

# 1 **Epigenetic clock and DNA methylation studies of roe deer** 2 **in the wild**

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## 16 **Abstract:**

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18 DNA methylation-based biomarkers of aging (epigenetic clocks) promise to lead to new  
19 insights in the evolutionary biology of ageing. Relatively little is known about how the natural  
20 environment affects epigenetic aging effects in wild species. In this study, we took advantage  
21 of a unique long-term (>40 years) longitudinal monitoring of individual roe deer (*Capreolus*  
22 *capreolus*) living in two wild populations (Chizé and Trois Fontaines, France) facing different  
23 ecological contexts to investigate the relationship between chronological age and levels of  
24 DNA methylation (DNAm). We generated novel DNA methylation data from n=90 blood  
25 samples using a custom methylation array (HorvathMammalMethylChip40). We present three  
26 DNA methylation-based estimators of age (DNAm or epigenetic age), which were trained in  
27 males, females, and both sexes combined. We investigated how sex differences influenced the  
28 relationship between DNAm age and chronological age through the use of sex-specific  
29 epigenetic clocks. Our results highlight that both populations and sex influence the epigenetic  
30 age, with the bias toward a stronger male average age acceleration (i.e. differences between  
31 epigenetic age and chronological ages) particularly pronounced in the population facing harsh  
32 environmental conditions. Further, we identify the main sites of epigenetic alteration that have  
33 distinct aging patterns across the two sexes. These findings open the door to promising  
34 avenues of research at the crossroad of evolutionary biology and biogerontology.

35  
36 **Keywords:** DNA methylation; environment; growth; life-history; senescence  
37

## 38 **Introduction**

39 The last decades have seen an increasing interest for the study of ageing in the wild  
40 (Monaghan et al. 2008; Fletcher and Selman 2015; Gaillard and Lemaître 2020). The starting  
41 point of this infatuation is undeniably the compilation of evidence reporting that - in  
42 populations of animals in the wild - senescence occurs in demographic performance (actuarial  
43 senescence: Brunet-Rossinni & Austad, 2006; Nussey et al., 2013; reproductive senescence:  
44 Lemaître & Gaillard, 2017; Nussey et al., 2013), phenotypic performance (body mass:  
45 Douhard et al., 2017; Nussey et al. 2011; foraging efficiency: Lecomte et al., 2010; MacNulty  
46 et al., 2009) and physiological traits (e.g. immune parameters, Nussey et al. 2012;  
47 haematological parameters, Jégo et al. 2014; steroid levels, Sugianto et al. 2020). Nowadays,  
48 the age-specific decline in demographic and physiological performance is considered to be the  
49 rule rather than the exception in the wild, at least in mammals and birds (Gaillard & Lemaître,  
50 2020; Nussey et al., 2013; but see also Zajitschek et al., 2020).

51 Animal populations in which individuals are monitored from birth to death in the wild  
52 provide a unique (but largely untapped) resource for studying individual differences in health  
53 and mortality risk at old ages (Gaillard and Lemaître 2020; Lemaître et al. 2020b). Multiple  
54 lines of evidence emphasize the relevance of such longitudinal and individually-based data.  
55 First, the vast majority of the current research in biogerontology focused on inbred laboratory  
56 organisms with no or low genetic variation and maintained under controlled conditions (e.g.  
57 *Caenorhabditis elegans*, *Drosophila melanogaster*, laboratory rodents, Partridge, 2010).  
58 Studies performed on those species have led to major breakthrough in the mechanisms  
59 regulating the ageing process from the molecular to the individual level (López-Otín et al.  
60 2013; Kennedy et al. 2014). However, their findings can be difficult to extrapolate to species  
61 living in more complex environments (Briga and Verhulst 2015), with diverse genetic  
62 background and much longer lifespan and thereby different life history strategies, such as  
63 humans (Perlman 2016). Even if studies of non-human primates kept in captive conditions are  
64 increasing (Languille et al. 2012; Jasinska 2020), the full diversity of mammalian species  
65 displaying life-history traits and life styles similar to the ones observed in humans (i.e.  
66 whether there are socially monogamous, long-lived, provide extensive periods of parental  
67 care or create tight social bounds with conspecifics) is yet to be considered. In addition, the  
68 study of the ageing process in the wild enables - by essence - to investigate the role played by  
69 the environment, an important piece of the ageing conundrum. Similar to what has been  
70 described in human populations (e.g. Robine et al., 2012 in the context of climatic variables),  
71 there is increasing evidence that environmental factors modulate ageing patterns in the wild

72 (Nussey et al. 2007; Holand et al. 2016). For instance, it is increasingly recognized that the  
73 social environment can have a major influence on health and mortality risk at late ages  
74 (Berger et al. 2018; Snyder-Mackler et al. 2020), by notably interacting with some hallmarks  
75 of ageing (e.g. telomere dynamics in Seychelle warblers, *Acrocephalus sechellensis*,  
76 Hammers et al., 2019). In addition, while mammalian females generally live longer than  
77 males in the wild (Lemaître et al. 2020c), as commonly observed in humans or laboratory  
78 rodents (Austad and Fischer 2016; Zarulli et al. 2018), the exact mechanisms modulating  
79 these sex differences in survival are yet to be deciphered (Tower 2017; Marais et al. 2018). In  
80 that context, the focus on wild populations can be particularly relevant as the magnitude of  
81 sex differences in lifespan is likely modulated by environmental conditions, in interaction  
82 with the sex differences in genetic background (Lemaître et al. 2020c; Tidière et al. 2020).  
83 Finally, widening the scope of model species for ageing research can provide important  
84 insights for ‘healthspan extension’, notably by targeting wild animal populations displaying  
85 extended lifespan compared to the one expected for their body size (Austad 2010) and by  
86 including more appropriate senescence metrics (Lemaître et al. 2020a; Ronget and Gaillard  
87 2020). To reach these goals, accurate markers of both chronological and biological ages on a  
88 wide range of organisms are required. The pan tissue epigenetic clock based on DNA  
89 methylation (see Horvath, 2013) is a promising indicator of biological age in humans (Paoli-  
90 Iseppi et al. 2017; Parrott and Bertucci 2019; Bell et al. 2019).

91 DNA methylation (DNAm) of cytosine residues within CpG dinucleotides (5-methyl-  
92 cytosine) across the genome constitutes a key epigenetic DNA modification tightly linked to  
93 the ageing process (Horvath and Raj 2018). Indeed, DNA methylation patterns accurately  
94 predict chronological age in humans (Horvath 2013; Jung and Pfeifer 2015) and captive  
95 mammals reared in laboratory conditions. Such strong relationship between age and DNAm  
96 has been found in many cell types (e.g. white blood cells, brain, liver; see Horvath, 2013). A  
97 comparative analysis of methylomes indicates that methylation can also be used to assess  
98 reliably physiological aging across mammals (Wang et al. 2020). The discrepancy between  
99 pigenetic age and chronological age (epigenetic acceleration) is associated in humans with a  
100 wide range of metabolic, infectious and degenerative diseases (Horvath et al. 2014; Horvath  
101 and Levine 2015), as well as cancer (Levine et al. 2015) and mortality (Marioni et al. 2015;  
102 Chen et al. 2016; Christiansen et al. 2016). We hypothesize that DNA methylation profiles  
103 integrates environmental effects that might modulate the pace of the epigenetic clock. To  
104 address this hypothesis. we studied epigenetic ageing in the wild and in a sex-specific way.

105           In this study, we took advantage of a unique long-term (>40 years) longitudinal  
106 monitoring of individual roe deer (*Capreolus capreolus*) living in two populations facing  
107 different ecological contexts to investigate the relationship between chronological age and  
108 levels of DNA methylation. All roe deer used in this study have been captured within their  
109 first year of life, when age can be accurately assigned (Hewison et al. 1999). First, we  
110 expected that the epigenetic clock built from peripheral blood leucocyte DNA should provide  
111 an accurate estimation of chronological age in roe deer in the wild. Second, we performed an  
112 epigenome wide association analysis (EWAS) to identify CpGs that were the most likely to be  
113 associated with aging in roe deer. Third, based on evidence that the pace of epigenetic age is  
114 modulated by environmental factors and provides reliable information on time to death (see  
115 above), we expected that the *Average age acceleration* (i.e. average difference between  
116 DNAm age and chronological age) would be higher in the population facing harsh  
117 environmental conditions than in the population facing favorable environmental conditions.  
118 Finally, since male roe deer show higher initial adult mortality and rate of actuarial  
119 senescence than females (Gaillard et al. 2004), we expected that the *Average age acceleration*  
120 would be higher for males than for females. Moreover, thanks to the epigenome wide  
121 association analysis, we expected to identify specific CpGs displaying sex-specific DNAm  
122 aging profiles.

123

## 124 **Methods**

### 125 *Study populations*

126 We sampled roe deer living in two enclosed forests with markedly different environmental  
127 contexts: Trois Fontaines (TF) and Chizé (CH). The Trois Fontaines forest (1,360 ha) is  
128 located in north-eastern France (48°43'N, 4°55'E) and is characterized by a continental  
129 climate, moderately severe winters and warm and rainy summers. This site has rich soils and  
130 provides high quality habitat for roe deer (Pettorelli et al. 2006). In contrast, the Chizé forest  
131 (2,614 ha) is located in western France (46°50'N, 0°25'W) and is characterized by temperate  
132 oceanic climate with Mediterranean influences. This site has a low productivity due to poor  
133 quality soils and frequent summer droughts (Pettorelli et al. 2006), and thereby provides a  
134 quite poor habitat for roe deer in most years. Individuals from these two populations have  
135 been intensively monitored using a long-term Capture-Mark-Recapture program since 1975  
136 and 1977 (for Trois Fontaines and Chizé, respectively). In each site, 10-12 days of capture  
137 using drive-netting are organized every year between December and March (see Gaillard et  
138 al., 1993 for details on capture sessions), which allows capturing and measuring about half the

139 population every year. Once a roe deer is captured, its sex and body mass (to the nearest 50g)  
140 are recorded and a basic clinical examination is performed. All individuals included in our  
141 analyses were of known age because they were either caught as newborn in spring (see  
142 Delorme et al. 1988 for further details) or as c.a. 8 months old during winter captures, when  
143 they still have their milk teeth (most often incisors and always premolars, Flerov 1952).

144

#### 145 *Roe deer blood samples and dna extraction*

146 In 2016 and 2017, we collected blood samples (up to 1mL per kg of body mass) from the  
147 jugular vein. Within 30 min of sampling, the blood was centrifuged at 3000 g for 10 min and  
148 the plasma layer was removed before washing the cells with an equivalent volume of 0.9%  
149 w/v NaCl solution. After a second centrifugation, the intermediate buffy coat layer,  
150 comprising mainly leukocytes, was collected in a 1.5-mL Eppendorf tube and immediately  
151 frozen at  $-80^{\circ}\text{C}$  in a portable freezer (Telstar SF 8025) until further use.

152 We extracted genomic DNA from leucocytes using the Macherey-Nagel NucleoSpin@  
153 Blood QuickPure kit. DNA purity was assessed using a Nanodrop ND-1000  
154 spectrophotometer (Thermo Scientific, Wilmington DE, USA). For all samples, the purity  
155 absorption range was 1.7 - 2.0 for the 260/280 nm ratio and  $> 1.8$  for the 260/230 nm ratio.  
156 We selected 96 samples by balancing the numbers of individuals among ages, and between  
157 populations and sexes. DNA concentration was determined spectrophotometrically using the  
158 Qubit assay kit. DNA samples were then diluted in ultrapure water to reach a concentration of  
159  $\sim 70 \text{ ng}\cdot\mu\text{l}^{-1}$  and displayed in a microplate to complete the DNA methylation protocol (see  
160 below). For 6 samples, the concentrations obtained after dilution were too low compared to  
161 the expected concentrations of  $70 \text{ ng}/\mu\text{l}$  and were excluded from the dataset. The 90 roe deer  
162 samples analysed in this study correspond to 79 individuals aged from 8 months to 13.5 years  
163 of age. This age range encompasses most of the roe deer lifespan as individuals older than 15  
164 years of age are rarely observed in the wild (the oldest age ever recorded for a roe deer  
165 monitored in the wild being 17.5 years old, Gaillard et al. 1998).

166

#### 167 *DNA Methylation data*

168 We generated DNA methylation data using the custom Illumina chip  
169 "HorvathMammalMethylChip40". The mammalian methylation array is attractive because it  
170 provides very high coverage (over thousand X) of highly conserved CpGs in mammals. Two  
171 thousand out of 38k probes were selected based on their utility for human biomarker studies:  
172 these CpGs, which were previously implemented in human Illumina Infinium arrays (EPIC,

173 450K) were selected due to their relevance for estimating age, blood cell counts, or the  
174 proportion of neurons in brain tissue. The remaining 35,988 probes were chosen to assess  
175 cytosine DNA methylation levels in mammalian species. Each probe is designed to cover a  
176 certain subset of species, such that overall all species have a high number of probes (Arneson,  
177 Ernst, and S. H., unpublished data). The particular subset of species for each probe is  
178 provided in the chip manifest file can be found at Gene Expression Omnibus (GEO) at NCBI  
179 as platform GPL28271. The SeSaMe normalization method was used to define beta values for  
180 each probe (Zhou et al. 2018).

181

### 182 *Statistical analyses*

183 We first aimed to detect the function providing the best fit of the relationship linking  
184 DNAm age and chronological age. We thus compared three models corresponding to (1) an  
185 absence of relationship (constant model), (2) a constant increase of DNAm with age (linear  
186 model), and (3) a non-linear increase of DNAm with age (quadratic model, Table S1). The  
187 most parsimonious model was selected using the Akaike Information Criterion (AIC). We  
188 calculated AIC weights (AICw) to assess the relative likelihood that a given model was the  
189 best among the three fitted models (Burnham and Anderson 2002). We selected the model  
190 with the lowest AIC, but when the difference in AIC (denoted  $\Delta AIC$ ) between two competing  
191 models was less than two units, we retained the simplest model in accordance with parsimony  
192 rules (Burnham and Anderson 2002).

193 Second, we analyzed factors that could explain the between-sample variation in the  
194 average age acceleration. We computed the 'Average age acceleration' as the difference  
195 between DNAm age and chronological age following Horvath (2013). We then investigated  
196 whether the average age acceleration was influenced by sex, population and roe deer body  
197 mass (measured at capture) for the three main life stages in terms of survivorship in roe deer  
198 (Gaillard et al. 1993): juvenile (< 1 year of age), prime-age (1 to 8 years of age), and  
199 senescent (>8 years of age). For both prime-age and senescent life stages we ran a set of 14  
200 models with the Average age acceleration as the dependent variable and sex, population and  
201 body mass as the independent variables (see Table S2 for a full list of models). To avoid  
202 fitting over-parameterized models, we did not include any three-way interactions. Due to the  
203 low number of individuals of 1 year of age ( $N=8$ ), we only fitted 4 models for the juvenile life  
204 stage, the constant model (i.e. no detectable influence of any independent variable) and  
205 models including either a linear effect of body mass, sex differences, or population  
206 differences. In all cases, the best fitting model was selected using AIC (see above).

207 Third, we investigated further how sex influenced the relationship between DNAm age  
208 and chronological age through the use of sex-specific epigenetic clocks. For this purpose, we  
209 first built an epigenetic clock (called ‘female clock’) using data from females’ samples only,  
210 and then investigated the relationship between the female clock and both male and female  
211 chronological age. We performed a similar analysis with an epigenetic clock built using data  
212 from males’ samples only (called ‘male clock’). The 90 samples analyzed in our study  
213 correspond to 79 different individuals (i.e. 11 individuals were sampled both in 2016 and  
214 2017). To account for this pseudo-replication problem (sensu Hurlbert 1984) we thus  
215 replicated all analyses using linear mixed-effects models, which included a random effect of  
216 individual roe deer, using the R-package *lme4* (Bates et al. 2015). For all models, results were  
217 qualitatively unchanged (see Electronic Supplementary Material, Table S3) and for the sake  
218 of simplicity, we only report results from simple linear models below.

219 Fourth, we performed an epigenome wide association study of chronological age in  
220 roe deer. Unfortunately, a good genome assembly is not available for roe deer. Therefore, we  
221 performed an EWAS analysis based on the related White-tailed deer, *Odocoileus virginianus*,  
222 (Ovir.te\_1.0) genome assembly). In total, 32,767 probes from the  
223 HorvathMammalMethylChip40 were aligned to loci that are proximal to 6,314 genes in the  
224 Ovir.te\_1.0 genome assembly. Due to the high inter-species conservation of the probes on the  
225 array, findings can probably be extrapolated to roe deer, and even humans or other  
226 mammalian species. To assess the potential mechanism, we used a multivariate regression  
227 model to identify the CpGs that have a distinct pattern of DNAm aging between the sexes.  
228 We used two multivariate models. In the first model, DNAm levels of an individual CpGs  
229 were regressed on sex-specific chronological age to identify the loci with DNAm aging that  
230 are shared between sexes (“Age” main effect), and also the basal sex difference that is  
231 independent of chronological age (“Sex” main effect). In the second model, we included an  
232 interaction term to identify the CpGs with distinct DNAm aging between males and females.

233

## 234 **Results**

### 235 *Relationship between DNAm and chronological age*

236 The model that best described the relationship between DNAm and chronological age was the  
237 quadratic model (Table 1a; **Figure 1a**; Table S1). This might be due to the fact that DNA  
238 methylation accumulates at a faster rate during the growth and development of juveniles than  
239 later in life when roe deer have reached their full size (at 2 years of age, roe deer have reached  
240 > 90% of their asymptotic mass, Hewison et al. 2011). Accordingly, the model that best

241 described the relationship between DNAm and chronological age in adults only (2 to 14 years  
242 of age) was the linear model (slope of  $0.79 \pm 0.02$ ,  $N=82$ ,  $R^2 = 0.93$ ; **Figure 1b**; Table S1).  
243 For a subset of 25 adults from the Trois-Fontaines population, the exact date of birth was  
244 known meaning that it was possible to compute age at a very fine-scale resolution (i.e. in  
245 days). The use of this chronological age data measured in days slightly improved the fit of the  
246 relationship compared to the one obtained with the chronological age measured in years but  
247 the slope was left unchanged (slope of  $0.79 \pm 0.04$ ,  $N= 25$ ,  $R^2 = 0.95$ ; **Figure 1c**).

248

#### 249 *Factors affecting the average age acceleration*

250 When focusing on juveniles only, the average age acceleration was best explained by  
251 body mass (Table S2) as shown by the positive relationship between these two variables  
252 (slope of  $0.09 \pm 0.03$ ,  $R^2 = 0.59$ ;  $N=8$ , **Figure 2a**). On the contrary, for adults, the constant  
253 model of the average age acceleration was selected, even if we observed a trend for a higher  
254 average acceleration in males than in females (difference in intercepts of  $0.36 \pm 0.20$ ,  $N=82$ ,  
255 **Figure 2b**). We then investigated in more details the factors potentially explaining the  
256 average age acceleration in adults by running separate analyses for prime-age and senescent  
257 adults. In prime-aged adults, the selected model was again the constant model of the average  
258 age acceleration, whereas in senescent individuals, the selected model included a sex by  
259 population interaction (Table S2). The Average age acceleration was higher in males at Chizé  
260 ( $0.44 \pm 0.37$ ) than both in females at Chizé ( $-1.06 \pm 0.23$ ) and in males at Trois-Fontaines ( $-$   
261  $1.32 \pm 0.14$ ) (Table 1b; **Figure 2c**).

262

#### 263 *Influence of sex on the relationship between DNAm age and chronological age in adults*

264 When using the female clock, a trend for interactive effects of age and sex occurred  
265 (Table 2a). As expected, the fit was much better in females (slope of  $0.85 \pm 0.01$ ,  $N=42$ ,  
266  $R^2=0.99$ ) than in males (slope of  $0.76 \pm 0.05$ ,  $N=40$ ,  $R^2=0.86$ ). Interestingly, most male's  
267 DNAm located above the line where DNAm age exactly matched chronological age, meaning  
268 that males are biologically older than their chronological age estimated using the female clock  
269 (**Figure 3a**). The opposite pattern occurred when using the male clock (Table 2b, **Figure 3b**).  
270 Beyond 7 years of age, females were consistently biologically younger than their  
271 chronological age estimated from the male clock (**Figure 3b**). As expected, the fit was also  
272 much better for males (slope of  $0.73 \pm 0.02$ ,  $N=40$ ,  $R^2=0.98$ ) than for females (slope of  $0.48 \pm$   
273  $0.03$ ,  $N=42$ ,  $R^2=0.86$ ) when using the male clock.

274



275 *Epigenome wide association study of chronological age*

276 The EWAS results revealed that chronological age alters DNAm in a large number of Loci  
277 (**Figure 4**). At a genome significance level ( $p < 10^{-8}$ ), a total of 1992 loci showed DNAm  
278 aging. The top affected CpGs with DNAm aging were proximate to GRHL2 5'UTR ( $z =$   
279 12.5), ADRB1 exon ( $z = 12.3$ ), and PURA 3'UTR ( $z = -11.4$ ) (**Figure 4A**). Aging-associated  
280 CpGs in deer blood were distributed in all genic and intergenic regions that can be defined  
281 relative to transcriptional start sites (**Figure 4B**). However, promoter regions had a higher  
282 proportion of hypermethylated CpGs compared to others. This result paralleled a higher  
283 positive association of CpG islands with age than non-island CpGs (**Figure 4C**).

284 Transcriptional factor enrichment analysis suggested TFAP2C motifs are  
285 hypermethylated with age in the leucocyte's DNA of roe deer (**Figure 4D**). This motif is  
286 involved in cell-cycle arrest, germ cell development, and it is implicated in several types of  
287 cancer (Bryant et al. 2012; Penna et al. 2013). Understanding the functional outcome of this  
288 change in roe deer will require further studies.

289 Gene level enrichment analysis of the significant CpGs highlighted changes in  
290 development, the nervous system, O-Glycan metabolism, cancer, and immune system, all of  
291 which are associated with aging biology in humans and other species (**Figure 4E**). The  
292 analysis suggested that aging mediated hypermethylation is marked by H3K27Me3 and  
293 potentially regulated by polycomb protein EED targets. EED is a member of the multimeric  
294 Polycomb family protein complex that maintains the transcriptional repressive states of genes.  
295 These proteins also regulate H3K27Me3 marks, DNA damage, and senescence states of the  
296 cells during aging (Ito et al. 2018).

297

298 *CpGs whose aging patterns depend on sex*

299 The epigenetic age acceleration was faster in male than in female roe deer. At genome-wide  
300 significance ( $p < 10^{-8}$ ), age and sex altered DNAm of 1726 and 1022 CpGs, respectively  
301 (**Figure 5A**). This suggests both age and sex have a large effect size on DNAm levels. For  
302 sex, differentially methylated CpGs (DMCs) were biased in specific scaffold chromosomes,  
303 which are expected to be the homologs of sex chromosomes in humans and other mammals.  
304 For the interaction of age and sex, only three CpG proximate to RAI2 5'UTR, FAM155B  
305 exon, and ZIC3 exon had  $p$ -values  $< 10^{-8}$ . At a 5% false discovery rate (FDR), a total of 22  
306 CpGs showed a statistically significant interaction of age and sex in roe deer blood samples.  
307 This suggests sex influences DNAm aging in roe deer.

308 We overlaid the CpGs that interacted between sexes on a scatter plot of aging z-scores  
309 of male and female roe deer. The analysis identified five kinds of interaction (Figure 5B). The  
310 strongest pattern was for CpGs that were hypermethylated with age in males, but not females.  
311 CpGs proximate to RAI2, FAM155B, and ZIC3 (Figure 5C) genes had the aforementioned  
312 pattern. In contrast, some CpGs such as BCOR promoter and EFNB1 downstream only  
313 influenced DNAm aging in females (Figure 5C).

314

## 315 Discussion

316 Our findings highlight a very tight correlation between epigenetic and chronological age in  
317 two populations of roe deer intensively monitored in the wild. The quality of the fit of the  
318 selected model describing the relationship between epigenetic and chronologic age over the  
319 roe deer entire lifespan was particularly high (with a correlation coefficient of 0.975 leading  
320 to a  $R^2$  of 0.95; Median absolute difference: 0.588 years), which adds to the increasing  
321 evidence that the epigenetic tool constitutes an accurate method to estimate age in vertebrate  
322 populations in the wild (Paoli-Iseppi et al. 2017). So far, a wide range of technics based on  
323 tooth wear are generally used to assign age in wild mammals (Morris 1972, Pérez-Barbería et  
324 al. 2014 for a review in red deer, *Cervus elaphus*). In roe deer, tooth wear that leads the first  
325 molar height to decline with increasing age throughout the lifespan allows assessing age of  
326 individual roe deer (Veiberg et al. 2007). However, this method is much less accurate than the  
327 epigenetic clock ( $R^2= 0.69$  vs. 0.94 when a linear regression is used, Fig. S1).

328 Interestingly, the fit of the roe deer epigenetic clock outperforms the few epigenetic  
329 clocks previously developed from other mammalian populations. All studies performed so far  
330 have investigated the relationship between epigenetic and chronologic age through linear  
331 regressions, and did not account for non-linearities. The linear regression provided a better fit  
332 in roe deer here (i.e. a coefficient of correlation of 0.97) than that reported in humpback  
333 whales (*Megaptera novaeangliae*, correlation of 0.89, Polanowski et al. 2014); wood mice,  
334 (*Apodemus sylvaticus*, correlation of 0.92, Little et al. 2020) or Bechstein's bat (*Myotis  
335 bechsteinii*, correlation of 0.80, Wright et al. 2018), which might be due to the broad age  
336 range we included in the analysis or to differences in the biological tissue used to extract  
337 DNA (e.g. leukocytes vs wing or ear punches). Overall, the epigenetic clocks used in our  
338 study constitute a particularly accurate method for estimating age in roe deer on the basis of  
339 leucocyte DNA.

340 We found that the relationship between epigenetic and chronologic ages was better  
341 described by a quadratic than linear model when juveniles were included, but any deviation

342 from a linear model vanished when considering only adults. This discrepancy and the  
343 negative second order term of the quadratic model clearly indicate that the relationship  
344 between epigenetic and chronological ages is steeper in growing juveniles than in adults. A  
345 similar pattern has been reported in humans where the rate of change in DNA methylation  
346 profiles (also called ‘tick rate’) was higher during the developmental period than during  
347 adulthood, when a constant tick rate seems to be the rule (Horvath 2013). The growth period  
348 is associated with a high rate of mitotic division and constitutes a particularly demanding life  
349 stage in terms of resource allocation in mammals (Reiss 1989). Although overlooked for a  
350 while, the ageing consequences of a fast growth during early life are increasingly investigated  
351 (Metcalf and Monaghan 2003) and recent evidence suggests that fast growth can shorten  
352 lifespan on the long-run (Lee et al. 2013; Kraus et al. 2013), even though the exact  
353 physiological mechanisms underlying this association are likely to be multiple and complex  
354 (Metcalf and Monaghan 2003; Monaghan and Ozanne 2018). In roe deer, individuals reach  
355 their asymptotic mass around the age of 4 years in both males and females (Hewison et al.  
356 2011). However, juveniles (i.e. at eight months when individuals are captured for the first  
357 time) have already gained about two-third of their adult body mass (Hewison et al. 2011),  
358 with a high amount of individual variation, which makes winter juvenile body mass a reliable  
359 measure of growth intensity. We found that the average age epigenetic acceleration increases  
360 with juvenile body mass, suggesting that individuals who allocate substantially in their  
361 growth are biologically older than their chronological age indicates, which might contribute to  
362 explain why, in roe deer, a fast-post-weaning growth is associated with a steeper rate of body  
363 mass senescence (Douhard et al. 2017). A positive association between DNA methylation  
364 profiles (as measured with the Horvath pan tissue clock) and height has also been observed  
365 among teenagers (Simpkin et al. 2016), which suggests that the discrepancies between  
366 biological and chronological age following fast growth might be widespread across mammals  
367 and also offer new perspectives for the study of the relationships between growth and ageing.

368 Despite the limited sample size, our analyses suggest that for a given age, male roe  
369 deer from the Chizé population are biologically older than females, a difference that is  
370 particularly pronounced at old ages. As the epigenetic age acceleration is associated with  
371 mortality risk in humans (e.g. Marioni et al. 2015), this result is in line with previous roe deer  
372 survival analyses, which showed that males at Chizé have a shorter lifespan than females -  
373 especially when born during years of strong environmental harshness (Garratt et al. 2015).  
374 Alike humans or laboratory rodents, there is compelling evidence that mammalian males in  
375 the wild display shorter lives than females (Lemaître et al. 2020c), as predicted by several

376 (and non-mutually exclusive) evolutionary theories (e.g. heterogametic sex hypothesis,  
377 mother's curse hypothesis, sex differences in life history strategies, see Austad and Fischer  
378 2016; Marais et al. 2018 for reviews). However, the magnitude of sex differences in lifespan  
379 remains highly variable among populations and species (Lemaître et al. 2020c). This  
380 discrepancy might partly result from the high variation in environmental conditions faced by  
381 populations in the wild (Lemaître et al. 2020c; Tidière et al. 2020). More specifically, harsh  
382 environmental conditions (e.g. low availability in resources, high pathogen richness) are  
383 expected to amplify the survival cost of male reproductive expenditure (e.g. allocation to  
384 sexual traits, territory defence) due to the acute resource-based allocation trade-offs between  
385 reproduction and survival insurance mechanisms (Kirkwood and Rose 1991; Kirkwood  
386 2017). At Chizé, environmental conditions are much harsher than at Trois Fontaines due to  
387 low quality resources, which might explain the clear epigenetic age acceleration in old males  
388 from this population.

389 More generally, our findings suggest that the epigenetic age acceleration might  
390 constitute a relevant biological marker of sex differences in health and biological conditions  
391 in mammals. For sex-related CpGs, the top enriched datasets were related to X-linked and  
392 Gonosomal inheritance. Other basal sex differences were related to the nervous system (e.g.  
393 synapse function), cognition (e.g. intellectual disability, spatial learning), and development  
394 (e.g. nervous system, teeth, muscles). For interaction, the enrichment analysis suggested a  
395 difference in heparan sulfate glucosaminoglycan biosynthesis between male and female  
396 aging. In humans, Alzheimer's disease patients have higher distribution and localization of  
397 heparan sulfate glucosaminoglycan in neurons, microglia, and often colocalize with amyloid  
398 plaques (Su et al. 1992). Moreover, these macromolecules are involved in aging through  
399 neurogenesis (Yamada et al. 2017), and skin homeostasis (Bucay et al. 2020). Our results  
400 suggest a sex difference in heparan sulfate glucosaminoglycan biosynthesis during aging,  
401 which could also contribute to sex differences in human neurodegenerative disorders.  
402 Moreover, our analysis suggests DNAm underlie some of these differences. While  
403 mammalian females undeniably live longer than males (Lemaître et al. 2020c), studies that  
404 have sought to decipher the genetic and physiological correlates of these sex differences in  
405 survival have remained rather inconclusive. For instance, other biological markers of aging  
406 such as telomere length or immune performance do not show clear differences between males  
407 and females in wild mammals (Peters et al. 2019; Remot et al. 2020). We emphasize that  
408 using the epigenetic age acceleration across longitudinal follow-ups of mammalian  
409 populations does constitute to date the most promising approach to i) estimate accurately the

410 chronological age of individual mammals, (ii) assess precisely sex differences in  
411 physiological condition, iii) disentangle the complex ecological and biological origins of these  
412 differences, and iv) establish reliable predictions in terms of individual trajectories. In the  
413 current context of a growing age in human populations associated with pronounced sex  
414 differences in the occurrence of age-associated diseases in the elderly (Austad and Fischer  
415 2016; Clocchiatti et al. 2016), this research avenue is extremely promising.

416

#### 417 **Author contributions**

418 SH and JFL conceived of the study and wrote the article. The remaining authors helped with  
419 the statistical analyses and data collection. All authors reviewed and edited the article.

420

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424

425 *Ethics.* The protocol of capture and blood sampling under the authority of the Office National  
426 de la Chasse et de la Faune Sauvage (ONCFS) was approved by the Director of Food,  
427 Agriculture and Forest (Prefectoral order 2009–14 from Paris). The land manager of both  
428 sites, the Office National des Forêts (ONF), permitted the study of the populations  
429 (Partnership Convention ONCFS-ONF dated 2005-12-23). All experiments were performed  
430 in accordance with guidelines and regulations of the Ethical Committee of Lyon 1 University  
431 (project DR2014-09, June 5, 2014).

432

433 *Competing interests.* SH is a founder of the non-profit Epigenetic Clock Development  
434 Foundation which plans to license several of his patents from his employer UC Regents. The  
435 other authors declare no conflicts of interest.

436

437

438

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627 46:e123–e123  
628  
629

630 **TABLES**

631

632 Table 1: Parameters of the selected models discussed in the main text. (a) Quadratic model  
 633 describing the relationship between DNAm age and chronological age in roe deer ( $N=90$ ;  $R^2$   
 634 = 0.95). (b) Best model explaining variation in the Average age acceleration for senescent roe  
 635 deer (i.e. >8 years of age) ( $N=23$ ;  $R^2 = 0.35$ ) (\* $p<0.05$ , \*\* $p<0.01$ , \*\*\*  $p<0.001$ ).  
 636

Dependent Variables		Estimate	SE	t
(a) DNAm age	Intercept	0.29	0.21	1.39
	Age	1.13	0.07	15.002***
	Age <sup>2</sup>	-0.02	0.006	-3.98***
(b) Average age acceleration	Intercept	-1.09	0.24	-4.48***
	Sex	1.53	0.53	2.92**
	Population	0.04	0.43	0.1
	Sex*Population	-1.81	0.75	-2.40*

637

638

639 Table 2: Parameters of the models testing for an interaction between chronological age and  
 640 sex using the female (a) or (b) the male clock (\* $p<0.05$ , \*\* $p<0.01$ , \*\*\*  $p<0.001$ ).  
 641

Dependent Variables		Estimate	SE	t
(a) DNAm age [Female epigenetic clock]	Intercept	1.03	0.2	5.17***
	Age	0.85	0.03	32.205***
	Sex	0.8	0.3	2.38**
	Age * Sex	-0.08	0.04	-1.89
(b) DNAm age [Male epigenetic clock]	Intercept	3.35	0.18	18.58***
	Age	0.48	0.02	20.25***
	Sex	-1.93	0.27	-7.10***
	Age * Sex	0.25	0.04	6.10***

642

643 **CAPTIONS FOR FIGURES**

644

645 **Figure 1: Epigenetic clock for roe deer built with individual methylation profiles from**  
646 **white blood cells DNA.** (a) Epigenetic clock for known-aged individuals between 8 months  
647 and 14 years of age ( $N=90$ ;  $R^2=0.95$ ); (b) Epigenetic clock for adult roe deer (i.e. >1 year old,  
648  $N=82$ ;  $R^2=0.93$ ); (c) Epigenetic clock for individuals where the exact age in days was known  
649 ( $N=25$ ;  $R^2=0.95$ ). The epigenetic age (DNAmAgeLoo) is expressed in years. In all graphs, the  
650 dashed line corresponds to the regression line  $y = x$ .

651

652 **Figure 2: Average age acceleration in the epigenetic clock of wild roe deer.** (a) Relationship  
653 between the average age acceleration and the body mass for juveniles (i.e. 8 months,  $N=8$ ), (b)  
654 Sex-differences in the *average age acceleration* between adult males and adult females (i.e. > 1  
655 years old,  $N=82$ ) (c) Average age acceleration for senescent individuals (i.e. > 8 years old,  
656  $N=23$ ) split by sex and population.

657

658 **Figure 3: Sex-specific epigenetic clock in roe deer.** (a) Relationship between DNAm age  
659 estimated with the female clock and both male and female chronological ages (b) Relationship  
660 between DNAm age estimated with the male clock and both male and female chronological  
661 ages. In all graphs, the dashed line corresponds to the regression line  $y = x$ . Females are  
662 displayed in light blue and males in dark blue.

663

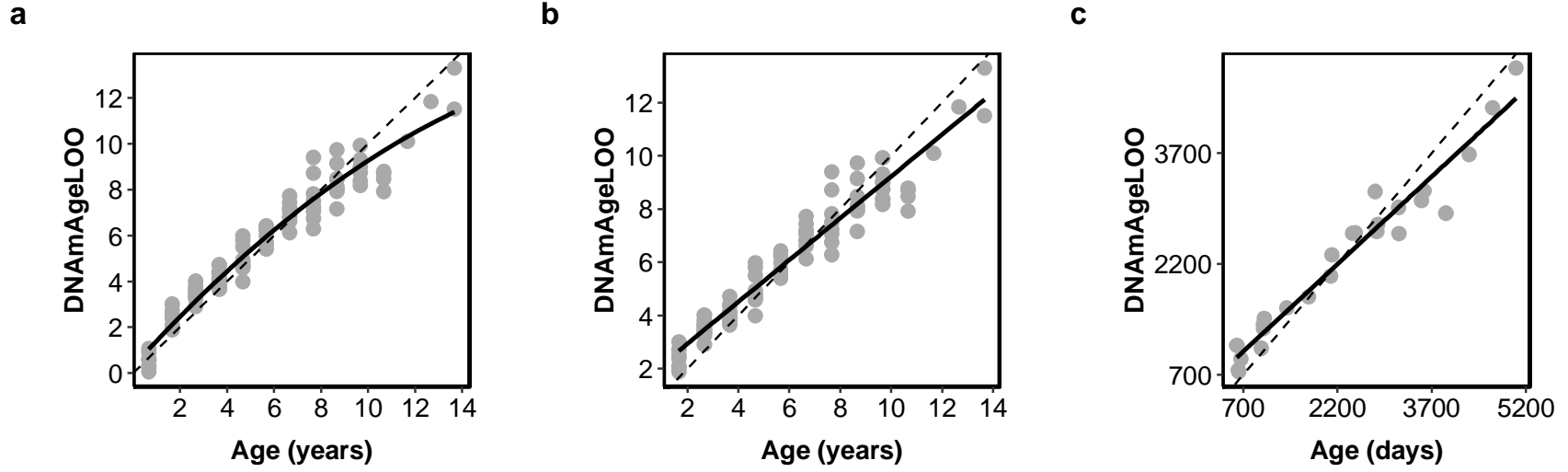
664 **Figure 4: Epigenome-wide association (EWAS) of chronological age in the blood of roe**  
665 **deer.** A) Manhattan plot of the EWAS of chronological age. Since the genome assembly is not  
666 available for roe deer, the coordinates are estimated based on the alignment of Mammalian  
667 array probes to White-tailed deer (*Ovir.te\_1.0*) genome assembly, a related species to roe deer.  
668 The direction of associations with  $p < 10^{-8}$  (red dotted line) is highlighted by red  
669 (hypermethylated) and blue (hypomethylated) colors. The top 30 CpGs were labeled by the  
670 neighboring genes. B) Location of top CpGs in each tissue relative to the closest transcriptional  
671 start site. Top CpGs were selected at  $p < 10^{-8}$  and further filtering based on z score of  
672 association with chronological age for up to 500 in a positive or negative direction. The grey  
673 color in the last panel represents the location of 32767 mammalian BeadChip array probes  
674 mapped to *Ovir.te\_1.0* genome. C) CpG islands have a higher positive association with age  
675 (hypermethylation) than other sites. D) Transcriptional motif enrichment for the top CpGs in  
676 the promoter and 5'UTR of the neighboring genes. The motifs were predicted using the MEME  
677 motif discovery algorithm, and the enrichment was tested using a hypergeometric test. E)  
678 Enrichment analysis of the top CpGs in cat blood. The analysis was done using the genomic  
679 region of enrichment annotation tool (McLean et al. 2010). The gene-level enrichment was  
680 done using GREAT analysis (McLean et al. 2010) and human Hg19 background. The top 3  
681 enriched datasets from each category (Canonical pathways, diseases, gene ontology, human  
682 and mouse phenotypes, and upstream regulators) were selected and further filtered for  
683 significance at  $p < 10^{-4}$ .

684

685 **Figure 5 : Sex influence on DNAm aging.** A) Manhattan plots of DNAm aging loci that are  
686 shared between sexes (Aging main effect), basal sex differences (Sex main effect), and the  
687 interaction of sex and aging. The analysis is done by multivariate regression models with or  
688 without (to estimate the main effect) interaction term for age and sex. For sex, the male is the  
689 reference variable to estimate the direction of change. Sample sizes: Males, 45 ; Females, 49.  
690 The coordinates are estimated based on the alignment of Mammalian array probes to White  
691 tailed deer (*Ovir.te\_1.0*) genome assembly. The red line in the Manhattan plot indicates  $p < 1e-$

692 3. B) Scatter plots DNAm aging between male and female roe deer. The highlighted CpGs are  
693 the loci with statistically significant interaction between species at a 5% FDR rate. In total, five  
694 categories of interaction were defined based on the aging z-score of each sex. C) DNAm aging  
695 in selected loci with a statistically significant sex interaction. D) Enrichment analysis of the  
696 genes proximate to CpGs related to age (shared between sexes), sex, and age:sex interaction.  
697 The gene-level enrichment was done using GREAT analysis (McLean et al. 2010) and human  
698 Hg19 background. The top CpGs were selected at a 5% FDR rate and based on Beta values of  
699 association for up to 500 in a positive or negative direction.

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FIGURE 1

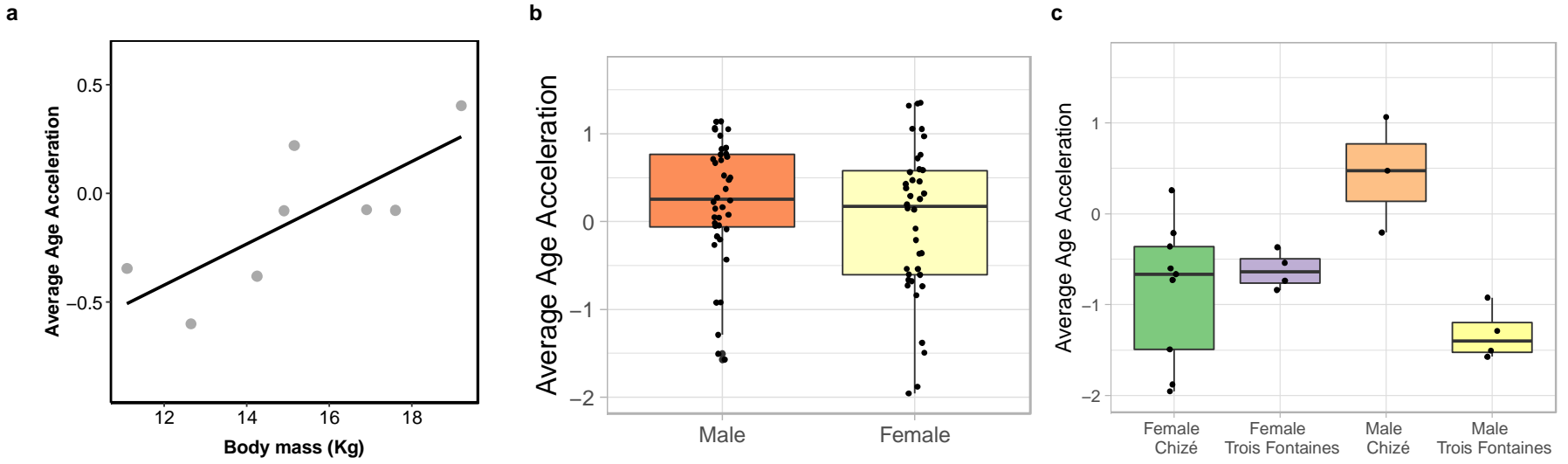
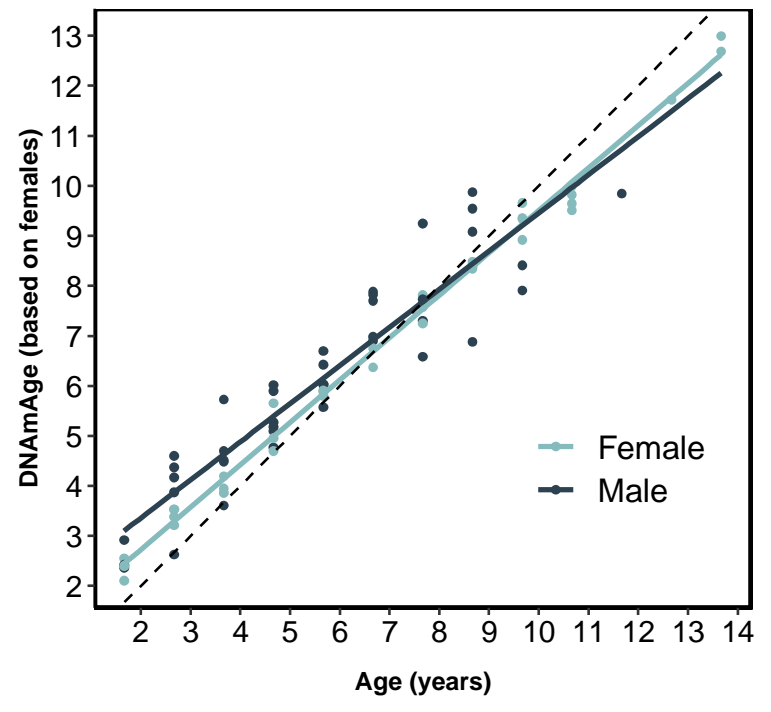


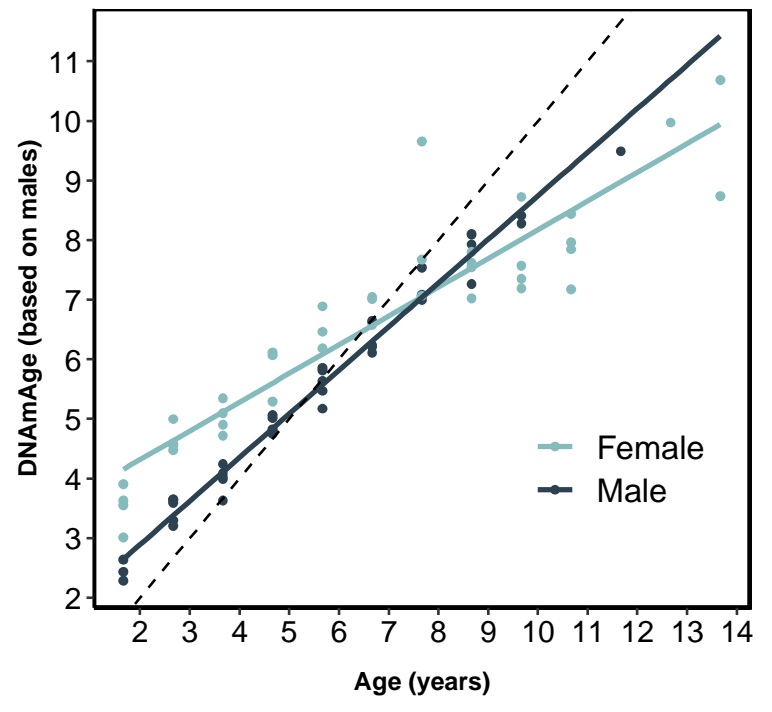
FIGURE 2

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**a**

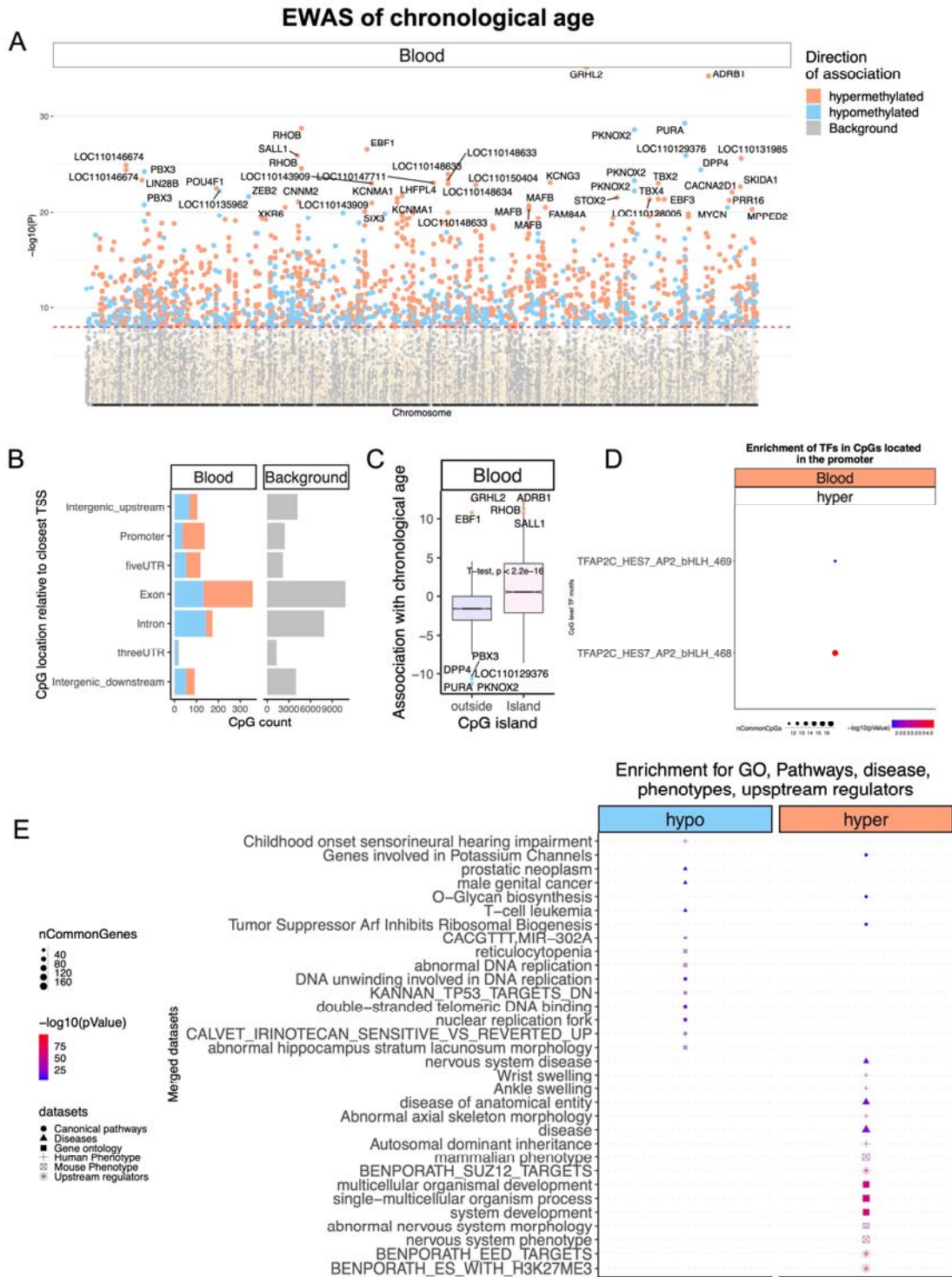


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FIGURE 3



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FIGURE 4



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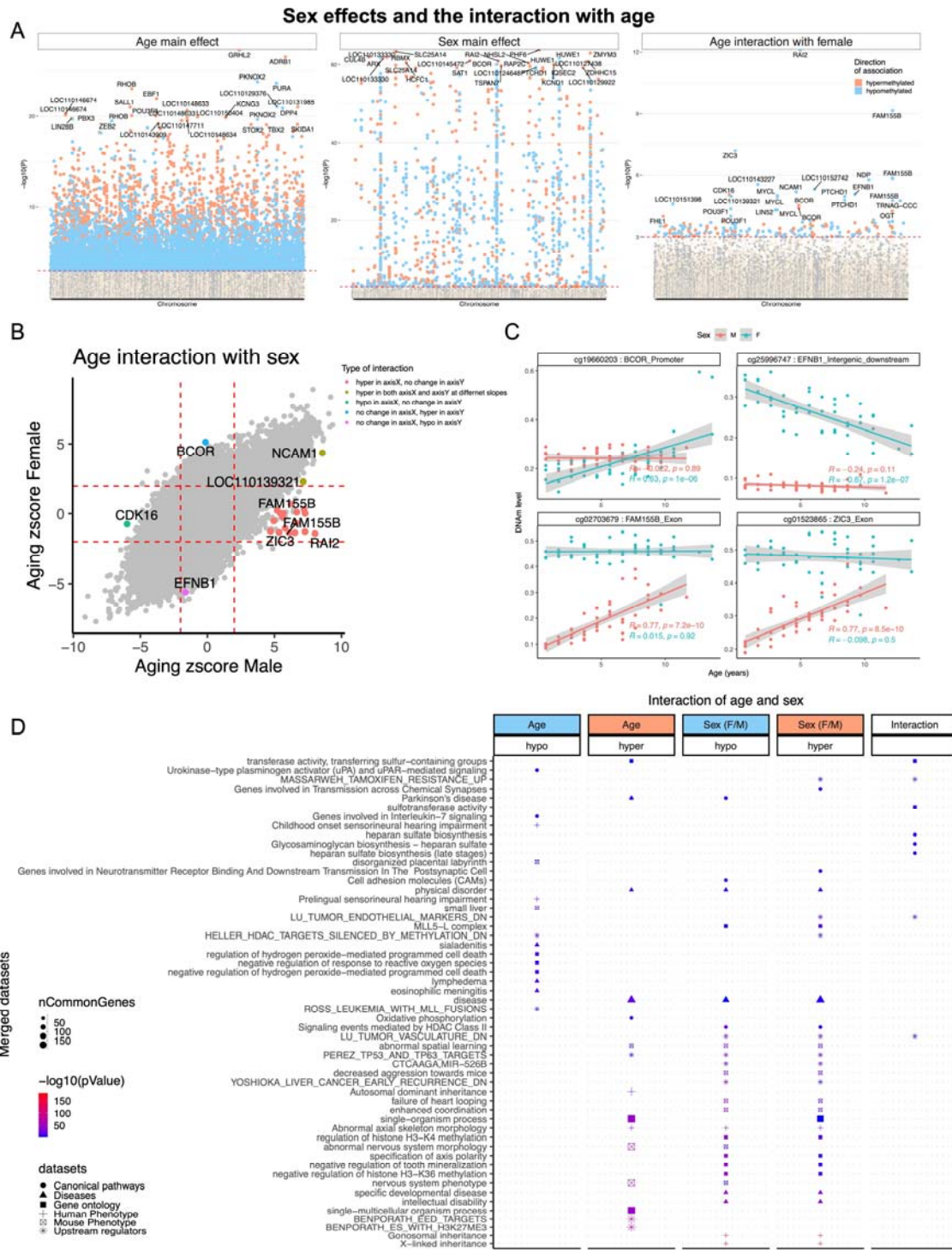


FIGURE 5

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ELECTRONIC SUPPLEMENTARY MATERIAL

Table S1: Model selection procedure from the set of linear models fitted to test the relationship between DNAm age and chronological age for all individuals in the dataset (A) or for adults only (B). The selected model is highlighted in bold, k is the number of parameters in the model,  $\Delta$ AIC is the difference in AIC between the candidate model and the selected model. The AIC weight (AICw) is calculated to measure the relative likelihood that a given model is the best among the set of fitted models.

			k	AIC	$\Delta$ AIC	AICw
<b>A</b>	All individuals (N=90)	Constant	2	448.09	260.46	0.00
		Linear	3	200.72	13.09	0.00
		<b>Quadratic</b>	<b>4</b>	<b>187.63</b>	<b>0.00</b>	<b>1.00</b>
<b>B</b>	Individuals older than 1-year-old (N=82)	Constant	2	334.37	165.86	0.00
		<b>Linear</b>	<b>3</b>	<b>169.06</b>	<b>0.55</b>	<b>0.43</b>
		Quadratic	4	168.51	0.00	0.57

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743 Table S2: Model selection procedure from the set of linear models fitted to test the  
 744 relationship between the Average age acceleration in the epigenetic clock and chronological  
 745 age, sex, population and body mass for adults (A), juveniles (B), Prime-aged individuals (C)  
 746 and senescent individuals (D). The selected model is highlighted in bold, k is the number of  
 747 parameters in the model,  $\Delta AIC$  is the difference in AIC between the candidate model and the  
 748 selected model. The AIC weight (AICw) is calculated to measure the relative likelihood that a  
 749 given model is the best among the set of fitted models.  
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		k	AIC	$\Delta AIC$	AICw
<b>A</b>	<b>Constant</b>	<b>2</b>	<b>222.78</b>	<b>1.14</b>	<b>0.25</b>
	Body mass	3	224.35	2.70	0.60
	Sex	3	221.65	0.00	0.00
	Population	3	224.73	3.08	0.69
	Body mass + Sex	4	223.64	2.00	0.45
	Body mass + Population	4	226.32	4.68	1.04
	Sex + Population	4	223.63	1.99	0.44
	Body mass + Sex + Population	5	225.61	3.96	0.88
	Body mass*Sex	5	225.50	3.85	0.86
	Body mass*Population	5	228.24	6.59	1.47
	Sex*Population	5	221.96	0.31	0.07
	Body mass*Sex + Population	6	227.48	5.83	1.30
	Body mass*Population + Sex	6	227.59	5.95	1.33
	Sex*Population + Body Mass	6	223.93	2.28	0.51
<b>B</b>	Constant	2	7.72	5.14	0.06
	Body mass	<b>3</b>	<b>2.57</b>	<b>0.00</b>	<b>0.83</b>
	Sex	3	9.65	7.08	0.02
	Population	3	7.15	4.58	0.08
<b>C</b>	<b>Constant</b>	<b>2</b>	<b>109.50</b>	<b>0.00</b>	<b>0.25</b>
	Body mass	3	111.01	1.50	0.12
	Sex	3	111.37	1.86	0.10
	Population	3	110.25	0.74	0.17
	Body mass + Sex	4	112.48	2.97	0.06
	Body mass + Population	4	112.22	2.72	0.07
	Sex + Population	4	112.11	2.60	0.07
	Body mass + Sex + Population	5	113.95	4.45	0.03
	Body mass*Sex	5	114.06	4.56	0.03
	Body mass*Population	5	114.07	4.56	0.03
	Sex*Population	5	113.07	3.56	0.04
	Body mass*Sex + Population	6	115.64	6.14	0.01
	Body mass*Population + Sex	6	115.73	6.23	0.01
Sex*Population + Body Mass	6	114.93	5.42	0.02	

<b>D</b>						
		Constant	2	64.93	64.93	0.04
		Body mass	3	65.24	65.24	0.03
		Sex	3	65.33	65.33	0.03
		Population	3	65.80	65.80	0.03
		Body mass + Sex	4	63.66	63.66	0.08
		Body mass + Population	4	67.23	67.23	0.01
	Individuals older than 8-years-old (N=23)	Sex + Population	4	65.15	65.15	0.04
		Body mass + Sex + Population	5	65.65	65.65	0.03
		Body mass*Sex	5	61.97	61.97	0.18
		Body mass*Population	5	68.50	68.50	0.01
		<b>Sex*Population</b>	<b>5</b>	<b>61.07</b>	<b>61.07</b>	<b>0.28</b>
		Body mass*Sex + Population	6	63.94	63.94	0.07
		Body mass*Population + Sex	6	67.15	67.15	0.01
		Sex*Population + Body Mass	6	62.07	62.07	0.17

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757 Table S3: Quadratic model describing the relationship between DNAm age and chronological  
 758 age in roe deer (N=90). Contrary to Table 1, the model was fitted with roe deer identity  
 759 included as a random effect. Results are qualitatively unchanged (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*  $p <$   
 760  $0.001$ ).

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	Estimate	SE	t
Intercept	0.28	1.31	1.39
Age	1.13	0.08	14.90***
Age <sup>2</sup>	-0.02	0.006	-4.02***

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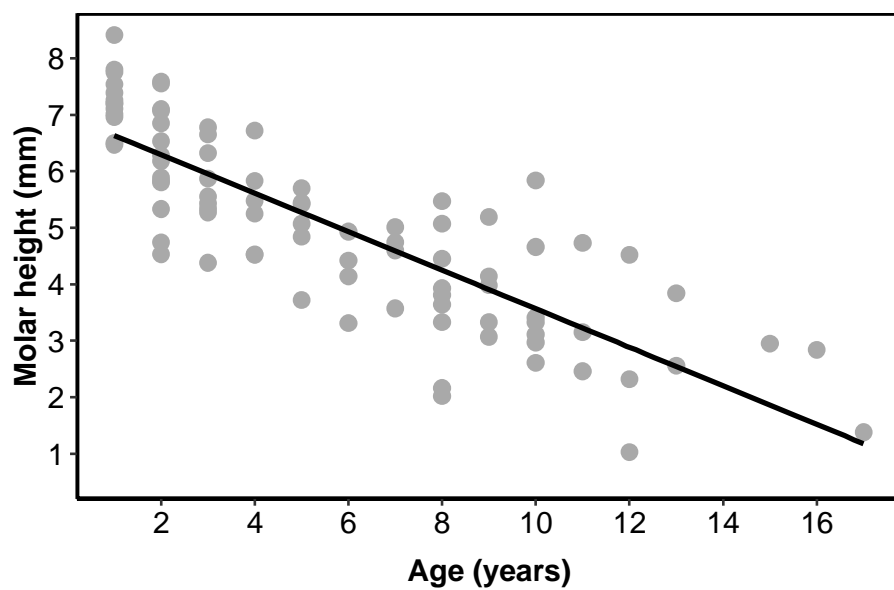
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767 Table S4: Parameters of the models including the interaction between chronological age and  
768 sex using the female (a) or the male (b) clock; and of the models including the interaction  
769 between chronological age and population using the Trois Fontaines (c) or the Chizé (d)  
770 clock. Contrary to Table 2, these models were fitted with the roe deer identity as a random  
771 effect. Results are qualitatively unchanged (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*  $p < 0.001$ ).  
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Dependent Variables		Estimate	SE	t
(a) DNAm age [Female epigenetic clock]	Intercept	1.03	0.2	5.09***
	Age	0.85	0.03	30.91***
	Sex	0.7	0.3	2.41**
	Age * Sex	-0.07	0.04	-1.57
(b) DNAm age [Male epigenetic clock]	Intercept	3.32	0.18	18.40***
	Age	0.48	0.02	19.86***
	Sex	-1.92	0.27	-7.10***
	Age * Sex	0.25	0.04	6.06***

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781 FIGURE S1: Relationship between molar height (M1, in mm) and age (in years) for roe deer

782 from Chizé and Trois-Fontaines (slope  $\pm$  se:  $-0.34 \pm 0.02$ ,  $N=88$ ,  $R^2= 0.69$ ).

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