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INVITED REVIEW

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DNA methylation markers of age(ing) in non-model animals

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Abstract

Inferring the chronological and biological age of individuals is fundamental to population ecology and our understanding of ageing itself, its evolution, and the biological processes that affect or even cause ageing. Epigenetic clocks based on DNA methylation (DNAm) at specific CpG sites show a strong correlation with chronological age in humans, and discrepancies between inferred and actual chronological age predict morbidity and mortality. Recently, a growing number of epigenetic clocks have been developed in non-model animals and we here review these studies. We also conduct a meta-analysis to assess the effects of different aspects of experimental protocol on the performance of epigenetic clocks for non-model animals. Two measures of performance are usually reported, the R^2 of the association between the predicted and chronological age, and the mean/median absolute deviation (MAD) of estimated age from chronological age, and we argue that only the MAD reflects accuracy. R^2 for epigenetic clocks based on the HorvathMammalMethylChip4 was higher and the MAD scaled to age range lower, compared with other DNAm quantification approaches. Scaled MAD tended to be lower among individuals in captive populations, and decreased with an increasing number of CpG sites. We conclude that epigenetic clocks can predict chronological age with relatively high accuracy, suggesting great potential in ecological epigenetics. We discuss general aspects of epigenetic clocks in the hope of stimulating further DNAm-based research on ageing, and perhaps more importantly, other key traits.

KEYWORDS
ageing, DNA methylation, epigenetic clock, non-model

1 | INTRODUCTION

Age is a central concept in ecology and evolution. Demographic information is essential to assess population viability, and its main fitness components, reproduction, and survival are usually age-dependent (Heydenrych et al., 2021). However, determining the chronological age in wild individuals is fraught with challenges, relying on methods with low accuracy (e.g., morphological characteristics, such as otolith measurements in fish, Boehlert, 1985), limited resolution (e.g., estimating elephant age from footprint dimensions, Western et al., 1983), or require lethal sampling (e.g., eye lens protein content; in kangaroos, Augusteyn et al., 2003). The exact age is only known in a few populations where individuals have been tracked since birth. Consequently, there is a general need for

reliable, efficient, non-lethal techniques to estimate the chronological age of individuals in wild populations.

In an evolutionary context, biological ageing is the decline in Darwinian fitness with chronological age, resulting in an irreversible physical deterioration of biological functions. Biological age refers to an individual's somatic integrity, and as such is a relative measure of the impact of senescence on an individual. Here, we loosely define biological age for an individual as the age at which the life expectancy of the focal individual equals the life expectancy of the average individual (Figure 1). Interestingly, individuals of the same chronological age often display large variations in their biological age and life expectancy (Figure 1a,b, Lowsky et al., 2014). Consequently, biological age is a better predictor of variation in mortality and morbidity than chronological age (Figure 1c). As a result, inferring biological age is conceptually related to the general challenge of measuring body condition (e.g., Frauendorf et al., 2021). Although ageing has been intensively studied, the root cause(s) of ageing and individual variation in the rate of ageing remain an enigma. The first step towards progress in this area is the reliable quantification of biological age from ageing biomarkers, thus potentially facilitating novel insights into the processes contributing to ageing (Baker & Sprott, 1988).

A wide variety of phenotypic and molecular biomarkers have been assessed to develop indicators of biological age (Xia et al., 2017). Epigenetic modifications of DNA can regulate gene expression, either by modulating chromatin structure, or through interactions during DNA transcription (Gibney & Nolan, 2010). A substantial effort has been directed towards studying epigenetic modifications in the context of ageing in recent years, and a rapidly growing body of studies has uncovered associations between age and the state of the epigenome (Bellizzi et al., 2019). The primary epigenetic process studied is DNA methylation (DNAm), which refers to the addition of a methyl group to a cytosine followed by a guanine separated by the phosphate backbone, usually referred to as a CpG site (Moore et al., 2013). In eukaryotes, DNAm is found only at cytosine residues, and has been associated with a repressed chromatin state and promoter silencing (Bird & Wolffe, 1999). Recent work on epigenetics in relation to ageing capitalized on earlier studies demonstrating that genome-wide DNAm levels change with chronological age, which has led to the suggestion that DNAm may contribute to overall senescence (Wilson et al., 1987).

Predictable longitudinal changes in DNAm in specific genomic regions have served as the basis for epigenetic 'clocks', that is, DNAm-based predictors of chronological age. Epigenetic clocks are inferred from regressions of the degree of DNAm at a set of CpG sites against the chronological age of individuals employing supervised machine learning methods, such as penalized regression models (Tibshirani, 1996; Zou & Hastie, 2005). These models aim to select the CpG sites whose DNAm levels best predict chronological age (see Rutledge et al., 2022 Box 1). Presently, epigenetic clocks are viewed as the most accurate biomarker-based predictors of chronological age and predicting chronological age in humans with an absolute mean deviation of only a few years (Bell et al., 2019; Jylhävä et al., 2017).

Epigenetic clocks are useful for estimating the chronological age of individuals when their age is unknown, for example, in forensics (Park et al., 2016), conservation (Barratclough et al., 2021) and to establish age-at-death of archaeological remains (Liu et al., 2023). In ageing research, where chronological age usually is known, estimates of epigenetic age (Figure 1a) serve as a predictor of an individual's health, that is, biological age, due to the observation that epigenetic age increases relative to chronological age in humans suffering from a variety of diseases, including Alzheimer's (Horvath & Ritz, 2015) and Parkinson's disease (Levine et al., 2018). Epigenetic age has also been shown to predict all-cause mortality (Christiansen et al., 2016; Marioni et al., 2015) and has proven a better predictor of an individuals' health than chronological age, thereby functioning as a true biological age estimator (as in Figure 1c).

Epigenetic clocks may facilitate insights into the process of ageing due to the already established functional aspects of DNAm, such as association with a repressed chromatin state and modulating of gene expression (Tate & Bird, 1993). Epigenetic

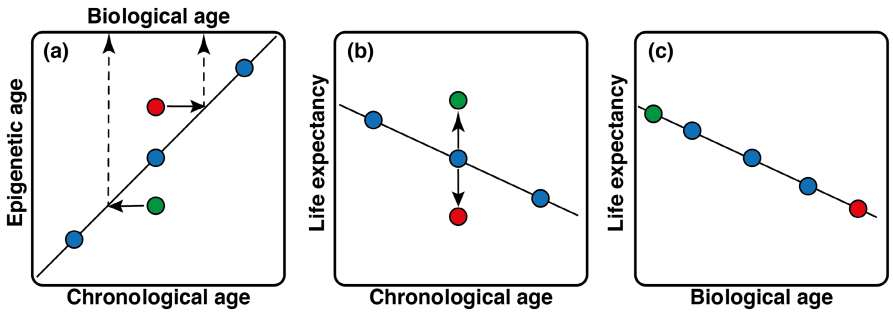

FIGURE 1 Schematic representation of the relation between chronological age, biological age, epigenetic age (= chronological age predicted by the epigenetic clock), and life expectancy. Coloured circles represent individuals. (a) Epigenetic age in relation to chronological age (bottom axis) in the population is represented by the solid line, and vertical arrows indicate the biological age (top axis) of two individuals whose epigenetic age deviates from their chronological age (red individual with positive age acceleration and green individual with negative age acceleration). (b) Life expectancy plotted for individuals as in panel A decreases with increasing chronological age, but individuals with a high biological age for their chronological age have a shorter life expectancy and vice versa. (c) Biological age fully predicts life expectancy leaving no additional variation to be explained by chronological age.

ageing variation may be associated with the ageing phenotype, and provide insights into the underlying mechanisms responsible for differences in the rate of ageing. For example, caloric restriction slows actuarial senescence in rodents (Simons et al., 2013), but the underlying mechanisms remain unknown despite a substantial research effort. Petkovich et al. (2017) recently showed that caloric restriction lowered the epigenetic age compared with mice fed ad libitum, potentially opening a new window into our understanding of the mechanism driving the decrease in ageing from caloric restriction. In this manner, epigenetic clocks can aid in elucidating how the environment and other factors affect life-history traits, fitness, and lifespan.

While most of what we know about epigenetics is based on humans and mice, the field is attracting increasing attention in evolutionary ecology, as evidenced by recent reviews focusing on practical aspects of the study of epigenetics (Husby, 2022; Laine et al., 2022), and pitfalls and promises of avian epigenetic studies in natural populations (Sepers et al., 2019). We here aim to provide an overview of studies available that explored age-related changes in DNAm in non-model animals and a critical review of epigenetic clocks inferred for non-model animals. The review is supported by a meta-analysis in which we identify factors affecting the performance of epigenetic clocks. Specifically, we assessed the inferred epigenetic clocks' performances in relation to the overall experimental set-up using two frequently reported metrics: the R^2 of the association between DNAm-based (predicted) age and chronological age and the absolute deviation between predicted and chronological age (Mean/Median Absolute Deviation-MAD). Lastly, we highlight the future potential and challenges we see in the study of epigenetics in relation to ageing and other aspects of ecology and evolution in natural systems as we are convinced that there is tremendous potential in the use of DNAm within a much wider framework.

2 | METHODS

2.1 | Literature search

The meta-analysis was conducted to determine which experimental and methodological aspects affected the performance of inferred epigenetic clocks. We performed a systematic literature search for publications up to July 2022. Studies relevant to the meta-analysis were identified from all databases in Web of Science™ and Scopus™ the following search string (TS=(Epigenetic clock*) AND TS=(animal* OR fish OR mammal* OR bird* OR avian OR reptile* OR amphibian* OR primate* OR vertebrate*) NOT TS=(cancer) NOT TS=(circadian) NOT TS=(plant*)). Review articles were excluded. The search yielded 458 studies. We also identified 17 relevant studies from citations and eight from the pre-print repository bioRxiv.com. Among the 483 studies identified, 56 were duplicates. The abstracts of the remaining 427 studies were screened according to the following criteria.

1. The targeted species were non-model organisms. In practice, this resulted in the exclusion of studies of humans and mice.
2. The study's objective was to either infer epigenetic clocks or characterize specific temporal changes in DNAm.

After screening abstracts, 72 studies were retained and subjected to additional full-text screening, among which data for the meta-analysis were retrieved from 51 studies. From those 51, 12 studies were removed from the meta-analysis for R^2 as they did not infer an epigenetic clock, four did not report their sample size and one did not report the R^2 (Figure 2). Moreover, 22 studies were excluded from the meta-analysis for MAD, 12 because they did not infer an epigenetic clock and 10 because they did not report MAD or sample size (Figure 2).

Our literature search resulted in the inclusion of data from 51 studies that characterized age-related variation in DNAm in non-model animals, published between 2014 and July 2022. One clear trend was the rapid increase in the number of studies published each year, especially after 2018 (Figure 3a). The majority of studies targeted mammals (Figure 3b), likely due to the development of the HorvathMammalMethylChip40 (Arneson et al., 2022), which includes ~36000 CpG sites conserved among mammals. Although the specific objective varied among individual studies, most aimed to infer an epigenetic clock (Figure 3c).

From the 51 relevant studies, a total of 40 studies inferred 43 different epigenetic clocks. However, because of missing data, only 34 studies were included in the meta-analysis, which yielded 38 effect sizes for the meta-analysis of R^2 and 33 effect sizes for the one for MAD (Figure 2, Table S3). The remaining studies either did not infer epigenetic clocks but instead aimed to identify regions, genes or CpG sites that differentially methylate with age or did not include the necessary information for inclusion in the meta-analysis (sample size, R^2 or MAD; Table S3). The studies that did not infer epigenetic clocks were nevertheless retained to offer a full overview of the research of DNAm in relation to age in non-model animals.

2.2 | Data extraction

We extracted the R^2 value of 42 epigenetic clocks, 38 of which were included in the meta-analysis (Figure 2). Mean or median absolute error (MAE) between the DNAm-based (predicted) age and chronological age has been used in combination with the R^2 to assess clock performance. Moving forward, we will refer to these error measures as Mean/Median Absolute Deviation (MAD) since we believe that the term 'error' suggests measurement error, whereas these deviations may in themselves be robust estimates providing valuable information. Mean squared error and standard deviation values were not taken into consideration as there is no straightforward way to transform those values to MAD.

Additionally, for each study, we recorded the publication year, targeted species, the number of species and sample size of individuals from each species, age range of the individuals sampled,

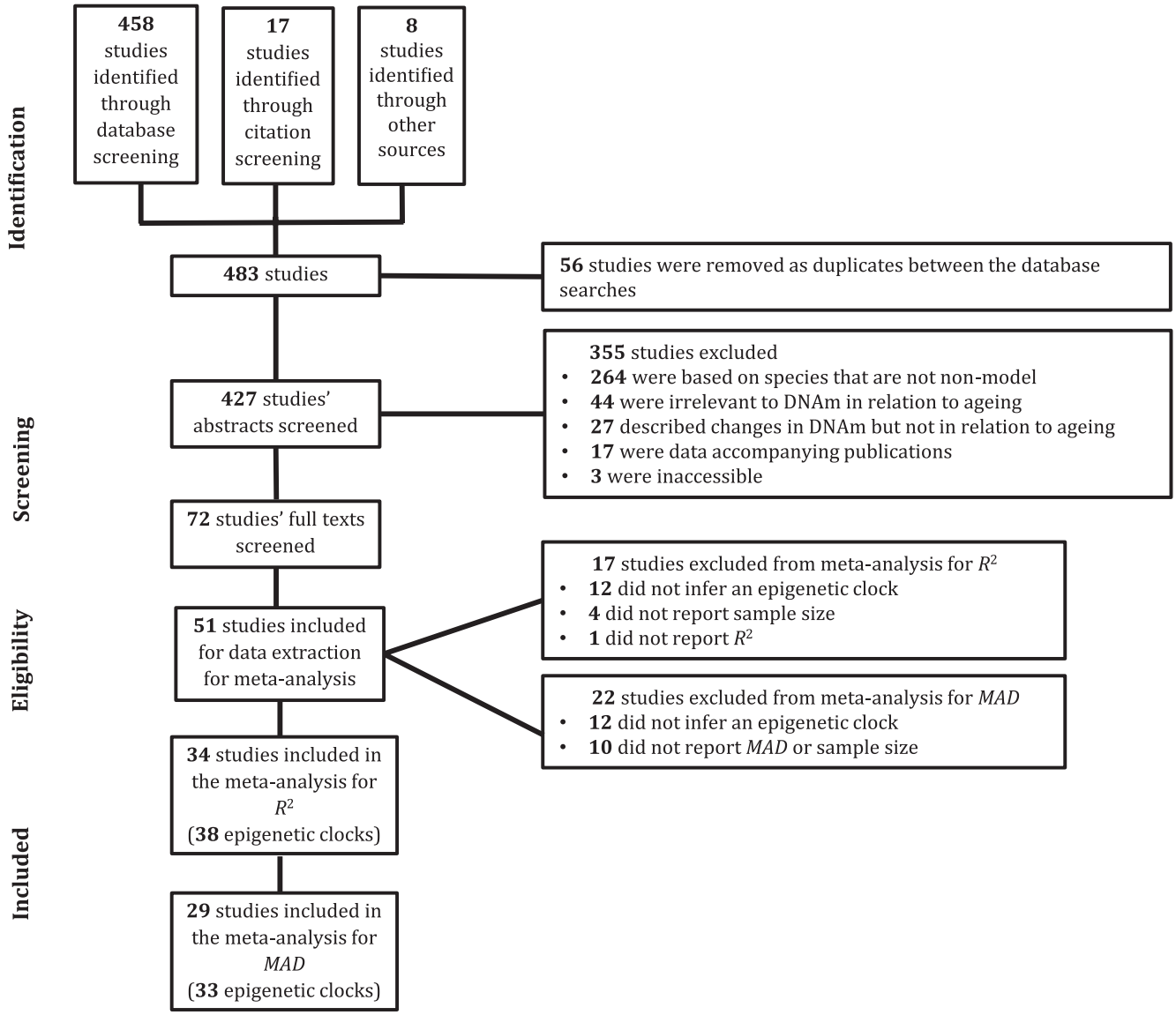

FIGURE 2 Flow chart detailing the literature search and screening process following guidelines for reporting items for systematic reviews and meta-analyses (PRISMA) (Moher et al., 2009). To minimize error, we used a double-screening process during literature search, screening and data extraction.

taxonomic information on the species at the class level, whether the sampled individuals were from a wild or captive population, the method employed to quantify DNAm, source tissue and the specific goal of the study (Table S3). For studies that were first identified in pre-print form, the initial date the study became available was recorded but for convenience the peer-reviewed published version is reported here. For studies that inferred an epigenetic clock, we also recorded the number of CpG sites employed to define the clock. To minimize error, we used a double-screening process during literature search, screening, and data extraction.

Several aspects potentially affect the accuracy of an epigenetic clock, such as the genetic and environmental context as well as different experimental approaches and bioinformatic pipelines. The specific aspects assessed in the present analysis were (1) the method used to quantify DNAm, (2) the type and number of tissue(s) sampled, (3) whether the animals sampled were wild or captive, (4) the number of individuals, and (5) CpG sites used to infer the specific epigenetic clock, as well as (6) the age range of the animals sampled.

2.3 | Overview of selected studies

The goal of the meta-analysis was to determine which aspects of the studies affected the accuracy of inferred epigenetic clocks. We first describe the selected papers, followed by a formal analysis of all potential modulators of the R^2 values of the relation between epigenetic and chronological age as well as the MAD of the epigenetic clocks.

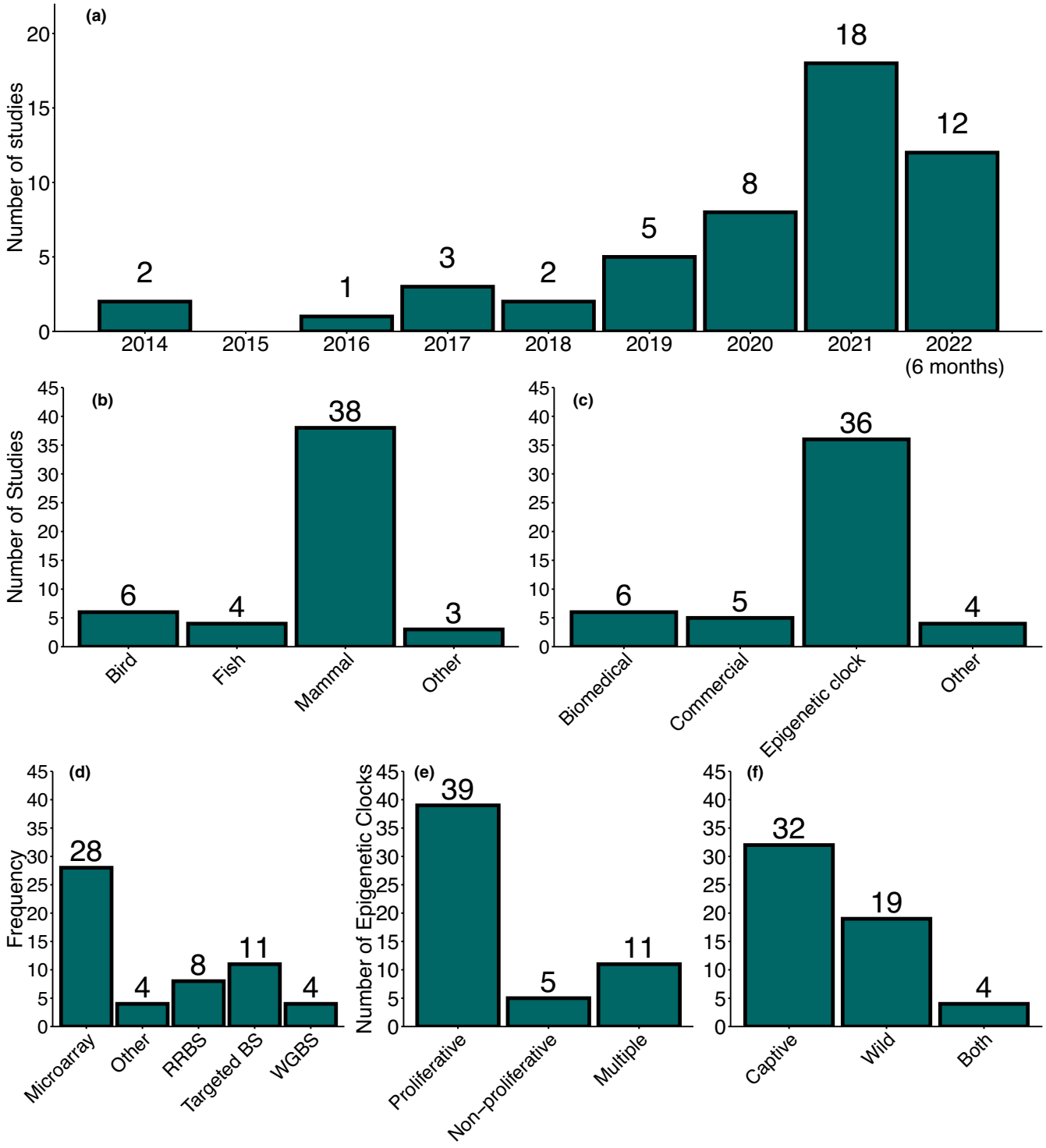

FIGURE 3 Description of all studies exploring DNAm in relation to ageing in non-model animals that were identified ($N = 51$). (a) Number of studies describing DNAm changes in relation to ageing per year included in this review. The year of publication is the year the studies first appeared online, either as pre-print or as an accepted publication. (b) Number of studies studying DNAm in relation to ageing in non-model animals in different taxonomic classes. (c) Applications of different studies. The majority of the studies with biomedical and commercial purposes still inferred epigenetic clocks but not for the sake of ageing research. (d) Frequency of studies/epigenetic clocks ($N = 55$) developed using different methods to quantify age-dependent methylation. 'Other' includes Multiplex BS, DREAM and the MethylFlash™ Methylated DNA Quantification Kit (Epigentek) (e) Number of epigenetic clocks ($N = 55$) made using each tissue type and (f) population type.

2.4 | Methods of DNAm quantification

Seven different methods of DNAm quantification were employed: Microarray, Targeted Bisulfite Sequencing (Targeted BS), Reduced Representation Bisulfite Sequencing (RRBS), Whole Genome Bisulfite Sequencing (WGBS), Multiplex Bisulfite Sequencing, Digital Restriction Enzyme Analysis of Methylation (DREAM) and the MethylFlash™ Methylated DNA Quantification Kit (Epigentek Group Inc.). The

last two methods were each used in a single study and were categorized together under 'other methods' (Figure 3d). The most frequent method used for DNAm quantification was Microarray, followed by Targeted BS, RRBS, WGBS and 'other'. All but one (Illumina Infinium Methylation 450K array; Guevara et al., 2020) studies under 'Microarray' used the HorvathMammalMethylChip40. For an overview of contemporary methods to measure DNAm as well as the advantages and disadvantages of each method, see Husby (2022, Table 1) and Laine (2022, Box 1).

2.5 | Tissues

Reported epigenetic clocks are based on either one or multiple species, as well as on one or multiple tissues. Data from epigenetic clocks that were trained on one species and tested on another species (mostly humans) were not included in the analysis as they were not strictly referring to non-model animals. Of the studies investigating changes at the CpG level in relation to age (including the studies inferring an epigenetic clock), 39 were based on proliferative tissues, 25 of which used blood, 11 were based on multiple tissues, and five on non-proliferative tissues (Jin et al. 2014 in muscle tissue of a teleost, Anastasiadi & Piferrer, 2019 in the muscle of European seabass, Jin et al., 2014 in Chinese Jinhua pig liver, Sun et al. 2021 in the hypothalamus region in white-throated sparrow brains, Horvath et al., 2022 in kidney in naked mole rats and Lowe et al., 2020 in the liver of naked mole rats; Figure 3e). As proliferative tissues, we consider tissues containing cells with the ability to regenerate and self-renew, such as skin tissue. As non-proliferative, we consider tissues that are composed of cells in a non-dividing state, such as the liver and kidney (Krafts, 2010).

TABLE 1 Model output, including estimates (posterior mean), estimation error, and probability of direction (p_d) for each variable for the R^2 of epigenetic clocks.

	Estimate	95% CI	p_d
Fixed effects			
Intercept	2.33 ± 0.60	(1.18, 3.52)	0.998
Targeted BS	−0.52 ± 0.26	(−1.02, −0.02)	0.979
RRBS	−0.61 ± 0.24	(−1.06, −0.14)	0.993
Other method	−0.77 ± 0.29	(−1.33, −0.19)	0.994
Wild	−0.14 ± 0.18	(−0.50, 0.22)	0.789
Wild & Captive	0.26 ± 0.30	(−0.35, 0.86)	0.814
Non-proliferative tissue	0.56 ± 035	(−0.14, 1.25)	0.942
Multiple tissues	0.20 ± 0.24	(−0.27, 0.67)	0.802
Age range	0.06 ± 0.09	(−0.12, 0.25)	0.769
Individuals sampled	−0.06 ± 0.12	(−0.30, 0.18)	0.706
Random effects			
Phylogeny	0.17 ± 0.14	(0.01, 0.49)	
Study	0.40 ± 0.07	(0.28, 0.53)	

Note: 'Intercept' includes Microarray, captive, and proliferative tissue. Here, we consider p_d-values between 0.95 and 0.97 as providing 'weak evidence', a p_d-value between 0.97 and 0.99 as 'moderately strong evidence', and a value greater than 0.99 as 'strong evidence'. Values of below 0.95 were considered as no discernible evidence/effects.

2.6 | Captive versus wild populations

In total, 32 and 19 studies were based on samples collected from captive and wild populations respectively. Four studies included samples from both captive and wild populations (Figure 3f).

2.7 | Statistical analyses

All analyses were conducted with R v4.1.2 (R Core Team, 2021) in RStudio v2022.02.2.

Pearson correlation coefficients were computed to assess the linear relationship between R^2 and MAD and the number of individuals sampled, the number of CpG sites employed to infer the epigenetic clocks and the age range of the individuals in each sudy as well as the relationship between the number of CpG sites employed to infer the epigenetic clocks and the number of inividuals sampled.

The goal of the meta-analysis was to test for the effects of method, tissue type, population type, age range, number of individuals sampled, and clock CpGs on the R^2 and method, tissue type, population type, number of individuals sampled, and clock CpGs on the MAD of the epigenetic clocks. To achieve this goal, we fitted fully Bayesian models with the *brms* package (v2.17.0; Bürkner, 2017, 2018), interfaced with the Markov Chain Monte Carlo (MCMC) sampler *RStan* (Stan Development Team, 2023).

From the included studies, we extracted R^2 values, to the positive roots (R) of which we applied Fisher's z-transformation

$$Z = \frac{1}{2}\log\left(\frac{1+R}{1-R}\right).$$

We modelled the z-value of each study as a draw from a normal density:

$$Z_i \sim \text{normal}\left(\mu_i, \sigma_i^2\right),$$

where the mean was given by a study-specific linear predictor

$$\mu_i = \alpha_i + X_i\beta$$

and a study-specific standard deviation ($sd = \sigma$) given by the observed standard error of the study with sample size N_i (inividuals sampled), that is,

$$\sigma_i = 1/\sqrt{N_i - 3}.$$

As is common in meta-analytic studies (Gelman et al., 2021), the study-specific intercepts α_i were modelled as normal 'random effects', that is,

$$\alpha_i \sim \text{normal}\left(\alpha, \tau^2\right)$$

where τ is the group-level variation.

The regression parameters β were treated as 'fixed'.

MAD estimates were nondimensionalized by dividing by age range A and subsequently normalized by log-transformation as follows:

$$Y_i \stackrel{\text{def}}{=} \log\left(MAD_i / A_i\right)$$

while an approximate (large sample) standard error SE was calculated using results from Pham-Gia and Hung (2001) and the first-order approximation $\text{var}(\log(x)) \approx \sigma_x^2 / \mu_x^2$, as follows:

$$\text{SE}\left(Y_i\right) \approx \frac{1}{4}\sqrt{\frac{A_i}{MAD_i}\frac{(\pi-2)}{\pi}\frac{(N_i-1)}{N_i^2}}$$

Just like the Z_i above, we treated the Y_i as draws from normal densities with a mean given by a study-specific linear predictor with random intercept, and a standard deviation given by $\text{SE}(Y_i)$.

For the population intercepts α and regression parameters β, we used normal priors with means of zero and $sd = 10$ for α and $sd = 1.0$ for β. For the sd τ of the random intercepts, we used *brms*' default prior, a half-t density with three degrees of freedom.

For each model, we ran four chains each comprised of 1000 warm-up iterations, followed by 2500 sampling iterations, thus yielding 10,000 posterior samples per model. Mixing and convergence of MCMC chains were monitored from the trace plots and $\widehat{R}$ values, which were all close to 1.00. Model fitting was also evaluated by inspecting posterior predictive checks, using the *pp_check* function in the package *brms* 2.18.0 (Figure S3). We chose the model that best explained the coefficient of determination or MAD of epigenetic clocks by comparing leave-one-out information criteria to determine model performance. Marginal means of categorical predictor variable levels were estimated using the *emmeans* R package 1.8.5 (Lenth et al., 2023). Estimated marginal means predict what the marginal means of the response variables per variable level would be if the data set were balanced.

We estimated the probability of direction (p_d), that is, the posterior probability that a parameter is positive or negative, whichever is the most probable, to test hypotheses regarding model parameters, that is, in pairwise comparisons between methods. Following Makowski et al., 2019, we consider p_d-values between 0.95 and 0.97 as providing 'weak evidence', a p_d-value between 0.97 and 0.99 as 'moderately strong evidence', and a value greater than 0.99 as 'strong evidence'. Values of below 0.95 were considered as no discernible evidence/effects.

As our data set included both studies that used *median* and studies that used *mean* absolute deviation, the type of error measure was entered in the model but was found to explain negligible variation (posterior mean estimate = 0.05). The two types of deviation estimates were treated as equal in the meta-analysis and are both referred to as *MAD*.

The number of CpG sites used to infer the epigenetic clocks could not be added as a predictor to the initial models as multiple studies did not report this information, significantly reducing the number of available effect sizes. We therefore constructed reduced models for R^2 and MAD with only method and number of CpG sites as covariates. The method was included as the only other covariate because it was a significant predictor in the full models and to avoid over-parameterizing the models. WGBS was removed from all models as three of the four studies that utilized this method did not infer an epigenetic clock and the fourth study did not report the R^2 (Raddatz et al., 2021).

Lastly, to estimate the degree of phylogenetic heterogeneity among the species in our data set, we estimated a phylogenetic tree from the open Tree of Life using the R package *rotl* 3.0.14 (Figure S2, Michonneau et al., 2016). Using R package *ape* 5.7-1 (Paradis & Schliep, 2019) we estimated a phylogenetic covariance matrix to include in our models, assuming Brownian motion evolution. For clocks based on multiple species, we randomly selected a single representative species to include in the phylogenetic estimation. Models with and without the phylogenetic covariance matrix were then compared using the function *loo_compare*, which is part of the R package *loo* 2.5.1 (Vehtari et al., 2017).

3 | RESULTS

3.1 | Performance of epigenetic clocks

The R^2 of the relation between epigenetic age and chronological age ranged from 0.56 to 0.98 (Figure 4a) while the MAD as a proportion of age range varied from 0.019 to 0.311 (Figure 4b). Note that high performance of an epigenetic clock is characterized by a high R^2 but a low MAD, and, as expected, there was a negative correlation between R^2 and scaled MAD (Figure S1; $r_s = -0.50$; $p < 0.01$).

3.2 | Methods of DNAm quantification

The R^2 of the inferred epigenetic clocks was found to covary with multiple factors when assessed in isolation (Figure S4). However, the model of R^2 identified the method of DNAm quantification as the only factor with a significant effect on R^2, with the Microarray method yielding significantly higher R^2 values when compared with the other methods of DNAm quantification (Figure 8, Table 1).

MAD was also found to change with several variables (Figure S5) and the method was found to have a significant effect. Both Microarray and RRBS performed significantly better than Targeted BS (Figure 9, Table 2).

3.3 | Tissues

Non-proliferative tissues produced epigenetic clocks with higher R^2 values, and this association approached statistical significance (Figure 8, Table 1). However, tissue type was not found to significantly affect the MAD of the epigenetic clocks included in our meta-analysis (Figure 9, Table 2).

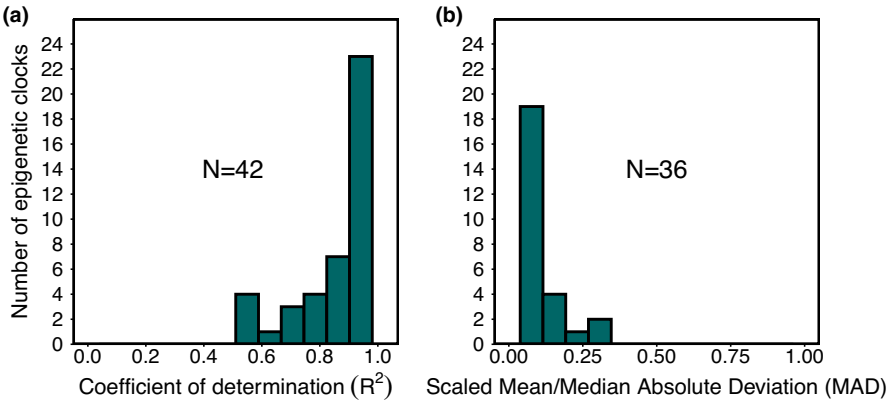

FIGURE 4 (a) Frequency distribution of R^2 values between chronological and epigenetic age of included epigenetic clocks ($N = 42$). (b) Frequency distribution of MAD scaled to the age range of the individuals in the study ($N = 36$).

TABLE 2 Model output, including estimates (posterior mean), estimation error, and probability of direction (p_d) for each variable for the *MAD* of epigenetic clocks.

	Estimate	95% CI	p_d
Fixed effects			
Intercept	−2.22 ± 0.65	(−3.51, −0.95)	0.999
Targeted BS	1.01 ± 0.39	(0.22, 1.77)	0.992
RRBS	−0.16 ± 0.36	(−0.88, 0.53)	0.665
Other method	0.48 ± 0.40	(−0.32, 1.26)	0.885
Wild	0.50 ± 0.29	(−0.07, 1.06)	0.957
Wild & Captive	0.31 ± 0.37	(−0.42, 1.04)	0.803
Non-proliferative tissue	−0.04 ± 0.41	(−0.83, 0.77)	0.541
Multiple tissues	0.41 ± 0.32	(−0.25, 1.03)	0.897
Individuals sampled	−0.18 ± 0.13	(−0.43, 0.08)	0.924
Random effects			
Phylogeny	0.33 ± 0.26	(0.01, 0.96)	
Study	0.55 ± 0.10	(0.38, 0.76)	

Note: 'Intercept' includes Microarray, captive, and proliferative tissue. Here, we consider p_d-values between 0.95 and 0.97 as providing 'weak evidence', a p_d-value between 0.97 and 0.99 as 'moderately strong evidence', and a value greater than 0.99 as 'strong evidence'. Values of below 0.95 were considered as no discernible evidence/effects.

3.4 | Captive versus wild populations

Epigenetic clocks based on samples from captive or wild populations were not found to differ in R^2 (Figure 8, Table 1). However, there was weak evidence pointing to epigenetic clocks made by sampling wild populations having lower MAD when compared with the ones made by sampling captive ones (Figure 9, Table 2).

3.5 | Age range

The age range of the individuals sampled to infer the epigenetic clocks was not found to significantly affect the R^2 (Figure S4d, Figure 7c, Table 1). (Note that because MAD was scaled to the age range testing its association with age range is not informative.)

3.6 | Number of CpG sites

Epigenetic clocks were based on 2–573 CpG sites, but most clocks were based on fewer than 100 CpG sites (Figure 5; median = 46; IQR = 74.5). A significant positive association was observed between the number of individuals sampled and the number of CpG sites used to infer the epigenetic clocks (Figure 6; $r = 0.52$; $p < 0.01$, $N = 28$). No significant association was detected between the R^2 and the number of CpG sites employed to infer the epigenetic clock (Figure 7b; Table S1; $p_d = 0.841$). However, there was moderately strong evidence for a negative association between the MAD and the number of CpG sites used to infer the clocks (Figure 7e; Table S2; $p_d = 0.984$). We here note that this analysis is necessarily restricted to *between* study variation. In comparison, it is likely that *within* studies, the R^2 estimates would decrease, and MAD estimates would increase when CpG sites were omitted from the epigenetic clock.

3.7 | Sample size

The association between the R^2 of the epigenetic clocks and the number of individuals sampled over all methods pooled together was not significant (Figure 7a; Table 1; $p_d = 0.706$). Although the MAD decreased with increasing number of individuals included, the relationship was not found to be significant (Figure 7d; Table 2; $p_d = 0.924$). Sample size per method was too small to estimate the method effects, but R^2 was consistently high for clocks made using Microarray technology (Figure 7a) and MAD consistently low (Figure 7d), regardless of the number of individuals sampled.

3.8 | Phylogeny

The estimated difference in expected log pointwise predictive density between the models with and the model without the phylogenetic covariance matrix for R^2 was −1.0 (SE = 0.9, BF = 0.472) and for MAD was −1.3 (SE = 0.9, BF = 0.606), providing weak evidence in favour of the models with the phylogenetic covariance matrix included.

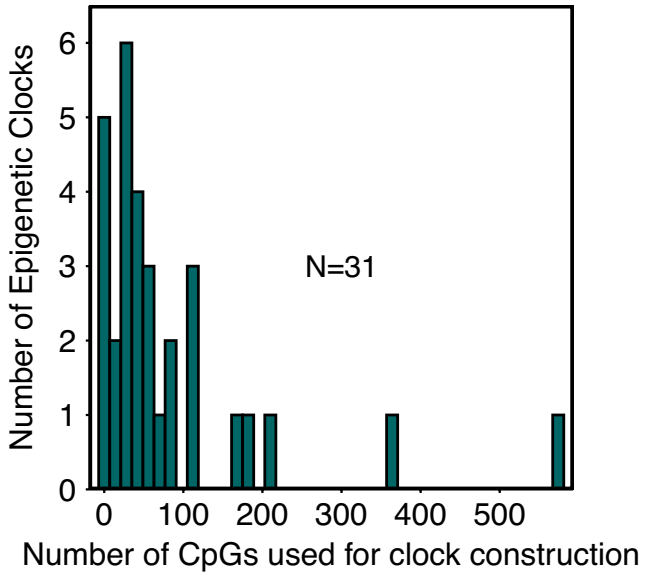

FIGURE 5 Frequency distribution of the number of CpG sites used to generate the epigenetic clocks ($N = 31$). Sample size differs from that of R^2 as some studies failed to report the number of CpG sites used to infer the epigenetic clocks, see Table S3 for details.

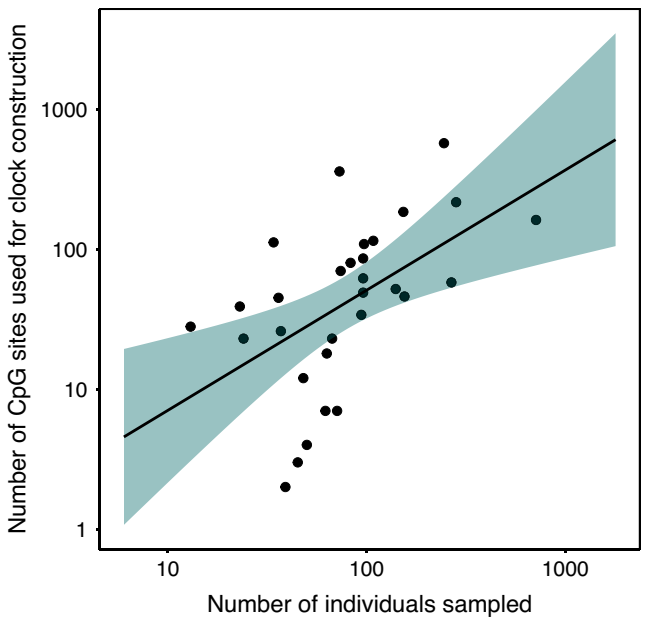

FIGURE 6 Number of CpG sites used for clock inference and its relationship to the number of individuals sampled (both in $\log_{10}$ scale). Solid line represents the best-fit regression through the data and the colored area is 95% CI.

4 | DISCUSSION AND REVIEW

The rapidly increasing accessibility of methods to obtain reliable epigenetic data has led to a surge in DNAm-based studies aimed at measuring epigenetic ageing in non-model animals. These studies have clearly shown the high potential in DNAm-based information in conservation, veterinary, evolutionary, and commercial applications. Some studies have even suggested anti-ageing interventions using epigenetic reprogramming (Lu et al., 2020; Ocampo et al., 2016; Yang et al., 2023). The aim of this review was to assess which parameters affected the accuracy of epigenetic clocks in non-model animals and to discuss more generally the potential and challenges we see in the study of epigenetic clocks of biological age and other traits of interest.

4.1 | Performance metrics

Since the development of the first epigenetic clock (less than 10 years ago), two metrics have been reported to characterize clock performance, namely the R^2 of the association between epigenetic (predicted) and chronological age, and the MAD expressed in years (Horvath, 2013). In a methodological context, accuracy is defined as the extent to which estimates are close to the true value. In this sense, the MAD represents accuracy, and the R^2 does not. The R^2 may however serve as a proxy of accuracy, as confirmed by the positive correlation between R^2 and MAD (Figure S1). Nevertheless, we here note that a high R^2 does not necessarily imply a low MAD. While most studies included in the meta-analysis reported the R^2, fewer reported the MAD, and we recommend reporting the MAD for all future epigenetic clock studies.

While there are data available to assess epigenetic clock *accuracy*, data on clock *precision*, defined as the extent to which repeated measurements of the same samples or data yield the same result (i.e., repeatability) were not available. We note however that when an epigenetic clock yields a high R^2 with chronological age when applied to an independent data set, this implies that precision is also high.

4.2 | DNAm quantification methods

DNAm can be quantified using techniques differing in DNA input, resolution, genome coverage and cost (reviewed in Husby, 2022), and any of these factors may affect the quality of the data and thereby the predictive accuracy of epigenetic clocks. To reduce the high costs related to measuring DNAm across the entire genome, multiple techniques targeting specific regions (usually CpG-rich regions) have been developed. Indeed, most epigenetic clocks in our data set were inferred using such targeted DNAm quantification techniques. However, as revealed by our analysis, apparently accurate epigenetic clocks have been inferred using information from a small fraction of the genome.

We found that Microarray technology (all but one of which were the HorvathMammalMethylChip40, Arneson et al., 2022) yielded on average higher R^2 values compared with other methods of DNAm quantification (Figure 8, Table 1) while RRBS yielded epigenetic clocks with the lowest MAD, just outperforming Microarray studies (Figure 9, Table 2). This may be due to RRBS' higher

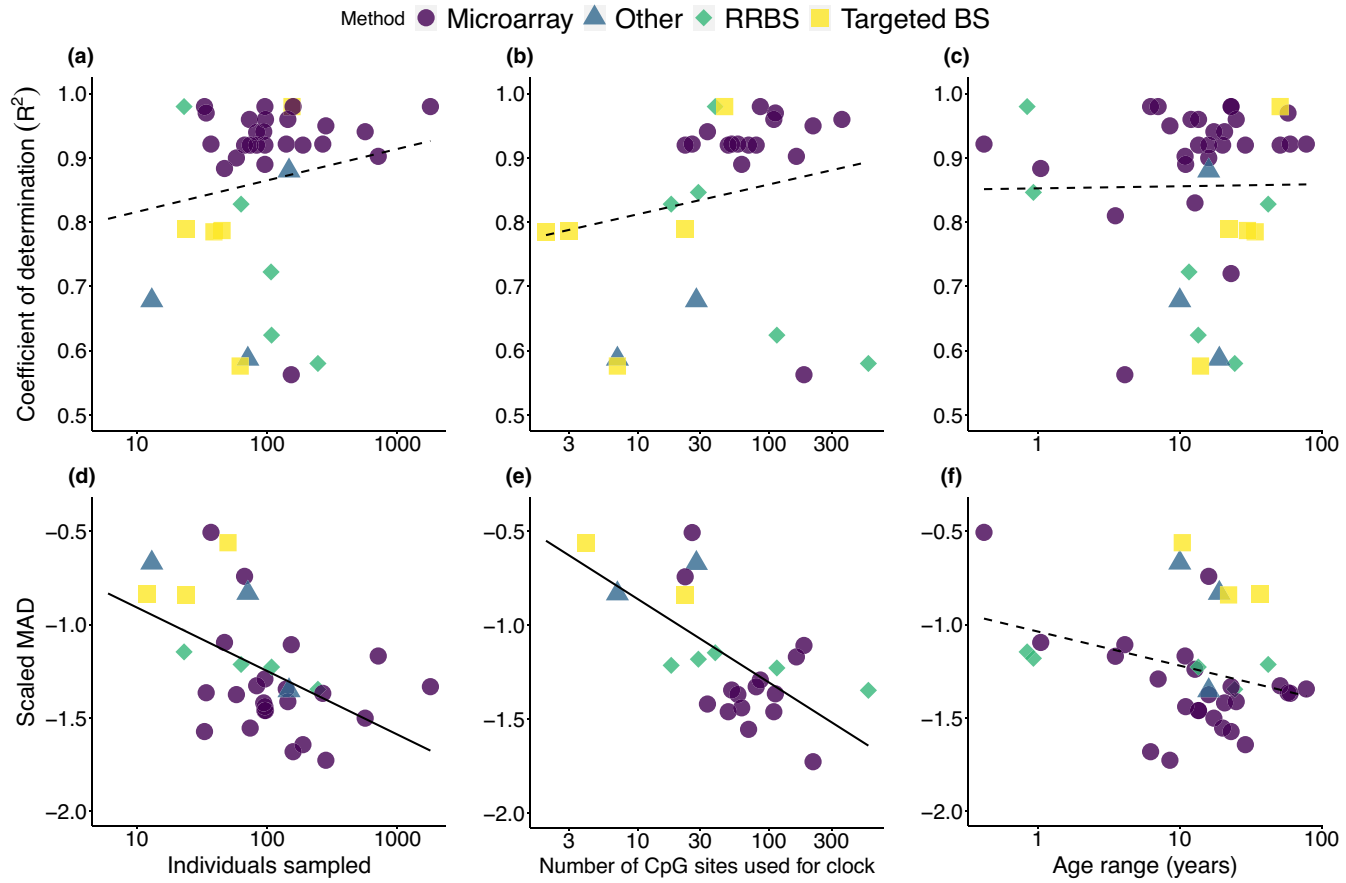

FIGURE 7 Relationship between raw data for R^2 and (a) the number of individuals sampled, (b) the number of CpG sites used for the inference of the epigenetic clocks, (c) the age range in years (all in $\log_{10}$ scale). Relationship between MAD scaled to age range and (d) the number of individuals sampled, (e) the number of CpG sites used for the inference of the epigenetic clocks, and (f) the age range in years (all in $\log_{10}$ scale). Symbols indicate the method used to measure DNAm. Lines represent the best-fit regression through the raw data, with solid lines indicating a significant relationship and dashed lines indicating a non-significant relationship (but note that the correlation between MAD and the number of individuals sampled did not quite reach significance in the full model).

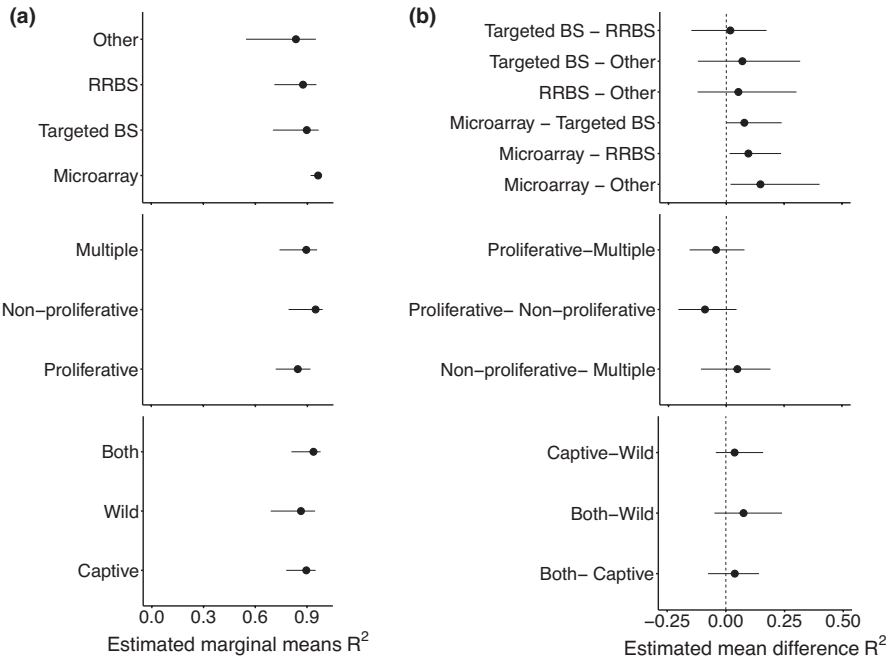

FIGURE 8 Estimated marginal means (a) and estimated mean pairwise differences (b) of the R^2 of the epigenetic clocks ($N = 38$) inferred with each of the model variables. Points show the posterior mean and error bars show the 95% CI.

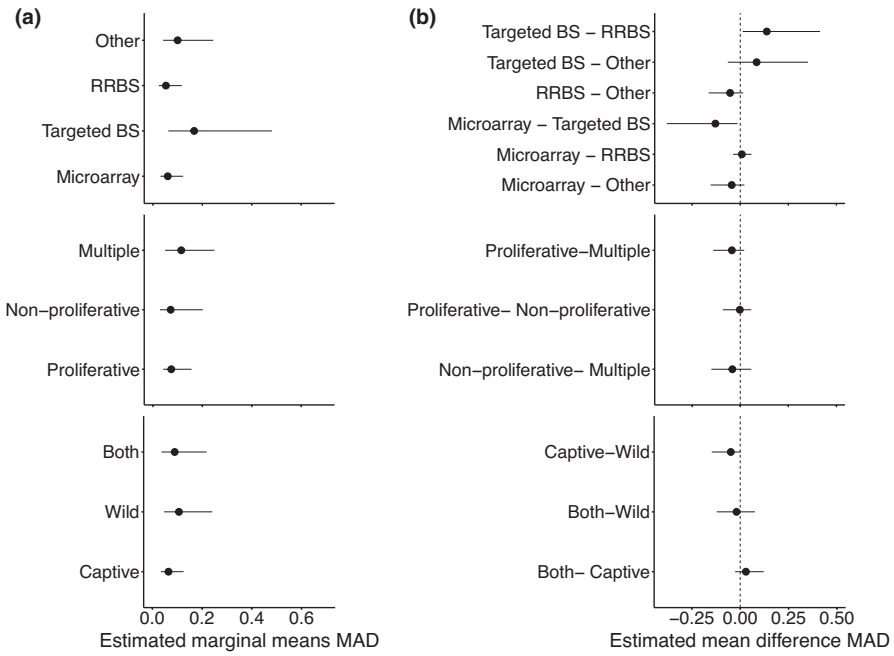

FIGURE 9 Estimated marginal means (a) and estimated mean pairwise differences (b) of the *MAD* of the epigenetic clocks ($N = 33$) inferred with each of the model variables. Points show the posterior mean and error bars show the 95% CI.

resolution, as Microarray-based methods typically only probe a subset of CpG sites in the genome. The most widely used methylation array, HorvathMammalMethylChip40 has been developed to leverage epigenetic age clocks in all mammal species, regardless of the existence of a reference genome and to ensure that biological insights gained in one species can apply to others (Arneson et al., 2022). Surprisingly, this array was recently used to infer an epigenetic clock in non-mammalian species, namely, African clawed frogs and Western clawed frogs (Zoller et al., 2022). The success in developing usable epigenetic clocks for many species using this chip suggests there can be great benefits in developing chips in taxonomic groups other than mammals, or taxonomic sub-groups of mammals, for example, primates or rodents. On the other hand, one chip, from one lab, seems insufficient to evaluate the potential of methylation chips in general. Furthermore, the requirements of a methylation chip are likely to depend on the information one is aiming to obtain. For example, in the frog study, only 4635 of the ~36,000 CpG sites on the chip mapped to the frog genomes (in contrast to for example, 29,846 CpG sites on the chip mapping to the roe deer genome, Lemaître et al., 2021). While this amount of sites may be sufficient to develop an epigenetic clock ($R^2 = 0.64$, MAD = 2.05 years, Zoller et al., 2022), it is but a small fraction of all CpG sites in the genome. We take the lower R^2 value of this clock (when compared with mammal clocks that were based on the HorvathMammalMethylChip40, Table S3) as a preliminary indication that the chip is less suitable to predict chronological age in non-mammalian taxons. More specifically, tailored methylation chips may enable further fine-tuning of epigenetic clocks and aid in identifying the genomic regions where methylation variation is functionally related to the specific characteristics of interest. Nevertheless, widely applicable, microarray-based DNAm quantification methods targeting conserved sequences between species or classes are likely to be important for the future of the field.

4.3 | Tissues

Age-associated DNAm changes are tissue-specific, at least in humans and rodents (Slieker et al., 2018) giving rise to both opportunities and challenges for the field. Given that epigenetic clocks developed from multiple tissues may provide a better representation of the cumulative effect of age on the epigenetic state of the individual, epigenetic clocks based on multiple tissues could potentially have higher accuracy as demonstrated by our analysis (Figure 9a). One could also expect proliferative and non-proliferative tissues to differ in the ability to predict chronological age, as reported in humans (Zhang et al., 2019). This is because cells in proliferative and non-proliferative tissues will vary widely in the extent to which their age matches organismal chronological age. However, neither the single versus multiple tissue comparison nor the proliferative versus non-proliferative tissue comparison revealed significant differences in R^2 (Figure 8b, Table 1) or MAD (Figure 9b, Table 2). Therefore, we note that a critical evaluation of the above hypotheses would require more data and especially within-study comparisons are likely to increase the statistical power in these tests.

On the other hand, tissue-dependent DNAm changes with age also offer opportunities. Ageing trajectories can differ between traits and fitness components, a phenomenon known as mosaic or heterogeneous ageing (Briga & Verhulst, 2021; Moorad & Ravindran, 2022). This heterogeneity is widespread and poorly understood. The observation that age-specific changes in DNAm are organ-specific offers an opportunity to investigate mosaic ageing among organs and tissues on a molecular level. For example, DNAm of testes and ovaries may provide information on the causes of changes in reproduction and link ageing phenotypes to specific genes through their methylation. We recognize however that such studies will generally require lethal sampling, limiting studies to

cross-sectional designs, which is a significant drawback compared with longitudinal studies due to selective survival biasing estimates of ageing trajectories (Van De & Verhulst, 2006). Potentially, DNAm changes in specific organs can also be detected in tissues that can be sampled repeatedly, such as blood, but the extent to which DNAm patterns in blood parallel those in other tissues remains unanswered (Husby, 2020). However, recent research in birds has revealed that temporal changes in DNA methylation are at least to some extent tissue-general showing that temporal DNAm changes in red blood cells can mirror those in other, less-accessible tissues that might require lethal sampling (Lindner et al., 2021). Nevertheless, such an indirect approach will require careful validation and will reduce statistical power compared with direct measurements, simply because the correlation is expected to be lower.

4.4 | Captive versus wild populations

Much of our understanding of ageing comes from captive model species, living in laboratory conditions. Because ageing rates can be modulated by environmental conditions (Briga & Verhulst, 2015; Reichard, 2016), it cannot be assumed that findings based on animals in captivity can be generalized to wild populations. For example, it is known that the rate of ageing varies between captive and wild individuals in ruminants (Lemaître et al., 2013). Captive individuals usually live in uniform environments with similar diets and the absence of predators and other challenges that shape individual ageing trajectories in free-ranging populations. This may lead to captive individuals of the same chronological age having less variation in epigenetic age, resulting in better performance of the epigenetic clocks compared with populations where there is more variation in the rate of ageing. When there is such a difference in homogeneity, DNAm markers developed using captive individuals may still yield a reliable yard stick to age wild animals, but this remains to be investigated. On the other hand, stronger viability selection in wild populations may well reduce phenotypic variation, and consequently the underlying epigenetic variation. This may cause individuals of the same chronological age to have *less* variation in epigenetic age, resulting in higher R^2 of the epigenetic clock, compared with populations with weaker viability selection. Thus, it is difficult to predict how the accuracy of DNAm clocks will compare between captive and wild populations. Our meta-analysis provided weak evidence that epigenetic clocks based on samples collected from captive individuals performed better than clocks inferred from wild individuals when measured by MAD ($p_d = 0.957$, Figure 9, Table 2), providing support for the first hypothesis. Still, studies sampling both wild and captive individuals of the same species and comparing the rate of epigenetic ageing of the two populations will be of particular interest to gain better insights into age-dependent DNAm differences between captive and wild populations (Beal et al., 2019). Such research may also shed new light on the mechanisms mediating environmental modulation of ageing.

4.5 | Age range

Increasing the age range of the training set as well as the similarity of ages represented in the training and test data sets can improve the accuracy of an epigenetic clock (Zhang et al., 2019). However, our analysis did not reveal a significant effect of age range on the R^2 (Figure 7c, Figure S4d). The reason for this could be that the age range only accounts for the highest and lowest ages in the sample, but does not provide any insight into the distribution of ages within that range as the standard deviation of the age distribution directly impacts the R^2. Additionally, higher age ranges might include very young and/or very old individuals, whose age is generally less accurately estimated as shown before in humans (Horvath, 2013; Levine et al., 2018) and beluga whales (Bors et al., 2021).

4.6 | Number of CpG sites

It is generally presumed that epigenetic clock accuracy is increased with more CpG sites included in calculating the clock (Han et al., 2020; Horvath, 2013; Zbieć-Piekarska et al., 2015); indeed such an effect seems inevitable, at least when considering *within* individual studies, and when individual CpG effects are mainly additive. In a sense, this is illustrated by the finding that there was a significant positive correlation between the number of individuals sampled and the number CpG sites included in the clock (Figure 6). This is likely due to the increase in statistical power when sampling more individuals to train an epigenetic clock. This yields smaller effect sizes statistically significant, increasing the number of informative CpG sites and the epigenetic clock fit to the training data. The number of CpG sites used to generate the epigenetic clocks in our review was highly variable (Figure 5) and significantly predicted clock performance as measured by MAD (Figure 7e, Table S2) but not by R^2 (Figure 7b, Table S1). Indeed, for some species clocks with a high R^2 were derived using only a few CpG sites (e.g., bottlenose dolphin: 2 sites, $R^2 = 0.79$, Beal et al., 2019; humpback whales: 3 sites, $R^2 = 0.79$, Polanowski et al., 2014); however, these clocks also had some of the highest MAD estimates (Figure 7b,e). Publication bias is a potential concern in this context, as shown for example for epigenetic clocks of mortality risk and disease in humans (Fransquet et al., 2019). This is an issue because it seems reasonable to expect that in particular clocks based on very few CpG sites will have a higher chance of being published when apparent accuracy is high. It will therefore be very interesting to see whether clocks based on a few CpG sites will accurately predict chronological age for new, independent data sets. On the other hand, the lack of a significant correlation between the R^2 as well as the MAD of the epigenetic clocks and the number of individuals sampled over all methods pooled together (Tables 1 and 2) argues against publication bias in the studies analysed. However, more data is required for a formal analysis of publication bias.

A puzzling observation on the DNAm clocks for humans and mice is that very few CpG sites are shared between different clocks for the same species while achieving comparable accuracy

(Galkin et al., 2020; Thompson et al., 2018). A possible explanation is that DNAm is highly correlated among many sites, which, in combination with slight differences in the methods to quantify DNAm and different machine learning algorithms and significance cut-offs, may easily yield different sets and numbers of age-related sites. It is nevertheless of interest to consider how this observation may affect the potential of the use of epigenetic clocks for evolutionary ecology. Estimating chronological or biological age based on DNAm is in essence a statistical exercise, the outcome of which is a tool to be used for further study. In this case, what matters in practice is the accuracy of the clock, not how it is developed. On the other hand, it remains to be assessed if different clocks with similar accuracy are equally robust with respect to spatial and temporal variation in populations of wild animals.

Recently, methods that enable researchers to access and study whole methylomes have been developed, making it possible to evaluate DNA methylation in various genomic contexts. DNAm of several genomic features, such as transcription start sites, enhancers, and gene bodies is thought to influence binding and therefore function of regulatory proteins as well as play a major role in development and differentiation (Jones, 2012). Moreover, DNAm of repeat regions, such as transposable elements and centromeric regions can aid in their downregulation that results in genome stability (Smith & Meissner, 2013). The variety of ways by which DNA methylation affects the transcriptome and links the genome and its epigenetic state to the phenotype will depend on the extent to which it predicts variation in the transcriptome. Similarly, how the development of clocks for various characteristics will contribute to an understanding of the mechanisms causing phenotypic variation will also rely on the extent to which they predict variation in the transcriptome. This in turn is likely to depend on how epigenetic clocks are constructed. For example, selecting CpG sites in or near promoters to construct a clock seems likely to yield a better match to the transcriptome than selecting from CpG sites located genome-wide, regardless of their functional importance. Thus, when the aim is beyond estimating chronological or biological age, to uncover underlying mechanisms of ageing, clocks based on CpG sites located in several relevant genomic regions may be more informative than clocks based on all sites for which DNAm variation is known, even when such clocks are less accurate when it comes to predicting chronological or biological age.

4.7 | Sample size

Optimal sample size for calibration of epigenetic clocks estimated on the basis of simulations on human and zebra fish data revealed a minimum calibration population size of 70, but ideally, at least 134 samples are needed to infer accurate epigenetic clocks (Mayne et al., 2021). The same study also revealed that with larger sample sizes, the number of selected CpG sites per model also increased, which is in line with our findings (Figure 6), but not with the findings of a recent review on piscine epigenetic clocks (Piferrer & Anastasiadi, 2023). Possibly because of this positive association, in addition to more accurate estimates, it was found that a higher training sample size can significantly increase the accuracy of epigenetic clocks in humans (Bell et al., 2019; Zhang et al., 2019). Nevertheless, our analysis revealed that depending on the method of DNAm quantification used, even studies with smaller sample sizes can yield epigenetic clocks with high predicting ability when measured by R^2 but not by MAD (Figure 7a,d).

Lastly, we note that the vast majority of available DNAm clocks on non-model animals are based on cross-sectional data, that is, data sets in which each individual is sampled only once. If DNAm varied consistently between individuals independent of age, for which there is some evidence (Jimeno et al., 2019; Liu et al., 2019), longitudinal data will potentially have substantially higher power to detect DNAm – chronological age correlations. However, this remains to be investigated.

4.8 | Phylogeny

While the large variation in clock accuracy can at least in part be attributed to methodological factors (e.g., sample size, species, or class-specific similarities in aspects of experimental protocol not accounted for in our analysis), there may in addition be real differences between species in the extent to which DNAm changes with age. The addition of the phylogenetic correlation matrix was found to have a small but positive effect on the models' predictive performance. This indicates that the epigenetic clocks of closely related species exhibit more similar levels of accuracy than expected by chance, suggesting that aspects of epigenetic regulation are conserved across species. Thus, the rate of change in epigenetic clocks may be influenced by factors that are shared among different lineages, such as environmental conditions, lifespans, life-history traits, or genetic factors. For example, it seems plausible that DNAm will change faster with age in populations with shorter lifespan, as found for telomere shortening rate (Tricola et al., 2018). Although the above suggests that phylogeny may influence the performance of epigenetic clocks, further studies using a broader range of species and perhaps larger sample sizes are required to disentangle biological and methodological drivers of this variation.

5 | CONCLUSIONS AND PERSPECTIVES

We have here conducted, to our knowledge, the first comprehensive review and meta-analysis on DNAm in relation to ageing in non-model animals. Despite the extensive list of available publications and plethora of different experimental set-ups in our data set, our assessment was heavily biased towards a single DNAm quantification method (Microarray) and a taxonomic class (mammals). Based on our quantitative analysis, one of the key findings is that as predicted theoretically, MAD is a more effective measure of accuracy for epigenetic clocks compared to R^2. Moreover, we show that epigenetic clocks can predict age, sometimes with high accuracy, suggesting

great potential for the field of ecological epigenetics in relation to age(ing). We expect a democratization of the use of DNAm assays in evolutionary and conservation ecology research and beyond as better (and, hopefully, more affordable) methods of DNAm quantification continue to become available.

The use of DNAm-based epigenetic clocks in wild populations has so far mostly been aimed at inferring chronological age, which is likely to be of practical importance in conservation studies and projects where age is essential but difficult to establish using other methods. With respect to future applications in the field, a major question is whether, as in humans, DNAm-based age clocks provide information on biological age when chronological age is known. If this were to be confirmed, this would open up exciting avenues of research in evolutionary ecology in general, and ageing research in particular. The finding that caloric restriction in rodents, which typically slows ageing and prolongs lifespan, also affected epigenetic age is encouraging in this respect (Gensous et al., 2019; Petkovich et al., 2017). Other promising findings are that hibernation in yellow-bellied marmots and castration in sheep were found to decelerate epigenetic ageing (Pinho et al., 2022; Sugrue et al., 2021). In this context, we once again highlight the need for longitudinal, experimental studies with manipulations that are known to affect healthspan and lifespan. Working with longitudinal samples holds the potential to point to sites that are truly variably methylated with age as indicated by the change in DNAm between the two (or more) samples taken during an individual's lifespan. Pinpointing where in the genome such DNAm changes occur can help locate conserved sites related to ageing between species or even classes. Such studies may also contribute significantly to our understanding of the mechanisms underlying variation in ageing in wild populations, can help evaluate already existing hypotheses about ageing as well as improve our understanding of how wild animals have evolved to regulate the ageing process.

5.1 | DNA methylation markers of 'other things'

In addition to the use of DNAm to develop markers of chronological and biological age, DNAm information can be employed to develop markers for other biological characteristics. Collecting epigenetic data is still costly, but in well-studied populations the same data can be used to study multiple traits of interest, thereby spreading the costs over multiple projects. This potential is evident from associations between DNAm and other characteristics of interest. For instance, a study in wild great tits detected a correlation between the level of urbanization and DNAm (Watson et al., 2020). Changes in genome-wide DNAm levels during early development in response to ambient temperature variation were detected in wild zebra finches (Sheldon et al., 2020) and age acceleration as measured by an epigenetic clock was correlated with male dominance rank in baboons (Anderson et al., 2021). These insights can for example be employed to study longitudinal changes in a DNAm social dominance marker along the social history of individual baboons to address the question whether scoring high on an epigenetic social dominance marker is a consequence or a predictor of obtaining a high social rank. In the context of ageing studies, it would be interesting to develop clocks that predict remaining lifespan rather than chronological age. Summarizing, the potential to develop DNAm markers for diverse characteristics is, in our view, an exciting avenue to explore further, widening the scope for ecological epigenetic research applications considerably.

AUTHOR CONTRIBUTIONS

The authors confirm their contribution to the paper as follows: Study conception and design: M. Tangili, A.J. Slettenhaar, S.Verhulst; Data collection: M. Tangili, A.J. Slettenhaar; Analysis and interpretation of results: M. Tangili, I. Pen, P.J. Palsbøll, S.Verhulst, H.L Dugdale; Draft manuscript preparation: M. Tangili, S.Verhulst. All authors reviewed the results, edited the draft version, and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interest for this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article

BENEFIT-SHARING STATEMENT

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