



Brief Report

# Accurate Epigenetic Aging in Bottlenose Dolphins (*Tursiops truncatus*), an Essential Step in the Conservation of at-Risk Dolphins

Ashley Barratclough <sup>1,\*</sup>, Cynthia R. Smith <sup>1</sup>, Forrest M. Gomez <sup>1</sup>, Theoni Photopoulou <sup>2</sup>, Ryan Takeshita <sup>1</sup>, Enrico Pirotta <sup>3,4</sup>, Len Thomas <sup>2</sup>, Abby M. McClain <sup>1</sup>, Celeste Parry <sup>1</sup>, Joseph A. Zoller <sup>5,6</sup>, Steve Horvath <sup>5,6</sup> and Lori H. Schwacke <sup>1</sup>

<sup>1</sup> Conservation Medicine Division, National Marine Mammal Foundation, San Diego, CA 92106, USA; cynthia.smith@nmmf.org (C.R.S.); forrest.gomez@nmmf.org (F.M.G.); ryan.takeshita@nmmf.org (R.T.); abby.mcclain@nmmf.org (A.M.M.); celeste.parry@nmmf.org (C.P.); lori.schwacke@nmmf.org (L.H.S.)

<sup>2</sup> Centre for Research into Ecological and Environmental Modelling, University of St Andrews, St Andrews KY16 9LZ2, UK; tp14@st-andrews.ac.uk (T.P.); len.thomas@st-andrews.ac.uk (L.T.)

<sup>3</sup> Department of Mathematics and Statistics, Washington State University, Vancouver, WA 98686, USA; pirotta.enrico@gmail.com

<sup>4</sup> School of Biological, Earth and Environmental Sciences, University College Cork, T23 N73K Cork, Ireland

<sup>5</sup> Department of Biostatistics, Fielding School of Public Health, University of California, Los Angeles, CA 90095, USA; jaz18@g.ucla.edu (J.A.Z.); SHorvath@mednet.ucla.edu (S.H.)

<sup>6</sup> Department of Human Genetics, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA

\* Correspondence: ashley.barratclough@nmmf.org



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**Abstract:** Epigenetics, specifically DNA methylation, allows for the estimation of animal age from blood or remotely sampled skin. This multi-tissue epigenetic age estimation clock uses 110 longitudinal samples from 34 Navy bottlenose dolphins (*Tursiops truncatus*), identifying 195 cytosine-phosphate-guanine sites associated with chronological aging via cross-validation with one individual left out in each fold ( $R^2 = 0.95$ ). With a median absolute error of 2.5 years, this clock improves age estimation capacity in wild dolphins, helping conservation efforts and enabling a better understanding of population demographics.

**Keywords:** DNA methylation; epigenetics; aging; bottlenose dolphin; chronological age

## 1. Introduction

Determining chronological age in cetaceans is an ongoing challenge, with knowledge of age being critical to interpreting biological data, understanding population demographics, and predicting survival. Previous age estimation methodologies have involved invasive tooth extraction for growth layer analysis, morphometrics, and pectoral flipper radiography [1,2]. These methods require physical examinations and vary in accuracy according to age demographic. Interdisciplinary approaches to conservation medicine, through the application of tools developed in human medicine and applied to marine mammals, are needed to accurately expand the capacity of aging. Epigenetic aging is a novel technology utilizing DNA methylation patterns as biomarkers of age [3]. DNA methylation is the addition of methyl groups  $\text{CH}_3$  to cytosine–phosphate–guanine sites (CpG sites) in the DNA sequence. In humans, analysis of the CpG site modification demonstrated a close correlation with chronological age; therefore, analysis of epigenetic modifications can provide an estimation of age [3,4]. Epigenetic age acceleration occurs when the estimated age exceeds the chronological age [5]. Exploring DNA methylation in humans has identified drivers of epigenetic age acceleration including environmental stressors, lifestyle and disease. The initial application for chronological age estimation of bottlenose dolphins (*Tursiops truncatus*) focused on two cytosine–phosphate–guanine sites (CpG) (of

17 screened) producing an  $R^2$  of 0.74 and a root mean squared error of 5.14 years when estimating chronological age [6]. Similarly, for nine odontocete species combined, a median absolute age prediction error of 2.57 years was produced using 142 CpG sites [7]. Creating species-specific epigenetic clocks with a wide range of known age animals improves the accuracy of age estimation [8]. The present study stands out in its use of a longitudinal dataset from the U.S. Navy Marine Mammal Program (Navy). Since 1959, the Navy has expanded knowledge in bottlenose dolphin health and physiology [9,10]. The extensive tissue archive, paired with daily observational and medical records for individual dolphins, provides a unique opportunity for scientific research. The application of DNA methylation technology to bottlenose dolphins promises to improve our understanding of species-specific aging drivers, as well as potential preventative measures for the reversal of DNA methylation and increased survival [11].

## 2. Materials and Methods

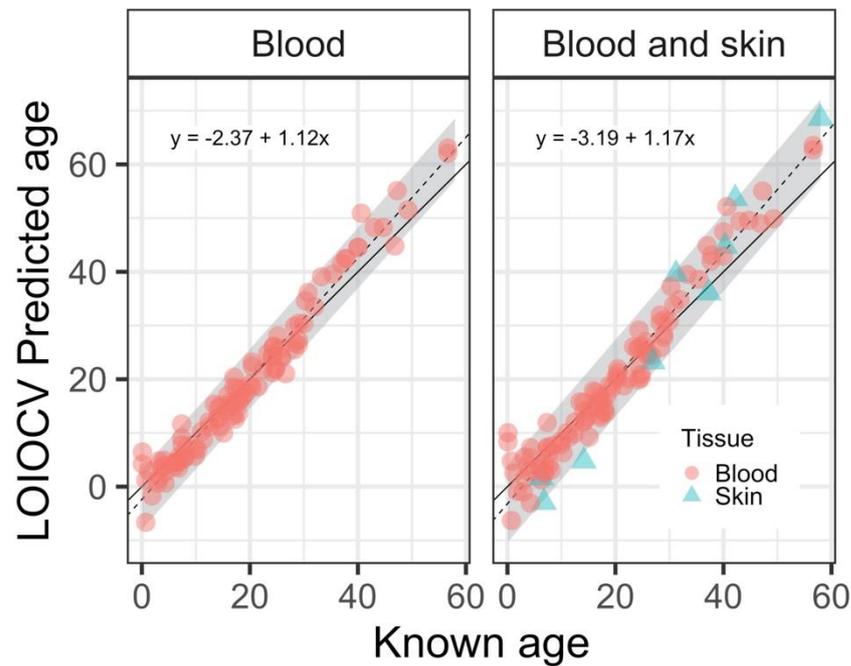
A total of 110 samples (101 blood buffy coat and 9 skin) were analyzed from 34 different dolphins (19 female, 15 male). Of these, 24 had exact birth dates, and the remaining 10 were estimated (within 2–4 years) via morphometric measurement and, where available, combined with tooth growth layer group analysis ( $n = 2$ ). Sample ages ranged between 1 month and 58 years. Each dolphin was selected according to life history and health status, with 2–5 samples per dolphin spaced at least 5 years apart. Dolphins were selected according to their age and lifespan to ensure a representative coverage of bottlenose dolphin lifespan (up to 60 years of age). The longitudinal measures helped to validate expected changes over the lifespan. Health status was assessed, and dolphins were classified as healthy or unhealthy. Due to epigenetic changes occurring over longer periods of time, chronic health concerns were defined as having a duration of > 6 months; therefore, acute episodes of illness were not included. Samples were collected during routine animal care, under the authorization of U.S. Code, Title 10, USC 7524. The Navy is accredited by AAALAC International, and adheres to the national standards of the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the Animal Welfare Act. Ethical approval was granted by the University of St Andrews' Animal Welfare and Ethics Committee (SEC20015). Archived blood buffy coat samples were from 1992 to 2020. Skin samples were collected from fresh carcasses during necropsy using standard protocols. Buffy coat and skin samples were archived at  $-80\text{ }^{\circ}\text{C}$ . Samples were submitted to the Technology Center for Genomics and Bioinformatics, University of California at Los Angeles, for DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Each DNA sample was 20  $\mu\text{L}$  with a concentration of 250 ng.

DNA methylation data were generated using a custom mammalian methylation array (HorvathMammalMethylChip40) with 37,492 CpGs [12]. An elastic net regression model was used to select the CpG sites associated with chronological age [13]. The elastic net was run on twenty random training sets (each including 2/3 of the data) to select the elastic net mixing parameter ( $\alpha$ ). The value of  $\alpha$  that returned the lowest mean squared error for the corresponding test sets was selected. The regularization parameter ( $\lambda$ ) was then chosen via cross-validation (CV), using individual IDs as the folds to account for repeated measurements (cross-validation with one individual left out in each fold, LOIOCV). Details are given in the Supplementary Material.

## 3. Results

The final model retained 112 CpG sites for the blood clock and 195 CpG for the multi-tissue clock (Figure 1, Figures S1 and S2). The lists of CpG sites selected in each clock are provided in the supplemental information.  $R^2$  (coefficient of determination) values for the LOIOCV predictions were 0.97 and 0.95 for the blood and multi-tissue clocks, respectively, compared to  $R^2 = 0.74$  previously reported for both dolphin and beluga clocks [6,10]. The final epigenetic clocks were highly accurate, with a median absolute LOIOCV prediction error of 2.0 years for the blood clock and 2.5 years for the multi-tissue clock. The accuracy

was likely achieved by longitudinal samples from a wide range of ages, facilitated by the long lifespans of Navy dolphins.



**Figure 1.** Regression of known chronological age against estimated age for blood ( $n = 101$ ,  $R^2 = 0.97$ , median absolute error of 2.0 years) and multi-tissue ( $n = 110$ ,  $R^2 = 0.95$ , median absolute error of 2.5 years) clocks generated by leave-one-individual-out-cross-validation (LOIOCV). Each point represents a single bottlenose dolphin sample. Solid lines represent the 1:1 line; dashed lines represent the fitted line. The grey ribbon is the 95% prediction interval.

#### 4. Discussion

This robust epigenetic clock validates the use of blood and skin tissue to support the precise estimation of age in bottlenose dolphins. In wild, free-ranging dolphins, blood can be obtained during a hands-on veterinary examination, but skin can be sampled remotely without requiring restraints. Estimation of chronological age from remotely sampled skin is pivotal in advancing cetacean conservation, particularly in large, free-swimming whales where temporary capture and restraint are currently not feasible. Knowledge of animals' age aids conservation and management efforts by improving our understanding of population demographics, as well as age-specific rates of morbidity, mortality and reproduction.

To establish the chronological aging clock in bottlenose dolphins, this study focused on blood buffy coat samples to ensure the most accurate results, due to the expected higher quality of DNA obtained from this tissue. An additional objective was to validate the use of DNA extracted from skin for epigenetic aging analysis. Whilst our sample size for skin was small, it provided us with validation, from known-age dolphins, to move forward with the next phase of the project, which is analyzing DNA extracted from wild dolphin skin samples. Currently, the blood clock is more accurate than the multi-tissue clock; however, a skin-specific clock can be produced with additional samples and compared with the multi-tissue clock to see which is most accurate for future use. The recently published odontocete clock produced an  $R^2$  of 0.81 for a skin-only clock with a mean absolute error of 7.76 years [7]. We hypothesize that a more accurate species-specific skin clock with an error equivalent to the multi-tissue clock of approximately 2 years can be created with an increased skin sample size. This study confirmed, as demonstrated in the cluster dendrogram (Figure S1), methylation patterns are tissue-specific in cetaceans; therefore, creating both species- and tissue-specific clocks will provide the most accurate results.

Establishing this accurate bottlenose dolphin species-specific clock was facilitated by the high proportion of known-age Navy dolphins and long lifespans, enabling longitudinal sampling. With some dolphins contributing up to five samples and a maximum age of 58 years old, this longitudinal approach has allowed repeated measures to be accounted for within our statistical analysis, producing the highly correlated  $R^2$  of 0.95. This longitudinal sample approach has not been feasible in previous cetacean studies due to the lack of known-age individuals or, in wild cases, lack of access to repeated samples. Using only known-age dolphins would have reduced both our sample size and our range of samples across the dolphin lifespan. While it may have produced a more accurate clock for a smaller age demographic, our aim was to apply the clock across the full lifespan; therefore, older animals without exact birth dates were included. Sample selection for this study was focused on establishing the chronological clock; however, the selection of both healthy and unhealthy individuals will enable the next phase of the project.

The next phase of this project will investigate the biological aging component of DNA methylation, using additional wild dolphin samples and health information to identify CpG sites associated with specific health parameters and cumulative stress. Due to the standard of care provided to the Navy dolphins, chronic, advanced health conditions are an unusual occurrence. Utilizing wild dolphin samples in the next phase of this project, will likely aid in the identification of CpG sites that are methylated in association with biological aging. Wild dolphins may have more advanced disease states than Navy dolphins due to lack of veterinary intervention and increased environmental stress. A comparison between known healthy individuals and unhealthy individuals will aid the identification of CpG sites involved in biological aging. If future conservation efforts are able to determine biological age from a skin biopsy, this could provide new insight into population health without hands-on veterinary examinations.

Finally, future epigenetic research should aim to predict individual dolphin lifespan by estimating the average remaining lifespan from DNA methylation patterns similar to what has been accomplished for humans [14]. These epigenetic estimators of mortality and morbidity risk could become useful for identifying environmental stress factors. This would enable epigenetics to provide insight into survivability by improving the understanding of demographics, and potential for population growth [14]. From a conservation perspective, knowledge of age for threatened and endangered species is one of the biological keys to determining population survival.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/jzbg2030030/s1>, Additional Statistical methods description with associated figures: Figure S1: Cluster Dendrogram for data quality control, Figure S2: Mean squared error plotted against the elastic net mixing parameter,  $\alpha$ , for blood and mixed tissue, Figure S4: Modelled median relationship between absolute prediction error and known age resulting from a quantile non-parametric additive model. References [10,13,15–17] were used in Supplementary Materials.

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