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Brief Report

Accurate epigenetic aging in bottlenose dolphins (Tursiops truncatus), an essential step in the conservation of at-risk dolphins.

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Abstract: Epigenetics, specifically DNA methylation, allows for estimation of animal age from blood or remotely sampled skin. This multi tissue epigenetic aging clock uses 110 longitudinal samples from 34 Navy bottlenose dolphins (*Tursiops truncatus*), identifying 195 cytosine-phosphate-guanine sites associated with chronological aging via leave-one-individual-out-cross-validation (R2=0.95). With a median absolute error of 2.5 years this clock improves age estimation capacity in wild dolphins, expanding conservation efforts, enabling better understanding of population demographics.

Keywords: DNA methylation; epigenetic aging; bottlenose dolphin; chronological age;

1. Introduction

Determining chronological age in cetaceans is an ongoing challenge, with knowledge of age critical to interpreting biological data, understanding population demographics and predicting survival. Previous aging methodologies have included invasive tooth extraction for growth layer analysis, morphometrics, and pectoral flipper radiography to estimate age [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 to marine mammals, are needed to expand the capacity of aging accurately.

DNA methylation patterns lend themselves for accurately estimating chronological 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.

Initial application for chronological age estimation of bottlenose dolphins (Tursiops truncatus) focused on two cytosine-phosphate-guanine sites (CpG) (of 17 screened) and produced estimates within 5 years of actual age [6]. Similarly, for nine odontocete species combined, a median absolute age error of 2.57 years was produced using 142 CpG sites [7]. Creating species-specific epigenetic clocks with a wide range of known age

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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. 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 reversal of DNA methylation and increased survival [11].

2. Materials and Methods

A total of 110 samples (101 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 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. The longitudinal measures helped to validate expected changes over the lifespan. In addition, 30 of the 110 samples were selected from 10 individuals with chronic health abnormalities (>6 months) to assess biological aging. 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 buffy coat samples were from 1992–2020. Skin samples were collected from fresh carcasses during necropsy using standard protocols. Buffy coat and skin samples were archived at -80ºC. 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 20µl and concentration 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 (α). The value of αthat returned the lowest mean squared error for the corresponding test sets was selected. The regularization parameter (λ) was chosen via cross-validation (CV), using individual IDs as the folds to account for repeated measurements (leave-one-individual-out CV, LOIOCV). Details are given in 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, Figure S1-S2). R2 values for the LOIOCV predictions were 0.97 and 0.95 for the blood and multi-tissue clocks, respectively, compared to R2 = 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 predicted age for blood (n=101, R2= 0.97, median absolute error 2.0 years) and multi-tissue (n=110, R2= 0.95, median absolute error 2.5 years) clocks generated by leave-one-individual-out-cross-validation (LOIOCV). Each point

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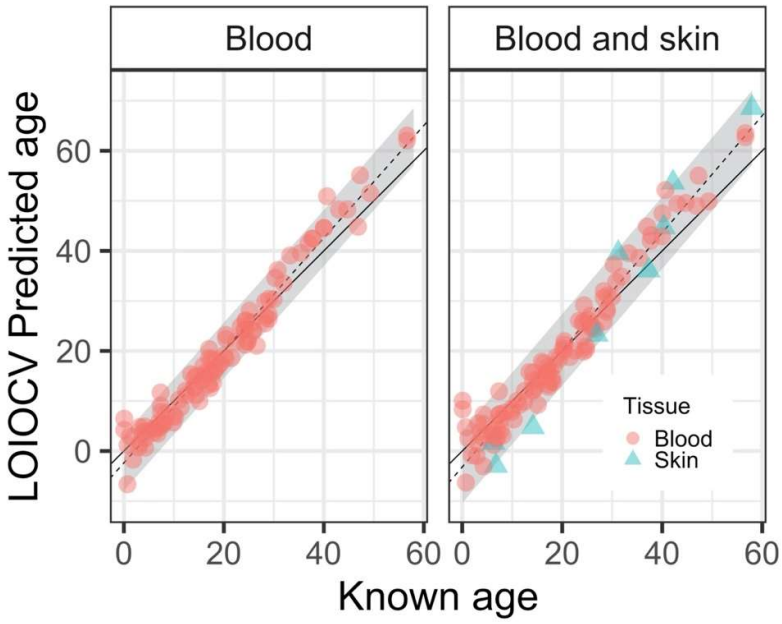
represents a single bottlenose dolphin sample. Solid lines represent the 1:1 line, dashed lines represent the fitted line. The grey ribbon is the 95% prediction interval.



4. Discussion

This robust dolphin epigenetic clock validates the use of blood and application of skin to support precise age estimation. In wild, free-ranging dolphins, blood can be obtained during a hands-on veterinary examination, but skin can be sampled remotely without requiring restraint. 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 age aids conservation and management efforts by improving our understanding of population demographics, as well as age-specific rates of morbidity, mortality and reproduction.

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.

Finally, future epigenetic research should aim to predict individual dolphin lifespan by estimating the average time to death 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 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 www.mdpi.com/xxx/s1, Additional Statistical methods description with associated figures: Figure S1: Cluster Dendogram for data quality control, Figure S2: Mean squared error plotted against the elastic net mixing parameter, α, for blood and mixed tissue, Figure S3: Mean squared error plotted against the log of the elastic net regularization parameter, λ, 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.

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