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Biological clocks as age estimation markers in animals: a systematic review and meta-analysis

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ABSTRACT

Various biological attributes associated with individual fitness in animals change predictably over the lifespan of an organism. Therefore, the study of animal ecology and the work of conservationists frequently relies upon the ability to assign animals to functionally relevant age classes to model population fitness. Several approaches have been applied to determining individual age and, while these methods have proved useful, they are not without limitations and often lack standardisation or are only applicable to specific species. For these reasons, scientists have explored the potential use of biological clocks towards creating a universal age-determination method. Two biological clocks, tooth layer annulation and otolith layering have found universal appeal. Both methods are highly invasive and most appropriate for *post-mortem* age-at-death estimation. More recently, attributes of cellular ageing previously explored in humans have been adapted to studying ageing in animals for the use of less-invasive molecular methods for determining age. Here, we review two such methods, assessment of methylation and telomere length, describing (*i*) what they are, (*ii*) how they change with age, and providing (*iii*) a summary and meta-analysis of studies that have explored their utility in animal age determination. We found that both attributes have been studied across multiple vertebrate classes, however, telomere studies were used before methylation studies and telomere length has been modelled in nearly twice as many studies. Telomere length studies included in the review often related changes to stress responses and illustrated that telomere length is sensitive to environmental and social stressors and, in the absence of repair mechanisms such as telomerase or alternative lengthening modes, lacks the ability to recover. Methylation studies, however, while also detecting sensitivity to stressors and toxins, illustrated the ability to recover from such stresses after a period of accelerated ageing, likely due to constitutive expression or reactivation of repair enzymes such as DNA methyl transferases. We also found that both studied attributes have parentally heritable features, but the mode of inheritance differs among taxa and may relate to heterogamy. Our meta-analysis included more than 40 species in common for methylation and telomere length, although both analyses included at least 60 age-estimation models. We found that methylation outperforms telomere length in terms of predictive power evidenced from effect sizes (more than double that observed for telomeres) and smaller prediction intervals. Both methods produced age correlation models using similar sample sizes and were able to classify individuals into young, middle, or old age classes with high accuracy. Our review and meta-analysis illustrate that both methods are well suited to studying age in animals and do not suffer significantly from variation due to differences in the lifespan of the species, genome size, karyotype, or tissue type but rather that quantitative method, patterns of inheritance, and environmental factors should be the main considerations. Thus, provided that complex factors affecting the measured trait can be accounted for, both methylation and telomere length are promising targets to develop as biomarkers for age determination in animals.

Key words: animals, biological clocks, tooth layers, otoliths, epigenetics, methylation, telomeres, biomarker, age determination, meta-analysis.

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CONTENTS

I. Introduction . . . 2
II. Methods . . . 4
(1) Literature search and review . . . 4
(2) Meta-analysis . . . 5
III. Biological clocks and age . . . 6
(1) DNA methylation . . . 6
(*a*) What is DNA methylation? . . . 6
(*b*) Changes with age . . . 6
(*c*) DNA methylation as a biomarker of animal age . . . 7
(*i*) Fishes (classes Agnatha, Chondrichthyes, and Osteichthyes) . . . 7
(*ii*) Amphibians (class Amphibia) . . . 9
(*iii*) Reptiles (class Reptilia) . . . 9
(*iv*) Birds (class Aves) . . . 13
(*v*) Mammals (class Mammalia) . . . 13
(*i*.1) Aquatic mammals (infraorder Cetacea) . . . 13
(*i*.2) Bats (order Chiroptera) . . . 13
(*i*.3) Carnivores (order Carnivora) . . . 13
(*i*.4) Elephants (order Proboscidea, suborder Elephantiformes) . . . 14
(*i*.5) Marsupials (infraorder Marsupialia) . . . 14
(*i*.6) Primates (order Primates) . . . 15
(*i*.7) Rodents (order Rodentia) . . . 15
(*i*.8) Shrews (order Eulipotyphla) . . . 15
(*i*.9) Ungulates (orders Artiodactyla and Perissodactyla) . . . 15
(2) Telomeres . . . 16
(*a*) What are telomeres? . . . 16
(*b*) Changes with age . . . 17
(*c*) Telomeres as a biomarker of animal age . . . 17
(*i*) Fishes (classes Agnatha, Chondrichthyes, and Osteichthyes) . . . 23
(*ii*) Amphibians (class Amphibia) . . . 23
(*iii*) Reptiles (class Reptilia) . . . 23
(*iv*) Birds (class Aves) . . . 23
(*v*) Mammals (class Mammalia) . . . 24
(*i*.1) Aquatic mammals (infraorder Cetacea and order Sirenia) . . . 24
(*i*.2) Bats (order Chiroptera) . . . 24
(*i*.3) Carnivores (order Carnivora) . . . 24
(*i*.4) Elephants (order Proboscidea, suborder Elephantiformes) . . . 24
(*i*.5) Marsupials (infraorder Marsupialia) . . . 25
(*i*.6) Primates (order Primates) . . . 25
(*i*.7) Rodents (order Rodentia) . . . 25
(*i*.8) Shrews (order Eulipotyphla) . . . 25
(*i*.9) Ungulates (orders Artiodactyla and Perissodactyla) . . . 25
IV. Meta-analysis results . . . 25
(1) Methylation-based clocks . . . 25
(2) Telomere-based clocks . . . 27
(3) Methylation *versus* telomeres . . . 27
V. Discussion . . . 27
VI. Conclusions . . . 31
VII. Acknowledgements . . . 31
VIII. Data availability statement . . . 31
IX. References . . . 31
X. Supporting information . . . 39

I. INTRODUCTION

Age is a key factor in the study of animal populations, as many critical biological attributes change over time. Age classes are often linked to biological functionality in terms of developmental stage (Noren & Edwards, 2007), reproductive maturity (Charpentier *et al.*, 2008), future reproductive potential (Jones *et al.*, 2014), reproductive success

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(Bradley & Safran, 2014), disease burden (Starr & Saito, 2014), and risk of mortality (Pérez-Barberìa *et al.*, 2014). These attributes typically differ between discreet age classes and the fraction of a population contained within the different age classes therefore assists conservation biologists to estimate their future fitness (Pérez-Pereira, López-Cortegano & García-Dorado, 2022). Multiple approaches have been used with varying degrees of success to determine the age of animals and often rely upon either external features that change predictably over time, a mark–recapture approach (Clutton-Brock & Sheldon, 2010), or biochemical attributes.

The first, and most common method, uses morphometrics or physical measurements of the skull (Smuts, Anderson & Austin, 1978), body (Marker & Dickman, 2003), scales (Isermann, Wolter & Breeggemann, 2010), wingspan (Erdem *et al.*, 2021), or mass to determine age by comparing measures to an established chart. While this method is particularly useful for juveniles and young adults it generally loses resolution when applied to adults of varying age classes in species where growth arrests at adulthood. To overcome this, morphometrics have also been complemented with other physical attributes such as degree of gumline recession (Hiller & Tyre, 2014) or the condition of the pelage (Marker & Dickman, 2003). Drawbacks of this approach are (*i*) that it requires the capture and prolonged immobilisation of the animal to record the measures, (*ii*) few and sparsely detailed validation studies exist (which may be difficult to conduct), and (*iii*) there is high inter-observer error, particularly in cases where a reasonable amount of expertise is needed to discern age based on observed traits.

The mark–recapture approach either relies upon distinct natural markings and external features that are documented, or artificial markings applied to young individuals upon first encounter that are used to verify the identity and age of the individual upon subsequent encounters. Natural markings that can be used to identify individuals include, for example, pigmentation patterns on the flukes of humpback whales [*Megaptera novaeangliae* (Borowski)] (Polanowski *et al.*, 2014). Artificial markings, on the other hand, include manmade tools of identification such as uniquely numbered bands (Sherley *et al.*, 2014) and geolocators (Saino *et al.*, 2015) attached to birds, tracking collars (Marker *et al.*, 2003) attached to cheetahs [*Acinonyx jubatus* (Schreber)], or teracycline marking (Cailliet, 1990) in fishes. A major pitfall in the use of these methods is that they rely upon the eventual re-encounter of the same individual and require the attachment or injection of a marker to a live animal. In addition, the lifespan of many animals far outreaches the average lifespan and career of conservationists, making long-term tracking unsuitable in some cases.

For these reasons, conservationists have frequently sought a biomarker or method of age determination that is universally applicable to most species, provides a direct measure of age, and does not require extensive experience or detailed individual validation studies. While several biochemical, physiological, and haematological attributes have been suggested (Short, Williams & Bowden, 1987; Cailliet, 1990; Chaney *et al.*, 2003), the most promising method for achieving this is to identify measurable features of biological clocks that change on an annual basis. A biological clock can be defined as: an innate mechanism that controls the physiological activities of an organism which change on a daily, seasonal, yearly, or other regular cycle. Two biological clocks have found widespread applications in the estimation of animal age. In fish, histological features of internal structures such as growth rings in otoliths, small stones found within the auditory system, have been used to determine age by counting stria that form from each annual deposition of a new layer (Campana, 2001). Similarly, the annual cycle of depositing a new layer of dentin (Lockyer, 1993; Lockyer *et al.*, 2007) or cementum (Wittwer-Backofen, Gampe & Vaupel, 2004) on teeth can be used to determine the age of mammals. Tooth cementum annulation (TCA; reviewed in Viciano, López-Lázaro & Tanga, 2022) was first documented across diverse species of mammals and reptiles in the 19th century (Owen, 1840) and has been a reliable method of age estimation in animals for nearly 75 years. TCA has been used to determine the age of more than a hundred species in the class Mammalia alone: across 10 orders and 38 families (see online Supporting Information, Table S1). There are, however, still several shortcomings to these methods, e.g. otoliths are only effective in fish, tooth histology is only accurate in mammals, and both methods are extremely invasive, either requiring the extraction of a tooth or only being appropriate in *post-mortem* age-at-death determination. Birds, on the other hand, represent a unique conundrum as they lack both teeth and otoliths, requiring the exploration of alternatives such as the measurement of pentosidine accumulation in the skin of lilac-breasted rollers, *Coracias caudatus* (Linnaeus), among others (Chaney *et al.*, 2003).

With the dawn of the molecular revolution many methods have been developed that use DNA to ascertain and study critical individual characteristics as well as population attributes within most species. Such attributes include sex (Morin *et al.*, 2005), parentage (Double *et al.*, 1997), population assignment (Kim & Sappington, 2013), hybridization (Brelsford, Milá & Irwin, 2011), speciation (Linck, Freeman & Dumbacher, 2020), and migration (Merlin & Liedvogel, 2019). As natural ageing is in many respects a programmed process under genetic control (Horvath, 2013), or results from unrepaired environmental damage to DNA (Kujoth *et al.*, 2005), there is great potential to exploit these changes to determine age. Some age-related changes, summarised in Fig. 1, include fluctuating levels of transcripts (Cook *et al.*, 2006), changes to the DNA sequence itself, e.g. mitochondrial mutations (Zapico & Ubelaker, 2016), and epigenetic modifications (Jones, Goodman & Kobor, 2015). Epigenetics is a collective term for mechanisms that modify DNA and DNA packaging, independent of changes to the genetic sequence (Bird, 2007).

Studies seeking to establish a genetic method for age estimation have identified several potential targets among the epigenetic regulatory elements including methylation

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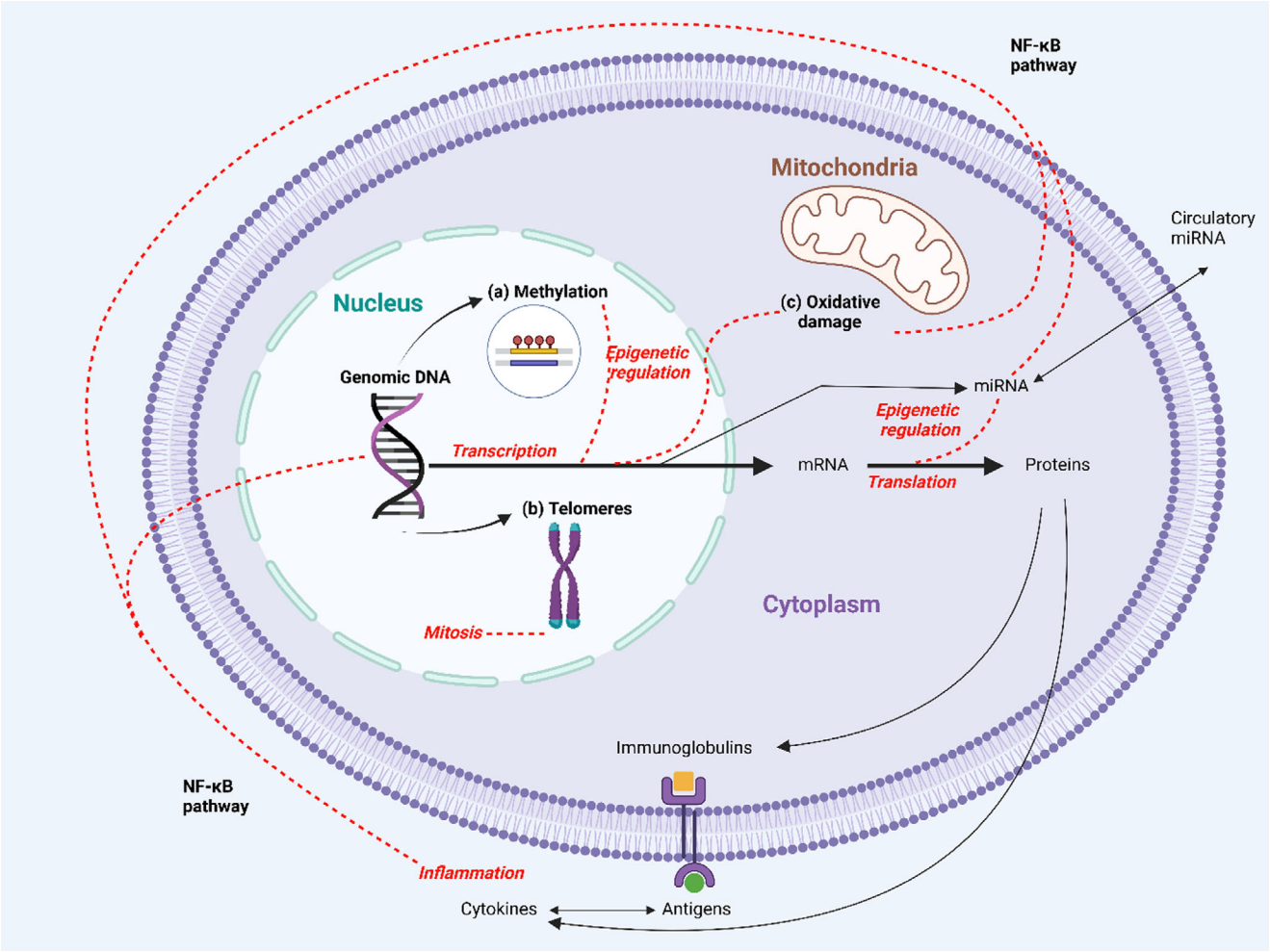


Fig. 1. An animal cell indicating the mechanisms of cellular ageing at the molecular level that are currently used as molecular biomarkers for age. Molecules are labelled in plain text and processes in red italics. Movement of molecules is indicated by lines with arrows; regulatory effects on molecular pathways are indicated by broken red lines. The main pathway of gene expression is shown by thicker arrows in the centre, and comprises the two phases transcription and translation, that turn information stored as DNA into functional molecules including proteins and various types of RNA. (a) DNA methylation levels at several sites change with age. (b) Telomeres have roles in many age-related cellular processes and shorten over time and with age. (c) Oxidative damage due to chronic inflammation through the constitutive activation of the nuclear factor-kappa B (NF-κB) pathway leads to mitochondrial mutations that accumulate over time. miRNA, microRNA; mRNA, messenger RNA. Image created in BioRender.com.

(Johansson, Enroth & Gyllensten, 2013) and telomere shortening (Thomas & Zapico, 2017). Both methylation and telomere shortening were initially tested in humans (Aubert & Lansdorp, 2008; Gopalan *et al.*, 2017) but have since been evaluated as valid methods of less-invasive age determination (Carroll *et al.*, 2018) that is transferable across most species (Jarman *et al.*, 2015; De Paoli-Iseppi *et al.*, 2017*a*). This review provides a synopsis and meta-analysis of two genetic biological clocks for age estimation, methylation and telomeres, in terms of (*i*) what they are, (*ii*) what changes they undergo throughout a lifespan, and (*iii*) the potential use of these features as applied to biological age determination in wildlife, evidenced by a meta-analysis of the literature.

II. METHODS

(1) Literature search and review

A systematic approach was used to search for and synthesise the available literature, as depicted in the PRISMA flow diagrams in Figs S1 and S2 (Haddaway *et al.*, 2022). Literature for methylation-based studies was searched for in the *Scopus* (www.scopus.com) and *Dimensions* (www.dimensions.ai) databases using the following Boolean search string: ('Epigenetics' OR 'Methylation') AND ('age' OR 'aging') AND ('determination' OR 'model') AND ('Animals' OR 'wild'). This process was repeated for telomere-based studies by substituting 'Telomere' for 'Methylation'. Search results

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were exported in the comma separated value format and the literature was subsequently summarised, guided by citation networks visualised using CitNetExplorer 1.0.0 and VOSviewer 1.6.16 (van Eck & Waltman, 2017). Results retrieved from *Scopus* were converted to the appropriate format with the R package *Scopus2CitNet 0.1.0.0* in RStudio 1.4.1106 (RStudio Team, 2021), running R version 4.0.5 (R Core Team, 2020). Further studies were identified from the reference lists of articles and free term 'citation searches' on *Google Scholar*. Other background information beyond the studies for systematic review was retrieved with free term searches using 'DNA methylation', 'telomere length', or 'biological clocks for age estimation' and are not included in the PRISMA statements. Studies were summarised based on species covered, publication year, method used, number of sites assayed (methylation), and sample size (N). Individual models reported in studies were summarised by tissue type, species, and method used, with their associated correlation coefficient (R^2), F-test statistic, t-test statistic, or chi-squared (χ^2) test statistic and P-value. Methylation studies that reported more than one model usually presented models using the same method, but different tissue types or species tested, while a few telomere studies reported multiple models generated to compare different measurement methods on the same samples. Where appropriate statistical measures were not reported, values were calculated from data in the online supplement or data extracted from plots using *WebPlotDigitizer* version 4.6 (Rohatgi, 2022). The data set used in this review is available online (see Section 0. Data availability statement). Deviation between predicted and known age are reported as mean error of prediction (MEP) due to variation in reported measure in studies e.g. median absolute error (MAE) or root mean-square error (MSE). Genome information for species, or their closest relative, was retrieved from the *Animal Genome Size Database* (Gregory, 2023), *Animal Chromosome Count Database* (Román-Palacios *et al.*, 2021), the *Bird 10,000 Genomes (B10K) Project* (Zhang, 2015), and/or the *Bird Chromosome Database* (Degrandi *et al.*, 2020).

(2) Meta-analysis

To conduct a meta-analysis, models reported in studies that were adequately similar in terms of tissue sample and method of detection/assay were identified from the systematic review, including 78 studies for methylation and 109 studies for telomeres. Models reported in studies with a sufficient sample size ($N \geq 15$) were included for consideration in the meta-analysis. For methylation, pan-species models with human samples were excluded from further analyses to focus only on non-human models. An additional two methylation models were also excluded (Ito, Yoshimura & Momoi, 2017; Horvath *et al.*, 2022*d*). The first was excluded because neither the correlation coefficient nor the data plots for the combined model were reported, the latter was excluded due to a discrepancy in the reported sample size for their *Macropus* model (59 in the table but 65 in the figure). The final data set included 60 methylation-based models across the five vertebrate classes: fishes ($N = 3$), amphibians ($N = 2$), reptiles ($N = 3$), birds ($N = 3$), mammals ($N = 49$). For mammals this included aquatic mammals ($N = 6$), bats ($N = 2$), carnivores ($N = 9$), elephants ($N = 2$), marsupials ($N = 3$), primates ($N = 10$), rodents ($N = 4$), shrews ($N = 1$), and ungulates ($N = 12$). For methylation we classified models into three groups. Group 1 ($N = 48$) models were those that collected data for a single species and used those data to create a molecular model for predicting age in the same species. For Group 2 ($N = 8$), the models used a large sample size in a related species to create a model, and subsequently tested the model in another species using fewer samples. Group 3 models ($N = 4$) developed and tested a single model using samples from multiple related species such as cetaceans, bats, cod, or pinnipeds. For the included methylation studies the correlation coefficient and sample size were used to calculate the Fisher's-Z_r transform and the variance (v) as a means of approximating the correlation coefficient effect size (r) for cross-study meta-analysis.

Two telomere studies were excluded from the main analyses, the first was completely excluded due to small sample sizes (Whittemore *et al.*, 2019) while the second was analysed separately, as for methylation, due to the pooling of samples for two species (group 2) of marten (Pauli *et al.*, 2011). Due to the large number of studies on birds, 12 avian models were selected at random for inclusion in the cross-taxa analyses ($N = 60$) while the full set of bird models ($N = 51$) were analysed separately to avoid phylogenetic bias. The first telomere data set, for cross-taxa analyses, included 60 models across the five vertebrate classes: fishes ($N = 6$), amphibians ($N = 3$), reptiles ($N = 6$), birds ($N = 12$), mammals ($N = 33$). For mammals this included aquatic mammals ($N = 2$), bats ($N = 6$), carnivores ($N = 13$), elephants ($N = 1$), primates ($N = 2$), rodents ($N = 4$), and ungulates ($N = 5$). Telomere models for cross-taxa analyses were also grouped, as for methylation, with group 1 studies ($N = 59$) representing studies on single species while group 2 studies ($N = 1$) included models based on pooled samples for multiple species.

The second telomere data set, on studies conducted exclusively in birds, included 51 models and were grouped based on key avian taxonomic groupings. This included specific taxonomic groupings such as: chats and flycatchers ($N = 6$); corvids ($N = 4$); cuckoos ($N = 1$); finches ($N = 3$); geese and waterfowl ($N = 1$); gulls, shorebirds, and waders ($N = 4$); juncos, buntings, and sparrows ($N = 2$), magpies ($N = 1$); manakins ($N = 1$); parrots ($N = 1$); penguins ($N = 3$); seabirds ($N = 11$); swallows ($N = 5$); swifts ($N = 1$); tits ($N = 3$); warblers ($N = 3$); and wrens ($N = 1$). For the included telomere studies statistical methods varied, thus effect size was calculated as before or adapted to the tests used in the study e.g. F- or χ^2-test statistics.

The individual meta-analyses were conducted in RStudio 1.4.1106 (RStudio Team, 2021), running R version 4.0.5 (R Core Team, 2020) with the package *meta 5.5-0* (Schwarzer, Carpenter & Rücker, 2015) and effect sizes calculated by *compute.es 0.2-2* (Del Re, 2013). The relative anticipated levels of author bias resulting from multiple

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publications by the same authors was assessed using a custom PYTHON script, *Author Bias Computation* (*ABCal*) version 1.0.1 (see Section 0). Meta-analyses were run with grouping based on class, study group, tissue type, and method. A separate meta-analysis was run between biological clocks, rather than studies, to compare the efficacy of either methylation or telomere length for determining age in animals.

Heterogeneity was measured between studies within groups as well as globally using standard heterogeneity percentages (I^2), tau-squared (τ^2), and the *P*-value for the χ^2-test statistic. Potential causes of heterogeneity were explored with a meta-regression of individual factors such as author, publication year, taxonomic class, tissue type, experimental method and either genome size (methylation) or karyotype (telomere). Type I error was avoided by having adequate sample sizes ($N > 50$), carrying out one-tailed analyses when possible, and reporting significance at standard alpha values along with correlation coefficients (R^2). To avoid potential type I error due to multiple testing, introduced by collinearity, each factor was measured in a separate and independent regression. Publication or small study bias, resulting in asymmetry, was assessed in R. The relationships between statistical power, effect size and sample size (N) for study methods were explored using G*Power version 3.1 (Faul *et al.*, 2009; Kang, 2021) and are graphically depicted in Fig. S3. A separate meta-regression analysis was run between study methods, rather than models, to compare the efficacy of either methylation or telomere length for determining age in animals.

III. BIOLOGICAL CLOCKS AND AGE

(1) DNA methylation

(a) *What is DNA methylation?*

DNA methylation is an epigenetic process that modifies DNA through the addition of a methyl group (CH_3) to the 5′ cytosine of cytosine–guanine pairs (CpGs) (Fig. 2), as part of DNA packaging as well as the regulation of gene expression. Active methylation of unmethylated CpGs is catalysed by two DNA methyltransferase enzymes, DNMT3A and DNMT3B (Chédin, 2011), that add a methyl group to the fifth carbon of cytosines to form 5-methylcytosine. Methylation status of methylated CpGs is maintained between replicative cycles through the enzymatic activity of DNA methyltransferase 1 (DNMT1), an enzyme that is able to recognise hemi-methylated DNA after cell division and add a methyl group to the fifth carbon of cytosines on unmethylated daughter strands (Sen *et al.*, 2010).

DNA is demethylated *via* one of two mechanisms, either active or passive demethylation (Guo *et al.*, 2014). Passive demethylation occurs at cell division, where the newly synthesised daughter strands lack a methyl group at the newly incorporated cytosines and methylation cannot be re-established by DNMT1 prior to subsequent division (Kagiwada *et al.*, 2013). As passive demethylation only occurs during highly active sequential cycles of cell division it is most common during embryogenesis and early development (Andergassen *et al.*, 2021), as well as certain types of malignant growths (Gomez *et al.*, 2007; Zhang *et al.*, 2020).

By contrast, active demethylation is an enzymatic process (Ooi & Bestor, 2008) involving TET methylcytosine dioxygenase enzymes, such as TET2, and thymine DNA glycosylase (TDG). TET enzymes demethylate DNA by repeatedly adding a hydrogen to the methyl group on the fifth carbon of cytosines, first forming 5-hydroxymethylcytosine, followed by 5-formylcytosine, and finally 5-carboxylcytosine (Fig. 2C). This then serves as the substrate for TDG, which decarboxylates 5-carboxycytosine back to an unmethylated cytosine (Dalton & Bellacosa, 2012). This process is typically associated with the uncoiling of DNA to provide access to promotor and enhancer elements for transcription factors such as nuclear factor kappa B (Tilstra *et al.*, 2011; Balistreri *et al.*, 2013), as well as polymerase complexes responsible for transcription and replication (Attwood, Yung & Richardson, 2002).

The methylation of CpGs is variable across the genome and is a heritable trait through parental imprinting (Andergassen *et al.*, 2021) (Fig. 3), as well as trans-generational epigenetic inheritance (Fitz-James & Cavalli, 2022), while still being subject to environmental influence (McEwen *et al.*, 2017), and complements other genetic evolutionary processes (Lind & Spagopoulou, 2018; Parrott & Bertucci, 2019; Cavalli & Heard, 2019). Those located within CpG islands, dense clusters of consecutive CpGs, tend to be hypermethylated and occur in proximity to regulatory elements of genes or transposable elements to maintain genomic stability. Those located outside such islands, within genes or in non-regulatory elements, are most often hypomethylated (Johansson *et al.*, 2013).

(b) *Changes with age*

The changes that occur to methylation during the natural ageing process have formed the subject of multiple extensive reviews of both human and rodent models (Horvath & Raj, 2018) and will therefore only be dealt with briefly. Epigenetic remodelling or reprogramming in the form of altered methylation status is most active during embryogenesis (Andergassen *et al.*, 2021) and gametogenesis (Guo *et al.*, 2014) (Fig. 4), while methylation status is generally more stable in differentiated tissue. Gradual changes in methylation status in differentiated tissue have, however, been observed (Bell *et al.*, 2012) and are sub-classified based on causation into two fundamental processes: epigenetic drift (Jones *et al.*, 2015) and an epigenetic clock (Horvath & Raj, 2018). The tendency for an inconsistent increase in variability in the epigenomes of individuals denotes epigenetic drift, whereas the epigenetic clock comprises site-specific changes that consistently relate to age across individuals (Hannum *et al.*, 2013). Studies have revealed that during ageing a genome-wide trend of hypomethylation occurs while CpG islands tend to become hypermethylated (Hannum *et al.*, 2013; Jones *et al.*, 2015) and that within genes, nearly

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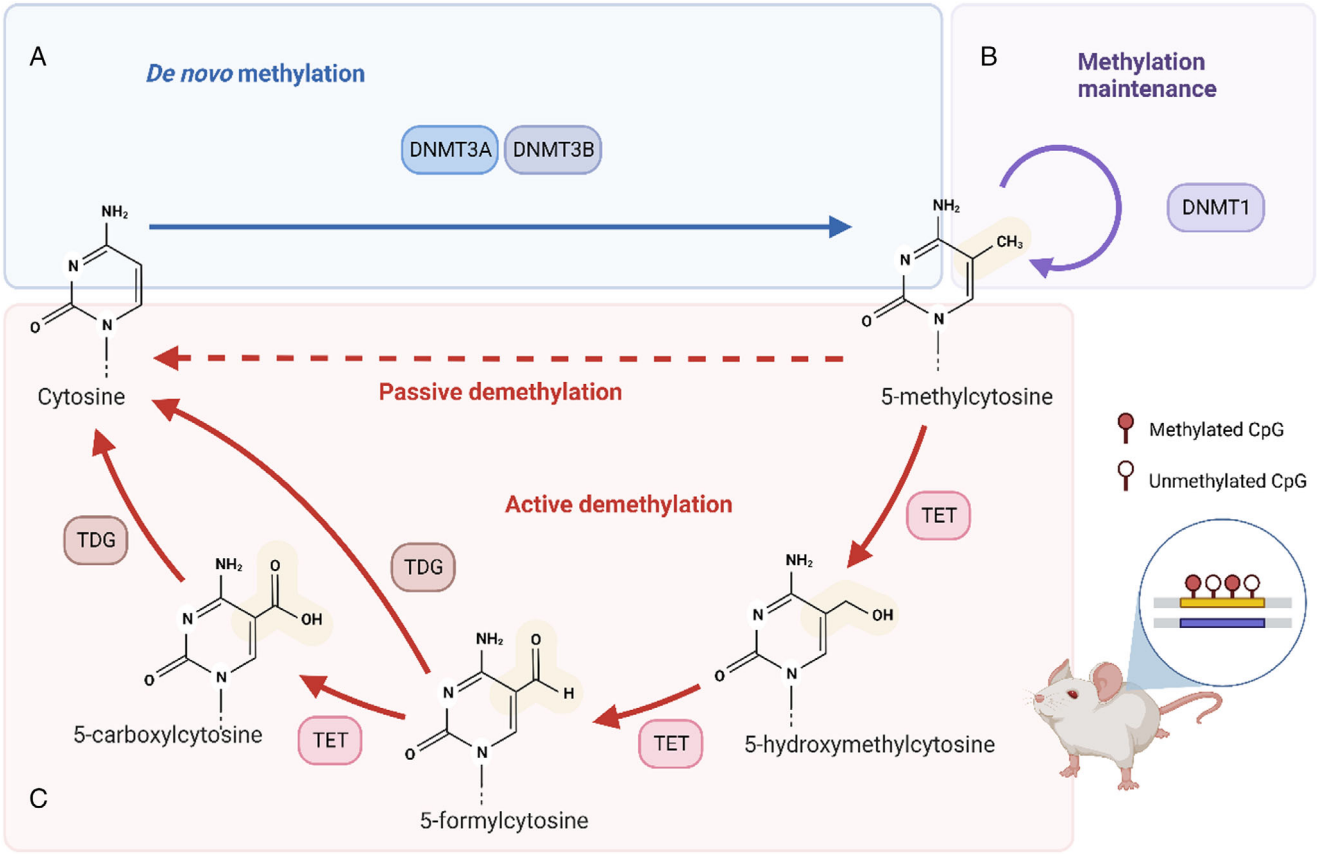


Fig. 2. Diagram of the regulation of methylation. (A) Active *de novo* methylation occurs at unmethylated cytosines through the activity of two DNA methyltransferases 3 (DNMT3A and DNMT3B) that are able to add a methyl group to unmethylated DNA at carbon 5 of cytosines to form 5-methylcytosine. (B) Maintenance of methylation is achieved through the enzymatic activity of DNA methyltransferase 1 (DNMT1) that is able to recognise hemi-methylated DNA after cell division and adds a methyl group to carbon 5 of cytosines on newly synthesised daughter strands. (C) DNA is demethylated through either active or passive demethylation. Passive demethylation happens when cells divide, and the newly synthesised daughter strands do not contain a methyl group at the newly incorporated cytosines. Active demethylation is an enzymatic process involving Tet methylcytosine dioxygenase (TET) enzymes and thymine DNA glycosylase (TDG). TET enzymes sequentially hydroxylate the methyl group on carbon 5 of cytosines to form 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine while TDG decarboxylates 5-carboxycytosine back to an unmethylated cytosine. CpG, cytosine–guanine pair. Image created in BioRender.com.

a third of all CpG sites are influenced by age (Johansson *et al.*, 2013).

(c) *DNA methylation as a biomarker of animal age*

Given its consistency, the epigenetic clock is a promising avenue for chronological age prediction (Lowe *et al.*, 2018; Hong *et al.*, 2019), particularly within the field of conservation (Rey *et al.*, 2020). Methylation levels are usually quantified by bisulfite sequencing (Ashapkin, Kutueva & Vanyushin, 2020), although several methods exist, and the selection of the most appropriate method depends on the relevant application (Kurdyukov & Bullock, 2016). Bisulfite sequencing methods typically provide data in the form of β-methylation values ranging from 0 to 1, and has been subject to extensive statistical validation to optimise sample sizes (Mayne, Berry & Jarman, 2021*b*) and model methods (Snir, vonHoldt & Pellegrini, 2016), including the development of useful tools to design new assays and analyse CpGs (Statham & Csárdi, 2008; Thompson *et al.*, 2009; Wei *et al.*, 2021).

Thus far, methylation studies have successfully been used to establish age models in several animal species and taxa, including: whales (Polanowski *et al.*, 2014; Tanabe *et al.*, 2020; Bors *et al.*, 2021), fishes (Mayne *et al.*, 2020*a*, 2021*a*), dolphins (Beal *et al.*, 2019), birds (De Paoli-Iseppi *et al.*, 2019), non-human primates (Ito *et al.*, 2018), rodents (Stubbs *et al.*, 2017; Lowe *et al.*, 2020), canids (Thompson *et al.*, 2017), bats (Wilkinson *et al.*, 2021) and equids (Larison *et al.*, 2021*a*,*b*). The results for these studies are summarised in Table 1.

(i) Fishes (classes Agnatha, Chondrichthyes, and Osteichthyes). Methylation studies for age estimation have also been expanded to other aquatic species. The first study explored the utility of this biomarker in zebrafish [*Danio rerio* (Hamilton)], as a model organism (Mayne *et al.*, 2020*a*). This

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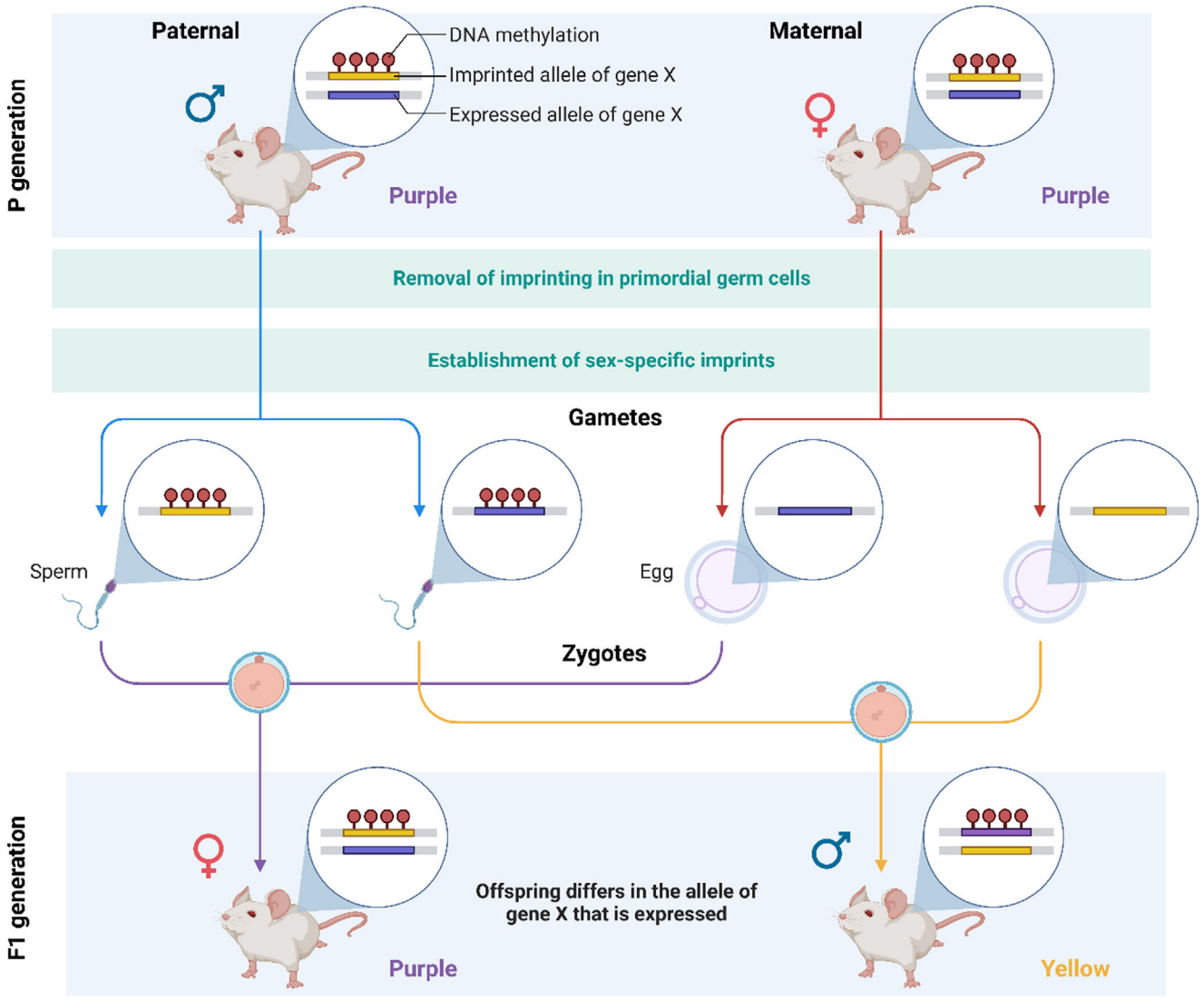


Fig. 3. Diagram of parental imprinting showing the heritability of methylation for a paternally imprinted gene. The top panel shows the parental (P) generation of mice that have identically imprinted (yellow) and expressed (purple) alleles for a hypothetical gene X. Through the removal of inherited imprints in primordial germ cells and the subsequent establishment of sex-specific imprints the alleles inherited paternally are methylated and not expressed, while the maternally inherited alleles are unmethylated and will be expressed. When a zygote is formed through the union of two gametes, the resulting first filial (F1) generation will differ in which allele is expressed, with some expressing the purple allele whilst others express the yellow allele. Image created in BioRender.com.

study used multiplexed methylation-sensitive PCR (MS-PCR) to assay 26 of 29 CpGs shown to follow an age-related decline and was able to establish a predictive model with a standard error of weeks. There was, however, a higher error rate in prediction with a separate test cohort and the authors concluded that MS-PCR may not be sensitive enough to establish age estimation models. The first study on a wild population (Anastasiadi & Piferrer, 2020) assayed methylation in European seabass [*Dicentrarchus labrax* (Linnaeus)]. This study constructed an age clock from 48 CpGs with an error of prediction of 2–3 years, however, the methodology required deep tissue samples and euthanasia similar to otolith age determination. The methods developed in zebrafish were later replicated by the same authors (Mayne *et al.*, 2021*a*) to produce two non-lethal age determination models from skin/fin samples for three threatened fish species: Australian lungfish [*Neoceratodus forsteri* (Krefft)], Murray cod [*Maccullochella peelii* (Mitchell)], and Mary River cod [*Maccullochella mariensis* (Rowland)]. The lungfish clock used 31 CpGs while the cod clock used 26 CpGs, and both had a standard error of prediction of less than a year. The results from a validation test sample showed significantly less error than in zebrafish, which may be related to the different lifespans of these species. Age-related

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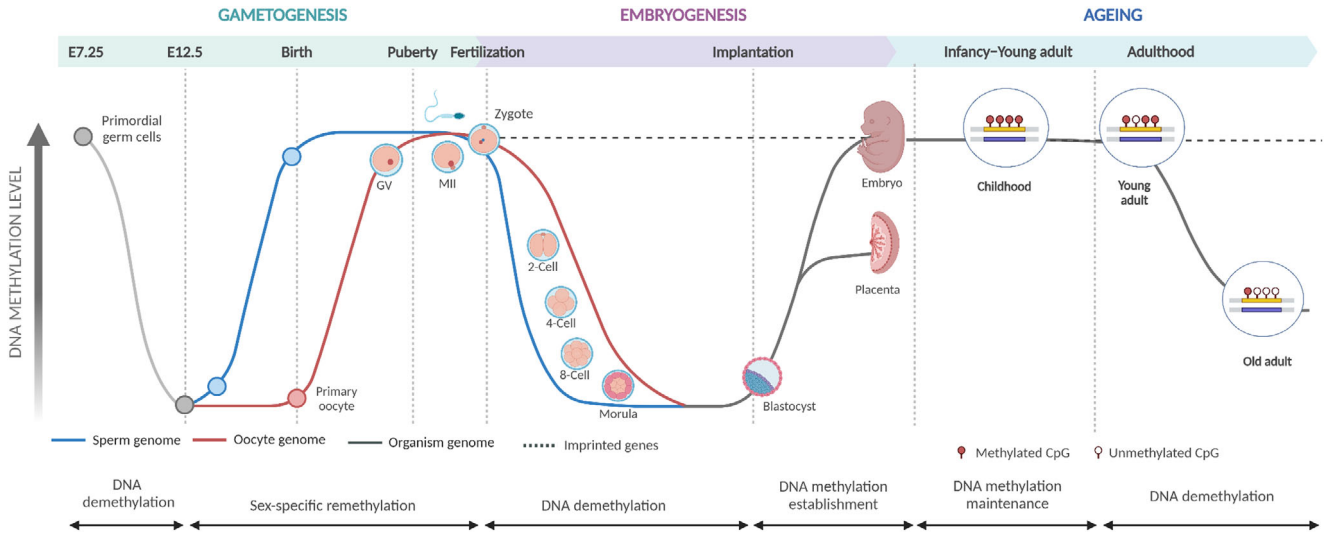


Fig. 4. Diagram of the reprogramming of methylation during key life stages from gametogenesis to embryogenesis and the resulting changes that occur during natural ageing. During gametogenesis the inherited genomic DNA is actively demethylated in primordial germ cells and sex-specific methylation re-established by *de novo* methylation when gametes, either sperm or egg cells, are created. After a zygote is formed from the union of two gametes, passive genomic demethylation takes place due to the rapid replication and division of cells while the methylation of imprinted genes is actively maintained. Once the blastocyst stage/implantation is reached, global methylation is re-established in the embryo. After birth, methylation is maintained throughout childhood from infancy to early adulthood in most tissues with the exception of primordial germ cells forming gametes. During the ageing process methylation is maintained at imprinted genes while global DNA demethylation occurs throughout the remainder of the genome. CpG, cytosine–guanine pair. Image created in BioRender.com.

methylation changes have also been observed in long snout seahorses [*Hippocampus reidi* (Ginsburg)], across different early developmental stages up to adulthood (Suarez-Bregua *et al.*, 2021).

(ii) Amphibians (class Amphibia). The first age clock using methylation for amphibians was recently established in Western clawed frogs [*Xenopus tropicalis* (Gray)] using bisulphite sequencing of the whole genome from web/skin tissue (Morselli *et al.*, 2023). A total of 331 age-correlated CpGs were identified, from which an age clock was established that was able to classify individuals into young, middle, or old age classes with high accuracy; the largest errors occurred in very young or very old individuals. A study published in parallel (Zoller *et al.*, 2022), on both Western clawed frog and African clawed frogs [*Xenopus laevis* (Daudin)], used the same methylation array chip previously developed in mammals, found thousands of conserved CpGs and constructed both individual as well as combined clocks for age. In the Western clawed frog, the mean error of prediction for age was less than a year, while the error for African clawed frog was ∼3 years. The observed difference in accuracy for predictions was likely due to the differences in lifespan as African clawed frogs have a higher average lifespan. A major difference between this and the former study is that the latter models were based on pan-tissue data, including organ and muscle tissue, as the frogs were killed.

(iii) Reptiles (class Reptilia). Early methylation studies in reptiles, done within the context of eco-toxicology (Parrott *et al.*, 2014; Nilsen *et al.*, 2016; Cocci *et al.*, 2018) and sexual differentiation (Venegas *et al.*, 2016), found evidence of an age-related decline in methylation. In the American alligator [*Alligator mississippiensis* (Daudin)], liquid chromatography–tandem mass spectrometry (LC-MS/MS) was used to quantify global methylation patterns in sub-adult and adult groups. The results from both studies revealed a global decline in genomic methylation of ∼8% (Parrott *et al.*, 2014) and 7–9% (Nilsen *et al.*, 2016) respectively. Interestingly both studies also identified a site-directed relationship with the decline in global methylation. This indicates that, while global hypomethylation occurs with ageing, the rate of epigenetic ageing may vary across the genome within a species where specific genes or genomic sites change more slowly or more rapidly than global changes. Subsequently, age clocks based on methylation were investigated in three species of sea turtle, species that have a high infant mortality, are often migratory, and long lived: making long-term tracking for scientific study particularly difficult. The first study exclusively evaluated lifespan prediction in two sea turtles, Australian flatback sea turtle [*Natator depressus* (Garman)] and leatherback sea turtle [*Dermochelys coriacea* (Vandelli)], using CpG promotor density estimates by polymerase chain reaction (PCR) and sequencing (Mayne *et al.*, 2020*b*). The authors were able to predict the lifespan of both turtles as ∼50 and 90 years respectively, in line with previous estimates, and provided the first evidence that methylation studies may be suitable for ageing reptilian species. A subsequent study, using known-age samples from green sea turtle [*Chelonia mydas* (Linnaeus)], developed a methylation-based

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Table 1. Summary of studies that assayed global or regional methylation in relation to development or age in animals. Studies that used the data to create a molecular model for predicting animal age, which were included in the meta-analysis, are indicated with an asterisk (*). Species are grouped by class to match the text and listed alphabetically. For each species the genome size in picograms or billions of base-pairs (bbp) is given along with estimates for the average lifespan. For each study, the method of measuring methylation is indicated: a, bisulfite sequencing; b, enzyme-linked immunosorbent assay (ELISA); c, liquid chromatography–tandem mass spectrometry (LC-MS/MS); d, methylation array chip; e, methylation-sensitive polymerase chain reaction (MS-PCR); f, cytosine–guanine (CpG) promotor density (lifespan). Where relevant, citations are also provided for studies on age determination using other biological clocks (tooth annulation/otolith) in the same/similar species. n.a., not available.

Species	Genome (bbp)	Lifespan (years)	Study	Method	Tooth annulation/otolith
(i) Fishes:					
Australian lungfish (*Neoceratodus forsteri*)*	60.73	80–85	Mayne *et al.* (2021*a*)	e	Høie *et al.* (2004)
European seabass (*Dicentrarchus labrax*)*	0.78	15–30	Anastasiadi & Piferrer (2020)	d	Secor *et al.* (1995)
Long snout seahorse (*Hippocampus reidi*)	0.45	1–4	Suarez-Bregua *et al.* (2021)	e	n.a.
Mary River cod (*Maccullochella mariensis*)*	0.83	80–92	Mayne *et al.* (2021*a*)	e	Høie *et al.* (2004)
Murray cod (*Maccullochella peelii*)*	0.83	75–114	Mayne *et al.* (2021*a*)	e	Høie *et al.* (2004)
Zebrafish (*Danio rerio*) – model organism	1.80	1–4	Mayne *et al.* (2020*a*)	e	Higgs *et al.* (2002)
(ii) Amphibians:					
African clawed frog (*Xenopus laevis*)*	3.10	5–15	Zoller *et al.* (2022)	d	n.a.
Western clawed frog (*Xenopus tropicalis*)	3.11	3–12	Morselli *et al.* (2023)	a	n.a.
Western clawed frog (*Xenopus tropicalis*)*	3.11	3–12	Zoller *et al.* (2022)	d	n.a.
(iii) Reptiles:					
American alligator (*Alligator mississippiensis*)*	2.49	30–50	Parrott *et al.* (2014)	c	n.a.
American alligator (*Alligator mississippiensis*)*	2.49	30–50	Nilsen *et al.* (2016)	c	n.a.
Australian flatback sea turtle (*Natator depressus*)	2.64	60–100	Mayne *et al.* (2020*b*)	f	n.a.
Green sea turtle (*Chelonia mydras*)*	2.64	70–80	Mayne *et al.* (2022)	e	n.a.
Leatherback sea turtle (*Dermochelys coriacea*)	2.70	45–50	Mayne *et al.* (2020*b*)	f	n.a.
Loggerhead sea turtle (*Caretta caretta*)	2.70	47–67	Cocci *et al.* (2018)	b	n.a.
(iv) Birds:					
Barn swallow (*Hirundo rustica*)	1.31	1–4	Saino *et al.* (2019)	a	n.a.
Black grouse (*Lyrurus tetrix*)*	1.32	4–5	Soulsbury *et al.* (2018)	a	n.a.
Common tern (*Sterna hirundo*)*	1.44	9–10	Meyer *et al.* (2023)	a	n.a.
Short-tailed shearwater (*Ardenna tenuirostris*)	1.18	19–38	De Paoli-Iseppi *et al.* (2017*b*)	a	n.a.
Short-tailed shearwater (*Ardenna tenuirostris*)*	1.18	19–38	De Paoli-Iseppi *et al.* (2019)	a	n.a.
(v) Mammals:					
Aquatic:					
Antarctic minke whale (*Balaenoptera bonaerensis*)	3.29	100–114	Tanabe *et al.* (2020)	a	Hohn (1980)
Beluga whale (*Delphinapterus leucas*)*	3.29	35–50	Bors *et al.* (2021)	a	Goren *et al.* (1987)
Beluga whale (*Delphinapterus leucas*)	3.29	35–50	Robeck *et al.* (2021*b*)	d	Goren *et al.* (1987)
Bottlenose dolphin (*Tursiops truncatus*)*	3.03	15–16	Beal *et al.* (2019)	a	Kimura (1980)
Bottlenose dolphin (*Tursiops truncatus*)*	3.03	15–16	Robeck *et al.* (2021*a*)	d	Kimura (1980)
Bottlenose dolphin (*Tursiops truncatus*)*	3.03	15–16	Robeck *et al.* (2021*b*)	d	Kimura (1980)
Commerson's dolphin (*Cephalorhynchus commersonii*)*	3.27	10–18	Robeck *et al.* (2021*b*)	d	Myrick *et al.* (1983)
Common dolphin (*Delphinus delphis*)*	3.03	35–40	Robeck *et al.* (2021*b*)	d	Myrick *et al.* (1983)
Harbour porpoise (*Phocoena phocoena*)*	3.46	8–13	Robeck *et al.* (2021*b*)	d	Myrick *et al.* (1983)
Humpback whale (*Megaptera novaeangliae*)*	3.29	45–50	Horvath *et al.* (2022*c*)	d	Ohsumi *et al.* (1965)
Humpback whale (*Megaptera novaeangliae*)*	3.29	45–50	Polanowski *et al.* (2014)	a	Ohsumi *et al.* (1965)
Killer whale (*Orcinus orca*)*	3.18	50–80	Robeck *et al.* (2021*b*)	d	Clark *et al.* (2000)
Pacific white-sided dolphin (*Lagenorhynchus obliquidens*)*	3.46	40–45	Robeck *et al.* (2021*b*)	d	Kasuya & Matsui (1984)
Rough-tooth dolphin (*Steno bredanensis*)*	3.03	32–48	Robeck *et al.* (2021*b*)	d	Myrick *et al.* (1983)
Short-finned pilot whale (*Globicephala macrorhynchus*)*	3.29	45–60	Robeck *et al.* (2021*b*)	d	Kasuya & Matsui (1984)
Sperm whale (*Physeter catodon*)	3.29	50–70	Polanowski *et al.* (2014)	a	Ohsumi *et al.* (1965)
Bats:					
Bechstein's bat (*Myotis bechsteinii*)*	2.72	15–21	Wright *et al.* (2018)	a	Linhart (1973)
Big brown bat (*Eptesicus fuscus*)*	2.25	13–20	Wilkinson *et al.* (2021)	d	Linhart (1973)
Common noctule (*Nyctalus noctule*)*	2.26	5–12	Wilkinson *et al.* (2021)	d	Linhart (1973)
Common vampire bat (*Desmodus rotundus*)*	2.66	12–30	Wilkinson *et al.* (2021)	d	Linhart (1973)

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Table 1. *(Cont.)*

Species	Genome (bbp)	Lifespan (years)	Study	Method	Tooth annulation/otolith
Egyptian fruit bat (*Rousettus aegyptiacus*)*	2.11	10–22	Wilkinson *et al.* (2021)	d	Linhart (1973)
Fish-eating bat (*Myotis vivesi*)*	2.72	8–10	Wilkinson *et al.* (2021)	d	Linhart (1973)
Greater horseshoe bat (*Rhinolophus ferrumequinum*)*	2.68	20–30	Wilkinson *et al.* (2021)	d	Linhart (1973)
Greater mouse-eared bat (*Myotis myotis*)*	2.72	6–13	Wilkinson *et al.* (2021)	d	Linhart (1973)
Greater sac-winged bat (*Saccopteryx bilineata*)*	3.16	1–6	Wilkinson *et al.* (2021)	d	Linhart (1973)
Greater spear-nosed bat (*Phyllostomus hastatus*)*	2.62	10–18	Wilkinson *et al.* (2021)	d	Linhart (1973)
Grey-headed flying fox (*Pteropus poliocephalus*)*	2.32	2–5	Wilkinson *et al.* (2021)	d	Linhart (1973)
Indian flying fox (*Pteropus giganteus*)*	2.18	31–44	Wilkinson *et al.* (2021)	d	Linhart (1973)
Jamaican fruit bat (*Artibeus jamaicensis*)*	2.74	9–19	Wilkinson *et al.* (2021)	d	Linhart (1973)
Large flying fox (*Pteropus vampyrus*)*	2.37	15–30	Wilkinson *et al.* (2021)	d	Linhart (1973)
Lesser short-nosed fruit bat (*Cynopterus brachyotis*)*	2.27	20–30	Wilkinson *et al.* (2021)	d	Linhart (1973)
Little brown bat (*Myotis lucifugus*)*	2.72	7–31	Wilkinson *et al.* (2021)	d	Linhart (1973)
Little golden-mantled flying fox (*Pteropus pumilus*)*	2.32	10–17	Wilkinson *et al.* (2021)	d	Linhart (1973)
Mexican free-tailed bat (*Tadarida brasiliensis*)*	2.94	8–12	Wilkinson *et al.* (2021)	d	Linhart (1973)
Pale spear-nosed bat (*Phyllostomus discolor*)*	2.52	6–9	Wilkinson *et al.* (2021)	d	Linhart (1973)
Pallid bat (*Antrozous pallidus*)*	2.67	9–14	Wilkinson *et al.* (2021)	d	Brook *et al.* (2019)
Proboscis bat (*Rhynchonycteris naso*)*	2.72	1–6	Wilkinson *et al.* (2021)	d	Linhart (1973)
Rodrigues flying fox (*Pteropus rodricensis*)*	2.23	28–30	Wilkinson *et al.* (2021)	d	Linhart (1973)
Seba's short-tailed bat (*Carollia perspicillata*)*	2.92	10–17	Wilkinson *et al.* (2021)	d	Linhart (1973)
Small flying fox (*Pteropus hypomelanus*)*	2.33	9–20	Wilkinson *et al.* (2021)	d	Linhart (1973)
Southern long-nosed bat (*Leptonycteris curasoae*)*	2.36	10–30	Wilkinson *et al.* (2021)	d	Linhart (1973)
Straw-coloured fruit bat (*Eidolon helvum*)*	2.03	15–22	Wilkinson *et al.* (2021)	d	Linhart (1973)
Velvety free-tailed bat (*Molossus molossus*)*	2.41	2–5	Wilkinson *et al.* (2021)	d	Linhart (1973)
Carnivores:					
Australian sea lion (*Neophoca cinerea*)*	3.15	17–25	Robeck *et al.* (2023)	d	Rust *et al.* (2019)
Bobcat (*Lynx rufus*)	2.92	7–13	Cantrell *et al.* (2020)	b	Crowe (1972)
California sea lion (*Zalophus californianus*)*	3.15	15–25	Robeck *et al.* (2023)	d	Rust *et al.* (2019)
Cheetah (*Acinonyx jubatus*)	2.56	10–15	Le Clercq *et al.* (2018)	e	Marker & Dickman (2003)
Cheetah (*Acinonyx jubatus*)*	2.56	10–15	Raj *et al.* (2021)	d	Marker & Dickman (2003)
Domestic cat (*Felis catus*)*	2.91	12–18	Qi *et al.* (2021)	e	Grue & Jensen (1979)
Domestic cat (*Felis catus*)*	2.91	12–18	Raj *et al.* (2021)	d	Grue & Jensen (1979)
Domestic dog (*Canis familiaris*)	2.80	10–13	Ito *et al.* (2017)	a	Grue & Jensen (1979)
Domestic dog (*Canis familiaris*)*	2.80	10–13	Thompson *et al.* (2017)	a	Grue & Jensen (1979)
Domestic dog (*Canis familiaris*)*	2.80	10–13	Horvath *et al.* (2022*e*)	d	Grue & Jensen (1979)
Fisher (*Pekania* [*Martes*] *pennanti*)	n.a.	10–14	Lachance *et al.* (2015)	b	Arthur *et al.* (1992)
Gray wolf (*Canis lupus*)*	2.81	14–16	Thompson *et al.* (2017)	a	Walleser *et al.* (2016)
Harbour seal (*Phoca vitulina*)*	2.94	25–35	Robeck *et al.* (2023)	d	Bowen *et al.* (1983)
Harp/Greenland seal (*Pagophilus groenlandicus*)*	2.94	20–30	Robeck *et al.* (2023)	d	Bowen *et al.* (1983)
Lion (*Panthera leo*)*	2.95	10–16	Raj *et al.* (2021)	d	White & Belant (2016)
Pacific walrus (*Odobenus rosmarus divergens*)*	2.40	20–40	Robeck *et al.* (2023)	d	Stewart *et al.* (1996)
Ringed seal (*Phoca hispida*)	2.94	15–28	Sareisian (2014)	b	Stewart *et al.* (1996)
Snow leopard (*Panthera unica*)	3.54	10–13	Qi *et al.* (2021)	e	McCarthy *et al.* (2005)
Steller sea lion (*Eumetopias jubatus*)*	3.15	20–30	Robeck *et al.* (2023)	d	Rust *et al.* (2019)

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Table 1. *(Cont.)*

Species	Genome (bbp)	Lifespan (years)	Study	Method	Tooth annulation/otolith
Tiger (*Panthera tigris*)*	2.71	8–10	Raj *et al.* (2021)	d	Sharma *et al.* (2021)
Elephants:					
African elephant (*Loxodonta africana*)*	4.44	60–70	Prado *et al.* (2021)	d	Spinage (1976)
Asian elephant (*Elephas maximus*)*	4.03	48–60	Prado *et al.* (2021)	d	Spinage (1976)
Marsupials:					
Eastern grey kangaroo (*Macropus giganteus*)	4.21	6–8	Horvath *et al.* (2022*d*)	d	Grue & Jensen (1979)
Gray short-tailed opossum (*Monodelphis domestica*)*	4.17	1–3	Horvath *et al.* (2022*d*)	d	Pekelharing (1970)
Red kangaroo (*Macropus rufus*)*	3.13	6–20	Horvath *et al.* (2022*d*)	d	Grue & Jensen (1979)
Red-necked wallaby (*Macropus rufogriseus*)	5.92	7–15	Horvath *et al.* (2022*d*)	d	Grue & Jensen (1979)
Tasmanian devil (*Sarcophilus harrisii*)*	3.63	5–8	Horvath *et al.* (2022*d*)	d	Pekelharing (1970)
Western grey kangaroo (*Macropus fuliginosus*)	3.91	10–29	Horvath *et al.* (2022*d*)	d	Grue & Jensen (1979)
Primates:					
Baboon (*Papio cynocephalus x P. anubis*)*	3.53	20–30	Anderson *et al.* (2021)	a	Wada *et al.* (1978)
Chimpanzee (*Pan troglodytes*)*	3.76	32–39	Guevara *et al.* (2020)	d	Wittwer-Backofen *et al.* (2004)
Chimpanzee (*Pan troglodytes*)*	3.76	32–39	Ito *et al.* (2018)	a	Wittwer-Backofen *et al.* (2004)
Common marmoset (*Callithrix jacchus*)*	3.43	7–16	Horvath *et al.* (2021*b*)	d	Scott *et al.* (1980)
Rhesus macaque (*Macaca mulatta*)*	3.59	25–40	Horvath *et al.* (2021*a*)	d	Newham *et al.* (2021)
Vervet monkey (*Chlorocebus pygerythrus*)*	4.19	15–30	Jasinska *et al.* (2022)	d	Scott *et al.* (1980)
Rodents:					
Mouse (*Mus musculus*)–Model organism	3.25	1–1.5	Spiers *et al.* (2016)	a	Grue & Jensen (1979)
Mouse (*Mus musculus*)–Model organism	3.25	1–1.5	Stubbs *et al.* (2017)	a	Grue & Jensen (1979)
Naked mole-rat (*Heterocephalus glaber*)*	2.90	22–31	Lowe *et al.* (2020)	a	Grue & Jensen (1979)
Naked mole-rat (*Heterocephalus glaber*)*	2.90	22–31	Horvath *et al.* (2022*a*)	d	Grue & Jensen (1979)
Naked mole-rat (*Heterocephalus glaber*) *	2.90	22–31	Kerepesi *et al.* (2022)	a	Grue & Jensen (1979)
Wood mouse (*Apodemus sylvaticus*) *	3.29	1–4	Little *et al.* (2020)	a	Grue & Jensen (1979)
Shrews:					
Masked shrew (*Sorex cinereus*)*	2.91	1–2	Cossette *et al.* (2022)	d	Grue & Jensen (1979)
Ungulates:					
Cattle (*Bos taurus*)*	3.70	10–20	Kordowitzki *et al.* (2021)	d	Grue & Jensen (1979)
Common zebra (*Equus quagga*)*	3.15	20–25	Larison *et al.* (2021*a*)	d	Penzhorn (1982)
Domestic horse (*Equus caballus*)*	3.15	25–30	Larison *et al.* (2021*a*)	d	Penzhorn (1982)
Domestic horse (*Equus caballus*)*	3.15	25–30	Horvath *et al.* (2022*b*)	d	Penzhorn (1982)
Domestic pig (*Sus scrofa domesticus*)*	3.00	15–20	Schachtschneider *et al.* (2021)	d	Grue & Jensen (1979)
Domestic sheep (*Ovis aries*)*	3.30	10–12	Sugrue *et al.* (2021)	d	Saxon & Higham (1968)
Grévy's zebra (*Equus grevyi*)*	3.15	20–25	Larison *et al.* (2021*a*)	d	Penzhorn (1982)
Roe deer (*Capreolus capreolus*)*	3.41	7–10	Lemaître *et al.* (2022)	d	Grue & Jensen (1979)
Somali wild ass (*Equus africanus somaliensis*)*	4.12	27–40	Larison *et al.* (2021*a*)	d	Penzhorn (1982)

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clock for age determination that would be transferable across several species of marine turtles (Mayne *et al.*, 2022). This model, based on 17 CpGs, was able to predict age with an error rate of 3–4 years among adult individuals. The method was developed also to include sites that could be assayed in hawksbill sea turtle [*Eretmochelys imbricata* (Linnaeus)], loggerhead sea turtle [*Caretta caretta* (Linnaeus)], and olive Ridley sea turtle [*Lepidochelys olivacea* (Eschscholtz)]; in addition to previously studied flatback and leatherback turtles. Ecotoxicology studies on methylation in loggerhead sea turtles found similar patterns in global methylation as compared to studies conducted in alligators (Cocci *et al.*, 2018).

(iv) Birds (class Aves). Similar to reptiles, many of the early methylation studies in birds had a focus on ecotoxicology (Brandenburg, 2016; Romano *et al.*, 2017) or other aspects of animal ecology. For example, several studies have focussed on the association between methylation levels and migration or breeding phenology (Saino *et al.*, 2017; Liebl *et al.*, 2021), temporal variation on an annual basis in both methylation levels (Viitaniemi *et al.*, 2019; Mäkinen *et al.*, 2019) and the expression of key regulatory elements within the methylation circuitry (Sharma *et al.*, 2018; Trivedi *et al.*, 2019). While these studies were not focussed on age estimation, they did establish the heritability of methylation (Romano *et al.*, 2017) and inter-generational resemblance of methylation patterns (Saino *et al.*, 2019). Studies aimed at exploiting methylation to model age found that 67 CpGs in 13 genes, previously used in mammals, were not well conserved in a long-lived seabird species, the short-tailed shearwater [*Ardenna tenuirostris* (Temminck)], with only 12 CpGs showing a weakly correlated relationship with age (De Paoli-Iseppi *et al.*, 2017*a*). The same authors were, however, able to construct an age clock for this species with seven newly developed CpGs that predicted age with a mean error of 2–3 years (De Paoli-Iseppi *et al.*, 2019). A similar trend of age–dependent changes was later observed in the black grouse [*Lyrurus tetrix* (Linnaeus)] that found four CpGs within one gene to be highly correlated with age (Soulsbury *et al.*, 2018). A recent study in the common tern [*Sterna hirundo* (Linnaeus)], identified several genomic regions that showed shifts in methylation equivalent to epigenetic clocks that changed predictably over time within individuals in a sex-specific manner mostly affecting females (Meyer *et al.*, 2023).

(v) Mammals (class Mammalia)

(*i*.1) *Aquatic mammals (infraorder Cetacea).* The first study to develop an age-estimation model in wildlife based on methylation was conducted on humpback whales and assayed 37 CpGs in eight genes, of which seven CpGs had a significant linear correlation with age (Polanowski *et al.*, 2014). This allowed the creation of a model able to estimate the age of several whale species with a standard error of prediction of ∼3 years. A similar study on humpbacks was reported more recently (Horvath *et al.*, 2022*c*) and used a conserved site mammalian methylation array which was able to reduce the MAE to 2 years. Several similar models have been developed in other whale species as well. Two of the genes assayed by Polanowski *et al.* (2014) were assessed in the Antarctic minke whale [*Balaenoptera bonaerensis* (Burmeister)] in 17 CpGs (Tanabe *et al.*, 2020). The authors, however, only found a significant relationship between methylation and age in one gene and concluded that the methylation–age relationship identified in one species may not be conserved between species. An analysis of 37,491 CpGs using a conserved site mammalian methylation array (Arneson *et al.*, 2022) applied to Beluga whale [*Delphinapterus leucas* (Pallas)] found 23 highly age-correlated CpGs, of which 15 were in genes that enabled the development of an age-estimation model able to determine age with comparable accuracy to the model developed in humpback whales (Bors *et al.*, 2021). Similar methods were later applied to bottlenose dolphins [*Tursiops truncatus* (Montagu)] in a study that replicated previous findings in humpback whales by assaying 17 CpGs in three genes on skin biopsies (Beal *et al.*, 2019). The authors found two of the genes to have comparable methylation levels between species while the third was significantly hypomethylated. Two of the assayed CpGs had significant age-correlated methylation which enabled the construction of a linear ageing model that could accurately predict the age of bottlenose dolphins with a standard error of ∼4 years (Beal *et al.*, 2019). Another study on the same species was able to construct a combined age-estimation model from skin and blood samples with an error in prediction of ∼2–3 years (Robeck *et al.*, 2021*a*).

These studies culminated in the recent validation of a methylation array chip which assayed between 79 and 142 CpGs, depending on tissue type, in nine species of cetaceans to construct a single age-estimation model that is transferable across species (Robeck *et al.*, 2021*b*). This study included four additional species of dolphins, two additional species of whales, and porpoises. The dolphins included the common dolphin [*Delphinus delphis* (Linnaeus)], Commerson's dolphin [*Cephalorhynchus commersonii* (Lacépède)], Pacific white-sided dolphin [*Lagenorhynchus obliquidens* (Gill)], and rough-tooth dolphin [*Steno bredanensis* (Cuvier)], as well as the harbour porpoise [*Phocoena phocoena* (Linnaeus)]. The whales included the killer whale [*Orcinus orca* (Linnaeus)] and short-finned pilot whale [*Globicephala macrorhynchus* (Gray)]. The final model generated from this study was able to predict age accurately in all tested species with a standard error of between 3 and 4 years, depending on tissue type, similar to findings in individual, species-specific studies.

(*i*.2) *Bats (order Chiroptera).* Among the only true flying mammals in the order Chiroptera, which includes bats, megabats, and microbats, two studies have aimed at developing methylation-based clocks for age (Wright *et al.*, 2018; Wilkinson *et al.*, 2021). The first used methylation levels at seven CpGs in three genes to construct an age clock from wing skin samples from Bechstein's bat [*Myotis bechsteinii* (Kuhl)] able to determine age with a MEP of 2–3 years (Wright *et al.*, 2018). A pan-species model was later developed using 162 CpGs from a conserved mammalian methylation array able to predict the age of 26 species within the order Chiroptera with a MEP of less than a year (Wilkinson *et al.*, 2021).

(*i*.3) *Carnivores (order Carnivora).* In carnivores, age clocks were assessed for canids (Ito *et al.*, 2017; Thompson

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et al., 2017, Horvath *et al.*, 2022*e*), felines (Cantrell *et al.*, 2020; Raj *et al.*, 2021; Qi *et al.*, 2021), mustelids (Lachance *et al.*, 2015) and pinnipeds (Sareisian, 2014; Robeck *et al.*, 2023). The first model in the domestic dog, *Canis familiaris* (Linnaeus) assayed CpGs in six genes and constructed a clock from the four highly age-correlated CpGs, able to predict age with a MEP of 34–37 months (Ito *et al.*, 2017). The number of breeds included was not stated but likely less than 50 (based on sample size) and the authors postulated that the error in clocks could be improved by screening for age-correlated CpGs to assay prior to modelling age using methylation. The second study (Thompson *et al.*, 2017), which also included both domestic dog and gray wolf [*Canis lupus* (Linnaeus)], was able to construct age clocks for each species from 41 and 67 CpGs respectively with a MEP of ∼10 months. This study also used fewer than 50 breeds but represented a significant improvement in the accuracy of the prediction. Furthermore, a relationship between size (mass) and accelerated epigenetic age was detected in line with the knowledge that smaller breeds live longer while larger breeds have shorter lifespans. A follow-up study in domestic dog by some of the same co-authors (Horvath *et al.*, 2022*e*) was able further to improve the epigenetic clock (MEP of 5–6 months) and predict age-to-death by assaying CpGs in 93 dog breeds.

The first study in felines (Qi *et al.*, 2021) studied methylation in multiple CpGs in two genes, across several breeds of domestic cat [*Felis catus* (Linnaeus)]. Significant correlates with age were detected using high-resolution melting analyses combined with MS-PCR for both genes and a combined ageing model with a MEP of 3.5–4 years was constructed for both healthy and chronically ill individuals. The authors also tested this method in a smaller sample of snow leopard [*Panthera uncia* (Schreber)] and found the trends observed in domestic cat to be sufficiently conserved to determine age in this species with an improved MEP of ∼2 years. The second feline study (Raj *et al.*, 2021), also in domestic cat, used a methylation array to identify 34 CpGs that have a conserved correlation with age and created a model with a MEP of ∼10 months, similar to that observed in canids. The authors further tested the model on smaller sample subsets ($N < 15$) of wild felines including cheetah, lion [*Panthera leo* (Linnaeus)], and tiger [*Panthera tigris* (Linnaeus)]. The cat clock was able to determine age in the related wild species with a MEP of 1–3 years.

Global methylation has also been studied in pinnipeds in relation to age in the ringed seal [*Phoca hispida* (Schreber)], using liver and kidney tissue, however, no clear difference was observed between the sub-adult and adult groups – possibly due to small sample sizes or tissue type (Sareisian, 2014). Similar studies were performed measuring global methylation using enzyme-linked immunosorbent assay (ELISA) in bobcats [*Lynx rufus* (Schreber)], and fishers [*Pekania pennanti* (Erxleben)], using liver samples (Lachance *et al.*, 2015; Cantrell *et al.*, 2020). While both identified the same trend of a decrease in methylation with age, supporting the possibility that these species may be well suited to the development of epigenetic clocks for age determination, neither was able to detect a statistically significant difference between young and mature individuals. This may indicate that site-directed methylation detection methods, such as bisulfite sequencing, are more useful for this purpose than ELISA or MS-PCR (Le Clercq, Dalton & Kotzé, 2018). This was illustrated by a later study in pinnipeds (Robeck *et al.*, 2023) that included six species: three species of sea lion, the Australian sea lion [*Neophoca cinerea* (Péron)], California sea lion [*Zalophus californianus* (Lesson)], and Steller sea lion [*Eumetopias jubatus* (Schreber)]; two seal species, harbour seal [*Phoca vitulina* (Linnaeus)], and harp/Greenland seal [*Pagophilus groenlandicus* (Erxleben)]; as well as one species of walrus, the Pacific walrus [*Odobenus rosmarus divergens* (Linnaeus)]. This study did find several age-correlated changes in methylation in pinnipeds that enabled the construction of an epigenetic clock, from combined skin and blood samples, able to predict age with a MEP of 1–1.5 years.

(*i*.4) *Elephants (order Proboscidea, suborder Elephantiformes)*. Methylation has been used to model age in both extant genera of elephants in the family Elephantidae: the African elephant [*Loxodonta africana* (Blumenbach)] and the Asian elephant [*Elephas maximus* (Linnaeus)]. Prado *et al.* (2021) created three epigenetic models of age, one for each of the genera as well as one general model for all elephants. For the African elephant, 2341 CpGs from a methylation array were found to be significantly correlated with age while the Asian elephant only had approximately a quarter as many. Nevertheless ∼366 of these age-correlated CpGs were shared by both species. The most accurate clock was reconstructed for the African elephant with a MEP of 2–3 years while the clock for Asian elephants had a MEP of 3–4 years. The combined clock for both species performed similarly to the latter, with a MEP of 3–4 years. Overall, this study illustrated that the accuracy of clocks may be improved by a higher number of targets rather than just increased sampling, while also highlighting that substantial differences may exist in age-related CpGs between closely related species.

(*i*.5) *Marsupials (infraorder Marsupialia)*. The first study to assay methylation in relation to age in marsupials (Horvath *et al.*, 2022*d*) was conducted in gray short-tailed opossum [*Monodelphis domestica* (Wagner)], a South American species with a pseudo-pouch, similar to those of male thylacines [*Thylacinus cynocephalus* (Harris)]. This study used a universal mammal array to develop a clock able to predict age with a MEP of 3–4 months. This model was subsequently validated in several other marsupials including the Tasmanian devil [*Sarcophilus harrisii* (Boitard)], eastern grey kangaroo [*Macropus giganteus* (Shaw)], western grey kangaroo [*Macropus fuliginosus* (Desmarest)], red kangaroo [*Macropus rufus* (Desmarest)], and red-necked wallaby [*Macropus rufogriseus* (Desmarest)] from the Australasian region. Despite the taxonomic and phylogenetic distance in the relatedness of these species, the model developed for opossums was well conserved in other marsupials and was able to predict the age of these species with a MEP of 12–13 and 11–13 months for Tasmanian devils and kangaroos/wallabies respectively.

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(*i*.6) *Primates (order Primates)*. Epigenetic clocks for age have been studied extensively in humans, *Homo sapiens sapiens* (Linnaeus) (Jarman *et al*., 2015; De Paoli-Iseppi *et al*., 2017*a*). Such studies were expanded into non-human primates including hybrid baboons (*Papio cynocephalus* × *P. anubis*) (Anderson *et al*., 2021), chimpanzees [*Pan troglodytes* (Blumenbach)] (Ito *et al*., 2018; Guevara *et al*., 2020), rhesus macaques [*Macaca mulatta* (Zimmermann)] (Horvath *et al*., 2021*a*), and monkeys (Horvath *et al*., 2021*b*; Jasinska *et al*., 2022). Ito *et al*. (2018) developed the first primate age clock in chimpanzees based on methylation differences in several CpGs analysed in three genes. The most accurate model used two CpGs and was able to predict age with a mean error of prediction of 5–6 years. Improved results were later achieved using a human methylation array (Guevara *et al*., 2020) owing to the high amounts of largely conserved DNA between humans and chimps. This study was able to construct a highly accurate age clock from as little as 10 CpGs with an error rate comparable to that observed in humans (2–3 years). A study conducted on a hybridised baboon population, between congeneric yellow baboon [*Papio cynocephalus* (Linnaeus)], and Anubis baboon [*Papio anubis* (Lesson)], used genome-wide methylation analysis refined to ∼573 CpG sites to create a model with predictive precision of 1–2 years.

In the rhesus macaque, three clocks were constructed using blood, skin, and multi-tissue methylation data (Horvath *et al*., 2021*a*). The most accurate models were derived from blood and multi-tissue data with a MEP of between 12 and 18 months, while the model from skin samples had a MEP of ∼2 years. The pan-tissue models in macaques were based on 71 CpGs from a custom mammalian methylation array. These clocks were also tested on vervet monkeys [*Chlorocebus pygerythrus* (Cuvier)] and were able to predict age with a MEP of 2–3 years using blood or multi-tissue models, and ∼8 years using skin. More detailed analyses of this species were published in a subsequent companion article (Jasinska *et al*., 2022) where clocks were constructed using blood, liver, and brain tissue. Overall performance was improved using species-specific data, with liver and cortex models found to be the most accurate with a MEP of 3–6 months while the blood-derived clock had a MEP of approximately 1 year. A similar study in common marmosets [*Callithrix jacchus* (Linnaeus)] constructed an age clock from blood samples from over 1000 age-related CpGs able to estimate age with a MEP of 8–9 months (Horvath *et al*., 2021*b*). The studies on macaques as well as vervet and marmoset monkeys also compared the accuracy of these models on human data and between species. They found most models to retain a relatively high predictive power across multiple primates and tissue types, despite the ∼12 million years divergence distance between macaques and vervets and 29 million years between humans and vervets (Horvath *et al*., 2021*a*; Jasinska *et al*., 2022).

(*i*.7) *Rodents (order Rodentia)*. Laboratory murine mouse models such as *Mus musculus* (Linnaeus) were used to construct age clocks in multiple tissue types (Stubbs *et al*., 2017). This was later replicated in two wild rodent species, the wood mouse [*Apodemus sylvaticus* (Linnaeus)], and naked mole-rat [*Heterocephalus glaber* (Rüppell)]. In wood mouse (Little *et al*., 2020), ear punches from a laboratory-reared and formerly wild population (Clerc *et al*., 2019) were used to develop an age clock based on 9 CpGs in four genes. The resulting clock provided the first evidence with a high accuracy for the prediction of age in wild mice with a MEP of 59 days. For the naked mole-rat, 23 CpGs that are age correlated were used to construct an initial age clock using liver tissue (Lowe *et al*., 2020). To enable non-lethal age determination of wild individuals this clock was validated using skin samples, with a resulting model able to predict age with a MEP of 3–4 years. The model was found to have slightly lower accuracy between skin and liver samples, indicating that skin may age slower. More recent studies in the same species used reduced-representation bisulfite sequencing (Kerepesi *et al*., 2022) and a universal mammalian methylation array (Horvath *et al*., 2022*a*) to construct more accurate models. The first was able to construct an age-prediction model from blood samples that could predict age with a MEP of 1.1 years (Kerepesi *et al*., 2022) while the second constructed a pan-tissue model able to predict age with a MEP of less than 1 year (Horvath *et al*., 2022*a*).

(*i*.8) *Shrews (order Eulipotyphla)*. To date, one study has explored epigenetic ageing in shrews (Cossette *et al*., 2022), an order closely related to Carnivora, Chiroptera, and Pholidota (pangolins), in the superorder Laurasiatheria. Using a conserved mammalian methylation array, this study explored both the utility of methylation in estimating the age of masked shrew, *Sorex cinereus* (Kerr), as well as the possible divergence in epigenetic ageing between two highly differentiated mainland and insular island populations. The epigenetic clock, constructed from 26 age-correlated CpGs, was able to predict age with a MEP of 0.1 years, however, some evidence was found for the accelerated ageing of island populations. Further epigenome-wide association studies also identified several genes without significant age associations that have differential methylation and possibly expression patterns that may contribute to the morphological differences between mainland and island populations.

(*i*.9) *Ungulates (orders Artiodactyla and Perissodactyla)*. In hooved mammals, distant relatives of whales and other aquatic mammals, age clocks were established in several species of both even-toed (Artiodactyla) and odd-toed (Perissodactyla) ungulates. In odd-toed ungulates, studies included wild and domesticated species of the family Equidae. Epigenetic clocks were modelled in common zebra [*Equus quagga* (Boddaert)] using 70 CpG sites for blood and 99 CpGs for blood and skin pooled samples (Larison *et al*., 2021*a*). The clock developed using blood samples outperformed the skin-based model and was able to predict age with a MEP of 6–7 months, as compared to a MEP of 1–2 years. The blood model was tested further in other species in the genus *Equus*, including domestic horses [*E. caballus* (Linnaeus)], Grévy's zebras [*E. grevyi* (Oustalet)], and Somali wild asses [*E. africanus somaliensis* (Noack)], and achieved results comparable to the skin-based model. A second study on domestic horses created three epigenetic clocks based

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on data derived from blood, liver, and pan-tissue sampling (Horvath *et al.*, 2022*b*). The most accurate model was derived from liver tissue with a MEP of 7–8 months while both the blood and pan-tissue models had a MEP of ∼1 year.

Studies conducted on even-toed ungulates included species from the families Bovidae (Kordowitzki *et al.*, 2021; Sugrue *et al.*, 2021), Cervidae (Lemaître *et al.*, 2022), and Suidae (Schachtschneider *et al.*, 2021). A study in roe deer [*Capreolus capreolus* (Linnaeus)], from wild populations in Eastern France, used the same methods as for equines to create sex-specific clocks for age determination with a MEP of 6–8 months (Lemaître *et al.*, 2022). Interestingly, this study also identified acceleration methylation ageing in males and several CpGs that showed differential methylation patterns based on sex. Due to the basal genetic similarities between this species and white-tailed deer [*Odocoileus virginianus* (Zimmermann)], this method may also be transferable to other deer species. Among domesticated species, epigenetic clocks were constructed for cattle [*Bos taurus* (Linnaeus)], domestic sheep [*Ovis aries* (Linnaeus)], and domestic pig [*Sus scrofa domesticus* (Linnaeus)]. Epigenetic clocks constructed for cattle and domestic sheep were able to estimate age with a MEP of 8–9 and 3–5 months, respectively (Kordowitzki *et al.*, 2021; Sugrue *et al.*, 2021), based on between 80 and 140 CpGs with conserved age-related methylation patterns across tissue types. For domestic pig an age-estimation clock was constructed from more than 900 CpGs with a MEP of 1–2 months (Schachtschneider *et al.*, 2021).

(2) Telomeres

(*a*) *What are telomeres?*

Early work in cytogenetics identified knobs of heterochromatin, visualised at the end of linear chromosomes (Fig. 5), which could be considered the natural ends of chromosomes (McClintock, 1941). These ends would later be called telomeres, from the Greek words for 'end' (telos) and 'part' (meros). These studies would pave the way towards the discovery of basic telomere structure, several decades later, and the advancement of science and technology towards the further characterization of their molecular composition and biology (Shay & Wright, 2019).

The telomeres are formed by a tandem repeat of a short nucleotide sequence. In humans, this sequence was identified to be a repeat of 5′-TTAGGG-3′ (Moyzis *et al.*, 1988) which is conserved in most eukaryotes, including all mammals (Gomes *et al.*, 2011). The full length of the telomere sequence varies; from 10 to 15 kilobases (kb) in humans (de Lange *et al.*, 1990) to 20–50 kb in laboratory mice (Kipling & Cooke, 1990). The tandem repeat sequence is partially double stranded (Fig. 6), ending with a free 3′ overhang which protrudes past the 5′ strand by ∼50–500 nucleotides (Wu, Takai & De Lange, 2012). It was initially unclear how the 3′ strand is formed (Palm & de Lange, 2008), as telomerase had already been excluded (Yuan *et al.*, 1999), but further studies elucidated the role of several nucleases (Wu *et al.*, 2012) including Exonuclease I. The 3′ strand, containing the triple G of the repeat is rich in guanosine and thus termed the G-strand, whilst the 5′ strand is termed the C-strand. The 5′-end is well defined and mostly ends with the ATC-5′ codon, in contrast to the variable 3′-end (Sfeir *et al.*, 2005). The telomere sequence folds in upon itself to form a laureate structure (Fig. 6) as a higher order chromatin configuration consisting of two distinct loops, the double-stranded DNA telomere loop (T-loop) and the single-stranded displacement loop (D-loop; Cleal, Norris & Baird, 2018). The D-loop is formed when the 3′ overhang of the free end invades and displaces a 50–300 nucleotide region of the telomeric sequence to close the loop, thereby protecting the complex from recognition by DNA repair

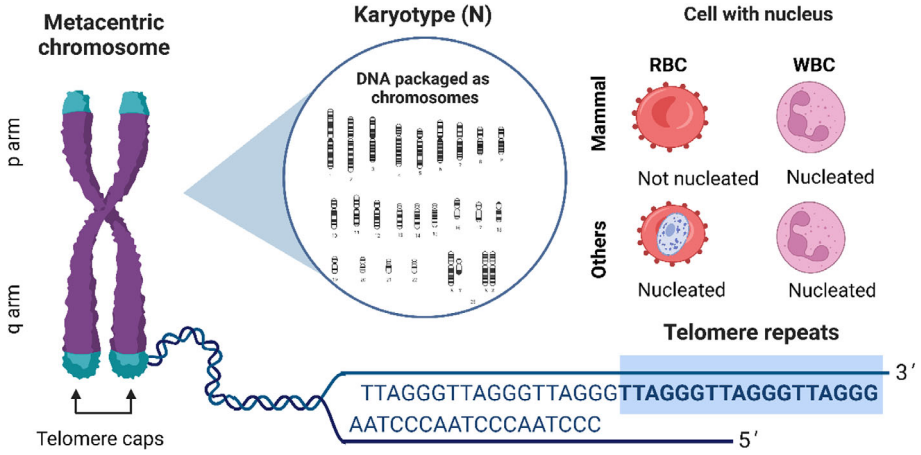


Fig. 5. Picture of metacentric chromosomes showing the telomere caps at either end. On the right the typical mammalian cell is shown with the nucleus highlighted. The nucleus contains the genomic DNA which is packaged in densely coiled structures called chromosomes for which the karyotype (chromosomal organisation) is illustrated in the central circle. The ends of the chromosomes (blue) are capped with telomeres – non-coding repeats of the same 6–8 base pair sequence – to protect the coding genomic DNA (purple) from damage or loss during replications. Cells from the tissue most commonly used in telomere length studies are also indicated showing the nucleated red blood cells (RBCs) found in non-mammalian species as well as the nucleated white blood cells (WBCs).

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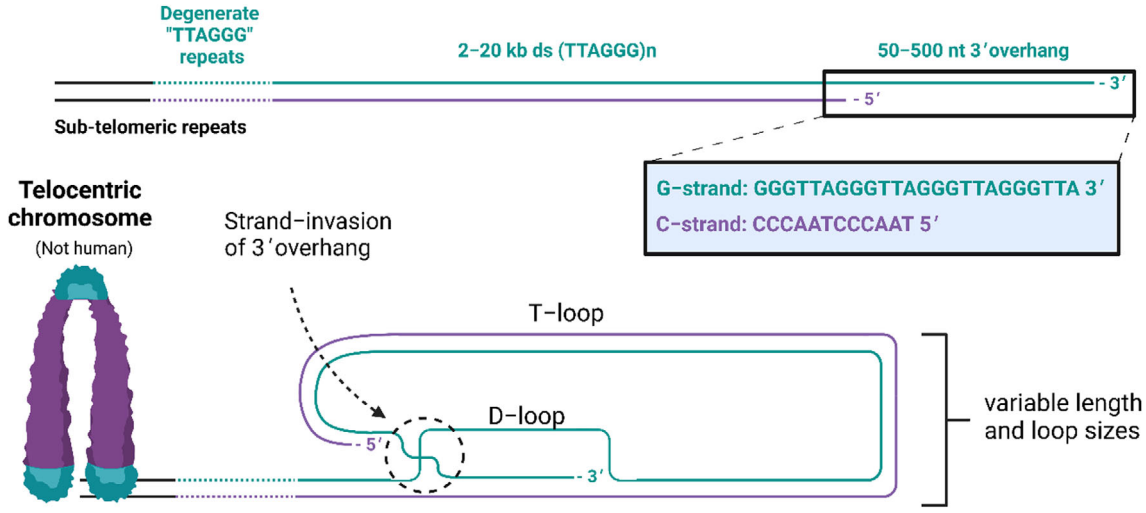


Fig. 6. Segments and higher chromatin structure of telomeric DNA. The DNA strand at the distal end of a chromosome ends is formed by sub-telomeric repeats, followed by degenerate tandem repeats, adjacent to the double stranded (ds) telomeric tandem repeats of 5′-TTAGGG-3′ that span ∼2–20 kilobase pairs (kb). The terminal ends of the telomere DNA comprise the longer, guanine rich, G-strand that forms the 3′ overhang of ∼50–500 nucleotides (nt), as well as the complementary C-strand of the 5′ terminal DNA. The terminal ends of the telomere strand bend back to form a hairpin-like loop, known as the telomere loop (T-loop), where the 3′ overhang inserts itself between a double-stranded segment forming the displacement or D-loop. The structure of telocentric chromosomes found in non-human and non-mammalian species is also illustrated. Image created in BioRender.com.

machinery as single strand breaks (Oganesian & Karlseder, 2011; Kazda *et al.*, 2012; Wu *et al.*, 2012).

Telomere length is maintained through the activity of telomerase (Fig. 7), a reverse transcriptase that elongates telomeres (Greider & Blackburn, 1985), as well as by the alternative lengthening of telomeres (ALT) pathway (Cesare & Reddel, 2010), which relies upon homologous recombination between telomeres. Telomerase is a ribonucleoprotein complex with RNA subunits that elongate chromosomes by adding telomeric repeat sequences to the terminal ends of chromosomes. This complex comprises two catalytic subunits, telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC), which serve as the template to synthesise telomeric repeat sequences, as well as a stabilising protein complex of Dyskerin protein and nucleolar protein family A member proteins (Greider & Blackburn, 1985).

(*b*) *Changes with age*

Similar to methylation, telomeres undergo several phases of alteration throughout gametogenesis, following zygote formation, during embryogenesis, and throughout the lifespan of an organism in different tissues (Fig. 8). For example, telomeres in oocytes are typically shorter than in somatic tissue and undergo activation following zygote formation to elongate throughout the two- and four-cell phases until blastocyst formation (Zhao *et al.*, 2014). This initially happens independent of telomerase, which has been found to be absent from spermatozoa, oocytes and morula stage cells (Wright *et al.*, 1996), followed by an upregulation of telomerase during the morula–blastocyst phase (Schaetzlein *et al.*, 2004). Elongation in the absence of telomerase is likely due to extensive genome-wide demethylation providing a favourable milieu for telomere lengthening via an ALT mechanism (Liu *et al.*, 2007). After birth, telomerase activity is restricted to germline cells and stem cells.

As DNA polymerase would not be able to replicate the segment of DNA to which it binds, telomeres serve as barriers protecting coding sequences located towards the end of chromosomes. In the absence of telomerase, however, telomeres are also subject to the gradual loss of sequence due to the terminal replication problem, and telomere length has since become a well-studied attribute of cellular ageing (Olsson, Wapstra & Friesen, 2018). The terminal replication problem is attributed to DNA polymerase requiring an RNA primer to initiate replication. During replication the RNA primer, which binds to the terminal nucleotides of a replicating strand, dissociates from the newly synthesised strand without being incorporated resulting in a daughter strand that is shorter than the template strand. Consequently, telomere length is typically lost at a rate of 30–50 bp per cell division and ultimately leads to cellular senescence. Furthermore, telomeres have been shown to be highly susceptible to oxidative damage due to reactive oxygen species (ROS), for example because of high stress levels, persistent inflammation, or in response to environmental toxins (Zhao *et al.*, 2014).

(*c*) *Telomeres as a biomarker of animal age*

Following the observations of telomere length reflecting age and cellular senescence in humans there has been increased

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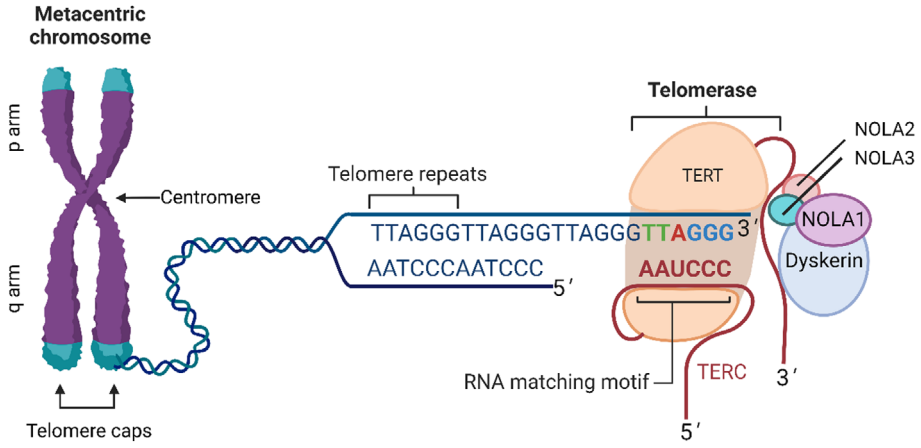


Fig. 7. Diagrammatic representation of the telomerase complex binding to the single–stranded loop of telomeres for elongation. The telomerase complex has a telomerase RNA component (TERC) that contains an RNA matching motif to incorporate nucleotides of the telomeric repeat sequence to the strand being elongated in the 5′ to 3′ direction. The telomerase reverse transcriptase (TERT) element enzymatically catalyses the addition of the newly synthesised telomeric repeat to the existing strand. Other elements of the telomerase complex are also indicated, including small nucleolar ribonucleoproteins Dyskerin, and the three nucleolar protein family A member proteins (NOLA1, NOLA2, and NOLA3). Image created in BioRender.com.

interest in its ability to determine age in animals (Haussmann & Vleck, 2002; Horn, Robertson & Gemmell, 2010) and such correlations have been illustrated in several non-human species including birds (Tricola *et al.*, 2018; Vedder *et al.*, 2022), aquatic mammals (Garde *et al.*, 2010; Izzo *et al.*, 2011), and reptiles (Hatase *et al.*, 2008). Telomere length has been assayed using several methods (Mensà *et al.*, 2019) ranging from terminal restriction fragment (TRF) analyses followed by southern blotting to fluorescence-based methods that include *in situ* hybridization (FISH), flow cytometry coupled FISH (Flow-FISH), and quantitative real time polymerase chain reaction (qPCR). Animal studies that assayed telomere length, included in this review and meta-analysis, are summarised in Table 2. As telomeres and age have been the subject of several reviews (Dunshea *et al.*, 2011; Wilbourn *et al.*, 2018; Remot *et al.*, 2021) this section will highlight a few key studies across

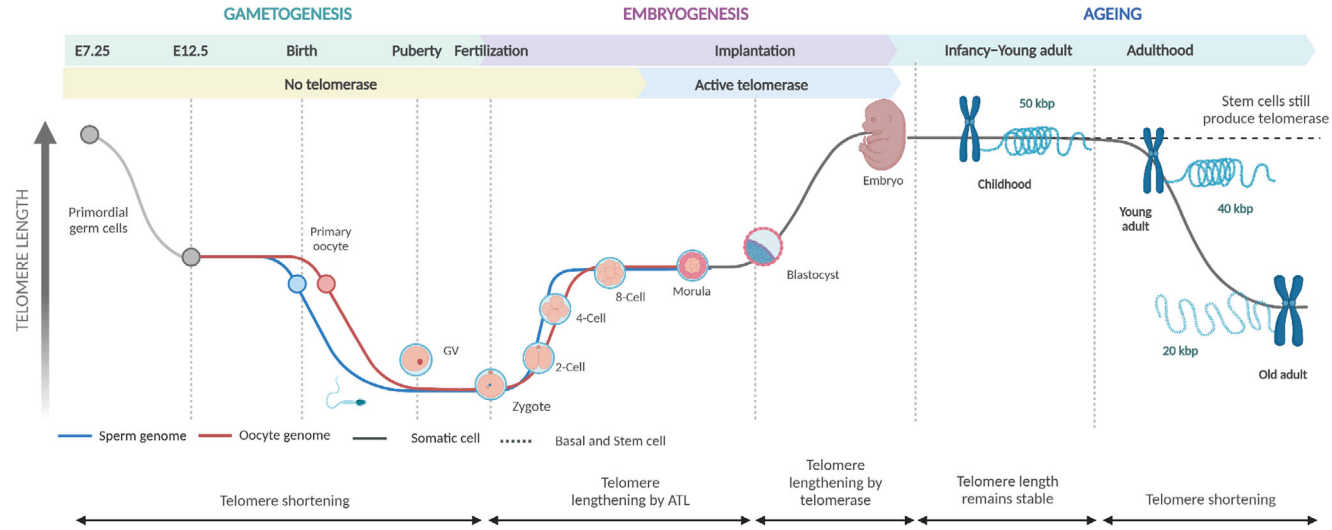


Fig. 8. Diagrammatic illustration of the alterations to telomere length during key life stages from gametogenesis to embryogenesis and the resulting changes that occur during natural ageing in most mammals. During gametogenesis the telomeres shorten in primordial germ cells and gametes, either sperm or egg cells, due to the absence of telomerase. After a zygote is formed from the union of two gametes, passive genomic demethylation happens which aids in telomere lengthening by enabling alternative telomere lengthening (ALT). Between the morula and blastocyst stage, telomerase expression is reactivated, and active telomere lengthening occurs prior to implantation. After birth, telomere length remains stable in most tissues throughout childhood from infancy to early adulthood. During the ageing process telomere length is maintained in stem cells while telomere shortening happens throughout the remainder of somatic cells. Image created in BioRender.com.

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Table 2. Summary of studies that assayed animal telomere length in relation to age, the environment, or transgenerational heredity. Studies that used the data to create a molecular model for animal age, which were included in the meta-analysis, are indicated with an asterisk (*) for across-class analyses and a double asterisk (**) for the birds-only analyses. Species are grouped by class and listed alphabetically. For each species the karyotype of diploid chromosomes is given along with estimates for the average lifespan. For each study, the method of measuring telomere length is indicated: a, fluorescent *in situ* hybridization–flow cytometry (Flow-FISH); b, terminal restriction fragment (TRF) length & Southern blot; c, quantitative real-time polymerase chain reaction (qPCR); d, quantitative-fluorescent *in situ* hybridization (Q-FISH). Where relevant, citations are also provided for studies on age determination using other biological clocks (tooth annulation/otolith) in the same/similar species. n.a., not available.

Species	Karyotype	Lifespan (years)	Study	Method	Tooth annulation/otolith
(i) Fishes:					
Common carp (*Cyprinus carpio*)*	100	11–13	Izzo *et al.* (2014)	c	Brown *et al.* (2011)
Common carp (*Cyprinus carpio*)*	100	11–13	Izzo (2010)	c	Brown *et al.* (2011)
European chub (*Squalius cephalus*)*	50	25–30	Molbert *et al.* (2021)	c	Brown *et al.* (2011)
European seabass (*Dicentrarchus labrax*)*	48	15–30	Horn *et al.* (2008)	b	Secor *et al.* (1995)
Rainbow trout (*Oncorhynchus mykiss*)*	60	4–6	Panasiak *et al.* (2020)	d	Payan *et al.* (1998)
Siberian sturgeon (*Acipenser baerii*)*	248	40–60	Simide *et al.* (2016)	c	Campana (2001)
Turquoise killifish (*Nothobranchius furzeri*)	36	4–8 months	Hartmann *et al.* (2009)	b, c	Reichenbacher *et al.* (2007)
Zebrafish (*Danio rerio*) – model organism	48	1–4	Lund *et al.* (2009)	b	Higgs *et al.* (2002)
Zebrafish (*Danio rerio*) – model organism	48	1–4	Anchelin *et al.* (2011)	a, b	Higgs *et al.* (2002)
(ii) Amphibians:					
Australian green tree frog (*Litoria caerulea*)*	26	16–21	Lundsgaard *et al.* (2022)	c	n.a.
Natterjack toad (*Epidalea calamita*)*	22	10–15	Sánchez-Montes *et al.* (2020)	c	n.a.
(iii) Reptiles:					
Algerian psammodromus (*Psammodromus algirus*)*	38	3–5	Burraco *et al.* (2020)	c	n.a.
American alligator (*Alligator mississippiensis*)	32	30–50	Bae *et al.* (2021)	c	n.a.
American alligator (*Alligator mississippiensis*)*	32	30–50	Scott *et al.* (2006)	b	n.a.
Frilled lizard (*Chlamydosaurus kingii*)*	32	10–20	Ujvari *et al.* (2017)	c	n.a.
Loggerhead sea turtle (*Caretta caretta*)*	56	47–67	Hatase *et al.* (2008)	c	n.a.
Leatherback sea turtle (*Dermochelys coriacea*)*	56	45–50	Plot *et al.* (2012)	c	n.a.
Przewalski's toadhead agama (*Phrynocephalus przewalskii*)	46	3–10	Zhang *et al.* (2018)	c	n.a.
Water python (*Liasis fuscus*)*	36	20–30	Ujvari & Madsen (2009)	b	n.a.
(iv) Birds:					
Chats & flycatchers:					
African stonechat (*Saxicola torquatus*)*,**	80	4–5	Apfelbeck *et al.* (2019)	b	n.a.
Bluethroat (*Luscinia svecica*)	82	1–5	Sørheim (2020)	c	n.a.
Collared flycatcher (*Ficedula albicollis*)*,**	80	5–9	Stier *et al.* (2020)	c	n.a.
Collared flycatcher (*Ficedula albicollis*)*,**	80	5–9	Voillemot *et al.* (2012)	c	n.a.
European pied flycatcher (*Ficedula hypoleuca*)**	80	2–15	Kärkkäinen *et al.* (2019)	c	n.a.
European pied flycatcher (*Ficedula hypoleuca*)**	80	2–15	Kärkkäinen *et al.* (2021)	c	n.a.
European stonechat (*Saxicola rubicola*)*,**	80	4–5	Apfelbeck *et al.* (2019)	b	n.a.
Corvids:					
Florida scrub-jay (*Aphelocoma coerulescens*)*,**	80	4–9	Tricola *et al.* (2018)	b	n.a.

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Table 2. *(Cont.)*

Species	Karyotype	Lifespan (years)	Study	Method	Tooth annulation/otolith
Jackdaw (*Corvus monedula*)**	80	3–5	Bauch *et al.* (2021)	b	n.a.
Jackdaw (*Corvus monedula*)**	80	3–5	Salomons *et al.* (2009)	b	n.a.
Mexican jay (*Aphelocoma ultramarina*)*,***	80	9–15	Tricola *et al.* (2018)	b	n.a.
Cuckoos:					
Great spotted cuckoo (*Clamator glandarius*)**	78	3–6	Soler *et al.* (2015)	c	n.a.
Eagles:					
White-tailed eagle (*Haliaeetus albicilla*)	66	21–25	Hansen *et al.* (2022)	c	n.a.
Finches:					
Bengalese finch (*Lonchura striata*)*,***	78	4–7	Tricola *et al.* (2018)	b	n.a.
Pine siskin (*Spinus pinus*)	78	5–10	Vernasco & Watts (2022)	c	n.a.
Zebra finch (*Taeniopygia guttata*)*,***	78	2–3	Heidinger *et al.* (2012)	c	n.a.
Zebra finch (*Taeniopygia guttata*)*,***	78	2–3	Tricola *et al.* (2018)	b	n.a.
Geese & waterfowl:					
Barnacle goose (*Branta leucopsis*)*,***	80	14–24	Pauliny *et al.* (2012)	b	n.a.
Gulls, shorebirds & waders:					
Common ruff (*Calidris pugnax*)*,***	86	4–5	Tricola *et al.* (2018)	b	n.a.
Dunlin (*Calidris alpina*)*,***	88	5–9	Pauliny *et al.* (2006)	b	n.a.
Oystercatcher (*Haematopus longirostris*)*,***	72	12–17	Tricola *et al.* (2018)	b	n.a.
Yellow-legged gull (*Larus michahellis*)**	66	18–20	Noguera & Velando (2021)	c	n.a.
Juncos, buntings & sparrows:					
Dark-eyed junco (*Junco hyemalis*)*,***	82	3–11	Bauer *et al.* (2018)	c	n.a.
Dark-eyed junco (*Junco hyemalis*)	82	3–11	Graham (2018)	c	n.a.
House sparrow (*Passer domesticus*)	76	3–5	Meillère *et al.* (2015)	c	n.a.
Savannah sparrow (*Passerculus sandwichensis*)*,***	74	2–6	Tricola *et al.* (2018)	b	n.a.
Magpies:					
Eurasian magpie (*Pica pica*)**	82	3–4	Soler *et al.* (2015)	c	n.a.
Manakins:					
Wire-tailed manakin (*Pipra filicauda*)**	78	10–15	Vernasco *et al.* (2021)	c	n.a.
Ovenbirds:					
Thorn-tailed Rayadito (*Aphrastura spinicauda*)	82	4–9	Quirici *et al.* (2016)	c	n.a.
Parrots:					
African Grey parrot (*Psittacus erithacus erithacus*)*,***	66	60–80	Aydinonat *et al.* (2014)	c	n.a.
Penguins:					
Adelie penguin (*Pygoscelis adeliae*)*,***	96	10–20	Tricola *et al.* (2018)	b	n.a.
King penguin (*Aptenodytes patagonicus*)**	72	26–41	Reichert *et al.* (2015)	c	n.a.
Magellanic penguin (*Spheniscus magellanicus*)*,***	68	20–30	Cerchiara *et al.* (2017)	c	n.a.
Seabirds:					
Black guillemot (*Cepphus grille*)*,***	50	10–11	Tricola *et al.* (2018)	b	n.a.
Common tern (*Sterna hirundo*)*,***	68	9–10	Bauch *et al.* (2014)	b	n.a.
Common tern (*Sterna hirundo*)*,***	68	9–10	Haussmann *et al.* (2003)	b	n.a.
Common tern (*Sterna hirundo*)*,***	68	9–10	Tricola *et al.* (2018)	b	n.a.
Common tern (*Sterna hirundo*)	68	9–10	Vedder *et al.* (2017)	b	n.a.
Common tern (*Sterna hirundo*)	68	9–10	Vedder *et al.* (2022)	b	n.a.
Cory's shearwater (*Calonectris borealis*)*,***	80	20–25+	Bauch *et al.* (2020)	b	n.a.
	80	20–25+	Bauch *et al.* (2022)	b	n.a.

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Table 2. *(Cont.)*

Species	Karyotype	Lifespan (years)	Study	Method	Tooth annulation/otolith
Cory's shearwater (*Calonectris borealis*)					
Great frigatebird (*Fregata minor*)*,***	78	30–34	Juola *et al.* (2006)	b	n.a.
Great frigatebird (*Fregata minor*)*,***	78	30–34	Tricola *et al.* (2018)	b	n.a.
Leach's storm petrel (*Oceanodroma leucorhoa*)*,***	74	20–36	Tricola *et al.* (2018)	b	n.a.
Northern fulmar (*Fulmarus glacialis*)*,***	78	30–42	Tricola *et al.* (2018)	b	n.a.
Red-footed booby (*Sula sula*)*,***	78	24–40	Tricola *et al.* (2018)	b	n.a.
Thick-billed murre (*Uria lomvia*)*,***	80	20–29	Tricola *et al.* (2018)	b	n.a.
Starlings:					
Spotless starling (*Sturnus unicolor*)	80	8–9	Soler *et al.* (2017)	c	n.a.
Swallows:					
Barn swallow (*Hirundo rustica*)**	80	1–4	Parolini *et al.* (2015)	c	n.a.
Barn swallow (*Hirundo rustica*)**	80	1–4	Tricola *et al.* (2018)	b	n.a.
Mangrove swallow (*Tachycineta albilinea*)*,***	78	4–8	Tricola *et al.* (2018)	b	n.a.
Sand martin (*Riparia riparia*)*,***	80	2–9	Pauliny *et al.* (2006)	b	n.a.
Tree swallow (*Tachycineta bicolor*)*,***	78	4–8	Tricola *et al.* (2018)	b	n.a.
Swifts:					
Alpine swift (*Tachymarptis melba*)*,***	78	10–15	Bize *et al.* (2009)	c	n.a.
Thrushes:					
Eastern bluebird (*Sialia sialis*)	80	6–10	de la Iglesia (2018)	c	n.a.
Eurasian blackbird (*Turdus merula*)	80	3–21	Ibáñez-Álamo *et al.* (2018)	c	n.a.
Tits:					
Blue tit (*Cyanistes caeruleus*)**	80	3–10	Sudyka *et al.* (2014)	c	n.a.
Great tit (*Parus major*)	80	2–3	Grunst *et al.* (2020)	c	n.a.
Great tit (*Parus major*)*,***	80	2–3	Salmón *et al.* (2016)	c	n.a.
Great tit (*Parus major*)*,***	80	2–3	Tricola *et al.* (2018)	b	n.a.
Warblers:					
American redstart (*Setophaga ruticilla*)**	78	5–10	Angelier *et al.* (2013)	c	n.a.
Great reed warbler (*Acrocephalus arundinaceus*)**	80	2–6	Asghar *et al.* (2015)	c	n.a.
Seychelles warbler (*Acrocephalus sechellensis*)*,***	78	5–17	Barrett *et al.* (2013)	c	n.a.
Seychelles warbler (*Acrocephalus sechellensis*)	78	5–17	Spurgin *et al.* (2018)	c	n.a.
Wrens:					
Purple-crowned fairywren (*Malurus coronatus*)	72	3–5	Eastwood *et al.* (2019)	c	n.a.
Purple-crowned fairywren (*Malurus coronatus*)*,***	72	3–5	Roast *et al.* (2022)	c	n.a.
(v) Mammals:					
Aquatic:					
Dugong (*Dugong dugon*)*	48	50–70	Cherdsukjai *et al.* (2020)	c	Marsh, Heinsohn & Marsh (1984)
Humpback whale (*Megaptera novaeangliae*)*	44	45–50	Olsen *et al.* (2014)	c	Ohsumi *et al.* (1965)
Bats:					
Bechstein's bat (*Myotis bechsteinii*)*	44	15–21	Foley *et al.* (2018)	c	Linhart (1973)
Big brown bat (*Eptesicus fuscus*)*	50	6–19	Ineson *et al.* (2020)	c	Linhart (1973)
Common bent-wing bat (*Miniopterus schreibersii*)*	46	13–20	Foley *et al.* (2018)	c	Linhart (1973)
Greater horseshoe bat (*Rhinolophus ferrumequinum*)*	58	20–30	Foley *et al.* (2018)	c	Linhart (1973)
	44	6–13	Foley *et al.* (2018)	c	Linhart (1973)

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Table 2. *(Cont.)*

Species	Karyotype	Lifespan (years)	Study	Method	Tooth annulation/otolith
Greater mouse-eared bat (*Myotis myotis*)*					
Greater mouse-eared bat (*Myotis myotis*)	44	6–13	Foley *et al.* (2020)	c	Linhart (1973)
Little brown bat (*Myotis lucifugus*)*	44	7–31	Ineson *et al.* (2020)	c	Linhart (1973)
Carnivores:					
American black bear (*Ursus americanus*)*	74	18–23+	Kirby *et al.* (2017)	c	Marks & Erickson (1966)
American marten (*Martes americana*)*	38	6–15	Pauli *et al.* (2011)	c	Belant *et al.* (2011)
Australian sea lion (*Neophoca cinerea*)*	36	17–25	Izzo *et al.* (2011)	c	Rust *et al.* (2019)
Domestic cat (*Felis catus*)*	38	12–18	Brümmendorf *et al.* (2002)	a	Grue & Jensen (1979)
Domestic cat (*Felis catus*)*	38	12–18	McKevitt *et al.* (2003)	b	Grue & Jensen (1979)
Domestic dog (*Canis familiaris*)	78	10–13	Dutra *et al.* (2020)	c	Grue & Jensen (1979)
Domestic dog (*Canis familiaris*)*	78	10–13	Fick *et al.* (2012)	c	Grue & Jensen (1979)
Domestic dog (*Canis familiaris*)*	78	10–13	McKevitt *et al.* (2002)	b	Grue & Jensen (1979)
European badger (*Meles meles*)*	44	6–16	Beirne *et al.* (2014)	c	Ahnlund (1976)
European badger (*Meles meles*)	44	6–16	van Lieshout *et al.* (2019)	c	Ahnlund (1976)
European badger (*Meles meles*)	44	6–16	van Lieshout *et al.* (2021)	c	Ahnlund (1976)
Grizzly bear (*Ursus arctos horribilis*)*	74	20–25	Wong (2017)	b	Craighead *et al.* (1970)
Harp/Greenland seal (*Pagophilus groenlandicus*)*	32	20–30	Garde *et al.* (2010)	b, c	Bowen *et al.* (1983)
Meerkat (Suricata suricatta)	36	12–14	Cram *et al.* (2018)	c	Rausch & Pearson (1972)
Pacific marten (*Martes caurina*)*	38	6–15	Pauli *et al.* (2011)	c	Belant *et al.* (2011)
Polar bear (*Ursus maritimus*)*	74	25–30	Wong (2017)	c	Christensen-Dalsgaard *et al.* (2010)
Spotted hyena (*Crocuta crocuta*)*	40	12–25	Lewin *et al.* (2015)	b	Grue & Jensen (1979)
Elephants:					
Asian elephant (*Elephas maximus*)*	56	48–60	Buddhachat *et al.* (2017)	c	Spinage (1976)
Marsupials:					
Tasmanian devil (*Sarcophilus harrisii*)	14	5–8	Smith *et al.* (2020)	c	Pekelharing (1970)
Primates:					
Baboon (*Papio hamadryas*)*	42	27–37	Baerlocher *et al.* (2003)	a	Wada *et al.* (1978)
Baboon (*Papio hamadryas*)	42	27–37	Karere *et al.* (2019)	c	Wada *et al.* (1978)
Chimpanzee (*Pan troglodytes*)	48	32–39	Tackney *et al.* (2014)	c	Wittwer-Backofen *et al.* (2004)
Chimpanzee (*Pan troglodytes*)	48	32–39	Eisenberg *et al.* (2017)	c	Wittwer-Backofen *et al.* (2004)
Crab-eating macaque (*Macaca fascicularis*)*	42	15–30	Lee *et al.* (2002)	a, b	Wada *et al.* (1978)
Rodents:					
American red squirrel (*Tamiasciurus hudsonicus*)	46	3–8	Dantzer *et al.* (2020)	b	Adams & Watkins (1967)
Columbian ground squirrel (*Urocitellus columbianus*)*	32	5–10	Viblanc *et al.* (2022)	c	Adams & Watkins (1967)
Damaraland mole-rat (*Fukomys damarensis*)*	74	15–16	Leonida *et al.* (2020)	d	Grue & Jensen (1979)
Eastern chipmunk (*Tamias striatus*)*	38	3–8	Tissier *et al.* (2022)	c	Adams & Watkins (1967)
Hottentot mole-rat (*Cryptomys hottentotus*)*	54	11–15	Leonida *et al.* (2020)	d	Grue & Jensen (1979)
Shrews:					
Iberian shrew (*Sorex granarius*)	42	10–14 months	Zhdanova *et al.* (2010)	d	Grue & Jensen (1979)
Ungulates:					
Domestic horse (*Equus caballus*)*	64	25–30	Denham *et al.* (2019)	b	Penzhorn (1982)
Moose (*Alces alces*)	68	15–25	Fohringer *et al.* (2022)	c	Rolandsen *et al.* (2008)

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Table 2. *(Cont.)*

Species	Karyotype	Lifespan (years)	Study	Method	Tooth annulation/otolith
Roe deer (*Capreolus capreolus*)*	70	7–10	Wilbourn *et al.* (2017)	c	Grue & Jensen (1979)
Soay sheep (*Ovis aries*)	54	10–12	Fairlie *et al.* (2016)	c	Saxon & Higham (1968)
Soay sheep (*Ovis aries*)*	54	10–12	Froy *et al.* (2021)	c	Saxon & Higham (1968)
Water buffalo (*Bubalus bubalis*)*	50	25–35+	Seibt *et al.* (2019)	c	Grimsdell (1973)

the same taxonomic range as those done comparing methylation to age.

(i) Fishes (classes Agnatha, Chondrichthyes, and Osteichthyes). In fishes, telomere length has been studied in relation to ecotoxicology and environmental stressors in addition to ageing, and the validity of telomeres as proxies of life history in ectotherms has been the subject of considerable research (Friesen, Wapstra & Olsson, 2021). With regards to age, a relationship has been illustrated in several species including model organisms such as zebrafish (Anchelin *et al.*, 2011) and several wild populations such as rainbow trout [*Oncorhynchus mykiss* (Walbaum)] (Panasiak, Dobosz & Ocalewicz, 2020), European seabass (Horn *et al.*, 2008), common carp [*Cyprinus carpio* (Linnaeus)] (Izzo *et al.*, 2014), turquoise killifish [*Nothobranchius furzeri* (Jubb)] (Hartmann *et al.*, 2009), and Siberian sturgeon [*Acipenser baerii* (Brandt)] (Simide *et al.*, 2016). Although most of these studies used non-lethal sampling techniques to explore telomere length as an alternative to otolith ageing techniques, a few did include lethal sampling and even explored telomere length in the head kidney, an organ unique to teleost fishes (Panasiak *et al.*, 2020, 2022).

(ii) Amphibians (class Amphibia). Two amphibian species have been studied in the context of telomere length changes throughout their lifespan. One was principally focussed on differential changes in the Australian green tree frog [*Litoria caerulea* (White)], as a result of early life exposure to ultraviolet (UV) and different periods of light (Lundsgaard, Cramp & Franklin, 2022). The authors found that differences in early life exposure led to differences in telomere length later in life, as well as a relatedness to several key aspects that influence long-term survival in frogs. The other specifically studied telomere shortening in a wild population of naturally ageing natterjack toads [*Epidalea calamita* (Laurenti)], over a period of 3 years (Sánchez-Montes *et al.*, 2020). Their study found significant telomere shortening in the first 2–3 years of life in most of the studied individuals and a general trend of telomere shortening throughout the lifespan of toads.

(iii) Reptiles (class Reptilia). The observation that telomere length is affected by temperature or light exposure in ectotherms was replicated in both experimental and wild populations of reptiles. Under experimental conditions, telomeres were found to shorten faster and result in shortened lifespans in Przewalski's toadhead agama [*Phrynocephalus przewalskii* (Strauch)] (Zhang *et al.*, 2018). In the wild, Algerian psammodromus [*Psammodromus algirus* (Linnaeus)] lizards showed a cline of variable telomere length across a latitudinal gradient of their habitat (Burraco *et al.*, 2020). In terms of age-related telomere studies, telomere length has been shown to decline with age in American alligators (Scott *et al.*, 2006; Bae *et al.*, 2021), as well as loggerhead sea turtles (Hatase *et al.*, 2008; Plot *et al.*, 2012). Conversely, telomeres were found to increase in length in tropical water python [*Liasis fuscus* (Peters)] (Ujvari & Madsen, 2009) and follow a curvilinear relationship in frilled lizards [*Chlamydosaurus kingii* (Gray)] with both increases and decreases across their full lifespan (Ujvari *et al.*, 2017).

(iv) Birds (class Aves). Birds represent the best studied class of wild vertebrates in terms of telomeres (Criscuolo, Dobson & Schull, 2021) with most studies falling into one of four broad categories: ecotoxicological studies, stress-associated studies, migration-related studies, and age-model studies. As in other organisms, telomere length has been shown to decrease at an increased rate in response to toxins or contaminants in birds, including Arctic-breeding black-legged kittiwakes [*Rissa tridactyla* (Linnaeus)] (Blévin *et al.*, 2016), and Cory's shearwaters [*Calonectris borealis* (Cory)] (Bauch *et al.*, 2022). In stress-associated studies, telomere length dynamics were compared to environmental stressors including noise (Dorado-Correa *et al.*, 2018; Grunst *et al.*, 2020), an urban environment (Salmón *et al.*, 2016; Ibáñez-Álamo *et al.*, 2018), or anthropogenic activity (Caccavo *et al.*, 2021) as well as social factors such as brood size (Voillemot *et al.*, 2012; Quirici *et al.*, 2016; Criscuolo, Zahn & Bize, 2017, Noguera & Velando, 2021), brood parasitism (Soler *et al.*, 2015), proximity to predators (Kärkkäinen *et al.*, 2019), and social isolation (Aydinonat *et al.*, 2014). In most cases, telomere length was negatively impacted by stress and cohort-wide increases in telomere loss were observed in response to measured stressors. It should, however, be noted that many of these studies solely included birds in their early life stages and did not access telomere length changes across the full lifespan of all species.

In terms of the relationship between telomere length and attributes of migration differences have been observed for the differential timing of breeding (Graham, 2018) and migration (Vernasco & Watts, 2022) as well as return rate (Angelier *et al.*, 2013) and reproductive success (Eastwood *et al.*, 2019). Studies have also illustrated that habitat quality

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differentially affects telomere shortening within species with different breeding habitats (Angelier *et al.*, 2013) and that generally advanced shortening is a consequence of the metabolic stain that migration creates when comparing migratory and resident populations of the same species (Bauer *et al.*, 2018).

Studies with a focus on cross-sectional changes in telomere length with increased age found varied results depending on the study species (Tricola *et al.*, 2018). For example, small effects were observed for an age correlation in Magellanic penguin [*Spheniscus magellanicus* (Forster)] (Cerchiara *et al.*, 2017), and other penguins (Reichert *et al.*, 2015) while an intermediate effect was observed in several other bird species (Bize *et al.*, 2009; Vedder *et al.*, 2017; Bauer *et al.*, 2018; Roast *et al.*, 2022). The largest effect was observed in three species: common tern (Haussmann, Vleck & Nisbet, 2003), great frigatebird [*Fregata minor* (Gmelin)] (Juola *et al.*, 2006), and sand martin *Riparia riparia* (Linnaeus) (Pauliny *et al.*, 2006). Although telomere length declines were evident in birds from most lineages the rate of decline varies and appears to be correlated with maximum lifespan (Tricola *et al.*, 2018).

(v) Mammals (class Mammalia)

(*i*.1) *Aquatic mammals (infraorder Cetacea and order Sirenia)*. Telomere length was found to decline with age in humpback whales but showed a high level of variation among individuals of the same age, resulting in a model with high error (∼28 years) in the predicted ages. The authors attributed this to the low sample size for calibration as well as the sensitivity of the qPCR assay as even small experimental deviations significantly altered the assay values (Olsen *et al.*, 2014). A recent study that included dolphins found a significant decline in telomere length with age, however, the study was limited by small sample sizes ($N < 10$) with no or few replicates across the same age. One key finding was that telomere length tended to decrease at an increased rate in species with shorter lifespans (Whittemore *et al.*, 2019). In wild dugong [*Dugong dugon* (Müller)], relatives of manatees in the herbivorous aquatic mammal order Sirenia, telomere length was highly correlated with age and showed less variability at the individual level (Cherdsukjai *et al.*, 2020).

(*i*.2) *Bats (order Chiroptera)*. Telomeres have been studied in at least six species of bats including several members of the long lived *Myotis* genus (Foley *et al.*, 2018, 2020; Ineson *et al.*, 2020). In both greater horseshoe bat [*Rhinolophus ferrumequinum* (Schreber)] and common bent-wing bat [*Miniopterus schreibersii* (Kuhl)] telomere length was found to decrease rapidly with age while telomeres in *Myotis* species, including Bechstein's bat, were well maintained throughout their lifespan (Foley *et al.*, 2018). Due to the absence of telomerase in mature blood cells this was likely due to the upregulation of genes involved in the ALT pathway (Foley *et al.*, 2020). A study on two American bat species (Ineson *et al.*, 2020) found telomere length to be correlated with age in both big brown bats [*Eptesicus fuscus* (Beauvois)] and little brown bats [*Myotis lucifugus* (Le Conte)]. The authors did, however, note that the relative predictive power for unknown-age individuals was low and the relationship between telomere attrition and age in *M. lucifugus* was correlated with advanced ageing in response to white nose syndrome, a common fungal infection in some bats.

(*i*.3) *Carnivores (order Carnivora)*. Some of the first studies in non-model animals with regards to telomeres and ageing used domesticated companion animals including the domestic dog and cat. In canids, telomere length was found to shorten at an advanced rate (McKevitt *et al.*, 2002) and to be correlated with the maximum lifespan of breeds (Fick *et al.*, 2012). Similar results were obtained from longitudinal studies in felids (Brümmendorf *et al.*, 2002; McKevitt *et al.*, 2003) including the observation that telomere length varies in sub-populations of leucocytes. Other studies on other members of the suborder Feliformia included the spotted hyena [*Crocuta crocuta* (Erxleben)], and meerkat [*Suricata suricatta* (Schreber)]. For both species, studies focussed on socio-ecological factors affecting variable telomere attrition primarily in relation to social rank and found higher ranking members to have a slower rate of telomere attrition and a corresponding longer life expectancy than low-ranking members (Lewin *et al.*, 2015; Cram *et al.*, 2018).

A study on two species of martens, the American marten [*Martes americana* (Turton)], and Pacific marten [*M. caurina* (Merriam)], specifically used tooth cementum annulation to determine age for cross-validation of a telomere-based PCR method of assigning age (Pauli *et al.*, 2011). The authors were able to establish a model that can assign the age of individuals to one of five functionally relevant classes with ∼80% accuracy, which improved to over 90% when including morphometric data. Several studies in the related European badger [*Meles meles* (Linnaeus)] found diverse factors to contribute to telomere biology in these species including age-related declines (Beirne *et al.*, 2014), disease-related declines (Beirne *et al.*, 2014), transgenerational effects (van Lieshout *et al.*, 2021), and differential fitness (van Lieshout *et al.*, 2019).

Other studied carnivores include several species of bears (Wong, 2017; Kirby, Alldredge & Pauli, 2017) and pinnipeds (Garde *et al.*, 2010; Izzo *et al.*, 2011) for which a small to intermediate effect for age was observed.

(*i*.4) *Elephants (order Proboscidea, suborder Elephantiformes)*. Telomere length has been studied in relation to age in the Asian elephant using 122 samples across the full lifespan of the species (Buddhachat *et al.*, 2017). A significant decline in telomere length was found ($P < 0.05$) independent of any observed effect for sex-linked differences in ageing rate. Furthermore, this study found telomere length to be markedly increased in younger individuals in comparison to shorter lived species, while the general trend and rate of telomere shortening was consistent in both Asian elephants and shorter-lived species. Another study (Whittemore *et al.*, 2019) evaluated differential telomere shortening in species with different lifespans, including Sumatran subspecies of Asian elephants (*Elephas maximus sumatranus*), but was limited in resolution by small sample sizes ($N = 4$). Nevertheless,

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this study did detect a decline in telomere length and differential rates of attrition among species of varied lifespans.

(*i*.5) *Marsupials (infraorder Marsupialia)*. Few studies have been conducted on telomere dynamics in marsupials. One study, in Tasmanian devils, focussed on telomere length as a predictive risk factor for facial tumours and found longer telomeres to have a protective role that delays the age of onset for facial tumours in different populations (Smith *et al*., 2020). Studies on sexual dimorphism in telomere length in Tasmanian devils and other marsupials have illustrated that while telomeres shorten during oogenesis they tend to elongate during spermatogenesis and the inherited length therefore shows strong parental effects. In facial tumours, however, all cells showed characteristically short telomeres even though telomerase activity is typically upregulated in cancer cells (Bender *et al*., 2012).

(*i*.6) *Primates (order Primates)*. Beyond the cornucopia of telomere studies conducted in humans, telomeres have been assayed in relation to age in at least three other primates. The first analysed telomere length in an ageing population of laboratory-kept crab-eating macaque [*Macaca fascicularis* (Raffles)] using flow-FISH (Lee *et al*., 2002). This study highlighted two important aspects of ageing in primates. Firstly, telomere length was shown to decline with age in a predictable manner and, secondly, the ratios of different leucocyte sub-types changes with age. A similar study in baboons (Baerlocher *et al*., 2003, 2007) further supported these findings. In chimpanzees, studies have been done on telomere dynamics in relation to sex and age. A study on female chimpanzees found telomere length is increased as compared to humans, however, the rate of telomere attrition is conserved in both species and declines predictably with age (Tackney *et al*., 2014). In male chimpanzees, the heritability of telomere length based on paternal age at conception was explored and an increase in the telomeres of spermatozoa with increased paternal age was observed with consequently high levels of paternal inheritance of telomere length (Eisenberg *et al*., 2017).

(*i*.7) *Rodents (order Rodentia)*. Studies on telomeres in rodents revealed that food availability differentially affects telomere attrition rates in dormouse species (Hoelzl *et al*., 2016*a*) while hibernation tends to increase telomere length (Hoelzl *et al*., 2016*b*). A similar trend was also observed for post-hibernation differences in telomere length among female Columbian ground squirrels [*Urocitellus columbianus* (Ord)] (Viblanc *et al*., 2022). Experimental studies in glucocorticoid-treated mothers with offspring in the American red squirrel [*Tamiasciurus hudsonicus* (Erxleben)] (Dantzer *et al*., 2020) found no evidence of maternal stress as measured by endocrine responses adversely affecting telomere length in offspring either during parturition, lactation, or rearing. This indicates that stress-associated telomere attrition in the young may be independent of a biological maternal effect. Further evidence of telomere elongation in hibernating rodent species was recently established in a longitudinal study of Eastern chipmunks [*Tamias striatus* (Linnaeus)] (Tissier *et al*., 2022). In the non-hibernating mole-rat, two species were, however, found to have shortening telomeres with advanced age in mature bone marrow cells (Leonida *et al*., 2020).

(*i*.8) *Shrews (order Eulipotyphla)*. Studies on telomeres in shrews included species such as the Iberian shrew [*Sorex granaries* (Miller)], however the complex biology of telomeres in these species, including telocentric chromosomes and the inclusion of ribosomal DNA between telomere repeats, makes them unsuitable as a biomarker of age (Zhdanova *et al*., 2010).

(*i*.9) *Ungulates (orders Artiodactyla and Perissodactyla)*. Several ungulate species for which methylation clocks of age have been established were also studied in telomere–age studies. Among deer species, telomeres of both roe deer and moose [*Alces alces* (Linnaeus)] from cross-sectional studies of populations from different ecoregions found telomere length changes with age to be highly correlated with geographic location, likely due to differential exposure to environmental stressors and food availability (Wilbourn *et al*., 2017; Fohringer *et al*., 2022). In Soay sheep [*Ovis aries* (Linnaeus)], a recently domesticated breed of sheep, longitudinal studies of a largely unmanaged population found evident differences in telomere shortening based on biological sex (Watson *et al*., 2017); while no evidence was found for an effect of parental age at conception (Froy *et al*., 2017) despite the high heredity (Froy *et al*., 2021) previously linked to individual lifespans (Fairlie *et al*., 2016) in this species. Telomere length was also found to decrease with age in domesticated horses (Denham, Stevenson & Denham, 2019) and domesticated water buffalo [*Bubalus bubalis* (Linnaeus)] (Seibt *et al*., 2019).

IV. META-ANALYSIS RESULTS

(1) Methylation-based clocks

Results from the meta-analysis of studies using methylation as a biological clock for age determination are summarised in Fig. 9. The global pooled cross-study effect was significant in favour of methylation being an accurate method of age prediction ($R^2 = 0.92$, $k = 60$, $P < 0.01$). No significant differences were observed in effect size between classes ($Q = 5.53$, df = 4, $P = 0.24$), however, a separate meta-analysis with clustering based on groups found a significant difference in effect sizes between the groups (Fig. S4; $Q = 10.13$, df = 2, $P < 0.01$). Further subgroup differences were also evident for effect size between models based on tissue type (Fig. S5; $Q = 17.52$, df = 6, $P < 0.01$) and study method (Fig. S6; $Q = 232.24$, df = 4, $P < 0.01$).

Between-study heterogeneity was high ($I^2 = 97\%$, $\tau^2 = 0.29$, $P < 0.01$). This heterogeneity was less for within-group analyses for groups 2 and 3 (Fig. S4) indicating that much of the heterogeneity was likely due to the smaller sample size used to validate models in related species not used to train the model. There was, however, still a high level of heterogeneity in group 1. Results from meta-regressions assessing individual factors as possible sources of heterogeneity are summarised in Table 3. As expected, heterogeneity and author were correlated with more than half of the

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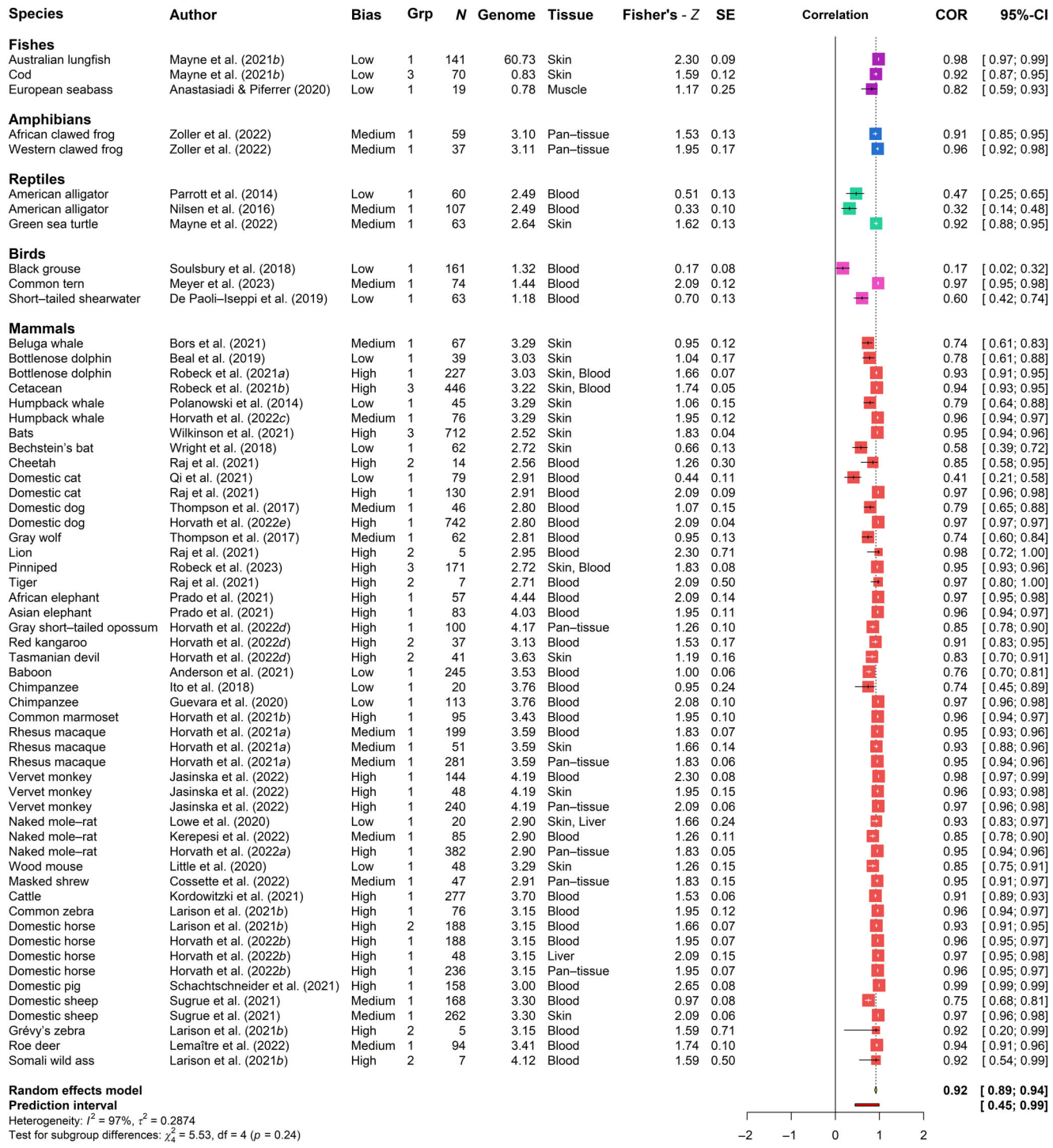


Fig. 9. Forest plot and results for the meta-analysis of studies using methylation to estimate animal age grouped by class. The pooled correlation from the random effects model is indicated (yellow diamond) along with measures of between-study heterogeneity and subgroup differences. The prediction interval is indicated with a maroon bar. Key study attributes such as sample size (*N*), genome size (in bbp), and tissue type are indicated along with the computed level of possible bias. CI, confidence interval; COR, correlation; *N*, sample size; SE, standard error.

heterogeneity being accounted for by within-study variances. A relationship with heterogeneity was also illustrated for both year and study method ($P < 0.01$) as well as class ($P < 0.05$) and sample size ($P < 0.10$), however, no relationship was found with maximum lifespan estimates, tissue type, or genome size. There were some significant indicators for

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Table 3. Summary of results for multiple meta-regressions for heterogeneity. Lifespan estimates are derived from the lower, average/middle, and upper estimates of average lifespans reported in Tables 1 and 2. Significance: $*P < 0.10$, $**P < 0.05$, and $***P < 0.01$

Factor	Z-value	P-value	Degrees of freedom (df)	Correlation (R^2)
Methylation:				
Author	3.10	<0.01***	40	0.724
Year	2.65	<0.01***	8	0.467
Bias	20.32	<0.01***	2	0.297
Sample size (N)	1.90	0.057*	1	0.049
Tissue	14.61	0.762	6	<0.01
Method	9.58	<0.01***	4	0.492
Class	5.65	0.022**	4	0.126
Genome size (bbp)	1.63	0.103	1	0.030
Lifespan:				
Lower	11.70	0.290	1	0.054
Middle	10.80	0.390	1	<0.01
Upper	10.32	0.497	1	<0.01
Telomeres (All):				
Author	4.66	<0.01***	46	0.513
Year	3.07	0.060*	17	0.147
Bias	5.63	0.251	2	0.013
Sample size (N)	8.94	0.057*	1	0.043
Tissue	6.83	0.368	7	0.057
Method	2.91	0.225	3	0.020
Class	3.54	0.499	4	<0.01
Karyotype (diploid)	2.26	0.024**	1	0.065
Lifespan:				
Lower	4.48	0.172	1	0.014
Middle	4.08	0.149	1	0.019
Upper	3.87	0.139	1	0.020
Telomeres (Birds):				
Author	3.123	<0.01***	24	0.378
Year	0.008	0.991	1	<0.01
Sample size (N)	6.553	0.507	1	<0.01

asymmetry and publication bias or small study effects (t-statistic = −2.30, df = 58, $P = 0.03$). The quantification of potential author bias using *ABCal* identified several studies (28 models) with a higher risk of bias considering they share a significant number of authors and co-authors, compared to medium- (17 models) and low-risk (15 models) studies. The computed risk of bias also accounted for a significant ($P < 0.01$) amount of the heterogeneity. Notably, eight authors contributed to more than five of the included studies while four contributed to more than 10.

(2) Telomere-based clocks

Results for the meta-analysis of studies using telomeres as a biological clock for age determination across taxa are summarised in Fig. 10 and the separate analysis for birds in Fig. 11. The global pooled cross-study effect was significant in favour of telomere length being an accurate method of age prediction ($R^2 = 0.31$, $k = 60$, $P < 0.01$). A significant difference was observed in effect size between classes ($Q = 46.43$, df = 4, $P < 0.01$), between study groups (Fig. S7; $Q = 28.96$, df = 1, $P < 0.01$), between models based on tissue used (Fig. S8; $Q = 522.36$, df = 7, $P < 0.01$), and study method (Fig. S9; $Q = 44.10$, df = 3, $P < 0.01$). When analysing only studies in birds, the pooled effect was slightly larger ($R^2 = 0.43$, $k = 51$, $P < 0.01$) with a similar prediction interval to cross-taxa studies.

Between-study heterogeneity was high for both cross-taxa studies ($I^2 = 100\%$, $\tau^2 = 0.076$, $P < 0.01$) as well as birds alone. Results from the meta-regression assessing possible contributors to heterogeneity are summarised in Table 3. As for methylation, a correlation was detected for heterogeneity and author (as well as year) accounting for more than half of the heterogeneity, however, no relationship was evident for study method, tissue type, or lifespan. A small effect was observed for sample size and karyotype, however, both only accounted for <10% of the observed heterogeneity. There were some significant indicators for asymmetry and publication bias or small study effects for the cross taxa analyses (t-statistic = 3.33, df = 58, $P < 0.01$), as for methylation, but not for the birds alone (t-statistic = −0.18, df = 49, $P = 0.86$). The quantification of potential author bias using *ABCal* identified a near-equal number of studies (26 models) with a higher risk of bias for the between-study meta-analyses considering they share a significant number of authors and co-authors. This was, however, not found to be a significant contributor to the heterogeneity of studies ($P = 0.25$). This risk of bias was generally lower than for methylation studies as most of the highly represented authors only contributed to 2–4 of the included models.

(3) Methylation *versus* telomeres

Results for the comparison between telomere length and methylation as biomarkers for age determination are indicated in Table 4 and Fig. S10. The overall effect size for methylation-based studies was higher than that observed for telomeres, 0.92 *versus* 0.31, with a narrower prediction interval. The observed difference was statistically significant (Z-value = 21.58, $P < 0.01$) while no significant difference was found between the sample size distributions of the two methods (t-statistic = 0.422, df = 104.5, $P = 0.67$).

V. DISCUSSION

Our systematic review on the use of biological clocks as markers for age determination in wildlife clearly illustrates that several biological clocks are well suited to this purpose. Two methods, tooth cementum annulation and otoliths, have been in use for nearly a century, however, both methods have limitations on their use in studying age in living animals as part of continued conservation efforts. We therefore identified and synthesised the molecular mechanisms and existing

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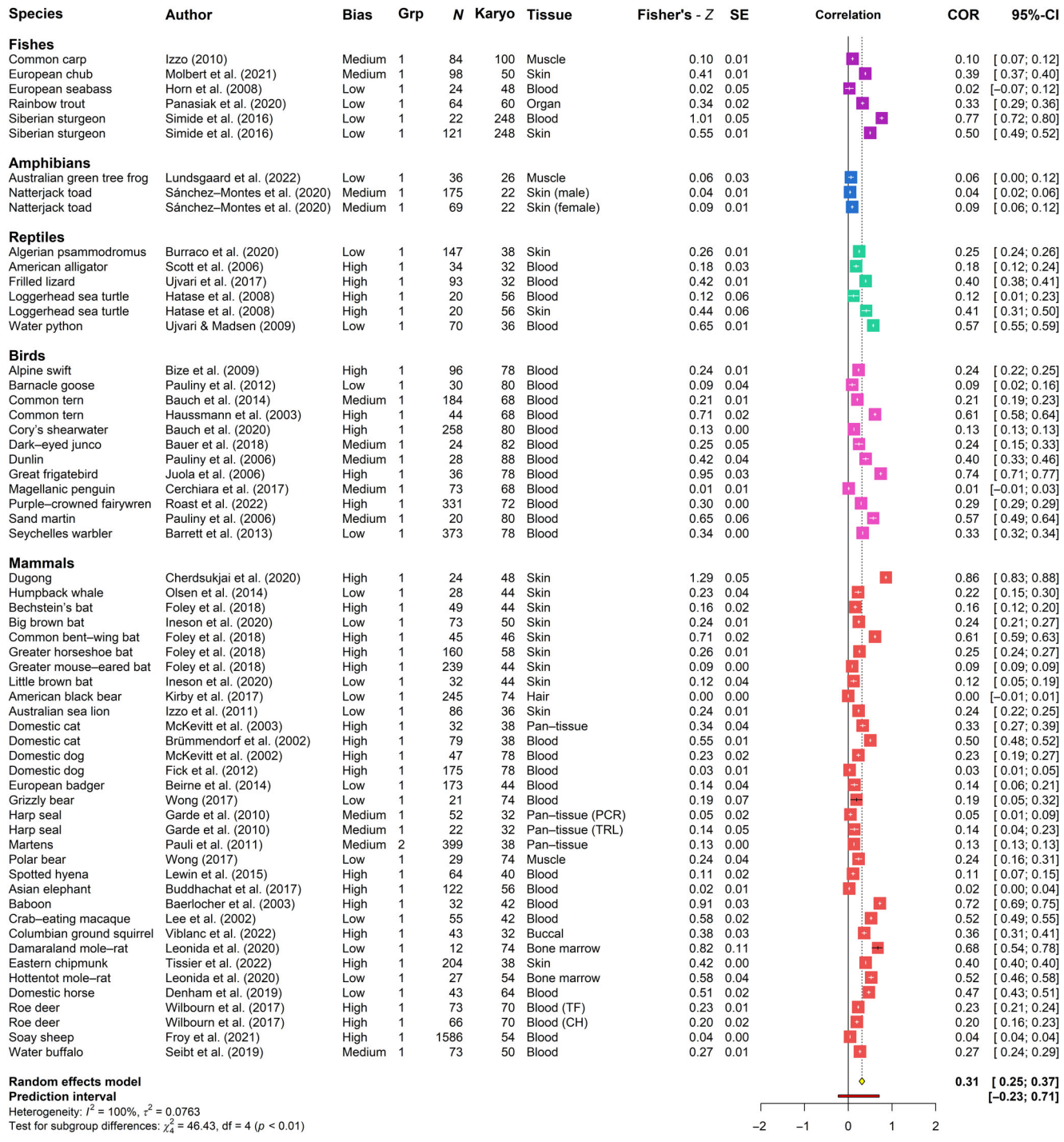


Fig. 10. Forest plot and results for the meta-analysis of studies using telomere length to estimate animal age grouped by class. The pooled correlation from the random effects model is indicated (yellow diamond) along with measures of between-study heterogeneity and the prediction interval is indicated with a red bar. Key study attributes such as sample size (*N*), karyotype (Karyo), and tissue type are indicated along with the computed level of possible bias. CH, Chizé region, France; CI, confidence interval; COR, correlation; *N*, sample size; PCR, polymerase chain reaction; SE, standard error; TF, Trois-Fontaines region, France; TRL, terminal restriction length.

primary evidence for the use of two genetic biological clocks, methylation and telomere length, that are suitable alternatives for studying age in living animals.

Methylation has previously been studied in several organisms in the context of eco-toxicology (Sareisian, 2014; Nilsen *et al.*, 2016; Romano *et al.*, 2017), relating changes in methylation to exposure to environmental pollutants, as well as

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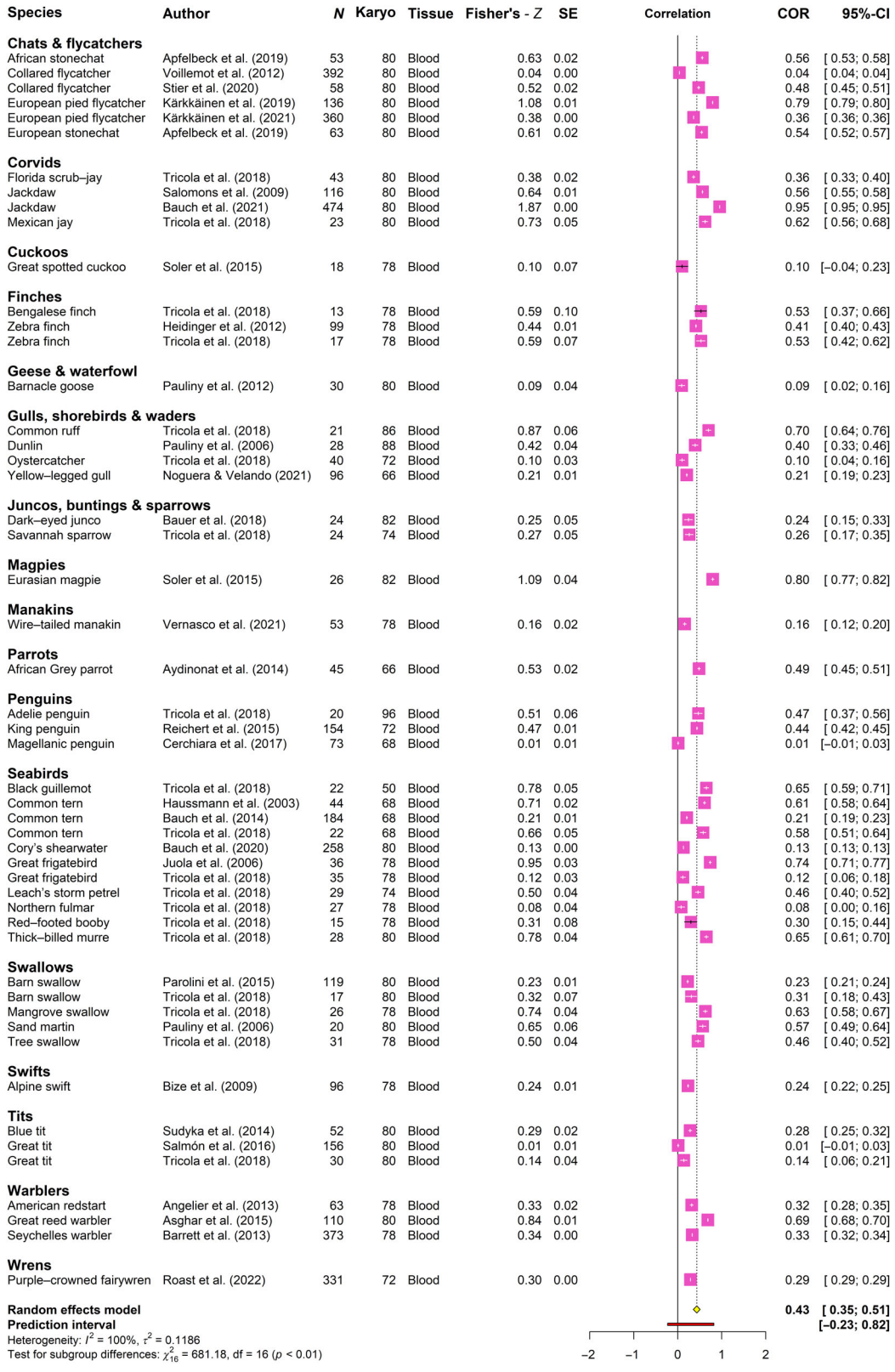


Fig. 11. Forest plot and results for the meta-analysis of studies using telomere length to estimate age in birds. Key study attributes such as sample size (*N*), karyotype (Karyo), and tissue type are indicated. The pooled correlation from the random effects model is indicated (yellow diamond) along with measures of between-study heterogeneity and the prediction interval is indicated with a red bar. CI, confidence interval; COR, correlation; *N*, sample size; SE, standard error.

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Table 4. Results from the comparison of meta-analysis results from studies on methylation and telomeres for age determination in animals. A fixed-effect method was used with model ($k = 2$) as a moderator. The overall z-value and P-value indicate that the observed effect sizes and variances between the methylation studies ($\mathcal{N} = 60$) and telomere studies ($\mathcal{N} = 60$) is statistically significant (***$P < 0.01$) with the methylation studies having a substantially higher estimation efficiency than telomeres

Meta-analysis	$\mathcal{N}$	Estimate	Standard error (σ_M)
Methylation	60	0.92	0.29
Telomeres	60	0.31	0.07
***Z*-value**		**21.58*****	
***P*-value**		**<0.01*****	

***$P < 0.01$.

several developmental or embryological studies (Suarez-Bregua *et al.*, 2021). More recently, however, many studies have shifted in focus towards the potential use of methylation to determine age in wildlife. This followed the great successes in human and murine mouse models and the accumulating evidence that methylation is conserved across most vertebrate (Jarman *et al.*, 2015), and even invertebrate (Macchi, Edsinger & Sadler, 2022), species. Thus far, methylation clocks for age determination have been developed or tested in more than 50 species across six classes in less than 10 years, including reptiles and bird species for which other biological clocks were not suitable.

Variable methods have been applied to several different tissue types to establish these models ranging from studies that used only a small, targeted selection of genes or subsets of CpGs to studies that used methylation arrays to assay thousands of CpGs at once to identify suitable targets empirically. Our review and meta-analysis illustrate that methylation is generally a highly accurate method able to predict age within weeks, months or years in many species and does not suffer significantly from variation due to changes in the lifespan of the organism or the tissue type. By contrast, one study did find a lack of age correlates in liver tissue (Sareisian, 2014) which may be related to the higher regenerative capacity of liver cells causing differential ageing in the liver compared to other tissue. Although our meta-analysis identified substantial study heterogeneity, this was mostly confined either to differences in sample size or to differences in the sensitivity of the specific study method used to assay methylation. Sample size requirements have been explored (Mayne *et al.*, 2021*b*) but remain fundamentally tied to effect size and the number of predictors used. In the case of methylation studies, calibrated with highly age-correlated CpGs, the effect sizes are generally large and sample sizes of 40 of above should be well suited for adequate statistical power, as measured by β-values (Fig. S3).

It should be noted that several authors did suggest that the accuracy of prediction is not always linear across the lifespan of organisms and that age estimates of older individuals are typically under- or overestimated. This does not, however, reduce the utility of this method to classify animals into functionally relevant age classes for conservation purposes (Wright *et al.*, 2018). Several other potential confounding factors have also been identified that may need to be taken into consideration in studies analysing methylation for age determination. For example, some evidence suggests that ageing of the epigenome in terms of methylation may be dependent on biological sex (Beal *et al.*, 2019; Meyer *et al.*, 2023), with females ageing slower, and transgenerational epigenetic inheritance in offspring may be linked to maternal age or brood size in some species (De Paoli-Iseppi *et al.*, 2017*a*). Methylation levels were also found to decrease during moulting and periods of fasting and return to normal later, highlighting the importance of taking life histories into account at the time of sampling (Poganik *et al.*, 2023). Other methods of correction such as accounting for leucocyte composition when blood cells are used have also been suggested (Adalsteinsson *et al.*, 2012), however, while variation in leucocyte ratios with age has been illustrated in some primates it is absent in others (Sato *et al.*, 2005), and the heredity or repeatability of such ratio changes is low across different generations for cross-sectional studies (Webb *et al.*, 2020).

Here we found that telomeres have been studied in relation to age in many of the same species as methylation, however, the total number of publications are nearly double the number of methylation studies with approximately half of the published studies focused on bird species. The abundance of studies on birds is likely attributable to the fact that, as jawless terrestrial vertebrates, neither tooth cementum annulation nor otoliths are suitable for age estimation in these species (Chaney *et al.*, 2003). Telomere length showed a moderate effect size in relation to age and the efficacy in relating telomere changes to age was affected by the average lifespan of some species, consistent with previous findings (Dantzer & Fletcher, 2015; Tricola *et al.*, 2018). Some studies have however found that telomere length can be heritable (Reichert *et al.*, 2015) and that both social (Lewin *et al.*, 2015; Cram *et al.*, 2018) and environmental factors can differentially affect telomere shortening rates within populations (Salmón *et al.*, 2016; Dorado-Correa *et al.*, 2018). While similar effects have been observed on methylation, telomere length tends to be permanently altered, likely due to a lack of telomerase activity in most tissue, while methylation levels recover after periods of stress (Poganik *et al.*, 2023) due to DNMT enzymes being persistently expressed. As a result, methylation outperformed telomere length in its ability to change predictably with age.

Both methylation and telomere length show distinct patterns of inheritance that vary across taxa. For example, methylation is known to follow parental imprinting in subsets of genes where different patterns of methylation are inherited from different parents (Andergassen *et al.*, 2021) while global changes can result in offspring that resemble the maternal methylome (Romano *et al.*, 2017; Saino *et al.*, 2019). Telomere length, however, showed strong paternal inheritance in mammals (Eisenberg *et al.*, 2017) but maternal inheritance in birds (Horn *et al.*, 2011; Reichert *et al.*, 2015), possibly

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indicating that the parental inheritance of telomere length is driven by the heterogamous sex as most male mammals are XY while most female birds are ZW. Interestingly, paternal inheritance has also been illustrated in sand lizard species that follow a similar ZZ/ZW pattern of sex chromosome heterogamy (Olsson *et al.*, 2011). More studies are needed to elucidate if similar patterns are absent from vertebrate species that either lack sex chromosomes or are completely homogamous, including most amphibians and mammals with unusual sex-determination systems (Saunders & Veyrunes, 2021).

Using attributes of cellular and molecular ageing as biomarkers for age across many taxa is fairly complicated. It requires not only a high level of consistency but also substantial heritability in the trait to ensure comparability in the measurements from a cross-sectional study accessing levels in age groups for different generations: as opposed to longitudinal studies that track changes in specific individuals across their lifetime. Furthermore, it needs to be correlated with all relevant biological and environmental factors that contribute to variability. Biological factors include biological sex, diet, stress, differences in physiology between ectotherms and endotherms, and differences in growth patterns such as the arrested growth in mammals and birds compared to continuous growth in most fish, reptiles, and amphibians. Environmental factors may include the effects of temperature, latitude, environmental stressors, and possible exposure to toxins or contaminants. When controlling for such complex variables, both methylation and telomere length are promising targets for the development of methods to determine age in animals.

VI. CONCLUSIONS

(1) The first biological clocks used to determine age in animals were tooth cementum/dentin annulation and otoliths but are mostly applicable to toothed vertebrates and fish and remain superior tools for age estimation in the *post-mortem* interval.
(2) Methylation is a conserved molecular ageing attribute able to predict age across most classes, orders and infraorders using as little as two CpGs in calibrated clocks.
(3) Telomere shortening is another biological clock with sufficient age prediction capabilities to measure age in animals but is less conserved.
(4) Methylation may be superior to telomere length in terms of accuracy and cross-taxa portability; however, costs may be higher depending on the method.

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VIII. DATA AVAILABILITY STATEMENT

Data and code used in this study are available for download from the Zenodo repository at the following link: https://doi.org/10.5281/zenodo.7893443. The custom PYTHON script, ABCal *version* 1.0.1., used to quantitate possible bias by virtue of authorship contributions is freely available on GitHub (https://github.com/LSLeClercq/ABCal).

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X. SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Summary of all species within the class Mammalia for which tooth cementum annulation (TCA) has been used as biological clock method for age determination.

Fig. S1. PRISMA flow diagram for search, screening, and inclusion of methylation studies for age determination in animals.

Fig. S2. PRISMA flow diagram for search, screening, and inclusion of telomere studies for age determination in animals.

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Fig. S3. Relationship between power and sample size for different effect sizes in a multiple linear regression with a minimum of three predictors at a significance of 95% ($\alpha = 0.05$).
Fig. S4. Forest plot and results for the meta-analysis of studies using methylation to estimate animal age with subgrouping based on the defined groups.
Fig. S5. Forest plot and results for the meta-analysis of studies using methylation to estimate animal age with subgrouping based on tissue type.
Fig. S6. Forest plot and results for the meta-analysis of studies using methylation to estimate animal age with subgrouping based on study method.
Fig. S7. Forest plot and results for the meta-analysis of studies using telomere length to estimate animal age with subgrouping based on defined groups.
Fig. S8. Forest plot and results for the meta-analysis of studies using telomere length to estimate animal age with subgrouping based on tissue type.
Fig. S9. Forest plot and results for the meta-analysis of studies using telomere length to estimate animal age with subgrouping based on study method.
Fig. S10. Forest plot indicating and comparing the results obtained from independent meta-analyses of studies using methylation *versus* telomere length to model the relationship with ageing in animals.

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