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RESOURCE ARTICLE

# Age prediction of green turtles with an epigenetic clock

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## Abstract

Age is a fundamental life history attribute that is used to understand the dynamics of wild animal populations. Unfortunately, most animals do not have a practical or nonlethal method to determine age. This makes it difficult for wildlife managers to carry out population assessments, particularly for elusive and long-lived fauna such as marine turtles. In this study, we present an epigenetic clock that predicts the age of marine turtles from skin biopsies. The model was developed and validated using DNA from known-age green turtles (*Chelonia mydas*) from two captive populations, and mark-recapture wild turtles with known time intervals between captures. Our method, based on DNA methylation levels at 18 CpG sites, was highly accurate with a median absolute error of 2.1 years (4.7% of maximum age in data set). This is the first epigenetic clock developed for a reptile and illustrates their broad applicability across a broad variety of vertebrate species. It has the potential to transform marine turtle management through a nonlethal and inexpensive method to provide key life history information.

KEYWORDS
DNA ageing, epigenetics, population management, wildlife management

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# 1 | INTRODUCTION

DNA methylation has been used to predict age in a wide range of mammals, fish, and birds (De Paoli-Iseppi et al., 2017). These studies develop what are referred to as "epigenetic clocks", where specific CpG sites can be used collectively to predict age from DNA methylation levels. DNA methylation is one of the main epigenetic modifications involved in regulating gene expression. Genes found in regions with more methylated DNA recruit proteins such as histones, and modify chromatin structure, leading to reduced expression (Moore et al., 2013).

In vertebrates, the most common form of DNA methylation is the addition of a methyl group to cytosine residues within cytosine-phosphate-guanine (CpG) sites (Bird, 1993). DNA methylation has been shown to be predictive of age in a range of somatic tissues in humans and mice (Horvath, 2013; Stubbs et al., 2017). This is ideal in a wildlife management setting as it can potentially be used as a nonlethal method to predict age. However, epigenetic clocks are currently not available for every species. DNA sequencing for measuring methylation levels has become cost effective and high throughput, making monitoring of wild animal populations with epigenetic clocks a realistic proposition (Heather & Chain, 2016; Li et al., 2019). However, epigenetic clocks do have their limitations and can be confounded by other factors. For example, sex can potentially confound analyses which is why studies either remove the sex chromosomes from the analysis or have an equal sex ratio (Horvath, 2013; Stubbs et al., 2017; Thompson et al., 2017). It is also important to note other factors such as population and environmental factors can influence DNA methylation and may impact age-specific models (Nilsen et al., 2016; Parrott et al., 2014; Polanowski et al., 2014).

Marine turtles are iconic, globally distributed large reptiles that traverse vast distances in the ocean. This lengthy and largely maritime life history makes it difficult to characterise marine turtle population demography and dynamics. This is significant since all marine turtle species are currently considered vulnerable or endangered (Mazaris et al., 2017). Methods such as mark-recapture are widely employed for marine turtles to estimate survival probabilities, abundance, and dispersal behaviour. However, key life history attributes, including age and growth rates remain difficult to determine (Casale et al., 2007; Hoenig, 1983).

Practical and nonlethal methods for age prediction do not exist for marine turtles. Skeletochronology can only be carried out on a deceased individual and therefore is unlikely a practical method for wildlife management (Avens & Snover, 2013; Guarino et al., 2004; Snover, 2002; Tomaszewicz et al., 2015). A nonlethal and practical method for age prediction of marine turtles would enable better understanding of their population dynamics, including population growth rates, survival probabilities, mortality rates, and life expectancies (Caughley, 1966; Cole, 1954; Müller et al., 2004). Estimates of these population characteristics would allow overall risk of extinction for these iconic organisms to be assessed more accurately. In this study, we present an epigenetic clock for marine turtles. The epigenetic clock was developed and validated using skin biopsies collected from known age green turtles (*Chelonia mydas*) but restricted to CpG sites conserved among all marine turtles. By restricting the epigenetic clock to CpG sites common to all marine turtles, the method can later be adapted to other marine turtles through validation with known age individuals.

# 2 | MATERIALS AND METHODS

## 2.1 | Known age marine turtle data set

Skin biopsy samples from 63 green turtles of known age (range 1–43.8 years) were collected from the Cayman Islands and Kélonia, Réunion (Table 1). Sample collection of Cayman Island turtle skin biopsies was approved by Cayman Islands Department of Agriculture Animal Welfare & Control. These samples are from captive animals and therefore age is known. Skin biopsies from Kélonia, Réunion were conducted under permits from Kélonia, Réunion Island Prefecture and Department of the Environment (09-1405/SG/DRCTCV; DA 2017-01). These turtles too were captive and have originated from wild populations. In addition to known age samples, biopsies were collected from two wild turtles during a mark-recapture investigations at Ningaloo, Western Australia, providing two paired samples of known time intervals. These samples were collected under

**TABLE 1** Sample sizes by locations of turtle skin biopsies used for reduced representation bisulphite sequencing. Carapace lengths and widths for individual turtles are provided in Table S1

| Sample origin | Species and total samples | Age range (years) | Sex distribution |
|---|---|---|---|
| Cayman Turtle Centre, Cayman Islands | Green turtle: 51 | 1–43 | Female: 31<br>Male: 10<br>Unknown: 10 |
| Turtle observatory, Kélonia, Réunion, France | Green turtle: 12 | 1–34 | Male: 2<br>Unknown: 10 |
| Western Australia mix origins, Australia | Flatback turtle: 1<br>Hawksbill turtle: 1<br>Leatherback turtle: 1<br>Loggerhead turtle: 1<br>Olive Ridley turtle: 1 | NA | Unknown: 5 |

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permits from the Department of Biodiversity, Conservation and Attractions (U10/2020-2022) and the CSIRO Large Animal Ethics Committee (AEC: 14-07).

In addition, one skin biopsy from each of the following species was included in the data set: flatback turtle (*Natator depressus*), hawksbill turtle (*Eretmochelys imbricata*), leatherback turtle (*Dermochelys coriacea*), loggerhead turtle (*Caretta caretta*), and olive ridley turtle (*Lepidochelys olivacea*). The collection of these samples was approved by Western Australia's Department of Biodiversity, Conservation and Attractions (U10/2020-2022). One sample from each species was used to identify CpG sites that are present in all marine turtle species to develop a future universal epigenetic clock for marine turtles. The purpose of this approach was that transferring the model to other marine turtle species will be possible when samples from sufficient known-age individuals become available. We therefore provide an epigenetic clock with CpG sites common to all marine turtles.

## 2.2 | Reduced representation bisulphite sequencing

The DNA from all samples was extracted using the DNeasy Blood and Tissue Kit (Qiagen) as instructed in the manufacturer's protocol. A total of 72 marine turtle skin biopsy samples were used for reduced representation bisulphite sequencing (RRBS; Table 1). RRBS libraries were prepared using *MspI* digestion as previously described (Smallwood et al., 2011). Bisulphite conversion efficiency was determined by using the spiked unmethylated Lambda DNA (Promega, cat no. D1521). This DNA is unmethylated and therefore all CpG sites should be converted. RRBS data was aligned (GenBank: J02459) and the level of methylation was determined the same as described in the next section. The Ovation RRBS Methyl-Seq system from NuGEN was used to construct the libraries. Libraries were sequenced on an Illumina NovaSeq with 100 bp single-end 200 cycling at the Australian Genome Research Facility (AGRF). The raw demultiplexed RRBS sequencing reads are provided online on the CSIRO data access portal at: https://data.csiro.au/collections/collection/CIcsiro:49784v1

## 2.3 | Sequencing data analysis

Demultiplexed fastq files were quality checked using FastQC version 0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and were trimmed using TRIMMOMATIC version 0.38 with a sliding window of 4–15 bp and a minimum read length of 36 bp (Bolger et al., 2014). Trimmed reads were aligned using BS-Seeker2 version 2.0.3 with default settings and BOWTIE2 version 2.3.4 to the green turtle genome from NCBI (assembly: rCheMyd1.pri) (Guo et al., 2013; Langmead & Salzberg, 2012; Rhie et al., 2020; Wang et al., 2013). BS-Seeker2's methylation module was used with default settings for methylation calling. DNA methylation calling returns values as a percentage (methylated reads/total reads). CpG sites with a mean inadequate coverage of <2 reads or a clustering of >100 reads was removed from downstream analysis as previously described (Stubbs et al., 2017). CpG sites with methylation levels in 90% of samples (482,578 CpG sites) were used for downstream analyses.

## 2.4 | Universal marine turtle epigenetic clock

CpG sites that were captured in all species and had adequate coverage, as described above, were included in model generation. The genomic locations of these sites were identified by BS-Seeker2's methylation module. Green turtle samples of known age were randomly assigned (Table S1) to either a training data set (46 samples), or a testing data set (17 samples) and was split evenly as much as possible by sex and age using the createDataPartition function in the caret R package (Kuhn, 2008). Age was transformed to a natural log to fit a linear model. Using an elastic net regression model, the age of the turtles was regressed over the percentage of methylation of CpG sites. The glmnet function in the glmnet R package was used to apply the elastic net regression model (Friedman et al., 2010). An elastic net regression model is a common method to identify the best predictors of age from a large data set of potential candidates (Horvath, 2013; Mayne, Korbie, et al., 2020; Stubbs et al., 2017; Thompson et al., 2017). It is best suited for high dimensional data with few samples (Friedman et al., 2010). This is the case with genomic data sets including DNA methylation data sets which have more CpG sites than samples. The glmnet function was set to a 10-fold cross validation and an $\alpha$-parameter of 0.5 (optimal between a ridge and lasso model). The performance of the model was assessed using Pearson correlations, absolute error, and relative error rates. All statistical analyses were carried out in R version 4.0.4 (R Core Team, 2013).

## 2.5 | Testing for differences in epigenetic age error between populations and sexes

Population and sex could not be considered as factors in the model as has been done in a previous epigenetic clock (Anastasiadi & Piferrer, 2020). Sex is difficult to determine in turtles for younger ages and only two populations are represented in this study. However, we did test if the model was biased towards a sex or population once it was developed. To test for differences in absolute error rate between samples of different populations and sex we used a one-way ANOVA. Since the age of the samples were not evenly distributed across location and sex, age was used as a blocking factor. A blocking factor is used to account for other factors and reducing known sources of variability for more sensitive analyses. By doing so this reduces the bias of age prediction error be attributed to source population.

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## 2.6 | Development of a multiplex PCR assay

The human and mammalian epigenetic clocks use the Illumina methylation arrays, and on a large scale are cost-effective but do not exist for nonmammalian species (Horvath, 2013; Lu et al., 2021). Some studies have used RRBS to develop an epigenetic clock (Stubbs et al., 2017; Thompson et al., 2017). Although these studies have produced accurate models, RRBS is expensive and is therefore not practical for direct application in most wildlife management studies. RRBS is expensive because it captures a large part of the genome, but epigenetic clocks only require a selected number of CpG sites. The motivation in this study was to make a cost-effective epigenetic clock for marine turtles. This would be carried out by using multiplex PCR; a high-throughput method that targets small amplicons (fragments of DNA) for DNA sequencing. The initial motivation for RRBS was to identify the CpG sites that are predictors of age.

Known age samples, along with two pairs of samples from two individuals of a mark-recapture study were bisulphite treated (Supporting Information) using a modified version of a previous protocol (Clark et al., 2006). Human methylated and nonmethylated DNA (Zymo, Cat no. D5014) was used to assess the efficiency of the bisulphite treatment. This kit contains DNA known to be methylated and unmethylated within the death-associated protein kinase gene (DAPK1, primers provided by the manufacturer). To test efficiency DNA from these controls was aligned to GenBank Accession no. NM_004938. The fully methylated DNA should have no conversion of cytosines whereas the unmethylated will have conversion to thymines.

Primers were designed using a 500 bp flanking region of the CpG sites identified from the elastic net regression model in the RRBS data (Table S2). PrimerSuite (www.primer-suite.com) was used to design the primers and has been used previously for multiplex PCR of bisulphite converted DNA (Korbie et al., 2015; Lu et al., 2017; Mayne, Espinoza, et al., 2021; Mayne, Korbie, et al., 2020). The parameters of PrimerSuite were 55°C annealing temperature, one assay pool, bisulphite converted DNA, and with the two fusion sequences (gacatggttctaca and cagagacttggtct). The cytosines are unmethylated in the primers. One target assay was chosen to reduce complexity in the laboratory and cost of running multiple pools. The fusion sequences were for barcoding with the Fluidigm 384 barcodes (Fluidigm cat no. 100–4876).

The Primers were tested in singleplex reaction using the GoTaq Hot Start Polymerase (Promega, cat no. M8296). Annealing temperatures ranging from 55–60°C were tested, with 56°C found to produce the strongest band for both singleplex and multiplex reactions. PCR products were visualised on a 2% agarose gel. Barcoding of samples was carried with oligonucleotides with attached Illumina adaptors (Fluidigm, 100–4876). Samples were pooled equally by volume. Sequencing was carried out on an Illumina MiSeq, 300 cycle, with 100× coverage to generate 400,000 reads per sample. Both multiplex and barcoding, master mixes, and cycling conditions are provided in Tables S3–S6.

Multiplex PCR sequencing data was hard clipped by 15 bp at both the 5' and 3' end to remove adaptor sequences with SEQKIT version 1.2 (Shen et al., 2016). Reads were aligned to the green turtle genome using BISMARK version 0.20.0 with default parameters and bowtie2 as an aligner (Krueger & Andrews, 2011; Langmead & Salzberg, 2012). The methylation calling was done with the bismark_methylation_extractor function which returned methylation values as a percentage. The model was recalibrated using the glmnet function with CpG sites maintained after the multiplex PCR design. The α-parameter was set to 0 in glment to force all sites to be used in the model. Here, the optimal sites had already been identified by RRBS and the model needed to be recalibrated with multiplex PCR data. The multiplex PCR data is publicly available at https://data.csiro.au/collection/csiro:53843

## 2.7 | Principal component analysis

Principal component analysis (PCA) was carried with the data scale set to unit variance with the FactoMineR R package (Lê et al., 2008). PCAs were carried out on all DNA methylation percentages with all CpG sites and age associated CpG sites. This information is detailed in the relevant sections of the results.

# 3 | RESULTS

## 3.1 | Universal marine turtle age markers

On average, 45.3 million reads per RRBS sample were aligned to the green turtle genome with an alignment rate of 88.6%. Bisulphite conversion was found to be >99% as determined by spiked unmethylated cl857 Sam7 Lambda DNA. This resulted in a total sum of 1,261,168 CpG sites with an average coverage of six reads per CpG site across all samples. Global methylation was 65.5%, lower than what is found in mammals (70%–80%), but similar to the common snapping turtle (*Chelydra serpentina*) where two-thirds of CpG sites have a methylation >75%. (Li & Zhang, 2014; Ruhr et al., 2021). Global methylation did not significantly correlate with age (Pearson correlation = 0.10, $p$-value = .67). However, DNA methylation at 8225 CpG sites exclusively in green turtles correlated with age (FDR < 0.05). A total of 844 CpG sites were common to all turtle species. The overlap of 844 CpG sites does not reflect evolutionary divergence or sequence conservation but rather a reflection of the extent of the turtle genomes covered by sequencing. RRBS involves the fragmentation of DNA by restriction enzymes. Although all species had an alignment rate of >85% to the green turtle genome, the reads between species did not fully overlap. This is most probably due to genomic variation resulting in different cut site locations. Whole genome sequencing would be a better method to determine genome conservation. Of the 844 CpG sites, 51 were significantly correlated with age (FDR < 0.05).

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The elastic net regression model returns the minimum number of sites to predict age and was run with the 844 CpG sites common to all turtle species. The glmnet function returned a minimum $\lambda$-value of 0.1007724 based on the training data. The regression model returned an optimal 29 CpG sites that are conserved in all marine turtle species to predict age. These sites were found to be conserved by aligning reads from each species to the green turtle genome and using the BS-Seeker2 call methylation module. Using this model with 29 CpG sites we detected a high correlation between the chronological and predicted age (Figures S1A,B) in both the training (Pearson's correlation = 0.93, $p$-value $< 2.20 \times 10^{-16}$) and testing (Pearson's correlation = 0.90, $p$-value = $7.53 \times 10^{-7}$) data sets. We also found a low median absolute error rate (MAE) of 2.57 years in the testing data set (Figure S1C). This translates to a relative error of 4.8% when compared to the maximum age in the data set, which is comparable to mammalian studies (4.2%–8.5%) (Horvath, 2013; Stubbs et al., 2017; Thompson et al., 2017). No statistical significance in absolute error rate was found between training and testing data sets ($t$ test, two-tailed, $p$-value = 010).

We also performed the elastic net regression model using the age-associated 8225 CpG sites that were found in green turtles but not necessarily in other species. Here, we found a similar correlation and MAE in the testing data set compared to the universal marine turtle clock (Pearson correlation = 0.93; MAE = 2.55 years). However, there was an increase to an optimum of 38 CpG sites for this specific green turtle epigenetic clock. Of the 38 CpG sites; 25 were part of the 29 "universal marker" panel described above. Since there was little difference between age prediction models, we chose to focus on sites conserved in all marine turtle species. The increase from 29 to 38 CpG sites may potentially be contributed by the larger pool of CpG sites that was available to the elastic net regression algorithm (844 vs. 8225). We also found no difference in absolute error rates in location (Cayman Island compared to Réunion, one-way ANOVA, $p$-value = .97) and sex (one-way ANOVA, $p$-value = .84). Multitissue and multispecies epigenetic clocks generally have more CpG sites than models with only working with single tissue or one species (Horvath, 2013; Lu et al., 2021). In this study, it may seem counterintuitive that the species-specific clock had more CpG sites. However, unlike other studies, the calibration data remained the same, but the pool of CpG sites for selection increased with green turtles only. One potential reason for the increase in CpG sites is that the model selected CpG sites that may be able to capture more subtle differences in methylation that may be better predictors of age. However, due to the lack of known age for other marine turtle species, this cannot be determined. A PCA of the 8225 CpG sites shows that the samples separated by location (Figure S2).

## 3.2 | Age-association of DNA methylation across the genome

Previous studies have shown that DNA methylation at any CpG site have some level of age-association (Mayne, Korbie, et al., 2020; Stubbs et al., 2017). We examined this with the elastic net regression model, where it was restricted to use 29 CpG sites randomly chosen throughout the genome. This was carried out 10,000 times and produced an average MAE of 10.0 years (Figure S3). This histogram shows that collectively some CpG sites chosen at random can be predictive of age. However, specific CpG sites are required to achieve the best performing model.

## 3.3 | Multiplex PCR followed by sequencing

The motivation for employing multiplex PCR was to reduce cost and increase throughput for application in routine turtle monitoring. To reduce the complexity of the multiplex PCR assay, we sought to design an assay based on a single multiplex. However, the maximum multiplex size that met our selection criteria without producing high levels of primer dimer was 18 of the 29 CpG sites. Examples of singleplex and multiplex reactions are provided in Figure S4. Two previous studies have shown it is still possible to generate precise and accurate predictive models of age with fewer biomarkers than initially intended for the assay (Mayne, Espinoza, et al., 2021; Mayne, Korbie, et al., 2020). It is important to note a reference genome is required to obtain the flanking sequences to carry out primer design for multiplex PCR. Fortunately, the green sea turtle reference genome was available for this study. The 63 known age green turtle samples were subject to the multiplex PCR method (see Methods S1). On average, per sample, 421,000 reads (339,000–449,000 reads) were aligned to the targeted amplicons with an alignment rate of 98.4%. Each amplicon had a mean coverage of 14,354 reads. No statistical difference was found with read coverage and absolute error rate in the testing data set (Pearson's correlation = −0.06, $p$-value = .6395). The model was retrained using the 18 CpG sites in the multiplex PCR assay (Table S7). The methylation at the 18 CpG sites were found to collectively separate the samples by age (Figure 1a). It should also be noted that the Kélonia, Réunion samples had a larger cohort of younger samples (<10 years, Table S1) than the Cayman Island samples. Despite this no difference was in absolute error rate was observed between locations (Cayman Island compared to Réunion, one-way ANOVA, $p$-value = .97). Methylation levels at six CpG sites positively correlated with age and 12 negatively correlated with age (Figure 1b and Table S7).

The multiplex PCR assay produced a similar performance as a predictor of chronological age as the RRBS in both the training (Pearson's correlation = 0.94, $p$-value $<2.20 \times 10^{-16}$) and testing (Pearson's correlation = 0.91, $p$-value $< 7.21 \times 10^{-7}$) data sets (Figure 2a,b). No statistical difference was observed between the error rates of the training (MAE = 1.2 years) and testing (MAE = 2.1 years) data sets (Figure 2c, $p$-value = .65, $t$ test, two-tailed). The testing data set had a median relative error rate of 13.3%. No statistical significance was found with age and the relative error rate (Pearson's correlation = 0.02, $p$-value = .89), suggesting relative error rate is not associated with age (Table 2). The relatively low correlation in the 10–20 year old range may not be reflective of the true

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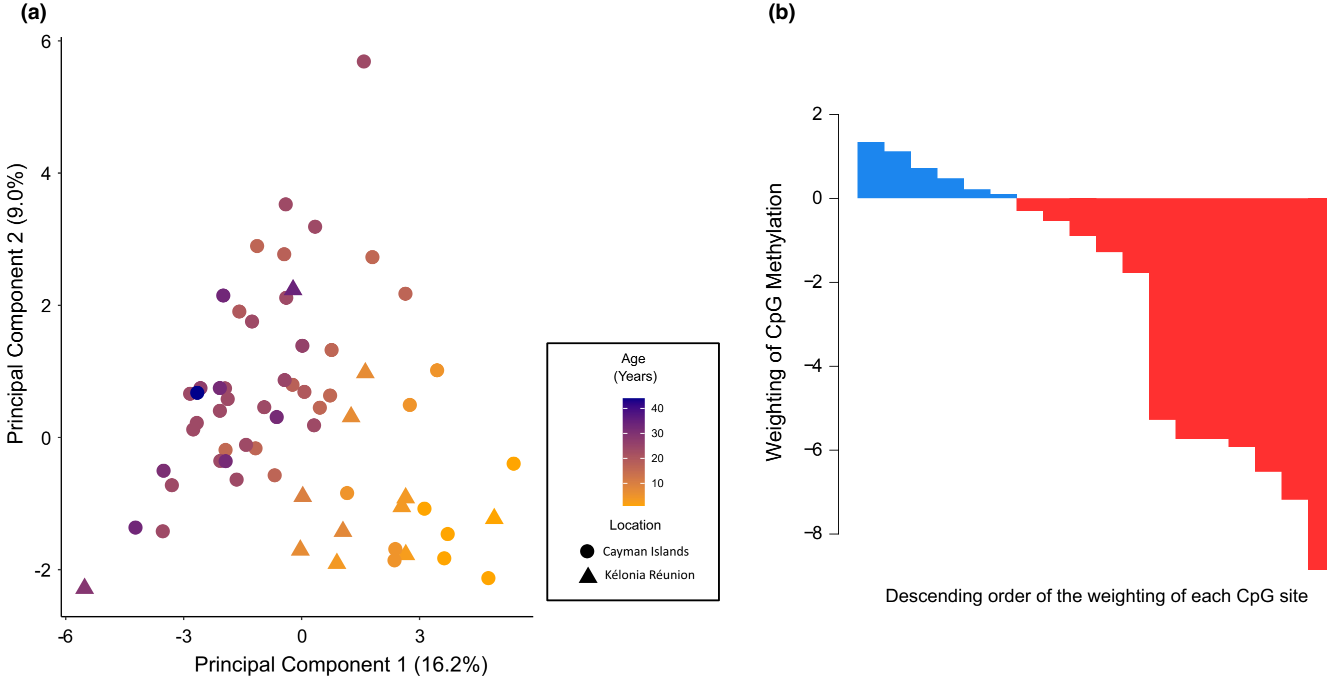


FIGURE 1 Distribution of samples by age associated CpG sites. (a) Principal component analysis of known age green turtles using the 18 age associated CpG sites. Principal component 1 separates the samples by age. (b) Magnitude and directionality of the age associated CpG sites. The plot illustrates that DNA methylation is not unidirectional and some sites either become hypermethylated or hypomethylated with age

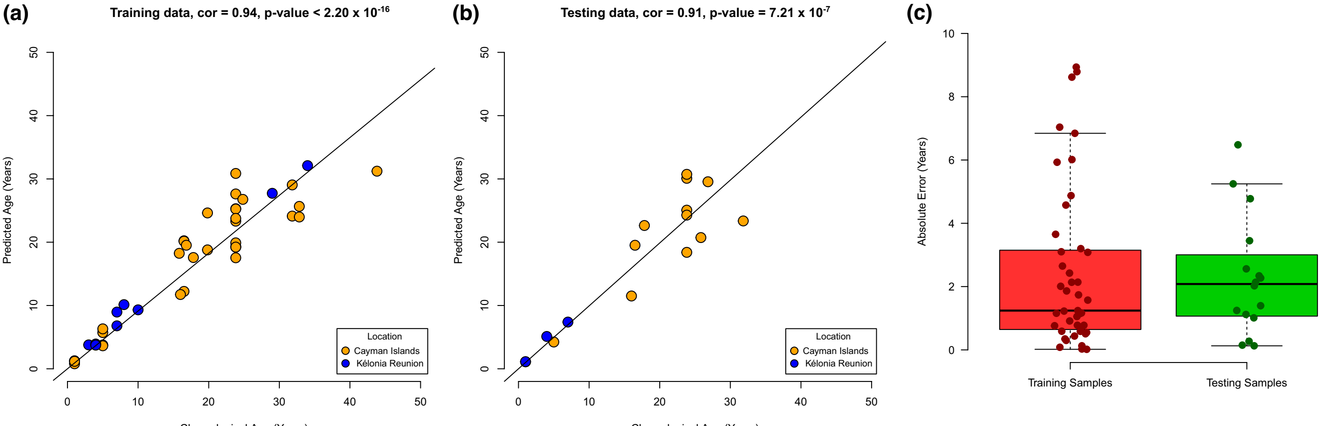


FIGURE 2 Performance of the epigenetic clock with the multiplex PCR assay. Pearson correlation between the chronological and predicted age in the (a) training and (Pearson's correlation = 0.94, $p$-value $< 2.20 \times 10^{-16}$), (b) testing data set (Pearson's correlation = 0.91, $p$-value $< 7.21 \times 10^{-7}$). (c) Absolute error rate in the training (median = 1.2 years) and testing (median = 2.1 years) data sets. As described in the manuscript there was no statistical difference observed in the performance of the training and testing data sets. This suggests a lack of overfitting which would otherwise result in a poor performance in other samples that were not involved in data calibration

TABLE 2 The performance of the epigenetic clock at increasing age intervals in the testing data set. The absolute and relative error rates for each age range is reported as the median. Table S1 provides both chronological and epigenetic age for all the samples in this study

| Age range | Total samples | Correlation | Median absolute error (Years) | Median relative error (%) |
|---|---|---|---|---|
| ≤10 | 5 | 0.98 | 0.67 | 15.6 |
| 10–20 | 4 | 0.67 | 4.1 | 22.4 |
| >20 | 8 | 0.82 | 3.1 | 11 |

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performance of the model as it contains a high number of individuals at a similar age (16–16.8 years, Table S1). A visual representation of the error rates in the testing data set at different age ranges is provided in Figure S5.

### 3.4 | Longitudinal validation

We also tested the model on two wild unknown-aged recaptures of green turtles from Ningaloo, Western Australia. The first was recaptured 2.3 years after initial capture and the turtle age was predicted to be 19.1 and 21.4 years on its first and second capture, respectively. The second was sampled 3.0 years apart and the turtle's age was estimated to be 11.7 and 16.3 years for its first and second captures, respectively.

## 4 | DISCUSSION

Mammals have been the primary focus of the development of epigenetic clocks (Lu et al., 2021; De Paoli-Iseppi et al., 2017). Although there has been a recent increase in the development of epigenetic clocks for fishes, the largest vertebrate class, and birds (Anastasiadi & Piferrer, 2020; Bertucci et al., 2021; Mayne, Korbie, et al., 2020). This study is the first to develop an epigenetic clock for reptiles. It targets the world's most widely distributed reptile, the endangered green turtle, a species that exemplifies the challenges of obtaining basic life-history information to support effective risk assessment and management.

There are several potential limitations of our green turtle epigenetic clock. First, like many other epigenetic clocks for wild animals it was developed and validated with a relatively low sample size ($n = 63$). This reflects the difficulty of obtaining known age samples for marine turtles because of their longevity and maritime life-history. Ideally, a sample size of at least 134 samples is required for epigenetic clocks with high statistical power (Mayne et al., 2021). A larger sample size would decrease the MAE, the relative error, and the standard deviation. Although the overall MAE in this study was 2.1 years, the error was found to be higher in older samples (Table 2, Table S1, Figure S5). This suggests the priority to obtain older age samples to improve the model. However, it should be noted that other epigenetic clocks with low samples sizes (<70 samples) still produce reliable epigenetic clocks (Polanowski et al., 2014; Wright et al., 2018). Moreover, without date of birth, the exact age of an individual can be difficult to define. This can result in an increase in error in the epigenetic age prediction as an individual may be stated as 1 year old but could range between 1 year and 1 day to 1 year and 365 days. However, another consideration is that absolute age may not be necessary for an epigenetic clock to provide useful information for management, so long as an epigenetic clock can place animals into age classes, it may be sufficient for downstream population analyses (Caughley, 1966, 1977). For example, in close-kin mark-recapture studies, age order provides a valuable increase in ability to estimate population size (Bravington et al., 2014). This means that epigenetic clocks need not be highly accurate or precise for all applications (Jarman et al., 2015). It should also be noted here that there is no other nonlethal alternative to predict the age of green turtles. Although an improved model can be made when more samples become available, our model offers a significant advancement over available alternatives for the management of green turtles.

At the time of this study, only the green turtle genome was available. Ideally, if all genomes were available, we would have performed genome pairwise alignments to determine the full list of conserved CpG sites. However, with the lack of genomic resources available we used RRBS on each species and aligned the data using the green turtle genome. This most probably returned a lower representation of the true number of CpG sites conserved between species. This is most probably due to different cut sites during RRBS, resulting in poor overlapping coverage. Another potential limitation is the use of just one individual for each species, which due to individual variation in cut sites, may result in apparently poor CpG conservation amongst species. When genomes for all species become available a more comprehensive analysis of CpG conservation between species can be done. The RRBS sequencing coverage was also low compared to other studies (Mayne, Korbie, et al., 2020; Stubbs et al., 2017; Thompson et al., 2017). A low sequencing coverage may result in other potential age associated CpG sites not being identified. Nevertheless, the aim of the RRBS was to identify the best predictors of age for the application with multiplex PCR, and despite the potential limitations, a practical model that is likely to meet end-user needs was derived.

The longevity of marine turtles makes it difficult to obtain a calibration sample set from known-age individuals across the entire lifespan. This highlights an issue for epigenetic clock development, that calibration data is necessary but often unavailable. In this study, the aim was to develop a universal clock for all marine turtles. However, only known age green sea turtles were available and therefore the true transferability of the clock cannot be tested until an extensive known-age data set becomes available for the other species. Since this is unlikely to occur soon, an alternative and more practical approach to validation may be to use recapture time intervals rather than absolute known ages to confirm the precision of the model. If the intervals of the epigenetic age prediction error are consistently within range to the known timepoints, it could be used as an alternative, albeit more ad hoc, validation. We performed this kind of longitudinal validation by sampling of two individuals at different time points. From only two cases, no valid statistical conclusion can be drawn, but the good agreement between observed and expected ages in this preliminary result (within the model's performance error rate as detailed in Table 2), suggests that this approach could be valuable when applied to other populations. The model can be further validated when other recaptures become available. We emphasise that the utility of epigenetic clocks derived for marine turtles must be considered in the context of specific management needs and the virtually nonexistence of viable alternative methods for estimating age.

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The second limitation of the model was calibrating the model with only two captive populations. Different animal populations are known to have unique genome-wide epigenetic signatures (Ardura et al., 2017; Hu & Barrett, 2017; Tönges et al., 2021), and potentially such signal may confound efforts to isolate age-predictive CpG sites. However, despite demonstrating that indeed at a genome-wide scale our study populations exhibited distinct epigenetic signatures, we showed that were no differences between them with the CpG sites used in the age-predictive model. The selection of the most strongly age informative CpG sites should focus the epigenetic clock on CpG sites that are strongly age affected in any population.

A final caveat for the application of our model relates to the age range to which it can be reliably applied. The oldest sample in this study was 43 years old, but marine turtles are capable of living up to 90 years (de Magalhães & Costa, 2009; Mayne et al., 2020). Simulations have shown that epigenetic clocks are most accurate with age ranges on which they are trained (Mayne, Berry, et al., 2021). Therefore, the performance of the model may decrease beyond 43 years, the maximum chronological age within the training data. Green turtles first breed at c. 20 years and have a maximum lifespan of 75 years, although this may vary between populations (Blechschmidt et al., 2020; Jensen et al., 2018; Tacutu et al., 2018). Turtles older than 40 years may represent demographically significant components of wild populations because of the higher fecundity that is often observed in older cohorts (Esteban et al., 2017). The lack of age markers in turtles historically has made such a hypotheses difficult to test, but it further emphasises the importance of future validation with older known age turtles.

The growing number of epigenetic clocks across vertebrate species is revealing similar probably regulatory functions for age-related CpG sites. Similar gene ontology terms have been found in epigenetic clocks from a range of mammals (Horvath, 2013; Stubbs et al., 2017; Thompson et al., 2017). For example, in canids, age related CpG sites are enriched for genes relating to histone modification (Thompson et al., 2017). More insight into the biological processes that relate CpG sites to age will become available as more epigenetic clocks are developed for a wider variety of species, including nonvertebrates. The current study highlights the potential of DNA methylation as a predictor of age in a major clade of vertebrates where it has not previously been demonstrated. This is important for wild reptile management as the measurement of DNA methylation can be cost effective and high throughput, making it a practical solution for many species.

## 5 | CONCLUSION

Our study shows that epigenetic clocks can be used in a reptilian species in a nonlethal manner to estimate age. Lack of estimates for this important life history parameter have been a significant constraint for research on marine turtle population biology in the past. This method paves the way for applications to fundamental ecological questions needed to inform conservation management of endangered species—including but not limited to, growth rates, population size estimates, and mortality rates. Future applications include using the model to construct population specific growth curves by measuring length and predicting age – critical analyses that have rarely been available to support the management of this iconic group of organisms.

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### CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

### AUTHOR CONTRIBUTIONS

B.M. performed all laboratory work, bioinformatic analysis, performed the machine learning analysis to generate each age estimation model and wrote the manuscript. W.M., V.B., F.C., K.B., M.B., M.A.V., and A.D.T. were involved in sample collection, study design and provided discussion into the manuscript. D.K. designed the primers for the multiplex PCR and was involved in the study design. O.B. and S.J. were involved in study design and provided discussion into the manuscript.

### DATA AVAILABILITY STATEMENT

The raw demultiplexed RRBS sequencing reads are provided online on the CSIRO Data Access Portal at: https://data.csiro.au/collections/collection/CIcsiro:49784v1and the multiplex PCR data can be found at https://data.csiro.au/collection/csiro:53843.

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