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RESOURCE ARTICLE

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Age estimation in a long-lived seabird (*Ardenna tenuirostris*) using DNA methylation-based biomarkers

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Abstract

Age structure is a fundamental aspect of animal population biology. Age is strongly related to individual physiological condition, reproductive potential and mortality rate. Currently, there are no robust molecular methods for age estimation in birds. Instead, individuals must be ringed as chicks to establish known-age populations, which is a labour-intensive and expensive process. The estimation of chronological age using DNA methylation (DNAm) is emerging as a robust approach in mammals including humans, mice and some non-model species. Here, we quantified DNAm in whole blood samples from a total of 71 known-age Short-tailed shearwaters (*Ardenna tenuirostris*) using digital restriction enzyme analysis of methylation (DREAM). The DREAM method measures DNAm levels at thousands of CpG dinucleotides throughout the genome. We identified seven CpG sites with DNAm levels that correlated with age. A model based on these relationships estimated age with a mean difference of 2.8 years to known age, based on validation estimates from models created by repeated sampling of training and validation data subsets. Longitudinal observation of individuals re-sampled over 1 or 2 years generally showed an increase in estimated age (6/7 cases). For the first time, we have shown that epigenetic changes with age can be detected in a wild bird. This approach should be of broad interest to researchers studying age biomarkers in non-model species and will allow identification of markers that can be assessed using targeted techniques for accurate age estimation in large population studies.

KEYWORDS
age, birds, DNA methylation, DREAM, epigenetics

1 | INTRODUCTION

Understanding the age structure of populations is a key aspect of animal ecology and conservation. Age estimate information can help to determine animal mortality, susceptibility to parasites, reproductive life history and the impact of anthropogenic activities (Froy, Phillips, Wood, Nussey, & Lewis, 2013; Gianuca, Phillips, Townley, & Votier, 2017; Musick, 1999; Scott, 1988). However, measuring the chronological age of many wild animals is a difficult task due to the lack of external changes that reflect age. Some animals have quantifiable physical changes as they increase in age, for example, tooth length in deer (Pérez-Barbería, Duff, Brewer, & Guinness, 2014) and growth rings in fish otoliths (Buckmeier, Irwin, Betsill, & Prentice, 2002; Campana, 2001; Gunn et al., 2008). However, few of these can be measured without capturing or even killing the animal. The impact and ethics of these interventions on animals is often the subject of debate (Festa-Bianchet, Blanchard, Gaillard, & Hewison, 2002;

Nelson, 2002). Other animals can show general changes with life stage, for example, plumage variation in some seabirds (Weimerskirch, Lequette, & Jouventin, 1989), or larval stage of arthropods and molluscs (Cobb & Wahle, 1994; Ernande, Clobert, McCombie, & Boudry, 2003), but these often only provide age information for immature individuals. This lack of accessible chronological age information limits our understanding of many wild animal species, and it is only through long-term, expensive tracking or marking studies that age data can be collected and used effectively.

Molecular biomarkers of age have recently been the focus of an increasing number of studies (Ito, Udono, Hirata, & Inoue-Murayama, 2018; Maegawa et al., 2017; Wright et al., 2018). Neither telomere length nor DNA damage markers have been successfully used for chronological age estimation in a wild animal population, so there is interest in developing alternative molecular age biomarkers (Dunshea et al., 2011; Jarman et al., 2015). One promising avenue is measuring epigenetic modification controlling changes in gene expression that occur during animal ageing. Epigenetic regulation of gene expression can occur at several different levels and can include histone modification, non-coding RNA (ncRNA) and DNA methylation (DNAm). DNAm, the addition of a methyl group to a cytosine followed by a guanine (CpG site), has been examined in the most detail, and recent evidence supports the use of this epigenetic modification for individual age determination (Hannum et al., 2013; Horvath, 2013; Vidal-Bralo, Lopez-Golan, & Gonzalez, 2016).

Here, we refer to two types of changes in DNAm with age that could be used to estimate age in wild animals. "Epigenetic drift" generally refers to broad DNAm signals at sites distributed across the genome, which in mammals, birds and fish has been reported to decline with age (Gryzinska, Blaszczak, Strachecka, & Jezewska-Witkowska, 2013; Jakubczak, Listos, Dudko, Abramowicz, & Jeżewska-Witkowska, 2016; Shimoda et al., 2014). Drift signals can also be enriched in CpG islands and enhancers (Slieker et al., 2016). "Clock-type" markers are specific CpG sites that show a strong correlation with known chronological age. Correlations observed in this category can be tissue-specific and can involve an increase (hypermethylation) or decrease (hypomethylation) with age (Horvath, 2013; Slieker, Relton, Gaunt, Slagboom, & Heijmans, 2018). Clock-type CpG age markers have recently been referred to as "age-related DNA methylation positions" (aDMPs) (Lowe et al., 2018; Slieker et al., 2018). aDMPs are generally located within the promoter or first exon of a gene (Bekaert, Kamalandua, Zapico, Voorde, & Decorte, 2015; Grönniger et al., 2010; Horvath, 2013; Sziráki, Tyshkovskiy, & Gladyshev, 2018; Zbieć-Piekarska et al., 2015). Epigenetic drift is thought to occur due to a decline or imperfect replication of DNAm by an epigenetic maintenance system with increasing age (Horvath, 2013; Horvath & Raj, 2018). However, the mechanisms for specific "clock-type" aDMP change have not yet been characterized.

Very little is known about DNAm in most non-model species, especially birds. Available studies have mostly focused on model species such as the Red junglefowl (*Gallus gallus*) (Gryzinska et al., 2013; Hu et al., 2013; Li et al., 2011) and Japanese quail (*Coturnix japonica*) (Andraszek, Gryzińska, Wójcik, Knaga, & Smalec, 2014). These studies show a distribution of DNAm in the genome similar to that observed in mammals. Epigenetic drift is the only age-related DNAm change that has been reported in birds. Gryzinska et al. (2013) observed DNAm changes between chickens aged between 1 day and 32 weeks using a colorimetric immunoenzymatic-based protocol. We have previously reported that the DNAm status of several mammalian clock-type age-related genes was not conserved in homologous regions of a seabird (De Paoli-Iseppi et al., 2017).

Here, we used known-age individuals from a long-term study of Short-tailed shearwater (*Ardenna tenuirostris*) to investigate age-related changes. The shearwater has high breeding site and partner fidelity and is long-lived, making it an ideal species in which to study population status and chronological ageing in a seabird population. Fisher Island (Tasmania, Australia) is the site of a long-term banding study of this species and as such can be used to collect known-age blood and feather samples for the investigation of DNAm and chronological age (Bradley, Skira, & Wooller, 1991). Epigenetic age estimates of seabirds would be particularly valuable for use in population viability analyses and could further our understanding of environmental effects on animal performance or foraging (Velarde & Ezcurra, 2018). For the first time, we have used digital restriction enzyme analysis of methylation (DREAM) to assess DNAm in a non-model vertebrate. We identified seven aDMPs in DNA extracted from 71 whole blood samples. A model relating methylation at these aDMPs to age was made and the precision evaluated using the mean absolute difference (MAD) between the estimated and known chronological ages. Our study is the first to identify DNAm changes with chronological age in a wild seabird and will provide a foundation for further study of age-related DNAm in non-mammalian vertebrates.

2 | METHODS

2.1 | Samples and DNA extraction

In sampling trips between 2015 and 2018, blood samples were collected from adult (November–December) and chick (March) *A. tenuirostris* from Fisher Island (40°13′00.7″S 148°14′20.7″E), Tasmania, under Department of Primary Industries, Parks, Water and Environment (DPIPWE) permit: FA15230 and University of Tasmania Animal Ethics Committee permits: A14277 and A0016107. Blood was collected onto Whatman FTA® Micro (WB120210) cards and stored as previously described (De Paoli-Iseppi et al., 2017). DNA was extracted from a 3 mm punch of immobilized blood using an Epicentre MasterPure™ (MCD85201) DNA Purification Kit according to the manufacturer's instructions. We examined blood DNA in two high-throughput sequencing runs of a total of N = 71 known-age individuals. Age was determined by recording the band number of birds first marked as chicks and was rounded to whole years as all sampling occurred in a short time window each year. Run 1 consisted of 35 known-age animals (5–21 years old, mean = 12.14 years). Two individuals aged 8 and 14 years old were replicated within this run. Run 2 consisted of DNA from 36 additional known-age samples

(6–21 years old, mean = 14.18 years). Run 2 contained three technical replicates from Run 1 (6, 12 and 21 years old), and three within-run replicates aged 8, 14 and 21 years old. Several birds were recaptured in sampling trips in different years allowing us to perform some limited longitudinal observations (Run 2: $N = 3 \times 2$ samples and $N = 4 \times 2$ samples at 1- and 2-year resights, respectively). In total, $N = 63$ known-age shearwater were used to calibrate the model following removal of replicates. Bird sex was determined by *CHD-1* gene amplification in blood DNA using a previously described method (Faux, McInnes, & Jarman, 2014). Sample details for each age group and known-age distribution are shown in Table 1 and Supporting Information Figure S1, respectively.

2.2 | Analysis of genome-wide "CCCGGG" methylation

We examined DNAm at CpG sites throughout the genome using DREAM of 71 Short-tailed shearwater whole blood DNA samples (Jelinek & Madzo, 2016). Briefly, genomic DNA (1 μg) extracted from shearwater blood FTA samples was sequentially cut with two enzymes that recognize the "CCCGGG" sequence motif in DNA (Figure 1). Methyl-sensitive *SmaI* first cuts only unmethylated sites leaving blunt 5'-GGG ends. Then, *XmaI* cleaves the remaining methylated sites leaving 5′-CCGGG ends. Thus, unique sequences are made for methylated or unmethylated CpG sites. Following this sequential digest, DNA was used to create sequencing libraries using NEBNext Multiplex Oligos for Illumina Index Primer Sets 1–3 and standard Illumina protocols. Blunt-end ligation is done using NEBNext Adaptor (10 μm) and T4 DNA ligase with hairpin loop cleavage with USER enzyme. Dual size selection for 250–450 bp fragments was done using AMPure XP beads. Unique barcodes were then added to DNA from individual samples with 12× rounds of PCR using AmpliTaq Gold DNA Polymerase (see Supporting Information Table S1). Individual barcoded samples were analysed for correct library size distribution (250–450 bp) using high sensitivity DNA 1000 kits on the Bioanalyzer 2100. Two microlitres of each sample was also quantified using a Qubit 2.0 to ensure equal volumes were pooled in the final library. Libraries were run at 2–4 ng/μl on the Illumina NextSeq 500 platform with a 15%–25% PhiX control at the Ramaciotti Centre for Genomics (UNSW, Sydney, Australia).

2.3 | Statistical analysis and construction of an age prediction model

2.3.1 | Sequencing data analysis pipeline

Raw DNA sequence reads were run through an in-house data analysis pipeline in the following steps.

1. *Quality filtering* Demultiplexed Fastq sequences were filtered with a maximum expected error (maxee) rate of 0.5 and converted to Fasta format (Edgar & Flyvbjerg, 2015).
2. *Dereplication* A database of unique reads from all samples was generated (dereplication) using trimmed sequences and the USEARCH10 command "fastx_uniques" (Edgar, 2010), with a min_unique size = 150.
3. *Methylated and non-methylated motif databases* These dereplicated sequences were duplicated to contain the unique sequence with either the 5′-GGG or 5′-CCGGG motif, in separate databases (GG or CC databases).
4. *Motif database hits* Each sample was then compared to each database using the "usearch-global" command with 97% identity and required an exact match to the first 2 bp of the relevant motif (id_prefix = 2). Hits for each sequence against both methylation databases were recorded.
5. *DNAm level calculation* The methylation level for each sample was then calculated as the count of the methylated signature divided by the total number of hits for a specific CpG marker, and the value was recorded between 0 and 1. A value of 0 is unmethylated (i.e., all sequences from that site match the GG sequence generated by methyl-sensitive *SmaI*) and 1 is methylated (i.e., all sequences from that site match the CC sequence generated by *XmaI*).

Methylation scores were retained for read depths between 20 and 2,000 reads. Scores that were calculated outside of this range were converted to a "NA." To retain potentially informative markers in the final analysis, markers with less than seven NA values across all samples were imputed using the mean of the remaining non-NA values for the marker. This method ensured that potential age-related markers would not be omitted based on missing scores and that imputed values would have a relatively small effect on any correlations observed. Since variation is required to find correlations

TABLE 1 Short-tailed shearwater (*Ardenna tenuirostris*) sample details

Group	Age range (years)	Samples	Longitudinal data	Male	Female	Replicates
Chicks	0.15	2	—	1	1	—
Young breeders	5–9	19	—	8	9	WR: $N = 2$, BR: $N = 1$
Middle age	10–18	42	6	24	18	WR: $N = 2$, BR: $N = 1$
Old	19+	9	1	3	6	WR: $N = 1$, BR: $N = 1$
Total	0.15, 5–21	71	7 pairs[b]	36[a]	34	WR: $N = 5$, BR: $N = 3$

Notes. BR: between run; WR: within run.
[a]1 sample failed sexing assay (depleted DNA). [b]Refers to 14 samples.

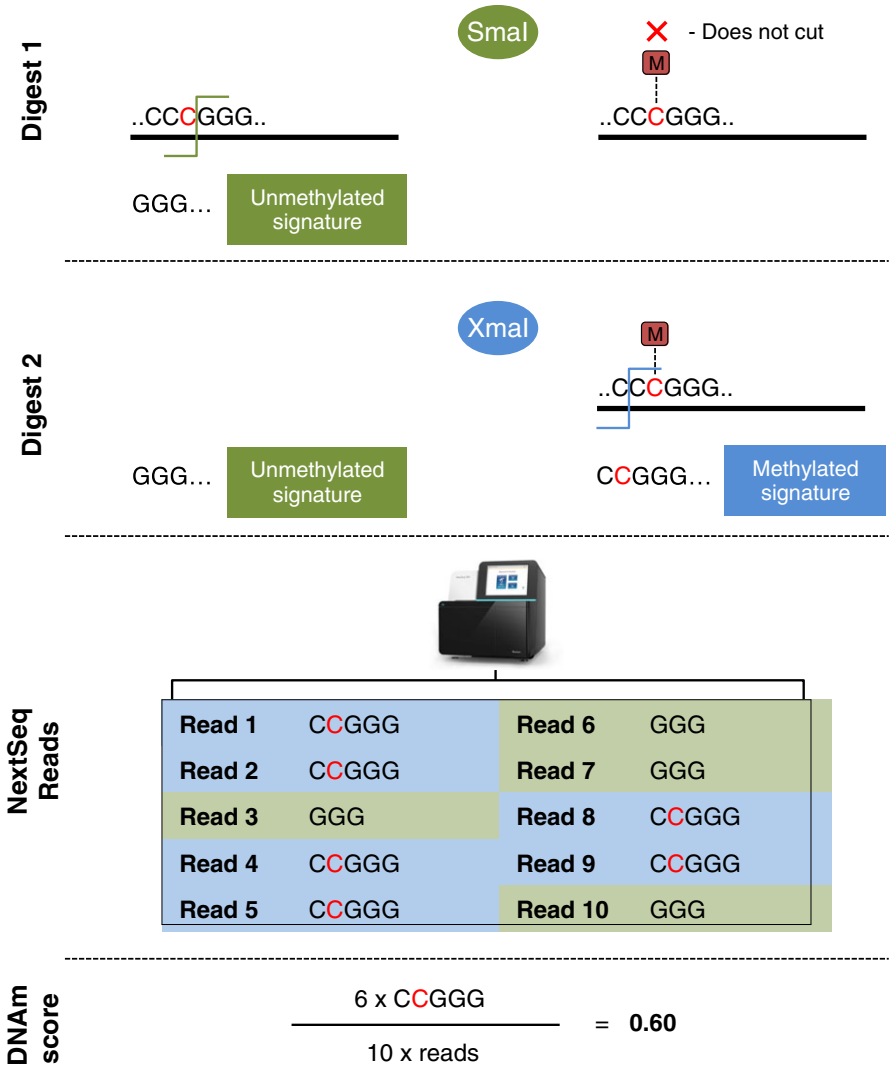

FIGURE 1 Digital restriction enzyme analysis of methylation (DREAM). A schematic showing an example of sequential digestion and sequencing of a single "CCCGGG" site for one sample. In digest 1, a methylation-sensitive enzyme (*SmaI*) is used to generate unmethylated signatures (GGG). *SmaI* does not cut methylated cytosines (methylated cytosines are indicated by red text and a floating, red "M" box). Following this, *XmaI* is added to the sample in digest 2 and generates methylated signatures (CCGGG). All samples are then run on a next-generation sequencing platform (e.g., Illumina NextSeq), and read counts of each signature are counted. In this example, for ten reads of a unique CpG marker, six contain the methylation signature giving a DNAm score of 0.6

with age, we removed markers that had a DNAm standard deviation of less than 5% across all samples. A small run effect was observed, so the mean DNAm difference between run 1 and 2 replicates was used to adjust the score of each marker in run 2.

2.3.2 | Predictor selection and age estimation model

Markers that passed filtering were then used to fit penalized lasso regularization paths to each predictor using the R package "GLMNET" (Friedman, Hastie, & Tibshirani, 2010). The penalty value used to select coefficients, lambda 1 standard error (λ1se), was calculated after repeated runs (100×) of the default *k*-fold cross-validation function of glmnet (cv.glmnet, 10-fold) with an alpha = 1 (lasso). This method randomly subsets the data each cycle and assesses the linear relationship between age and DNAm. Following repeated runs of this function, a mean λ1se value was generated. The λ1se value generally selects CpG sites for the simplest model with an error similar to the best model (λ minimum), given the cross-validation uncertainty.

Individual markers that passed the λ1se cut-off were inspected visually using simple linear regression, and markers that had an $R^2 < 0.2$ or showed small changes in DNAm range (<15%) were removed from further analysis. Remaining age-related CpG sites were then incorporated into a multiple linear regression model. To test the selected markers, the original data set was randomly split into 75/25% training ($N = 47$) and test ($N = 16$) data sets, respectively. Training set DNAm values for each aDMP were used to create a multiple linear regression model. The model was then tested with remaining samples in the test set. This random sub-sampling method was run for 100 iterations. By substituting the calculated methylation values for each of the individual shearwaters used in the training and test sets into the equation, we obtained the predicted epigenetic age. MAD, the uncertainty of age estimates expressed in years, between the known and estimated age was then calculated. The 77 bp sequence following the CG motif was analysed by BLASTn searches of bird genomes available on the NCBI database to identify any regions conserved between species (Altschul, Gish, Miller, Myers, & Lipman, 1990).

2.4 | Global DNA methylation analyses

2.4.1 | Global analysis of 2,338 CpG sites using DREAM

The mean DNAm of 2,338 CpG sites identified using DREAM was analysed by age group in years as follows: Chicks: 0.12–0.15 (N = 2), Young breeder: 5–9 (N = 16), Middle: 10–18 (N = 39) and Old: 19+ (N = 6). CpGs were analysed using a one-way ANOVA followed by post-test for multiple comparisons (Tukey's HSD). Mean DNAm differences were calculated in both the chick and young breeder context and analysed as above. Significance was set at $p < 0.05$.

2.4.2 | Colorimetric DNA methylation analysis

We also measured epigenetic drift in global DNAm using a commercially available methylated DNA quantification assay for relative 5-mC content (Abcam, Colorimetric, ab117128). Briefly, 42 shearwater blood DNA samples (chicks, 5–21 years old, mean = 10.9 years) were analysed in duplicate, alongside the supplied positive (5 ng) and negative controls. Methylated DNA was captured and detected using diluted (1:1,000, 1:2,000) 5-mC antibodies. Following the addition of a developing solution, colour change was monitored and quantified at 450 nm (Tecan Spark). Using the mean absorbance values of the duplicates, relative 5-mC for each sample was calculated as follows: (((Sample OD − Negative control OD)/DNA input (ng))/(((Positive control OD − Negative control OD) × 2)/Positive control input (5 ng))) * 100. Analysis of duplicate colorimetric data was done using a one-way ANOVA with Šidák correction for multiple comparisons for each age group in years as above.

3 | RESULTS

3.1 | Sequencing metrics

Quality analysis of DREAM libraries showed bands in the expected post-clean-up range (range = 194–974 bp, mean = 451 bp; Bioanalyzer gel and electropherogram traces are shown in Supporting Information Figure S2a–d). A total of 125 million sequences (mean of 1,761,622 per sample) passed initial bioinformatic QC (maxee = 0.5 and matched restriction site motif; Supporting Information Table S2). The sum of reads from sequences with a mean high read depth (>2,000×) represented approximately 6% (mean = 84,518 reads) of the total mean sequences per sample. Following filtering and dereplication, we identified 93,884 unique sequences that were used to create a database of reference sequences (i.e., markers for specific CpG sites) for sample matching (Supporting Information Figure S3). Following the pipeline filtering described, a total of 2,338 unique CpGs were used for lasso analysis (GLMNET).

3.2 | Development and testing of an age prediction model in the short-tailed shearwater

DNAm data from seven CpG sites obtained using DREAM were included in the age prediction model based on our selection criteria (Figure 2a–g). Information on removed CpG sites with weaker age correlations is provided in Supporting Information Table S3 (e.g., just below our mean λ1se cut-off of 1.2; see Supporting Information Figure S4). To investigate potential sex-related DNAm effects in the seven aDMPs used in the age prediction model, separate linear regressions were done for each sex (Supporting Information Figure S5a-g). Sex had a significant effect on DNAm age correlation in a single aDMP in isolation (M1801, p = 0.0031, Bonferroni corrected), with males driving the association (Supporting Information Figure S5c). However, there was no sex-specific effect when the methylation scores for all seven aDMPs were then used to create the age estimation model (Figure 3, sex regression slopes and diagnostics are shown in Supporting Information Figures S5h and S6, respectively). Read depth had a mean of 51× for these CpG sites (Supporting Information Figure S7). The MAD between the known and estimated age reports the uncertainty in age estimates expressed in years. Following repeated cross-validation, the seven aDMP age assay provided epigenetic age estimates in training subsamples with a MAD of 2.34 ± 1.73 (*SD*) years (mean R^2 = 0.605, range: 0.46–0.72) (Supporting Information Figure S8a). In the validation test subsamples, the age estimates had an increased error; across all age estimates, MAD = 2.81 ± 2.08 years (mean R^2 = 0.404, range: 0.03–0.80) (Supporting Information Figure S8b). The significant y-intercept of 5.13 indicated that the predicted ages were overestimated for chicks and young birds and underestimated for older individuals, and may indicate a non-linear relationship. The training set MAD ranged from 1.17 to 6.25 years, whilst in the test set, MAD ranged from 1.58 to 7.86 years. The MADs for each year and grouped age, as described in the methods, are shown in Figure 4. Between-run replicates for seven age-related CpG sites showed a mean DNAm score difference of 11.29% (range: 3.79%–12.80%) and 6.83% (range: 4.21%–11.04%) pre- and post-run adjustment, respectively (Supporting Information Table S4). Within-run replicates showed a MAD in DNAm of 8.65% (range: 5.18%–11.91%) for the age-related markers.

3.3 | Biomarker sequence and gene conservation

The seven aDMPs we identified were used to search for conserved regions in available bird genomes and scaffolds using BLASTn. Of these seven markers, four had low *E* values and >50% query cover indicating a reasonable match with a known sequence in the available avian databases (Table 2). Marker 1,071 matched with the *G3BP1* region in the Zebra finch (*Taeniopygia guttata*) genome; however, the query cover was only slightly above 50%. Marker 1934 had a 100% query cover match with an uncharacterized locus in the Mallard (*Anas platyrhynchos*) genome. Marker 2083 matched against scaffold 4,695 in the North Island brown kiwi (*Apteryx australis mantelli*) genome. Finally, marker 3,169 had a 100% query cover match to the *DHH* gene in several species with the top hit to the Eurasian blue tit (*Cyanistes caeruleus*) genome.

3.4 | Longitudinal observations of DNA methylation in resighted individuals

We observed that 6/7 (85%) age estimates for resighted individuals sampled 1 or 2 years apart showed the expected positive increase in

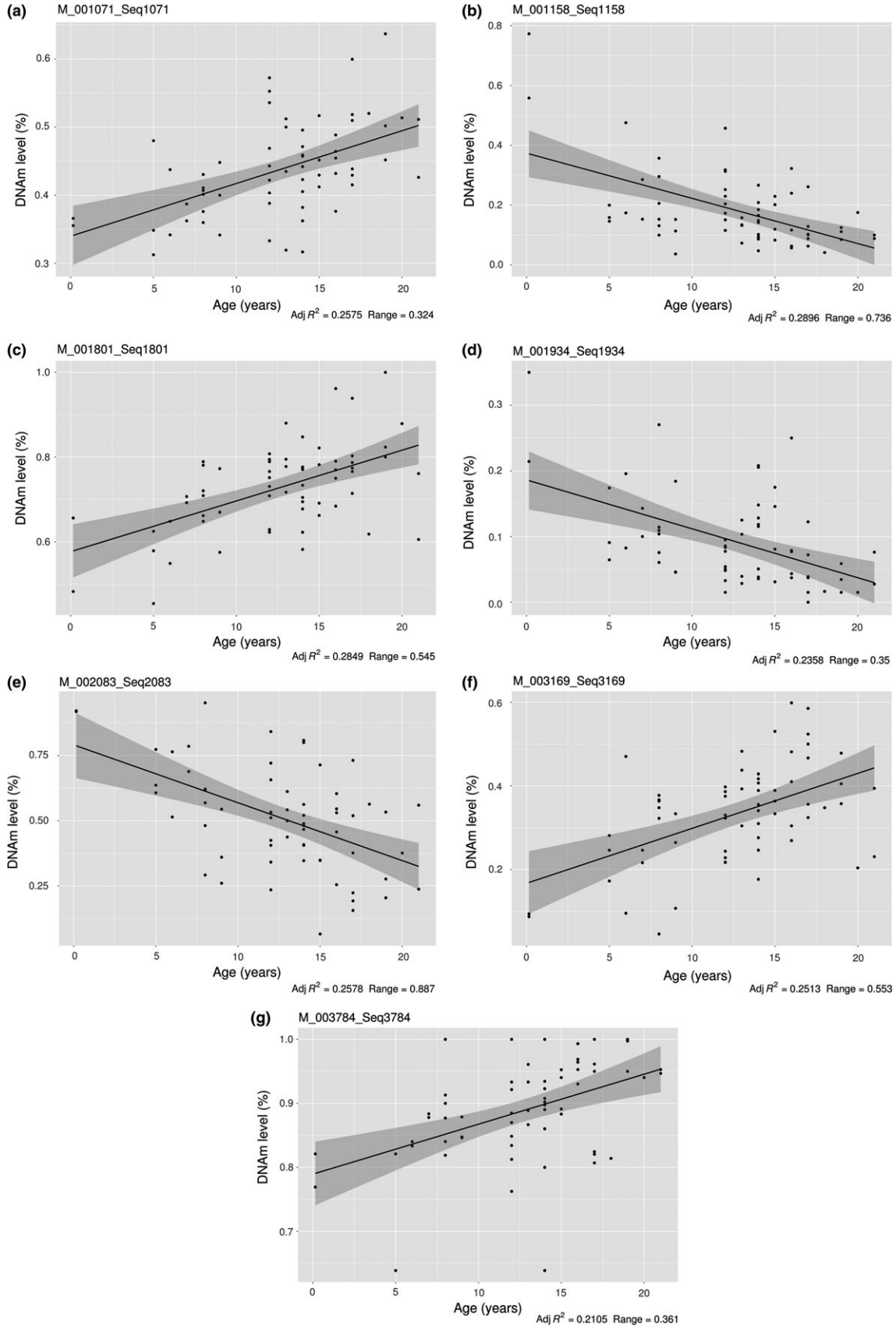

FIGURE 2 (a–g) DNA methylation of selected CpG sites showing a relationship with chronological age. Linear regression of DNAm and chronological age for each CpG selected using lasso penalization from a total of $N = 63$ Short-tailed shearwater blood samples. Sequence details for each CpG are shown in Table 2

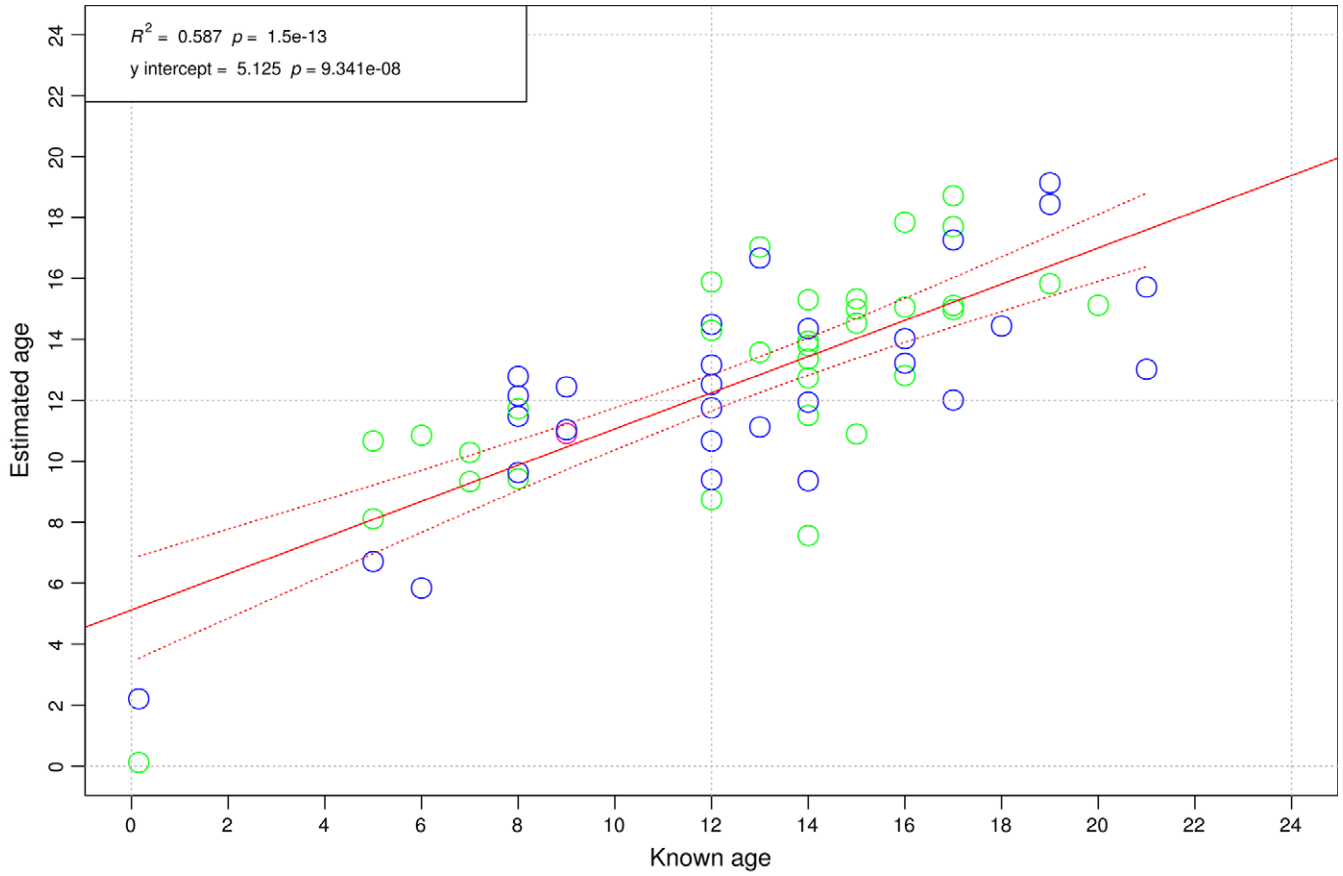

FIGURE 3 Full multiple linear regression model. Multiple linear regressions for predicted ages of *N* = 63 Short-tailed shearwater from quantification of DNA methylation at seven CpG sites. 95% confidence limits of the placement of the regression line are shown. Females are shown in blue (*N* = 30), males are shown in green (*N* = 32), and a single unknown sex (*N* = 1, 9 years old) is shown in pink

predicted age relative to their known age from leg bands (Figure 5). At many individual aDMPs, the longitudinal samples did not follow the expected DNAm trend (Supporting Information Figure S9a-b). However, when combined into the model, only one individual showed a negative change in estimated age from two samples taken at 15 and 17 years of age. The MAD between estimated and known age for 2-year resights was 0.74 years (*N* = 8) and 0.87 years (*N* = 6) for 1-year resights.

3.5 | DNA methylation of 2,338 CpGs using DREAM assay

We show that a large proportion of the 2,338 CpG sites that passed the filtering cut-off are highly methylated, with 50.2% of CpGs showing DNAm levels greater than 80% across all ages (Figure 6a). We also observed a small, but non-significant linear change in DNAm from young animals to old. The mean DNAm was 0.712, 0.724, 0.725 and 0.729, for chicks, young breeders, middle and old birds, respectively (Figure 6b). The difference in mean DNAm, relative to chick levels, for each individual CpG site is shown in Figure 6c. This shows that relative to older birds, chicks are less methylated at low DNAm levels (approximately <10%) and more methylated at high DNAm levels (approximately >90%).

3.6 | Global 5-mC using colorimetric assay

Relative 5-mC was quantified against the supplied 5 ng positive control. Global blood DNAm levels of the Short-tailed shearwater were combined into age groups as described in the methods. Chicks and young breeders showed similar relative 5-mC levels (mean = 0.725, *N* = 4 and mean = 0.727, *N* = 15, respectively). Both of these groups had slightly higher relative 5-mC than that observed in middle-aged birds (mean = 0.614, *N* = 17) and old birds (mean = 0.498, *N* = 5). Following adjustment for multiple comparisons, no significant differences were observed between the age groups (Figure 7).

4 | DISCUSSION

Seabirds exhibit little or no external physical changes with age, and there are currently no reliable biomarkers of chronological age in most long-lived seabirds beyond fledging. The identification of an accurate age biomarker would be a substantial advance in our ability to understand seabird age-related demographics. Seabird age estimation using molecular methods is currently not possible. DNAm changes with age have been reported for both wild and model mammalian species in several tissues, indicating that DNAm age biomarkers may be useful in birds. In this study, we quantified the DNAm profile of known-age

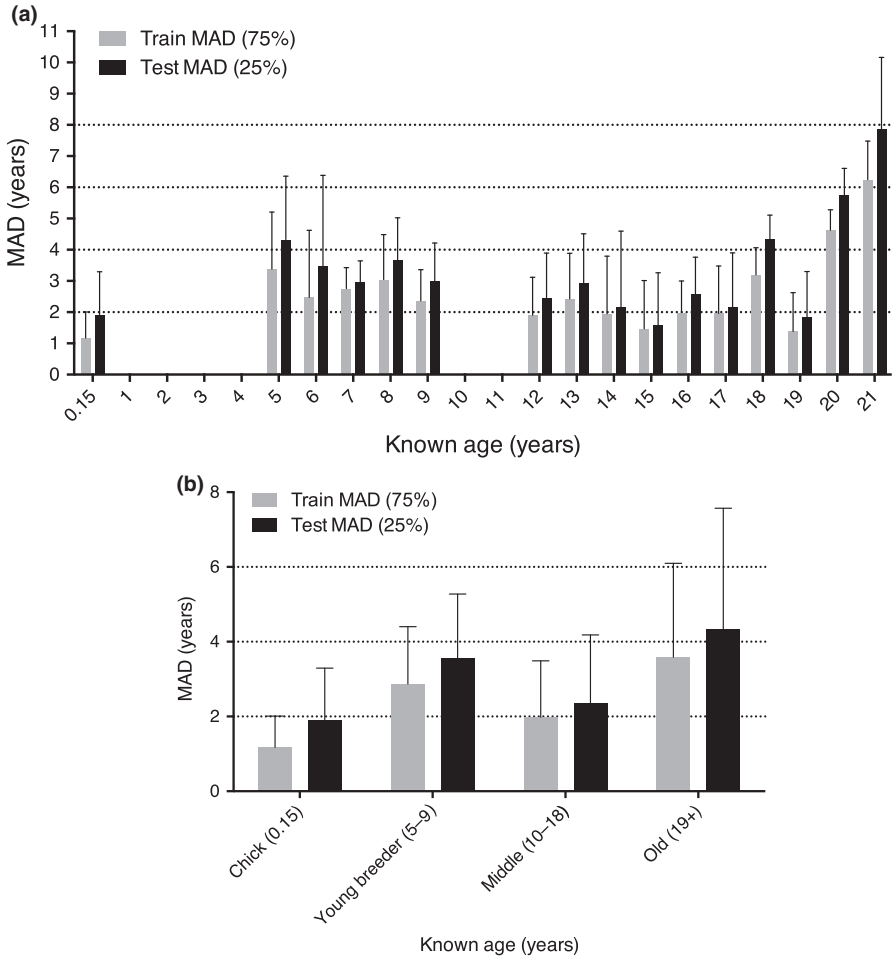

FIGURE 4 Yearly and age-class grouped mean absolute deviation (MAD). Determined by the absolute difference between the estimated and known age, the MADs are shown (a) for each year of age for known-age animals included in the model and (b) for each of the described age groups

Short-tailed shearwaters using DREAM. We present evidence for DNAm changes with chronological age in seven CpG sites.

4.1 | Age-related biomarkers in birds

Previous bird ageing research has focused primarily on telomere length assays and pentosidine accumulation in collagen. Studies of terminal telomere restriction fragments (TRFs) have shown that telomere length can shorten with increasing age and that the rate of change corresponds to lifespan in several species (Bize, Criscuolo, Metcalfe, Nasir, & Monaghan, 2009; Juola, Haussmann, Dearborn, & Vleck, 2006; Tricola et al., 2018). However, this trend is not consistent amongst all birds, with some species showing increases in TRF with age, as in the Leach's storm-petrel (*Oceanodroma leucorhoa*) (Haussmann et al., 2003), and no decline in length, or both as reported for the Magellanic penguin (*Spheniscus magellanicus*) (Cerchiara et al., 2017). For individuals in some avian species, change in telomere length can be tracked longitudinally and correlate with reproductive timing; however, the use of TRF for cross-sectional analysis of age has yet to be demonstrated (Bauer et al., 2018).

Pentosidine is a less frequently studied age biomarker for birds. It forms cross-links between amino acid residues in collagen and accumulates with age in birds (Fallon, Cochrane, Dorr, & Klandorf, 2006; Iqbal, Probert, Alhumadi, & Klandorf, 1999). Pentosidine has been shown to accumulate in a linear fashion in terrestrial birds and some seabirds including California gulls (*Larus californicus*) (Chaney, Blemings, Bonner, & Klandorf, 2003) and Double-crested cormorants (*Phalocrocorax auritus*) (Fallon et al., 2006). This technique has yielded age estimates with a precision of 2–4 years in wild birds (Chaney et al., 2003; Fallon et al., 2006; Rattiste et al., 2015). However, in a study of another long-lived seabird, the Bridled tern (*Onychoprion anaethetus*), no correlation between pentosidine levels and age was found (Labbé, 2017). It is not known how pentosidine levels may respond to the effects of changing biological age or environmental stressors. As a result of the limited success in age estimation by these methods, our research aimed to build upon recent successes in mammals by assessing DNAm estimates of age in the Short-tailed shearwater.

We previously established that specific aDMPs from mammals were not conserved in the shearwater (De Paoli-Iseppi et al., 2017). We therefore sought to identify bird-specific aDMPs or a global DNAm signature associated with age using DREAM of whole blood samples. This is the first epigenetic age assay developed for use in a seabird, and one of the few used in a wild species. Using the DREAM method, we identified seven novel aDMPs in shearwaters. Following repeated cross-validation of our known-age samples to train and test the age estimation model, we reported a test set MAD for all ages of 2.81 ± 2.08 years. The linear relationship with age in these CpG sites

TABLE 2 Age-related CpG site sequences and BLASTn results (birds only)

CpG	Sequence (Illumina NextSeq 77 bp)[a]	Adj. R^2	DNAm range (%)	DNAm direction	Sequence GC content (%)	BLAST species	BLAST region	Query cover (%)	Ident. (%)	*E* value
1071	**CCCGGG**GAACATAACCAGGGCCCGAGGAACTGAACCAGAG CTCCAGGAACAAAACCAGAGCCAGAGGAACTAAACAA	0.258	0.32	Pos	54.5	*Taeniopygia guttata (Zebra finch)*	G3BP1 (mRNA)	51	86	4.10E−02
1158	**CCCGGG**AAGCTGCCAAAAGCGCGAAGCCGCTGGCGG ACGCGGAGGTAAGAGCTGAGAGGGACGTGCCGGTGCCTCG	0.290	0.74	Neg	70.5	*Apteryx australis mantelli (North Island brown kiwi)*	Genomic scaffold 187	46	86	1.40E−01
1801	**CCCGGG**AGATGCCCGGAAATGTAGCCGTGCCGCGGCT GGCCCATCCGGCAGCGCCGTCCCCCGCTCTGCCGGCGTCT	0.285	0.55	Pos	75.3	*Lonchura striata domestica (Society finch)*	AKAP10 (mRNA)	42	88	1.40E−01
1934	**CCCGGG**TTTGAATTACTATTGAATAAGCAGCAATGAAA TCTCTATCAAAATAATCAGTACTTCCAAAAACCACAAAC	0.236	0.35	Neg	33.8	*Anas platyrhynchos (Mallard)*	Uncharacterized LOC106014977 (ncRNA)	100	97	1.00E−28
2083	**CCCGGG**CCCAGGGCCAGCTGCCGGGCTTGGCCCCAGGAGG AGGAGGAGAAGGAGGAGGAAGGCAGGATCTCCAAGGC	0.258	0.89	Neg	71.4	*Apteryx australis mantelli (North Island brown kiwi)*	Genomic scaffold 4,695	64	84	8.00E−05
3169	**CCCGGG**CCGGGGCCCGGTGGGGCGGCGGGTTGGGGCG GCGGCAGCTCTCGCCGCTGCTCTACAAGCAGTTCGTGC	0.251	0.55	Pos	79.2	*Lepidothrix coronata (Blue-crowned manakin)*	DHH (mRNA)	100	99	8.00E−30
3784	**CCCGGG**GACCGGGTGCACATCTGGGCTGAGAGATGCTG AAAGCAAAGAGCAGCCGGAGCGCTGGGAGAGCGGTGGGA	0.211	0.36	Pos	66.2	*Cyanistes caeruleus (Eurasian blue tit)*	ANAPC16 (mRNA)	35	93	1.40E−01

[a]Restriction site is shown in bold with CpG sites underlined.

is not as strong as those reported for whales (Polanowski, Robbins, Chandler, & Jarman, 2014) or dogs (Thompson, vonHoldt, Horvath, & Pellegrini, 2017), but was similar to that reported for a bat species (Wright et al., 2018). We also observed variation in MAD for different age classes, with birds aged 5–9 years and 19+ providing less accurate age estimates compared to other groups (Figure 4a). Additionally, the significant y-intercept in our model (Figure 3) causes an overestimation of age in younger individuals. A single marker (M1801) showed evidence for male-driven DNAm age correlation. Due to the reduced sample size when comparing by sex only, more known-age samples would be required to confirm the lack of association in females and ideally, whole genome information could determine if this marker is located on a sex chromosome.

However, the biggest limitation in developing our model was the low number of young non-breeding bird samples that we could capture in the field, which hinders our understanding of the rate of DNAm change between chicks and early breeders (5–9 years), and with more samples, this may be correctable in future. The shearwaters studied here typically do not return to their island of birth until their first year of breeding at age five (Bradley et al., 1991; Bradley, Wooller, Skira, & Serventy, 1989). However, for unknown reasons we did not recover many individuals in the 5–9 early breeder age range. The larger DNAm variability in these young animals could be due to the stressful effects of the first year of breeding. Shearwaters lay one of the largest eggs relative to body mass of all seabirds, and individuals face challenges including incubatory fasting and intermittent foraging (Wooller, Bradley, Skira, & Serventy, 1990). Additionally, both migration and parenthood can reduce body condition, and evidence suggests that these birds may undergo intermittent breeding if an individual determines its body condition is too low (Bradley, Wooller, & Skira, 2000).

Despite some uncertainty in ages estimated with our model, this approach could discriminate between relevant age classes (e.g., young and old adults). These epigenetic age estimates, in combination with other parameters including sex and weight, could be used to examine the effect of climate change on population viability (Lee, 2017). Recent studies also highlight other areas in which estimated age data could be informative, including post-pest eradication monitoring of island-breeding seabird populations (Brooke et al., 2018), parasite load in the Blue tit (Aguilar et al., 2016) and modelling the impacts of longline fisheries on effective population size (Cortés, García-Barcelona, & González-Solís, 2018; Mills & Ryan, 2005).

Obtaining a broad age range of samples from long-lived, known-age birds is difficult as extensive banding studies are rare. Whilst the Fisher Island shearwater population has been followed for several decades, the youngest and oldest adult individuals we recovered were 5 and 21 years old, respectively. The oldest individual, at 21 years old, represents a little over half of the maximum reported lifespan for this species of 39 years. However, research on age-dependent survival on Fisher Island birds shows few animals living beyond 25 years post-first breeding, which would place our oldest individual at closer to 70% of the expected lifespan of approximately 30 years (Baylis, Sunnucks, & Clarke, 2018; Bradley et al., 1989). The

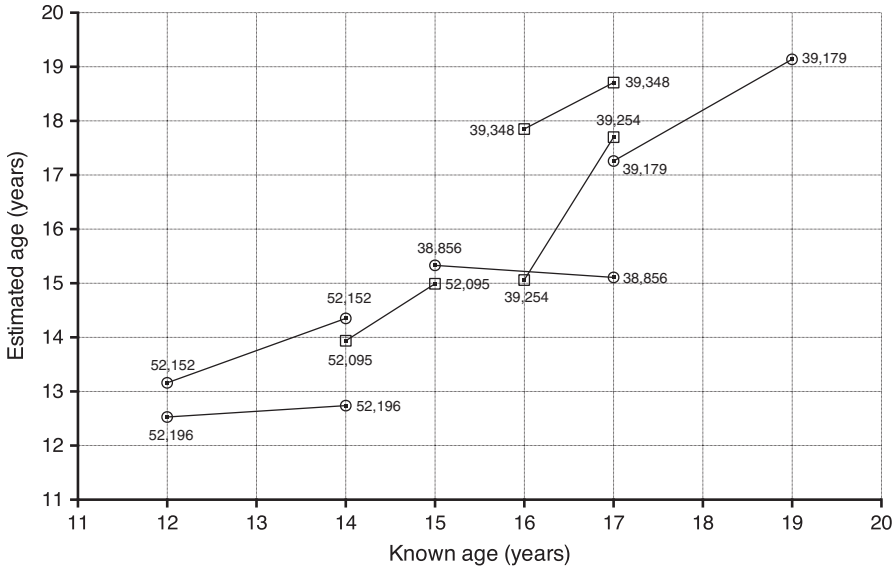

FIGURE 5 Longitudinal DNAm data for 1- and 2-year resights. The estimated epigenetic age vs. known age (from leg bands) for each individual bird with longitudinal resights (1-year $N = 3$; 2-year $N = 4$)

relationship we have observed with age should be investigated further for older individuals; however, previous studies in mammals have primarily shown linear correlations with age (Maegawa et al., 2010; Polanowski et al., 2014; Spiers et al., 2016). Although no recaptures were made within the 1- to 4-year age range, as these non-breeding birds are not at the nesting sites, the relationship of adults to the DNAm level of the chicks suggests birds at these ages will have a similar trend to the rest of the calibration range.

We quantified "epigenetic drift" in DNAm levels observed across all 2,338 CpG sites included in our analysis. We did not identify a significant trend with chronological age. However, we did observe some interesting differences between young and old age groups at the lower and upper limits of DNAm. In contrast to mammalian and the only other bird study, we found no clear trend of DNA hypomethylation in older animals compared to that in younger individuals (Gaudet et al., 2003; Gryzinska et al., 2016; Portela & Esteller, 2010). The lack of statistical significance could be due to the analysis of this relatively small subset of total CpGs in the bird genome.

Immunoenzymatic analyses of chicken 5-mC levels have shown decreased global methylation with age (Gryzinska et al., 2013). Using the same method, we found no relationship between relative 5-mC levels and age in 42 known-age shearwater whole blood samples. However, we observed a non-significant trend towards decreasing methylation across age groups. Our study of age-related global DNAm in shearwaters is only the second of this phenomenon in birds, and further work will be required to determine whether this approach could be suitable for age estimation in other bird species.

4.2 | Measuring methylation in non-model organisms

Despite the identification of several thousand unique CpG sites using the DREAM method, the 20× read depth requirement for DNAm calculation resulted in the exclusion of many sites from further analysis. A small percentage of the total reads was also lost to repetitive elements. There is little doubt that as technologies improve sequencing depths will increase, and direct analysis of CpG DNAm will be possible (Rand et al., 2017; Slatko, Gardner, & Ausubel, 2018). Improvements in bioinformatics will also help to validate DNAm markers and predict age in large data sets (Vidaki, Ballard, Aliferi, Miller, & Barron, 2017). The DREAM technique has been used previously to identify DNAm changes following compound exposure in zebrafish embryos (Bouwmeester et al., 2016) and caloric restriction in mice (Maegawa et al., 2017). A similar method, EpiRADSeq, also uses a methylation-sensitive restriction enzyme (*HpaII*) and NGS to quantify DNAm in CpG sites (Schield et al., 2016). This technique differs from DREAM in that only a single methylation-sensitive enzyme is used in combination with a frequent cutter (*PstI*). *HpaII* recognizes a "CCGG" motif, which is likely to lead to higher genomic coverage of CpG sites due to increased cut frequency. However, DNAm scores generated using this method are relative to the count of unmethylated EpiRADSeq reads only. This is avoided when using a dual methylation-sensitive digest as in DREAM, as reads are generated for both methylated and unmethylated CpGs (Jelinek & Madzo, 2016). Reduced representation bisulphite sequencing can also be used to quantify CpG DNAm, but does require a higher quantity of initial genomic DNA (Meissner et al., 2005). The output of these various techniques depends upon several molecular, platform and bioinformatic factors and choices, which is discussed in detail elsewhere (O'Leary, Puritz, Willis, Hollenbeck, & Portnoy, 2018). Our results now show that the DREAM method can also be used to quantify global DNAm and screen for aDMPs in non-model animals. The primary limitation in applying this method is the high read depth required per CpG site, particularly in organisms with relatively high quantities of repetitive DNA. This makes it cost-prohibitive as a method for applying to population-wide samples, but certainly effective as a screening method for identifying aDMPs.

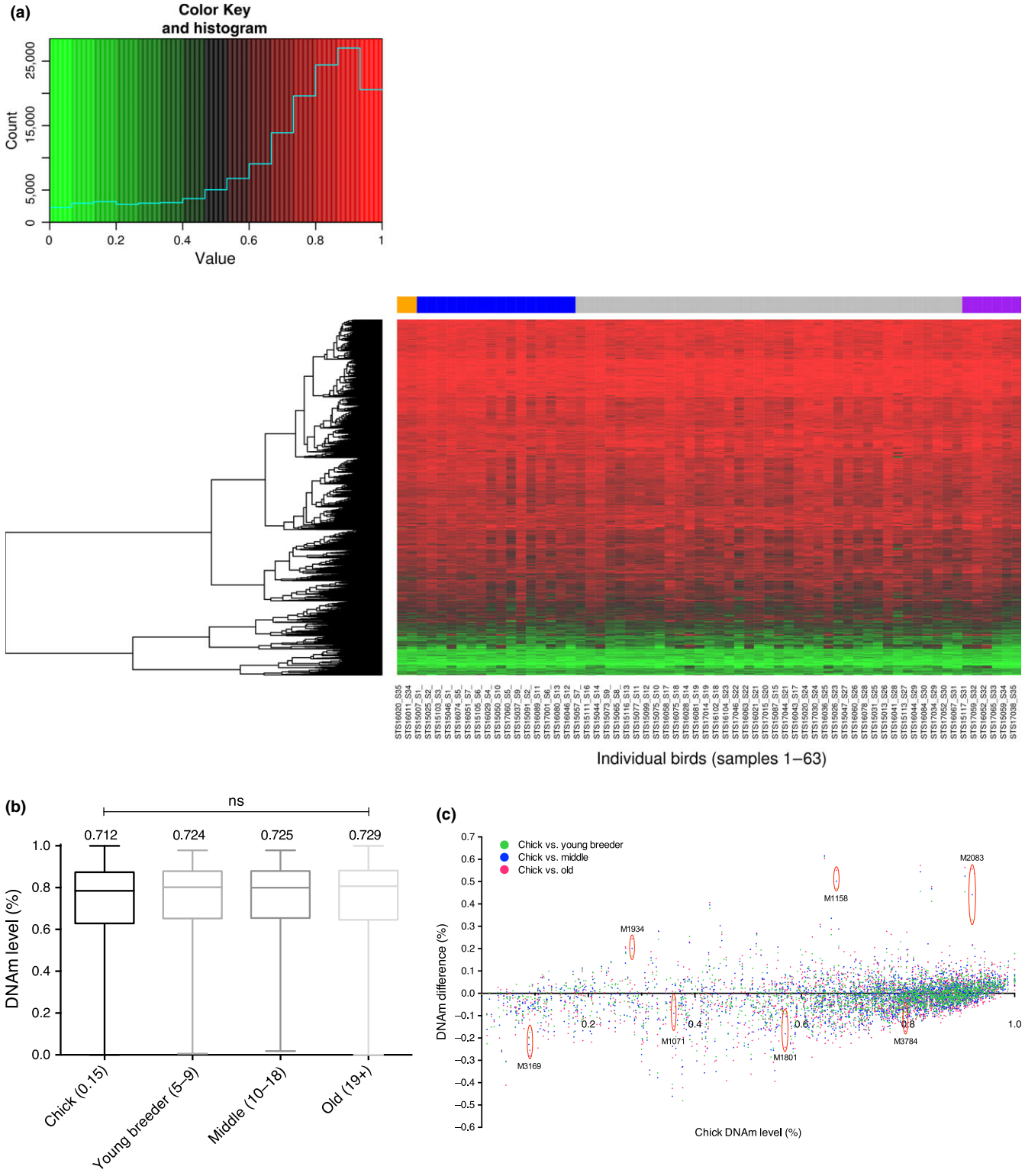

FIGURE 6 DNA methylation in chicks, young, middle and old shearwater. (a) Heatmap of 2,338 CpGs analysed using DREAM in shearwater. Age groups are defined by column header colours, chicks are shown in orange, young breeders (5–9 years) in blue, middle (10–18 years) in grey and old (19+ years) in purple. A colour key indicates the DNAm value ranging from 0 (unmethylated, green) to 1 (methylated, red). (b) The mean DNA methylation (DNAm) level of each of 2,338 CpG sites (N = 63 birds) for each age group: chicks (N = 2), young breeders (5–9 years, N = 16), middle-aged (10–18 years, N = 39) and old (19+ years, N = 6). A post-ANOVA test for linear trend did not indicate that the small increase in DNAm was significant. (c) The difference in mean DNAm for each CpG in a chick context (high to low) vs. older age groups, that is a positive value indicates higher DNAm in chicks and vice versa. Age-related CpGs used in the model are circled in red and are labelled as shown in Figure 2

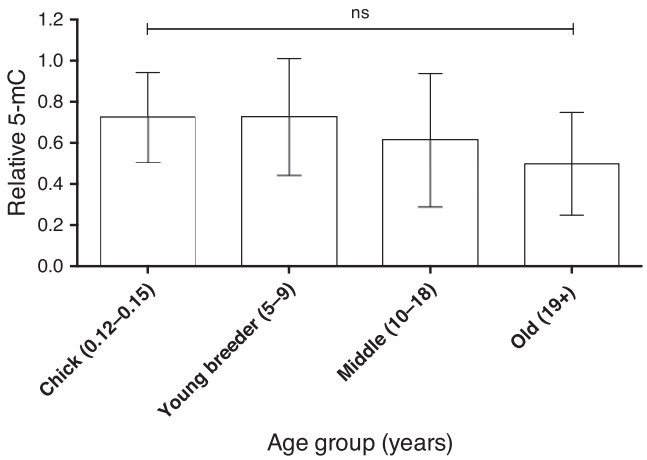

FIGURE 7 Global 5-mC of known-age shearwater using colorimetric assay. Relative global 5-mC content was assayed using a colorimetric-based assay and analysed with a plate reader. A trend towards global hypomethylation was observed in $N = 42$ known-age Short-tailed shearwater; however, this result was not significant following correction for multiple comparisons ($p > 0.05$)

Once aDMPs are identified by DREAM, targeted DNAm scoring assays could be developed to reduce costs for high-throughput applications.

An additional limitation to the simple analysis of shearwater DREAM and indeed most non-model NGS data is the limited genomic resources available for further analyses. Multiplex restriction site PCR (mRS-PCR) could be used to obtain both up- and downstream sequence around an aDMP of interest (Sarkar, Turner, & Bolander, 1993; Weber, Bolander, & Sarkab, 1998). This method can generate larger reference sequences for use in targeted bisulphite assays such as EpiTYPER, pyrosequencing or other NGS-based techniques (Ehrich et al., 2005). More sequence information may also result in more accurate comparative genomic analyses against bird genomes that are currently undergoing scaffold alignment. The genes *DHH* and *G3BP1* were identified as conserved age-related sequences from our data, and these could be used in future as part of a targeted gene assay in shearwater (Table 2; M1071 and M3169). Whilst we cannot comment on any potential functional effects of DNAm, *DHH* and *G3BP1* encode for signalling molecules in cell morphogenesis and a DNA-unwinding enzyme, respectively. Two other markers also showed high conservation with other bird species; however, these hits were either unassigned (M2083) or uncharacterized (M1934). These factors limit our ability to identify biomarkers that have the potential to be used in closely related species, and design a cost-effective, targeted age assay.

5 | CONCLUSIONS

This study demonstrates that seabird age estimates can be generated from a DNAm age assay. This minimally invasive method could be used to produce age estimates for Short-tailed shearwaters from chicks to 21 years old. This is the first time an epigenetic assay has been applied to a wild seabird and could be used in future to estimate population age structure. Further refinement of this method could result in the identification, validation and use of target genes, similar to that in mammals, for related seabird species and see wider use for monitoring and conservation.

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AUTHOR CONTRIBUTIONS

All authors conceived the ideas and designed methodology; R.D.P., A.M.P., C.R.M. and M.A.H. collected samples; R.D.P. and A.M.P. did the genetics laboratory work; R.D.P., B.E.D. and S.N.J. analysed the data; and R.D.P. led manuscript writing. All authors contributed to drafts and gave final approval for publication.

DATA ACCESSIBILITY

DREAM count data, adjusted DNAm values for 2,338 CpGs, fasta pipeline and variable selection R scripts used in this publication have been deposited in the Dryad Digital Repository at [https://doi.org/10.5061/dryad.n4h3672]. Sample details and raw Illumina sequence data (FASTQ) are available from NCBI/SRA using accession: PRJNA507458, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA507458.

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SUPPORTING INFORMATION

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

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