.e., ones that increased in methylation with body
206 mass in both systems; Fig. S2–5) and (2) had methylation rates for the majority of wild mouse
207 samples (we failed to acquire sufficient read counts for methylation rate measurement for all 11
208 CpG sites from the gene *Gm7325* in 9% of all wild mouse samples). Following these principles,
209 we chose in total 53 CpG sites. This revised clock was similarly validated with the independent
210 lab dataset and then used to estimate epigenetic age for all wild house mice for which methylation
211 rates at the CpG sites included in the clock were successfully measured (*n*=201; 93% of all wild
212 mouse samples) using linear modeling.

213

214 To test for the effect of covariates on predicted epigenetic age in the lab mouse validation dataset,
215 we fit a linear model with epigenetic age as the dependent variable and chronological age, sex,
216 cage, and sequencing run ID as predictor variables. A Wilcoxon rank sum test with 1,000
217 permutations was used to test whether the predicted epigenetic age of wild mice ranked as adults
218 was older than that of wild mice ranked as juveniles. The ability of the clock to detect an increase
219 in age among wild mice sampled on two consecutive occasions was tested using a binomial test.
220 This test used a null hypothesis p=0.5 that predicted age was higher at the later time-point than the
221 earlier time point (i.e. mice are equally likely to increase or decrease in epigenetic age over time).
222 We also used a linear model to test whether time between sampling points predicted absolute
223 change in epigenetic age. Age category at the first time-point and sex were also included as
224 predictors. Mice with less than 25 days between time-points were excluded from this longitudinal
225 analysis as the mean absolute error (MAE) of the clock in the validation dataset was 25 days (Fig.
226 1B).
227
228 To explore whether lab and wild mice might differ in epigenetic age for a given chronological age,
229 we made use of the fact that body mass is a good predictor of age when mice are young, in both
230 lab and wild settings (Spangenberg et al., 2014; Jax, 2022a; Jax, 2022b; Gerber et al., 2021; Gray
231 et al., 2015). We used a linear model to test whether source (lab/wild) predicted epigenetic age
232 when controlling for the effects of body mass (a proxy of age). Epigenetic age was the dependent
233 variable and source, body mass, sex and an interaction between body mass and sex (as the
234 relationship between mass and age is somewhat sex-dependent, Jax, 2022a; Jax, 2022b; Gerber et
235 al., 2021; Gray et al., 2015) were fitted as predictor variables. Female wild mice with signs of

236 ongoing or recent pregnancy (those recorded as suspected pregnant) were excluded, as body mass
237 will be a less accurate age proxy in these individuals, who are significantly heavier than other
238 females (one sample per Animal ID; non-pregnant n=48, suspected pregnant n=9; linear model,
239 $F_{1,54}$= 29.28, p<0.001). Lastly, to explore whether the *rate* of epigenetic ageing differed between
240 lab and wild mice we used two methods. First, we used a linear model which tested whether the
241 increase in epigenetic age observed between two consecutive time-points in repeat-sampled mice
242 varied as a function of the time between samples, source population (lab/wild) and their
243 interaction. Second, we used Levene's test to ask whether variation in the rate of epigenetic ageing
244 differed according to source. Here, we used the ratio between change in epigenetic age and change
245 in time as the response variable, and source (lab/wild) as the explanatory variable.
246

247 *Ethical statement*

248 Wild mouse work was conducted under Home Office license PPL PB0178858 held at the
249 University of Oxford, and with research permits from the Islands Conservation Advisory
250 Committee (ICAC), and Natural Resources Wales.
251

252 **Results**

253 *Construction of a non-invasive epigenetic clock*

254 We used faecal samples from C57BL/6 laboratory mice (*Mus musculus*, n=50, one sample per
255 animal) from two animal facilities to generate a DNA methylation-based epigenetic clock. We first
256 built an epigenetic clock using 73 targeted CpG sites from five genes that were previously

257 associated with age in laboratory mice (*Hsf4*, *Gm9312*, *Kcns1, Gm7325,* and *Prima1;* Table. S1;
258 Han et al., 2018). Elastic net regression identified 22 CpG sites from the five targeted genes; three
259 from *Hsf4*, six from *Gm9312*, six from *Kcns1,* one from *Gm7325* and one from *Prima1* (Table
260 S2). The epigenetic clock built using methylation patterns across these sites had a mean absolute
261 error (MAE) of 7 days (Pearson's r=0.996, p<0.001; Fig. 1A). We validated the clock by applying
262 it to an independent set of C57BL/6 mice that were not used in training the clock (n=15, one sample
263 per animal). Among these lab mice, predicted (epigenetic) age was also strongly correlated with
264 chronological age (Pearson's r=0.935, p<0.001, MAE=23 days; Fig. 1B). Neither sex, cage nor
265 sequencing run had a significant effect on epigenetic age (linear model: chronological age
266 $F_{1,8}$=63.797, p<0.001; sex $F_{1,8}$=0.328, p=0.583; cage $F_{3,8}$=0.040, p=0.989; sequencing run
267 $F_{1,8}$=0.057, p=0.817). This demonstrates that non-invasive faecal samples can be used to generate
268 an epigenetic clock in laboratory mice with equivalent or higher accuracy in estimating
269 chronological age compared to a clock previously derived using blood samples (Han et al., 2018;
270 MAE=35–41 days in two validation datasets).
271
272 As our aim was to develop a lab-based epigenetic clock that could be used to predict age in wild
273 mice, we next inspected methylation rates of targeted CpG sites (n=73) against body mass (a proxy
274 for age) in lab and wild mice. The majority of CpG sites showed similar trends (i.e., increase in
275 methylation rate with body mass; Fig. S2–5). However, nine CpG sites from genes *Prima1* and
276 *Gm9312* showed contradicting patterns, where methylation rate increased in wild but decreased in
277 lab mice with body mass (all CpG sites from *Prima1*, CpG sites at positions 10, 33, 116, 218, and
278 224 from *Gm9312*; Fig. S2, S3), and as such we excluded these CpG sites. Further, we failed to

279 acquire sufficient read counts and thus methylation rates for the 11 CpG sites from the gene
280 *Gm7325* in 20 wild mouse samples (9% of all wild mouse samples), and consequently decided to
281 remove this gene from the clock model. We then used the above described approach to build an
282 epigenetic clock with the remaining 53 CpG sites from four genes (Table S3), whose methylation
283 rates showed a positive linear relationship with body mass (Fig. S2–4). For this clock, elastic net
284 regression identified 11 CpG sites from genes *Hsp4* and *Kcns1* (Table S3). Here, the slope deviated
285 more from 1 (where 1 would indicate perfect positive linear relationship between chronological
286 and epigenetic age) than did the first clock (slope estimate 0.81 ± 0.02 standard error vs 0.98 ±
287 0.01 standard error in training set; Fig 1A, 1C). However, the clock still had a high accuracy with
288 a MAE of 19 days in the training set (Pearson's r=0.979, p<0.001; Fig. 1C) and 24 days in the
289 validation set (Pearson's r=0.939, p<0.001; Fig. 1D). This second clock was then used for further
290 analyses in the present study.
291

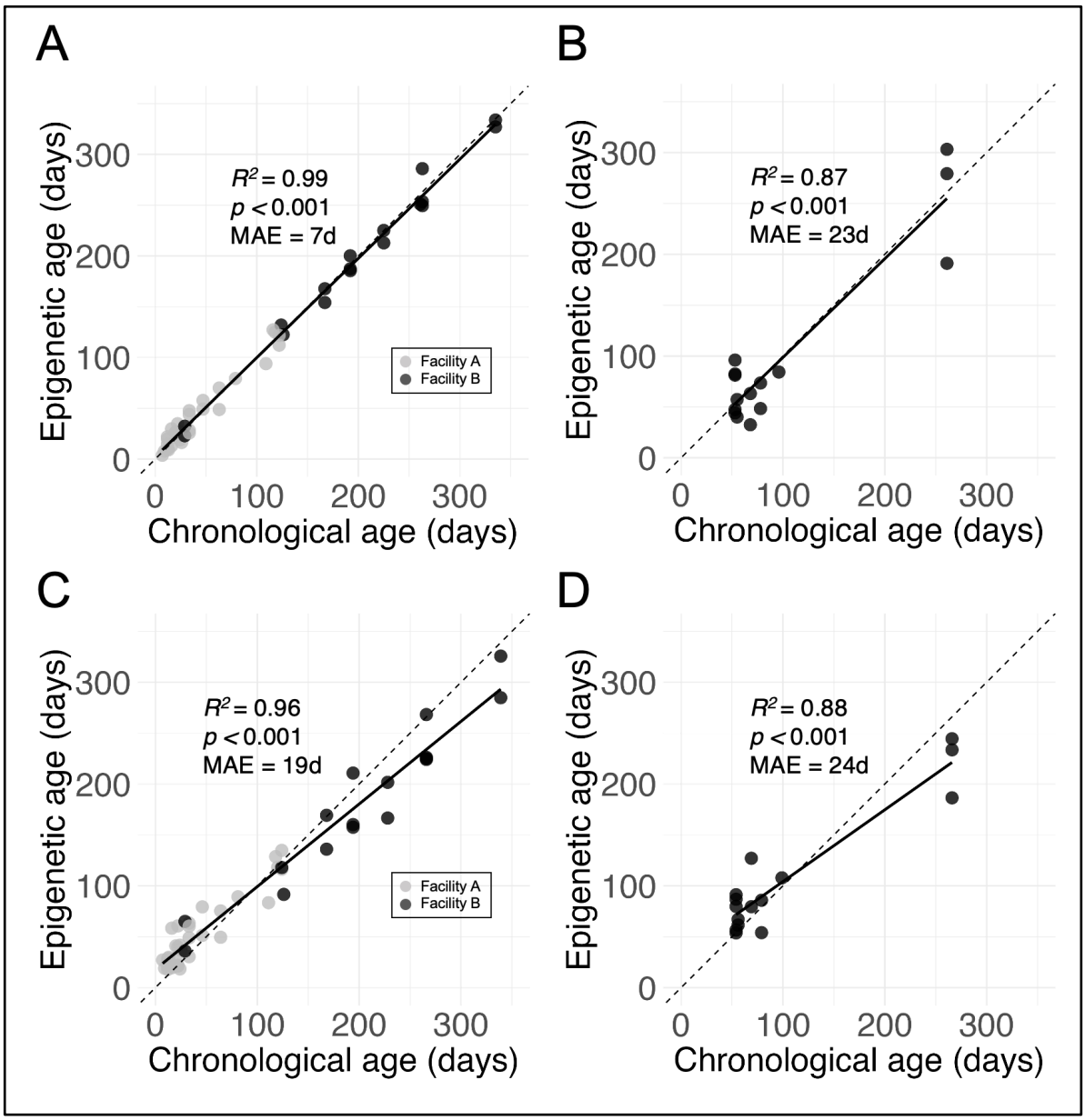


Figure 1. The relationship between DNA methylation-based (epigenetic) age and chronological age in C57BL/6 mice (**A**, **C**) used to train the epigenetic clock model or (**B**, **D**) an independent set of mice (a validation set) not used in building the epigenetic clock model. Two epigenetic clocks were built: one with 73 input CpG sites from five genes (**A**, **B**), and one with 53 input CpG sites from four genes (**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).

301 *Chronological age prediction in wild mice*

302 We next applied the lab-mouse derived epigenetic clock (Fig. 1C, 1D) to 205 faecal samples from
303 119 wild house mice to test if it can be used to estimate chronological age in wild individuals of
304 unknown age. Mice of all available body sizes were included with the aim of capturing as much
305 age variation as possible (body mass range 5.9–43.0g, mean 19.0, median 19.8). The epigenetic
306 age of wild mice varied from -26 to 460 days (mean 227, median 207; 2 out of 205 samples (1%)
307 had a negative epigenetic age).

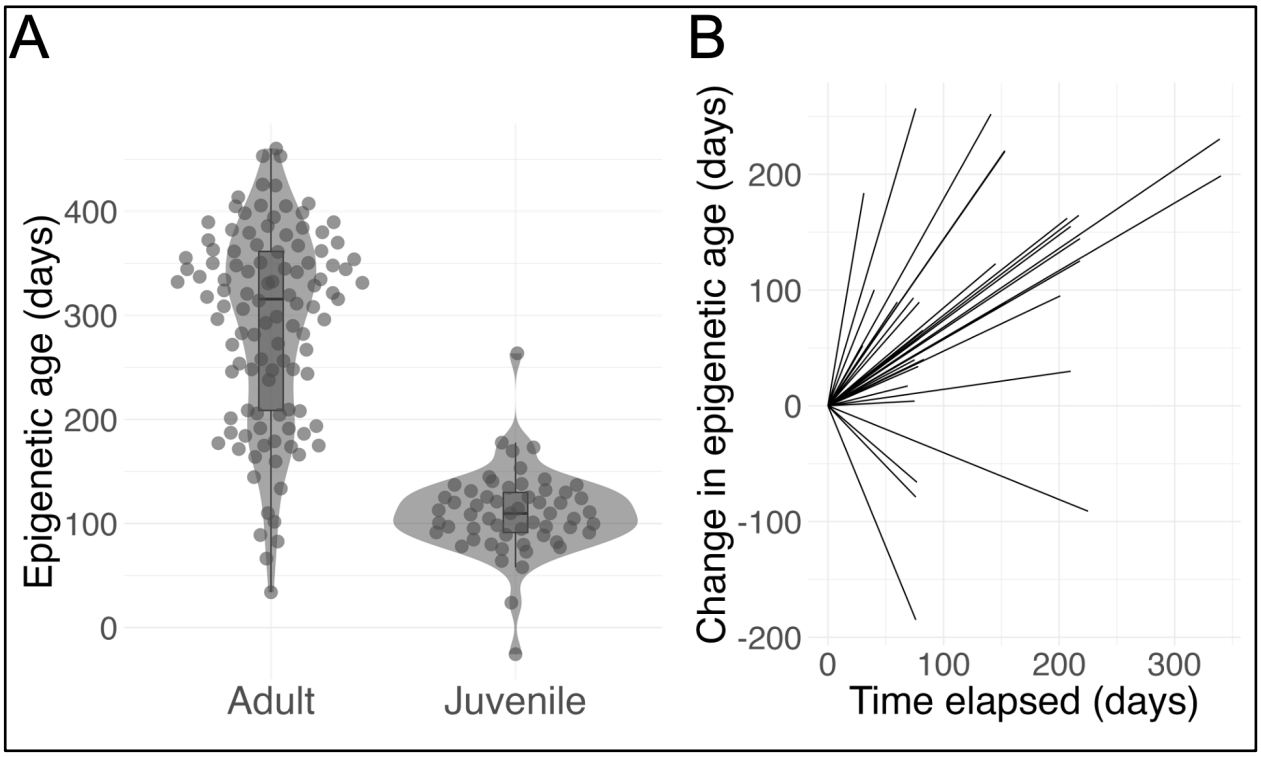
308

309 Mice assigned as adults in the field using external characteristics were estimated by the epigenetic
310 clock model to be significantly older than those assigned as juveniles (permutational Wilcoxon
311 rank sum test, $p<0.001$, $n=166$; Fig. 2A). Moreover, among 35 wild mice sampled twice between
312 30 and 340 days apart (mean 129, median 81), a great majority (31; 89%) were epigenetically older
313 at the latter timepoint (binomial test for H_0 p=0.5, $p<0.001$). In general, the number of days
314 between sampling time-points positively predicted change in epigenetic age (linear model,
315 $F_{1,17}=8.516$, $p=0.010$; Pearson's $r=0.406$, $p=0.01$, $n=35$), but there was wide variation in the
316 slope observed among individuals (Fig. 2B). Rate of epigenetic ageing
317 ($\frac{absolute\ change\ in\ epigenetic\ age}{days\ elapsed}$) was not predicted by any investigated variables (sex $F_{1,18}=0.366$,
318 $p=0.552$; reproductive status $F_{11,18}=0.653$, $p=0.763$, body mass $F_{1,18}=0.046$, $p=0.834$, season
319 $F_{2,18}=0.370$, $p=0.696$, and trapping area $F_{1,18}=1.986$, $p=0.176$ at first sampling point). Together
320 these results indicate that an epigenetic clock trained with samples from inbred lab mice can be

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

322 precise.

323



324

325 **Figure 2.** (**A**) Epigenetic age of wild mice phenotypically characterised as adult (*n*=109) and

326 juvenile (*n*=57) predicted with an epigenetic clock built using C57BL/6 lab mice. Age category

327 was assigned in the field using body size and appearance (see Methods). Median epigenetic age

328 was 316 days in adults and 110 days in juveniles. Circles are individual samples. (**B**) Change in

329 epigenetic age between two timepoints in wild mice sampled twice between 30 and 340 days apart

330 (*n*=35). Lines represent individual mice. Epigenetic age increased with time for 31 out of 35 (89%)

331 mice.

332

333 *Wild mice are multiple times older epigenetically than laboratory mice*

334 We next assessed whether epigenetic age for a given chronological age differed between wild and
335 laboratory mice. In the absence of known chronological age for wild mice, we used body mass to
336 provide an upper limit age estimate for individuals classed as juveniles. Others have reported that
337 12–13-day-old wild house mice from mainland Europe weigh around 7g (range 3.6–10.5g, mean
338 6.8; Gerber et al., 2021) and another study showed that 14-day-old wild-derived but captive house
339 mice from Gough Island (home to the largest wild house mice recorded) weigh around 8.5g (range
340 ~7–10.5g, raw data not available; Gray et al., 2015; Fig. S1). Thus, irrespective of context, house
341 mice between 12–14 days typically are expected to weigh 7–10.5g (Gerber et al., 2021; Gray et
342 al., 2015). We therefore examined epigenetic age from a cross-sectional set of juvenile wild
343 Skokholm Island mice that fall within this body mass range and thus which we expect to be no
344 more than 25 days old (*n*=19, body mass 6.1–10.4g with a mean of 8.4g). Among these individuals,
345 the mean epigenetic age estimate was 106 days (range -26–173, median 110; 1 sample had a
346 negative epigenetic age), which is more than four times their expected chronological age (Fig. S1).
347
348 To explore whether wild mice were epigenetically older than lab mice beyond early life, we
349 explored the relationship between epigenetic age and body mass across mice of all sizes. While
350 the reliability of body mass as an indicator of age declines after initial growth during first few
351 weeks of life, it continues to increase with chronological age in both C57BL/6 lab and wild mice
352 beyond this time and thus can be used as a rough estimate of age in adults as well (Jax, 2022a; Jax,
353 2022b; Gerber et al., 2021; Gray et al., 2015). In a cross-sectional subset of 57 lab and 99 wild
354 mice (excluding wild mice with visual indication of ongoing/recent pregnancy, see Methods, and
355 those with unknown sex), source (lab/wild) predicted epigenetic age (linear model,

356 $F_{1,135}$=93.0143, p<0.001; controlling for an interaction term between sex and body mass,
357 $F_{1,135}$=0.8519, p=0.357), with wild mice having a higher mean epigenetic age than that of lab mice
358 consistently across investigated range of body mass (Fig. 3A). In line with this finding, wild mice
359 also had higher rates of DNA methylation in the targeted genes (Fig. S2–4), explaining the higher
360 epigenetic age profile.
361
362 To further examine whether older epigenetic age profiles among wild mice might be due to
363 accelerated ageing through exposure to environmental stressors, such as food shortage or climatic
364 variation, we studied the rate of epigenetic ageing across lab and wild mice for which two
365 timepoints were available. In lab mice (n=15), the rate of epigenetic ageing was similar to that
366 observed when originally validating the clock using the cross-sectional data (longitudinal data:
367 slope estimate = 0.77 ± 0.15 standard error; cross-sectional data: slope estimate = 0.80 ± 0.08
368 standard error; Fig. 1B). This rate was slightly shallower in wild mice (n=35; slope estimate = 0.49
369 ± 0.19 standard error), but not significantly so (linear model: $F_{1,48}$=0.085, p=0.772, Fig. 3B). Lab
370 mice included were all adults, while the longitudinal data of wild mice included juvenile mice;
371 however, exclusion of juvenile mice (<15g of body mass) did not appear to have a strong effect
372 on these trends (Fig. S6). Further, although the rate of epigenetic ageing appeared to be more
373 variable among wild than lab mice, the variance in epigenetic ageing rate did not differ statistically
374 across settings (Levene's test, $F_{1,48}$=3.140, p=0.083; correlation between change in epigenetic age
375 and days elapsed: lab mice, Pearson's r=0.81, p<0.001; wild mice, Pearson's r=0.48, p=0.003; Fig.
376 3B).
377

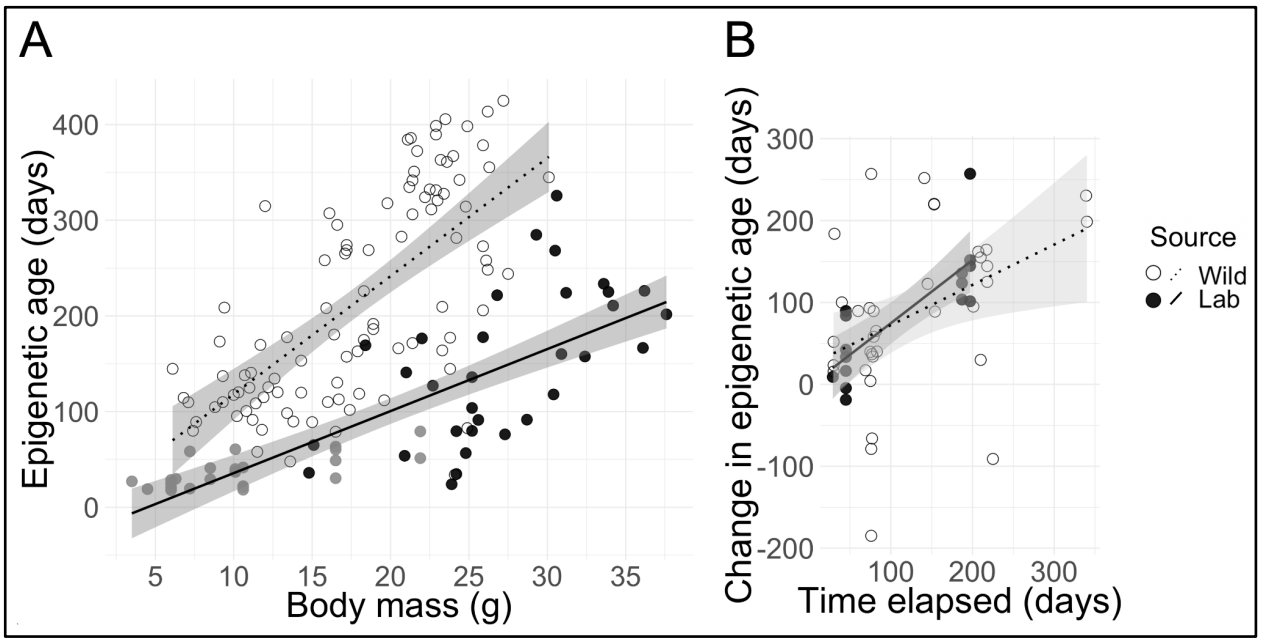


Figure 3. Wild mice have elevated epigenetic age compared to lab mice for a given body mass, but do not show a different rate of epigenetic ageing in adulthood (**A**) Epigenetic age of wild (*n*=99) and laboratory mice (*n*=57) in relation to body mass. Empty circles are wild mice and filled circles are lab mice; black circles are lab mice for which body mass was recorded during sample collection, and grey circles are lab mice for which body mass was estimated post hoc (see Methods). (**B**) Change in epigenetic age in relation to time elapsed in laboratory (*n*=15) and wild mice (*n*=35) sampled twice a minimum 25 days apart. Body mass varies 19.2–26.3g for lab mice and 7.0–37.7g for wild mice. Empty circles are wild mice and filled circles are lab mice. Lines are linear regression lines (*dashed* = wild mice, *solid* = lab mice) and shading indicates 95% confidence intervals.

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. The clock effectively distinguished wild juveniles from adults, and typically predicted increases in age over time among repeat-captured individuals at a

395 similar success rate (~90% individuals predicted older at later time point) to the wild baboon study
396 of Anderson et al (2021)).
397
398 However, while the clock accurately predicted age (with error of ± 25 days) in an independent set
399 of laboratory mice, we observed high variation among wild mice in how their epigenetic age
400 changed over chronological time, suggesting our clock had reduced accuracy in this different
401 ecological context. Others have had better success in applying clocks built with captive individuals
402 to wild individuals (e.g. Mayne et al., 2022 in green turtles; Robeck et al., 2021 in cetaceans;
403 correlation between chronological and epigenetic age in these studies 0.67–0.98 *vs* 0.41 between
404 change in time and change in epigenetic age in our study). However, these studies have built
405 epigenetic clocks using samples from captive individuals where there is genetic and environmental
406 variation, such as zoos or outdoor enclosures. Our clock was built with samples from inbred lab
407 mice housed under very stable environmental conditions, but applied to wild mice that are outbred
408 and exposed to a highly variable temperate climate. Studies of different lab strains have also
409 confirmed that epigenetic clocks may behave differently in different genetic backgrounds. For
410 instance, Han et al demonstrated DBA/2 mice to be up to twice as old epigenetically as C57BL/6
411 mice (Han et al., 2018).
412
413 Moreover, DNA methylation may be influenced by environmental factors (Zocher et al., 2021;
414 Parrott et al., 2014; Viitaniemi et al., 2019), and wild animals are generally exposed to more
415 variable environments. As such, the contrasting genetic and environmental backgrounds in our
416 mouse systems may partly explain why age estimates in wild mice based on a clock from lab mice

417 had low accuracy. Finally, while all samples from lab mice were preserved immediately after
418 defecation, the time between defecation and sample preservation varied in wild mice (where
419 samples were collected from traps which animals had been in overnight, up to 13 hours). It is
420 possible some DNA degradation by nuclease and gut microbes occurred before the samples were
421 preserved (in DNA/RNA Shield, which inactivates microbes and preserves DNA integrity),
422 affecting the methylation profiles.
423
424 The epigenetic age of wild mice from Skokholm Island varied from -26 to 460 days (mean 227,
425 median 207; 2 (1% of all 205) samples had negative epigenetic ages, -26 and -21 days). The
426 presence of negative predicted ages in wild individuals may be due to measurement error, but it
427 may also be due to the inherent biological differences between wild and lab populations. DNA
428 methylation-based age predictions may reflect the distinct age trajectories and relative age of wild
429 individuals compared to their lab counterparts, highlighting the importance of considering the
430 natural variation in epigenetic ageing processes across different populations.
431
432 Despite our epigenetic clock having a very close relationship with chronological age in laboratory
433 mice, several lines of evidence suggest these epigenetic age estimates for wild mice are
434 overestimates of their chronological age. First, when investigating juveniles, in which body mass
435 is an accurate predictor of age across both wild and lab mice (Jax, 2022a; Jax, 2022b; Gerber et
436 al., 2021; Gray et al., 2015), we found that wild juveniles were predicted to be several times older
437 than their expected chronological age from body mass (Gerber et al., 2021; Gray et al., 2015).
438 Second, by comparing the epigenetic age estimates to body mass in mice of all sizes, we found

439 that the estimated epigenetic age of wild mice was on average several times higher at any given
440 body mass than that of lab mice, with similar patterns seen in CpG site methylation rates. While
441 accelerated weight gain in *ad libitum*-fed lab mice may contribute to a lower epigenetic age among
442 adult lab mice compared to wild mice, these findings suggested that the more challenging
443 environment experienced by wild mice may accelerate epigenetic clocks. Our comparison of the
444 epigenetic age of wild vs lab mice is specific to a comparison with the C57BL/6 strain; however,
445 the difference between the epigenetic age of e.g., C57BL/6 and DBA/2 mice is substantially
446 smaller than the observed difference between C57BL/6 and wild mice (DBA/2 mice are only twice
447 as old epigenetically than are C57BL/6; Han et al., 2018). As such, while the choice of strain may
448 have influenced our findings, it is unlikely to entirely explain the difference between lab and wild
449 mice.
450
451 To test whether the older epigenetic age profile of wild mice could be explained by accelerated
452 ageing post-weaning (i.e., from when they are trappable) we investigated the rate of epigenetic
453 ageing using individuals captured and sampled twice over time. If anything, the rate of epigenetic
454 age appeared slightly slower and more variable in wild mice, though this observation relied on a
455 relatively small sample size and was not statistically significant. Moreover, as we observed
456 heightened epigenetic age in wild compared to lab mice even during the first ~2 weeks of life, we
457 suggest that peri- and early postnatal effects on offspring DNA methylation may vary between
458 laboratory and wild mice and contribute to their different epigenetic age profiles. Various human,
459 mouse, and other animal studies have demonstrated the association between prenatal maternal
460 experience (such as food shortage, diet, infection, substance exposure, and stress) and offspring

461 DNA methylation patterns, with differences from the prenatal (foetal) phase still detectable in later
462 life (Tobi et al., 2009; Heijmans et al., 2008; Lan et al., 2013; Richetto et al., 2017; Camerota et
463 al., 2021; Joubert et al., 2016; Kertes et al., 2016; Vangeel et al., 2017).
464
465 While our approach of training an epigenetic clock with lab individuals and using it to estimate
466 age in wild individuals did not allow precise estimation of chronological age, our results
467 demonstrate such an approach can still be effective in distinguishing between juvenile and adult
468 individuals. Such information may be useful in contexts where a faecal deposit is found but the
469 individual is not observed, such as in field-based projects of animals that are hard or impossible to
470 capture. At the same time, this method can provide interesting insights into biological ageing when
471 applied to wild animals of known chronological age or to individuals sampled longitudinally such
472 that changes in epigenetic age can be estimated (De Paoli-Iseppi et al., 2017; Powell & Proulx,
473 2003; Brivio et al., 2015). Considering the much greater variability in epigenetic ageing rates we
474 observed in wild compared to laboratory animals, our results suggest wild systems may provide
475 an informative environment in which to study drivers of epigenetic age acceleration. In the present
476 study we were not able to identify drivers of epigenetic ageing rates in the wild due to a small
477 sample size, but further work with larger samples sizes could address this important question.
478
479 We used faeces as a source of host DNA and demonstrated that an epigenetic clock can be built
480 with faecal samples at a similar or even improved accuracy to a previously published blood-based
481 mouse epigenetic clock (Han et al., 2018). As faecal samples can be collected non-invasively, a
482 given individual can be sampled over time without limitations to sampling frequency. Further, it

may be possible to collect faecal samples without capturing the animal, making this methodological approach particularly useful when estimating age in wild species that are endangered, hard to capture or even detect.

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 entirely wild mice. While this approach did not provide highly precise 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.

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Author information

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.

Data accessibility

Data Accessibility

Data will be made publicly available upon publication.

References

Ambatipudi S, Horvath S, Perrier F, et al. DNA methylome analysis identifies accelerated epigenetic ageing associated with postmenopausal breast cancer susceptibility. *Eur J Cancer*. 2017;75:299-307. doi:10.1016/j.ejca.2017.01.014

Anderson JA, Johnston RA, Lea AJ, et al. High social status males experience accelerated epigenetic ageing in wild baboons. *Elife*. 2021;10:e66128. Published 2021 Apr 6. doi:10.7554/eLife.66128

Berry RJ, Jakobson ME. Life and death in an island population of the house mouse. *Exp Gerontol.* 1971;6: 187–197.

524 Bors EK, Baker CS, Wade PR, et al. An epigenetic clock to estimate the age of living beluga
525 whales. *Evol Appl*. 2021;14(5):1263-1273. Published 2021 Feb 3. doi:10.1111/eva.13195
526 Brivio F, Grignolio S, Sica N, Cerise S, Bassano B. Assessing the Impact of Capture on Wild
527 Animals: The Case Study of Chemical Immobilisation on Alpine Ibex. *PLoS One*.
528 2015;10(6):e0130957. Published 2015 Jun 25. doi:10.1371/journal.pone.0130957
529 Camerota M, Graw S, Everson TM, et al. Prenatal risk factors and neonatal DNA methylation in
530 very preterm infants. *Clin Epigenetics*. 2021;13(1):171. Published 2021 Sep 10.
531 doi:10.1186/s13148-021-01164-9
532 Cao X, Li W, Wang T, et al. Accelerated biological ageing in COVID-19 patients. *Nat Commun*.
533 2022;13(1):2135. Published 2022 Apr 19. doi:10.1038/s41467-022-29801-8
534 De Paoli-Iseppi R, Deagle BE, McMahon CR, Hindell MA, Dickinson JL, Jarman SN. Measuring
535 Animal Age with DNA Methylation: From Humans to Wild Animals. *Front Genet*.
536 2017;8:106. Published 2017 Aug 17. doi:10.3389/fgene.2017.00106
537 Fairfield EA, Richardson DS, Daniels CL, Butler CL, Bell E, Taylor MI. Ageing European lobsters
538 (*Homarus gammarus*) using DNA methylation of evolutionarily conserved ribosomal
539 DNA. *Evol Appl*. 2021;14(9):2305-2318. Published 2021 Sep 23. doi:10.1111/eva.13296
540 Ferrari, Manuela; Lindholm, Anna K; König, Barbara (2015). The risk of exploitation during
541 communal nursing in house mice, *Mus musculus domesticus.* Animal Behaviour, 110:133-
542 143.
543 Flurkey, Currer, and Harrison, 2007. 'The mouse in biomedical research.' in James G. Fox (ed.),
544 American College of Laboratory Animal Medicine series (Elsevier, AP: Amsterdam;
545 Boston).

546 Friedman J, Hastie T, Tibshirani R. Regularization Paths for Generalized Linear Models via
547 Coordinate Descent. *J Stat Softw*. 2010;33(1):1-22.
548 Gardner ST, Bertucci EM, Sutton R, Horcher A, Aubrey D, Parrott BB. Development of DNA
549 methylation-based epigenetic age predictors in loblolly pine (Pinus taeda). *Mol Ecol*
550 *Resour*. 2023;23(1):131-144. doi:10.1111/1755-0998.13698
551 Gerber N, Auclair Y, König B, Lindholm AK. Population density and temperature influence the
552 return on maternal investment in wild house mice. *Front Ecol Evol.* 2021;8:602359.
553 https://doi.org/10.3389/fevo.2020.602359
554 Gray MM, Parmenter MD, Hogan CA, et al. Genetics of Rapid and Extreme Size Evolution in
555 Island Mice. *Genetics*. 2015;201(1):213-228. doi:10.1534/genetics.115.177790
556 Han Y, Eipel M, Franzen J, et al. Epigenetic age-predictor for mice based on three CpG sites. *Elife*.
557 2018;7:e37462. Published 2018 Aug 24. doi:10.7554/eLife.37462
558 Harvanek ZM, Fogelman N, Xu K, Sinha R. Psychological and biological resilience modulates the
559 effects of stress on epigenetic ageing. *Transl Psychiatry*. 2021;11(1):601. Published 2021
560 Nov 27. doi:10.1038/s41398-021-01735-7
561 Heijmans BT, Tobi EW, Stein AD, et al. Persistent epigenetic differences associated with prenatal
562 exposure to famine in humans. *Proc Natl Acad Sci U S A*. 2008;105(44):17046-17049.
563 doi:10.1073/pnas.0806560105
564 Ito H, Udono T, Hirata S, Inoue-Murayama M. Estimation of chimpanzee age based on DNA
565 methylation. *Sci Rep*. 2018;8(1):9998. Published 2018 Jul 3. doi:10.1038/s41598-018-
566 28318-9
567 Jarman SN, Polanowski AM, Faux CE, et al. Molecular biomarkers for chronological age in animal
568 ecology. *Mol Ecol*. 2015;24(19):4826-4847. doi:10.1111/mec.13357

569 JAX®. Body weight information for aged C57BL/6J. Available at: https://www.jax.org/jax-mice-
570 and-services/strain-data-sheet-pages/body-weight-chart-aged-b6 (Accessed: 30.11.2022)
571 JAX®. Body weight information for C57BL/6J. Available at: https://www.jax.org/jax-mice-and-
572 services/strain-data-sheet-pages/body-weight-chart-000664 (Accessed: 30.11.2022)
573 Joubert BR, Felix JF, Yousefi P, et al. DNA Methylation in Newborns and Maternal Smoking in
574 Pregnancy: Genome-wide Consortium Meta-analysis. *Am J Hum Genet*. 2016;98(4):680-
575 696. doi:10.1016/j.ajhg.2016.02.019
576 Joyce BT, Gao T, Zheng Y, et al. Epigenetic Age Acceleration Reflects Long-Term Cardiovascular
577 Health. *Circ Res*. 2021;129(8):770-781. doi:10.1161/CIRCRESAHA.121.318965
578 Kerepesi C, Meer MV, Ablaeva J, et al. Epigenetic ageing of the demographically non-ageing
579 naked mole-rat. *Nat Commun*. 2022;13(1):355. Published 2022 Jan 17.
580 doi:10.1038/s41467-022-27959-9
581 Kertes DA, Kamin HS, Hughes DA, Rodney NC, Bhatt S, Mulligan CJ. Prenatal Maternal Stress
582 Predicts Methylation of Genes Regulating the Hypothalamic-Pituitary-Adrenocortical
583 System in Mothers and Newborns in the Democratic Republic of Congo. *Child Dev*.
584 2016;87(1):61-72. doi:10.1111/cdev.12487
585 Lan X, Cretney EC, Kropp J, et al. Maternal Diet during Pregnancy Induces Gene Expression and
586 DNA Methylation Changes in Fetal Tissues in Sheep. *Front Genet*. 2013;4:49. Published
587 2013 Apr 5. doi:10.3389/fgene.2013.00049
588 Larison B, Pinho GM, Haghani A, et al. Epigenetic models developed for plains zebras predict age
589 in domestic horses and endangered equids. *Commun Biol*. 2021;4(1):1412. Published 2021
590 Dec 17. doi:10.1038/s42003-021-02935-z

591 Lemaître JF, Rey B, Gaillard JM, et al. DNA methylation as a tool to explore ageing in wild roe
592 deer populations. *Mol Ecol Resour*. 2022;22(3):1002-1015. doi:10.1111/1755-0998.13533
593 Mayne B, Mustin W, Baboolal V, et al. Age prediction of green turtles with an epigenetic clock.
594 *Mol Ecol Resour*. 2022;22(6):2275-2284. doi:10.1111/1755-0998.13621
595 Moore LD, Le T, Fan G. DNA methylation and its basic function. *Neuropsychopharmacology*.
596 2013;38(1):23-38. doi:10.1038/npp.2012.112
597 Morales Berstein F, McCartney DL, Lu AT, et al. Assessing the causal role of epigenetic clocks
598 in the development of multiple cancers: a Mendelian randomization study. *Elife*.
599 2022;11:e75374. Published 2022 Mar 29. doi:10.7554/eLife.75374
600 Parrott BB, Bowden JA, Kohno S, et al. Influence of tissue, age, and environmental quality on
601 DNA methylation in Alligator mississippiensis. *Reproduction*. 2014;147(4):503-513.
602 Published 2014 Mar 2. doi:10.1530/REP-13-0498
603 Peng C, Cardenas A, Rifas-Shiman SL, et al. Epigenetic age acceleration is associated with allergy
604 and asthma in children in Project Viva. *J Allergy Clin Immunol*. 2019;143(6):2263-
605 2270.e14. doi:10.1016/j.jaci.2019.01.034
606 Petkovich DA, Podolskiy DI, Lobanov AV, Lee SG, Miller RA, Gladyshev VN. Using DNA
607 Methylation Profiling to Evaluate Biological Age and Longevity Interventions. *Cell Metab*.
608 2017;25(4):954-960.e6. doi:10.1016/j.cmet.2017.03.016
609 Pinho GM, Martin JGA, Farrell C, et al. Hibernation slows epigenetic ageing in yellow-bellied
610 marmots. *Nat Ecol Evol*. 2022;6(4):418-426. doi:10.1038/s41559-022-01679-1
611 Polanowski AM, Robbins J, Chandler D, Jarman SN. Epigenetic estimation of age in humpback
612 whales. *Mol Ecol Resour*. 2014;14(5):976-987. doi:10.1111/1755-0998.12247

613 Powell RA, Proulx G. Trapping and marking terrestrial mammals for research: integrating ethics,
614 performance criteria, techniques, and common sense. *ILAR J*. 2003;44(4):259-276.
615 doi:10.1093/ilar.44.4.259
616 Prado NA, Brown JL, Zoller JA, et al. Epigenetic clock and methylation studies in elephants.
617 *ageing Cell*. 2021;20(7):e13414. doi:10.1111/acel.13414
618 R Core Team (2023). _R: A Language and Environment for Statistical Computing_. R Foundation
619 for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
620 Richetto J, Massart R, Weber-Stadlbauer U, Szyf M, Riva MA, Meyer U. Genome-wide DNA
621 Methylation Changes in a Mouse Model of Infection-Mediated Neurodevelopmental
622 Disorders. *Biol Psychiatry*. 2017;81(3):265-276. doi:10.1016/j.biopsych.2016.08.010
623 Robeck TR, Fei Z, Lu AT, et al. Multi-species and multi-tissue methylation clocks for age
624 estimation in toothed whales and dolphins. *Commun Biol*. 2021;4(1):642. Published 2021
625 May 31. doi:10.1038/s42003-021-02179-x
626 Spangenberg E, Wallenbeck A, Eklöf AC, Carlstedt-Duke J, Tjäder S. Housing breeding mice in
627 three different IVC systems: maternal performance and pup development. *Lab Anim*.
628 2014;48(3):193-206. doi:10.1177/0023677214531569
629 Stubbs TM, Bonder MJ, Stark AK, et al. Multi-tissue DNA methylation age predictor in mouse.
630 *Genome Biol*. 2017;18(1):68. Published 2017 Apr 11. doi:10.1186/s13059-017-1203-5
631 Sullivan IR, Adams DM, Greville LJS, Faure PA, Wilkinson GS. Big brown bats experience
632 slower epigenetic ageing during hibernation. *Proc Biol Sci*. 2022;289(1980):20220635.
633 doi:10.1098/rspb.2022.0635
634 Tangili M, Slettenhaar AJ, Sudyka J, et al. DNA methylation markers of age(ing) in non-model
635 animals. *Mol Ecol*. 2023;32(17):4725-4741. doi:10.1111/mec.17065

636 Thompson MJ, vonHoldt B, Horvath S, Pellegrini M. An epigenetic ageing clock for dogs and
637 wolves. *ageing (Albany NY)*. 2017;9(3):1055-1068. doi:10.18632/ageing.101211
638 Tobi EW, Lumey LH, Talens RP, et al. DNA methylation differences after exposure to prenatal
639 famine are common and timing- and sex-specific. *Hum Mol Genet*. 2009;18(21):4046-
640 4053. doi:10.1093/hmg/ddp353
641 Vangeel EB, Pishva E, Hompes T, et al. Newborn genome-wide DNA methylation in association
642 with pregnancy anxiety reveals a potential role for *GABBR1*. *Clin Epigenetics*. 2017;9:107.
643 Published 2017 Oct 3. doi:10.1186/s13148-017-0408-5
644 Viitaniemi HM, Verhagen I, Visser ME, Honkela A, van Oers K, Husby A. Seasonal Variation in
645 Genome-Wide DNA Methylation Patterns and the Onset of Seasonal Timing of
646 Reproduction in Great Tits. *Genome Biol Evol*. 2019;11(3):970-983.
647 doi:10.1093/gbe/evz044
648 Wilkinson GS, Adams DM, Haghani A, et al. DNA methylation predicts age and provides insight
649 into exceptional longevity of bats [published correction appears in Nat Commun. 2021
650 May 5;12(1):2652] [published correction appears in Nat Commun. 2022 Sep
651 7;13(1):5266]. *Nat Commun*. 2021;12(1):1615. Published 2021 Mar 12.
652 doi:10.1038/s41467-021-21900-2
653 Wright PGR, Mathews F, Schofield H, et al. Application of a novel molecular method to age free-
654 living wild Bechstein's bats. *Mol Ecol Resour*. 2018;18(6):1374-1380. doi:10.1111/1755-
655 0998.12925
656 Yousefzadeh, M., Henpita, C., Vyas, R., Soto-Palma, C., Robbins, P., & Niedernhofer, L. (2021).
657 DNA damage-how and why we age?. *eLife*, *10*, e62852.
658 https://doi.org/10.7554/eLife.62852

659 Zocher S, Overall RW, Lesche M, Dahl A, Kempermann G. Environmental enrichment preserves
660 a young DNA methylation landscape in the aged mouse hippocampus. *Nat Commun*.
661 2021;12(1):3892. Published 2021 Jun 23. doi:10.1038/s41467-021-23993-1