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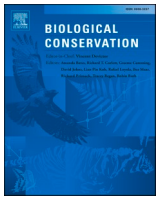

Estimating age and investigating epigenetic changes related to health across multiple bottlenose dolphin populations

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ABSTRACT

Epigenetic age estimation has the potential to influence conservation approaches for wildlife. DNA extracted from a small skin sample can be analyzed for methylation changes, which can be related to chronological age through an epigenetic clock to obtain a DNA-based age estimate (DNAm age). For a wide range of cetacean species, skin samples can be obtained remotely using dart biopsy. We tested multiple modeling approaches (elastic net regression, random forest regression, and a hybrid of elastic net and random forest classification) using DNA methylation data from 426 skin samples from observed-age wild bottlenose dolphins (*Tursiops* spp.), and 50 managed care dolphins, to develop an epigenetic clock. The hybrid model gave highly accurate age predictions for calf (<2 yrs., n = 28), subadult (2–15 yrs., n = 273) and adult (15–25 yrs., n = 110) age classes with median absolute errors (MAE) of 0.149, 1.46 and 3.25 yrs., respectively. In the oldest individuals (>25 yrs., n = 65), age predictions were less accurate, with MAE of 5.90 yrs. The overall MAE across all ages (n = 476) was 1.91 yrs. DNAm age has been linked to health in humans, with higher age residuals (i.e., higher DNAm age as compared to actual age) being associated with increased risk of morbidity and mortality. We compared dolphin age residuals with previously reported health scores that are predictive of one year mortality risk. Our findings suggest that analysis of DNA methylation patterns is a viable approach, not only for estimating age of dolphins, but potentially also to assess individual and population health.

1. Introduction

Analysis of DNA methylation is an epigenetic tool that can provide important information for wildlife research and conservation. DNA methylation involves the addition of methyl groups (CH_3) primarily to cytosine-phosphate-guanine (CpG) sites within the DNA. The degree of methylation of CpG sites has been shown to correlate closely with chronological age (time since birth), enabling the development of epigenetic aging clocks—predictive models where the degree of methylation of specific CpG sites is used to estimate age (Horvath and Raj, 2018). Accurate estimates of chronological age can be important for interpreting individual biological information such as physical exam findings, blood values, and morphometrics, and for understanding behavior and population demographics. Epigenetic aging clocks have been developed for multiple cetacean species (Polanowski et al., 2014; Beal et al., 2019; Robeck et al., 2021a, 2021b; Parsons et al., 2023). Those from blood samples have been shown to be more accurate than those from skin (Robeck et al., 2021a), but blood collection currently

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requires hands-on sampling, and thus is only feasible from animals in managed care or a limited number of small cetacean populations that are amenable to temporary capture. By contrast, skin samples can be obtained for most cetacean species via remote biopsy techniques (Noren and Mocklin, 2011).

A second important application for the analysis of DNA methylation patterns to wildlife conservation is the association of shifts in methylation with environmental or physiological stress (Rey et al., 2020). Human studies have demonstrated it is possible to detect epigenetic changes that may signal that an individual is physiologically compromised, such that survival or reproduction is impaired (Simpson and Chandra, 2021). Age estimated from DNA methylation (DNAm age) is termed "biological age", and can provide a proxy for health status. Positive age residuals, i.e., when an individual's biological age is greater than their chronological age, has been referred to as "age acceleration", and has been associated with increased risk of morbidity and mortality in people with multiple studies demonstrating this (Jain et al., 2022; Levine et al., 2018; Perna et al., 2016; Horvath, 2013). In humans, differences in biological age have been associated with lifestyle: increased DNAm age has been demonstrated for individuals with low activity levels, excessive alcohol consumption, or who smoke tobacco (Gao et al., 2016; Lind et al., 2018; Mamoshina et al., 2019; Perna et al., 2016; Zeilinger et al., 2013). In wildlife populations, assessment of age residuals could provide a tool to understand the impact of environmental stressors at both individual and population levels. Studies of cetaceans to date have either used samples from animals in managed care (Robeck et al., 2021a; Robeck et al., 2021b) or have been based on wild populations without inclusion of paired information on the health of the sampled individuals (e.g., Bors et al., 2020; Crossman et al., 2021; Parsons et al., 2023). Therefore, the ability to assess the relationship between health and DNA methylation patterns has been limited.

Bottlenose dolphins (*Tursiops* spp.) are considered a model species for understanding cetacean health, and for studying variation in aging rates in long-lived species (Schwacke et al., 2023; Venn-Watson et al., 2020). Substantial knowledge of bottlenose dolphin biology and physiology has been amassed through a focus on advanced veterinary care for populations in managed care (Houser et al., 2010; Venn-Watson et al., 2011), as well as hands-on veterinary health assessments and tagging studies made possible by the accessibility of nearshore populations (Barratclough et al., 2019; Wells, 2014; Wells et al., 2004). A recent study developed bottlenose dolphin health scores using a suite of health indices including blood-based markers, body mass index, and ultrasound examinations collected during hands-on veterinary assessments of free-ranging dolphins (Schwacke et al., 2023). Health scores range from 0.0 to 1.0 and represent a dolphin's 1–2 year survival probability. The health scores can be used to inform population demographics with improved understanding of the predicted individual survival and subsequent contribution to the population.

Our research builds on the value of bottlenose dolphins as a model species, using a robust sample size of skin biopsies from known-age individuals. The samples were previously collected from catch-and-release health assessment studies across eight free-ranging populations, providing an opportunity to enhance how DNA methylation analyses may contribute to an understanding not only of population demographics, but also population health. Specifically, we 1) produce a robust species-specific epigenetic clock to estimate chronological age based on skin samples, and 2) examine the association between age residuals and previously derived health scores from the same individuals.

2. Methods

2.1. Sample collection and DNA extraction

Skin samples (n = 426) from free-ranging bottlenose dolphins (Table 1) were obtained as part of catch-and-release health assessment studies or remote biopsy sampling. Samples were obtained between 1991 and 2022 and stored in a 20 % dimethyl sulfoxide (DMSO) solution saturated with sodium chloride. The samples were collected from eight management stocks. Four of Tamanend's bottlenose dolphin (*Tursiops erebennus*): the Charleston Estuarine System Stock near Charleston, South Carolina (CHS), the Central Georgia Estuarine System Stock near Sapelo, Georgia, the Southern Georgia Estuarine System Stock near Brunswick, Georgia, and the Indian River Lagoon Estuarine System Stock from the Florida east coast (IRL); and four of common bottlenose dolphin (*Tursiops truncatus*): the Sarasota Bay/Little Sarasota Bay Stock (SAR) and the St. Joseph Bay Stock (SJB) from the Florida west coast, the Mississippi Sound/Lake Borgne/Bay Boudreau Stock in Mississippi (MSS), and the Barataria Bay Estuarine System Stock in Louisiana (BAR). Due to limited sample numbers, samples from the Central and Southern Georgia Estuarine System Stocks were combined (SGE).

The samples were selected from free-ranging dolphins of known chronological age (n = 87; known birth date or observation as a calf during photo-identification studies) (Speakman et al., 2006), dolphins with an estimated age from dentinal analysis of growth layer groups (GLGs; n = 280) (Hohn et al., 1989), or from dolphins with an estimated age from dental radiography (n = 59) (Herrman et al., 2020). Hereafter, we refer to the samples with age information as "observed age" rather than actual age due to the varying degree of accuracy of the different age estimation methods. Samples were from 389 unique individuals, with 37 samples being duplicates from the same individuals sampled over multiple years. Two individuals were sampled over three different years, and one individual was sampled over four years.

DNA had been previously extracted from skin for some of the samples (n = 299) using a phenol-chloroform protocol as described in Rosel and Block (1996) or using a DNeasy Blood and Tissue Kit (Qiagen) following manufacturer's protocols and was archived at the NOAA Southeast Fisheries Science Center, Marine Mammal Molecular Genomics Laboratory in Lafayette, Louisiana (Rosel and Block, 1996). DNA was extracted from skin for this project for the remaining samples (n = 127), using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as above. DNA quality was assessed via gel electrophoresis and DNA

Table 1
Number of samples from each location and the method used to infer age: GLG = growth layer group in tooth, LH = life history, RAD = tooth radiograph age. The locations are: BAR = Barataria Bay, Louisiana, CHS = Charleston, South Carolina, IRL = Indian River Lagoon, Florida, MSS = Mississippi Sound, Mississippi, SGE = the combined South Georgia Estuary Stocks, SAR = Sarasota, Florida, SJB = St. Joe Bay, Florida, USN = United States Navy, San Diego.

Location	# samples	# GLG	# LH	# RAD	Age range (years)	Species	Included in training
BAR	111	44	8	59	1.3–42	*T. truncatus*	No
SGE	21	20	1	0	4.4–36	*T. erebennus*	No
CHS	65	65	0	0	2.0–33	*T. erebennus*	Yes
IRL	86	86	0	0	3.5–23	*T. erebennus*	Yes
MSS	11	11	0	0	5.5–30	*T. truncatus*	No
SAR	112	34	78	0	1.7–50	*T. truncatus*	Yes
SJB	20	20	0	0	2.5–32	*T. truncatus*	No
USN	50	10	40	0	0–58	*T. truncatus*	Yes
Total	476						

concentration was determined using a Qubit4 fluorometer (Invitrogen) or a Hoefer DyNAQuant 200 fluorometer (GE Healthcare). For each sample, approximately 300 ng of DNA was loaded into a 96-well plate and dried down using a SpeedVac (Savant); when dry, plates were sealed and submitted to The Clock Foundation (Torrance, CA) for DNA methylation analysis.

An additional 50 samples were obtained from dolphins at the U.S. Navy (USN) Marine Mammal Program as an additional reference population (Fig. 1), including 9 skin samples previously analyzed and reported (Barratclough et al., 2021). DNA for these 50 samples were extracted (using the DNeasy Blood and Qiagen Tissue Kit) and plated at The Clock Foundation. Of these 50 samples, 40 were from individuals born at the Navy facility, providing exact known age. The primary reason to include the Navy samples was the large number of exact known age dolphins and the wide age range (up to 58 years of age), providing additional samples for the older cohort (>25 yrs) which were lacking in the wild stock studies. All 50 of the Navy dolphin samples were from unique individuals.

2.2. DNA methylation analyses

DNA methylation was measured using a custom mammalian methylation array (HorvathMammalMethylChip40) with 37,492 CpG sites (Arneson et al., 2022). A beta value was created for each CpG site by obtaining the ratio of the fluorescence intensity of a methylated probe for that specific site to the total overall probe intensity (Du et al., 2010). The SeSAMe pipeline was used to normalize the raw data and produce an estimate of the degree of methylation, for each CpG site for each individual (Zhou et al., 2018). A detection p-value was also produced from the pipeline to accompany each normalized beta value: the p-value for a given CpG site can be interpreted as the probability that the signal overlaps with the background distribution of fluorescence (Zhou et al., 2018). Beta values range from zero to one, with zero indicating no methylation at that CpG site (Zhou et al., 2018).

2.3. Epigenetic clock for age prediction

To train the epigenetic clock for age prediction, we included data from the U.S. Navy dolphins, and from the CHS, SAR, and IRL studies populations (Table 1; Fig. 1). We did not include data from the remaining populations to train the clock because environmental stressors can alter DNA methylation processes and those populations were sampled as part of unusual mortality events or chemical spill investigations and thus maybe affected by additional environmental stress factors (for discussion see Schwacke et al., 2023). For example, dolphins in BAR and MSS were sampled as part of the injury assessment in the years following the *Deepwater Horizon* (DWH) oil spill.

As part of training the clock, we applied a 5-fold cross validation, dividing the dataset into five random subsets, each with 20 % of the samples. For dolphins sampled over multiple years, we assigned all of the individual's samples to the same cross-validation fold. Performance of the clock was evaluated by iteratively leaving out each of the five subsets, and then calculating the DNAm ages for the omitted samples. We calculated median and quartiles for absolute error (absolute difference between DNAm age and observed age). As error can vary by age, we stratified by age class using the following classifications: calf (age $\leq$ 2 years); subadult (2 $<$ age $\leq$ 15 years); adult (15 $<$ age $\leq$ 25 years); older (age $>$ 25 years). Age 15 was used as the delineation between subadult and adult age classes based on the assumption of both sexual and physical maturity at this age, for both female and male based on previous growth model analyses (McFee et al., 2010; Read et al., 1993). The threshold for the older age class ($>$ 25 years) was based on documentation of reproductive senescence in older ages (Karniski et al., 2018). Error in age estimation can be expected to increase with age, and the age distribution of samples was skewed with a greater number of samples available from younger dolphins (Table 2) (Barratclough et al., 2023). Therefore, we applied a log transformation to the age data prior to fitting the model. Following the 5-fold cross-validation, we estimated a final clock using all samples from all five subsets. We applied this final model to estimate DNA methylation ages for the remaining sites (SGE,

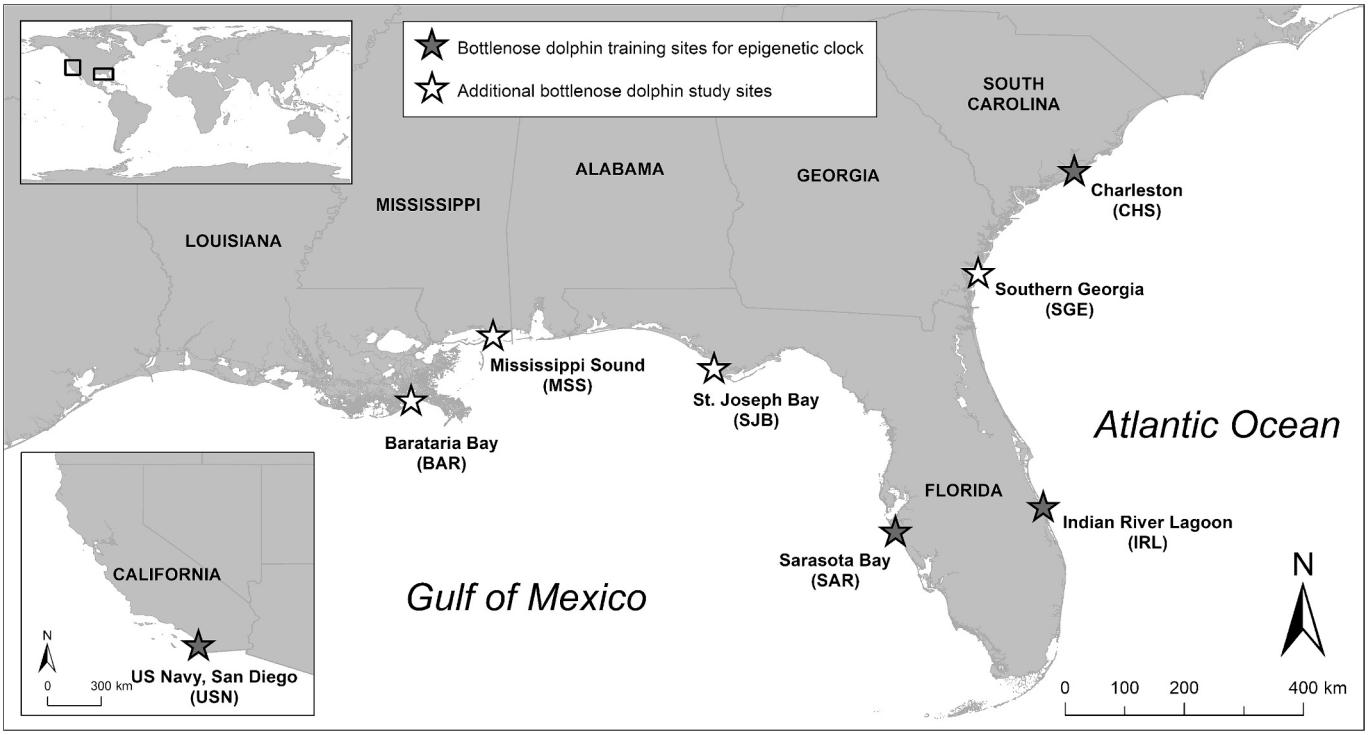

Fig. 1. Geographical locations where bottlenose dolphin skin samples were collected. Green stars indicate locations from which samples were included to train the epigenetic clock; white stars indicate locations where dolphins were sampled as part of unusual mortality event (UME) or chemical release investigations and were excluded for training the clock. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Median absolute error in years (lower quartile, upper quartile) of DNAm age stratified by age class from elastic net regression (ENR), random forest regression (RFR), and hybrid modeling approaches. Age classes are defined as ≤2 calf, 2 < subadult ≤15, 15 < adult ≤25, older >25 yrs.

Fitting method	Calf n = 28	Subadult n = 273	Adult n = 110	Older n = 65	Overall ages n = 476
ENR	0.11 (0.05,0.29)	1.41 (0.64,3.18)	4.11 (2.36,7.09)	6.4 (3.28,13.00)	2.17 (0.80,4.84)
RFR	0.43 (0.14,0.97)	1.74 (0.70,2.78)	2.94 (1.63,4.70)	10.14 (6.75,14.57)	2.16 (0.91,4.61)
Hybrid	0.149 (0.047,0.284)	1.46 (0.606,3.25)	3.25 (1.43,5.07)	5.90 (2.18,9.31)	1.91 (0.80,4.29)

SJB, MSS, and BAR).

We investigated multiple methods for fitting the epigenetic clock to the training data set. We first applied an elastic net regression (ENR), setting the alpha parameter to alpha = 0.12 as previously estimated by Barratclough et al. (2021), and determined the lambda penalty parameter using a leave-one-out cross validation. Consistent with previously reported results using ENR-fitted epigenetic clocks for skin samples, we found greater error in estimates for older age classes (Robeck et al., 2021a). We also investigated a non-parametric random forest regression (RFR). The RFR also performed well for calf, subadult, and adult age classes, but errors were large and negatively biased for the older age class. Therefore, we developed a hybrid model as described below for training the epigenetic clock.

Our hybrid approach for age prediction integrated a random forest classifier (RFC) and two ENR models. For the RFC, we set the number of trees to 1000. We fit one ENR model for the full spectrum of sample ages, and a second ENR using only samples from dolphins that were above the age of physical maturity (>15 years). We used alpha = 0.12 for both ENR models, and the lambda penalty parameter was determined using a leave-one-out cross validation. We first applied the RFC to classify dolphins as at or above the age of physical maturity. For dolphins classified as being below the age of physical maturity with a probability >0.50, age was predicted from the first ENR model. Ages for dolphins classified as being above the age of physical maturity were estimated as a weighted sum of the ages predicted from both ENR models with the weighting for the second model (using only physically mature animals) being the probability of being physically mature as estimated using the RFC, and the weighting for the first model being the complement of this probability. We calculated actual error as the difference between DNAm age and observed age, and absolute error as the absolute value of actual error. We additionally calculated proportional error as the difference between DNAm age and observed age, divided by the observed age. Results are reported as median absolute error (MAE) and median proportional error (MPE). We checked model fit by examining actual error by age class, fitting a generalized linear model (GLM) to actual error with age class as a factor.

For training both ENR and RFC/RFR clocks, we assigned sample weights based on the method used for estimation of chronological age. Our goal was to down-weight samples for which age was less certain. Samples from dolphins with ages derived from life history information (either known birth dates or observed as a young calf) were given weights of 1.0. Samples from Navy dolphins with unknown birth dates were given weights <1, based on the proportion of age estimated:

$$weight = 1 - \frac{age\ estimate\ at\ acquisition}{current\ age}.$$

Samples from free-swimming dolphins whose age was estimated via GLG were given weights <1 based on the expected error associated with the GLG age class, specifically the root mean squared error (RMSE). We used RMSE, which squares errors before averaging, rather than MAE because we wanted to give a relatively high penalty for large errors. The RMSE values used for GLG aging were 1.46, 6.34, and 17.0 yrs. for dolphins <16, 16–30 yrs. and > 30 yrs., respectively (Barratclough et al., 2023). For animals aged by GLG for the first time when the skin sample was taken for methylation analysis, the weight was calculated as

$$weight = 1 - \frac{RMSE}{estimated\ age}.$$

For animals aged by GLG at some previous time, the weight was increased to reflect the fact that some portion of their age was known:

$$weight = \left(\left(1 - \frac{RMSE}{estimated\ age}\right) \times \frac{estimated\ age}{current\ age}\right) + \frac{current\ age - estimated\ age}{current\ age},$$

where "estimated age" is the estimate at the time the GLG was taken, and "current age" is the estimated age plus the intervening time interval. None of the samples used for training had ages estimated via radiograph, therefore weighting was not needed for the radiograph samples.

All statistical analyses were conducted in R (R Core Team, 2023), using the glmnet package version 4.1–7 (Friedman et al., 2010) for ENR analyses and Ranger package version 0.15.1 (Liaw and Wiener, 2002) for RFC and RFR analyses.

2.4. Functional significance of predictive CpG sites

One advantage of RFR is the automatic calculation of the relative importance for each predictor variable. Variable importance is computed from permuting the out-of-bag data and represents how much the mean square error increases when the given variable is left out. We used the variable importance produced by the RFR analysis to investigate the contribution from the various CpG sites in predicting ages. We selected the CpG sites with a variable importance >0.001 for gene-based ontology enrichment analysis using the Genomic Regions Enrichment of Annotations Tool (GREAT) (McLean et al., 2010) with the Horvath-MammalMethylChip40 library provided as an explicit background set species assembly (Human: GRCh37 (UCSC hg19, Feb. 2009) and GREAT version 4.0.4). GREAT identifies overrepresented nearby annotated genes, and furthermore gene ontologies, for a set of genomic locations (CpGs in our case).

2.5. Analysis of age residuals and health

We modeled the relationship between DNAm age (response variable, assumed normally distributed) and observed age using a generalized additive model (GAM) with thin plate regression spline, basis dimension 4. We then calculated the residuals from the modeled fit, and used these calculated age residuals for two additional analyses. The purpose for using the residuals from the fitted GAM rather than the actual error (i.e., difference between DNAm age and observed age) was to discount any potential systematic change in residuals with age. GAM analyses used the gam function from the mgcv package in R (Wood, 2011).

Our first analysis examined the relationship of age residuals with health scores previously derived by Schwacke et al. (2023). These scores came from health data collected during the same catch-and-release studies as those where the skin samples were taken for epigenetic analysis. The data included markers of inflammation (neutrophil count, albumin, globulin, alkaline phosphatase, hemoglobin), metabolism (body mass index, cholesterol, glucose), neuroendocrine status (cortisol, triiodothyronine) and lung disease (scored from ultrasound). The health data were paired with known or estimated one-year survival outcomes

by Schwacke et al. (2023) and used to develop a statistical model, the "Veterinary Expert System for Outcome Prediction" (VESOP). VESOP produces health scores for each dolphin that range from 0.0 to 1.0 representing the dolphin's predicted one year survival probability(two year survival scores were also developed but were not used here). A total of 768 individuals were used to develop the VESOP model, and health scores were available for 401 samples for which we had epigenetic age and age residuals. Here, we modeled the health scores using a GAM for beta-distributed data (beta family with a logit link function) with geographic sampling site as a random effect and age residual as a thin plate regression spline with basis dimension 4. We tested models with only sampling site, and with sampling site plus age residual, and used AIC to select the most parsimonious model.

Second, we were interested in whether dolphins exposed to oil from the *Deepwater Horizon* oil spill had higher age residuals compared to actual (observed) age. To examine this, we created a factor covariate to classify dolphins that were alive in 2010 at the time of the DWH spill and sampled in areas heavily impacted by the spill (BAR or MSS). We assigned these dolphins to the "DWH" cohort; dolphins sampled in other locations and dolphins with observed birth dates after 2010 were assigned to the "non-DWH" cohort. We then used a *t*-test to examine potential differences in age residuals between the two cohorts. Due to the large error in DNAm age predictions for the oldest age class and the lack of samples from the oldest age class in the DWH cohort, we excluded this class for the t-test.

3. Results

3.1. Epigenetic clock for age prediction

Of the three modeling approaches, the hybrid model (Fig. 2) produced the smallest median absolute error (MAE) overall for estimating chronological age (1.91 years; Table 2). However, relative performance among the three models varied between age classes: for calves and juveniles, ENR had a slightly lower MAE than the hybrid model; for adults, RFR had a lower MAE than the hybrid model; while for older animals the hybrid model had the lowest MAE (Table 2). Regardless of the modeling approach used, MAE was highest for the older age category (Table 2, Fig. 2, Supplementary Fig. 3), but proportional errors across age classes were similar. For the hybrid model, median proportional errors (MPEs) were 0.16, 0.24, 0.15, and 0.17 for calf, subadult, adult, and older age categories, respectively. Our check of model fit by age class using a GLM indicated that actual error differed among age class ($p = 0.0001$); as can be seen from Fig. 2, this is because the age predictions are negatively biased for the oldest age class.

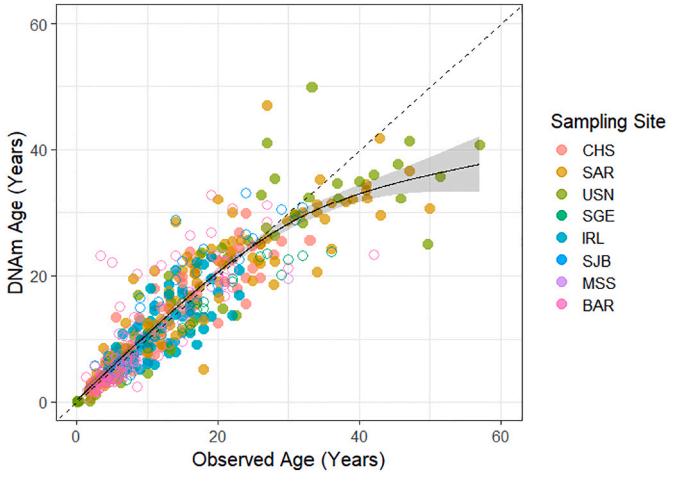

Fig. 2. Observed age versus DNAm predicted age from the hybrid model using 5-fold cross-validation. Symbols colored by sampling site; filled circles represent samples used to train the model. Hollow circles represent samples from populations sampled as part of unusual mortality event or chemical spill investigations, which were not used in model training. Solid line represents the fitted generalized additive model of DNAm vs. observed age; shaded ribbon represents pointwise 95 % confidence interval on the predicted relationship. Dashed line represents 1:1 relationship (perfect agreement). The locations are as follows: BAR = Barataria Bay, Louisiana, CHS = Charleston, South Carolina, IRL = Indian River Lagoon, Florida, MSS = Mississippi Sound, Mississippi, SGE = South Georgia Estuary Stocks, SAR = Sarasota, Florida, SJB = St. Joe Bay, Florida, USN = United States Navy, San Diego.

The RFR model contained 338 CpG sites with variable importance >0.001 (Fig. 3). Enrichment analysis of the genomic regions containing these CpG sites suggests that gene ontology processes related to synapse assembly and protein complex/cellular component disassembly are significantly enriched in the dolphin age model (Supplementary Table 1). Similar mouse phenotype (single knock-out) ontologies are significantly over-represented, including excitatory synapse assembly, decreased central nervous system synapse formation, and increased skeletal muscle cell apoptosis. Additional details can be found in the SI.

3.2. Analysis of age residuals and health

The GAM of DNAm age versus observed age successfully corrected for the negative bias in predicted DNAm age for older animals (Fig. 2), so that we could use the residuals from this analysis (the "age residuals") to look for patterns associated with health.

The beta GAM model with VESOP score (i.e., predicted survival probability) as the response that included age residual as an explanatory variable (Fig. 4) had a lower AIC score compared to the model with only the sampling site random effect (−1441.46 vs. -1440.41). Both age residual and sampling site were significant predictors of survival ($p = 0.043$ and $p < 0.001$, respectively.

Dolphins with the highest positive age residuals had the lowest survival probabilities (Fig. 4). Individuals with age residuals of 5–10 years and >10 years had mean survival probabilities of only 0.91 and 0.90, respectively. In comparison, age residuals of 0–5 yrs. and negative age residuals produced survival probabilities of 0.94 and 0.95, respectively, similar to the expected baseline survival probability (0.94) estimated by Schwacke et al. (2022).

Age residuals were higher for the DWH cohort ($p = 0.041$, Supplementary Fig. 4).

4. Discussion

Our analysis of a large set of samples (n = 476) from multiple wild bottlenose dolphin populations demonstrates that DNA methylation patterns can provide very accurate age estimation using skin samples. The ability to rely solely on remotely obtained skin samples is critical for application of the technique across a broad spectrum of cetacean species. This is of particular value in the application to large whales, which are intractable for the type of hands-on catch and release sampling that is currently required for collection of blood. Skin-based methylation clocks for bottlenose dolphins (Robeck et al., 2021a, 2021b) and other cetacean species (Bors et al., 2020; Peters et al., 2022; Parsons et al., 2023) have used smaller sample sizes and reported median errors only marginally higher than the overall MAE from our clock. Some have been as low as 2–3 years as compared to our overall MAE of 1.91 years. However, examining MAE across analyses of different datasets does not provide a true comparison of accuracy because errors can be heavily influenced by the age distribution of samples, with much smaller errors expected for the estimation of younger ages. For this reason, we stratified error estimates into four age classes. As expected, our results demonstrated lowest absolute errors in age prediction for the calf and subadult age classes (MAE = 0.149 and 1.46 years, respectively). Slightly higher errors were observed for adult dolphins between 15 and 25 years of age (MAE = 3.25 years), with the highest errors observed in the older age class (MAE = 5.90 years). Age predictions were also

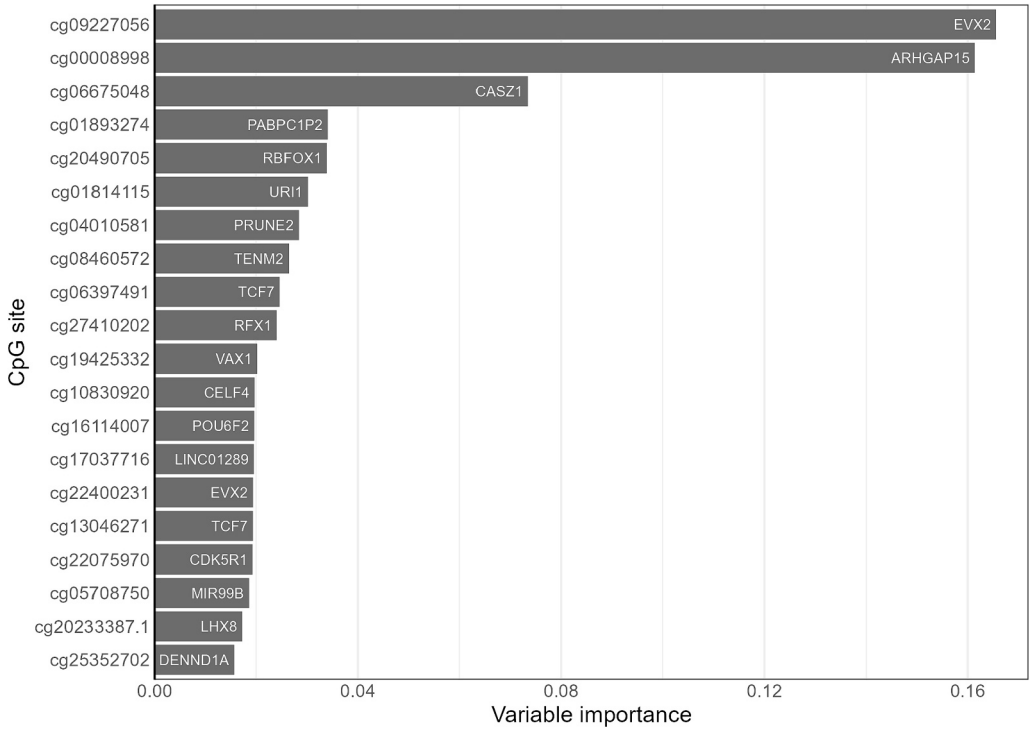

Fig. 3. Bar graph representing the top 20 CpG sites of importance generated by the RFR model with the degree of variable importance on the x axis and the corresponding CpG site on the y axis.

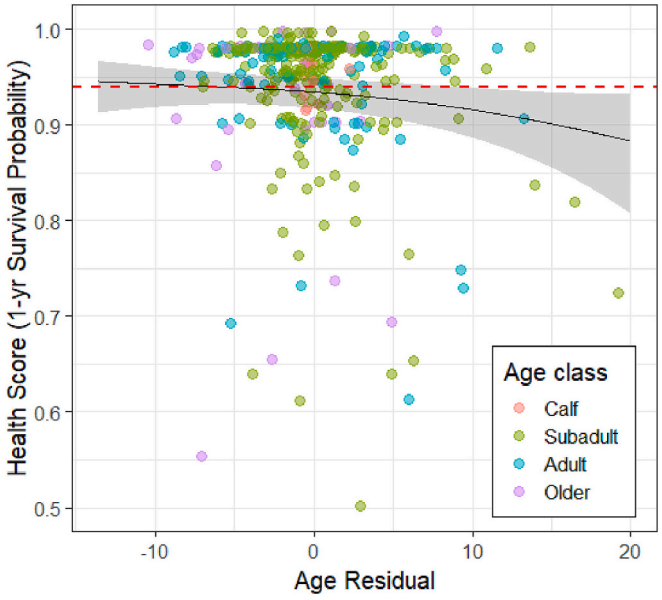

Fig. 4. Age residuals from GAM fit to DNAm age from observed age versus health scores derived from VESOP model. Solid black line represents fit of beta generalized additive model (GAM) with smooth function for age residual with shading indicating a 95 % confidence band on the smooth. Dashed red line represents expected non-calf survival from Schwacke et al. (2022). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

negatively biased for the oldest age class (Fig. 2). Although prior studies using cetacean skin have not reported errors stratified by age class, their visual representations of fitted models suggest that errors increased with increasing age for their datasets as well (Robeck et al., 2021a, 2021b; Parsons et al., 2023; Peters et al., 2022).

We hypothesize that multiple processes contribute to the reduction in accuracy of age estimates in older animals. It is possible that in older dolphins there is a reduced capacity for changes in DNA methylation, particularly in skin. Previous DNA methylation studies have suggested increased variability and reduced prediction accuracy when skin versus blood is used (Robeck et al., 2021a, 2021b), although these prior studies used different sample sets with a differing number of samples to assess accuracy. Another possibility is "cohort survivor bias", a phenomenon seen in cross-sectional studies in which individuals that age slower survive to older ages, and are therefore more likely to be sampled at these older ages (Beard, 1959; Nelson et al., 2020). Supporting this possibility, Venn-Watson et al. (2020) report age-related changes in hematologic and clinical chemistry measures that vary among Navy bottlenose dolphins, and classify different subgroups of "slow" and "accelerated" agers. Some of the variation in older age estimates could also be related to lower accuracy in the observed ages of the older dolphins that we used to train the clocks, as some of these observed ages were derived from previous GLG age estimates. Barratclough et al. (2023) found that GLG estimates are very accurate for bottlenose dolphins 15 years of age or younger (MAE = 0.5 years), but that estimates above that point are somewhat less accurate (MAE = 2.5 for dolphins 16–30 years). GLG estimates for dolphins >30 years are the least accurate (MAE = 11.5 years), and also negatively biased. If GLG estimates for some of our samples were imprecise or biased, it could have introduced variation into both the fitting and application of the epigenetic clock specifically for the oldest age class. However, we do not believe that this is the primary driver of the bias or higher variation in DNAm ages for older dolphins. To address the potential uncertainty in observed age from GLG analyses, we assigned lower weights to training samples commensurate with the proportion of the years of the dolphin's life that were unobserved and estimated from GLG analysis. Furthermore, only a small portion (N = 6, or 2 %) of the samples used to train the epigenetic clock had age estimates from GLG analyses that were >30 years, the age range for which the uncertainty in GLG estimates is most concerning. Only five (3 %) of the samples from the non-training sites had GLG estimates above 30 years. With such a large dataset, if age estimates for

this small portion of samples are indeed negatively biased, they unlikely have a substantial influence. Finally, similar negative bias for older age classes has been observed in human studies for which precise ages are known (Vidaki et al., 2017). Therefore, it is likely some contribution of all of the above processes explain the negatively biased estimates for the oldest age class of dolphins.

To improve the accuracy of age estimates of older age classes, we explored multiple modeling approaches. ENR remains the most commonly applied approach for fitting epigenetic aging clocks in both human and wildlife epigenetic literature, therefore we first attempted to fit a single ENR to the full spectrum of ages. While it resulted in very accurate fits for the calf and subadult age classes, the predictions for dolphins over the age of around 10–15 years was much less accurate and had a high degree of variability (Table 2, Supplementary Fig. 1). We hypothesized that the nonlinear relationship between age and beta values for many of the CpG sites contributed to this loss of predictive ability for the older age classes. Therefore, we explored a nonparametric RFR approach. With the RFR, we obtained estimates for the calf and subadult age classes with low error similar to the ENR results, but with improved accuracy for the adult age class (Table 2). However, the RFR performed very poorly for the upper extreme of ages, for which the number of samples was limited. This is not surprising in that RFRs, which do not assume a linear or any functional relationship, cannot extrapolate outside of the training set of values. When the number of training values is limited, as is the case for the oldest age class, the RF can be expected to perform poorly. This then led us to a hybrid modeling approach, using a RF to classify samples as above or below the age of physical maturity (15 years), when some growth or aging processes might be expected to change, and then applying an ENR, specifically fit for the appropriate age range, for the physically mature animals. As known-age samples in the older age range are less readily available, the hybrid model enabled more accurate age predictions in the older demographics as compared to using a single model across the full lifespan. Currently only two locations (USN and SAR) contribute to the majority of samples >30 years old; in the future, with additional longitudinal observations from other locations, the number of samples from older dolphins included in the clock can be increased.

Although the current model is less accurate for older-age dolphins (>25 years old), we note that this may not be important for some studies, depending on the study goal. A typical wild population will contain relatively few older animals: Schwacke et al. (2017) reported cumulative survival functions for wild bottlenose dolphins that give median age at death of around 15 with around 15 % of males and 30 % of females surviving to age 25. Hence studies focused on population-level questions related to aging may be relatively little affected. In addition, some life-history research questions focus on younger ages, where animals reach sexual or physical maturity, calves separate from their mothers, or initial social bonds form. The current clock is very accurate at these ages.

Many dolphins, particularly subadults and adults, sampled from sites heavily impacted by the DWH oil spill had high DNAm age relative to their observed age. Health assessment sampling in BAR and MSS was initiated just after the DWH disaster and documented chronic health conditions such as lung disease and markers of inflammation that have continued in the decade following the spill (Schwacke et al., 2022; Smith et al., 2017). We hypothesize that the large age residuals may be due to the chronic disease conditions in many of these dolphins, as well as in some individuals from the other populations. This was supported by the negative correlation between the DNAm age residuals and health scores from the VESOP model (Fig. 4). There was substantial variation in the health scores that was not explained by the DNAm residuals, but this is not surprising as the scores are estimated from a suite of hematological and biochemical measures in addition to physical exam and ultrasound. These relatively non-specific diagnostic markers could be indicative of chronic disease, but also could be related to acute infectious disease or injury. Peracute or acute conditions would not necessarily cause significant changes in DNA methylation patterns, with chronic disease more likely to influence DNA methylation based on human literature (Robertson, 2005). Regardless, our findings of an association between DNAm age and VESOP health score, which can be interpreted as a one-year survival probability, is consistent with the GrimAge assessment in humans, where increased age residuals indicate a reduced lifespan (Lu et al., 2019). Our findings suggest that analysis of DNAm patterns could be a viable approach for assessing wildlife population health and identifying populations affected by chronic or recurring stressors. Future research will aim to identify the CpG sites that drive the age residuals and explore any links with specific health conditions or environmental stressors.

A desirable attribute of an epigenetic analysis, especially for novel wildlife datasets, is to be able to identify the specific CpG sites of importance for each age class to enable functional interpretation via the GREAT analysis, and for future comparisons of differences in methylation profiles in relation to geographical location and individual health status (Farhadi et al., 2022). However, functional interpretation of methylation patterns can be speculative, as some methylation patterns stay consistent across tissues and species, while others are tissue-dependent (Christensen et al., 2009; Day et al., 2013). From the RFR model, the set of 338 CpG sites with variable importance >0.001 map to genomic regions enriched in genes and gene ontology terms associated with meiosis and gamete production, neuroplastin and its function in synapse formation/memory, skeletal muscle development and maintenance, disassembly of cellular machinery, organic cation transport, and myoblast proliferation. In comparison to previously reported CpG site of importance from bottlenose dolphin skin clocks (Robeck et al., 2021a; Robeck et al., 2021b), the only nearest-gene for the top seven CpG sites that our results share is the EVX2 gene: a homeobox gene shown to be important in neuronal specification during development (Juárez-Morales et al., 2016).

Another variable that could affect DNA methylation patterns and development of species-specific epigenetic clocks is the sex distribution of samples. For example, studies in humans have demonstrated that male sex is associated with increased epigenetic aging, which correlates with the fact that, in many species, males have shorter lifespans compared to females (Oblak et al., 2021). In dolphins, sexually dimorphic longevity is less understood, with annual survival for females >30 years at 0.88 (0.82–0.92) and for males >30 at 0.85 (0.72–0.90) (Schwacke et al., 2017). Where possible, we aimed to have an even distribution of male and female dolphins across the lifespan and across geographical locations. We did not observe differing aging rates when comparing male and female samples. Additional information is available in the SI Figs. 5 and 6.

Epigenetics is a viable conservation tool to facilitate the understanding of demographics and health for free-ranging cetaceans through analysis of DNA methylation patterns in skin. The wild dolphin skin clock developed in this study will be applied in the future to archived samples from dolphins of unknown age. This will aid conservation of bottlenose dolphin stocks via improved understanding of their life expectancy and population demographics in the different geographical regions. Identifying the presence of increasing age residuals in at-risk cetaceans can be used as a conservation tool to inform management decisions such as increasing funding to the most at-risk populations, as well as population demographic and survival predictions informing population status trajectories. For example, age residuals could be used as a measure of combined stress and its outcomes on health status and subsequently survival, as part of assessments of the population consequences of multiple stressors (Tyack et al., 2022). This was demonstrated in the compromised population in Barataria Bay, Louisiana with evidence of increased age residuals, which was in line with lower survival probability for these individuals. Obtaining DNA from the well-established field technique of remote skin biopsies will now allow age estimation, enabling application of this technology to larger, free-swimming cetaceans where catch-and-release techniques are not

viable and for future application to many endangered wildlife species, beyond cetaceans.

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CRediT authorship contribution statement

Ashley Barratclough: Writing – original draft, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Ryan Takeshita:** Writing – review & editing, Methodology, Investigation. **Len Thomas:** Writing – review & editing, Validation, Methodology, Formal analysis. **Theoni Photopoulou:** Writing – review & editing, Validation, Methodology. **Enrico Pirotta:** Formal analysis, Methodology, Writing – review & editing. **Patricia E. Rosel:** Conceptualization, Methodology, Resources, Writing – review & editing. **Lynsey A. Wilcox Talbot:** Data curation, Methodology, Writing – review & editing. **Nicole L. Vollmer:** Data curation, Methodology, Writing – review & editing. **Randall Wells:** Writing – review & editing, Data curation. **Cynthia R. Smith:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Teresa K. Rowles:** Writing – review & editing, Data curation. **Steve Horvath:** Methodology, Software, Writing – review & editing. **Lori Schwacke:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr Peter Tyack reports financial support was provided by Strategic Environmental Research and Development Program. Dr Ashley Barratclough reports financial support was provided by National Oceanic and Atmospheric Administration. Dr Lori Schwacke reports financial support was provided by Office of Naval Research. Dr Ashley Barratclough reports financial support was provided by Oiled Wildlife Care Network. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocon.2024.110570.

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