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MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Ageing methods for common dolphins (*Delphinus delphis*)

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Eva-Maria Franziska Hanninger

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Abstract

Accurate age estimation underpins demographic inference, population viability analyses, and conservation management for cetaceans, yet remains challenging for most odontocete species. Reliance on invasive tooth-based ageing has constrained the contexts in which age data can be obtained, limiting demographic resolution for species exposed to ongoing anthropogenic pressures. This thesis develops an integrated suite of molecular and radiographic ageing frameworks for common dolphins (*Delphinus delphis*), expanding the methodological toolkit available for age and life-history assessment across post-mortem, free-ranging, and live-stranding contexts. The thesis establishes the first species-specific epigenetic clock for common dolphins using DNA methylation from skin samples of stranded and bycaught individuals aged via dental growth layer groups. The clock achieved a median absolute error (MAE) of 1.80 years (Pearson's correlation (r) = 0.91, R^2 = 0.82) and remained reliable up to 16 years of age, with prediction errors increasing thereafter. In parallel, this thesis also establishes the first calibrated radiographic bone-ageing model for the species, demonstrating strong predictive performance using both univariate and multivariate approaches (canonical analysis of principal coordinates (CAP): females MAE = 1.35 years, Spearman's rank correlation (ρ) = 0.90, R^2 = 0.85; males MAE = 1.80 years, ρ = 0.94, R^2 = 0.84; polynomial regression: females MAE = 1.25 years, ρ = 0.93, R^2 = 0.90; males MAE = 1.08 years, ρ = 0.95, R^2 = 0.86). Radiographic estimates outperformed epigenetic predictions and remained reliable to approximately 20 years of age. The thesis further demonstrates that radiographic skeletal ossification can be used to infer sexual maturity with high accuracy (95.8% of females and 98.4% of males correctly classified), allowing two fundamental life-history parameters — chronological age and sexual maturity — to be resolved within a single non-invasive imaging framework. Collectively, these approaches provide practical tools for obtaining age and life-history information from free-ranging, stranded and bycaught animals, as well as live-stranding responses. By improving access to reliable age data across sampling contexts, the frameworks developed here support future population assessments for common dolphins, including evaluation of fisheries bycatch and cumulative anthropogenic impacts. More broadly, this thesis demonstrates the value of integrating molecular, skeletal, and traditional ageing approaches rather than relying on any single method in isolation. It further highlights the importance of long-term curated biological archives for advancing cetacean life-history research and establishes a transferable foundation for developing species-specific ageing models across odontocetes.

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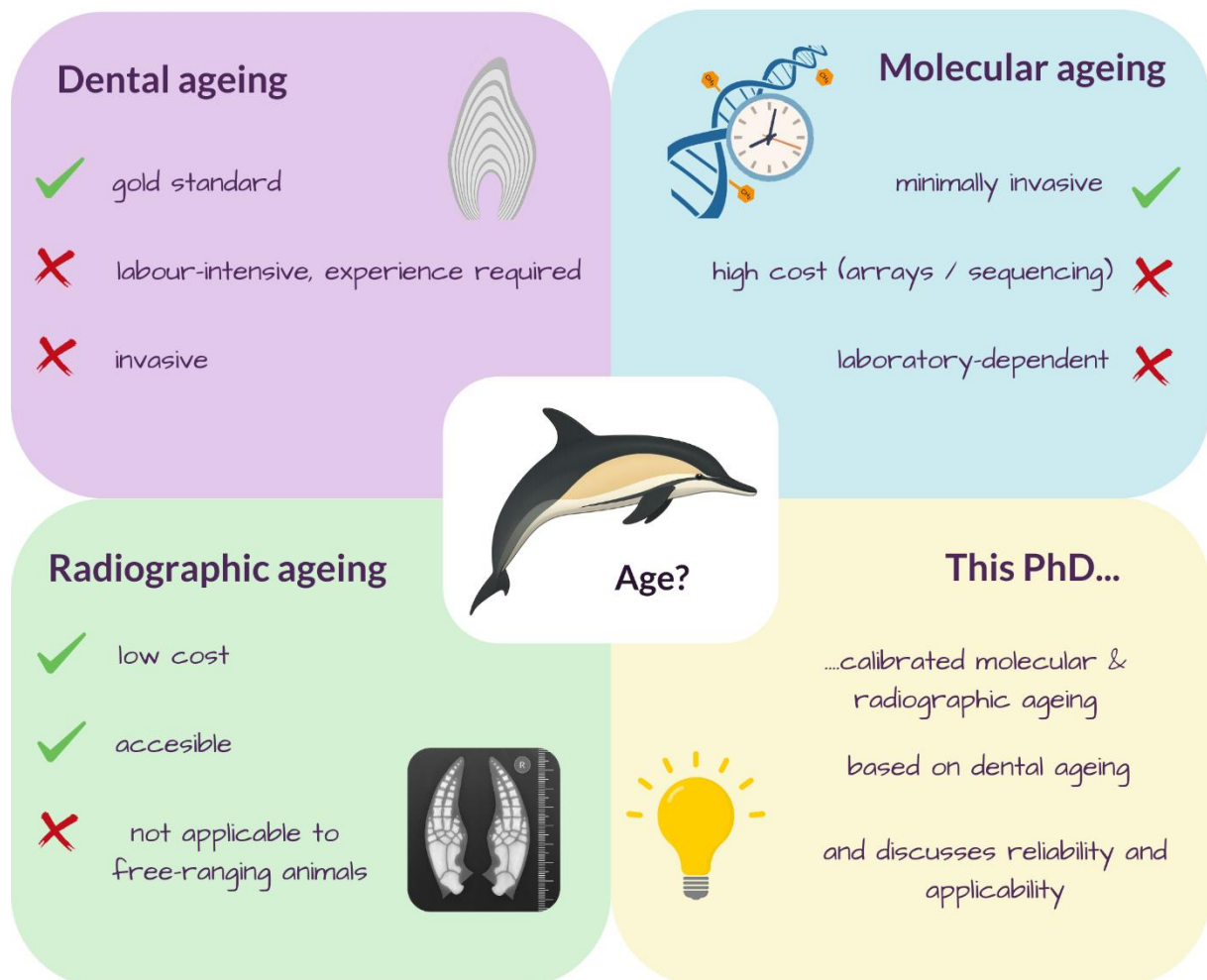
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List of abbreviations / acronyms

Abbreviation	Full term
CpG	Cytosine phosphate guanine site
DNA	Deoxyribonucleic acid
DNAmAge	DNA methylation age
DNAmAgeLOO	Leave-One-Out DNA methylation age prediction
LOOCV	Leave One Out Cross Validation
ENR	Elastic Net Regression
RFC	Random Forest Classifier
ASM	Age at sexual maturity
MLE	Maximum life expectancy
MAE	Median absolute error
PCA	Principal component analysis
DCC	Decomposition condition code
GLGs	Growth layer groups
AnAge	Animal Ageing and Longevity Database
kVp	Kilovoltage peak
mAs	Milliampere-seconds
ICC	Intraclass correlation coefficient
GLMM	Generalized linear mixed-effects model
AIC	Akaike Information Criterion
Δ AIC	Change in Akaike Information Criterion
HCl	Hydrochloric acid
M2	Metacarpal 2
M3	Metacarpal 3
M4	Metacarpal 4
M5	Metacarpal 5
D2P1	Digit 2, phalanx 1 (first phalanx of digit 2)
D3P1	Digit 3, phalanx 1 (first phalanx of digit 3)
D2P2	Digit 2, phalanx 2 (second phalanx of digit 2)
D3P2	Digit 3, phalanx 2 (second phalanx of digit 3)
D4P1	Digit 4, phalanx 1 (delta bone)

Chapter 1

Introduction



1. Age assessment across vertebrates

1.1. Age estimation approaches applicable across vertebrate taxa

Across vertebrates, age estimation approaches vary widely and reflect differences in anatomy, growth patterns, and the availability of age-informative biological structures. Some approaches are broadly applicable across vertebrate groups because they are not tied to hard tissues, whereas others rely on taxon-specific structures that record growth over time. A small number of approaches can be applied across diverse vertebrate taxa. These include demographic methods such as mark–recapture, which infer age indirectly through repeated observations of known individuals (Bright Ross et al., 2022; Ford, 1981; Loison et al., 1999), and molecular biomarkers of ageing, which estimate age from age-associated biological changes.

Molecular approaches include DNA methylation patterns (Bocklandt et al., 2011; De Paoli-Iseppi et al., 2017) and telomere length dynamics (Haussmann & Vleck, 2002; Rizvi et al., 2015; Vaiserman & Krasnienkov, 2021; Ye et al., 2023). While telomere length has been explored as an age-related biomarker, its utility for accurate age estimation is limited by substantial inter-individual variation, tissue specificity, and sensitivity to environmental and life-history factors (Der et al., 2012; Haussmann & Vleck, 2002; Vaiserman & Krasnienkov, 2021). In contrast, DNA methylation shows consistently stronger and more predictable associations with chronological age (Vaiserman & Krasnienkov, 2021).

DNA methylation describes the presence of methyl groups at cytosine–phosphate–guanine (CpG) sites and functions as an important regulator of gene expression in multicellular organisms (Bocklandt et al., 2011; De Paoli-Iseppi et al., 2017; Sen et al., 2016). Age-associated changes in DNA methylation arise through epigenetic drift, whereby stochastic and directional alterations accumulate with age, accompanied by declining fidelity of methylation maintenance and characteristic patterns of global hypomethylation and site-specific hypermethylation (Cruickshanks et al., 2013; Hayano et al., 2019; Pal & Tyler, 2016).

The strong correlation between age and methylation levels at specific CpG sites enables the calibration of epigenetic clocks, which are widely regarded as the most accurate biological age predictors currently available (De Paoli-Iseppi et al., 2017; Guevara & Lawler, 2018; Jylhävä et al., 2017; Peters et al., 2023). Epigenetic clocks have been developed for humans and a wide range of vertebrate species, including wildlife; however, their application is constrained by the

requirement for tissue samples from individuals of known age for calibration (Lu et al., 2023). Nevertheless, species-specific epigenetic clocks are now available for multiple wild mammalian species (e.g., Cantrell et al., 2020; Larison et al., 2021; Pinho et al., 2022; Prado et al., 2021) and other vertebrate taxa, including fish (e.g., Anastasiadi & Piferrer, 2020; Mayne et al., 2020; Suarez-Bregua et al., 2021; Weber et al., 2024), reptiles (e.g., Mayne, Tucker, et al., 2020; Nilsen et al., 2016; Shealy et al., 2025), amphibians (e.g., Bennett et al., 2025; Morselli et al., 2023; Zoller et al., 2022), birds (e.g., De Paoli-Iseppi et al., 2019; Saino et al., 2019; Soulsbury et al., 2018) and marsupials (Horvath, Haghani, Zoller, Raj, et al., 2022). A summary of representative studies is presented in Appendix 1 (Table A1.1).

1.2. Taxon-specific age estimation approaches

In contrast, many age-estimation methods rely on hard tissues or skeletal features that differ among vertebrate groups. In fishes, age estimation typically relies on the interpretation of primary growth increments in otoliths or the quantification of growth rings in other hard structures, including scales, opercular bones, vertebrae, and cross-sectioned dorsal or pectoral spines and fin rays (Das, 1994; Maceina et al., 2007; Politikos et al., 2021). In amphibians and reptiles, age is commonly assessed using body size, skeletochronological techniques, or reproductive indicators such as the progression of testis lobation (Halliday & Verrell, 1988; Szekely et al., 2025; Zug, 1990). In birds, age can be inferred by counting cellular layers within the circumferential lamellae of cortical bone (Drozdowska & Meissner, 2014; Klomp & Furness, 1992).

In mammals, age-estimation approaches include the assessment of annual growth layers in bones, teeth, and horns (Klevezal, 2007), as well as biochemical and radiometric techniques such as aspartic acid racemization and radiocarbon dating (Bada et al., 1980; Campana & Stewart, 2014; George et al., 1999; Kestelle et al., 2003; Masters et al., 1977; Matteucci et al., 2022; Yasunaga et al., 2017). Mammalian age estimation also frequently focuses on skeletal development and bone maturation. Bone formation and maturation proceed in a predictable sequence, providing a framework for inferring chronological age (Aghajanian & Mohan, 2018; Biga et al., 2019; Ratib et al., 2012; Wong et al., 2021).

The assessment of bone maturation enables evaluation of developmental changes in the skeleton in both living and deceased individuals. Holistic bone-age assessments typically integrate

ossification patterns across multiple skeletal elements (e.g., cranial sutures, long bones, ribs, and laryngeal cartilage), alongside degenerative indicators such as osteophyte formation, degenerative joint disease, spinal degeneration, and dental wear, particularly in adult individuals (Grabherr et al., 2009; Klevezal, 2007; Nemeskéri et al., 1960; Ruengdit et al., 2020). However, in living individuals, skeletal maturation can also be assessed non-invasively using forelimb radiographs, enabling age estimation until the attainment of physical maturity. Such approaches have been applied to humans (e.g., Baumann et al., 2009; Kim et al., 2017; Schmeling et al., 2006), as well as multiple mammalian species, such as black-tail deer (*Odocoileus hemionus*; Lewall & Cowan, 1963), cottontail rabbits (*Sylvilagus* spp.; Hale, 1949; Thomsen & Mortensen, 1946), opossums (*Didelphis virginiana*; Petrides, 1949), raccoons (*Procyon lotor*; Sanderson, 1961), red (*Vulpes fulva*) and gray foxes (*Urocyon cinereoargenteus*; Sullivan & Haugen, 1956), and even the extinct woolly mammoth (*Mammuthus primigenius*; Lister, 1999).

2. Age assessment in cetaceans

2.1. Traditional methods

2.1.1. Field-based age estimation approaches

Some external indicators of ageing have been described in cetaceans (marine mammals of the order Cetacea, including whales, dolphins, and porpoises), such as age-related speckling in bottlenose dolphins (*Tursiops truncatus*; Krzyszczyk & Mann, 2012), changes in dorsal fin shape in Irrawaddy dolphins (*Orcaella brevirostris*; Sheng et al., 2022), or dorsal fin height in male killer whales (*Orcinus orca*; Olesiuk et al., 1990). These traits are species-specific and not generally applicable across taxa. Indeed, cetaceans as a group exhibit few reliable external indicators of chronological age (Barratclough et al., 2023; Beal et al., 2019; Krzyszczyk & Mann, 2012). This limitation makes field-based age assessments largely reliant on body morphometrics for the broad classification of age classes such as neonate, juvenile, and adult. However, these indicators are indirect and not universally applicable. Although body length can assist in assigning broad age classes, substantial inter-individual variation in growth trajectories means that body length alone does not permit precise age estimation (Betty et al., 2019; Chivers, 2018).

For reasons outlined, robust field-based age assessment typically relies on longitudinal approaches, such as photo-identification and mark–recapture of known individuals from birth. The feasibility of these approaches is strongly influenced by species-specific movement patterns, social organisation, and the presence of stable, individually distinctive markings (Bamford & Robinson, 2016). While such methods have been successfully applied in intensively monitored populations, such as Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) in Shark Bay, Australia (Connor & Krützen, 2015), their application is limited in many other species. Common dolphins (*Delphinus delphis*) provide a clear example for which mark–recapture studies are notoriously difficult: individuals are often poorly marked (Bamford & Robinson, 2016; Hupman, 2016; Neumann et al., 2002) and exhibit a highly fluid fission–fusion social system (Neumann, 2001), both of which substantially reduce individual re-identification rates and long-term recapture probabilities.

2.1.2. Post-mortem and tissue-based age estimation approaches

Post-mortem age assessment techniques in cetaceans differ between mysticetes (baleen whales) and odontocetes (toothed whales, dolphins, and porpoises), reflecting differences in the availability of growth-recording hard tissues. In mysticetes, age estimation has been conducted through the analysis of growth layer groups (GLGs) in earplugs (Gabriele et al., 2010), as well as through radiocarbon dating and aspartic acid racemization of tissues such as tympanic bullae or eye lenses (George et al., 1999; Kastelle et al., 2003; Yasunaga et al., 2017). While the latter approach can also be applied to the teeth of odontocetes (Bada et al., 1980), age estimation here most commonly relies on the analysis of growth layer groups (GLGs) in decalcified and stained longitudinal tooth sections (e.g., Betty et al., 2023; Cooper et al., 2018; Evans et al., 2002, 2011; Hohn et al., 2016; Lockyer, 1993, 1995; Murphy et al., 2014). Growth layer group analysis in odontocetes is enabled by monophyodont (i.e., teeth erupt once and are not replaced) and homodont (i.e., teeth are morphologically similar) dentition (Loch et al., 2015; Uhen, 2009), such that teeth preserve a continuous postnatal growth record beginning with the neonatal line (Figure 1.1A), which marks age zero (Betty, 2019). This growth record is present in all normally developed teeth, except for some anterior and posterior underdeveloped ones (Hohn, 2009).

Growth layer groups (Figure 1.1B) are defined as repeating sequences of alternating opaque and translucent layers in dentine or cementum (Goren et al., 1987). Differences in optical density arise from variation in mineralisation, which is thought to reflect seasonal changes in physiology and nutrition (Ohsumi et al., 1963; Scheffer & Myrick, 1980). With the exception

of beluga whales (*Delphinapterus leucas*; e.g., Goren et al., 1987; Waugh et al., 2018), a single growth layer group generally represents one year of deposition in most marine mammals (Amano et al., 2011; Murphy et al., 2014; Pribanić et al., 2000; Scheffer & Myrick, 1980). This periodicity has been validated using tetracycline time-marking in captive individuals, in which fluorescent incorporation into mineralising dental tissues provides an independent temporal reference (Myrick et al., 1984; Myrick & Cornel, 2004). Additional validation has been achieved through radiocarbon dating (Campana & Stewart, 2014).

Despite their widespread use, GLG-based age estimates have several limitations. The method requires specialised equipment and substantial expertise, and age prediction error increases markedly in older individuals (Barratclough et al., 2023). Progressive tooth wear, compression of growth layer groups, and the presence of accessory lines (additional layers that may not correspond to annual deposition) can complicate interpretation (Barratclough et al., 2023; Murphy et al., 2014; Read et al., 2018). Moreover, because tooth extraction is invasive, sampling is typically restricted to post-mortem material, except in cases where procedures are conducted under anaesthesia (Barratclough, Wells, et al., 2019). Consequently, dental ageing cannot be applied to assess demography in free-ranging populations. For odontocetes, age data are therefore predominantly derived from stranded or bycaught individuals, which may not accurately represent the age structure of live populations and may introduce bias into survivorship estimates and population viability analyses (Betty et al., 2023).

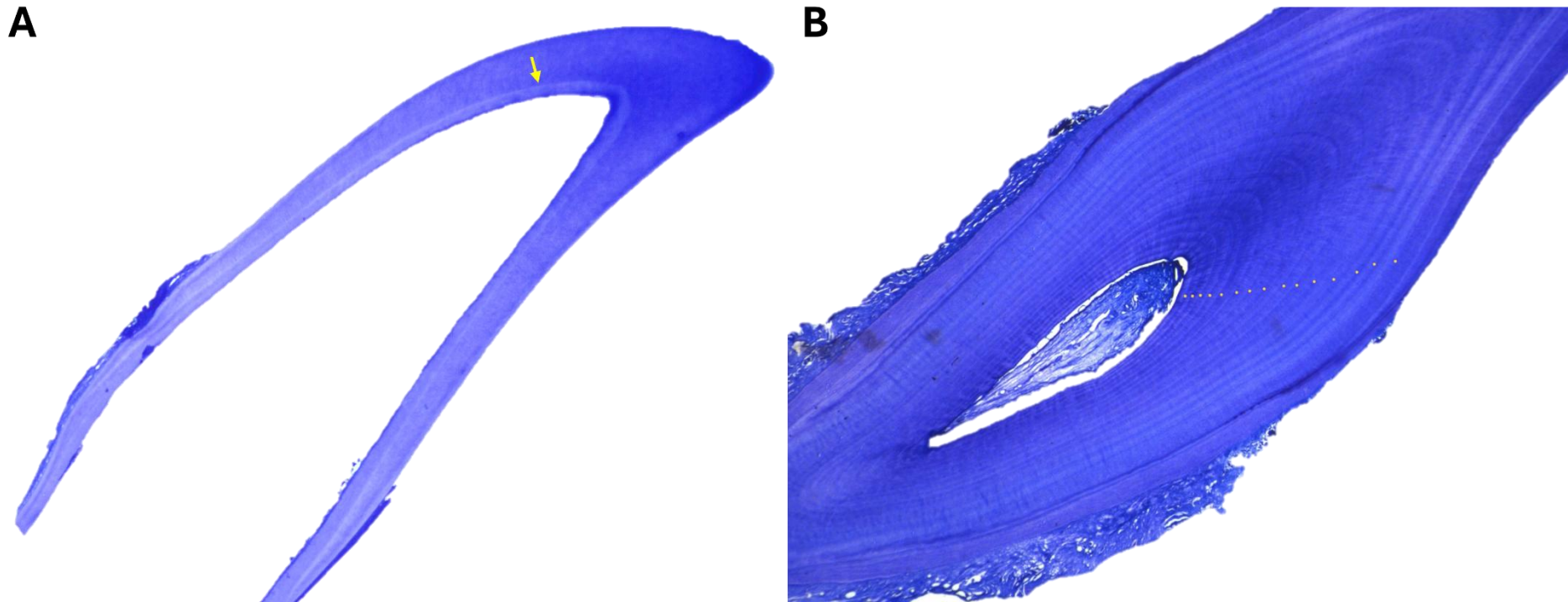


Figure 1.1: Longitudinal histological sections of teeth from two different common dolphins (*Delphinus delphis*) used for dental age estimation. **A)** Section showing the neonatal line (arrow). **B)** Section illustrating successive growth layer groups (GLGs) within the dentine, indicated by dotted markings, which are used to infer chronological age. Images were captured under a microscope at A) 12 $\times$ and B) 16 $\times$ magnification.

2.2. Emerging alternatives

2.2.1. Epigenetic clocks

In the absence of feasible mark–recapture or other longitudinal field studies, epigenetic ageing currently represents the only approach capable of providing direct estimates of age in free-ranging cetaceans. Epigenetic ageing is based on molecular methods that infer age from DNA methylation patterns measured in small skin or blood samples (Arneson et al., 2022; Lu et al., 2023). This makes epigenetic ageing uniquely suited for application in free-ranging cetaceans through minimally invasive biopsy sampling (Barratclough et al., 2025; Peters et al., 2023).

In cetaceans, several species-specific epigenetic clocks have been established for mysticetes, including humpback whales (*Megaptera novaeangliae*; Horvath, Haghani, Zoller, et al., 2022; Polanowski et al., 2014), North Atlantic fin whales (*Balaenoptera physalus*; García-Vernet et al., 2021), Antarctic minke whales (*B. bonaerensis*; Goto et al., 2020; Tanabe et al., 2020), North Atlantic right whales (*Eubalaena glacialis*; Lee, 2021), and bowhead whales (*Balaena mysticetus*; Parsons et al., 2023).

For odontocetes, multi-species epigenetic clocks have been developed, including the ‘*Multi-species Odontocete Epigenetic Ageing Clock*’, cross-validated across nine species (Robeck, Fei, Lu, et al., 2021), and more recently the ‘*Multi-species Cetacean Clock*’ calibrated using 13 species (Zoller et al., 2025). These frameworks enable age estimation across multiple odontocete species using a single model, providing a valuable tool where species-specific sample acquisition is limited (Robeck, Fei, Lu, et al., 2021). While age-predictive performance of multi-species clocks can remain comparable for closely related sister taxa (Peters et al., 2023), comparative evaluations consistently demonstrate superior accuracy of species-specific epigenetic clocks when sufficient calibration data are available (Barratclough et al., 2021; Peters et al., 2023).

However, the development of species-specific epigenetic clocks requires calibration using a sample population of known-age individuals, typically comprising at least ~70 samples (Mayne et al., 2021). As a result, to date only nine species-specific epigenetic clocks have been developed for odontocetes. These include three clocks for common bottlenose dolphins (Barratclough et al., 2021; Beal et al., 2019; Robeck, Fei, Haghani, et al., 2021), two for Indo-Pacific bottlenose dolphins (Peters et al., 2023; Yagi et al., 2024), one for killer whales (Parsons

et al., 2023), one for beluga whales (Bors et al., 2021), one for Hector's and Māui dolphins (*Cephalorhynchus hectori hectori* and *C. h. maui*; Hernandez et al., 2023), and one for Risso's dolphins (*Grampus griseus*; Mori et al., 2024).

In odontocetes, the development of species-specific epigenetic clocks is constrained by the availability of samples from individuals of known age. Consequently, most odontocete clocks have been calibrated using animals in human care (Barratclough et al., 2021; Robeck, Fei, Haghani, et al., 2021) or based on long-term observational studies of well-monitored wild populations (Beal et al., 2019; Parsons et al., 2023; Peters et al., 2023; Yagi et al., 2024). To date, only three odontocete epigenetic clocks have relied on growth layer group counts from stranded or bycaught individuals for initial calibration (Bors et al., 2021; Hernandez et al., 2023; Mori et al., 2024). Notably, concerns have been raised that uncertainty in dental ageing could compromise the robustness of epigenetic clock calibration, particularly in older individuals where growth layer groups may be confounded with accessory lines (Bors et al., 2021; Hernandez et al., 2023; Mori et al., 2024; Zoller et al., 2025).

2.2.2. Radiographic ageing

Similar to methods used in humans and other terrestrial mammals (Section 1.1), radiographic imaging has also been explored as a non-invasive alternative for age assessment in cetaceans. Approaches applied to date include radiographic measurement of the dental pulp-to-tooth ratio (Herrman et al., 2020) and evaluation of forelimb (i.e., pectoral flipper) bone maturation (Galatius et al., 2006; Ogden et al., 1981). However, dental radiography in bottlenose dolphins has only been shown to be reliable up to approximately 10 years of age (Herrman et al., 2020), and forelimb-based approaches were, until recently, calibrated only for the classification of broad age categories (Galatius et al., 2006; Ogden et al., 1981).

However, in 2019, a radiographic approach was presented to reliably age bottlenose dolphins of all age classes (Barratclough, Sanz-Requena, et al., 2019), which is possible because cetacean forelimbs exhibit numerous developmental and osteological differences compared to their terrestrial ancestors (Dawson, 2003). Cetaceans possess an increased number of phalanges per digit compared to the plesiomorphic phalangeal count of 2-3-4-5-4 for amniotes or 2-3-3-3-3 for mammals (Caldwell, 2002; Cooper, 2009; Cooper et al., 2007; Cooper & Dawson, 2009; Fedak & Hall, 2004; Richardson & Oelschläger, 2002; Sun et al., 2022), affecting digit I, II, and III in long-finned pilot whales (*Globicephala melas*; Figure 1.2), digit II and III in other

extant odontocetes and digit III and IV in extant mysticetes (Cooper et al., 2007; Richardson & Oelschläger, 2002).

Furthermore, the bone ossification of cetaceans is timed differently compared to wider terrestrial taxa (Mellor et al., 2009). Specifically, forelimb bone ossification is delayed in some cetaceans, resulting in poorly ossified digital elements in adults (Mellor et al., 2009). Due to the delay in bone maturation, adult forelimbs resemble those of juveniles (Mellor et al., 2009). This process is defined as paedomorphosis and has been described in vaquitas (*Phocoena sinus*; Mellor et al., 2009), harbour porpoises (*P. phocoena*; Galatius et al., 2006), killer whales (Mellor et al., 2009), as well as baleen whales (Flower, 1885). The mechanism behind paedomorphism in cetaceans is assumed to be an acceleration of sexual maturation relative to the rest of development (progenesis; Galatius et al., 2006).

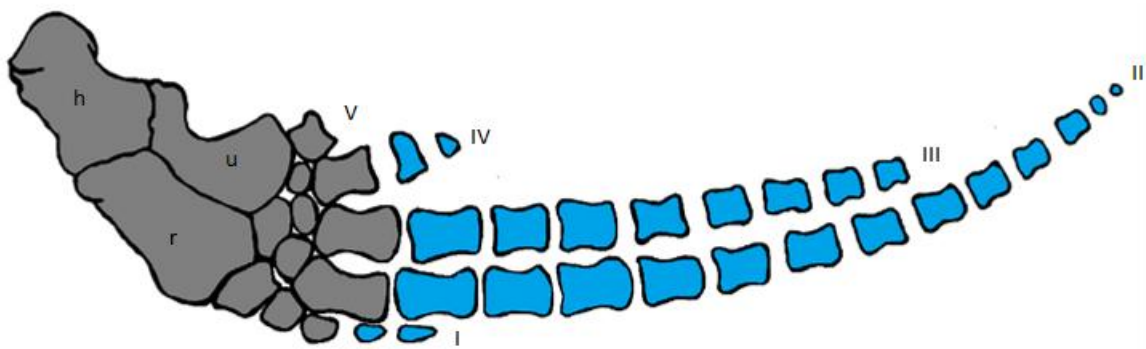


Figure 1.2: Hyperphalangy in pilot whale (*Globicephala melas*). The phalanges are depicted in blue. Abbreviations: h: humerus, r: radius, u: ulna. Illustration modified from Fedak & Hall (2004).

A further difference between cetaceans and their terrestrial ancestors is the physiological presence of delta bones (Cooper et al., 2007; Dawson, 2003). Delta bones are defined as triangular in shape with a convex and a concave longitudinal border (Dawson, 2003), and were initially described as a congenital deformity in humans (Choo & Mubarak, 2013; Wood & Flatt, 1977). In cetaceans, these bones present a physiological stage in the bone maturation of specific metacarpals and phalanges (Dawson, 2003). Delta bones ossify at later stages of life than long bones and are therefore inherently valuable for age assessment of elderly cetaceans (Barratclough, Sanz-Requena, et al., 2019).

Due to paedomorphosis, hyperphalangy, and the existence of delta bones in cetaceans, age-related changes continue to occur after sexual maturity and enable radiographic age assessment of bottlenose dolphins of all age classes (Barratclough, Sanz-Requena, et al., 2019). The 2019 study developed a scoring system for delta bone maturation and integrated it with assessments of long bone maturation and age-related degenerative, arthritic changes in older individuals (Barratclough, Sanz-Requena, et al., 2019). Although this represents a major advance in age assessment for bottlenose dolphins, the approach has not yet been applied to any other cetacean species.

Evidence from multiple odontocete taxa indicates that the sequence of bone maturation is broadly conserved, suggesting that this framework may have wider applicability. In bottlenose dolphins (Barratclough, Sanz-Requena, et al., 2019), Guiana dolphins (*Sotalia guianensis*; De Carvalho et al., 2015), striped dolphins (*Stenella coeruleoalba*; Calzada & Aguilar, 1996), harbour porpoises (Galatius et al., 2006), Dall's porpoises (*Phocoenoides dalli*; Ogden et al., 1981), and short-finned pilot whales (*G. macrorhynchus*; Lee, 1978; Ogden et al., 1981), epiphyseal ossification typically initiates in the humerus, followed by the proximal epiphyses of the radius and ulna, then the distal radial and ulnar epiphyses, and finally the metacarpals and phalanges in a proximodistal progression.

Despite this conserved sequence, the temporal progression of bone maturation varies among species and is shaped by life-history traits such as longevity, growth rate, and behaviour. Ossification timing may also be influenced by genetic background, environmental conditions, and endocrine factors, including thyroxine, growth hormone, and sex steroids (Almeida et al., 2017; Iuliano-Burns et al., 2009; Keller et al., 1970; Ogden et al., 1981; Ratib et al., 2012).

Variation in sex steroid concentrations further contributes to sex-specific differences in ossification timing. In most cetaceans, females attain sexual maturity earlier than males (Galatius et al., 2006), and pubertal increases in oestrogens and testosterone promote epiphyseal maturation (Almeida et al., 2017; Iuliano-Burns et al., 2009). In bottlenose dolphins, female forelimb bones grow more rapidly during early development, reach asymptotic growth earlier, and show earlier maturation compared to males (Barratclough, Sanz-Requena, et al., 2019). Similarly, in striped dolphins, vaquitas, and harbour porpoises, bone maturation patterns are comparable between sexes up to approximately 5–6 years of age, after which females exhibit

more advanced epiphyseal ossification than age-matched males (Calzada & Aguilar, 1996; Galatius et al., 2006; Mellor et al., 2009).

Accordingly, accurate age estimation based on skeletal maturation requires species-specific calibration using radiographs from individuals of known age (Barratclough, Sanz-Requena, et al., 2019), a requirement that can only be met through long-term biobanks that curate age-linked specimens alongside complementary matrices such as pectoral flippers, teeth, and gonadal tissue. Such repositories can provide calibration datasets spanning the full range of developmental stages and allow direct integration of skeletal, dental, and reproductive information from the same individuals.

3. Methodological gaps

While alternative methods for age estimation exist in odontocetes, these approaches have been calibrated for only a limited number of species, and age determination therefore still largely relies on post-mortem examination and histological processing of teeth. Epigenetic clocks presently exist for only a handful of species, and radiographic bone ageing has been formally developed only for bottlenose dolphins. The scarcity of individuals of known age has constituted a major limiting factor for the development and validation of both epigenetic and radiographic approaches. Common dolphins are among the taxa for which only post-mortem dental ageing is currently available, underscoring the need for a flexible and complementary toolkit that can be applied both to free-ranging individuals and in situations where tooth extraction is not feasible. Furthermore, for radiographic methods it remains unclear whether interspecific differences in morphology, anatomy, and developmental patterns constrain cross-species transferability, emphasising the need for comparative evaluation and cross-validation prior to application in novel taxa.

4. Common dolphins in New Zealand as research opportunity

The development, calibration, and cross-validation of ageing methods require large, well-characterised reference datasets spanning broad age ranges and both sexes. Such datasets are rarely available for cetaceans, as most life-history research relies on opportunistic samples from stranded or bycaught individuals, and only a small number of long-term programmes have combined this with the foresight to systematically collect, archive, and curate multiple tissue

types alongside detailed biological metadata. This challenge is compounded by the fact that odontocetes are long-lived relative to the duration of most scientific studies and funding cycles, rendering true longitudinal monitoring across the lifespan of individuals logistically difficult and rarely achievable.

The development of reliable ageing methods is particularly important for common dolphins given the conservation pressures faced by the species. Common dolphins are among the most heavily human-impacted delphinids globally (Bearzi et al., 2008; Peltier et al., 2016, 2021; Piroddi et al., 2011) and represent the most frequently bycaught dolphin species in Australasia (Abraham et al., 2021; Tulloch et al., 2020). Understanding the population-level consequences of such mortality requires robust demographic information, for which accurate age estimation is fundamental. Age data underpin estimates of age-specific survival, mortality, and reproductive parameters, including age at sexual maturity and calving intervals, and consequently contribute to population viability analyses used to assess population resilience and responses to anthropogenic pressures (Manlik et al., 2022; Verborgh et al., 2021). The availability of reliable and broadly applicable ageing methods is therefore important not only for individual age determination, but also for understanding the demographic consequences of fisheries interactions and informing the conservation and management of common dolphin populations.

Common dolphins in New Zealand represent an exceptional research opportunity in this regard due to the availability of a long-term comprehensive biobank housed at Massey University. Over the past three decades, a diverse selection of tissues has been systematically collected from stranded and bycaught individuals, with associated metadata including sex, reproductive status, and chronological age estimated using established post-mortem methods (Palmer et al., 2022, 2023). Specifically, dental age estimation based on growth layer group analysis (Appendix 1, Table A.1.2) has underpinned previous life-history studies (Palmer et al., 2022, 2023). The biobank spans a broad range of age classes and both sexes and provides a robust set of individuals for which key life-history parameters have been independently characterised alongside wider data on contaminants, health, and disease. Such datasets are essential for methodological research, as the development and validation of alternative age-assessment approaches require reference material from individuals of known age and reproductive status.

5. Rationale and significance of the study

This thesis addresses the need for complementary ageing methods that can be applied to live, free-ranging individuals or that provide more accessible and scalable alternatives to existing post-mortem approaches, while still delivering biologically meaningful estimates of age. It does so by calibrating and evaluating two new alternative ageing approaches for common dolphins: epigenetic age estimation based on DNA methylation patterns and radiographic assessment of skeletal maturation. Both approaches are calibrated and assessed using individuals of known chronological age derived from established dental growth layer group analyses. This thesis enables a direct comparison of molecular and radiographic ageing methods and discusses their respective advantages and limitations. As part of this discussion, the thesis further evaluates one specific advantage of radiographic ageing, namely whether radiographic flipper assessment can be applied to infer sexual maturity within a species-specific framework and thereby extend its utility beyond chronological age estimation alone.

Specifically, the objectives of the thesis are:

- Objective 1:** Calibration of the first species-specific epigenetic age estimation model for common dolphins using skin samples from stranded and bycaught individuals, and evaluation of how reliance on dental age estimates from deceased animals influences model performance.
- Objective 2:** Assessment of the cross-species applicability of the bottlenose dolphin radiographic ageing framework to common dolphins, considering morphological, anatomical, and developmental differences between the two species.
- Objective 3:** Calibration of a radiographic ageing framework for common dolphins that incorporates known morphological, anatomical, and developmental differences between common and bottlenose dolphins, and evaluation of radiographic ageing results in relation to epigenetic clock performance.
- Objective 4:** Assessment of the reliability of pectoral flipper radiographs for inferring sexual maturity, and derivation of probabilistic curves to estimate sexual maturity based on bone maturation stage.

6. Thesis structure

This thesis is organised into six chapters, comprising an overall introduction, four data chapters, and a general discussion chapter. The data chapters are written in publication format and comprise two published chapters (Chapters 2 and 3), one chapter in press (Chapter 5), and one currently under peer review (Chapter 4). This format has resulted in some unavoidable repetition, particularly in relation to background and materials and methods; however, every effort has been made to limit duplication where appropriate. The aims of each chapter are as follows:

Chapter 1 provides an overview of age estimation methods and establishes the need for complementary and alternative methods to assess chronological age in common dolphins.

Chapter 2 presents the first species-specific epigenetic age estimation model for common dolphins and evaluates the reliability of using dental age estimates as a calibration reference, as well as the implications of calibrating molecular ageing models using post-mortem skin samples. This chapter is a reformatted version of the following manuscript: Hanninger, E. F., Peters, K. J., Gerber, L., Barratclough, A., Betty, E. L., Palmer, E. I., Horvath, S., Stockin, K. A. (2025). Dental ageing offers new insights into the first epigenetic clock for common dolphins (*Delphinus delphis*). *Ecology and Evolution* 15, no. 11: e72424. <https://doi.org/10.1002/ece3.72424>.

Chapter 3 evaluates the cross-species applicability of a radiographic skeletal ageing framework originally developed for bottlenose dolphins to common dolphins. This chapter examines morphological and developmental differences between the two species, as well as the occurrence of age-related joint degeneration and its relevance for radiographic age estimation. Chapter 3 is a reformatted version of the following manuscript: Hanninger, E. F., Barratclough, A., Betty, E. L., Dittmer, K., Saltr , F., Perrott, M. R., Bowler, J., Peters, K. J., Stockin, K. A. (2026). Cross-species applicability of a bone-ageing method in delphinids. *Royal Society Open Science*, <https://doi.org/10.1098/rsos.252005>.

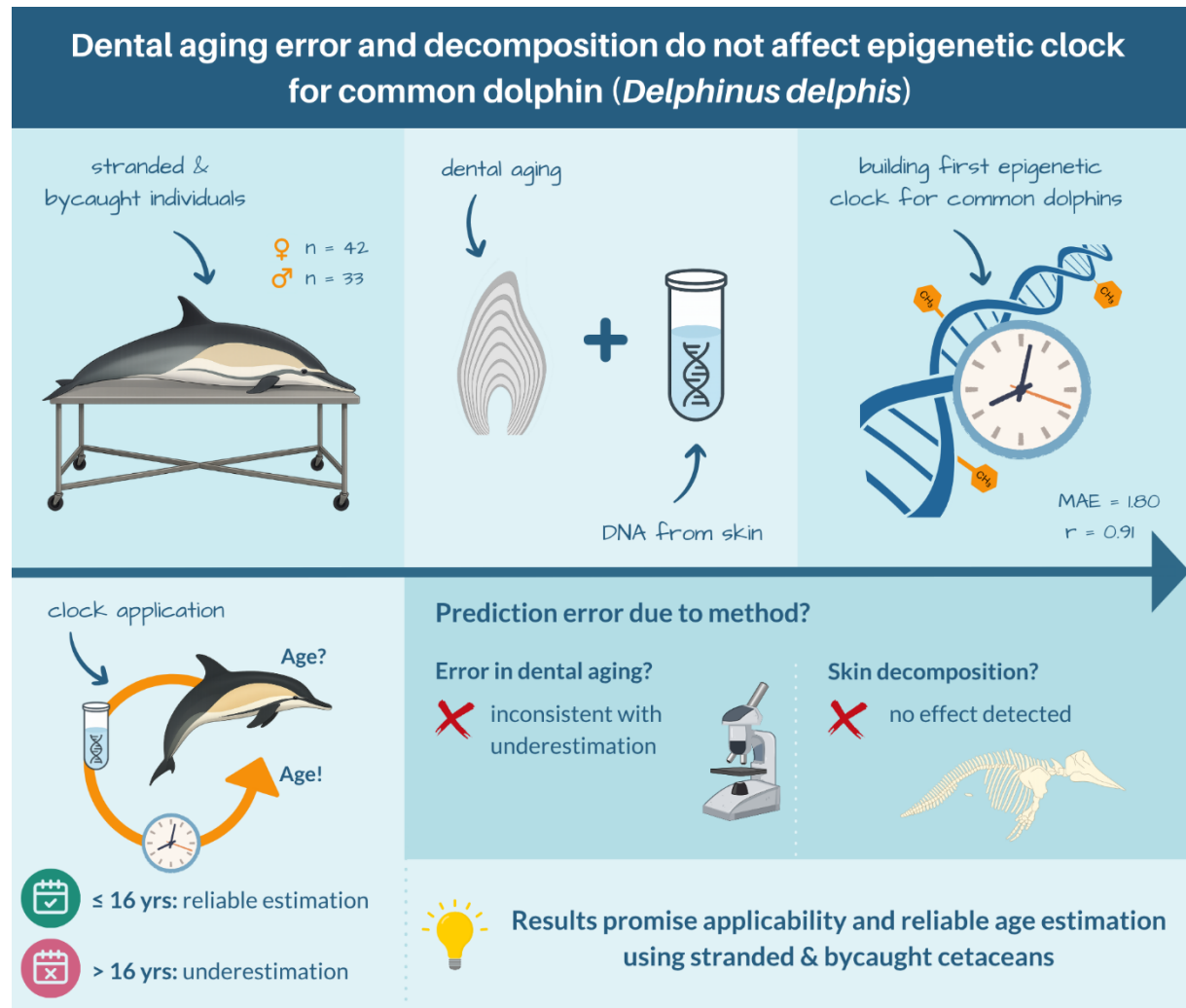
Chapter 4 presents the first radiographic pectoral flipper ageing framework for common dolphins and assesses its reliability through direct comparison with epigenetic clock performance. This chapter is a reformatted version of the following manuscript: Hanninger, E. F., Barratclough, A., Betty, E. L., Anderson, M. J., Perrott, M. R., Bowler, J., Palmer, E. I., Peters, K. J., Stockin, K. A. (in review). Radiographic assessment of bone maturation as a tool for age estimation in common dolphins (*Delphinus delphis*). *Marine Biology*.

Chapter 5 evaluates the use of pectoral flipper radiography for inferring sexual maturity status in common dolphins and derives probabilistic curves that enable estimation of sexual maturity based on skeletal maturation stage. This chapter is a reformatted version of the following manuscript: Hanninger, E. F., Barratclough, A., Betty, E. L., Anderson, M. J., Perrott, M. R., Bowler, J., Palmer, E. I., Peters, K. J., Stockin, K. A. (2026). Radiographic assessment of bone maturation: A tool to estimate sexual maturity in common dolphins (*Delphinus delphis*)? *Marine Mammal Science*, <https://doi.org/10.1111/mms.70175>.

Chapter 6 synthesises the findings of the data chapters by comparing the performance and reliability of the different ageing approaches, discussing their application for conservation and management, and outlining priorities for future research.

Chapter 2

Dental ageing offers new insights to the first epigenetic clock for common dolphins (*Delphinus delphis*)



This chapter is a reformatted version of the following manuscript:

Hanninger, E. F., Peters, K. J., Gerber, L., Barratclough, A., Betty, E. L., Palmer, E. I., Horvath, S., Stockin, K. A. (2025). Dental ageing offers new insights into the first epigenetic clock for common dolphins (*Delphinus delphis*). *Ecology and Evolution* 15, no. 11: e72424. <https://doi.org/10.1002/ece3.72424>.

STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Eva-Maria F. Hanninger		
Name and title of main supervisor:	Professor Karen A. Stockin		
In which chapter is the manuscript/published work?	Chapter 2		
Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:¹ Laboratory work was conducted by Eva-Maria Hanninger, Emma Betty, Emily Palmer, Katharina Peters, and Karen Stockin. Statistical analyses were conducted by E. Hanninger with support from K. Peters, Livia Gerber, and Ashley Barratclough. Conception, study design, and methodology were undertaken by E. Hanninger, K. Peters, and Karen Stockin. The manuscript was drafted by E. Hanninger. Review and editing of the final manuscript were performed by E. Hanninger, K. Peters, L. Gerber, A. Barratclough, E. Betty, E. Palmer, Steve Horvath, and K. Stockin. Funding acquisition was led by E. Hanninger and K. Stockin.			
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Abstract

Determining exact age in wild odontocetes is essential for understanding population dynamics, survival, and reproduction, yet remains logistically challenging. Although epigenetic ageing is emerging as a valuable approach, only nine species-specific clocks currently exist. Most have been calibrated using known-age animals in human care or well-studied wild populations. Only three previous studies have used dental ages from stranded or bycaught individuals. This is due to concerns that dental age inaccuracies, especially in older animals, may affect epigenetic clock performance. To explore this, we developed the first species-specific epigenetic clock for common dolphins (*Delphinus delphis*), analysing DNA methylation at 37,492 cytosine-phosphate-guanine sites in skin samples from stranded and bycaught dolphins with estimated dental ages. Elastic net models with Leave-One-Out Cross-Validation were applied to three subsets: the ‘relaxed’ subset (all individuals; $n = 75$, median absolute error (MAE) = 2.02, Pearson correlation (r) = 0.81, $R^2 = 0.66$), the ‘strict’ subset (excluding individuals with minimum dental age estimates only; $n = 73$, MAE = 2.29, $r = 0.81$, $R^2 = 0.66$), and the ‘restricted’ subset (excluding outliers with prediction errors >6 years; $n = 63$, MAE = 1.80, $r = 0.91$, $R^2 = 0.82$) to compare performance. Our models consistently underestimated the age of dolphins >16 years, even when minimum dental ages were applied, suggesting that absolute errors between dental and epigenetic estimates are unlikely to reflect the dental ageing error. Additionally, post-mortem decomposition condition code (DCC 1 to 3) did not affect age prediction, signalling promise for future epigenetic clocks calibrated with strandings and bycaught individuals.

1. Introduction

In wildlife management, age determination plays a vital role in understanding population structure, an individual's reproductive state, and lifespan (Barratclough, Sanz-Requena, et al., 2019; Betty et al., 2022). Such knowledge of population age structure provides crucial insights into population viability and resilience to both anthropogenic and environmental pressures (Betty et al., 2019; Heydenrych et al., 2021; Manlik et al., 2022; Palmer et al., 2022). More broadly, understanding life-history features is essential for effective conservation and management of protected species (Moore & Read, 2008), particularly those impacted by human-induced threats such as fisheries interactions, habitat degradation, and pollution

(Murphy et al., 2018, 2021; Peltier et al., 2021; Stockin et al., 2010; Tulloch et al., 2020). However, accurate age estimates are essential to ensure reliable assessment of key life-history parameters, such as age at sexual maturity (ASM) and inter-calving intervals (Manlik et al., 2022; Palmer et al., 2022; Verborgh et al., 2021). Yet obtaining such estimates is particularly challenging in odontocetes (toothed whales and dolphins), which exhibit limited external signs of ageing (Barratclough et al., 2023; Beal et al., 2019; Krzyszczyk & Mann, 2012). Although total body length can assist in assessing age class (i.e., neonate/juvenile/adult), growth varies greatly among individuals of the same species and age, making it unreliable for precise age estimation (Betty et al., 2019; Chivers, 2018). Instead, age estimation requires either long-term field studies (Connor & Krützen, 2015; Peters et al., 2023) or invasive methods, such as tooth extraction (Lockyer, 1993; Westgate & Read, 2007).

Typically, odontocetes are aged by quantification of growth layer groups in teeth, also known as “tooth ageing” (e.g., Betty et al., 2022; Cooper et al., 2018; Evans et al., 2002; Lockyer, 1993, 1995; Murphy et al., 2014; Westgate & Read, 2007). Tooth ageing requires trained, experienced readers and calibration using individuals of known age (Perrin & Myrick, 1980). Although growth layer groups are generally assumed to represent annual deposition in most species — with the exception of beluga whale (*Delphinapterus leucas*; e.g., Goren et al., 1987; Waugh et al., 2018) — their interpretation becomes increasingly challenging in older individuals. Contributing factors include varying degrees of tooth wear, compression of growth layer groups, and the presence of accessory lines (additional growth lines that may not correspond to annual deposition; Barratclough et al., 2023; Murphy et al., 2014; Read et al., 2018). Additionally, because of its invasive nature, tooth extraction is typically restricted to post-mortem investigations, except for procedures conducted under anaesthesia (Barratclough, Wells, et al., 2019), and accordingly does not permit assessment of demography in free-ranging populations (Betty et al., 2023). Consequently, age estimates are often derived from stranded or bycaught individuals, which may not accurately represent the age structure of free-ranging populations and may bias population viability analyses and survivorship estimates (Betty et al., 2023).

Emerging molecular methods offer a minimally invasive alternative that can be applied to free-ranging cetaceans, using samples such as skin or blood, for example, to assess epigenetic patterns of DNA methylation for age estimation (Bocklandt et al., 2011; Horvath, 2013; Horvath & Raj, 2018; Teschendorff & Horvath, 2025). Because of a phenomenon called

‘epigenetic drift’, genomic regions gain or lose cytosine methylation with age (Issa, 2014; Poulsen et al., 2007; Sen et al., 2016).

The Mammalian Methylation Consortium carried out epigenome-wide association studies of age in 348 mammalian species, including mysticetes and odontocetes (Haghani et al., 2023; Lu et al., 2023). The correlation between chronological age and DNA methylation at CpG sites (cytosine-phosphate-guanine) enables the development of epigenetic clocks, which are regarded as the most reliable predictors of age available (De Paoli-Iseppi et al., 2017; Guevara & Lawler, 2018; Jylhävä et al., 2017; Simpson & Chandra, 2021). The initial development of epigenetic clocks requires a highly accurate known age calibration population (Mayne et al., 2023). To date, 11 epigenetic clocks exist for odontocetes: three for common bottlenose dolphins (*Tursiops truncatus*; Barratclough et al., 2021; Beal et al., 2019; Robeck, Fei, Haghani, et al., 2021), two for Indo-Pacific bottlenose dolphins (*T. aduncus*; Peters et al., 2023; Yagi et al., 2024), one for killer whales (*Orcinus orca*; Parsons et al., 2023), one for beluga whales (Bors et al., 2021), one for Hector's and Māui dolphins (*Cephalorhynchus hectori hectori* and *C. h. maui*; Hernandez et al., 2023), one for Risso's dolphins (*Grampus griseus*; Mori et al., 2024), and 2 multi-species odontocete or cetacean clocks, which were cross-validated with 9 (Robeck, Fei, Lu, et al., 2021) and 13 species (Zoller et al., 2025).

Most odontocete clocks have been calibrated on animals in human care (Barratclough et al., 2021; Robeck, Fei, Haghani, et al., 2021), or long-term observational studies of well-monitored populations (Beal et al., 2019; Parsons et al., 2023; Peters et al., 2023; Yagi et al., 2024). At the time of our study, only three prior studies used growth layer groups of stranded and bycaught animals for the initial calibration of their epigenetic clocks (Bors et al., 2021; Hernandez et al., 2023; Mori et al., 2024). Notably, concerns have been raised about whether errors in dental ageing may affect the robustness of epigenetic clocks, particularly in older individuals where growth layer groups can be confused with accessory lines (Bors et al., 2021; Hernandez et al., 2023; Mori et al., 2024; Zoller et al., 2025).

Here, we present the first epigenetic clock for common dolphins (*Delphinus delphis*) — an essential tool for future population viability analysis (PVA) in a species highly impacted by human activities (Peltier et al., 2021; Piroddi et al., 2011; Stockin et al., 2009; Tulloch et al., 2020). Further, we provide three distinct models that enhance current understanding of the reliability of dental ageing for age calibration of methylated clocks. Our clock was developed

using dental age estimates from individuals spanning the full known lifespan of free-ranging common dolphins, estimated at approximately 30 years (Perrin, 2009), with reports of individuals in human care reaching >35 years (Murphy et al., 2014). As dental ageing can be less accurate in older individuals because of tooth wear and growth layer group compression, this approach allows us to evaluate how such uncertainties can affect clock accuracy. Specifically, we hypothesised that: (1) a clock model excluding individuals with uncertain dental age estimates would outperform one that includes all samples, and (2) a model on the basis of all samples would perform worse than previously published odontocete clocks calibrated using known-age individuals from long-term observational studies or human care.

2. Materials & methods

2.1. Sample collection

As part of ongoing longitudinal studies on health and life history, we accessed a 30-year tissue archive of aged common dolphins spanning 0 to 34 years of age, for which sex, body length, sexual maturity, reproductive stage, and radiographic bone ageing had been completed *a priori*. Dental age was assessed via growth layer groups in the dentine of thin, decalcified, and stained sections of teeth by at least two independent, experienced readers over three readings. If no agreement was reached, an additional tooth was prepared and examined by at least two readers until consensus was reached on the age estimate. In total, each animal had 1 to 3 teeth processed for ageing. Age estimates were further validated against additional indicators of physical and sexual maturity, including total body length, the number of ovarian *corpora albicans* scars on the basis of Palmer et al. (2022) and pectoral flipper radiographic ageing (adapted from Barratclough, Sanz-Requena, et al., 2019). These metrics allowed cross-verification of age estimates across multiple biological parameters.

Full-thickness skin samples were routinely collected from the trailing edge of the fluke during post-mortem examination. To ensure an adequate sampling size for the construction of a reliable epigenetic clock (Mayne et al., 2021), we selected skin from 84 common dolphin samples examined between 2000 and 2023. Sample selection was guided by multiple criteria, including dental age (Table A2.1) and sex ratio (females: $n = 47$; males: $n = 37$). We included individuals with dental ages spanning the maximum known lifespan of common dolphins, with equal age and sex distribution, as the sample availability would allow. Our samples originated from various mortality contexts, including bycatch ($n = 9$) and strandings (single stranding events: n

= 46; mass stranding events: $n = 28$), and one individual from human care. Only animals of decomposition condition category DCC 1–3 (IJsseldijk et al., 2019) were included in the study (Tables A2.1 and A2.2).

For 82 common dolphins, exact dental ages were ascertained. For the remaining two samples, only age ranges could be estimated. Here, we applied the minimum year of each age estimate to reduce the risk of age overestimation because of accessory lines. The age range of our samples spanned the estimated lifespan of the common dolphin, with the oldest free-ranging individual having a minimum dental age of 31 years. Additionally, we included a skin sample from a dolphin in human care (Kelly, Napier Marineland, held 1974 to 2008). Because of a pulp anomaly, only an age range could be determined, but Kelly was known to have spent 34 years in human care (Murphy et al., 2014).

2.2. DNA extraction

Genomic DNA was extracted from skin samples using a QuickDNA Miniprep Plus Kit (Zymo) following the protocol for solid tissue samples. DNA purity was assessed using a NanoDrop Microvolume Spectrophotometer (Thermo Fisher Scientific) by an automated measurement of absorbance at the wavelengths 260 and 280 nm (Desjardins & Conklin, 2010). DNA was considered pure with an absorbance ratio ~ 1.8 at 260 nm/280 nm (Desjardins & Conklin, 2010). DNA was purified using a DNA Clean & Concentrator Kit (Zymo) following the manufacturer's instructions and concentration was measured using a QUBIT 4 fluorometer (Thermo Fisher Scientific) aiming for a total DNA amount per sample of >250 ng.

2.3. DNA methylation data

A custom Infinium methylation array (HorvathMammalMethyl Chip40) assembled with 37,492 CpG sites was used to measure DNA methylation (Arneson et al., 2022). The array targets highly conserved CpG sites across mammals, allowing the assessment of DNA methylation in each mammal (Arneson et al., 2022). Sample quality control followed the Clock Foundation pipeline (Zoller & Horvath, 2024). Methylated (M) and Unmethylated (U) channels reporting background signal levels were marked as failed with the p-value threshold of $p > 0.05$. Additionally, the proportion of failed probes was counted for each sample. Samples with more than 1% of failed probes were considered fraction outliers. Further quality control steps included principal component analysis (PCA) performed on the centred, unnormalised beta-

value matrix to identify major sources of variance in the dataset (Mishra et al., 2017), as well as hierarchical clustering analysis, in which samples exceeding two standard deviations from the mean were marked as outliers (Zoller & Horvath, 2024). Nine samples were excluded from further analysis (see Results and Table A2.2).

2.4. Subsets

We developed the epigenetic clock using three distinct sample subsets. Details of sample selection for each subset are provided in Table A2.1, in addition to age distributions by sex (Figure A2.1). The first model, referred to as the ‘*relaxed*’ subset ($n = 75$; age range: 0–34 years) included all samples, also incorporating two individuals for whom only a minimum estimated dental age was available; both originated from individuals >30 years of age. The second model, termed ‘*strict*’, excluded these two individuals and only incorporated animals with confirmed dental ages ($n = 73$; age range: 0–31 years). Following initial analyses, a third model was generated using only samples from the ‘*relaxed*’ subset that showed an absolute error of less than 6 years between actual and predicted age. This ‘*restricted*’ subset ($n = 63$; age range: 0–26 years) automatically excluded the two individuals with the minimum age estimates. Except for three samples, all excluded individuals were ≥ 16 years old.

2.5. Age prediction

2.5.1. Elastic net regression with LOOCV

To build the epigenetic clocks, we used the `MammalMethylClock` R package, which incorporates functionalities for the assessment, development, and utilisation of epigenetic clocks (Zoller & Horvath, 2024).

The package uses pre-existing life-history data from the Animal Ageing and Longevity Database (AnAge; Human Ageing Genomic Resources, n.d.). In the case of *Delphinus delphis*, the AnAge data (female average age at attainment of sexual maturity (ASM): 4.8 years, male ASM: 4.3 years) did not align with previously published life-history data for common dolphins (Danil & Chivers, 2007; Murphy & Rogan, 2006; Palmer et al., 2022, 2023; Westgate & Read, 2007); therefore, we manually overwrote the data with an average value of 8.15, as reported for male and female reproduction in Aotearoa New Zealand common dolphins (female ASM: 7.5 years, male ASM: 8.8 years; Palmer et al., 2022, 2023). For the gestation period, we used 1.05

years, which was determined on the basis of the Aotearoa New Zealand common dolphins (Palmer et al., 2022). On the basis of these life-history features, we applied a log-linear transformation to our methylation data (Lu et al., 2023; Zoller & Horvath, 2024). This transformation accounts for accelerated epigenetic ageing processes before attainment of sexual maturity (ASM), providing a logarithmic function if age is lower than ASM and a linear form in case age is greater than ASM (Lu et al., 2023). We further used the function `fun_llin2.inv` to take species-specific gestation times into account.

To build a species-specific epigenetic clock, the package uses an elastic net regression (Zoller & Horvath, 2024), which is an algorithm for fitting generalised linear models with elastic-net penalties (Friedman et al., 2010). The elastic net algorithm works on very large datasets in which the number of predictor variables exceeds the number of observations (Friedman et al., 2010). Elastic nets are particularly useful in situations where there are many correlated predictor variables. We used an elastic net regression model (`glmnet` package) to determine the optimal mixing parameter (α) via cross-validation on 20 random subsets (each with 2/3 of the data). Models were fit across α values from 0 to 1 (in 0.1 steps), and the α with the lowest median mean squared error was selected (Barratclough et al., 2021). On the basis of this analysis, the elastic net was chosen (‘*relaxed*’ subset: $\alpha = 0.4$, ‘*strict*’ subset: $\alpha = 0.5$, ‘*restricted*’ subset: $\alpha = 0.7$) to remove any degeneracies and wild behaviour caused by extreme correlations (Friedman et al., 2010; Lu et al., 2023; Zoller & Horvath, 2024).

Cross-validation was completed with a Leave-One-Out Cross-Validation (LOOCV) analysis (Zoller & Horvath, 2024). This function uses n -fold internal cross-validation, with n being the number of samples (Zhang, 1993; Zou & Hastie, 2005). For each sample of the data, the LOOCV approach skips one sample, fits the epigenetic clock on the remaining data, and predicts the age of the omitted sample (Zhang, 1993; Zou & Hastie, 2005).

2.5.2. Hybrid epigenetic clock

For the ‘*relaxed*’ subset, we applied a hybrid DNA methylation age prediction approach integrating a random forest classifier (RFC) and two elastic net regression (ENR) models following Barratclough et al. (2024). Physical maturity status was classified using an RFC with 1000 trees, trained to distinguish physically mature dolphins (≥ 18 years for females, ≥ 20 years for males; Palmer, 2023). Two ENR models were trained to predict log-linearly transformed

ages, anchored on sex-specific sexual maturity thresholds (7.5 years for females, 8.8 years for males; Palmer et al., 2022, 2023). The first and second ENR were trained on all individuals and physically mature dolphins, respectively, to refine age predictions in older animals. For both ENRs, the optimal balance between LASSO and Ridge penalties (α) and the penalty parameter (λ) was selected by nested 5-fold internal cross-validation on the training data. Overall model performance was evaluated using 5-fold cross-validation. In each fold, the RFC and ENR models were retrained on the training data, and age predictions were generated as a weighted sum of the two ENR models, where the weight for the mature-only model corresponded to the RFC-predicted probability of being physically mature. A gestation adjustment of 1.05 years was applied to the final age estimates (Palmer et al., 2022).

2.5.3. Error metric assessment

The accuracy of the resulting epigenetic clocks was tested with Pearson correlation coefficients (r) and the calculation of median absolute errors (MAEs) between dental and predicted epigenetic age. We further tested the accuracy of the DNA age predictions by performing a linear regression of DNA predicted age against dental age (Barratclough et al., 2021; Beal et al., 2019; Peters et al., 2023). This analysis allowed us to assess how closely the DNA-predicted ages aligned with dental age estimates. A slope close to 1 and an intercept near 0 would indicate a strong agreement between predicted and observed values, whereas deviations may highlight systematic under- or overestimation. The coefficient of determination (R^2) was also examined to quantify the proportion of variance in DNA-predicted age explained by dental age.

2.5.4. Effect of tissue decomposition

To assess whether tissue decomposition influenced the accuracy of epigenetic age predictions, we compared the absolute age prediction errors from the ‘*relaxed*’ subset across decomposition condition categories DCC 1 to 3 (fresh to moderate) assigned post-mortem during sampling (IJsseldijk et al., 2019). Absolute prediction errors were calculated as the absolute difference between predicted and observed ages for each individual. We then used a Kruskal–Wallis rank sum test to assess differences in error distributions among decomposition condition categories, as the error data did not meet the assumptions of normality.

2.5.5. Effect of storage duration

To evaluate whether long-term storage of samples could bias epigenetic age estimates, storage time was calculated as the difference between the year of DNA methylation analysis (2023) and the year of sample collection, as indicated in the sample identifier. Absolute age prediction errors from across all samples were used as the response variable. Associations between storage time and prediction error were assessed using Spearman rank correlation (robust to deviations from normality) and linear regression, with and without chronological age included as a covariate. For the regression models, residuals were inspected using diagnostic plots and tested for normality using the Shapiro–Wilk test to confirm model assumptions.

2.5.6. Age predictions on the basis of other odontocetes clocks

Additionally, our clock's performance was tested in relation to that of other epigenetic clocks, using the `MammalMethylClock` R package, browsing the Clock database with the search criteria ‘odontocetes’ and ‘skin samples’. We applied previously published multi-species and species-specific odontocete clocks to our samples and compared the respective MAE and r -values between the resulting clocks.

2.6. Sex prediction

To predict the sex of each sample on the basis of the methylation data, we used the R package `glmnet` following the approach of Peters et al. (2023) using a binomial elastic net regression with $\alpha = 0.4$ without LOOCV. The sex was encoded as a binary outcome variable (0 = female, 1 = male; Peters et al., 2023). With a predicted probability >0.5 , the sample was male (Peters et al., 2023).

3. Results

Following Zoller & Horvath (2024), eight outliers collected between 2001 and 2008 were removed as part of the sample quality assessment (e.g., hierarchical clustering, failed probe fractions, and principal component analysis; Table A2.2). In addition to these technical outliers, one further animal (KS19-23Dd) was excluded because of inconsistencies between dental age, pectoral bone age, and number of *corpora albicans*. Accordingly, analyses continued with a total sample size of 75 animals.

3.1. Age prediction

3.1.1. Elastic net regression with LOOCV

The elastic net models with LOOCV showed a strong correlation between dental age and predicted age across the different subsets (Figure 2.1). For the ‘*relaxed*’ subset, the elastic net regression retained 38 CpG sites (Table A2.3) and produced an MAE of 2.02 years, with a correlation of $r = 0.81$ ($p = 1.39 \times 10^{-18}$) and an R^2 of 0.66 using LOOCV. In 12 individuals, the absolute difference between actual and predicted age exceeded 6 years (Table 2.1). Although predicted age was overestimated in three younger individuals (aged 11.5, 12 and 14 years), most discrepancies ($n = 9$) involved underestimation in older dolphins with a dental age greater than 16 years. The training set of the ‘*strict*’ subset retained 24 CpG sites (Table A2.3) and yielded a MAE of 2.29 years, with a correlation of $r = 0.81$ ($p = 2.33 \times 10^{-18}$) and an R^2 of 0.66 using LOOCV. Nine samples, all from individuals older than 16 years, had an absolute error exceeding 6 years, with the predicted ages consistently underestimated in all cases. For the ‘*restricted*’ subset, the elastic net model retained 22 CpG sites (Table A2.3) and demonstrated the best performance, achieving a MAE of 1.80 years, a correlation of $r = 0.91$ ($p = 1.48 \times 10^{-24}$), and an R^2 of 0.82 using LOOCV. In this subset, four individuals — each over 16 years of age — had an absolute error exceeding 6 years. Notably, all again were underestimated. A summary of the error metrics of the LOOCV is provided in Table 2.1. All elastic nets with LOOCV showed the tendency of overestimating age in younger individuals and underestimating age in older animals (‘*relaxed*’: regression slope: 0.58, regression intercept: 3.69; ‘*strict*’: regression slope: 0.56, regression intercept: 3.52; ‘*restricted*’: regression slope: 0.73, regression intercept: 1.69). The ‘*restricted*’ subset has the slope closest to 1, indicating that it exhibits the least amount of bias among the three, performing best in maintaining proportional predictions across ages.

3.1.2. Hybrid epigenetic clock

The hybrid model using the ‘*relaxed*’ subset produced a MAE of 2.61 years (Figure A2.2) with a correlation of $r = 0.73$ ($p = 6.16 \times 10^{-14}$). Linear regression of DNA predicted age against dental age yielded a slope of 0.5, an intercept of 4.15, and an R^2 of 0.54.

Table 2.1: Error metrics for elastic net regression models predicting epigenetic age in common dolphins (*Delphinus delphis*), evaluated with Leave-One-Out Cross-Validation (LOOCV) for the ‘relaxed’, ‘strict’, and ‘restricted’ subsets.

Metric	‘relaxed’	‘strict’	‘restricted’
Mean Absolute Error (AE)	3.42	3.22	2.21
Median AE	2.02	2.29	1.80
<i>n</i> samples with AE >6 years	12	9	4
<i>n</i> samples with AE >10 years	5	4	0
Maximum AE	16.31	17.33	7.77

3.1.3. Effect of tissue decomposition

We found no difference in absolute epigenetic age prediction error across the decomposition condition categories ($p = 0.746$, $\chi^2 = 1.23$, $df = 3$), indicating that fresh to moderate decomposition change had no overall effect on the accuracy of the epigenetic age estimates.

3.1.4. Effect storage duration

Storage duration in the ‘relaxed’ subset ranged from 0 to 23 years (median = 9 years). Spearman correlation indicated no association between storage time and prediction error ($\rho = 0.10$, $p = 0.38$). Similarly, linear regression showed that storage time did not predict error ($\beta = 0.029 \pm 0.064$ SE, $p = 0.65$, $R^2 = 0.003$). Residuals from this simple model deviated from normality; however, when chronological age was included as a covariate, residuals conformed to model assumptions (Shapiro–Wilk $p = 0.10$). In this adjusted model, storage time remained non-significant ($\beta = 0.048 \pm 0.045$ SE, $p = 0.30$), whereas age strongly predicted error ($\beta = 0.316 \pm 0.036$ SE, $p = 7.3 \times 10^{-13}$, $R^2 = 0.51$). Together, these results indicate that storage duration did not confound clock performance in our dataset.

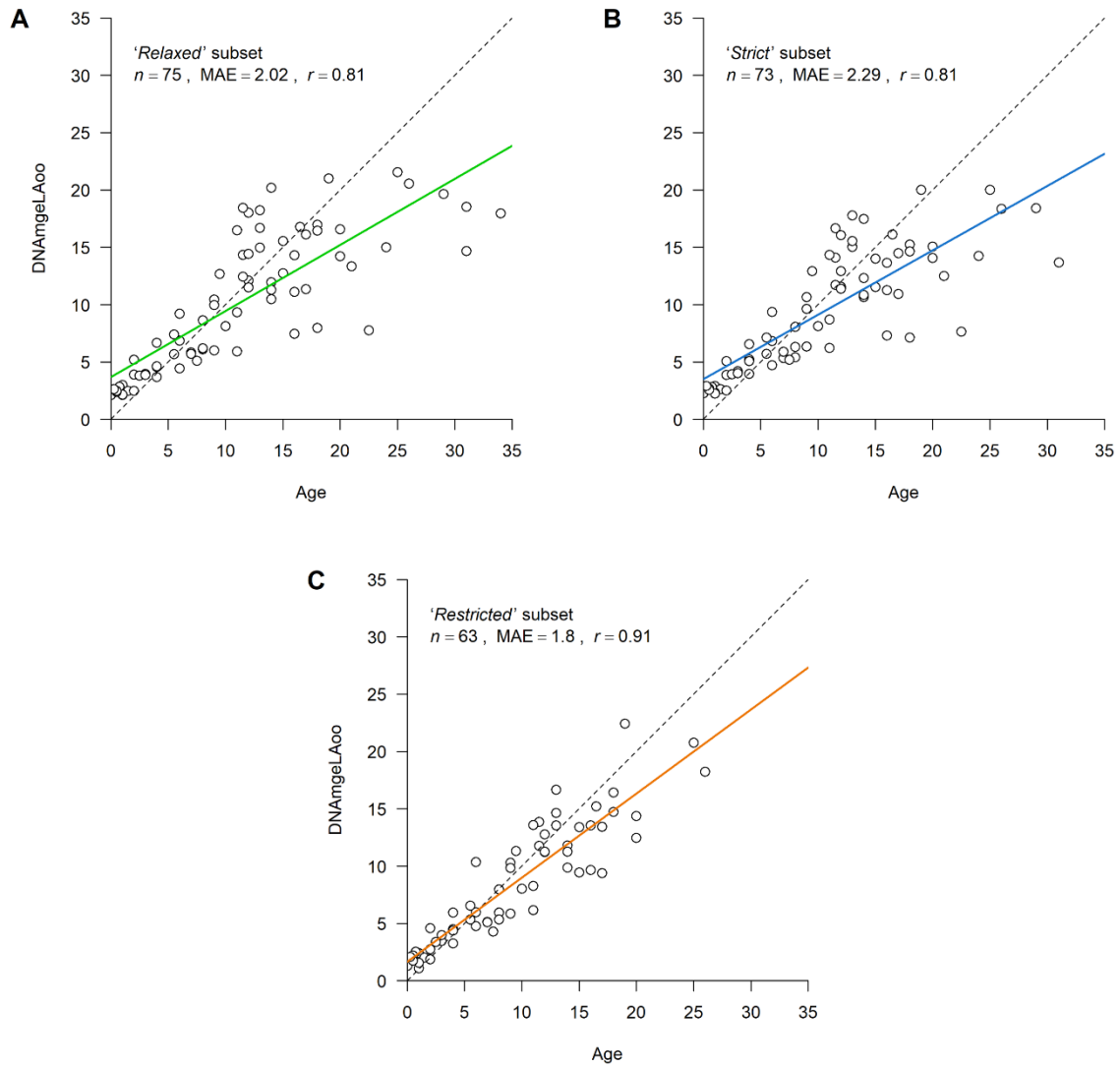


Figure 2.1: Epigenetic ages (DNAmAgeLOO) estimated using elastic net regression models with Leave-One-Out Cross-Validation (LOOCV) applied to skin samples of common dolphins (*Delphinus delphis*) for the **A)** ‘relaxed’, **B)** ‘strict’, and **C)** ‘restricted’ subsets. The ‘relaxed’ subset included all individuals, the ‘strict’ subset excluded those with only estimated age ranges or minimum ages, and the ‘restricted’ subset further removed outliers with absolute errors >6 years. Regression lines are shown in green (‘relaxed’), blue (‘strict’), or orange (‘restricted’), with the dotted diagonal indicating perfect correlation ($y = x$). Individual animals are shown as dots. Sample size (n), Pearson correlation (r), and median absolute error (MAE) are reported for each model.

3.1.5. Age predictions on the basis of other odontocetes clocks

Age predictions for our samples, generated using both species-specific and multi-species odontocete clocks from the Clock Database, are shown in Figure A2.3, with corresponding error metrics in Table A2.4. The multispecies odontocete clock (Robeck, Fei, Lu, et al., 2021) predicted the age of our samples with an accuracy of $r = 0.79$ for the ‘relaxed’, $r = 0.8$ for the ‘strict’, and $r = 0.85$ for the ‘restricted’ subset, and a MAE of 11.8, 11.84, and 12.42, respectively. The use of the multispecies odontocete blood and skin clock resulted in comparable MAE values, but the latter revealed lower age correlations (Table A2.4). Comparing our results to those of other species-specific clocks, the Hector's and Māui dolphin (*Cephalorhynchus hectori hectori* and *C. h. maui*; Hernandez et al., 2023) clock performed best for our samples regarding the MAE between actual and predicted age, with a MAE ranging from 2.71 to 3.01 depending on the subset of samples used (Table A2.4). Although both multispecies clocks showed the tendency to overestimate the age of our samples, the *Cephalorhynchus* clock consistently underestimated samples above the age of 6 years (see Text A2.1 and A2.2).

3.2. Sex prediction

The elastic net model retained 197 CpG sites and achieved 100% accuracy in predicting sex. The probability of males being correctly classified as male ranged from 0.9892 to 0.9953, whereas the probability of females being misclassified as male was very low, ranging from 0.0025 to 0.0078.

4. Discussion

Here, we present the first species-specific epigenetic clock for common dolphins, developed using DNA methylation profiles from skin samples of individuals with estimated dental ages. The elastic net regressions with LOOCV exhibited robust performance across all three models, with the ‘restricted’ subset (MAE = 1.80, $r = 0.91$, $R^2 = 0.82$) yielding the highest accuracy with a MAE equivalent to approximately $\pm 5.1\%$ of maximum life expectancy (MLE: 35 years, Murphy et al., 2014). The ‘relaxed’ and ‘strict’ models also performed strongly, with MAEs (2.02 and 2.29 years) equivalent to approx. $\pm 5.8\%$ and $\pm 6.5\%$ of maximum life expectancy, and correlation coefficients of 0.81.

Epigenetic clocks have been shown to estimate chronological age with high accuracy (Jylhävä et al., 2017). However, even when trained on extensive datasets encompassing all age groups, these clocks still exhibit variability among individuals, which could reflect differences in their biological ageing rates (Jylhävä et al., 2017). Variation in ageing rates might be influenced by numerous factors, including reproductive history (Shirazi et al., 2020), early-life adversities (Colich et al., 2020), nutrition (Fitzgerald et al., 2021; Weindruch et al., 2001), and exposure to environmental contaminants (Y. Liu et al., 2021). In cetaceans, 11 odontocete epigenetic clocks have been developed to date, using a range of sample types including skin, blood, multi-tissue, and faeces (Table A2.5). Notably, only three prior studies calibrated their epigenetic clocks using growth layer groups from stranded and bycaught animals, with concerns raised that reliance on tooth age calibration may compromise the robustness of epigenetic clocks (Bors et al., 2021; Hernandez et al., 2023; Mori et al., 2024; Zoller et al., 2025).

Although dental ageing provides robust age estimates for animals younger than 15 years, the reliability of this method decreases in older individuals (Barratclough et al., 2023; Betty et al., 2023; Murphy et al., 2014). Specifically, factors such as tooth wear, the compression of growth layer groups, and the presence of accessory lines can compromise the accuracy of age estimates (Barratclough et al., 2023; Betty et al., 2023; Murphy et al., 2014). Such factors generally result in dental ageing underestimation of older individuals (Barratclough et al., 2023), especially when ageing is restricted to a single tooth, as is typical in studies of animals under human care (Barratclough et al., 2023). To mitigate this, we processed up to three teeth per animal, further selected the least worn and straightest teeth, and assigned minimum age estimates when uncertainty remained because of accessory lines or compressed growth layer groups. Despite these precautions, our epigenetic clock repeatedly underestimated the ages of older individuals (i.e., >16 years) across all subsets.

The consistent underestimation of older dolphins suggests that discrepancies between dental and epigenetic age assessments are unlikely to stem from inaccuracies in the dental ageing method itself. Specifically, if dental ageing inaccuracies were the primary source of error, we would expect epigenetic age estimates to exceed dental ages in older individuals — which was not observed. Additionally, contrary to our first hypothesis, overall performance was similar across both the *‘relaxed’* and *‘strict’* subsets. Notably, the *‘relaxed’* model yielded a lower MAE and a regression slope marginally closer to 1, indicating more balanced age predictions across the lifespan. This result suggests that excluding samples with uncertain dental ages did

not substantially improve model performance. However, the performance of the ‘*relaxed*’ model was comparable to most previously published skin-based odontocete clocks calibrated using growth layer groups, though it fell marginally short of the accuracy reported for clocks developed from known-age individuals in long-term observational studies or human care (Text A2.2). We considered whether this performance gap could be explained by factors relating to tissue origin. Our samples originated from a variety of contexts, including mass and single strandings, bycatch events, and one individual under human care. Although mass strandings are typically unrelated to individual health problems (Cordes, 1982), single strandings may involve animals affected by illness, compromised health, or poor nutritional status (Arbelo et al., 2013; Boys, 2022). Although poor health has been linked to accelerated epigenetic ageing in other species (Newediuk et al., 2025), this contrasts with the consistent underestimation observed here in older individuals.

We also considered whether post-mortem decomposition or storage duration could influence methylation patterns. Although post-mortem changes may potentially affect methylation at specific loci, a comparison of live and deceased human samples showed comparable methylation-based age predictions (Dias et al., 2020). Consistent with this, we found no significant effect of decomposition condition on epigenetic age prediction error, suggesting that our clock is likely applicable to both live and deceased animals. Similarly, the duration of storage showed no significant association with prediction error, that is, long-term storage did not affect clock performance. Beyond biological and technical factors, the composition of the training dataset itself plays a critical role in shaping clock performance.

Although our study encompassed the full estimated lifespan of common dolphins, most samples were from individuals aged 0–20 years. Skin samples from older common dolphins remain rare, and the limited and fragmentary representation of older animals may have reduced the accuracy of age estimations in the upper age ranges. When designing epigenetic clocks for wildlife, it is crucial to minimise, where possible, age-related sampling biases (Newediuk et al., 2025). Specifically, to ensure accurate age predictions, samples should be evenly distributed across age classes, particularly from both young and aged individuals. This prevents poor performance of clocks because of narrow or skewed age distributions (Newediuk et al., 2025). However, the heterogeneous age distribution of stranded and bycaught individuals relies upon extensive tissue archives, which capture the full age spectrum of the species.

In our study, regression analyses revealed slopes smaller than 1 for all three models, indicating a systematic overestimation of age in younger individuals and underestimation in older individuals. This pattern is consistent across multiple species (Beal et al., 2019; Bors et al., 2021; Peters et al., 2023; Polanowski et al., 2014). Among our models, the ‘*restricted*’ model showed the slope closest to 1, suggesting the most balanced age predictions across the lifespan. The phenomenon of “regression to the mean” poses a well-recognised challenge for epigenetic clock models, particularly when predicting ages beyond the range of the training data. This statistical effect causes extreme values to shift toward the population mean, leading to underestimation of older individuals and overestimation of younger ones when these age groups are underrepresented in the training set. In human studies, this has prompted efforts to develop specialised clocks for centenarians (Dec et al., 2023). Indeed, this concept and its implications for research design have been widely discussed (Campbell & Kenny, 1999). Accordingly, when interpreting epigenetic age estimates for individuals at the extremes of the age spectrum, it is important to account for the influence of regression to the mean and its bias toward the average age of the training cohort.

Regression to the mean, because of the sparse representation of older individuals, is one explanation for the underestimation of age at higher values. Another plausible factor is the biological deceleration of DNA methylation change in later life. For example, human epigenetic clocks have been shown to progress rapidly during development and then decelerate, leading to a systematic underestimation of age in older individuals (Horvath, 2013; Kuzub et al., 2022; Marioni et al., 2019). If odontocetes exhibit a comparable biological deceleration, it would systematically bias clock estimates downward in older dolphins, regardless of sample distribution. This has also been hypothesised in bottlenose dolphins, where the use of hybrid models has been suggested as a strategy to address both uneven age distributions and life stage-dependent variation in methylation change (Barratclough et al., 2024).

Hybrid models aim to improve predictions across the lifespan by adjusting for shifts in methylation patterns after physical maturity. Barratclough et al. (2024) applied this strategy in bottlenose dolphins (*Tursiops truncatus*), combining a random forest classifier with two elastic net regression models to improve age prediction for older individuals. However, applying a similar approach to common dolphins yielded a poorer fit than elastic net regression alone, likely because of the small number of physically mature individuals in our dataset ($n = 14$), limiting the classifier's and mature-age model's effectiveness. Future approaches, such as

learning curves (Perlich et al., 2003) or bias–variance decomposition (Domingos, 2000), could be used to disentangle whether underperformance reflects model bias or limited sample size when sufficient sample sizes permit.

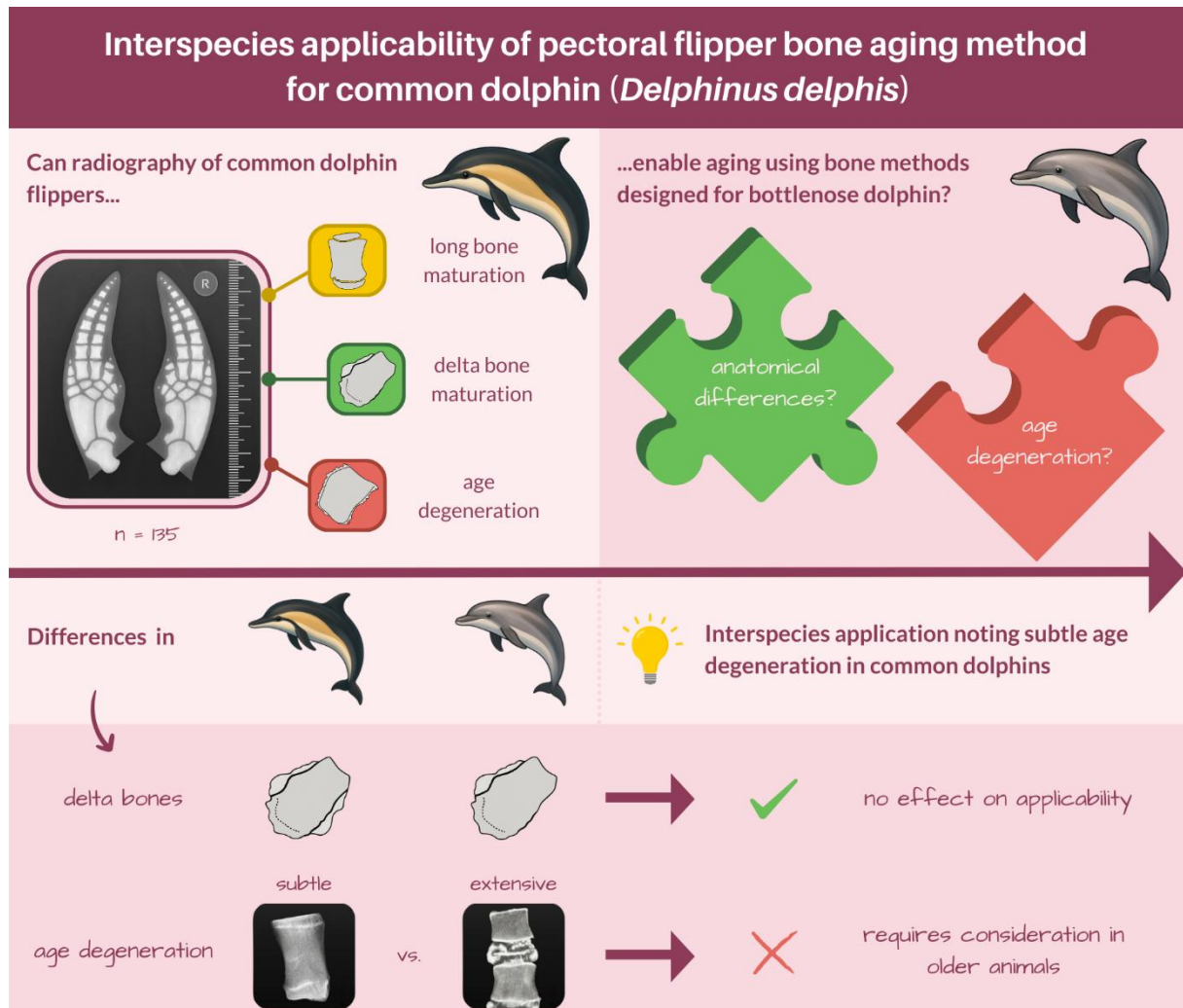
Despite limitations identified, our results highlight the promise of epigenetic clocks on the basis of dental ageing in addressing long-standing challenges in age estimation, particularly for species where invasive sampling or long-term observational data are not feasible. Beyond age estimation, our clock also accurately predicts sex on the basis of methylation patterns at CpG sites, achieving 100% accuracy, a finding consistent with previous studies (Bors et al., 2021; Peters et al., 2023; Robeck, Fei, Haghani, et al., 2021). Tools that enable accurate age and sex estimation are becoming increasingly critical, given the significant anthropogenic threats facing common dolphins globally (Abraham et al., 2021; Bilgmann et al., 2014; Fernández-Contreras et al., 2010; Hamer et al., 2007; Mannocci et al., 2012; Murphy et al., 2013; Mussi et al., 2021; Peltier et al., 2016, 2021; Piroddi et al., 2011; Tulloch et al., 2020; Vella et al., 2021), where several populations are in decline (Bearzi et al., 2008; Murphy et al., 2013; Piroddi et al., 2011). Understanding life-history parameters is critical for conservation management (Betty et al., 2019; Heydenrych et al., 2021; Manlik et al., 2022). For example, accurate age estimates underpin population viability analyses and are essential to quantify population-level impacts such as fisheries bycatch (Manlik et al., 2022; Palmer et al., 2022; Verborgh et al., 2021). Since post-mortem age assessment may bias survivorship estimates (Betty et al., 2023) used to evaluate fisheries impacts, our epigenetic clock provides a critical advance by enabling age estimation from living animals.

The ability to age live individuals strengthens conservation efforts globally. Life-history traits are comparable among common dolphin populations worldwide (Danil & Chivers, 2007; Murphy & Rogan, 2006; Palmer et al., 2022, 2023; Westgate & Read, 2007), and species-specific epigenetic clocks have been shown to accurately predict age in subspecies and closely related sister taxa (Barratclough et al., 2024, 2025; Peters et al., 2023; Robeck, Fei, Lu, et al., 2021). Our epigenetic clock has broad applicability for this cosmopolitan oceanic species and thus may further facilitate comparisons of epigenetic age acceleration across populations experiencing varying environmental pressures (Newediuk et al., 2025). By enabling reliable age and sex estimation, this tool can enhance conservation strategies and improve our understanding of population dynamics, both in Aotearoa New Zealand, and throughout the species' range. Finally, with concerns of decomposition and tooth ageing effects on clock

calibration addressed here, future methylated clocks should be considered for stranded and bycaught dolphin populations, especially those underpinned by longitudinal tissue archives that allow teeth estimates to be further cross-validated with wider life-history data.

Chapter 3

Cross-species applicability of a bone-ageing method in delphinids



This chapter is a reformatted version of the following manuscript:

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We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.			
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Abstract

Radiographic evaluation of flipper ossification is a reliable technique for age estimation in bottlenose dolphins (*Tursiops truncatus*), although its applicability to other odontocetes remains untested. Interspecific differences in flipper morphology and bone development may limit transferability or require adjustments for robust application. This study applies the bottlenose dolphin framework to common dolphins (*Delphinus delphis*) to characterise developmental patterns, describe morphological differences and evaluate the interspecies application of this method. Radiographs from 137 common dolphins aged 0–31 years were examined, with bone maturation scored at 16 anatomical sites on the pectoral flipper. Several differences were identified, including bilateral asymmetry between left and right flippers and the need to adjust long and delta bone scoring systems due to interspecific variation in bone maturation. Despite these differences, reproducibility between observers was high, and ossification scores correlated strongly with age, underscoring the reliability of the method. This study provides the groundwork for a species-specific bone ageing model for common dolphins by addressing interspecific variation in skeletal maturation. One caveat concerns the subtle expression of degenerative changes in this species compared with bottlenose dolphins, which may limit age estimation precision in older individuals but is unlikely to affect accuracy across most of the lifespan. Thus, our findings provide the first evidence that, despite morphological differences, the method can be successfully applied to common dolphins.

1. Introduction

Radiographic assessment of hand and wrist ossification is an established method for estimating chronological age in humans until the age of 18 years, particularly in paediatric and forensic applications (Baumann et al., 2009; Kim et al., 2017; Schmeling et al., 2006). This approach evaluates bone shape, structure, mineralisation, and physeal plate fusion and is most effective in minors, as ossification and physeal fusion follow a predictable sequence during childhood and adolescence until sexual and physical maturity (Baumann et al., 2009; Kim et al., 2017; Mughal et al., 2014; Schmeling et al., 2006). Given the value of radiographic bone assessments for estimating chronological age, similar approaches have been investigated for potential applications to wildlife species, including various terrestrial mammals, such as deer (Lewall & Cowan, 1963), foxes (Sullivan & Haugen, 1956), rabbits (Hale, 1949; Thomsen & Mortensen,

1946), opossums (Petrides, 1949), raccoons (Sanderson, 1961), and the extinct woolly mammoth (Lister, 1999).

In cetaceans, radiographic bone age assessments were historically limited to the classification of broad age categories (Galatius et al., 2006; Ogden et al., 1981). However, more recently, a radiographic approach was developed that enabled the reliable estimation of chronological age across all age classes in bottlenose dolphins (*Tursiops truncatus*; Barratclough, Sanz-Requena, et al., 2019). Crucially, the ability to estimate age in older individuals is supported by evolutionary adaptations of the cetacean forelimb, which undergoes prolonged and gradual ossification processes that continue well beyond sexual maturity (Barratclough, Sanz-Requena, et al., 2019). These adaptations reflect major developmental and osteological changes from the terrestrial ancestors of cetaceans. Key modifications include (i) paedomorphosis in pectoral flipper development — the retention of juvenile features into adulthood (Galatius et al., 2006; Mellor et al., 2009), (ii) hyperphalangy, an increased number of phalanges per digit compared to the typical phalangeal formula of 2–3–4–5–4 in amniotes or 2–3–3–3–3 in mammals (Cooper et al., 2007; Fedak & Hall, 2004; Richardson & Oelschläger, 2002), and (iii) the occurrence of delta bones (Cooper et al., 2007; Dawson, 2003). Delta bones are triangular with a convex and a concave longitudinal border (Dawson, 2003). While initially described as a congenital deformity in humans (Choo & Mubarak, 2013; Wood & Flatt, 1977), in cetaceans delta bones represent a normal physiological stage in the ossification of specific metacarpals and phalanges (Dawson, 2003). These bones ossify later in life and thus provide valuable markers for ageing older cetaceans (Barratclough, Sanz-Requena, et al., 2019). These anatomical adaptations have been used to develop a comprehensive radiographic scoring system that assessed long bone ossification, alongside delta bone maturation, and the occurrence of age-related degenerative changes, such as joint surface wear and bone remodelling in 16 specific anatomical bone locations of the pectoral flipper (Barratclough, Sanz-Requena, et al., 2019). Such an integrated approach enabled age estimation across all life stages, marking a significant advance in the non-invasive ageing of bottlenose dolphins (Barratclough, Sanz-Requena, et al., 2019). However, comparable frameworks have been lacking for other cetacean species due to the limited availability of radiographs from known-age individuals.

While the sequence of bone maturation in cetaceans appears to be consistent across species — beginning in the humerus, followed by the radius and ulna, and progressing distally through the metacarpals and phalanges (Barratclough, Sanz-Requena, et al., 2019; Calzada & Aguilar,

1996; De Carvalho et al., 2015; Galatius et al., 2006; Hui, 1977; K. E. Lee, 1978; Ogden et al., 1981) — the timing of these ossification stages differs between sexes and varies with species-specific life history traits. Sex- and species-specific differences reflect underlying life history variation, including hormonal changes during puberty, which drive bone maturation (Almeida et al., 2017; Iuliano-Burns et al., 2009). Accordingly, accurate age estimation requires species-specific calibration using radiographs from known-age individuals (Barratclough, Sanz-Requena, et al., 2019). However, the need for species-specific calibration arises not only from differences in life history traits, but also the existing radiographic approach developed for bottlenose dolphins might not be directly transferable to other cetacean species. In particular, it is not clear whether interspecific anatomical differences in pectoral flipper morphology (Cooper et al., 2007) and bone maturation limit the broader applicability of this method beyond bottlenose dolphins, or whether its implementation in other species requires specific adjustments to account for such variation.

To develop a radiographic ageing model calibrated for common dolphins (*Delphinus delphis*), several species-specific knowledge gaps must first be addressed. For example, the degree of hyperphalangy varies among cetacean species — including differences between bottlenose and common dolphins (Cooper et al., 2007) — raising the question of whether the same anatomical bone locations are equally suitable for assessing bone maturation in both species. The ossification patterns of delta bones (Barratclough, Sanz-Requena, et al., 2019; Dawson, 2003) have also not been systematically assessed across cetaceans, leaving uncertainty about potential interspecific differences and whether common dolphins follow a distinct trajectory of delta bone maturation. Bilateral symmetry in bone development likewise warrants investigation, as asymmetry could bias age estimates. It is, therefore, important to determine whether assessing bone maturation from a single flipper provides an accurate representation of skeletal development, or whether bilateral asymmetry may influence results. Although previous research in odontocetes, including common dolphins, has found no evidence of marked asymmetry (Barratclough, Sanz-Requena, et al., 2019; De Carvalho et al., 2015; Hui, 1977; Ogden et al., 1981), the extent to which this pattern holds across different age classes remains insufficiently characterised. Finally, the extent and characteristics of age-related degeneration in pectoral flippers remain poorly documented in common dolphins (Cooper & Dawson, 2009; Nganvongpanit et al., 2017; Turnbull & Cowan, 1999) and, more broadly, there is a lack of comparative research examining how such degeneration varies among cetacean species.

Here, we applied the radiographic scoring scheme established by Barratclough, Sanz-Requena, et al. (2019) for bottlenose dolphins to common dolphins to investigate species-specific differences in skeletal maturation. The sample comprised individuals with documented life-history data, and their age estimates were derived from growth layer group (GLG) examination of extracted, thin-sectioned teeth. We describe the sequence of bone maturation in common dolphins and highlight morphological variation in bone development, with emphasis on delta bone ossification, bilateral symmetry, and age-related degeneration. Finally, we assess the applicability of the anatomical scoring locations defined by Barratclough, Sanz-Requena, et al. (2019) to common dolphins.

2. Materials & methods

2.1. Sample collection

2.1.1. Pectoral flipper collection

Pectoral flippers ($n = 137$ pairs) were collected during routine post-mortem examinations of stranded and bycaught common dolphins (*Delphinus delphis*) from 2002 to 2025. Flippers were surgically dissected at the scapulohumeral joint by severing all soft tissue attachments between the scapula and humerus (Calzada & Aguilar, 1996; Stockin et al., 2008; Watson et al., 2008). Following removal, the flippers were immediately frozen at -20°C until subsequent examination. To prevent rotational misalignment of the carpal cuboidal bones via planar position, each set of pectorals was stored flat in alignment with Barratclough, Sanz-Requena, et al. (2019). Ensuring planar positioning prior to frozen storage enabled radiography of pectoral flippers while frozen.

2.1.2. Dental age assessment

Life-history assessments (sex, age, body length, and sexual maturity) were completed *a priori* as part of longitudinal studies on life history (Palmer et al., 2022, 2023). Specifically, dental age was determined by counting GLGs in the dentine of thin, decalcified, and stained tooth sections (Palmer, 2023). Teeth were independently assessed by a minimum of two experienced readers in three sessions. In cases where age estimates did not align between readers, an additional tooth (up to three per individual) was processed and re-read to establish a consensus (Palmer, 2023). Dental age was assessed for 120 animals. The remaining 17 individuals could not be assigned a dental age for several reasons: teeth had not yet erupted in foetuses (extracted

in utero or aborted prior to first breath, confirmed by pulmonary atelectasis — the absence of air in the foetal lung before first respiration; Colegrove et al., 2016; $n = 5$) and some neonates (characterised by at least one of the following: presence of vibrissal hairs, an unhealed navel, foetal folds, soft and folded dorsal fin and tail flukes, or unerupted teeth; Díaz López et al., 2018; Puig-Lozano et al., 2020; $n = 7$) or no suitable teeth were available for sectioning ($n = 5$). Dental age ranged from 0 to 31 years of age, with sex distribution reported in Appendix 3 (Figure A3.1).

2.2. Radiology

To ensure consistency across results, we used the same x-ray machine (Shimadzu, Auckland Radiology Group), with standardised settings of 40 kilovoltage peak (kVp) and 4 milliampere-seconds (mAs), and a source-to-sample distance of 100 cm. The radiographic settings were optimized *a priori*, aiming for the lowest possible radiation dose while maintaining diagnostic image quality. The same setup was applied to all pectoral flippers across both sexes and all age classes to ensure consistent visualization.

2.3. Radiographic assessment of bone maturation

Following Barratclough, Sanz-Requena, et al. (2019), radiographs were systematically reviewed in a proximal-to-distal sequence, starting with the radius and ulna and then progressing through the metacarpals and their associated phalanges from cranial to caudal. We assessed bone maturation at sixteen anatomical locations (Figure 3.1, Table 3.1) previously identified as age-informative in bottlenose dolphins. Although common dolphins may have a greater number of phalanges in digits II and III compared to bottlenose dolphins (Cooper et al., 2007), we retained those 16 locations because distal phalanges progressively decrease in size, and beyond the second phalanx of digits II and III, the physeal growth stage becomes increasingly difficult to assess visually. Carpal bones and metacarpal I were not included since they lack distinct ossification stages and show poor correlation with age (Barratclough, Sanz-Requena, et al., 2019). Proximal radius and ulna, as well as the humerus, were further excluded because these elements could not be consistently visualised in a standard radiographic plane (Barratclough et al., 2024; Ogden et al., 1981). All radiographs were assessed blind by at least one experienced reader, with a subset of 40 radiographs (chosen from different developmental stages) additionally evaluated by two independent readers (EMFH and AB) to assess scoring

consistency. We further examined both pectoral flippers to assess lateralisation in bone maturation, with any morphological deviations from the original scoring scheme recorded. Scores for each anatomical location and pectoral flipper were then summed to derive a total radiographic score for each specimen (Barratclough, Sanz-Requena, et al., 2019).

Individual bone maturity scores were assigned according to the criteria developed by Barratclough, Sanz-Requena, et al. (2019), with separate schemes applied to long bones and delta bones (Table 3.1). Long bone development was classified based on the presence and progression of ossification centres, physal thinning, partial or complete physal fusion, and, at advanced stages, age-related degenerative changes such as osteophyte formation and joint remodelling (Table 3.2, Barratclough, Sanz-Requena, et al., 2019). Maturation stages were scored from –1 to 8 following Barratclough, Sanz-Requena, et al. (2019), with half scores assigned to bones that had clearly begun but not yet completed the transition between two defined stages (definitions and schematics provided in Appendix 3, Figure A3.2). Most long bones were scored at both the proximal and distal epiphyses unless otherwise noted (Table 3.1). A single score representing the higher of the two epiphyseal values was applied for D2P2 and D3P2, consistent with Barratclough, Sanz-Requena, et al. (2019) and supported by our results (Appendix 3, Table A3.1). Specifically, using the maximum epiphyseal score marginally increased the correlation between age and total radiographic score compared with treating proximal and distal epiphyses separately, averaging the two values, or using the minimum score.

Delta bones (*'M5'* and *'D4PI'*) were scored as entire structures, since they typically lack defined proximal and distal epiphyses (Barratclough, Sanz-Requena, et al., 2019). Their development follows a unidirectional, surface-specific ossification pattern that differs markedly from long bones (Table 3.3). Ossification begins with the formation of a secondary ossification centre adjacent to the primary centre on the proximal side, while the axial border starts to straighten, producing the emerging triangular (“delta”) outline (Barratclough, Sanz-Requena, et al., 2019). Mineralisation and consolidation then progress across the proximal surface before extending distally once proximal consolidation is complete (Barratclough, Sanz-Requena, et al., 2019). Later stages are characterised by increased mineral density and surface uniformity, culminating in complete consolidation and loss of the physal line (Barratclough, Sanz-Requena, et al., 2019). In some individuals, degenerative changes such as osteophyte formation or joint surface irregularity may be observed in the final stage (Barratclough, Sanz-Requena, et

al., 2019). Delta bone maturity was scored from –1 to 8 based on the morphological criteria defined by Barratclough, Sanz-Requena, et al. (2019), with half scores used to reflect transitional states (definitions and schematics provided in Appendix 3, Figure A3.3).

Table 3.1: Bones assessed for radiographic scoring in common dolphins (*Delphinus delphis*). Abbreviations, scoring locations, and bone types (long or delta) are provided. Long bones were typically scored at both proximal and distal epiphyses unless otherwise indicated. Delta bones (‘M5’ and ‘D4P1’) lack distinct epiphyseal ends and were scored as entire structures. For ‘D2P2’ and ‘D3P2’, proximal and distal scores were combined, as separate scoring did not improve age prediction accuracy.

Bone	Abbr.	Scoring location	Scoring system
Radius	–	Distal	Long bone
Ulna	–	Distal	Long bone
Metacarpal 2	M2	Proximal and distal	Long bone
Metacarpal 3	M3	Proximal and distal	Long bone
Metacarpal 4	M4	Proximal and distal	Long bone
Metacarpal 5	M5	Entire bone	Delta bone
First phalanx of digit 2	D2P1	Proximal and distal	Long bone
First phalanx of digit 3	D3P1	Proximal and distal	Long bone
First phalanx of digit 4	D4P1	Entire bone	Delta bone
Second phalanx of digit 2	D2P2	Proximal & distal (combined)	Long bone
Second phalanx of digit 3	D3P2	Proximal & distal (combined)	Long bone

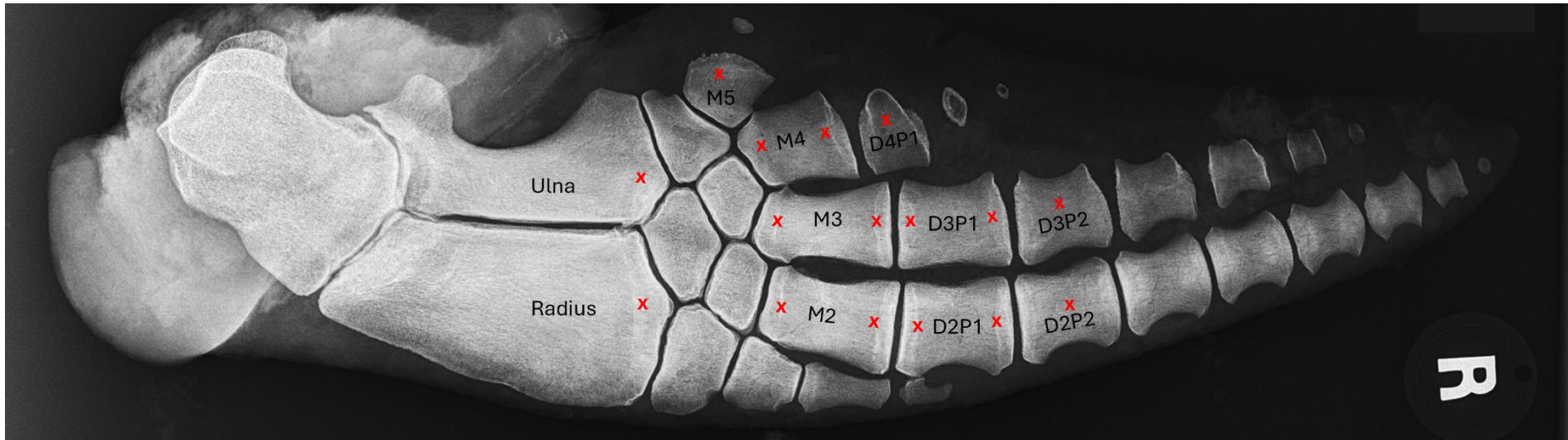


Figure 3.1: Scoring locations (red crosses) in a pectoral fin of a common dolphin (*Delphinus delphis*). The scoring locations include the distal radius and ulna, the proximal and distal surfaces of metacarpal 2 (M2), metacarpal 3 (M3), metacarpal 4 (M4) and the proximal and distal surfaces of the first phalanges of digit 2 and 3 (D2P1 and D3P1). The second phalanges of digit 2 and 3 (D2P2 and D3P2) received a combined score for the proximal and distal surfaces and metacarpal 5 (M5) and the first phalanx of digit 4 (D4P1) are delta bones and scored as such. Radiographs were acquired using a setting of 40 kVp and 4 mAs, with a source-to-sample distance of 100 cm.

Table 3.2: Long bone scoring system applied to common dolphins (*Delphinus delphis*) following Barratclough, Sanz-Requena, et al. (2019).

Score	Description
-1	No primary ossification centre visible
0	The primary centre is present, but no secondary ossification centre is visible
1	An epiphyseal ossification centre is present but only occupies <50% of the width of the adjacent metaphysis
2	The secondary ossification centre is well established, ranging between 50–100% of the metaphyseal width; the physis is evident as a distinct radiolucent space, while the secondary centre appears less mature and irregular in depth across its width; maturation progresses in a midline-to-abaxial direction
3	Thinning of the radiolucent physis but no evidence of fusion of the metaphysis and the secondary ossification centre; mineral density increases in the subchondral bone on both sides of the physis; late stage 2 can resemble early stage 3 with the distinction being based on whether the physis width is irregular (stage 2) or uniform (stage 3)
4	Osseous bridges begin to form; in the radius and ulna this starts midline and proceeds abaxially; the abaxial margins remain open, and both early bridge formation and >90% bridging qualify as stage 4
5	Complete closure is present, accompanied by a faint hyperdense “ghost” physeal line or epiphyseal scar spanning at least 50% of the plate; the former epiphysis may not reach the full transverse width of the bone, but closure is complete
6	The physeal line is remodelled, with <50% to no evidence of the hypermineralised transverse remnant, and the former epiphysis extends across the full transverse width of the bone
7	Complete fusion occurs with no evidence of a previous ghost physeal plate and flattening of the physeal surfaces with accentuated pointed edges
8	Arthritic changes are visible, including osteophytes, osteolysis, articular calcification, and in some cases fusion of metacarpals or phalanges

Table 3.3: Delta bone scoring system applied to common dolphins (*Delphinus delphis*) following Barratclough, Sanz-Requena, et al. (2019).

Score	Description
-1	Not present
0	Very small oval shape, undefined delta shape
1	Axial surface starts to be linear, consistent with delta shape
2	One flat surface; areas of slight irregular perimetral surface typically on the proximal side with a degree of adjacent mineralisation which can oppose the epiphyseal surface but not necessarily show definitive osseous bridge formation; bone density is clearly reduced in peripheral new bone in comparison to the primary ossification centre
3	Increased mineralisation of adjacent proximal cartilaginous surface, consolidation may not be present
4	Area increases in consolidation and mineralisation, slightly less dense than the body of the delta bone; a defined hypermineralised physeal plate line is present; the abaxial aspect can show the initiation of the new straight lateral border rather than a horseshoe shape
5	Density of the secondary ossification centre is similar to primary bone and initial mineralisation is present on the distal surface, with the proximal side showing a defined straight lateral border rather than a horseshoe shape; individual variation may result in non-linear borders; therefore, emphasis should be placed on the secondary ossification centres on the proximal and distal surfaces
6	Smooth proximal surface with matching density to the primary ossification centre, distal surface still shows some incongruent mineralisation; physeal line is still visible
7	Smooth proximal surface with complete consolidation of the secondary ossification centre on both sides and dissolution of the physeal line
8	Degenerative changes present

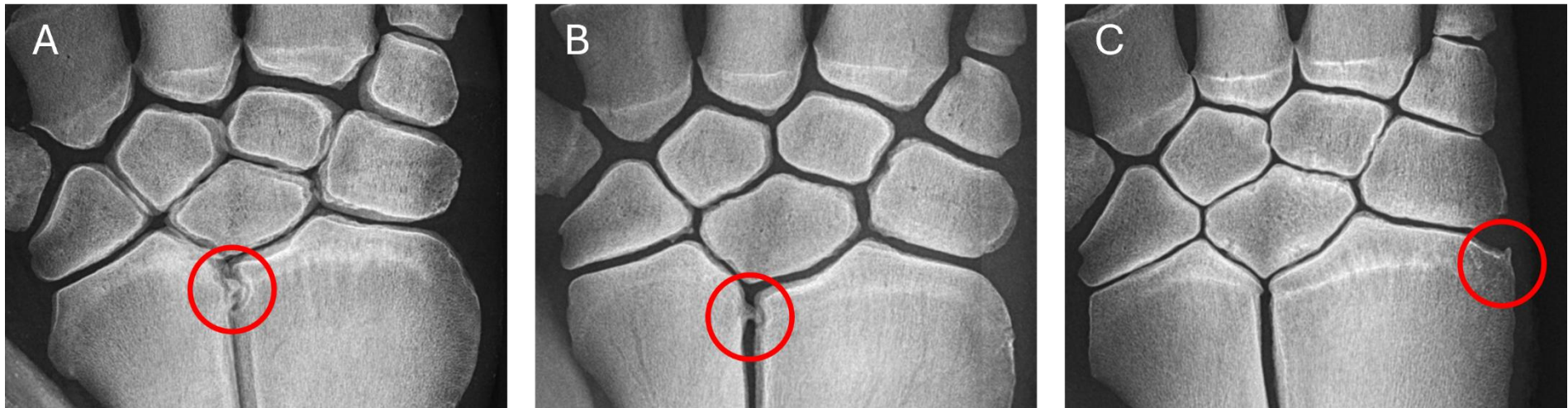


Figure 3.2: A) & B) Osseous projection (red circles) observed along the distal interosseous margin between the radius and ulna of two common dolphins (*Delphinus delphis*). This bony outgrowth appears irregular and projects into the interosseous space, suggestive of localized cortical remodelling or marginal bone proliferation. C) Irregular, peripheral bony prominence (red circle) arising from the dorsolateral aspect of the distal radius of a common dolphin, extending beyond the cortical contour near the radiocarpal joint. The projection has a pointed appearance and may reflect localized appositional bone growth or remodelling at the dorsal rim. Radiographs were acquired using a setting of 40 kVp and 4 mAs, with a source-to-sample distance of 100 cm.

2.4. Age-related degenerative changes

As a bone maturation score of 8 required distinguishing degenerative changes from normal variation, potential age-related lesions in common dolphins were morphologically compared to those reported by Barratclough, Sanz-Requena, et al. (2019). In bottlenose dolphins, such degeneration included bone or cartilage changes associated with osteolysis or proliferative remodelling (Barratclough, Sanz-Requena, et al., 2019). In addition to these previously described changes, we observed putative bone remodelling in the interosseous region between the ulna and radius ($n = 19$; Figure 3.2A&B), and between the radius and adjacent carpal bone ($n = 7$; Figure 3.2C). These anomalies have not been previously described and were considered potentially age-related; however, they were frequently inconsistent with the overall age appearance of the full radiograph, warranting further investigation through bone histopathology. To this end, a subsample of affected bones ($n = 24$) was dissected, fixed in 10% neutral buffered formalin, demineralised using a standard HCl-based solution, and processed for histopathological examination (An & Martin, 2003). Routine bone histological techniques were employed to assess tissue integrity, cellular architecture, and the presence of pathological changes (An & Martin, 2003). This analysis aimed to determine whether the anomalies represented normal anatomical variation or age-related conditions such as osteoarthritis or bone resorption (Aho et al., 2017; Giordano et al., 2016; Pritzker et al., 2006; Sulzbacher, 2013).

2.5. Statistical analyses

2.5.1. Observer reliability

We assessed potential observer-related bias in radiographic scoring by fitting generalized linear mixed-effects models (GLMMs) in R (`lme4` package; Bates et al., 2015) using the subset of 40 radiographs that were scored independently by two observers. For each of the anatomical scoring locations, as well as for the total bone score (sum of all locations), we fitted a baseline model with a random intercept for sampling location to account for clustering effects. We marginally adjusted the scores (+0.01) to enable modelling under a Gamma distribution with a log link, as this distribution cannot accommodate zero values. We used the model outputs to estimate intraclass correlation (ICC) and design effect via the `performance` package, quantifying the proportion of variance explained by location-level clustering (Shrout & Fleiss, 1979). To evaluate the potential influence of observer identity, we fitted a second model for

each scoring variable, including observer ID as a fixed effect. Model diagnostics and visualizations were performed using the `performance` and `flexplot` packages (Fife, 2022; Lüdtke et al., 2021). Model comparisons were done to determine whether accounting for observer identity improved model fit. Competing models were evaluated using differences in Akaike Information Criterion (ΔAIC) and Bayes Factors.

2.5.2. Correlation with age

We quantified the relationship between ossification scores at each anatomical location and dental age using Spearman's rank correlation coefficient (ρ ; Spearman, 1904). We ran independent analyses for males and females, incorporating all available sites from both flippers.

2.5.3. Sequence of ossification

To examine the relative timing of ossification between bones, we constructed pairwise precedence matrices. This allowed us to identify whether one element consistently reached a higher maturation stage before another across individuals, thereby capturing the typical developmental order of bones and highlighting systematic differences between sexes or flipper sides. Our approach follows the logic of event-pairing (Bininda-Emonds et al., 2002; Jeffery et al., 2005; Wilson & Sánchez-Villagra, 2009), in which all possible pairs of developmental events are compared to one another.

For each individual (excluding 13 animals with ossification scores of 8, since age-related degeneration does not necessarily follow a fixed anatomical pattern), we compared every possible pair of bones: if bone i had a higher maturation score than bone j , this contributed 1 to $P(i > j)$; if the reverse was true, it contributed 0; and equal scores contributed 0.5. We averaged these pairwise outcomes across all animals, yielding a square precedence matrix in which rows represent bone i and columns bone j , with each entry giving the probability (expressed as a percentage) that bone i is more mature than bone j .

We visualised the ordered matrices as heatmaps with percentage labels, where darker shading indicates stronger evidence that the row element tends to ossify earlier than the column element. This procedure condenses complex individual-level scoring data into reproducible sequence diagrams that summarise the relative order of ossification events in a transparent and comparable way.

2.5.4. Bilateral asymmetry

We compared paired total radiographic scores ($n = 137$) by calculating left–right differences ($L - R$). To evaluate whether one side is systematically more advanced, we applied a paired permutation test with 100,000 random sign-flips on the signed differences (Good, 2005). This approach makes no distributional assumptions, is robust to zero-inflated data with many ties and was therefore preferred over paired t-tests or Wilcoxon signed-rank tests.

To quantify the magnitude of asymmetry, we calculated the absolute difference ($|L - R|$). We report the absolute mean difference for all dolphins to show population-level asymmetry, and for dolphins with non-zero differences to show the typical severity when asymmetry occurred. We estimated a 95% confidence interval (CI) for these means by non-parametric bootstrap resampling with 5,000 replicates, using the bias-corrected and accelerated method, which adjusts for both bias and skewness in the bootstrap distribution (Efron, 1987). Finally, we ran an exact sign test (Hollander et al., 2015) on the subset of dolphins with non-zero differences to test whether asymmetries were biased toward the left or right flipper.

3. Results

3.1. Sequence of ossification

Bone maturation in common dolphins progressed in a proximal-to-distal and cranial-to-caudal direction (Figure 3.3). Ossification began in the radius, followed by the ulna and proximal metacarpals II and III. It then progressed distally into the metacarpals and phalanges, with digit II maturing earlier than digit III, and digit III earlier than digit IV. Both delta bones, as well as the distal metacarpal IV, were the last bones to mature.

Nonetheless, deviations from this sequence were observed in 19.7% of examined animals ($n = 27$), in which advanced maturation occurred in metacarpals II and/or III compared to radius and ulna. These deviations appeared either in isolation ($n = 15$) or together with the first phalanges of the same digits ($n = 12$). Most cases ($n = 22$; females: $n = 12$, males: $n = 10$) were observed in younger dolphins, particularly those with a maximum total bone score of 28 (62.9% of 35 individuals at this maturation stage; Figure 3.4A&B) and with a dental age of two years or younger. This group included two fetuses and five neonates. Although less frequent, discrepancies were also identified in five older dolphins (aged 7 to 16.5 years) with total bone

scores ranging between 53.5 and 85.5. Four of these animals exhibited complete physal fusion in proximal metacarpals II and/or III while the radius and/or ulna still retained open lateral physal margins (Figure 3.4C). In one individual, metacarpal II and its first phalanx had already reached a score of 6, whereas the ulna remained at stage 5.

3.2. Bilateral asymmetry

Asymmetry in bone maturation was observed in 22 of 137 common dolphins (16.1%). In fourteen cases, the left flipper showed more advanced development, whereas in six cases the right flipper was more advanced. In two individuals (KS19-18Dd and KS22-73Dd), asymmetries in both flippers balanced out so that the total bone scores equalized. Most asymmetries occurred in younger animals ($n = 19$) with total radiographic scores below 35 and dental ages under 2.5 years, including two foetuses and six neonates (Figure 3.5A). In these cases, the affected bones were in developmental stages 0–2 and left–right differences were generally minor: the maximum discrepancy in total radiographic score was 2.5 points, with most individuals ($n = 14$) differing by only one point or less.

More pronounced asymmetries were noted in three individuals. A 12-year-old dolphin (KS21-41Dd) exhibited the largest overall discrepancy, with bone development in the left flipper five points ahead of the right (Figure 3.5B). A second individual (KS15-16Dd, 26 years) showed asymmetry consistent with putative unilateral bone degeneration. The third case (KS23-54Dd; Figure 3.5C) involved a suspected congenital deformity with anomalous metacarpal anatomy, in which the proximal physis of metacarpal III was absent, resulting in left–right differences. The morphology of the latter case suggests a congenital anomaly — rather than trauma, as no external scarring was evident — involving either premature closure of the growth plate or delayed segmentation or ossification of the cartilaginous precursor (e.g., Aparisi Gómez et al., 2021; Barik & Farr, 2021; Maroteaux et al., 1984; Mortier, 2003).

Despite these individual observations, bilateral asymmetry was not significant at the population level. Specifically, left and right flippers did not differ systematically across the full dataset (permutation test, $p = 0.45$). Absolute left–right differences were small overall (mean = 0.20; 95% CI: 0.12–0.35), while among the 20 dolphins with measurable asymmetry, the mean difference was 1.4 points (95% CI: 1.0–2.1). Asymmetries were not directionally biased (14 left > right vs 6 right > left; binomial test, $p = 0.12$).

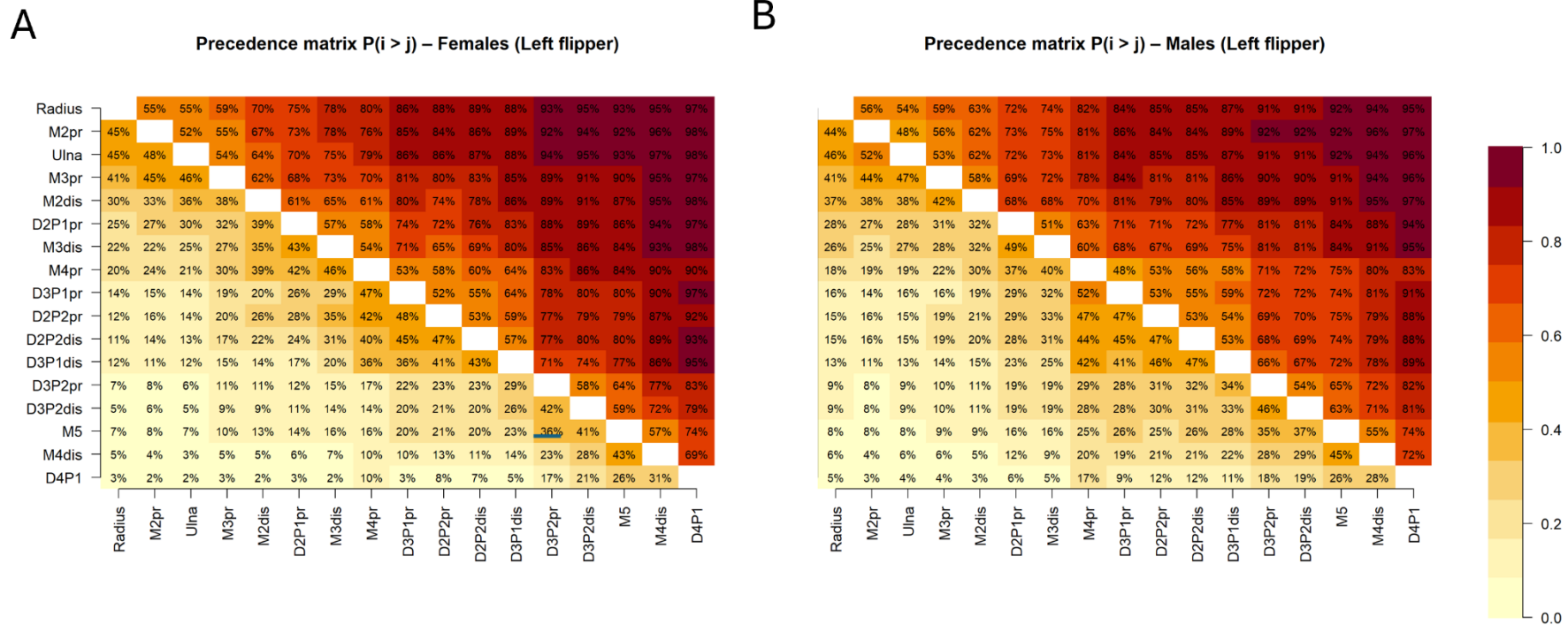


Figure 3.3: Pairwise precedence matrices of bone ossification in the left flipper of common dolphins (*Delphinus delphis*), showing bone ossification patterns for both sexes. Each square shows the percentage of individuals in which the bone on the row reached a higher ossification stage than the bone on the column, based on pairwise event-pair comparisons. Darker shading indicates stronger evidence that the row element tends to ossify earlier than the column element. Values were averaged across individuals (excluding those with degeneration scores of 8) to yield the probability $P(i > j)$. Results are presented separately for **A)** females and **B)** males, providing population-level summaries of relative ossification order for each sex. Abbreviations: M2–M5 = metacarpals II–V; pr/dis = proximal/distal epiphysis; D2–D4 = digits II–IV; P1/P2 = first/second phalanx.

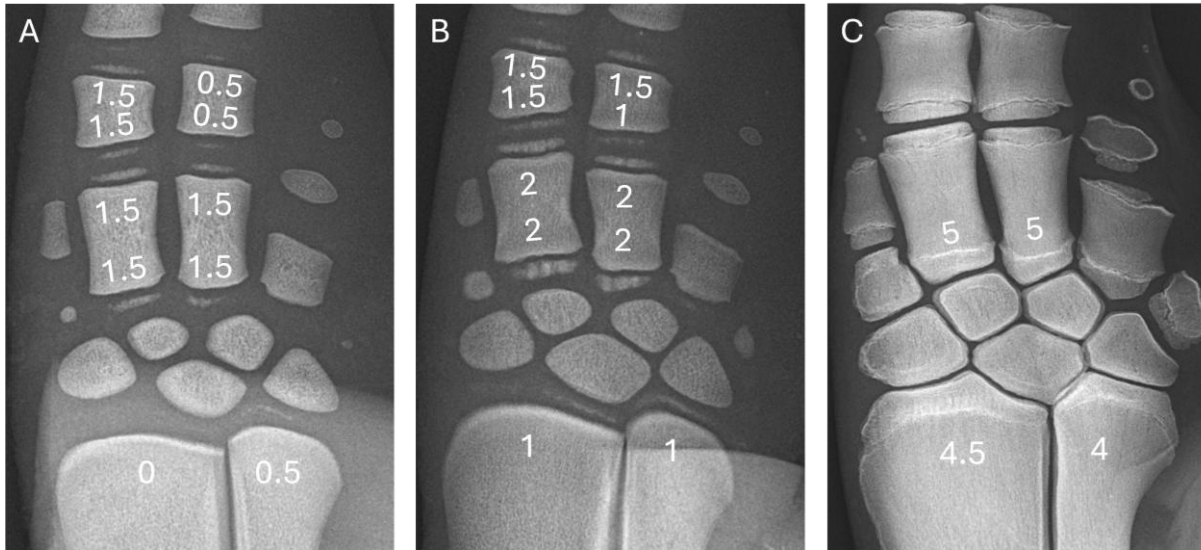


Figure 3.4: Exemplar common dolphin (*Delphinus delphis*) radiographs in which bone development did not follow a typical proximo-distal progression. Bone maturation scores are indicated on the radiographs. **A)** Absence of secondary ossification centres in the distal radius, while metacarpals II and III and the phalanges exhibit more advanced development. **B)** Advanced maturation observed in metacarpals II and III and the phalanges, compared to the radius and ulna. **C)** Complete fusion of the proximal growth plates in metacarpals II and III, despite unfused growth plates in the radius and ulna, which remain open. Radiographs were acquired using a setting of 40 kVp and 4 mAs, with a source-to-sample distance of 100 cm.

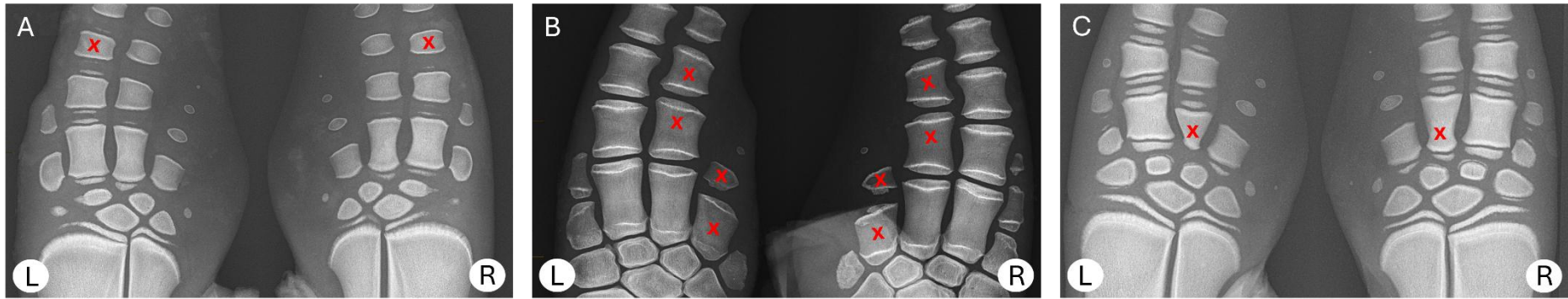


Figure 3.5: Exemplars of bilateral differences in bone development in common dolphins (*Delphinus delphis*). Locations where the developmental stage differs between the left (L) and right (R) flippers are marked with a red cross. **A)** Presence of secondary ossification centres at both the proximal and distal epiphyses of the second phalanx of digit II in the left flipper, and absence of these centres in the corresponding bones of the right flipper. **B)** Advanced fusion of the growth plate in metacarpal IV and the phalanges of digit III in the left flipper compared to the right, along with a higher stage of delta bone development in the first phalanx of digit IV. **C)** Absence of a discernible physis in the third metacarpal of the left flipper, where the metaphyseal and epiphyseal regions appear fused with a smooth cortical transition. Adjacent metacarpals and the corresponding element on the right flipper display open physes, consistent with the expected developmental stage. Radiographs were acquired using a setting of 40 kVp and 4 mAs, with a source-to-sample distance of 100 cm.

3.3. Modifications to the bottlenose dolphin scoring framework

3.3.1. Bone maturation of long bones

In common dolphins, traces of the physal scars (i.e., ghost lines) were sometimes visible beyond the stage 5 characteristics described by Barratclough, Sanz-Requena, et al. (2019). In that scheme, stage 5 is defined by complete physal closure with a ghost line or scar spanning at least 50% of the former plate. Beyond stage 5, the physal line is progressively remodelled, with reduced visibility and eventual loss of the hypermineralised transverse remnant. Our observations showed overlap between these criteria, as ghost lines occasionally persisted despite features of more advanced bone maturation, including incorporation of the former epiphysis across the full transverse width of the bone (stage 6) or a flattening of the physal surfaces with accentuated pointed edges (stage 7).

3.3.2. Bone maturation of delta bones

A subset of common dolphins ($n = 40$; 29.2%) showed a divergent maturation pattern in their delta bones, characterised by simultaneous development of proximal and distal secondary ossification centres (Figure 3.6E-H). This contrasts with the unidirectional sequence described in bottlenose dolphins, where distal mineralisation is not expected until after proximal consolidation is complete (Barratclough, Sanz-Requena, et al., 2019). In common dolphins, however, distal ossification frequently began before the proximal centre had fully consolidated. This atypical pattern resembled long bone development and occurred in either ‘M5’ ($n = 9$), ‘D4P1’ ($n = 21$), or in both bones within the same individual ($n = 10$). It was confined to delta bone stages 1–4 and, among individuals scored within this range, was present in 19 of 44 cases (43.2%) for ‘M5’ and 31 of 73 cases (42.5%) for ‘D4P1’.

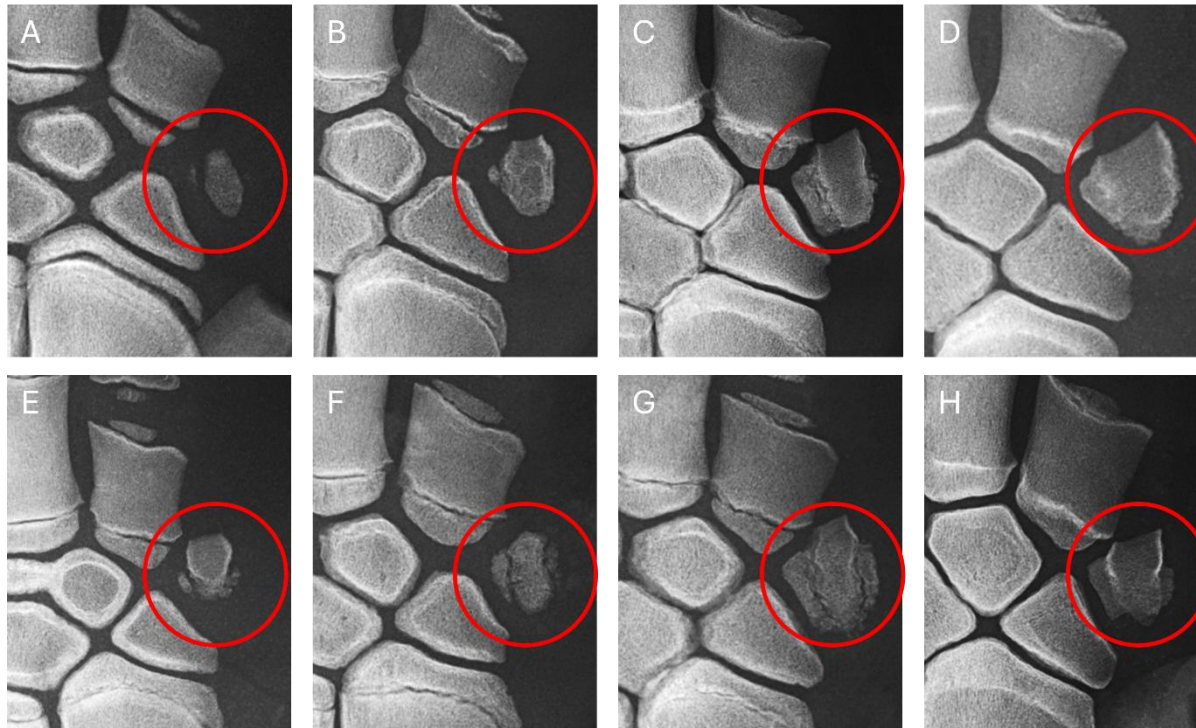


Figure 3.6: Exemplars of common dolphin (*Delphinus delphis*) delta bone development based on metacarpal V (red circles), showing stages 1 to 4 for **A)–D)** the typical unidirectional ossification pattern and **E)–H)** cases with simultaneous proximal and distal ossification. Bone ossification stages are shown as follows: **(A, E)** Stage 1; **(B, F)** Stage 2; **(C, G)** Stage 3; **(D, H)** Stage 4. Radiographs were acquired using a setting of 40 kVp and 4 mAs, with a source-to-sample distance of 100 cm.

3.4. Age-related degenerative changes

Putative signs of age-related degeneration were radiographically identified in 13 animals assigned a score of 8. These cases showed loss of cortical definition at articular surfaces (Figure 3.7A), irregular articular contours (Figure 3.7B), and narrowing of joint spaces (Figure 3.7C). Although 22 individuals ($n = 44$ flippers) were 15 years old or older, degenerative changes in common dolphins were subtle and less pronounced than those described for bottlenose dolphins. We did not document extreme cases of age-related degeneration, including extensive cartilage mineralisation, as previously described in bottlenose dolphins (Barratclough, Sanz-Requena, et al., 2019).

The putative bone remodelling observed in common dolphin radiographs (Figure 3.2A–C) but not reported in bottlenose dolphins was excluded from scoring, as histological analysis of 24 affected individuals revealed considerable variation in underlying pathology. Histological evaluation indicated that these changes could not reliably be interpreted as evidence of age-related degeneration. Features suggestive of degenerative joint disease, such as cartilage fragmentation and irregularity (Aho et al., 2017; Pritzker et al., 2006), were present in 6 cases (25%). Two additional individuals (8.3%) exhibited potentially age-related but non-degenerative changes, including increased ossification of ligamentous structures. Other findings included periosteal new bone formation likely due to prior periostitis ($n = 1$; 4.2%), prominent blood vessels radiographically mimicking bone narrowing ($n = 6$; 25%), increased cartilage thickness of uncertain origin at intraosseous ligament attachment sites ($n = 4$; 16.7%), and a possible healed infarct ($n = 1$; 4.2%). In four cases (16.7%), no histological abnormalities were detected, indicating normal anatomical variation.

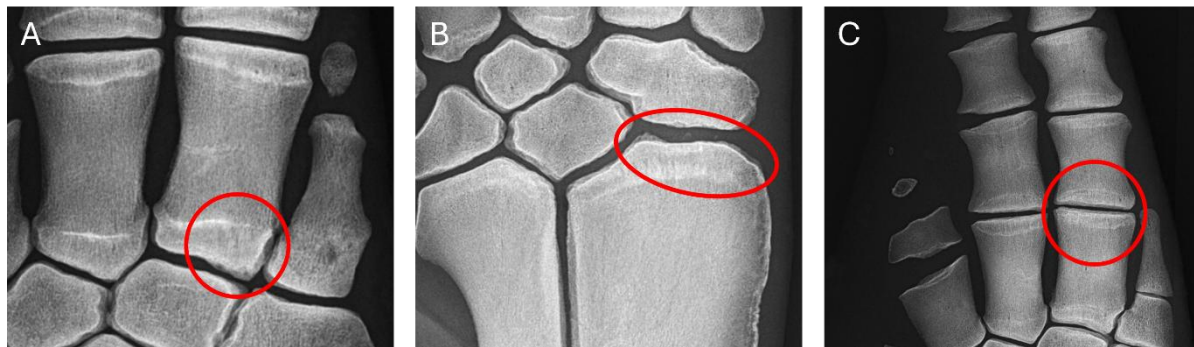


Figure 3.7: Exemplars of common dolphin (*Delphinus delphis*) bones assigned to stage 8 based on the scoring scheme by Barratclough et al. (2019). Stage 8 is defined by degenerative changes in bone or cartilage associated with osteolysis or proliferative remodelling. **A)** Loss of normal cortical definition in the distal end of metacarpal IV (red circle). **B)** Irregular and undulating distal articular surface of the radius, with loss of smooth cortical contour and evidence of surface remodelling (red circle). **C)** Narrowing of the metacarpophalangeal joint space, with close apposition of articular surfaces (red circle). Radiographs were acquired using a setting of 40 kVp and 4 mAs, with a source-to-sample distance of 100 cm.

3.5. Correlation with age

All assessed bone locations demonstrated strong correlations with dental age, with Spearman's rho values ranging from 0.88 to 0.92 ($p = 1.82 \times 10^{-27}$ to 9.2×10^{-22}) and 0.91 to 0.96 ($p = 1.09 \times 10^{-31}$ to 1.04×10^{-22}) for females and males, respectively, indicating that the anatomical sites established for bottlenose dolphins are also highly applicable to common dolphins.

3.6. Observer reliability

Despite identification of morphological differences from the original bottlenose dolphin scoring scheme and the need for some adjustments, scoring agreement between observers remained high. We found no evidence that observer differences systematically influenced the recorded scores. In the baseline Gamma GLMM with a random intercept for location, scores showed substantial variation between locations (ICC = 0.34), but adding observer identity as a fixed effect did not significantly improve model fit ($\Delta AIC < 2$; Bayes Factor ≈ 0.03). The estimated effect of Observer 2 corresponds to an approximate 1.1% increase in predicted scores ($\beta = 0.011$, SE = 0.025, $p = 0.662$).

4. Discussion

This study provides the first assessment of whether radiographic scoring schemes developed for bottlenose dolphins can be applied to common dolphins, despite well-documented differences in forelimb morphology (Cooper et al., 2007) and life-history traits (Palmer et al., 2022, 2023; Wells et al., 2025). By applying the Barratclough, Sanz-Requena, et al. (2019) *Tursiops*-derived system to a large dataset of common dolphin flippers of individuals with known dental age, we demonstrate that the framework is highly transferable, with consistent scoring across observers and strong correlations between ossification stage and chronological age. These results underscore the reliability of this approach for cross-species application, even in the face of morphological differences in maturation. Nevertheless, morphological and developmental differences between species must still be considered when applying this framework, since our results highlight several species-specific considerations for common dolphins.

4.1. Bilateral asymmetry

We observed bilateral asymmetry in approximately 16% of individuals, most frequently in younger animals (Figure 3.5A). This finding contrasts with bottlenose dolphins, where bilateral development has generally been described as symmetrical by Barratclough, Sanz-Requena, et al. (2019). However, the phenomenon is well documented in humans, where similar asymmetries in wrist radiographs of children are typically attributed to natural variability in maturation rates or, in some cases, nutritional deficiency or metabolic impairments (Baer & Durkatz, 1957; Francis, 1939; Francis & Werle, 1939; Torgersen, 1951). Such discrepancies tend to be most visible in younger individuals, when skeletal development progresses rapidly. Yet in humans, they do not compromise the accuracy of bone age estimation (Martin et al., 2010). The minor asymmetries (< 2.5 points of difference between left and right) observed in young (≤ 2 yrs) individuals are, therefore, unlikely to bias age estimation, although this remains to be confirmed using a calibrated age assessment model.

Larger left-right asymmetries were found in one older dolphin (KS15-16Dd, 26 yrs), resulting from the unilateral occurrence of age-related degeneration. In older humans, laterality may create differences in mechanical stress between hands (Steele, 2000), which can increase the risk of degenerative changes in the dominant hand (Solovieva et al., 2006). Evaluating only one side may therefore affect the reliability of age estimates in elderly individuals. Lateralized flipper use or locomotory behaviour has been documented in multiple cetacean species, in both wild and managed care contexts (Clapham et al., 1995; Clark & Kuczaj, 2016; Hoese, 1971; Johnson & Moewe, 1999; Kaplan et al., 2019; Marino & Stowe, 1997; Ridgway, 1986; Sobel et al., 1994), although such behaviour has not yet been reported in common dolphins. As unilateral age-related degeneration occurred only once in our dataset, these findings suggest that such asymmetries are unlikely to substantially influence age estimates.

Consistent with Hui (1977), these asymmetries were furthermore not significant at the population level, with left and right flippers showing no systematic differences. Even so, potential side-specific variation should be considered when developing or applying radiograph-based ageing models. In post-mortem examinations, assessment of both pectoral flippers remains advisable, as this helps detect possible unilateral degeneration and distinguish subtle age-related changes from normal individual variation. Evaluation of both flippers, together with gross external examination, may also reveal pathologies. For example, bilateral differences may

arise from trauma, congenital deformities, or infectious processes such as *Brucella ceti*, which has been associated with impaired bone growth and development (Roca-Monge et al., 2022). When radiographs are obtained post-mortem, access to both flippers generally is straightforward for delphinids, facilitating comparison in cases of unilateral anomalies. In live animals, by contrast, a unilateral approach remains appropriate to minimize handling time and radiation exposure, since the asymmetries observed in our study were only minor.

4.2. Framework adaptations

Our analysis revealed several differences from the bottlenose dolphin scoring system that required adjustment for use in common dolphins. For example, common dolphin long bones occasionally retained visible physal scars beyond the stage at which they are typically resorbed in bottlenose dolphins. Barratclough, Sanz-Requena, et al. (2019) analysed opportunistic radiographs from live bottlenose dolphins across multiple facilities, where variation in imaging protocols may have influenced the results. Moreover, obtaining a clear dorsoventral projection in a live dolphin presents inherent challenges in positioning the pectoral flippers, whereas this can be achieved more readily in amputated flippers imaged post-mortem. Nonetheless, despite differences in visibility of the physal scar and imaging context, the scoring system itself remained applicable to common dolphins. Emphasis should, however, be placed on bone shape and the degree of epiphysal coverage rather than on the ghost line alone.

By contrast, patterns in delta bone development reflected true interspecific divergence rather than potential methodological effects. Instead of the strictly proximal-to-distal ossification sequence described in bottlenose dolphins (Barratclough, Sanz-Requena, et al. 2019), almost a third of examined common dolphins ($n = 40$) exhibited simultaneous proximal and distal ossification centres affecting delta bones in early stages (Stages 1–4; Figure 3.6E–H). These differences, while biologically notable, did not compromise the functionality of the scoring system. In fact, delta bone development appeared more clearly defined in common dolphins than in bottlenose dolphins, where staging was comparatively ambiguous. In common dolphins, delta bones could be reliably staged regardless of the presence of distal mineralisation. When scoring Stages 1–4, emphasis should be placed solely on the maturation of the proximal ossification centre to ensure consistency across both unidirectional (Figure 3.6A–D) and bidirectional (Figure 3.6E–H) patterns of delta bone development.

Both the persistence of physal scars and the distinct delta bone trajectory were readily accommodated within the framework without loss of resolution in ossification staging. Importantly, scoring remained highly consistent between observers, with the 16-point scoring approach reducing subjectivity and individual variability. This high level of agreement reinforces the robustness and transferability of the bottlenose dolphin framework to common dolphins. Combined with strong correlations between ossification scores and chronological age, this provides a solid foundation for developing a calibrated radiographic ageing model for common dolphins.

4.3. Potential limitations

One potential limitation to transferability concerns the expression of age-related degenerative changes. In common dolphins, such changes were relatively subtle (Figure 3.7) compared with more advanced cartilage mineralisation and joint surface remodelling reported in bottlenose dolphins (Barratclough, Sanz-Requena, et al., 2019). This is striking given that our dataset spanned the full estimated lifespan of common dolphins, including cases up to 31 years of age, with over 16% of individuals aged 15 years and older, thus representing approximately half of the reported maximum life span. Animals that reached physical maturity (≥ 18 years for females, ≥ 20 years for males, Palmer, 2023) were included (females: $n = 8$, males: $n = 4$) but remain rare due to their infrequent strandings. Still, the low prevalence of degeneration is unexpected in this dataset. Although bottlenose dolphins have a longer life expectancy (48–57 years; Weigl, 2005; Wells & Scott, 2009) compared to ~ 30 years in common dolphins (Perrin, 2009), the occurrence of age-related degeneration, such as osteoarthritis, is generally thought to scale with maximum lifespan (van der Kraan, 2025). Consequently, both species would be expected to exhibit comparable patterns of skeletal ageing relative to their longevity, with osteoarthritic changes typically emerging once animals reach approximately 50–80% of their maximum life span (van der Kraan, 2025).

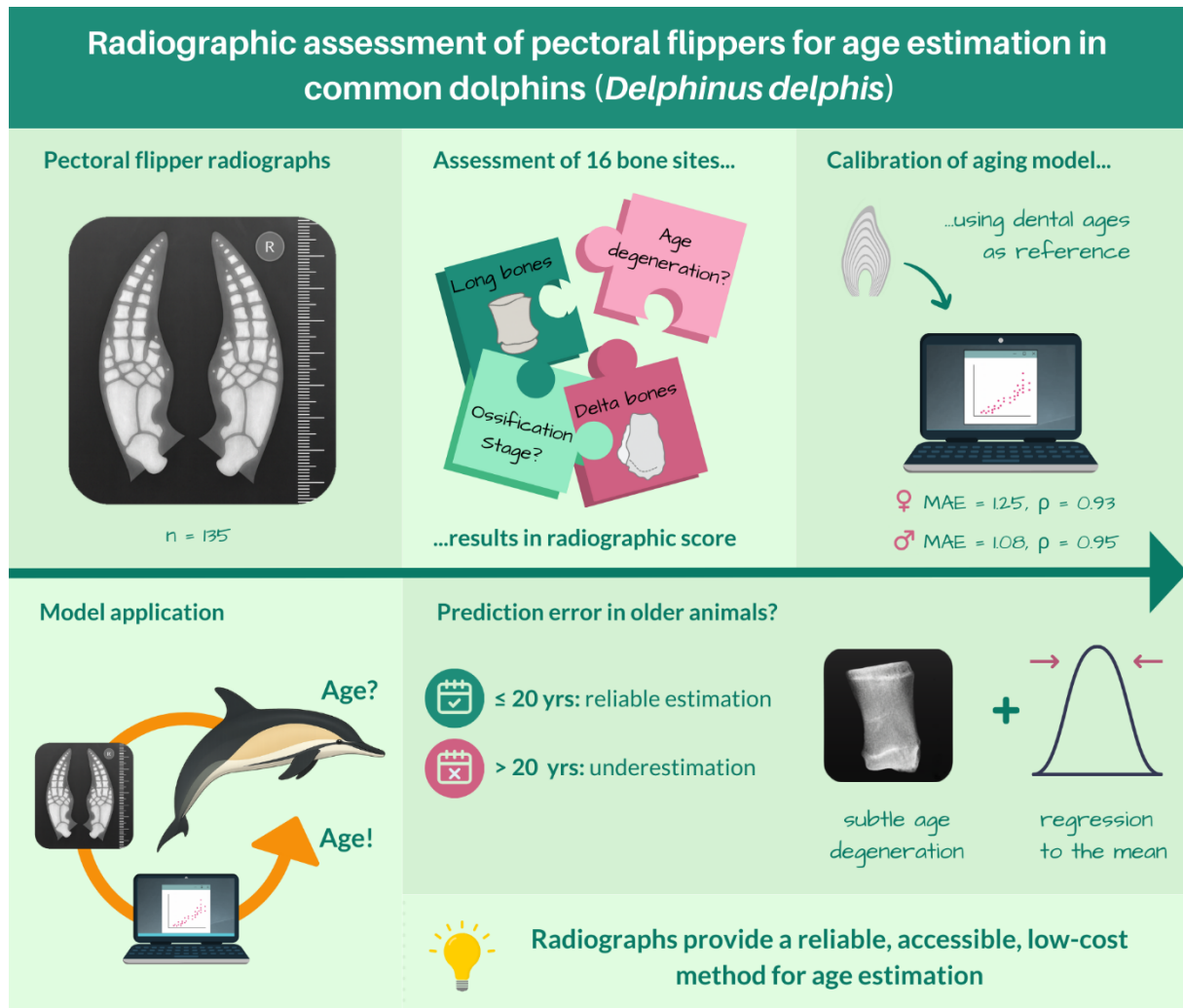
Differences in the timing of sexual maturity could, however, influence when skeletal tissues transition from development to maintenance, thereby affecting the onset of degenerative processes (Stewart et al., 2024). Furthermore, the reduced extent of bone degeneration in common dolphins may reflect species-specific biomechanical, ecological, or physiological factors, with locomotor differences also potentially contributing. The bottlenose dolphin study was based on individuals in human care (Barratclough, Sanz-Requena, et al., 2019). This raises

the possibility that environmental and/or behavioural differences between managed-care and free-ranging dolphins may contribute to variation in the prevalence of degenerative changes. However, this factor appears unlikely to solely account for the observed interspecific difference, as our assessment of radiographs from free-ranging bottlenose dolphins from New Zealand (Massey University, unpublished data, 2015) also reveal signs of age-related degeneration, consistent with the findings of Barratclough, Sanz-Requena, et al. (2019). Given the limited sample size of physically mature common dolphins, further research is required to determine whether the rarity of degenerative changes is genuine or a sampling artefact.

Clearly, the subtle expression of age-related degeneration in common dolphins must be considered when developing future pectoral flipper ageing models, as it may reduce the accuracy of estimates in physically mature individuals. Nonetheless, this limitation does not compromise most of the age range, and the strong correlation between bone ossification and dental age indicates that reliable estimates can be achieved for the majority of the lifespan. Furthermore, the demonstrated transferability of the bottlenose dolphin scoring framework provides a strong foundation for a calibrated radiographic model tailored to common dolphins. This offers a valuable alternative to traditional age-estimation techniques and supports broader comparative applications of radiographic ageing across odontocetes.

Chapter 4

Radiographic assessment of bone maturation: A tool for age estimation in common dolphins (*Delphinus delphis*)



This chapter is a reformatted version of the following manuscript:

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STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.			
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Name and title of main supervisor:	Prof Karen Stockin		
In which chapter is the manuscript/published work?	Chapter 4		
Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work: ¹ Laboratory work was conducted by Eva-Maria Hanninger, Emma Betty, Emily Palmer, Matthew Perrott, Joy Bowler, and Karen Stockin. Radiographic analyses were performed by E. Hanninger with support from Ashley Barratclough. Statistical analyses were conducted by E. Hanninger with support from Marti Anderson and Katharina Peters. Conception, study design, and methodology were developed by E. Hanninger, A. Barratclough, and K. Stockin. The manuscript was drafted by E. Hanninger. Review and editing were undertaken by E. Hanninger, A. Barratclough, E. Betty, M. Anderson, M. Perrott, J. Bowler, E. Palmer, K. Peters and K. Stockin. Funding acquisition was led by E. Hanninger and K. Stockin.			
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Abstract

We present the first radiographic ageing framework for common dolphins (*Delphinus delphis*), based on ossification and epiphyseal fusion patterns in the pectoral flipper, demonstrating higher reliability for chronological age estimation than currently available epigenetic approaches for this species. Using individuals of known dental age, we calibrated two modelling approaches to predict dental age from radiographic bone scores: 1) a univariate polynomial regression using a total bone score (sum of 16 scores across all assessed flipper bones), and 2) a multivariate canonical analysis of principal coordinates (CAP) incorporating 16 individual bone-score variables. Both approaches successfully predicted dental age from skeletal ossification patterns. For an age range of 0 to 24 years, polynomial regression demonstrated high predictive accuracy with median absolute errors (MAEs) of 1.25 years in females (Spearman's $\rho = 0.93$, $R^2 = 0.90$) and 1.08 years in males ($\rho = 0.95$, $R^2 = 0.86$). The CAP model yielded MAEs of 1.35 years in females ($\rho = 0.90$, $R^2 = 0.85$) and 1.80 years in males ($\rho = 0.94$, $R^2 = 0.84$). Notably, both radiographic bone ageing models achieved equal or lower median absolute errors and higher coefficients of determination than a recently developed epigenetic clock for common dolphins derived from the same population (MAE = 1.80, Pearson's correlation (r) = 0.91, $R^2 = 0.82$). When applying the bone ageing models to individuals of unknown dental age, both models produced age estimates consistent with expected life-history stages (foetus, neonate, juvenile, subadult, adult), although accuracy declined in dolphins above 20 years, likely as a consequence of subtle age-related variation in skeletal changes in this species. Radiographic ageing provides an accurate non-invasive tool for demographic assessment to support conservation management of common dolphins.

1. Introduction

Understanding key reproductive parameters, such as age at sexual maturity, gestation and lactation periods, and calving intervals, is central to understanding population viability. Crucially, these assessments rely on accurate age estimation, which provides the foundation for interpreting reproductive status, population structure, and longevity (Betty et al., 2022, 2023). Robust age data are, therefore, essential for evaluating population persistence, predicting responses to environmental and anthropogenic pressures, and informing effective conservation strategies (Betty et al., 2019; Heydenrych et al., 2021; Manlik et al., 2016; Moore & Read, 2008; Palmer et al., 2022).

Traditionally, chronological age assessment in odontocetes has mostly relied on counting growth layer groups (GLGs) in thin, stained tooth sections (e.g., Betty et al., 2023; Evans et al., 2002; Lockyer, 1993, 1995; Murphy et al., 2014; Palmer et al., 2022, 2023; Westgate & Read, 2007). Because tooth extraction is invasive, it is typically confined to post-mortem examinations, with the exception of procedures performed under anaesthesia (Barratclough, Wells, et al., 2019). In addition, GLG assessment requires experienced readers and access to specialised histology laboratories, which can be costly and time-consuming (Evans et al., 2011; Murphy et al., 2014). These logistical and financial constraints further limit the feasibility and scalability of traditional age estimation across populations and regions, highlighting the need for alternative approaches.

Epigenetic age estimation based on DNA methylation provides a feasible non-lethal alternative for ageing live animals (e.g., Barratclough et al., 2021; Hanninger et al., 2025; Hernandez et al., 2023; Peters et al., 2023; Robeck et al., 2021; Zoller et al., 2025), though it also has practical constraints. Implementing these methods requires either high-throughput sequencing or the use of Illumina methylation arrays (Arneson et al., 2022; X. Liu et al., 2024). At present, the relevant patent for array-based processing is held by the Clock Foundation in the United States (Arneson et al., 2022; Lu et al., 2023). Consequently, both the laboratory procedures and the potential need to ship samples internationally are costly and logistically demanding, limiting the accessibility of epigenetic ageing in many research and management contexts.

Such constraints have encouraged interest in methods that are more practical and broadly accessible. Pectoral flipper radiography offers one such option, as radiography is comparatively inexpensive and relies on equipment that is widely available, even in remote field settings. This technique allows age estimation, as recently demonstrated for bottlenose dolphins (*Tursiops truncatus*; Barratclough, Sanz-Requena, et al., 2019), and provides a non-invasive morphological alternative to tooth-based ageing. Based on the assessment of 16 specific flipper bone locations, the method evaluates ossification and epiphyseal fusion, which follow a predictable age-related sequence (Barratclough, Sanz-Requena, et al., 2019). The framework, so far only applied to bottlenose dolphins under human care, incorporates assessments of long-bone maturation, delta-bone development (a cetacean-specific physiological stage in the ossification of specific metacarpals and phalanges; Barratclough, Sanz-Requena, et al., 2019; Dawson, 2003) and the occurrence of age-related degeneration (Barratclough, Sanz-Requena, et al., 2019). However, while the radiographic age assessment framework for bottlenose

dolphins represents a major advance, differences in ossification timing between sexes and among species mean that models of ageing must be developed and calibrated on a sex- and species-specific basis, using radiographs from known-aged individuals (Barratclough, Sanz-Requena, et al., 2019).

Species-specific calibration is necessary because developmental trajectories are shaped by life-history traits, including hormonal changes during puberty (Almeida et al., 2017; Iuliano-Burns et al., 2009). Furthermore, the direct applicability of this framework to other cetacean species has remained uncertain, and equivalent models for species such as common dolphins (*Delphinus delphis*) have, until now, been lacking. Uncertainties about transferability arise from morphological differences in flipper anatomy (e.g., degree of hyperphalangy; Cooper et al., 2007), interspecific variation in delta bone maturation, and limited knowledge of the occurrence and extent of degenerative bone disease in other species (Hanninger, Barratclough, Betty, Dittmer, et al., 2026).

Recently, Hanninger, Barratclough, Betty, Dittmer, et al. (2026) addressed whether interspecific morphological differences would require modification of scoring criteria for application to common dolphins. While interspecific differences in morphology and bone maturation patterns were present, these differences did not prevent the scoring framework from being applied effectively. Importantly, scoring agreement between independent observers remained high, highlighting the robustness of the method. One potential limitation identified by the authors, however, was that age-related degeneration appeared comparatively more subtle in common dolphins, which could theoretically reduce accuracy in aged individuals (>20 yrs; Hanninger, Barratclough, Betty, Dittmer, et al., 2026).

Building on these findings, the present study develops a calibrated radiographic model for age assessment in common dolphins, providing a species-specific tool for age estimation. The model was developed using a 30-year tissue archive of stranded and bycaught individuals with known dental ages, sex, body length, and sexual maturity (Palmer et al., 2022, 2023). Radiographic bone scores for 137 individuals (aged from 0 to 31 years) were assessed *a priori* (Hanninger, Barratclough, Betty, Dittmer, et al., 2026) and used to construct a model spanning the full lifespan of free-ranging dolphins (~30 years; Perrin, 2009) represented in the sample. To account for sex-specific differences in ossification, separate models were developed for males and females. We present two complementary modelling approaches for age estimation.

The first approach followed Barratclough, Sanz-Requena, et al. (2019) using the total bone score for each radiograph (i.e., the sum of all individual scores) in a polynomial regression calibrated against dental age. This approach was applied to ensure comparability with previous bottlenose dolphin studies. Second, we used a multivariate approach — canonical analysis of principal coordinates (CAP, Anderson & Robinson, 2003; Anderson & Willis, 2003), where the 16 bone score values were treated as 16 individual inter-correlated variables. In this analysis, principal coordinates are calculated from Euclidean distances among all individuals on the basis of the 16 bone variables, in a holistic framework. The model seeks a linear combination of the principal coordinates to maximise discrimination among dolphin individuals along a gradient of known dental age. This approach allowed the multidimensional structure of flipper ossification to be characterised and related directly to chronological age.

2. Materials & methods

2.1. Sample collection

2.1.1. Life-history assessment

Dolphins assessed in this study ($n = 137$) are stranded or bycaught individuals examined postmortem between 2002 and 2025. Age, growth, and sexual maturity were assessed *a priori* to pectoral flipper examination as part of earlier life history research (Palmer et al., 2022, 2023). We included foetuses ($n = 5$; either extracted in utero or aborted prior to first breath, confirmed by pulmonary atelectasis; Colegrove et al., 2016), neonates ($n = 12$; live birth confirmed by inflated lungs and showing at least one of the following characteristics: presence of vibrissal hairs, an unhealed navel, foetal folds, soft and folded dorsal fin and tail flukes, or unerupted teeth; Díaz López et al., 2018; Puig-Lozano et al., 2020), juveniles ($n = 56$; lacking neonatal characteristics and with total body length less than average length at attainment of sexual maturity, i.e. <183 cm for females and <190 cm for males, Palmer et al., 2022, 2023), subadults ($n = 5$; adult-sized, i.e. ≥ 183 cm in females and ≥ 190 cm in males, but not yet sexually mature; Palmer et al., 2022, 2023), and adults ($n = 57$; sexually mature). Two additional individuals for which age class could not be determined were also included.

Sex (females: $n = 72$; males: $n = 65$) was determined anatomically during post-mortem examination. In rare cases where the genital region was damaged ($n = 2$), molecular sexing was conducted using multiplex PCR amplification of sex chromosome-specific loci (ZFX and SRY)

from skin samples (Gilson et al., 1998; Peters et al., 2023). PCR products were amplified with established primers (P153Z & P23EX and y53-3c & Y53-3D; Aasen & Medrano, 1990; Gilson et al., 1998) and visualised by gel electrophoresis.

Dental age was available for 120 animals (64 females, 56 males). The remaining 17 individuals lacked suitable teeth for GLG ageing (e.g., non-erupted teeth in foetuses and neonates, or teeth otherwise unsuitable for processing). The age was determined by counting GLGs in the dentine of thin, decalcified, and stained tooth sections (Palmer, 2023). Each tooth was independently read by at least two experienced readers across three sessions. If age estimates differed, an additional tooth was processed (up to a maximum of three teeth per individual) and re-read to reach consensus (Palmer, 2023). Dental age ranged from 0 to 31 years, and age distributions by sex are shown in Appendix 3 (Figure A3.1).

2.1.2. Pectoral flipper collection and radiography

Pectoral flippers ($n = 137$ pairs), collected *a priori* during post-mortem examinations, were stored flat, frozen, and subsequently radiographed in a standardised manner, using a single Shimadzu radiographic unit set at 40 kVp (kilovoltage peak) and 4 mAs (milliamperes-seconds), with a source-to-sample distance of 100 cm (Hanninger, Barratclough, Betty, Dittmer, et al., 2026).

2.2. Radiographic analysis

Radiographs were evaluated *a priori* in a proximal-to-distal sequence (Hanninger, Barratclough, Betty, Dittmer, et al., 2026), beginning at the distal radius and ulna and continuing through the metacarpals and phalanges, following Barratclough, Sanz-Requena, et al. (2019). Anatomical bone sites were selected based on their age-informative value in bottlenose dolphins (Barratclough, Sanz-Requena, et al., 2019), as well as their high correlation with age in common dolphins (Hanninger, Barratclough, Betty, Dittmer, et al., 2026). The scoring locations included the distal epiphyses of radius and ulna, proximal and distal epiphyses of metacarpals II–IV and the first and second phalanges of digits II and III, as well as two delta bones, namely metacarpal V and the first phalanx of digit IV. In contrast, carpal bones, metacarpal I, humerus, and the proximal epiphysis of radius and ulna were excluded, as they either lack clear ossification stages, show weak age associations, or could not be imaged reliably in plane (Barratclough, Sanz-Requena, et al., 2019; Hanninger, Barratclough, Betty, Dittmer,

et al., 2026; Ogden et al., 1981). Scoring protocols were adapted from Barratclough, Sanz-Requena, et al. (2019) and adjusted to reflect morphological variation observed in common dolphins (Hanninger, Barratclough, Betty, Dittmer, et al., 2026).

Long bones were assigned maturity stages ranging from –1 to 8, reflecting the progression from the initial appearance of ossification centres through physal narrowing, partial and complete physal fusion, and, at later stages, degenerative change. Half-scores were applied where intermediate conditions were observed. Long bones were generally scored at both the proximal and distal epiphyses. One exception in the original *Tursiops* framework involved the second phalanges of digits II and III, for which a single combined value was used; this corresponded to the higher of the two epiphyseal scores (Barratclough, Sanz-Requena, et al., 2019). Given that hyperphalangy differs between common dolphins and bottlenose dolphins (Cooper et al., 2007), we evaluated whether this combined-score approach was appropriate for our dataset. To do so, we repeated the analyses under four configurations: treating the proximal and distal scores for these phalanges as separate variables ('separate epiphyses'), using an averaged combined score ('combined score average'), applying the maximum score ('combined score max'), and considering only the minimum score ('combined score min').

Delta bones (metacarpal V and the first phalanx of digit four) were scored as single units on a -1 to 8 scale. In common dolphins, both delta bones sometimes exhibited simultaneous development of proximal and distal ossification centres; therefore, for stages 1–4, maturity was classified solely on the proximal ossification centre, regardless of distal development (Hanninger, Barratclough, Betty, Dittmer, et al., 2026). This approach differs from the scoring system originally developed for bottlenose dolphins, in which delta bone ossification follows a unidirectional sequence, with distal mineralisation occurring only after consolidation of the proximal centre (Barratclough, Sanz-Requena, et al., 2019). The classification scheme and criteria used to assign scores to long and delta bones are summarised in Tables 4.1 and 4.2, with schematic illustrations available in Appendix 3 (Figures A3.2 & A3.3). A total bone maturity score was calculated for each radiograph by summing the scores across all assessed locations. Depending on the scoring approach applied to the second phalanx of digits II and III, this total comprised either 16 individual bone scores when using the combined-score method, or 18 individual scores when treating the proximal and distal epiphyses of these phalanges separately.

Table 4.1: Long bone scoring system applied to common dolphins (*Delphinus delphis*) following Barratclough, Sanz-Requena, et al. (2019) with adjustments (*) made based on anatomical differences previously observed for common dolphins (Hanninger, Barratclough, Betty, Dittmer, et al., 2026).

Score	Description
-1	No primary ossification centre visible
0	The primary centre is present, but no secondary ossification centre is visible
1	An epiphyseal ossification centre is present but only occupies <50% of the width of the adjacent metaphysis
2	The secondary ossification centre is well established, ranging between 50–100% of the metaphyseal width; the physis is evident as a distinct radiolucent space, while the secondary centre appears less mature and irregular in depth across its width; maturation progresses in a midline-to-abaxial direction
3	Thinning of the radiolucent physis but no evidence of fusion of the metaphysis and the secondary ossification centre; mineral density increases in the subchondral bone on both sides of the physis; late stage 2 can resemble early stage 3 with the distinction being based on whether the physis width is irregular (stage 2) or uniform (stage 3)
4	Osseous bridges begin to form; in the radius and ulna this starts midline and proceeds abaxially; the abaxial margins remain open, and both early bridge formation and >90% bridging qualify as stage 4
5	Complete closure is present, accompanied by a faint hyperdense “ghost” physeal line or epiphyseal scar spanning at least 50% of the plate; the former epiphysis may not reach the full transverse width of the bone, but closure is complete
6	The physeal line is remodelled, with <50% to no evidence of the hypermineralised transverse remnant, and the former epiphysis extends across the full transverse width of the bone; *the visibility of the physeal scar was not a conclusive criterion in common dolphins and emphasis should be placed on the extension of the former epiphysis
7	Complete fusion occurs with no evidence of a previous ghost physeal plate and flattening of the physeal surfaces with accentuated pointed edges
8	Arthritic changes are visible, including osteophytes, osteolysis, articular calcification, and in some cases fusion of metacarpals or phalanges

Table 4.2: Delta bone scoring system applied to common dolphins (*Delphinus delphis*) following Barratclough, Sanz-Requena, et al. (2019) with adjustments (*) made based on anatomical differences previously observed for common dolphins (Hanninger, Barratclough, Betty, Dittmer, et al., 2026).

Score	Description
-1	Not present
0	Very small oval shape, undefined delta shape
1	Axial surface starts to be linear, consistent with delta shape; *mineralisation may be present adjacent to both the proximal and distal epiphyseal surface but does not show osseous bridge formation
2	One flat surface; areas of slight irregular perimetral surface typically on the proximal side with a degree of adjacent mineralisation which can oppose the epiphyseal surface but not necessarily show definitive osseous bridge formation; bone density is clearly reduced in peripheral new bone in comparison to the primary ossification centre; *mineralisation may be present adjacent to the distal epiphyseal surface and emphasis should be placed on the degree of proximal mineralisation
3	Increased mineralisation of adjacent proximal cartilaginous surface, consolidation may not be present; *mineralisation may be present adjacent to the distal epiphyseal surface and emphasis should be placed on the degree of proximal mineralisation
4	Area increases in consolidation and mineralisation, slightly less dense than the body of the delta bone; a defined hypermineralised physeal plate line is present; the abaxial aspect can show the initiation of the new straight lateral border rather than a horseshoe shape; *mineralisation may be present adjacent to the distal epiphyseal surface and emphasis should be placed on the degree of proximal mineralisation
5	Density of the secondary ossification centre is similar to primary bone and initial mineralisation is present on the distal surface, with the proximal side showing a defined straight lateral border rather than a horseshoe shape; individual variation may result in non-linear borders; therefore, emphasis should be placed on the secondary ossification centres on the proximal and distal surfaces
6	Smooth proximal surface with matching density to the primary ossification centre, distal surface still shows some incongruent mineralisation; physeal line is still visible
7	Smooth proximal surface with complete consolidation of the secondary ossification centre on both sides and dissolution of the physeal line
8	Degenerative changes present

2.3. Statistical analysis

2.3.1. Bone age modelling

i) Polynomial regression

To model the relationship between dental age and skeletal maturity, represented by total radiographic bone score, second-degree polynomial regression models were fitted following Barratclough, Sanz-Requena, et al. (2019). Analyses were conducted separately for females ($n = 64$) and males ($n = 56$) with known dental ages. Predictive performance was evaluated using leave-one-out cross-validation (LOOCV), whereby each individual was excluded in turn, the polynomial regression was refitted to the remaining individuals, and the fitted model was used to predict the excluded individual's age. This procedure produced one cross-validated age estimate per individual and provided an unbiased assessment of predictive accuracy.

Predicted ages obtained from LOOCV were compared with known dental ages to quantify performance. Accuracy was summarised using the mean, median (MAE), and standard deviation of absolute errors. The association between predicted and dental age was assessed using Spearman's rank correlation coefficient (ρ) as distributional assumptions for parametric correlation were not met. In addition, linear regression of predicted age on dental age was used to estimate the slope, intercept, and coefficient of determination (R^2). Following cross-validation, final polynomial models were refitted using all individuals with known dental ages to derive explicit regression equations, which were subsequently applied to estimate ages for individuals lacking known dental ages. For comparability with the previously published epigenetic clock model (Hanninger et al., 2025), we re-ran the full modelling and cross-validation procedure after excluding individuals with absolute age prediction errors >6 years, as flagged in the polynomial regression and/or CAP model. In the epigenetic analysis, this threshold corresponded to the upper tail of the prediction error distribution and was retained here to ensure methodological consistency across ageing approaches. Performance metrics are reported for both the full and reduced datasets.

Furthermore, as differences in bone maturation between the left and right pectoral flippers were observed in some individuals ($\sim 16\%$; Hanninger, Barratclough, Betty, Dittmer, et al., 2026), we repeated polynomial regressions for both sexes using five scoring strategies: the lowest total bone score of both flippers ('BSL'), the highest score ('BSH'), the average of both flippers

(‘ABS’), and the scores from the left (‘Left’) and right (‘Right’) flipper separately. These models were fitted under each configuration of the phalangeal scoring approach (combined scores for the second phalanges of digits II and III using averaged, maximum, or minimum values, or treating proximal and distal epiphyseal scores as individual values), yielding a set of alternative predictive models for each sex.

To assess robustness and practical equivalence across all modelling choices, pairwise comparisons were conducted among all resulting models. For each model pair, absolute differences between cross-validated age predictions ($|\text{model A} - \text{model B}|$) were calculated. Agreement between models was summarised using the mean absolute difference and Spearman’s rank correlation coefficient (ρ). Because the distributions of paired differences consistently deviated from normality, no parametric tests were applied. Practical equivalence was evaluated using a nonparametric bootstrap procedure (10,000 resamples) to estimate 90% confidence intervals for the mean absolute difference. Models were considered equivalent when the upper bound of this interval was ≤ 0.5 years. This threshold reflects the typical resolution of dental ageing, which generally cannot reliably distinguish differences smaller than approximately 0.5 years, except in very young individuals where a resolution of ~ 0.25 years may occasionally be achievable.

ii) Canonical analysis of principal coordinates

Canonical analysis of principal coordinates (CAP; Anderson & Robinson, 2003; Anderson & Willis, 2003) was used to assess whether variation in bone ossification scores across multiple skeletal locations could be used collectively to predict dental age. This approach was chosen because CAP can be used directly to measure and test the extent to which multivariate structure defined by a chosen resemblance measure (in this case, Euclidean distances based on the radiographic bone measurements) can be used to predict positions along a hypothesised variable (in this case, dental age). The canonical model also allows projection of new individuals into the canonical space to predict their positions along the age variable axis. Here, this analysis was applied to determine whether variation in skeletal maturation, captured by 16 (or 18) radiographic bone measurements, can be used to model dental age, and to obtain age predictions for individuals lacking direct age estimates.

All analyses were conducted in PRIMER version 7 (Clarke & Gorley, 2015) with the PERMANOVA+ add-on (Anderson et al., 2008) and were performed separately for females

and males. A resemblance matrix was constructed from ossification scores (16 or 18 variables, depending on the scoring approach), with Euclidean distances calculated on untransformed data. Canonical analysis was done on the distance matrix (in each case) to predict dental age. The analysis finds a canonical axis (CAP1) that is a linear combination of principal coordinates (PCO axes, calculated from the distance matrix; Gower, 1966) that achieves a maximum correlation and best discriminates positions along the dental age variable axis. The resulting canonical correlation (δ) captures and quantifies the strength of association between multivariate skeletal development and age.

Not all PCO axes are included in the analysis, but to avoid over-parameterization of the CAP model, a subset of m PCO axes is used. The value of m is chosen so as to minimize the leave-one-out residual sum of squares of the resulting CAP model (Anderson et al., 2008). Based on this assessment, $m = 9$ was selected for the final models for both females and males; this value captured the majority of age-related structure while avoiding unnecessary model complexity.

Model strength and significance were evaluated using squared canonical correlations (δ^2) for the chosen value of m , and permutation tests with 9,999 randomisations (Anderson & Robinson, 2003; Anderson & Willis, 2003). A plot of dental age vs the CAP axis (drawn separately for males and females) was used to visualise the CAP model. Finally, individuals without known ages were projected onto the CAP axis to obtain their predicted ages, enabling evaluation of their placement along the skeletal maturation trajectory defined by the training dataset.

To evaluate prediction accuracy, CAP-based age estimates were required for all individuals with known dental ages. Leave-one-out cross-validated (LOOCV) age estimates were extracted directly from the CAP analyses and compared with known dental ages to quantify predictive accuracy. Model performance was summarised using the mean, median (MAE), and standard deviation of absolute errors, together with Spearman's rank correlation coefficient (ρ) between dental and predicted bone ages. In addition, linear regression of predicted age against dental age was used to estimate the slope, intercept, and coefficient of determination (R^2). Consistent with the polynomial regression analysis, results are presented for both the full dataset and a subset excluding individuals with absolute prediction errors >6 years in either the polynomial regression or the CAP model, to evaluate robustness to extreme prediction discrepancies.

3. Results

3.1. Bone age modelling

3.1.1. Polynomial regression

All polynomial regression models showed highly comparable performance across both sexes, with only negligible differences in error metrics and predictive strength across alternative scoring strategies and flipper representations (Appendix 4, Tables A4.1 and A4.2). Furthermore, comparisons of age predictions among flipper representations ('BSL', 'BSH', 'ABS', 'Left', 'Right') revealed an exceptionally high degree of agreement across all scoring approaches. For females, pairwise comparisons among the 20 models (190 model pairs) showed consistently small differences in predicted ages. Mean absolute errors ranged from 0.02 - 0.13 years (median 0.06 years), and Spearman rank correlations were uniformly high (0.9990 - 1). The upper bounds of the bootstrap 90% confidence intervals for mean absolute error ranged from 0.02 - 0.17 years, well below the 0.5-year practical-equivalence threshold. For males, mean absolute errors ranged from 0.01 - 0.13 years (median 0.06 years), with likewise high Spearman's rank correlations (0.9976 - 0.9999). The upper bounds of the 90% confidence intervals ranged from 0.02 - 0.18 years, with all model comparisons staying within the 0.5-year threshold. Our results show near-complete consistency in the relative ranking of individuals across models and sexes. All models yielded highly similar age-prediction values, indicating that neither the scoring approach of the second phalanges of digit II and III, nor the occurrence of bilateral differences in bone maturation affected predictive outcomes meaningfully. For subsequent analyses, results are reported for the right flipper using the maximum combined score for the second phalanges of digits II and III. Outputs from all alternative scoring approaches and flipper representations are provided in Appendix 4 (Tables A4.1 to A4.3).

For females ($n = 64$), the model produced a second-degree polynomial. Age predictions achieved a mean absolute error of 2.03 years, with a median absolute error of 1.36 years and a standard deviation of 2.27 years (Table 4.3). Predicted ages were strongly correlated with dental ages (Spearman's $\rho = 0.91$). Linear regression yielded a slope of 0.82 and an intercept of 1.51 (Figure 4.1A), with dental age explaining approximately 80% of the variance in predicted age ($R^2 = 0.80$). Three individuals exhibited absolute prediction errors >6 years, with a maximum absolute error of 12.77 years. The largest discrepancy occurred in the oldest female (W08-17Dd; 29 years), who appeared radiographically younger relative to her dental age (predicted

age: 16.23 years), likely reflecting minimal observable age-related degeneration. In contrast, the other two individuals (absolute errors of 6.28 and 10.26 years) appeared radiographically more mature than expected for their dental age, suggesting advanced skeletal development relative to chronological age. To assess the influence of these pronounced discrepancies on overall model performance, the female model was re-run excluding these individuals (ID codes: W08-17Dd, KS19-17Dd, KS19-20Dd), as well as one additional outlier flagged by the CAP model (KS12-13Dd), resulting in a reduced sample size of 60 females (maximum age 20 years). Under this analysis, mean absolute error decreased to 1.56 years and median absolute error to 1.25 years. The standard deviation of absolute errors was 1.33 years, and the maximum absolute error decreased to 5.99 years. The correlation between predicted and observed ages increased ($\rho = 0.93$), with an improved regression slope of 0.91, intercept of 0.74, and $R^2 = 0.90$ (Figure 4.1B).

For males ($n = 56$), the model produced a second-degree polynomial. Predictions achieved a mean absolute error of 2.13 years, with a median of 1.40 years and a standard deviation of 2.21 years. Predicted ages were very strongly associated with dental age (Spearman's $\rho = 0.94$). Linear regression yielded a slope of 0.83 and an intercept of 1.38 (Figure 4.1C), with dental age explaining about 82% of the variance in predicted age ($R^2 = 0.82$). Three males exhibited absolute prediction errors >6 years, with a maximum absolute error of 10.95 years observed in the oldest male (KS23-45Dd; 31 years), who appeared radiographically younger relative to his dental age (predicted age: 20.05 years), likely reflecting limited observable age-related degeneration. The remaining two males showed comparatively advanced skeletal maturation relative to their dental age, with absolute prediction errors of 6.48 and 7.02 years. To assess the influence of these pronounced discrepancies on overall model performance, the model was re-run excluding these individuals (ID codes: KS09-13Dd, KS23-45Dd, KS23-50Dd), as well as one additional animal flagged by the CAP analysis (KS15-16Dd), reducing the sample size to 52 males (maximum age 24 years). This sensitivity analysis resulted in a second-degree polynomial (malesWO) with a mean absolute error of 1.71 years, a median absolute error of 1.08 years, and a standard deviation of absolute errors of 1.52 years. The maximum absolute error decreased to 6.23 years (observed in the oldest remaining male, KS23-46Dd; 24 years). The correlation between predicted and observed ages remained high ($\rho = 0.95$), with a regression intercept of 0.91, slope of 0.87, and $R^2 = 0.86$ (Figure 4.1D). Error metrics are summarised in Table 4.3. The corresponding polynomial equations are presented below, where y denotes predicted age (years) and x the total bone score.

$$\begin{aligned}
y(\text{females}) &= -1.09958 + 0.0495122 x + 0.00142868 x^2 \\
y(\text{femalesWO}) &= 0.111499 - 0.0111793 x + 0.0020191 x^2 \\
y(\text{males}) &= 0.0735636 + 0.0316692 x + 0.00131418 x^2 \\
y(\text{malesWO}) &= -0.698231 + 0.081646 x + 0.000822096 x^2
\end{aligned}$$

Equations for the other model configurations are provided in the Supplementary Materials (Table A4.3). Age predictions for animals with unknown chronological ages, estimated using these formulas, are provided in Table 4.4.

Table 4.3: Error metrics for age predictions in common dolphins (*Delphinus delphis*) generated from sex-specific polynomial models (F = females, M = males). Models were fitted using the total bone score of the right flipper. For each sex, models were additionally recalculated following exclusion of individuals with an age prediction error >6 years in the full model. These outlier-removed versions are denoted as F-WO (females without outliers) and M-WO (males without outliers).

Error metrics	F	F-WO	M	M-WO
Mean Absolute Error (AE)	2.03	1.56	2.13	1.71
Median AE	1.36	1.25	1.40	1.08
<i>n</i> samples with AE >6 years	3	0	3	1
<i>n</i> samples with AE >10 years	2	0	1	0
Maximum AE	12.77	5.99	10.95	6.23

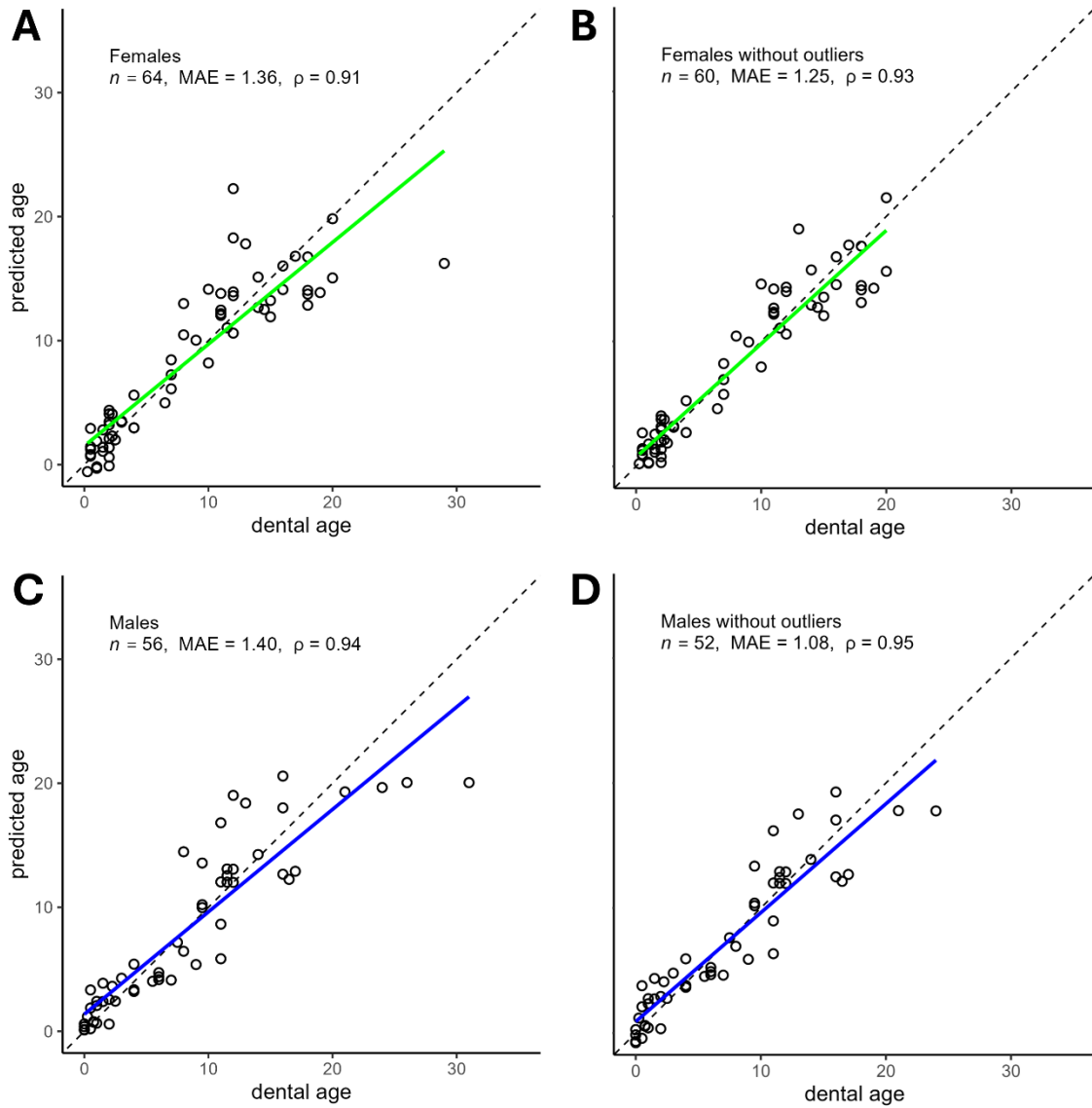


Figure 4.1: Relationship between dental age and predicted bone age in common dolphins (*Delphinus delphis*). Predicted ages were generated using second-degree polynomial regression of dental age on the total bone score of the right flipper, with leave-one-out cross-validation (LOOCV). **A)** Model based on the total bone score of the right flipper in females. **B)** Model based on the total bone score of the right flipper in females but excluding outliers with absolute age prediction errors >6 years. **C)** Model based on the total bone score of the right flipper in males. **D)** Model based on the total bone score of the right flipper in males but under exclusion of outliers with absolute age prediction errors >6 years. Each circle represents an individual. Solid-coloured lines show linear regressions between dental age and LOOCV-predicted age, while dashed lines represent the 1:1 line. Reported are sample size (n), median absolute error (MAE), and the Spearman rank correlation coefficient (ρ).

3.1.2. Canonical analysis of principal coordinates (CAP)

Canonical analysis of principal coordinates (CAP) revealed a strong association between bone ossification scores and dental age. Results are presented for the right pectoral flipper, using the maximum scores for the proximal and distal epiphyses of the second phalanges of digits II and III.

Using the full dataset, the first canonical axis (CAP1) showed a canonical correlation of $\delta = 0.93$ in both sexes, corresponding to a squared canonical correlation (δ_1^2) of 0.87, indicating that CAP1, drawn from the multivariate skeletal maturation patterns, explained approximately 87% of the variation in dental age (Figure 4.2A&B).

CAP-derived fitted values for individuals of known ages showed a mean absolute error of 2.23 years in females (median = 1.79 years; SD = 1.94 years), with a Spearman correlation of $\rho = 0.90$. Linear regression yielded a slope of 0.85, an intercept of 1.27, and an R^2 of 0.81. Two females (W08-17Dd, KS12-13Dd) exhibited age prediction errors >6 years. The maximum error was 10.84 years in females. Following exclusion of individuals with age prediction errors >6 years (as identified by either the polynomial regression or CAP model), model performance improved substantially. For females, the canonical correlation increased to 0.95, corresponding to a squared canonical correlation (δ_1^2) of 0.90 (Figure 4.2C). The mean absolute error between dental and predicted age was 1.90 years (SD = 1.60), with a median absolute error of 1.35 years. Spearman's rank correlation was $\rho = 0.90$. Linear regression of predicted age on dental age yielded a slope of 0.89, an intercept of 0.86, and $R^2 = 0.85$.

In males, the full dataset yielded a mean absolute error of 2.51 years (median = 1.85 years; SD = 2.71), with a Spearman correlation of $\rho = 0.93$. Linear regression of predicted age on dental age produced a slope of 0.76, an intercept of 1.64, and $R^2 = 0.74$. Age prediction error exceeded 6 years in four males (KS09-13Dd, KS15-16Dd, KS23-45Dd, KS23-50Dd), with a maximum error of 13.43 years. Following exclusion of these individuals, the canonical correlation increased to 0.95 ($\delta_1^2 = 0.90$; Figure 4.2D). The mean absolute error decreased to 1.90 years (SD = 1.55), with a median absolute error of 1.80 years. Spearman's ρ was 0.94, and linear regression yielded a slope of 0.88, an intercept of 0.82, and $R^2 = 0.84$. Error metrics are summarised in Table 4.5.

Finally, the CAP model was applied to individuals of unknown dental ages (Table 4.4). Foetal and neonatal specimens plotted below the neonatal range, and linear back-prediction from CAP1 produced negative age values.

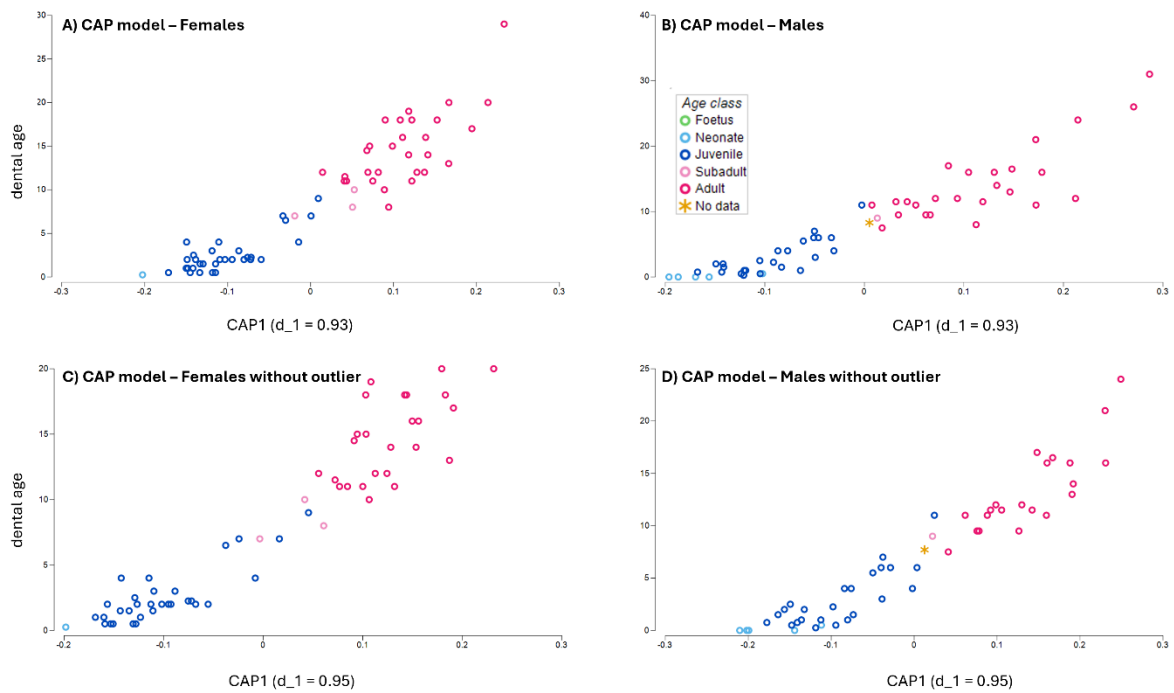


Figure 4.2: Canonical Analysis of Principal Coordinates (CAP) based on Euclidean distances calculated from 16 pectoral-flipper ossification variables of individual common dolphins (*Delphinus delphis*), showing their multivariate relationship with dental age (in years). Panels show CAP models for **A)** females and **B)** males fitted to the full calibration dataset, and **C)** females and **D)** males fitted after exclusion of the identified age outliers. In each panel, points represent individuals, coloured according to their assigned age class, as indicated (foetus, neonate, juvenile, subadult, adult, and unknown). CAP1 represents the first canonical axis; d_1 denotes the canonical correlation between CAP1 and dental age.

Table 4.4: Estimated ages for common dolphins (*Delphinus delphis*) with unknown or unavailable dental ages, derived using two independent approaches. Ages were predicted using (i) a sex-specific second-degree polynomial regression model based on total pectoral-flipper ossification scores of the right pectoral flipper, and (ii) a Canonical Analysis of Principal Coordinates (CAP) model. Individuals included foetuses, neonates, juveniles, subadults, and adults for which no validated dental ages (GLGs) were available. Both models were fitted on the full dataset. Negative age estimates reflect extrapolation at very early developmental stages where epiphyseal fusion scores fall outside the calibration range.

ID	Age class	Polynomial age	CAP age
WS02-44Dd	ND	0.07	-1.73
WS07-01DdA	Foetus	-0.58	-1.20
KS10-78Dd	Subadult	8.95	8.49
KS11-50DdA	Foetus	-1.10	-2.53
KS13-02Dd	Neonate	0.24	-1.54
KS14-62Dd	Neonate	0.06	-2.85
KS19-23Dd	Adult	20.91	19.98
KS21-01DdA	Foetus	0.07	-1.73
KS22-03DdA	Foetus	-1.10	-2.53
KS22-73Dd	Foetus	0.77	0.28
KS22-77Dd	Neonate	0.44	-2.07
KS24-68Dd	Neonate	-0.26	-1.00
KS24-88Dd	Juvenile	3.59	3.40
KS24-96Dd	Neonate	0.07	-1.73
KS25-11Dd	Neonate	0.39	-2.23
KS25-79Dd	Adult	14.39	14.02
KS25-80Dd	Adult	11.45	10.64

Table 4.5: Error metrics for age predictions in common dolphins (*Delphinus delphis*) generated using sex-specific CAP models (F = females, M = males). Models were fitted using the total bone score of the right flipper. For each sex, models were additionally recalculated following exclusion of individuals with an age prediction error >6 years in the full model. These outlier-removed versions are denoted as F-WO (females without outliers) and M-WO (males without outliers).

Error metrics	F	F-WO	M	M-WO
Mean Absolute Error (AE)	2.23	1.90	2.51	1.90
Median AE	1.79	1.35	1.85	1.80
<i>n</i> samples with AE >6 years	2	1	4	1
<i>n</i> samples with AE >10 years	1	0	2	0
Maximum AE	10.84	8.20	13.43	7.60

4. Discussion

4.1. Advantages of radiographic ageing

This study represents the first radiographic ageing method for common dolphins. Skeletal development of the pectoral flipper can be used to accurately estimate chronological age in this species. Specifically, the strong agreement between radiographically predicted ages and dental ages indicates that bone ageing provides a robust and reliable approach to age estimation in common dolphins. Indeed, both polynomial regression (females: MAE = 1.25, ρ = 0.93, R^2 = 0.90; males: MAE = 1.08, ρ = 0.95, R^2 = 0.86) and CAP models (females: MAE = 1.35, Spearman's ρ = 0.90, R^2 = 0.85; males: MAE = 1.80, ρ = 0.94, R^2 = 0.84) demonstrated comparable or improved predictive performance relative to a recently developed epigenetic clock derived from the same population (MAE = 1.80, r = 0.91, R^2 = 0.82; Hanninger et al., 2025). Furthermore, reliable age estimates up to approximately 20 years of age for both sexes were achieved with bone ageing, representing a substantial advance by comparison with epigenetic approaches, which demonstrated increasing prediction error for animals from ~16 years of age within the same population (Hanninger et al., 2025).

We acknowledge that bone-based models were fitted separately for females and males, whereas the epigenetic clock was developed using a pooled-sex framework. This structural difference should be considered when interpreting comparative performance. However, empirical evidence suggests that sex-specific stratification of DNA methylation clocks does not generally result in meaningful improvements in predictive accuracy (Carlsen et al., 2023; Sala et al., 2024). To address this structural difference, additional harmonised analyses were conducted. The epigenetic clock published by Hanninger et al. (2025) was refitted independently for females and males, and a pooled polynomial regression model combining both sexes was fitted for the skeletal ageing approach (Appendix 4, Text A4.1–A4.3, Figures A4.1 & A4.2). These analyses did not alter the comparative outcome: the skeletal ageing models continued to demonstrate lower absolute prediction error and stronger correlation than the methylation-based model. The superior performance of the radiographic bone ageing method, therefore, cannot be attributed solely to differences in sex stratification between modelling frameworks.

Instead, these differences might reflect the fact that these two methods extract fundamentally different types of information associated with the biology of an ageing organism. Radiographic models capture skeletal growth and maturation, which follow a relatively predictable developmental trajectory closely aligned with chronological age. In contrast, epigenetic clocks quantify age-related molecular changes that reflect biological age and are more sensitive to individual condition, health status, and life history (Barratclough et al., 2024). Although both skeletal development and epigenetic ageing can be influenced by environmental and physiological factors (e.g., Colich et al., 2020; Fitzgerald et al., 2021; Liu et al., 2021; Shirazi et al., 2020; Weindruch et al., 2001), epigenetic age is inherently more sensitive to cumulative stressors and variation in life experience, which can reduce concordance with chronological age (Jylhävä et al., 2017; M. E. Levine et al., 2018).

In addition to its demonstrated reliability, radiographic bone ageing offers substantial practical advantages for age estimation in common dolphins. Traditional tooth-based ageing requires tooth extraction followed by laboratory processing involving significant expertise, procedures that are time-consuming, costly, and not always feasible in field-based or resource-limited settings (Betty et al., 2023; Evans et al., 2002; Lockyer, 1993, 1995; Murphy et al., 2014; Palmer et al., 2022, 2023; Westgate & Read, 2007). Moreover, interpretation of growth layer groups becomes increasingly challenging in older individuals, where tooth wear and the accumulation of accessory lines reduce clarity and reliability (Barratclough et al., 2023).

Radiographic ageing avoids these constraints and has been shown to provide highly reliable age estimates in bottlenose dolphins, with improved performance relative to GLG-based assessments, particularly at older ages (Barratclough et al., 2023).

Radiographic bone ageing also offers practical advantages when considered alongside molecular age-estimation approaches. While epigenetic ageing enables minimally invasive age estimation in live animals, it depends on specialised and in some cases patented, molecular methods, resulting in substantial per-sample costs (e.g., Hanninger et al., 2025; Hernandez et al., 2023; Peters et al., 2023; Robeck et al., 2021; Zoller et al., 2025). By contrast, radiographs are relatively inexpensive, widely accessible through mainstream human medical facilities, and can be collected routinely in many post-mortem workflows. While on-site imaging during stranding events may not always be feasible, in cases where it is, digital radiographs can be examined in real time, providing immediate age estimates that are highly valuable during live health assessments. Together, these attributes support radiographic ageing as a powerful and cost-effective approach within the broader suite of current age-estimation methods.

4.2. Performance of the modelling approaches

To translate radiographic data into age estimates, we applied two modelling approaches. The first was a polynomial regression, adapted from the bottlenose dolphin framework (Barratclough, Sanz-Requena, et al., 2019). This approach showed a high level of stability, even though common dolphins differ anatomically from bottlenose dolphins in ways that could have influenced scoring, including a broader range of hyperphalangy (Cooper et al., 2007) and known lateralised differences in bone maturation (Hanninger, Barratclough, Betty, Dittmer, et al., 2026). We evaluated whether alternative assessments of the second phalanx of digits II and III, as well as different configurations for combining scores from both flippers, would influence predicted ages. Across all of these variations, the predicted ages remained essentially unchanged. Taken together, these findings indicate that the polynomial approach is robust to minor scoring variation, supporting the use of unilateral radiographs in live animals, where minimising handling time and radiation exposure is a priority. Accordingly, we presented results for the right flipper only using the maximum combined score for the second phalanges of digits II and III, consistent with the previously published bottlenose dolphin framework (Barratclough, Sanz-Requena, et al., 2019). The second modelling approach, CAP (Anderson & Robinson, 2003; Anderson & Willis, 2003), provided a multivariate view of skeletal

maturation capturing coordinated developmental patterns. Both approaches captured age-related variation in flipper development and demonstrated high and broadly comparable predictive performance. Together, these results demonstrate that both univariate (polynomial regression) and multivariate (CAP) approaches can be successfully used to estimate age from bone maturation in common dolphins.

The marginal differences in predictive performance between polynomial regression and CAP may be explained by differences in how the two approaches incorporate bone score information. Radiographic bone scoring showed good interobserver consistency in this study (Hanninger, Barratclough, Betty, Dittmer, et al., 2026), although natural morphological variation can lead to small differences in interpretation, particularly in transitional stages where ossification has not yet fully progressed into a defined maturation category. Polynomial regression aggregates information into a single total bone score by summing across all assessed sites, thereby reducing the influence of minor discrepancies at individual bones and limiting the impact of small scoring differences. In contrast, CAP treats each bone score as an independent variable within a multivariate framework, meaning that small inconsistencies at individual sites may introduce additional noise into the ordination structure. In addition, we used a linear CAP model, whereas the polynomial regression (including a squared term) clearly permits some non-linearity. Thus, this multivariate approach, capturing the holistic shapes in skeletal features across multiple measurements, may well be further improved by the use of a suitable nonlinear CAP model (e.g., Anderson et al., 2005; Millar et al., 2005).

For both the polynomial regression and CAP models, linear regression of predicted age against dental age yielded slopes <1 (polynomial regression: full dataset: females: 0.82, males: 0.83; without outliers: females: 0.91, males: 0.87; CAP: full dataset: females: 0.85, males: 0.76; without outliers: females: 0.89, males: 0.88), indicating compression of the predicted age range and reduced accuracy at older ages. Intercepts were positive across all models. For the polynomial regression, intercepts were 1.51 and 1.38 years in the full dataset and decreased to 0.74 and 0.91 years after outlier exclusion in females and males, respectively. For the CAP models, intercepts were 1.27 years in females and 1.64 years in males for the full dataset. Following exclusion of individuals with absolute prediction errors >6 years, intercepts decreased to 0.86 years in females and 0.82 years in males. In both modelling approaches, removal of extreme prediction discrepancies resulted in slopes shifting closer to 1 and intercepts closer to 0, indicating reduced systematic bias in the linear fit.

Patterns observed at older ages are a direct consequence of this regression behaviour interacting with species-specific skeletal ageing processes. Slopes below 1 result in age-range compression, such that predicted age increases more slowly than true age, leading to age underestimation. In the present study, this pattern appears to be driven by two factors. First, the sample contained relatively few animals above the age of attainment of physical maturity (females: $n = 8$; males: $n = 4$; ≥ 18 years for females and ≥ 20 years for males; Palmer, 2023). As the study relied entirely on opportunistic strandings, the limited number of confirmed older individuals was likely insufficient to offset regression-to-the-mean effects (Campbell & Kenny, 1999), reducing accuracy at the upper end of the age range.

Second, once physical maturity is reached (≥ 18 years for females, ≥ 20 years for males; Palmer, 2023), ongoing skeletal growth no longer provides a strong age-informative signal, and age estimation increasingly relies on age-related degenerative changes. These degenerative skeletal modifications appeared to be far more subtle in common dolphins than in bottlenose dolphins (Hanninger, Barratclough, Betty, Dittmer, et al., 2026). This may partly reflect differences in the mobility of the animals examined, given that the present study analysed free-ranging common dolphins, whereas the bottlenose dolphin study examined individuals under human care (Barratclough, Sanz-Requena, et al., 2019). Differences observed may also reflect species-specific life-history traits, including longer life expectancy in bottlenose dolphins and differences in the timing of sexual maturity relative to maximum lifespan (Hanninger, Barratclough, Betty, Dittmer, et al., 2026), which influence growth plate closure. Consequently, while the models of ageing examined here performed robustly across early and mid-life stages, age estimates likely become progressively more variable in geriatric individuals. Nevertheless, our models provided reliable age estimates for both sexes, with substantial age underestimation occurring only in animals older than 20 years.

4.3. Radiographic age estimation in foetuses and neonates

Most individuals lacking dental ages in our study were foetal or neonatal specimens with unerupted teeth, precluding tooth-based age determination. As foetal specimens occupy maturation states below those observed in postnatal individuals, both models assigned negative age estimates to foetuses, reflecting their position along a maturation gradient calibrated using postnatal individuals.

Both modelling approaches showed limited ability to distinguish late-term foetuses from very young neonates prior to tooth eruption. The polynomial regression correctly classified three of five foetuses as negatively aged, whereas the CAP model classified four of five foetuses as negatively aged. For neonates, one of six individuals was incorrectly classified as negatively aged by the polynomial regression, and six were classified as negatively aged by the CAP model. When extrapolated beyond the calibrated postnatal age range, both modelling frameworks position these individuals along a similar segment of the maturation gradient, limiting their ability to reliably discriminate between foetal and neonatal stages. Importantly, not all derived polynomial equations can yield negative predicted ages; some formulas remain strictly non-negative across the observed range of bone scores and therefore support only postnatal age estimation.

Beyond statistical reasons for the lack of discrimination, radiographic assessment revealed substantial overlap in skeletal maturation between foetuses and neonates, which contrasts with findings from bottlenose dolphins, where foetuses and neonates could be distinguished radiographically (Barratclough, Sanz-Requena, et al., 2019). However, such discrimination in *Tursiops* may have been driven by the inclusion of very early-stage foetuses (aborted approximately five to six months prior to parturition), whose skeletal development lay well below the neonatal range. In contrast, the present study assessed common dolphin foetuses predominantly with total body lengths above 90 cm, within the reported range of birth lengths for this species (82–101 cm) and close to the estimated mean length at birth (98.1 cm; Palmer, 2023). Consequently, skeletal development in late-term foetuses likely overlaps substantially with that of neonates, reducing the capacity to distinguish these categories radiographically.

Importantly, variation in ossification may not solely reflect gestational stage but may also arise from inter-individual differences in growth trajectories. Notably, the bottlenose dolphin study was based on animals in human care (Barratclough, Sanz-Requena, et al., 2019), where maternal condition, nutrition, and overall health are monitored and likely more consistent. In free-ranging common dolphins, a broader range of maternal factors may influence skeletal development in both foetuses and neonates, potentially accelerating or delaying bone maturation and increasing overlap in radiographic appearance. Such factors include maternal nutrition (Lewall & Cowan, 1963; Ratib et al., 2012; Thacher et al., 2000), underlying disease (e.g., *Brucella ceti* infection; Roca-Monge et al., 2022), and exposure to endocrine-disrupting environmental contaminants (Schwacke et al., 2012) known to adversely affect bone growth

(Brankovič et al., 2020; Shulhai et al., 2024; Yaglova & Yaglov, 2021). These environmental and health-related influences may therefore have contributed to the lack of differentiation observed in radiographic age estimates at the earliest life stages. Future research could aim to investigate how late into foetal development foetuses and neonates can be reliably distinguished.

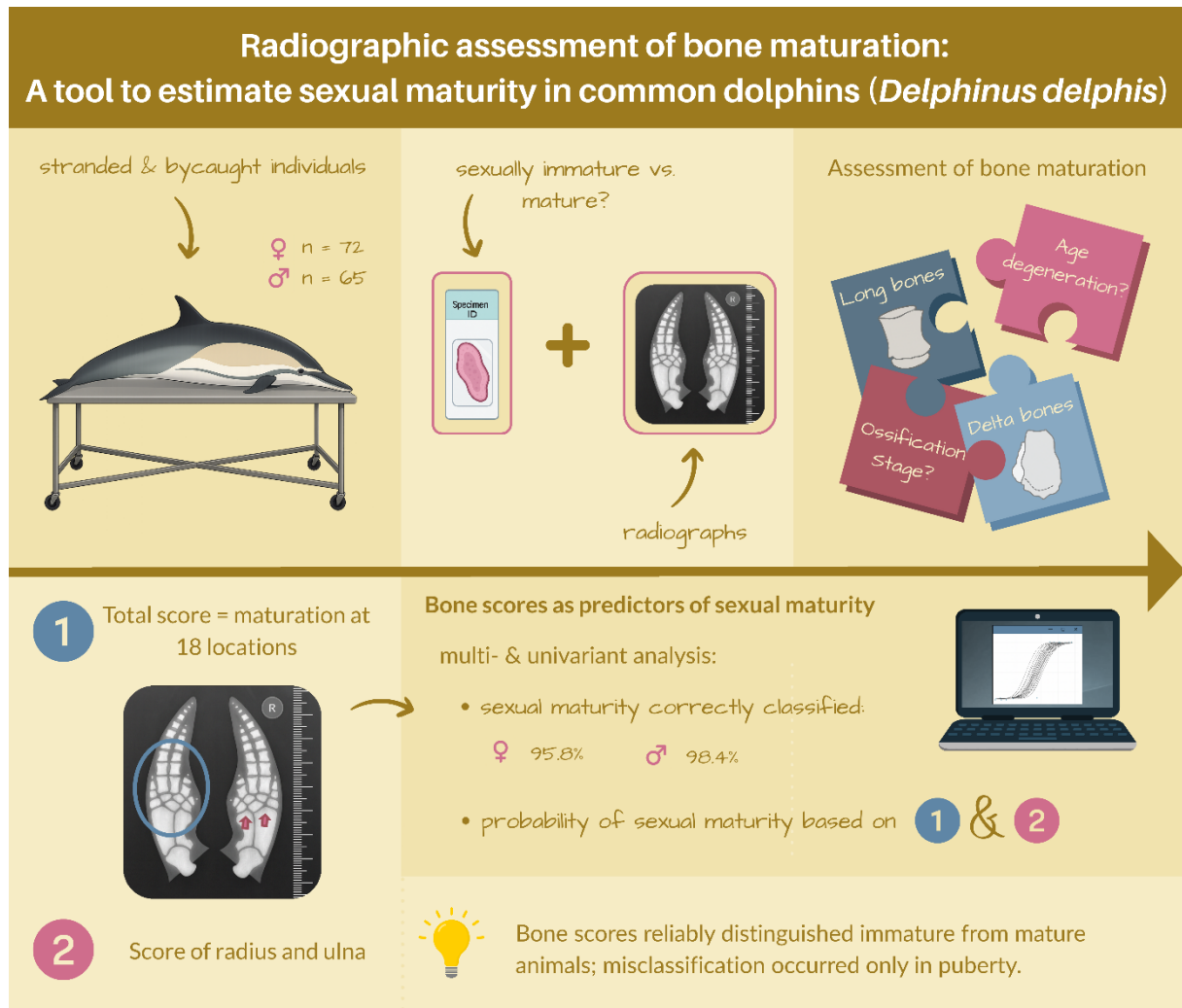
4.4. Conclusions

Radiographic scoring of the pectoral flipper is a reliable method for estimating age in common dolphins. This approach is quick, practical, accessible, and provides a comparatively low-cost alternative to tooth-based or molecular ageing methods. The ability to apply this method retrospectively to museums and archives is an additional advantage. Models developed here yielded reliable age estimates for individuals up to approximately 20 years of age in both sexes, with systematic underestimation occurring primarily in individuals older than 20 years. This represents a substantial advance, particularly given that epigenetic ageing approaches in this species show increasing prediction error from approximately 16 years of age onward. Together, these findings underscore the enduring value of morphological and developmental assessments for age estimation and demonstrate that radiographic assessment is a robust and informative tool for life-history research in common dolphins.

At present, this study represents only the second radiographic age-estimation framework developed for cetaceans and the first to explore epigenetic aging of the same individuals comparatively. Our findings highlight the broader relevance of radiographic ageing approaches for species in which other ageing methods are limited or unavailable and emphasise the importance of long-term, multi-tissue archives for advancing life-history research across cetaceans.

Chapter 5

Radiographic assessment of bone maturation: A tool to estimate sexual maturity in common dolphins (*Delphinus delphis*)?



This chapter is a reformatted version of the following manuscript:

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We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.			
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Name and title of main supervisor:	Karen Stockin		
In which chapter is the manuscript/published work?	Chapter 5		
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Abstract

Accurate inference of sexual maturity is fundamental to interpreting marine mammal life histories and population structure. Here, we evaluate skeletal metrics in the pectoral flipper of common dolphins (*Delphinus delphis*) as indicators of attainment of sexual maturity using Bayesian logistic regression models with a hierarchical ordinal (HOF-style) parameterisation and canonical analysis of principal coordinates (CAP). HOF models were used to relate sexual maturity to skeletal ossification, generating probabilistic response curves based on (i) a total bone score defined as the sum of discrete morphological maturation stages (scored from -1 to 8) across 16 flipper bones, and (ii) the minimum maturation stage (-1 to 8) reached by either the distal radius or ulna. For total bone scores, the predicted midpoint of sexual maturity (probability = 0.5) occurred at an ossification score of 74.85 points in females (95% credible interval (CI): 72.50–77.13) and 68.35 points in males (95% CI: 63.47–73.12), with a steeper transition in females. For the minimum radius–ulna score, midpoints were similar between sexes, at 4.90 in females (95% CI: 4.51–5.27) and 4.75 in males (95% CI: 4.32–5.16). CAP was applied as a complementary multivariate constrained ordination method to distinguish immature and mature individuals based on combined ossification patterns and to quantify leave-one-out classification performance. For the whole dataset, CAP demonstrated strong discrimination between maturity states (squared canonical correlation = 0.84), with 95.8% of females and 98.4% of males correctly classified and misclassification restricted to individuals near the maturity boundary. Together, these results demonstrate that radiographic skeletal ossification provides a robust proxy for sexual maturity in common dolphins in New Zealand.

1. Introduction

The attainment of sexual maturity is an influential life-history parameter for assessing population viability, as it can determine when individuals first contribute to reproduction and thus constrains lifetime reproductive output. Together with other life-history traits such as gestation length, lactation duration, pregnancy rates, calving intervals, life expectancy and age-specific survival rates, the timing of sexual maturity strongly shapes population growth trajectories (Betty et al., 2019, 2023; Botta et al., 2010; Evans & Hindell, 2004; Palmer et al., 2022, 2023; Rossi et al., 2017). Because the timing of sexual maturity strongly influences reproductive output and population growth, establishing a robust baseline estimate is essential for detecting shifts in maturity timing. Deviations from this baseline — towards earlier or later

maturation — may occur in response to environmental conditions, population density, or anthropogenic pressures, reflecting plasticity in life-history strategies (Clutton-Brock & Sheldon, 2010; Kemper et al., 2014; Murphy, 2004; Palmer et al., 2022, 2023). Accurate estimates of this parameter are, therefore, central to conservation and management, underpinning population models, informing assessments of reproductive potential, and improving predictions of population responses to environmental variability and anthropogenic pressures (Betty et al., 2023; Heydenrych et al., 2021; Manlik et al., 2022; Moore & Read, 2008).

Despite its importance, classification of sexual maturity in cetaceans remains challenging, particularly in free-ranging individuals, where direct behavioural or physiological observations are rarely feasible (Lanyon & Burgess, 2014). Consequently, post-mortem gonadal assessment remains the most widely applied method for classifying sexual maturity. In females, sexual maturity is typically categorised into binary classes of immature or mature, based on the gross morphological presence of *corpora lutea*, *corpora albicantia*, pregnancy, or lactation (Danil & Chivers, 2007; Grandi et al., 2022; Kemper et al., 2019; Palmer et al., 2022; Westgate & Read, 2007). In contrast, histopathological examination of the testes in males allows finer resolution of reproductive development, enabling the identification of a pubescent transitional state in addition to immature and mature categories (Betty et al., 2019; Murphy et al., 2005; Palmer et al., 2023). Application of this approach has enabled the description of key life-history parameters across multiple cetacean species, including estimates of mean age and body length at attainment of sexual maturity (e.g., Danil & Chivers, 2007; Grandi et al., 2022; Kemper et al., 2014; Palmer et al., 2022; Westgate & Read, 2007). However, post-mortem assessment is restricted to deceased individuals and requires access to specialised post-mortem facilities and histopathological laboratories (e.g., Betty et al., 2019, 2023; Palmer et al., 2022, 2023; Westgate & Read, 2007), making it unsuitable for many free-ranging populations and longitudinal studies.

While alternatives applicable to live animals exist, these methods also present practical constraints. Endocrinological approaches that rely on hormone measurements from remotely collected samples (e.g., blubber biopsies) require specialised infrastructure and extensive sample processing. This can limit their feasibility for routine or large-scale application (Burgess, Lanyon, Brown, et al., 2012; Burgess, Lanyon, & Keeley, 2012; Lanyon & Burgess, 2014). Ultrasonographic assessment of reproductive organs is also possible; however, it

requires hands-on physical examination and restraint and is therefore impractical for most free-ranging cetacean species (Lanyon & Burgess, 2014; Richard et al., 2017).

In this context, pectoral flipper radiography represents a complementary approach that can be applied in post-mortem or live-stranding contexts, where radiographs can be obtained during brief handling, but histological assessment is unavailable. Pectoral flipper radiography has been established as a reliable method for age estimation in cetaceans (Barratclough, Sanz-Requena, et al., 2019; Hanninger, Barratclough, Betty, Palmer, et al., 2026) and has further been proposed as an indicator of sexual maturity (De Carvalho et al., 2015; Hui, 1977; K. E. Lee, 1978; Ogden et al., 1981; Stockin et al., 2008). Skeletal maturation reflects hormonally mediated developmental processes, with increased circulating glucocorticoids driving ossification (Almeida et al., 2017; Iuliano-Burns et al., 2009; Nilsson et al., 2005). As a result, radiographic ossification can provide biologically informative maturity proxies using standardised imaging frameworks.

Two radiographic approaches have been proposed to infer sexual maturity. A holistic assessment of multiple flipper bones was suggested as a tool to predict sexual maturity in male common dolphins (*Delphinus delphis*; Hui, 1977), based on correlations between overall radiographic flipper maturation and testis weight. In females, Hui (1977) did not explicitly assess the onset of sexual maturity but instead examined whether increasing radiographic bone maturation was associated with increasing numbers of ovarian scars following attainment of sexual maturity; no clear relationship was identified. This likely reflects the fact that the number of *corpora albicantia* and *corpora lutea* does not increase linearly with age after maturity (Palmer, 2023) and is strongly influenced by reproductive history, pregnancy, and sampling timing, limiting its utility as a comparator for progressive skeletal maturation.

Alternatively, sexual maturity has further been inferred from the degree of ossification in the distal radius and ulna, reflecting hormonally mediated bone maturation during puberty. Rising oestrogen and testosterone concentrations accelerate epiphyseal maturation and promote progressive physeal bridging and closure in growth plates, thus enabling the inference of sexual maturity (Almeida et al., 2017; Iuliano-Burns et al., 2009).

In short-finned pilot whales (*Globicephala macrorhynchus*), sexual maturity was not observed prior to the onset of trabecular osseous bridging between the metaphysis and secondary

ossification centres of the distal radius and ulna (Lee, 1978). Based on preliminary and unpublished observations, the same radiographic state (corresponding to a stage 4 in bone maturation within an ordinal numbered morphological staging scheme) was later suggested to reflect sexual maturity in Dall's porpoise (*Phocoenoides dalli*; Ogden et al., 1981). Based on these findings, this ossification state has subsequently been applied in the context of age class assessment in other cetacean species (Stockin et al., 2008). The use of this ossification state defines a minimum radiographic criterion for the onset of sexual maturity. However, sexual maturity is a gradual, transitional process, and the alignment between radius-ulna ossification and sexual maturity varies among species and between sexes rather than conforming to a universal radiographic threshold such as radius-ulna stage 4.

This is demonstrated for several delphinid species, in which forelimb ossification stages have been interpreted relative to independently established life-history estimates of sexual maturity. In estuarine dolphins (*Sotalia guianensis*), individuals that had not yet reached the expected age for sexual maturity nonetheless exhibited a radius-ulna bone score of 4 points (De Carvalho et al., 2015). In bottlenose dolphins (*Tursiops truncatus*), sexual maturity was suggested to occur across a transitional range of radius-ulna scores between 4 and 5, rather than occurring at a single discrete value (Barratclough, Sanz-Requena, et al., 2019). In striped dolphins (*Stenella coeruleoalba*), full distal radio-ulnar fusion (stage 5) was reached at 5–6 years of age in females and 8–9 years in males (Calzada & Aguilar, 1996), substantially earlier than the reported mean age at sexual maturity in females for this species (~12 years; Calzada et al., 1996). Although sexual maturity was not directly assessed in that study, this comparison indicates that complete radio-ulnar fusion can precede reproductive maturity in this species.

Despite the widespread use of radiographic indicators and life-history benchmarks, no clear modelling framework currently exists for cetaceans that explicitly estimates the probability of maturity as a function of skeletal development. Existing studies have largely relied on descriptive associations or fixed radiographic thresholds, rather than formal probabilistic models that link radiographic traits to maturity status (Lee, 1978; Ogden et al., 1981). As a result, the extent to which radiographic indicators reliably predict maturity, within and among species, has not been evaluated. This gap creates the need for species-specific, probabilistic maturity models that integrate radiographic data with independent maturity classifications.

In this study, sexual maturity was assessed post-mortem using gonadal examination (Palmer et al., 2022, 2023). For modelling purposes, maturity status was treated as a binary classification (0 = immature vs. 1 = mature) for both sexes. The pubescent transitional stage identifiable histologically in males was not modelled due to the limited number of pubescent individuals ($n = 2$). We used a 30-year tissue archive of stranded and bycaught individuals with known dental age, sex, body length, and sexual maturity status (Palmer et al., 2022, 2023), for which radiographic bone scores had been assessed previously (Hanninger, Barratclough, Betty, Dittmer, et al., 2026).

The aim of this study was to determine whether radiographic patterns of pectoral flipper ossification can provide a reliable and biologically meaningful indicator of sexual maturity in common dolphins, both in terms of estimating maturity probability and distinguishing immature from mature individuals.

The probability of sexual maturity was evaluated using hierarchical logistic regression (HOF) models. These were applied to two radiographic frameworks: a holistic assessment of pectoral flipper bone maturation based on radiographic scores from 16 anatomical locations, and a threshold-based framework using the minimum bone score attained by either the radius or ulna for classification as sexually mature, as suggested by previous studies (Lee, 1978; Ogden et al., 1981).

In parallel, we used a multivariate framework based on canonical analysis of principal coordinates (CAP; Anderson & Robinson, 2003; Anderson & Willis, 2003), retaining all individual bone scores to test for maturity-related structure in multivariate space and to quantify leave-one-out classification success between maturity groups.

2. Materials & methods

2.1. Sample collection

2.1.1. Life-history assessment

Routine post-mortem examinations were performed on 137 common dolphins recovered from stranding and bycatch events between 2002 and 2025. Dental age and sexual maturity status were assessed *a priori* until 2021 as part of independent life-history research (Palmer et al., 2022, 2023), with additional assessments extending the dataset to 2025.

Sex determination was based primarily on gross examination of reproductive organs. Of the 137 individuals, 72 were female and 65 were male. In two cases, the genital region was scavenged, preventing reliable assessment, so sex was determined genetically using skin tissue via multiplex PCR targeting sex chromosome markers ZFX and SRY, following established protocols (Aasen & Medrano, 1990; Gilson et al., 1998; Peters et al., 2023).

Sexual maturity was assessed by gross and histological examination of the reproductive organs. Females were classified as sexually immature ($n = 41$) or mature ($n = 31$) based on the presence of *corpora albicantia* and/or *corpora lutea*, or evidence of pregnancy or lactation (Palmer et al., 2022). Males were classified according to the presence and extent of spermatogenesis (immature: $n = 38$; mature: $n = 25$; unknown: $n = 2$; Palmer et al., 2023). The immature category included two pubescent males in which spermatogonia and spermatocytes were present, but no spermatids and spermatozoa. Due to the limited number of pubescent individuals ($n = 2$), this transitional stage was not included as an independent category in the modelling.

Our assessment included foetuses ($n = 5$), defined as individuals extracted in utero or aborted prior to first breath, as confirmed by pulmonary atelectasis (Colegrove et al., 2016); neonates ($n = 12$), for which live birth was confirmed by inflated lungs and the presence of at least one neonatal characteristic, including vibrissae hairs, patent navel, foetal folds, soft and folded dorsal fin and tail flukes, or unerupted teeth (Díaz López et al., 2018; Puig-Lozano et al., 2020); juveniles ($n = 56$), lacking neonatal characteristics and with total body length below the average length at attainment of sexual maturity for this population (<183 cm in females and <190 cm in males; Palmer et al., 2022, 2023); subadults ($n = 5$), defined as adult-sized individuals (≥ 183 cm in females and ≥ 190 cm in males) that had not yet attained sexual maturity (Palmer et al., 2022, 2023); and adults ($n = 57$), defined as sexually mature individuals. Two additional individuals for which age class could not be determined due to no teeth being available were also included.

2.1.2. Pectoral flipper collection and radiography

For each of the 137 dolphins, the pectoral flippers were surgically dissected at the scapulohumeral joint by severing all soft tissue attachments between the scapula and humerus (Calzada & Aguilar, 1996; Stockin et al., 2008; Watson et al., 2008) during routine post-mortem examinations conducted between 2002 and 2025. Pectoral flippers were stored flat-frozen until imaging in ventrodorsal projection on a Shimadzu radiographic system operated at 40 kVp and

4 mAs, with a source-to-sample distance of 100 cm (Hanninger, Barratclough, Betty, Dittmer, et al., 2026).

2.2. Radiographic analysis

Radiographs were evaluated *a priori* in a standardised proximal-to-distal sequence using the flipper-bone maturity framework initially developed for bottlenose dolphins (Barratclough, Sanz-Requena, et al., 2019) and adapted to account for morphological differences in common dolphins (Hanninger, Barratclough, Betty, Dittmer, et al., 2026). Sixteen bone sites (Chapter 3, Figure 3.1) were selected for assessment based on their demonstrated correlations with dental age in previous radiographic ageing studies (Barratclough, Sanz-Requena, et al., 2019; Hanninger, Barratclough, Betty, Dittmer, et al., 2026). These included selected epiphyses of the radius, ulna, metacarpals, and phalanges, as well as two delta bones (metacarpal V and the first phalanx of digit IV).

Long bones were assigned maturity stages from –1 to 8, reflecting progressive ossification and physal fusion, with half scores used to capture intermediate stages (Appendix 3, Figure A3.2). Most long bones were scored at both the proximal and distal epiphyses. However, consistent with prior radiographic frameworks (Barratclough, Sanz-Requena, et al., 2019; Hanninger, Barratclough, Betty, Dittmer, et al., 2026; Hanninger, Barratclough, Betty, Palmer, et al., 2026), a single score was used to represent the greater of the two epiphyseal values for the second phalanx of digit II and III. Delta bones were scored as single units on a –1 to 8 scale (Appendix 3, Figure A3.3), with classification adjusted to reflect species-specific ossification patterns in common dolphins.

Following assignment of maturity stages to each of the 16 assessed bone sites, a total bone maturity score was calculated for each radiograph as the sum of all individual site scores. Full scoring criteria are provided in Barratclough, Sanz-Requena, et al. (2019) and Hanninger, Barratclough, Betty, Dittmer, et al. (2026), with schematic illustrations presented in Appendix 3 (Figures A3.2 and A3.3). The scoring methodology, including assessment of inter-observer reliability based on a subset of radiographs independently scored by two observers, is described in detail in Hanninger, Barratclough, Betty, Dittmer, et al. (2026).

2.3. Statistical analysis

2.3.1. Univariate analysis: HOF-Model

i) Probability of sexual maturity

Median bone age at attainment of sexual maturity was modelled using two predictors: (1) total bone maturity score and (2) minimum radius–ulna score. Models were based on radiographs of the right pectoral flipper only. Prior analyses comparing left and right flippers indicated no meaningful differences in bone maturation (Hanninger, Barratclough, Betty, Dittmer, et al., 2026; Hanninger, Barratclough, Betty, Palmer, et al., 2026) therefore, a single-flipper approach was applied.

The second predictor, the minimum radius–ulna score, was included as a complementary measure to describe the relationship between forearm ossification and maturity. In this model, the lower of the two scores was applied when the radius and ulna differed, representing the minimum ossification score that must be reached by at least one of the two bones for an individual to be considered sexually mature.

To estimate median bone age at attainment of sexual maturity, we applied Bayesian logistic regression with ‘HOF’ parameterisation (Huisman et al., 1993; Palmer et al., 2022), fitted with `rstan` and `rstanarm` packages in R. The HOF equation is as follows:

$$P(y = 1|x, mp, w) = \frac{1}{1 + \exp(-w(x - mp))}$$

where y is maturity status (0 = immature; 1 = mature), and x is bone score. The two model parameters are the slope w and the midpoint mp , representing the bone score at which the probability of sexual maturity is 0.5 (i.e., the median bone age at attainment of sexual maturity). Priors were set after inspection of the data (Table 5.1). Prior predictive testing was done to ensure that priors were biologically realistic.

Model performance was evaluated using leave-one-out (LOO) predictive density estimated by Pareto-smoothed importance sampling (PSIS; Vehtari et al., 2017), with re-estimation of influential points. Initial PSIS-LOO diagnostics indicated that most observations fell within acceptable Pareto-k ranges, although some cases returned high or very high Pareto-k values (in both females and males; Appendix 5, Table A5.1). These problematic observations occurred at

the extremes of the bone score distribution, involving very immature individuals with near-zero predicted probabilities of maturity and some of the oldest animals with probabilities effectively equal to one. In both situations, the steep logistic slope produced unstable importance weights, a known failure mode of PSIS-LOO when individual observations dominate the likelihood (Vehtari et al., 2017, 2024). To address this, subsequent analyses of total bone scores were restricted to scores of 40–90 in females (immature: $n = 16$; mature: $n = 20$) and 30–100 in males (immature: $n = 19$; mature: $n = 16$), thereby aligning the models more closely with the observed transition zone and balancing immature and mature sample sizes as far as possible. To further stabilise model behaviour, predicted probabilities were bounded away from 0 and 1 by applying a small symmetric regularisation term ($\epsilon \leq 0.1$) to the Bernoulli likelihood. This adjustment limited the influence of extreme observations, prevented skewing of the inferred transition, and resulted in consistently acceptable PSIS diagnostics. No such adjustment was required for models based on the minimum radius–ulna score, and these analyses were conducted using the full sample size (females: immature: $n = 42$; mature: $n = 31$; males: immature: $n = 37$; mature: $n = 25$).

ii) Comparison of sex-related differences in maturity parameters

To test for sex differences in maturity parameters, we compared posterior distributions of the midpoint (mp , the bone score at which the probability of maturity is 0.5) and the slope (w , the steepness of the transition between immature and mature). For each parameter, we calculated the posterior distribution of the difference in its value between females and males (parameter value for females minus the parameter value for males) and summarised the mean, standard deviation, 95% credible interval, and the posterior probability of this difference. Evidence for a difference was considered strong when the 95% credible interval for the difference did not include zero.

Table 5.1: Prior specifications for slope (w) and midpoint (mp) parameters in Bayesian logistic regression models of sexual maturity for common dolphins (*Delphinus delphis*). ‘Right’ = total bone score of the right pectoral flipper; ‘RUM’ = minimum score of radius and ulna. Priors were chosen based on observed data ranges and biological expectations of maturity transitions. Truncation to positive values ensured that maturity probability increased monotonically with bone development.

Sex	Model	Predictive prior	Justification
Females	‘Right’	$w \sim \text{Normal}^+(0.8, 0.2)$ $mp \sim \text{Normal}(76, 2)$	In females, maturity probability increases sharply between total bone scores of approximately 73 and 81, with the steepest transition occurring over ~ 5 –6 score units centred near a midpoint of ~ 76 ; the slope prior ($w \sim \text{Normal}^+(0.8, 0.2)$) reflects this steepness, corresponding to a 10–90% transition width of about 4–7 score units and constrained to positive values to enforce monotonicity; the midpoint prior ($mp \sim \text{Normal}(76, 2)$) centres the 50 % maturity probability near the empirical transition zone while allowing values of ≈ 72 –80 to capture uncertainty toward earlier maturation
	‘RUM’	$w \sim \text{Normal}^+(2.0, 0.2)$ $mp \sim \text{Normal}(5.0, 0.2)$	Transition from immature to mature is observed between scores 4–6; the slope prior centres on moderately steep transitions, truncated to positive values to reflect increasing maturity; the midpoint prior captures the transition midpoint near 5
Males	‘Right’	$w \sim \text{Normal}^+(0.40, 0.1)$ $mp \sim \text{Normal}(70, 3)$	Visual inspection and prior predictive checks indicated a gradual increase in maturity centred near $mp \approx 70$, with mixed outcomes spread over a broad range of total bone scores (no single score defines the transition); Using $\Delta x_{10-90\%} \approx 4.4/w$, $w = 0.40$ implies an approximately 11-unit 10–90% transition width, consistent with a gradual transition; the standard deviation (0.1) allows for both shallower and steeper slopes while truncation at $w > 0$ enforces monotonicity; the midpoint prior ($mp \sim \text{Normal}(70, 3)$) places the 50% maturity probability near 70, while allowing values of approximately 64–76 to capture uncertainty toward earlier or later maturation
	‘RUM’	$w \sim \text{Normal}^+(2.2, 0.2)$ $mp \sim \text{Normal}(5.0, 0.5)$	Transition zone is observed between ~ 4.5 –6; the slope prior centres on steep but biologically reasonable curves, truncated positive; the midpoint prior reflects the 50% maturity probability around 5, consistent with observed data

2.3.2. Multivariate analysis

Canonical analysis of principal coordinates (CAP; Anderson & Robinson, 2003; Anderson & Willis, 2003) was used to assess whether multivariate patterns of skeletal ossification, measured across multiple bone locations, were structured by sexual maturity status. Analyses were performed independently for females and males to account for sex-specific patterns of skeletal maturation. CAP was selected because it allows direct Clarke testing of group separation in multivariate resemblance space and provides a robust framework for classification and cross-validation.

All analyses included the full sample size of 137 individuals and were done using PRIMER v7 (Clarke & Gorley, 2015) with the PERMANOVA+ add-on (Anderson et al., 2008). A resemblance matrix was constructed from the 16 individual bone scores using Euclidean distances on untransformed data, as all bone score variables were in the same units and had the same measurement scale. Sexual maturity status (immature vs mature) was specified as a fixed grouping factor in the canonical analysis.

The number of principal coordinate axes retained (m) was chosen to maximise leave-one-out cross-validated classification success across candidate values, and values of $m = 3$ and $m = 7$ were selected for the final models for males and females, respectively. Model performance was evaluated using squared canonical correlations and leave-one-out cross-validation, with individuals classified into maturity groups based on their distances to group centroids in canonical space. Canonical coordinate scores (CAP1) were used for visualisation and interpretation of maturity-related structure.

To ensure comparability between modelling frameworks and to evaluate whether classification performance in the CAP analysis was influenced by extreme observations, we conducted a sensitivity analysis using the same truncated datasets previously applied to the HOF models. Truncation in the HOF framework was implemented in response to elevated Pareto- k values identified during PSIS-LOO cross-validation, which indicated that individuals at the extremes of the bone score distribution exerted disproportionate influence on model behaviour. We restricted the dataset to include only total bone scores of 40–90 in females (immature: $n = 16$; mature: $n = 20$) and 30–100 in males (immature: $n = 19$; mature: $n = 16$), thereby aligning the

models more closely with the observed transition zone and balancing immature and mature sample sizes as far as possible.

To maintain methodological consistency across analytical approaches, we re-ran the CAP analyses using these truncated datasets. For the truncated datasets, the number of principal coordinate axes retained (m) was re-estimated, resulting in $m = 5$ for females and $m = 2$ for males. This procedure ensured that CAP classification success was not artificially inflated by the inclusion of individuals positioned far from the maturity transition (e.g. foetuses, neonates, and the oldest fully mature animals). Leave-one-out classification accuracy and canonical correlations were recalculated for the truncated datasets and compared with results obtained from the full dataset.

3. Results

3.1. Univariate analysis: HOF-Model

The maximum observed total bone score in immature animals was 74 in females and 71 in males, while the minimum observed score in mature animals was 75 in females and 59 in males. Bayesian logistic regression estimated the midpoint of maturity at 74.85 (95% credible interval (CI): 72.50–77.13) in females and 68.35 (95% CI: 63.47–73.12) in males, corresponding to the scores at which the probability of sexual maturity reached 0.5. The slope was steeper in females, 0.85 (95% CI: 0.50–1.22; Figure 5.1A), compared with 0.38 (95% CI: 0.21–0.56) in males, indicating greater inter-individual variability and a more gradual transition in males (Figure 5.1B). The mean posterior difference in midpoints (female – male) was +6.50 (SD = 2.75), with a 95% CI from 1.03 to 11.92 and a 99.2% posterior probability that the female midpoint exceeded the male midpoint, indicating a consistently higher ossification score at the midpoint in females. The corresponding difference in slope parameters was +0.47 (SD = 0.21; 95% CI: 0.08–0.87), with a 99.0% posterior probability that the female slope was steeper, suggesting a sharper transition between immature and mature states. PSIS-LOO diagnostics indicated no problematic observations in the epsilon-adjusted HOF models (all Pareto- $k < 0.7$).

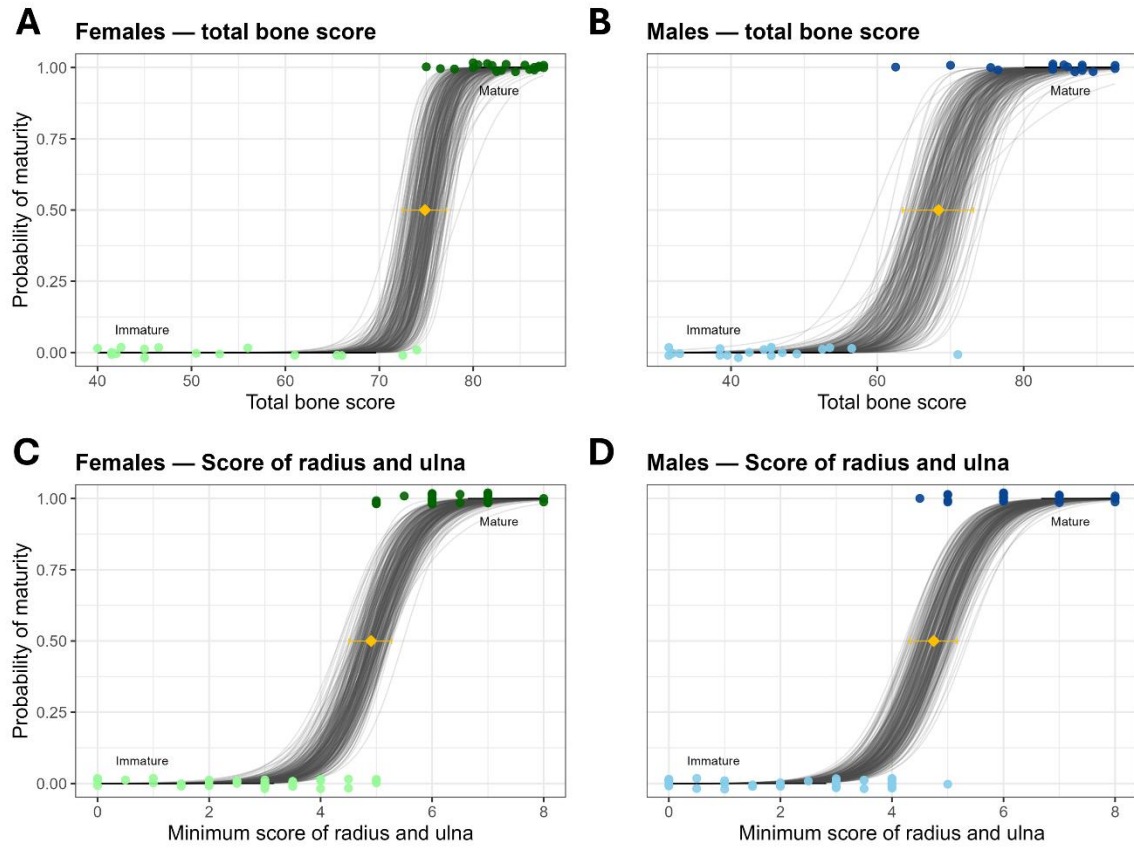


Figure 5.1: Bayesian logistic regression models describing the probability of sexual maturity in common dolphins (*Delphinus delphis*) based on ossification scores. **A)–B)** Models using the total bone score for the right pectoral flipper for females (**A**, green) and males (**B**, blue). **C)–D)** Models using the minimum score of the radius and ulna for females **C)** and males **D)**, where the lower of the two bone scores was used when they differed. Points represent individuals classified as immature (0) or mature (1) based on sexual maturity data. Black curves show posterior draws from the fitted models, and yellow diamonds mark the posterior median ($\pm$ 95 % credible interval) of the midpoint at which the probability of sexual maturity reaches 0.5.

For the minimum radius–ulna score, the maximum observed score in immature animals was 5, while the minimum observed score in mature animals was 5 and 4.5 in females and males, respectively. The estimated midpoint of maturity was 4.90 (95% CI: 4.51–5.27) in females and 4.75 (95% CI: 4.32–5.16) in males. Slopes were steep and similar across sexes, estimated at 2.54 (95% CI: 1.82–3.35; Figure 5.1C) for females and 2.29 (95% CI: 1.92–2.68; Figure 5.1D) for males. For the minimum radius–ulna score, there was no strong evidence for sex-specific differences in either the midpoint or the steepness of the maturity transition, with 95% credible intervals for sex differences overlapping zero (female – male midpoint difference: mean = +0.16, SD = 0.29, 95% CI = –0.40 to 0.71; female – male slope difference: mean = +0.26, SD = 0.44, 95% CI = –0.56 to 1.17). PSIS-LOO diagnostics indicated no problematic observations in the epsilon-adjusted HOF models (all Pareto- $k < 0.7$).

3.2. Multivariate analysis

3.2.1. Full dataset

Canonical analysis of principal coordinates revealed strong discrimination between immature and mature individuals based on multivariate patterns of skeletal ossification (Figure 5.2).

For females, the first canonical axis (CAP1) was strongly correlated with maturity status ($\delta = 0.91$), yielding a squared canonical correlation of $\delta^2 = 0.84$. Leave-one-out cross-validation showed a high degree of classification success for this CAP model, with 69 of 72 individuals being correctly assigned to their original maturity class, corresponding to an overall accuracy of 95.8% (4.17% misclassification error). The three misclassified individuals were immature females incorrectly assigned to the mature group (Table 5.2).

For males, the first canonical axis (CAP1) also showed a high canonical correlation ($\delta = 0.92$), corresponding to a squared canonical correlation of $\delta^2 = 0.84$. Leave-one-out cross-validation demonstrated excellent performance for this CAP model as well, with 62 of 63 individuals being correctly assigned to their original maturity class, resulting in an overall accuracy of 98.4% (1.59% misclassification error). The single misclassified individual (KS10-78Dd) was a pubescent male at the onset of the maturity transition (Table 5.2).

For both males and females, canonical coordinate scores along the canonical axis (CAP1) showed clear discrimination by maturity status, with immature individuals generally exhibiting

lower (negative) CAP1 values and mature individuals higher (positive) values (Figure 5.2). This constrained ordination shows visually how a clear shift in sexual maturity for each of the sexes was successfully captured by holistic changes across multiple bone structures in the CAP model (Figure 5.2). Projection of two previously unclassified individuals into the model canonical space for males resulted in assignment of one individual to the immature group (KS11-52Dd) and the other to the mature group (KS25-79Dd), based on their distances to group centroids (Figure 5.2B). KS25-79Dd was very close to the mature centroid (distance = 0.0107) and substantially farther from the immature centroid (distance = 0.2272), indicating a strong affinity with the mature group. In contrast, KS11-52Dd was closer to the immature centroid (distance = 0.0819) than to the mature centroid (distance = 0.1345) but positioned nearer the boundary between immature and mature groups along CAP1.

3.2.2. Truncated model

In the truncated model, the first canonical axis (CAP1) remained strongly correlated with maturity status ($\delta = 0.92$) in females, yielding a squared canonical correlation of $\delta^2 = 0.84$. Leave-one-out cross-validation showed a high degree of classification success, with 34 of 36 individuals correctly assigned to their original maturity class, corresponding to an overall accuracy of 94.4% (5.6% misclassification error). The two misclassified females (KS19-16Dd and KS23-09Dd) were immature individuals incorrectly assigned to the mature group (Table 2).

Similarly, in males, the correlation remained high ($\delta = 0.91$), yielding a squared canonical correlation of $\delta^2 = 0.84$, with 33 of 35 individuals correctly classified. This corresponds to an overall accuracy of 94.3% and a misclassification error of 5.7%. The two misclassified males were KS16-27Dd (incorrectly assigned to the immature group) and KS10-78Dd (incorrectly assigned to the mature group).

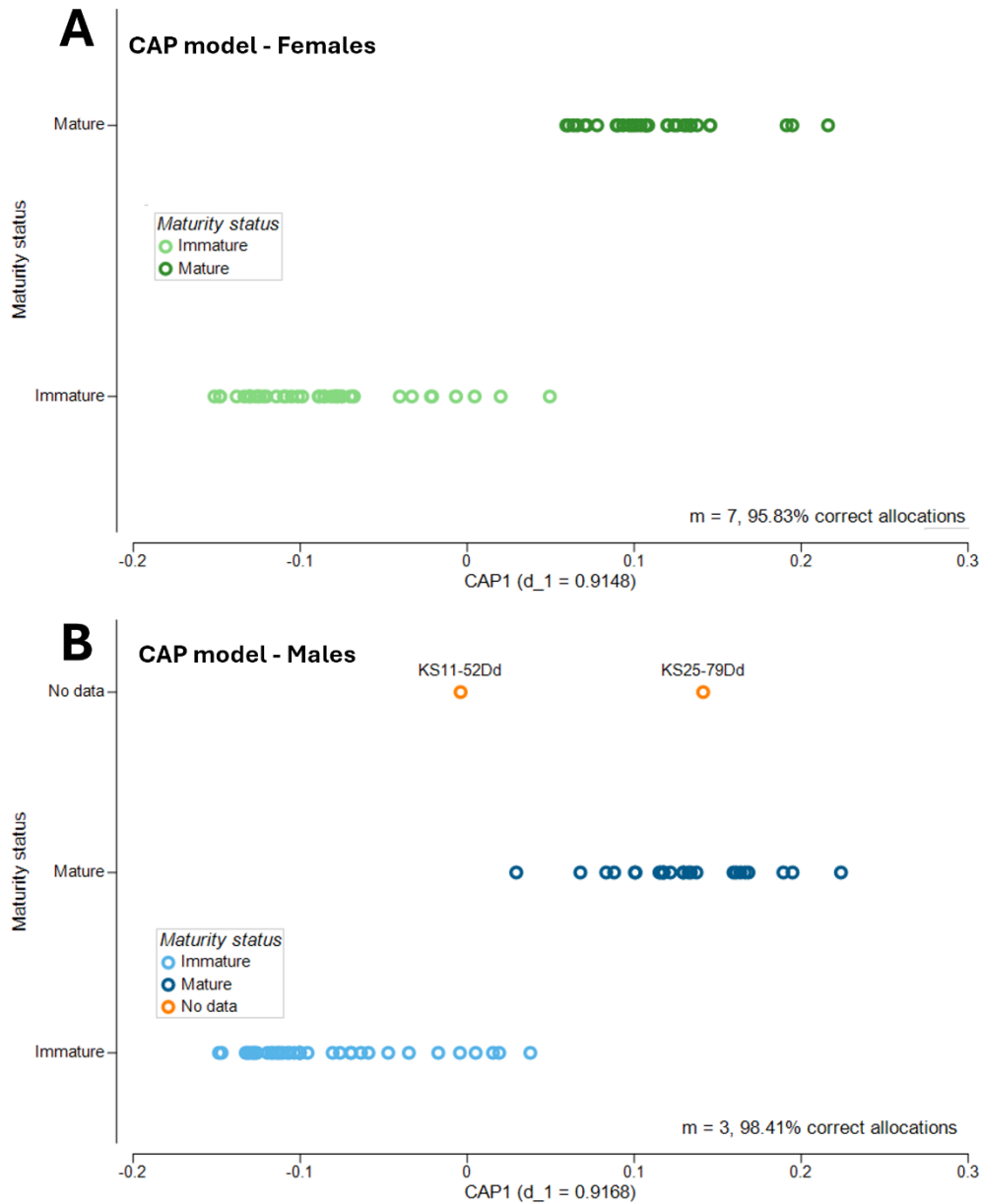


Figure 5.2: Canonical analysis of principal coordinates (CAP) of multivariate skeletal ossification data ($p = 16$ variables) in relation to sexual maturity status based on the full dataset. Analysis was done separately for **A**) female and **B**) male common dolphins (*Delphinus delphis*). Points represent individual dolphins plotted along the first canonical axis (CAP1), which maximises discrimination between immature (0) and mature (1) individuals based on Euclidean distances. Symbol colour indicates sex and maturity status (light green = immature females, dark green = mature females; light blue = immature males, dark blue = mature males; orange = individuals of unknown maturity status). The percentage of individuals correctly classified (as being immature or mature) under leave-one-out cross-validation was 95.8% for females and 98.4% for males.

Table 5.2: Characteristics of individuals misclassified by the CAP sexual maturity model. Sex is indicated as male (M) or female (F). Sexual maturity status (SM) is coded as 0 = immature and 1 = mature. Predicted sexual maturity (PSM) indicates the CAP-based classification (0 = immature, 1 = mature). Dental age is given in years where available (ND = not determined). Total body length (TBL) is reported in centimetres, and age class reflects the categorical classification based on independent biological criteria.

ID	Sex	SM	PSM	Age	TBL (cm)	Age class
KS10-78Dd	M	0	1	ND	208	Subadult
KS19-16Dd	F	0	1	9	173	Juvenile
KS23-09Dd	F	0	1	8	199	Subadult
KS24-59Dd	F	0	1	10	185	Subadult

4. Discussion

4.1. Model performance

Here, we have demonstrated that skeletal ossification can provide a robust and biologically informative signal of sexual maturity in common dolphins by integrating probabilistic modelling with multivariate discrimination of ossification patterns across multiple bone sites. Specifically, the HOF model provides a transparent framework for estimating the probability of sexual maturity from radiographic ossification scores, allowing uncertainty around maturity assignment to be explicitly quantified. As a complementary approach, the CAP model captures multivariate structure in skeletal development holistically based on the ossification patterns across multiple bones. This permits effective discrimination between immature and mature individuals. Together, these approaches provide useful insights into the relationship between skeletal maturation and gonadal maturity, enabling probabilistic inference at the individual level and robust discrimination (along with the assessment of model performance) among maturity classes within the study sample.

The CAP model achieved high overall classification accuracy and clear separation between immature and mature individuals based on multivariate ossification patterns. This demonstrates that skeletal development across multiple bone sites contains a consistent maturity-associated

signal that is robust within the study sample. For both the full dataset (females: $n = 73$; males: $n = 62$) and the truncated version (females: $n = 36$; males: $n = 35$), error rate was low, with only one male (KS10-78Dd; 1.6%) and three females (KS19-16Dd, KS23-09Dd, KS24-59Dd; 4.2%) deemed misclassified in the full dataset and two males (KS10-78Dd, KS16-27Dd; 5.7%) and two females (KS19-16Dd, KS23-09Dd; 5.6%) misclassified in the truncated model.

All misclassified individuals occurred close to the transition between immature and mature states, where discordance among maturity indicators can be expected. For example, KS10-78Dd was a pubescent male (spermatogonia and spermatocytes present; Palmer et al., 2023) and therefore lies within the physiological transition zone between immature and mature categories. KS16-27Dd was a sexually mature male (7.5 years of age) who was incorrectly classified as immature. Notably, this individual had attained sexual maturity at an age below the estimated mean age of sexual maturity for male common dolphins in New Zealand (8.8 years; Palmer et al., 2023). The three misclassified immature females all had dental ages (aged 8, 9 and 10) exceeding the reported mean age at sexual maturity in females (7.5 years; Palmer et al., 2022), but remained classified as juvenile or subadult based on the absence of ovarian scars and somatic development. In particular, KS19-16Dd remained juvenile despite the dental age of 9, with a comparatively small body size (173 cm) relative to the reported mean length at sexual maturity in females (183.4 cm; Palmer et al., 2022). Another female (KS24-59Dd) had a dental age of 10 years but demonstrated poor body condition (hollow upper flanks with visible lumbar transverse processes) and hepatocellular atrophy, indicative of nutritional stress, providing a plausible explanation for delayed sexual maturity (Rautou et al., 2008). Together, these cases indicate that our ‘misclassifications’ primarily occurred in individuals positioned near the boundary between immature and mature states, where skeletal, dental, and somatic indicators did not perfectly align. Such cases reflect ambiguity around the maturity transition as opposed to model failure.

The sensitivity analysis based on the truncated datasets yielded the same overall inference as the full-dataset CAP models: classification performance remained strong, and misclassifications continued to be restricted to the maturity transition zone, indicating that model behaviour was not driven by extreme observations. Because truncation reduced sample size, misclassification percentages were expected to appear higher even when only a small number of individuals were incorrectly assigned. Importantly, misclassification largely involved the same individuals across both analytical versions, with only minor differences: the

truncated model misclassified one fewer female (KS24-59Dd) but one additional male (KS16-27Dd) relative to the full dataset. Across both datasets, erroneous assignments occurred only among individuals close to the immature–mature boundary, reinforcing that uncertainty in CAP-based classification is confined to the biological transition zone rather than reflecting failure to distinguish clearly immature from fully mature individuals.

Unfortunately, sparse representation of pubescent individuals, particularly in males ($n = 2$), means that some degree of misclassification at the transition was unavoidable. Limited sampling within this boundary region constrains classification precision in the CAP framework, as well as probabilistic inference in the HOF models. In probabilistic models specifically, such limited sampling restricts estimation of the gradient and precise location of the transition, rather than indicating weak separation between immature and mature states. Classification precision at the transition could therefore be improved by increasing sampling of individuals within this boundary region and, for males, by explicitly incorporating a pubescent category within an ordinal modelling framework (e.g. Bayesian cumulative logit models; Palmer et al., 2023). However, this approach was not adopted here due to insufficient numbers of pubescent individuals.

4.2. Intraspecific differences in timing of sexual maturity

The timing of bone ossification differs between sexes. In most cetacean species, females reach sexual maturity earlier than males (Galatius et al., 2006). Rising oestrogen and testosterone levels during puberty promote bone maturation and epiphyseal closure (Almeida et al., 2017; Iuliano-Burns et al., 2009; Nilsson et al., 2005). In common dolphins in New Zealand, sexual maturity is attained at a mean age of 7.5 years in females and 8.8 years in males (Palmer et al., 2022, 2023). Females reached a 50% probability of sexual maturity at a higher total bone score than males, consistent with earlier pubertal hormone exposure leading to more advanced skeletal maturation by the time sexual maturity is attained.

In contrast to the total bone score, the minimum radius–ulna score showed no strong evidence for sex-specific differences in either the midpoint or the speed (steepness) of the maturity transition, indicating a broadly similar ossification pattern in females and males. The radius and ulna are among the earliest elements of the forelimb to complete epiphyseal fusion, and their development, therefore, reflects early stages of skeletal maturation. The absence of sex

differences in the radius–ulna model suggests that the higher midpoint observed for the total bone score in females is driven by more advanced maturation of later-developing elements, particularly the metacarpals and phalanges, rather than by differences in proximal forelimb ossification.

4.3. Interspecific differences in timing of sexual maturity

Sexual maturity is initiated by activation of the hypothalamic–pituitary–gonadal axis and rising sex-steroid concentrations (Spaziani et al., 2021). Although sex steroids accelerate bone maturation, epiphyseal closure occurs only after prolonged cumulative exposure and therefore typically follows the onset of reproductive maturity (Kumbar & Ballur, 2023; Nilsson et al., 2005; Rajdev et al., 2014). This pattern is well established in humans, where sexual maturity occurs before fusion of the distal radius (Cavallo et al., 2021; Ratib et al., 2012), and radiographic assessment of bone age from wrist radiographs is used to estimate remaining growth and predicted adult height (Greulich et al., 1959; Ratib et al., 2012).

Nevertheless, interspecific comparisons showed that the duration of skeletal growth is shaped by life-history traits, life expectancy, and species-specific growth trajectories, such that reproductive maturation may occur at different stages of long-bone ossification (Geiger et al., 2014; Kilborn et al., 2002). More generally, the proportion of the lifespan during which skeletal growth continues after reproductive maturation varies substantially among species (Kilborn et al., 2002). These biological factors thus provide plausible mechanisms by which the relationship between radius–ulna ossification and sexual maturity could differ among cetacean species.

A direct comparison across species remains constrained, however, because existing studies vary substantially in design, maturity classification, and analytical approach, and with no prior research formally modelling sexual maturity as a probabilistic outcome (Barratclough, Sanz-Requena, et al., 2019; De Carvalho et al., 2015; Lee, 1978; Ogden et al., 1981). Consequently, apparent interspecific differences cannot be disentangled from methodological differences among studies.

While biological differences among species may influence the alignment between skeletal development and reproductive maturation, the absence of a consistent modelling framework across studies currently precludes robust interspecific comparison. Consequently, the

applicability of radius–ulna ossification scores as universal indicators of sexual maturity cannot be evaluated across species and should not be assumed without species-specific validation. Interpretation of radiographic maturity indicators, therefore, requires methodologically consistent, species-specific modelling frameworks.

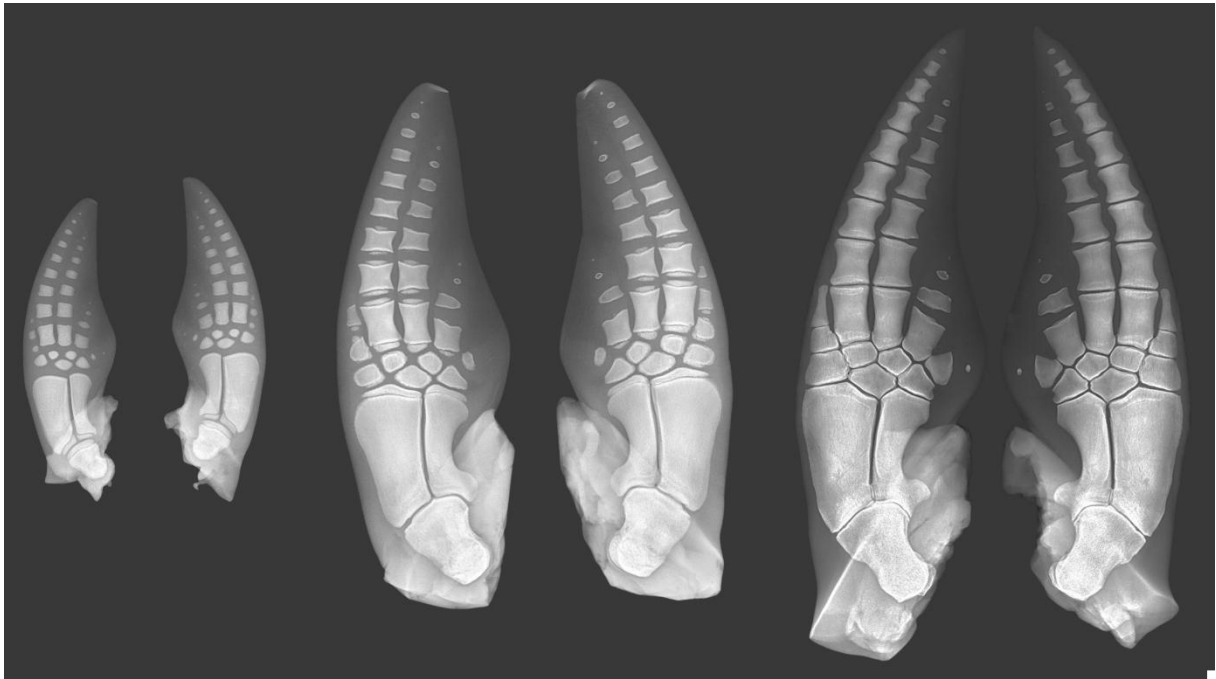
Beyond species-level considerations, this modelling framework should also be evaluated across different common dolphin populations, considering variation in life-history traits among populations (e.g., Danil & Chivers, 2007; Murphy & Rogan, 2006; Palmer et al., 2022, 2023; Westgate & Read, 2007). Life-history traits may diverge under population-level pressures such as environmental variation, density dependence, or anthropogenic impacts, consistent with plasticity in life-history strategies (Clutton-Brock & Sheldon, 2010; Kemper et al., 2014; Murphy, 2004; Palmer et al., 2022, 2023). As this framework was developed for common dolphins within New Zealand, its applicability to wider populations requires population-specific validation.

4.4. Conclusion

Radiographic assessment offers a practical opportunity to advance comparative life-history research in cetaceans. Specifically, radiography provides a standardised, non-destructive, and widely accessible means of quantifying skeletal development across age classes and species, allowing skeletal growth to be linked directly to the state of sexual maturity. By modelling sexual maturity as a binary biological state and expressing maturity as a probability function of radiographic ossification, the present study establishes a reproducible, species-specific predictive model framework for common dolphins. With validation across wider common dolphin populations and even additional species, radiographic approaches could facilitate robust comparisons of skeletal–reproductive relationships at both population and species level within cetaceans.

Chapter 6

General discussion



Representative radiographs of common dolphins at neonatal (<1 year), juvenile (2.25 years), and adult (31 years) life stages. Radiographs were acquired using a setting of 40 kVp and 4 mAs and a source-to-sample distance of 100 cm.

This PhD comprises four data chapters, two of which have been published (Chapters 2 and 3), one of which is in press (Chapter 5), and one of which is currently under peer review with an international journal (Chapter 4). The final chapter synthesises the ageing methodologies developed in this PhD (epigenetic ageing and radiographic bone ageing) for common dolphins (*Delphinus delphis*). Specifically, this chapter (1) outlines the overall contribution to knowledge provided by the thesis, (2) evaluates the relevance for conservation and life-history research, and (3) highlights priorities for future research.

1. Contribution to knowledge

1.1. Chapter 2

*Dental ageing offers new insights to the first epigenetic clock for common dolphins (*Delphinus delphis*)*

This chapter contributes new knowledge in several key ways, first and foremost by establishing the first species-specific epigenetic clock for common dolphins. Using dental ages from stranded and bycaught individuals, I demonstrate that accurate DNA methylation-based age estimation can be achieved (median absolute error (MAE) = 1.80 years, Pearson correlation (r) = 0.91, R^2 = 0.82). Secondly, this chapter challenges previous assumptions underlying odontocete epigenetic clocks that calibration using dental ages inherently compromises model performance. By systematically comparing models that differed in their treatment of dental-age uncertainty and potential outliers, this chapter shows that excluding individuals with only minimum age estimates does not meaningfully improve clock accuracy. The consistent underestimation of older individuals across all models further suggests that discrepancies between dental and epigenetic age estimates are unlikely to be driven primarily by dental ageing error but instead reflect biological processes or statistical constraints affecting epigenetic ageing at later life stages. This is a significant finding considering so few studies to date have applied epigenetics to their collections of bycaught and stranded animals because of prior unfounded concerns about the role of GLG dental ageing accuracy in epigenetic clock accuracy.

Thirdly, this chapter demonstrates that post-mortem decomposition state and long-term sample storage do not compromise epigenetic age prediction, thereby validating the use of archival tissues for clock calibration. Collectively, these findings expand the methodological foundation of cetacean epigenetic ageing by providing a robust molecular ageing tool for common dolphins

while directly addressing aspects of clock validation — such as GLG uncertainty and decomposition — that have previously limited the application of international tissue archives in epigenetic studies.

1.2. Chapter 3

Cross-species applicability of a bone-ageing method in delphinids

This chapter contributes new knowledge by delivering the first formal evaluation of whether a radiographic flipper-ageing framework developed for bottlenose dolphins (*Tursiops truncatus*) can be successfully applied to common dolphins. The original framework integrates assessments of long-bone maturation, delta-bone development, and age-related degenerative change. For the first time, this chapter rigorously tests the applicability of this approach to a cetacean species beyond bottlenose dolphins by identifying morphological and developmental differences in skeletal maturation and evaluating how these may influence cross-species transferability.

The chapter documents species-level differences in delta-bone maturation and in the occurrence and extent of degenerative disease, as well as subtle left–right asymmetry in pectoral flipper development in common dolphins relative to bottlenose dolphins. Based on these findings, revised scoring criteria were developed specifically for common dolphins via targeted modifications to the original bottlenose dolphin framework. These revisions accommodate species-specific morphology while maintaining a strong association with chronological age and low inter-observer error. However, the comparatively low prevalence and subtle expression of degenerative change in common dolphins are identified as potential limitations for ageing geriatric individuals. These are significant findings because the chapter shows that flipper-based radiographic ageing frameworks can be transferable across cetacean species when recalibrated for species-specific developmental patterns.

Collectively, this chapter establishes the foundation for a calibrated radiographic ageing model for common dolphins (implemented in Chapter 4) and provides a general framework for evaluating the transferability of flipper-based ageing approaches across cetacean species.

1.3. Chapter 4

Radiographic assessment of bone maturation: A tool for age estimation in common dolphins (*Delphinus delphis*)

This chapter contributes new knowledge by developing and validating a world-first radiographic bone-ageing framework for common dolphins, providing a species-specific, morphology-based method for estimating chronological age across the lifespan. Building on the skeletal assessments established in Chapter 3, it demonstrates that patterns of pectoral flipper ossification and epiphyseal fusion can be quantitatively calibrated against dental age to generate accurate age estimates in free-ranging dolphins. Using both univariate (polynomial regression) and multivariate (canonical analysis of principal coordinates, CAP) approaches, radiographic bone scores captured age-related skeletal development with high accuracy and biological coherence:

- CAP: females: median absolute error (MAE) = 1.35, Spearman's rank correlation (ρ) = 0.90, $R^2 = 0.85$; males: MAE = 1.80 years, $\rho = 0.94$, $R^2 = 0.84$
- Polynomial regression: females: MAE = 1.25 years, $\rho = 0.93$, $R^2 = 0.90$; males: MAE = 1.08 years, $\rho = 0.95$, $R^2 = 0.86$

This chapter further evaluates the reliability of this ageing method in relation to the epigenetic clock developed in Chapter 2. When assessed alongside the molecular ageing tool derived from the same population (MAE = 1.80 years, $r = 0.91$, $R^2 = 0.82$), radiographic bone ageing yielded lower prediction error, particularly at older ages, and remained reliable into later life stages. Whereas error in epigenetic age estimates increased beyond approximately 16 years, radiographic methods remained robust to approximately 20 years.

Collectively, these findings establish radiographic bone ageing as a practical and scientifically robust method for application in both post-mortem and live-stranding contexts, with clear relevance for life-history research, demographic inference, and conservation management. By developing this new radiographic approach for common dolphins, this chapter provides a validated alternative to both epigenetic and dental ageing techniques and expands the methodological toolkit available for age estimation in this species. This is a significant contribution to knowledge given that epigenetic clocks are increasingly treated as a benchmark approach for age estimation in cetaceans, yet are costly to implement, technically demanding,

and frequently constrained by intellectual-property restrictions. This chapter instead offers an inexpensive and logistically accessible alternative that facilitates broader uptake of age estimation in conservation and management contexts.

1.4. Chapter 5

Radiographic assessment of bone maturation: A tool to estimate sexual maturity in common dolphins (*Delphinus delphis*)

This chapter advances knowledge by formally establishing radiographic skeletal ossification as a probabilistic proxy for sexual maturity in common dolphins in New Zealand. It demonstrates that a single unified framework using radiography can be used to infer both chronological age (Chapters 3 and 4) and sexual maturity (Chapter 5). In this first study, the aim was to explicitly model maturity probability as a function of skeletal ossification using species-specific quantitative approaches. I integrated univariate probabilistic modelling of ossification scores with multivariate classification of holistic skeletal development to demonstrate that radiographic bone maturation captures a strong and biologically meaningful signal of sexual maturity in both sexes. High classification accuracy was achieved (95.8% in females and 98.4% in males), with misclassification confined to pubescent dolphins.

By extending radiographic flipper assessments beyond age estimation alone (Chapters 3 and 4), this chapter demonstrates, for the first time, that pectoral flipper radiography can simultaneously resolve two fundamental life-history parameters that underpin demographic and population-level analyses. This dual capability positions radiographic approaches as an efficient and integrative tool for cetacean life-history research in situations where direct histopathological assessment of dental age and reproductive tissues is not feasible. This is an especially significant advancement in countries where access to histological labs for teeth and gonad preparation is not available locally, regionally, or even nationally.

2. Implications for conservation management

Reliable estimation of chronological age is essential for cetacean conservation, as survival, recruitment, and population growth fundamentally depend on age. Age data underpin the description of population demographics, estimation of age-specific mortality, and inference of the timing of key life-history transitions (e.g. sexual maturity) that shape demographic

trajectories (Manlik et al., 2022; Palmer et al., 2022; Verborgh et al., 2021). In common dolphins, extensive life-history information has been derived from post-mortem dental ages, particularly from stranded and bycaught individuals (e.g., Danil & Chivers, 2007; Murphy et al., 2005; Palmer et al., 2022, 2023; Westgate & Read, 2007). However, reliance on dental ageing restricts age determination to situations where teeth can be extracted and processed histologically, limiting its applicability in contexts such as live-stranding responses and other scenarios where invasive sampling is not appropriate. Approaches that allow age estimation without tooth extraction, therefore, expand the circumstances under which age data can be obtained.

While dental ageing based on growth layer groups remains the reference method for age assessment in odontocetes (e.g., Betty et al., 2023; Cooper et al., 2018; Evans et al., 2002; Lockyer, 1993, 1995; Murphy et al., 2014; Westgate & Read, 2007), the complementary ageing frameworks developed in this thesis expand the methods to obtain age and life-history information for common dolphins. Epigenetic age estimation (Chapter 2) enables age prediction in free-ranging individuals through minimally invasive skin biopsy sampling, which can be performed remotely or in hand (Barratclough et al., 2025; Peters et al., 2023). Radiographic bone ageing (Chapters 3–5) provides a cost-effective and accessible approach for age estimation from pectoral flipper radiographs, thereby avoiding the need for invasive tooth extraction and laboratory-intensive histological processing. Importantly, radiography can also be applied during live-stranding responses using mobile X-ray equipment, extending its utility beyond post-mortem contexts. This enables the simultaneous assessment of two key life-history parameters by providing real-time age estimates and allowing inference of sexual maturity state from ossification patterns (Chapter 5). An additional advantage is that radiographic images can be shared internationally without requiring export permits, which are typically required for DNA samples used in epigenetic analyses.

Together, the epigenetic clock and the radiographic framework provide practical tools for obtaining age and associated life-history information from free-ranging biopsied individuals, stranded and bycaught animals, and live-stranding responses. By increasing access to reliable age information, these approaches strengthen population-level inference and support conservation assessments for species facing ongoing anthropogenic pressures (Manlik et al., 2016; Palmer et al., 2022; Verborgh et al., 2021).

Common dolphins are subject to substantial anthropogenic impacts in several regions, particularly through fisheries bycatch (Abraham et al., 2021; Bilgmann et al., 2014; Brophy et al., 2009; Geijer & Read, 2013; Mannocci et al., 2012; Peltier et al., 2016, 2021; Tulloch et al., 2020). For the Australasian population, concerns have been raised regarding cumulative anthropogenic impacts and the lack of detailed demographic assessment, prompting calls for population viability analyses to evaluate population vulnerability (Beaumont, 2024; Palmer, 2023). These analyses fundamentally depend on reliable age data to characterise age structure and age-specific demographic rates (Lacy, 1993; Manlik et al., 2016, 2022; Verborgh et al., 2021). By improving access to reliable age estimates across different sampling contexts, the approaches developed in this thesis facilitate future age-based population assessments by increasing the availability and accuracy of age data.

3. Study considerations & limitations

Considerations and limitations within this doctoral study apply across the radiographic ageing, skeletal development, sexual maturity, and epigenetic ageing frameworks presented in this thesis. Initial calibration of all approaches relied on opportunistic samples from stranded and bycaught individuals, which inevitably constrain age structure, health status, and life-history representation. Older and physically mature dolphins, as well as pubescent animals, are underrepresented, reflecting both their lower stranding probability and the demographic structure of free-ranging populations. For ageing analyses, this bias limits model calibration at the upper end of the age range and contributes to systematic age compression (i.e., regression to the mean; Chapters 2 and 4). Likewise, the scarcity of pubescent individuals restricts inference of the transition between sexually immature and mature life stages (Chapter 5).

The same sampling constraint also affects health-related variability within the dataset. Health status may influence both epigenetic ageing and skeletal maturation (e.g., Colich et al., 2020; Fitzgerald et al., 2021; Liu et al., 2021; Shirazi et al., 2020; Weindruch et al., 2001), potentially introducing additional variation into age estimates (Chapters 2-5). Epigenetic age is known to deviate from chronological age in relation to disease, physiological stress, and nutritional condition, while skeletal development and maturation can likewise be affected by compromised health or altered growth trajectories. Consequently, age-related signals derived from molecular or morphological markers may reflect not only chronological ageing but also individual health histories. Samples used in this thesis originated from a variety of contexts, including mass

strandings, single strandings, bycatch events, and even one individual formerly housed under human care. While mass strandings are generally considered unrelated to individual health problems, single strandings commonly involve animals affected by illness, compromised health, or poor nutritional status. Such heterogeneity is inherent to opportunistic datasets and may contribute to inter-individual variability that cannot be fully accounted for in cross-sectional analyses.

Beyond constraints related to sampling composition and health status, a further limitation concerns how age-related information is distributed across the lifespan. Early life is characterised by rapid and ordered biological change, yielding high age resolution, whereas later life involves slower change over longer intervals, such that chronological age advances faster than the age-informative signal. This may reflect both deceleration of epigenetic change (Chapter 2) with age (Horvath, 2013; Kuzub et al., 2022; Marioni et al., 2019) and increasing reliance on degenerative rather than growth-related skeletal traits (Chapters 3 and 4), which show substantial individual variability. Consequently, older individuals become more difficult to distinguish, limiting fine-scale age discrimination at advanced ages across all frameworks.

Lastly, an additional constraint relates to the reference standard used for model calibration. Both ageing approaches were calibrated using dental age estimates. This reflects the absence of common dolphins in long-term human care and the infeasibility of longitudinal recapture studies in this typically oceanic species. Dental ageing is most reliable in younger animals but accuracy declines in older individuals as tooth wear and compression of growth layer groups reduce precision, a limitation that is exacerbated when estimates rely on a single extracted tooth (as is common in studies of animals under human care; Barratclough et al., 2023). In my study, this limitation was addressed by processing up to three teeth per individual. Preference was given to the least worn and straightest specimens, and all teeth were read independently by a minimum of two experienced readers, with minimum age estimates assigned where uncertainty remained due to accessory lines or compressed growth layer groups. These mitigation measures provided a high level of confidence in the dental age estimates used for model calibration (Chapters 2-4). Furthermore, as demonstrated in the epigenetic ageing analyses (Chapter 2), age-range compression observed in older individuals persisted despite conservative dental age assignment, indicating that this pattern is unlikely to be driven by dental ageing error.

4. Future directions

Both the epigenetic and radiographic ageing models developed in this thesis would benefit from continued sample collection, particularly of older and pubertal individuals, to improve age estimation at the upper end of the lifespan and inference of sexual maturity around the pubertal transition.

For radiographic bone ageing in particular, a larger dataset would also support the development of an app-based tool capable of estimating age directly from radiographs, mirroring approaches already applied in human forensics and paediatrics (Sato, 2015). Such an application would reduce the subjectivity inherent in manual bone scoring and facilitate rapid, standardised age estimation, including in remote settings and during live-stranding responses.

Future research should further build on the frameworks developed here by investigating the biological factors that influence variation in epigenetic and skeletal ageing. For epigenetic ageing, this includes examining epigenetic age acceleration in relation to pathology/disease status and toxicological exposure, particularly heavy metals and emerging contaminants such as per- and polyfluoroalkyl substances (PFAS). These analyses would allow assessment of how epigenetic age deviates from chronological age in relation to health and environmental stressors. For radiographic bone ageing, future analyses should examine how variation in bone-based age estimates relates to nutritional stress, chronic infection, and contaminant exposure, which may alter skeletal maturation trajectories. Nutritional stress is currently under systematic assessment in this study population (Massey University, unpublished data), directly enabling future integration of bone ageing results with measures of energetic condition. Environmental contaminants and infectious diseases known to affect skeletal development, including *Brucella ceti*, represent additional factors that warrant targeted investigation (Roca-Monge et al., 2022). Integration of health-related information with the ageing results presented here is feasible, as most cases have already been reviewed by a board-certified veterinary pathologist, with detailed histology, microbiology and toxicological reports already available.

In addition, curation of pectoral flippers from wider odontocete species has occurred since the early 2000s and accordingly advanced as part of the Massey University BIOCET project, allowing future cross-species comparisons of skeletal maturation and ossification patterns. This will enable calibration of additional species-specific bone ageing models and improved understanding of bone development as an indicator of sexual maturity across taxa.

5. Conclusion

This thesis makes a significant contribution to the literature in the field of cetacean life-history research by expanding the methodological toolkit available for age assessment in common dolphins. My research demonstrates that dental, radiographic, and epigenetic ageing approaches each offer distinct and complementary advantages for age estimation in common dolphins, with differing applicability depending on sampling context and life stage. Dental ageing remains the reference method for chronological age determination, radiographic bone ageing provides a practical and cost-effective alternative that additionally allows inference of sexual maturity, and epigenetic ageing uniquely enables age estimation in free-ranging individuals through biopsy sampling. Importantly, all three methods show increasing uncertainty at older ages, reflecting tooth wear and growth layer group compression in dental ageing, variability in skeletal degeneration in bone ageing, and epigenetic deceleration in molecular clocks. These shared limitations underline the importance of integrating multiple ageing approaches, allowing age estimates to be cross-validated and life-history information to be interpreted in context rather than relying on any single method in isolation. This synthesis further highlights the critical value of long-term biobanks, as the availability of well-curated, archived dental, skeletal, and soft-tissue samples is essential for developing, validating, and cross-comparing ageing frameworks. Without sustained curation of such matrices, this research would not have been possible. Together, the frameworks developed in this thesis provide a flexible and complementary toolkit for age assessment in common dolphins and establish a foundation for more robust demographic inference in future research.

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Appendix 1

Introduction

1. Supplementary tables

Table A1.1: Summary of representative studies that used methylation data to develop molecular ageing models for predicting animal age.

Fish	References
European seabass (<i>Dicentrarchus labrax</i>)	Anastasiadi & Piferrer (2020)
Long snout seahorse (<i>Hippocampus reidi</i>)	Suarez-Bregua et al. (2021)
Short-lived killifish (<i>Nothobranchius furzeri</i>)	Giannuzzi et al. (2024)
Blackbelly rosefish (<i>Helicolenus dactylopterus</i>)	Weber et al. (2024)
Cownose ray (<i>Rhinoptera bonasus</i>)	Weber et al. (2024)
Red snapper (<i>Lutjanus campechanus</i>)	Weber et al. (2024)
Red grouper (<i>Epinephelus morio</i>)	Weber et al. (2024)
Zebrafish (<i>Danio rerio</i>)	Mayne, Korbie, et al. (2020)
Amphibians	
African clawed frog (<i>Xenopus laevis</i>)	Zoller et al. (2022)
Western clawed frog (<i>X. tropicalis</i>)	Bennett et al. (2025); Morselli et al. (2023); Zoller et al. (2022)
Reptiles	
American alligator (<i>Alligator mississippiensis</i>)	Nilsen et al. (2016); Parrott et al. (2014)
Australian flatback sea turtle (<i>Natator depressus</i>)	Mayne, Tucker, et al. (2020)
Leatherback sea turtle (<i>Dermochelys coriacea</i>)	Mayne, Tucker, et al. (2020)
Brown anole (<i>Anolis sagrei</i>)	Shealy et al. (2025)
Loggerhead sea turtle (<i>Caretta caretta</i>)	Cocci et al. (2018)
Birds	
Barn swallow (<i>Hirundo rustica</i>)	Saino et al. (2019)
Black grouse (<i>Lyrurus tetrix</i>)	Soulsbury et al. (2018)
Common tern (<i>Sterna hirundo</i>)	Meyer et al. (2023)
Great tit (<i>Parus major</i>)	Haller et al. (2025)
Gould's petrel (<i>Pterodroma leucoptera</i>)	Roman et al. (2024)
Domestic Chicken (<i>Gallus gallus domesticus</i>)	Raddatz et al. (2021)
King penguin (<i>Aptenodytes patagonicus</i>)	Cristofari et al. (2024)
Short-tailed shearwater (<i>Ardenna tenuirostris</i>)	De Paoli-Iseppi et al. (2019)

Table A1.1: Continued.

Aquatic mammals	References
Multi-species and multi-tissue methylation clocks for age estimation in toothed whales and dolphins	Robeck, Fei, Lu, et al. (2021)
Multi-species Cetacean Clock	Zoller et al. (2025)
Antarctic minke whale (<i>Balaenoptera bonaerensis</i>)	Goto et al. (2020); Tanabe et al. (2020)
North Atlantic fin whales (<i>B. physalus</i>)	García-Vernet et al. (2021)
North Atlantic right whales (<i>Eubalaena glacialis</i>)	Lee (2021)
Bowhead whales (<i>Balaena mysticetus</i>)	Parsons et al. (2023)
Beluga whale (<i>Delphinapterus leucas</i>)	Bors et al. (2021)
Bottlenose dolphin (<i>Tursiops truncatus</i>)	Barratclough et al. (2021); Beal et al. (2019); Robeck, Fei, Haghani, et al. (2021)
Indo-Pacific bottlenose dolphins (<i>T. aduncus</i>)	Peters et al. (2023); Yagi et al. (2024)
Humpback whale (<i>Megaptera novaeangliae</i>)	Horvath, Haghani, Zoller, Fei, et al. (2022); Polanowski et al. (2014)
Sperm whale (<i>Physeter catodon</i>)	Polanowski et al. (2014)
Killer whale (<i>Orcinus orca</i>)	Parsons et al. (2023)
Hector's and Māui dolphins (<i>Cephalorhynchus hectori hectori</i> and <i>C. h. maui</i>)	Hernandez et al. (2023)
Risso's dolphin (<i>Grampus griseus</i>)	Mori et al. (2024)
False Killer Whales (<i>Pseudorca crassidens</i>)	Martien et al. (2026)
Bats	
Bechstein's bat (<i>Myotis bechsteinii</i>)	Wright et al. (2018)
Big brown bat (<i>Eptesicus fuscus</i>)	Wilkinson et al. (2021)
Common noctule (<i>Nyctalus noctula</i>)	Wilkinson et al. (2021)
Common vampire bat (<i>Desmodus rotundus</i>)	Wilkinson et al. (2021)
Egyptian fruit bat (<i>Rousettus aegyptiacus</i>)	Wilkinson et al. (2021)
Fish-eating bat (<i>M. vivesi</i>)	Wilkinson et al. (2021)
Greater horseshoe bat (<i>Rhinolophus ferrumequinum</i>)	Wilkinson et al. (2021)
Greater mouse-eared bat (<i>M. myotis</i>)	Wilkinson et al. (2021)
Greater sac-winged bat (<i>Saccopteryx bilineata</i>)	Wilkinson et al. (2021)
Greater spear-nosed bat (<i>Phyllostomus hastatus</i>)	Wilkinson et al. (2021)
Grey-headed flying fox (<i>Pteropus poliocephalus</i>)	Wilkinson et al. (2021)
Indian flying fox (<i>P. giganteus</i>)	Wilkinson et al. (2021)
Jamaican fruit bat (<i>Artibeus jamaicensis</i>)	Wilkinson et al. (2021)
Large flying fox (<i>P. vampyrus</i>)	Wilkinson et al. (2021)
Lesser short-nosed fruit bat (<i>Cynopterus brachyotis</i>)	Wilkinson et al. (2021)
Little brown bat (<i>M. lucifugus</i>)	Wilkinson et al. (2021)
Little golden-mantled flying fox (<i>P. pumilus</i>)	Wilkinson et al. (2021)
Mexican free-tailed bat (<i>Tadarida brasiliensis</i>)	Wilkinson et al. (2021)
Pale spear-nosed bat (<i>Phyllostomus discolor</i>)	Wilkinson et al. (2021)
Pallid bat (<i>Antrozous pallidus</i>)	Wilkinson et al. (2021)
Proboscis bat (<i>Rhynchonycteris naso</i>)	Wilkinson et al. (2021)
Rodrigues flying fox (<i>P. rodricensis</i>)	Wilkinson et al. (2021)
Seba's short-tailed bat (<i>Carollia perspicillata</i>)	Wilkinson et al. (2021)
Small flying fox (<i>P. hypomelanus</i>)	Wilkinson et al. (2021)
Southern long-nosed bat (<i>Leptonycteris curasoae</i>)	Wilkinson et al. (2021)
Straw-coloured fruit bat (<i>Eidolon helvum</i>)	Wilkinson et al. (2021)
Velvety free-tailed bat (<i>Molossus molossus</i>)	Wilkinson et al. (2021)

Table A1.1: Continued.

Carnivores	References
Multi-tissue DNA methylation ageing clocks for sea lions, walruses, and seals	Robeck et al. (2023)
Ringed seal (<i>Phoca hispida</i>)	Sareisian (2014)
Bobcat (<i>Lynx rufus</i>)	Cantrell et al. (2020)
Cheetah (<i>Acinonyx jubatus</i>)	Raj et al. (2021)
Domestic cat (<i>Felis catus</i>)	Qi (2025); Qi et al. (2021); Raj et al. (2021)
Domestic dog (<i>Canis familiaris</i>)	Horvath, Lu, Haghani, Zoller, et al. (2022); Thompson et al. (2017); Wang et al. (2020)
Tsushima leopard cats (<i>Prionailurus bengalensis euptilurus</i>)	Qi et al. (2021)
Vermont bobcat (<i>Lynx rufus</i>)	Lachance et al. (2015)
Fisher (<i>Pekania pennanti</i>)	Lachance et al. (2015)
Gray wolf (<i>Canis lupus</i>)	Thompson et al. (2017)
Lion (<i>Panthera leo</i>)	Qi (2025); Raj et al. (2021)
Jaguar (<i>P. onca</i>)	Qi (2025)
Amur leopard (<i>P. pardus orientalis</i>)	Qi (2025)
Snow leopard (<i>P. uncia</i>)	Qi (2025); Qi et al. (2021)
Tiger (<i>P. tigris</i>)	Qi (2025); Raj et al. (2021)
American black bear (<i>Ursus americanus</i>)	Czajka et al. (2024)
Elephants	
African elephant (<i>Loxodonta africana</i>)	Prado et al. (2021)
Asian elephant (<i>Elephas maximus</i>)	Prado et al. (2021)
Marsupials	
Eastern grey kangaroo (<i>Macropus giganteus</i>)	Horvath, Haghani, Zoller, Raj, et al. (2022)
Gray short-tailed opossum (<i>Monodelphis domestica</i>)	Horvath, Haghani, Zoller, Raj, et al. (2022)
Red kangaroo (<i>M. rufus</i>)	Horvath, Haghani, Zoller, Raj, et al. (2022)
Red-necked wallaby (<i>M. rufogriseus</i>)	Horvath, Haghani, Zoller, Raj, et al. (2022)
Tasmanian devil (<i>Sarcophilus harrisii</i>)	Horvath, Haghani, Zoller, Raj, et al. (2022)
Western grey kangaroo (<i>M. fuliginosus</i>)	Horvath, Haghani, Zoller, Raj, et al. (2022)
Primates	
Pan-primate methylation clock	Horvath et al. (2021)
Baboon (<i>Papio cynocephalus</i> and <i>P. anubis</i>)	Anderson et al. (2021); Goldman et al. (2022)
Chimpanzee (<i>Pan troglodytes</i>)	Guevara et al. (2020); Ito et al. (2018); Pai et al. (2011)
Common marmoset (<i>Callithrix jacchus</i>)	Horvath, Zoller, Haghani, Lu, et al. (2021)
Rhesus macaque (<i>Macaca mulatta</i>)	Bronk et al. (2024); Goldman et al. (2022); Horvath, Zoller, Haghani, Jasinska, et al. (2021)
Vervet monkey (<i>Chlorocebus pygerythrus</i>)	Jasinska et al. (2022)

Table A1.1: Continued.

Rodents	References
Mouse (<i>Mus musculus</i>)	Hanski et al. (2024); Meer et al. (2018); Minter et al. (2022); Perez-Correa et al. (2022); Spiers et al. (2016); Stubbs et al. (2017)
Rat (<i>Rattus norvegicus</i>)	Levine et al. (2020)
Naked mole-rat (<i>Heterocephalus glaber</i>)	Horvath, Haghani, Macoretta, Ablaeva, et al. (2022); Kerepesi et al. (2022); Lowe et al. (2020)
Wood mouse (<i>Apodemus sylvaticus</i>)	Little et al. (2020); Wolf et al. (2025)
Yellow-bellied marmots (<i>Marmota flaviventris</i>)	Pinho et al. (2022)
Prairie voles (<i>Microtus ochrogaster</i>)	Sailer et al. (2020), (2024)
Shrews	
Masked shrew (<i>Sorex cinereus</i>)	Cossette et al. (2023)
Ungulates	
Cattle (<i>Bos taurus</i>)	Caulton et al. (2021); Kordowitzki et al. (2021)
Common zebra (<i>Equus quagga</i>)	Larison et al. (2021)
Domestic horse (<i>E. caballus</i>)	Horvath, Haghani, Peng, Hales, et al. (2022); Larison et al. (2021)
Domestic pig (<i>Sus scrofa domesticus</i>)	Schachtschneider et al. (2020), (2021)
Domestic sheep (<i>Ovis aries</i>)	Caulton et al. (2021); Sugrue et al. (2021)
Grévy's zebra (<i>E. grevyi</i>)	Larison et al. (2021)
Roe deer (<i>Capreolus capreolus</i>)	Lemaître et al. (2020)
Somali wild ass (<i>E. africanus somaliensis</i>)	Larison et al., (2021)
White-tailed deer (<i>Odocoileus virginianus</i>)	Czajka et al. (2024)
Red deer (<i>Cervus elaphus</i>)	Caulton et al. (2021)
Waipiti deer (<i>C. canadensis</i>)	Caulton et al. (2021)
Mountain goat (<i>Oreamnos americanus</i>)	Czajka et al. (2024)
Domestic goat (<i>Capra hircus</i>)	Caulton et al. (2021)

Appendix 2

Dental ageing offers new insights to the first epigenetic clock for common dolphins (*Delphinus delphis*)

1. Extended results

1.1. Age predictions based on other odontocetes clocks

Age predictions for our samples, generated using both multi-species and species-specific odontocete clocks from the Clock Database, are presented in Figure A2.3 and Table A2.4. The multi-species odontocete clock (Robeck, Fei, Haghani, et al., 2021) predicted the ages of our samples with correlation coefficients of $r = 0.79$ for the ‘relaxed’, $r = 0.80$ for the ‘strict’, and $r = 0.85$ for the ‘restricted’ subset, and median absolute errors (MAEs) of 11.80, 11.84, and 12.53 years, respectively. The use of the multi-species odontocete blood and skin clock resulted in comparable MAE values, but lower age correlations (Appendix 2, Table A2.4). When comparing our results with those of other species-specific clocks, the *Cephalorhynchus* clock (*C. hectori hectori* and *C. h. maui*; Hernandez et al., 2023) performed best for our samples in terms of MAE between actual and predicted age, with values ranging from 2.71 to 3.01 years depending on the subset of samples used (Appendix 2, Table A2.4). While both multi-species clocks showed a tendency to overestimate the ages of our samples, the *Cephalorhynchus* clock consistently underestimated samples above 6 years of age.

2. Extended discussion

2.1. Age predictions based on other odontocetes clocks

For the ‘relaxed’ subset, the skin and the skin/blood multi-species odontocete clock resulted in a similar MAE of 11.8 and 11.54 years, respectively (Figure A2.3 & Table A2.4). However, the skin clock ($r = 0.79$) demonstrated a better correlation between dental and predicted age, compared to the skin and blood clock ($r = 0.68$). This is surprising since methylation rates are highly conserved in blood and blood-based clocks are considered more precise (Robeck, Fei, Haghani, et al., 2021; Robeck, Fei, Lu, et al., 2021). Age prediction would have been expected to improve when applying the combined skin and blood clock to our samples. However, similar

observations have been previously reported, and it was assumed that the differences between the skin epigenome of different species might be larger than differences in the blood epigenome (Peters et al., 2023). Both, the skin and the skin/blood multi-species odontocete clocks showed the tendency of overestimating the age of our samples. While samples of common dolphins were used in the construction of the multispecies odontocete clock, the sample size was limited (skin samples: $n = 3$, blood samples: $n = 3$) and samples of longer-lived odontocetes such as bottlenose dolphins (*Tursiops truncatus*) were more strongly represented (skin samples: $n = 41$, blood samples: $n = 140$; Robeck, Fei, Lu, et al., 2021). This might explain the overestimation of age in our samples due to differences in life-history features and maximum lifespan. Our findings align with previous work (Barratclough et al., 2021; Peters et al., 2023) suggesting that the accuracy of epigenetic age estimation can be enhanced through the development of species-specific clocks.

When testing other available odontocete clocks, the epigenetic clock for *Cephalorhynchus* (*C. hectori hectori* and *C. h. maui*; Hernandez et al., 2023) performed best in estimating the age of our samples with a MAE of 3.01 for the ‘relaxed’ subset and a correlation of $r = 0.79$. However, the clock consistently underestimated the age of our samples for individuals with an age >6 years. For Hector’s dolphins, there is a lack of knowledge on life-history features, such as age at sexual maturity and reproductive rates (Hernandez et al., 2023). While currently unknown, lifespan has been estimated to be around 20 years (Hernandez et al., 2023; Slooten, 1991; Slooten & Lad, 1991). Given that the *Cephalorhynchus* clock is calibrated for a faster-ageing species, this may account for the underestimation observed in our common dolphin samples, which are believed to have a maximum lifespan of around 30 years (Perrin, 2009). While age at attainment of sexual maturity of Hector’s dolphins is currently unknown (Hernandez et al., 2023), our results suggest a similar ageing trajectory for Hector’s and common dolphins up until the age of 6. The similarity in epigenetic age estimates between Hector’s and common dolphins up to age 6 may reflect conserved early-life developmental processes, with divergence emerging later due to differences in timing of maturity and species-specific ageing trajectories.

2.2. Error metrics reported by previous literature

Error metrics reported by previously published odontocetes clocks are shown in Table A2.5. These clocks were developed using a range of tissue types, including skin, blood, multi-tissue, and faeces; however, our comparison focuses specifically on skin-based clocks. Clocks

calibrated using growth layer groups (Bors et al., 2021; Hernandez et al., 2023; Mori et al., 2024) report MAEs equivalent to approximately $\pm 10.45\%$ of maximum life expectancy (MLE) for Hector's and Māui dolphins (MLE: 20 years; Slooten, 1991) and $\pm 5\%$ for beluga whales (MLE: 60–70 years; Suydam, 2009). No median absolute error was reported for Risso's dolphins (Mori et al., 2024). The predictive performance of these clocks is reflected in a Pearson correlation coefficient of $r = 0.87$ for Hector's and Māui dolphins (Hernandez et al., 2023), a coefficient of determination of $R^2 = 0.74$ for beluga whales and an R^2 of 0.71 for Risso's dolphins (Mori et al., 2024). Clocks developed using long-term observational data show lower relative errors: Peters et al. (2023) reported an MAE of $\pm 3.13\text{--}4.2\%$ ($r = 0.86$) for Indo-Pacific bottlenose dolphin (MLE: 40–50 years; Wang & Yang, 2009), while Parsons et al. (2023) reported $\pm 2.25\%$ ($r = 0.96$) for killer whales (MLE: up to 90 years; Foote, 2008). In another observational study of bottlenose dolphins, Beal et al. (2019) reported an R^2 of 0.78, though no MAE was provided. A clock developed using samples from animals in human care achieved an MAE of $\pm 4.9\%$ of maximum life expectancy for bottlenose dolphins (MLE: 51.6 years; Weigl, 2005) and a correlation of $r = 0.95$ (Robeck, Fei, Haghani, et al., 2021).

2.3. User guide and limitations

This epigenetic clock provides a robust framework for age estimation in common dolphins (*Delphinus delphis*). It shows the highest accuracy within the age range represented by the majority of our training data (up to ~16 years), while estimates for older individuals remain informative but increasingly underestimate chronological age. This underestimation likely reflects both sparse representation of older dolphins in the calibration dataset and a biological slowing of methylation changes with age. Neither decomposition state nor storage duration influenced prediction accuracy, indicating that these factors did not confound our dataset and that the clock is likely applicable to live animals. Although the calibration dataset was derived from New Zealand dolphins, the broader utility of this clock is supported by the demonstrated reliability of epigenetic clocks in other species, which have proven consistent across populations and even transferable among closely related taxa.

3. Supplementary tables

Table A2.1: Common dolphin (*Delphinus delphis*) sample IDs included in the ‘relaxed’, ‘strict’, and ‘restricted’ subsets used for epigenetic clock model development. For each sample, the dental age and sex are provided, as well as the decomposition condition category (DCC), indicating carcass preservation at the time of sampling (IJseldijk et al., 2019).

ID	DCC	Dental Age	Sex	Subset		
				‘Relaxed’	‘Strict’	‘Restricted’
1	2	6	F	✓	✓	✓
2	2	19	M	✓	✓	✓
3	2	20	F	✓	✓	✓
4	N/A	13	M	✓	✓	✓
5	2	8	M	✓	✓	✓
6	2-3	25	F	✓	✓	✓
7	2	13	F	✓	✓	✓
8	3	9	F	✓	✓	✓
9	2	15	F	✓	✓	✓
10	2	7	F	✓	✓	✓
11	2	14	F	✓	✓	✓
12	1	16	F	✓	✓	✓
13	2	1	F	✓	✓	✓
14	2	3	F	✓	✓	✓
15	1	8	M	✓	✓	✓
16	2	11	F	✓	✓	✓
17	2	0	M	✓	✓	✓
18	2	2	F	✓	✓	✓
19	2-3	4	F	✓	✓	✓
20	2	0.75	M	✓	✓	✓
21	3	4	M	✓	✓	✓
22	2	1	M	✓	✓	✓
23	1	6	M	✓	✓	✓
24	3	6	M	✓	✓	✓
25	2	17	M	✓	✓	✓
26	1	2	M	✓	✓	✓

Table A2.1: Continued.

ID	DCC	Dental Age	Sex	Subset		
				<i>'Relaxed'</i>	<i>'Strict'</i>	<i>'Restricted'</i>
27	2	2	F	✓	✓	✓
28	2	11	M	✓	✓	✓
29	2	1.5	M	✓	✓	✓
30	2	2.5	M	✓	✓	✓
31	2	5.5	M	✓	✓	✓
32	2	16	M	✓	✓	✓
33	2	1	F	✓	✓	✓
34	3	3	F	✓	✓	✓
35	3	18	F	✓	✓	✓
36	3	14	F	✓	✓	✓
37	3	26	M	✓	✓	✓
38	2	0.5	F	✓	✓	✓
39	2	4	F	✓	✓	✓
40	2-3	0.5	F	✓	✓	✓
41	3	7.5	M	✓	✓	✓
42	2	11.5	M	✓	✓	✓
43	2	4	M	✓	✓	✓
44	2-3	7	F	✓	✓	✓
45	2-3	9	M	✓	✓	✓
46	2-3	5.5	F	✓	✓	✓
47	3	10	F	✓	✓	✓
48	2-3	12	F	✓	✓	✓
49	2	0.25	M	✓	✓	✓
50	2	11	F	✓	✓	✓
51	2	12	F	✓	✓	✓
52	2-3	15	F	✓	✓	✓
53	2	18	F	✓	✓	✓
54	2-3	8	F	✓	✓	✓
55	2	9	F	✓	✓	✓
56	1	20	F	✓	✓	✓
57	1	13	F	✓	✓	✓

Table A2.1: Continued.

ID	DCC	Dental Age	Sex	Subset		
				<i>'Relaxed'</i>	<i>'Strict'</i>	<i>'Restricted'</i>
58	2	14	F	✓	✓	✓
59	2	17	F	✓	✓	✓
60	1	16.5	M	✓	✓	✓
61	2	9.5	M	✓	✓	✓
62	2	11.5	M	✓	✓	✓
63	2	12	M	✓	✓	✓
64	2	22.5	F	✓	✓	X
65	1	29	F	✓	✓	X
66	3	18	F	✓	✓	X
67	2-3	16	F	✓	✓	X
68	3	14	M	✓	✓	X
69	2	12	M	✓	✓	X
70	3	11.5	M	✓	✓	X
71	1	21	M	✓	✓	X
72	2	31	M	✓	✓	X
73	1	24	M	✓	✓	X
74	2	≥ 34	F	✓	X	X
75	3	≥ 31	F	✓	X	X

Table A2.2: Sample quality control procedures applied to ensure data integrity for downstream DNA methylation analysis in common dolphins (*Delphinus delphis*). Samples were excluded based on probe detection p-values (>1% failed probes; fraction outliers), principal component analysis (PCA), and hierarchical clustering (HC), which were performed to identify technical outliers and samples with aberrant DNA methylation profiles. Additionally, sample KS19-23Dd was excluded as a life-history anomaly based on pectoral fin radiography and ovarian *corpora albicans* counts. DCC refers to the tissue decomposition condition category, indicating carcass preservation at the time of sampling (IJsseldijk et al., 2019).

ID	DCC	PCA Outlier	Fraction Outlier	HC Outlier	Life-history outlier
76	N/A	Yes	-	-	-
77	2	Yes	-	-	-
78	3	Yes	-	-	-
79	N/A	Yes	-	-	-
80	2	Yes	Yes	Yes	-
81	2	-	Yes	-	-
82	3	Yes	Yes	Yes	-
83	2	Yes	-	-	-
84	2-3	-	-	-	Yes

Table A2.3: Cytosine-phosphate-guanine (CpG) sites retained by the elastic net regression models for estimating epigenetic age in common dolphins (*Delphinus delphis*) for the ‘relaxed’, ‘strict’, and ‘restricted’ subsets. The subsets included different samples, as detailed in Table A2.1.

CpG site	Subset		
	‘Relaxed’	‘Strict’	‘Restricted’
Intercept	2.707102179	1.776196521	2.187039858
Probe-ID	Model coefficient	Model coefficient	Model coefficient
cg00008998	-0.174793681	-0.16416	-0.30144
cg01167424	-0.039862449	-	-0.01709
cg01814115	-0.394351964	-0.60808	-0.7008
cg02909927	-0.015346173	-	-
cg03738429	-0.391731339	-0.2613	-
cg04010581	-0.035275789	-0.10929	-0.10229
cg04051518	-0.130802155	-	-0.21781
cg04905851	-0.06589915	-	-
cg06796713	-	-	-0.34456
cg07804445	-0.592141011	-0.55117	-0.46492
cg08377921	-	-	-
cg08550913	-	-	-1.97353
cg08611749	-0.158115072	-0.00516	-
cg09058643	-0.167154015	-	-
cg09363187	-	0.176106	-
cg09461098	-0.680099807	-0.81789	-1.52703
cg09916017	0.235893107	-	-
cg10277282	0.040840017	-	-
cg11116727	-0.333141023	-0.38212	-0.11971
cg11260459	-	-	-0.36367
cg12053353	-0.229100545	-0.26633	-
cg12373771	-	-	0.021864
cg12584622	-0.026700007	-0.01643	-
cg13060540	-0.170448176	-0.13771	-
cg13260846	0.036436683	-	0.069161
cg13325480	-	-	-0.21954
cg14142471	-	-	-0.20964
cg15303324	-0.119960222	-0.19948	-
cg17037716	-0.011535008	-	-
cg17696987	-0.014918802	-	-

Table A2.3: Continued

CpG site	Subset		
	<i>'Relaxed'</i>	<i>'Strict'</i>	<i>'Restricted'</i>
Probe-ID	Model coefficient	Model coefficient	Model coefficient
cg18271715	-	-	-0.26503
cg18583520	-1.1124165	-0.64806	-
cg18734357	-	-	-0.01144
cg19355776	-	-	-0.0793
cg19591642	-0.080327164	-	-
cg20490705	-	-	-0.01144
cg20582188	-0.534397174	-0.56815	-0.70342
cg21454760	-0.125015485	-0.21864	-
cg21898068	-0.072178198	-0.08277	-
cg22189866	-0.344160257	-	-
cg22423049	-0.052793821	-	-
cg22572209	-0.462827113	-0.22744	-
cg23170538	-0.274028766	-0.01244	-
cg24888049	-0.168501987	-	-
cg25184118	-0.090402011	-	-
cg25254739	-0.399436757	-0.48822	-0.33018
cg25402449	-	-0.05742	-
cg26186239	0.002794655	-	-
cg26631126	-0.552342767	-0.76605	-0.78668
cg27038395	0.080454226	0.276721	-
cg27395516	-0.548172671	-0.27504	-

Table A2.4: Error metrics for age predictions of common dolphin (*Delphinus delphis*) samples using previously published odontocete epigenetic clocks. Metrics are reported separately for models trained on skin (S) and blood (B) tissue where applicable. Performance is summarised as median absolute error (MAE) and correlation coefficient (r), providing an assessment of how accurately these external models predict age in our dataset. The subsets included different samples, as detailed in Table A2.1.

Epigenetic Clock Name	Reference	Tissue	'Relaxed'		'Strict'		'Restricted'	
			MAE	r	MAE	r	MAE	r
Coef.OdontoceteSkinAge.LogLinear2	Robeck, Fei, Lu, et al., (2021)	S	11.8	0.79	11.84	0.8	12.53	0.85
Coef.OdontoceteBloodSkinAge.LogLinear2	Robeck, Fei, Lu, et al., (2021)	B/S	11.54	0.68	11.67	0.69	12.46	0.73
Coef.CetaceanCephalorhynchusHectoriHectoriSkinAge.LogLinear2	Hernandez et al., (2023)	S	3.01	0.79	2.91	0.79	2.71	0.87
Coef.CetaceanDelphinapterusLeucasSkinAge.LogLinear2	Bors et al., (2021)	S	7.09	0.77	7.05	0.79	6.62	0.86
Coef.Killerwhale.Skin	Parsons et al., (2023)	S	25.09	0.77	25.41	0.78	25.41	0.84
Coef.IndoPacificBottlenoseSkinClock	Peters et al., (2023)	S	4.09	0.67	4.03	0.7	3.71	0.74
Coef.BottlenoseSkin	Barratclough et al., (2021)	S	48.39	0.21	48.43	0.18	48.68	0.18
Coef.Bottlenose	Barratclough et al., (2021)	B/S	27.12	0.6	27.19	0.62	27.45	0.67
Coef.BottlenoseSkinAge.LogLinear2	Robeck, Fei, Haghani, et al., (2021)	S	5.32	0.68	5.23	0.69	4.75	0.77
Coef.BottlenoseBloodSkinAge.LogLinear2	Robeck, Fei, Haghani, et al., (2021)	B/S	31.03	0.64	31.25	0.66	31.32	0.71

Table A2.5: Error metrics reported in published studies for species-specific odontocete epigenetic clocks. Reported metrics include median absolute error (MAE, in years), Pearson’s correlation coefficient (r), residual error, and coefficient of determination (R^2), reflecting the accuracy of chronological age predictions in the respective datasets. Sample size (n) and the tissue type used for model calibration (skin or blood) are also listed for context. These values provide a comparative basis for evaluating the performance of the common dolphin (*Delphinus delphis*) clock developed in this study.

Species	Tissue	Method of calibration	n	MAE	Residual error	r	R^2	Reference
<i>C. h. hectori</i>	Skin	GLGs	48	2.09	-	0.87	-	Hernandez et al., (2023)
<i>D. leucas</i>	Skin	GLGs	67	2.9	-	-	0.74	Bors et al., (2021)
<i>G. griseus</i>	Skin	GLGs	30	-	-	-	0.71	Mori et al., (2024)
<i>T. aduncus</i>	Skin	Observational	65	2.1	-	0.86	-	Peters et al., (2023)
<i>T. truncatus</i>	Skin	Observational	39	-	4.83	-	0.78	Beal et al., (2019)
<i>T. truncatus</i>	Skin	In human care	87	2.53	-	0.95	-	Robeck, Fei, Haghani, et al., (2021)
<i>O. orca</i>	Skin	Observational	131	2.26	-	0.96	-	Parsons et al., (2023)
<i>T. truncatus</i>	Blood	In human care	140	1.46	-	0.97	-	Robeck, Fei, Haghani, et al., (2021)
<i>T. truncatus</i>	Blood/Multi-tissue	In human care	110	2.5	-	-	0.95	Barratclough et al., (2021)
<i>T. truncatus</i>	Multi-tissue	In human care	227	2.13	-	0.93	-	Robeck, Fei, Haghani, et al., (2021)
<i>T. aduncus</i>	Faeces	Observational	36	5.08	-	-	0.33	Yagi et al., (2024)

4. Supplementary figures

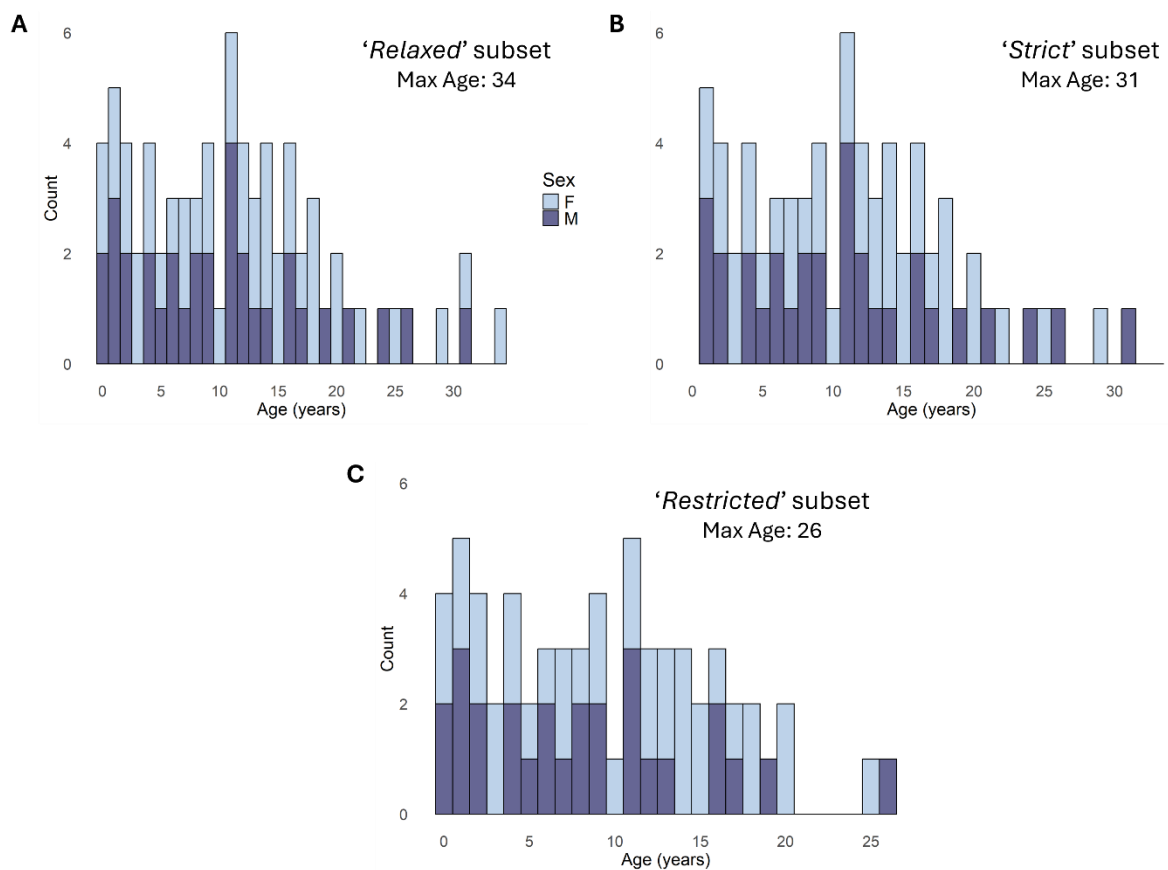


Figure A2.1: Dental age distributions by sex for common dolphins (*Delphinus delphis*) in the A) 'relaxed', B) 'strict', and C) 'restricted' subsets used for epigenetic model development. Max Age = maximum age in each subset.

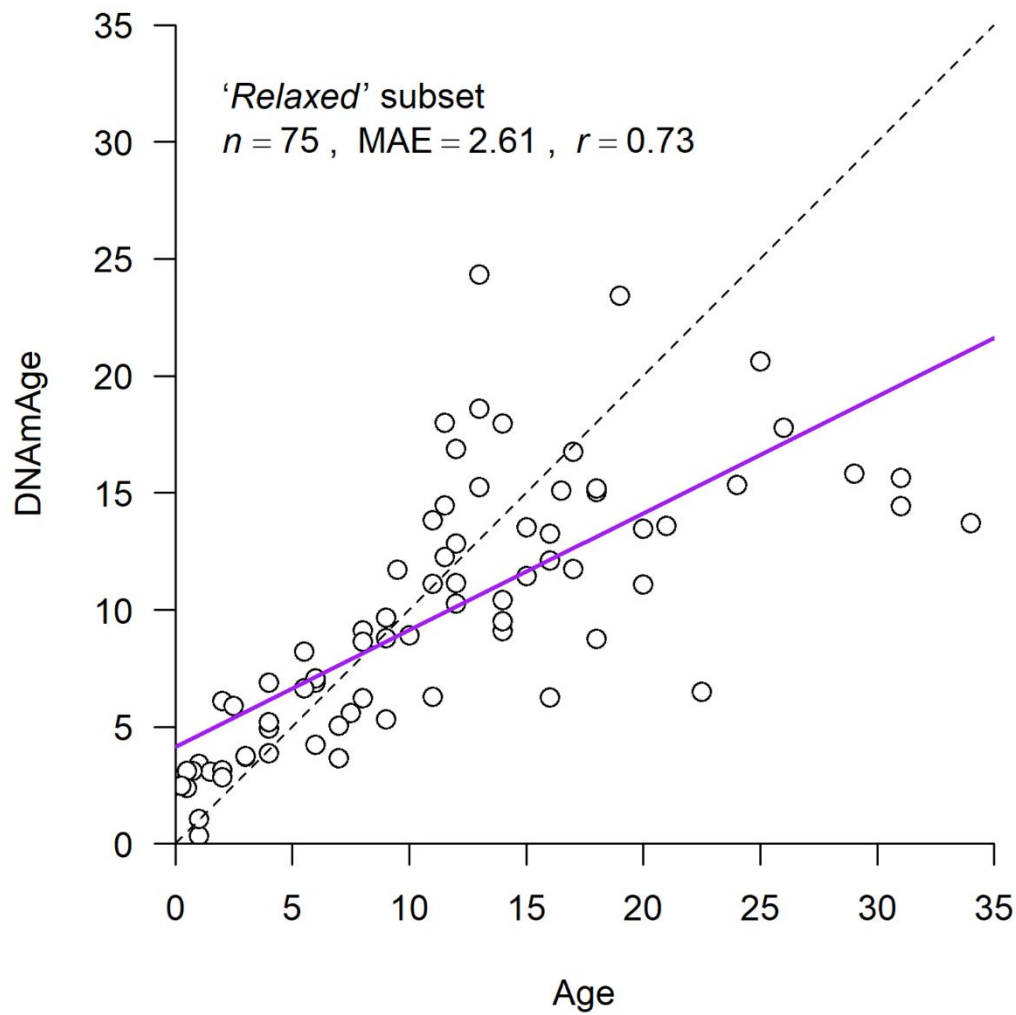


Figure A2.2: Relationship between observed chronological age and DNA methylation-predicted age (DNAmAge) for common dolphin (*Delphinus delphis*) in the ‘relaxed’ subset, estimated using a hybrid model. The hybrid approach combined a random forest classifier (RFC) to assign physical maturity status and two elastic net regression (ENR) models: one trained on all individuals and one on physically mature dolphins only. Results are based on 5-fold cross-validation, with RFC and ENR models retrained in each fold to avoid overfitting. Each point represents an individual dolphin; the solid purple line shows the fitted linear regression between observed and predicted ages, and the dashed line marks the 1:1 line of perfect prediction. Model performance metrics, such as sample size (n), median absolute error (MAE), and Pearson’s correlation coefficient (r), are displayed in the plot title.

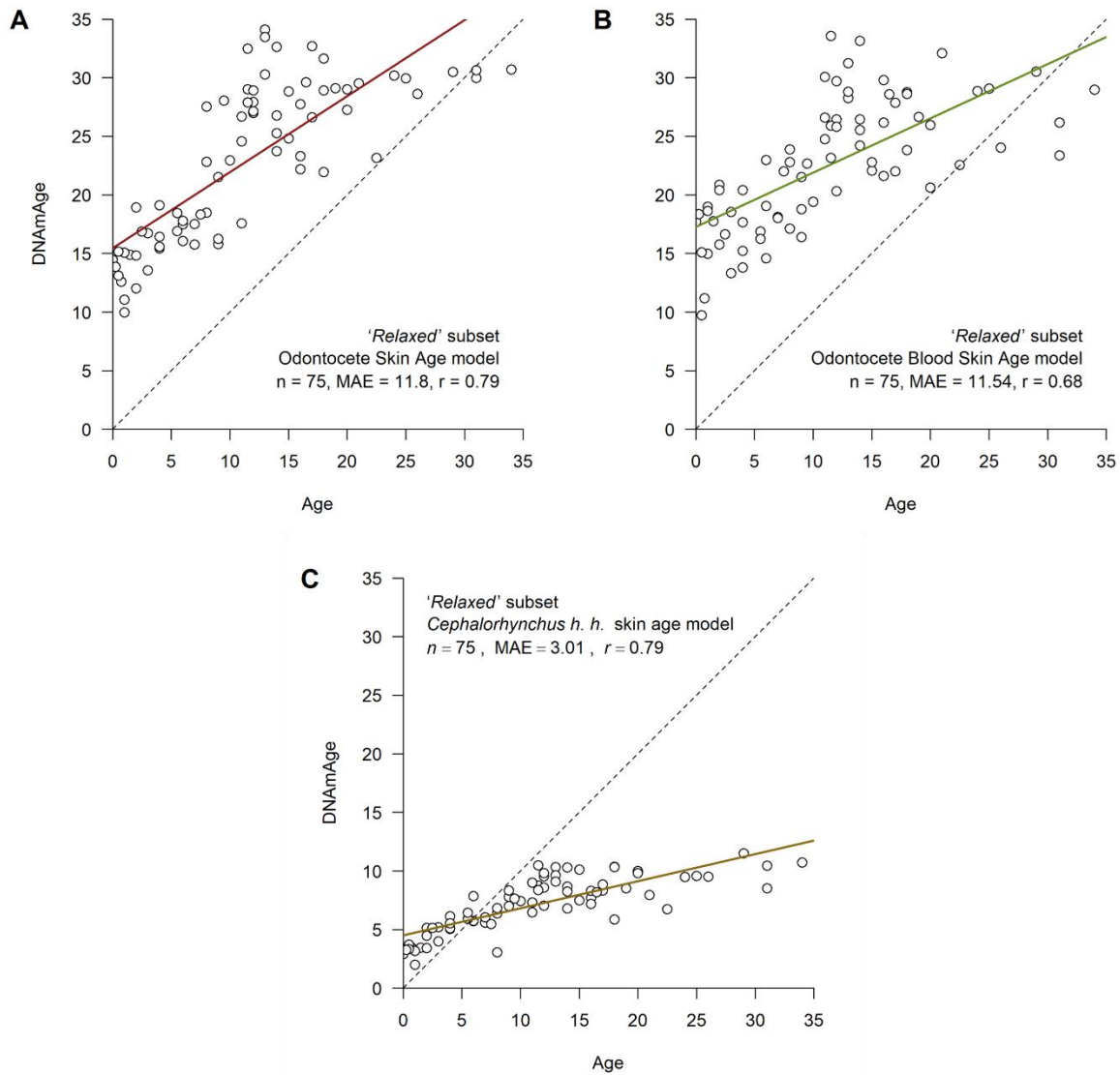


Figure A2.3: Epigenetic ages (DNAmAge) calculated for common dolphins (*Delphinus delphis*) from the 'relaxed' subset using the **A)** skin clock for multi-species odontocetes (Robeck, Fei, Lu, et al., 2021), **B)** skin and blood clock for multi-species odontocetes (Robeck, Fei, Lu, et al., 2021) and **C)** the *Cetacean Cephalorhynchus hectori hectori* Skin Clock (Hernandez et al., 2023). Regression lines are shown in colour. The dotted diagonal indicates a perfect agreement with $y = x$. Individual animals are represented by green dots. Pearson correlation (r) and median absolute error are given for each model.

Appendix 3

Cross-species applicability of a bone-ageing method in delphinids

1. Supplementary tables

Table A3.1: Spearman correlations between dental age and total ossification scores in common dolphins (*Delphinus delphis*). The total score was calculated as the sum across all bone locations; differences between methods relate only to the handling of ‘D2P2’ and ‘D3P2’: (i) *Maximum*: each bone represented by the higher of its proximal and distal epiphysis scores; (ii) *Average*: each bone represented by the mean of proximal and distal epiphysis scores; (iii) *Separate epiphyses*: proximal and distal epiphyses of each bone scored separately. Correlations and *p*-values are shown by sex and flipper side.

Method of Total Score Calculation	Sex	Side	Spearman ρ	<i>p</i> -value
Separate epiphyses	F	L	0.9205	5.46×10^{-27}
	F	R	0.9199	6.73×10^{-27}
Maximum	F	L	0.9217	3.49×10^{-27}
	F	R	0.9209	4.75×10^{-27}
Average	F	L	0.9216	3.55×10^{-27}
	F	R	0.9212	4.12×10^{-27}
Min	F	L	0.9210	4.52×10^{-27}
	F	R	0.9205	5.35×10^{-27}
Separate epiphyses	M	L	0.9481	1.56×10^{-28}
	M	R	0.9470	2.66×10^{-28}
Maximum	M	L	0.9489	1.04×10^{-28}
	M	R	0.9482	1.44×10^{-28}
Average	M	L	0.9479	1.66×10^{-28}
	M	R	0.9473	2.28×10^{-28}
Min	M	L	0.9477	1.89×10^{-28}
	M	R	0.9468	2.88×10^{-28}

2. Supplementary figures

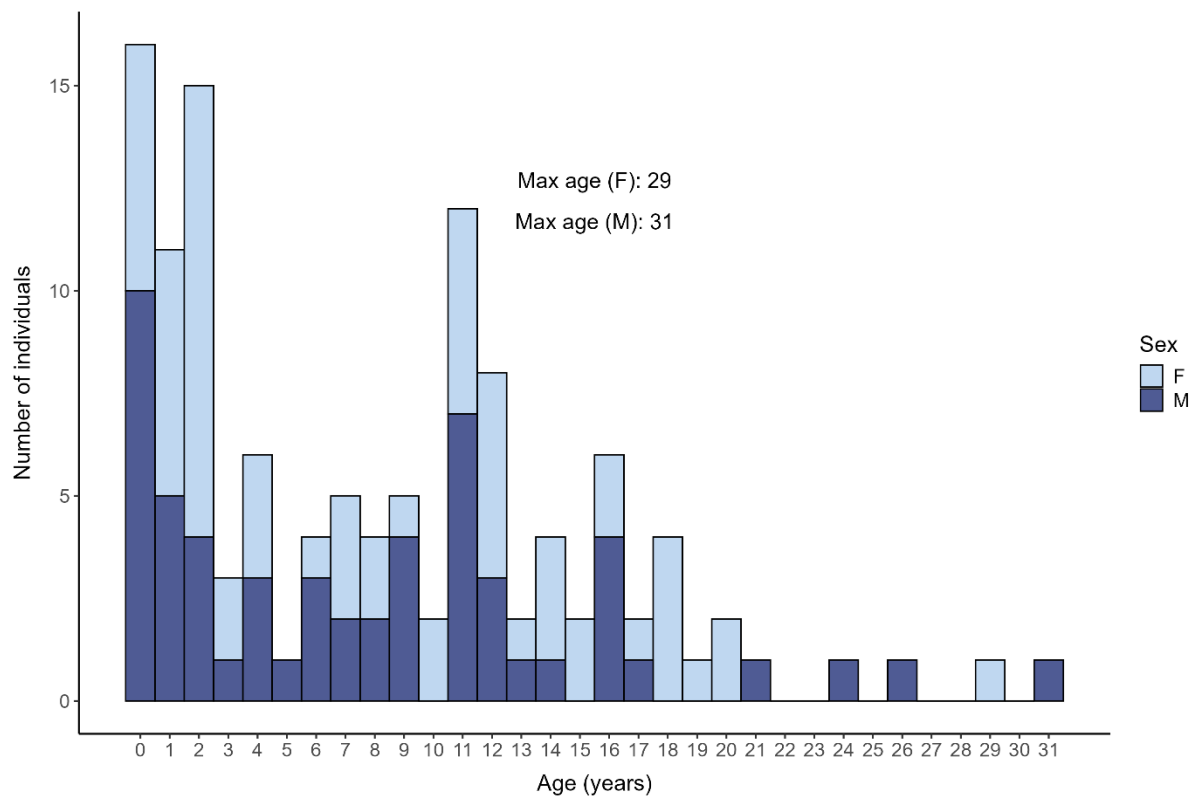


Figure A3.1: Dental age distributions by sex for common dolphins (*Delphinus delphis*) for which pectoral flipper bone maturation was assessed. Dataset shown does not include foetuses.

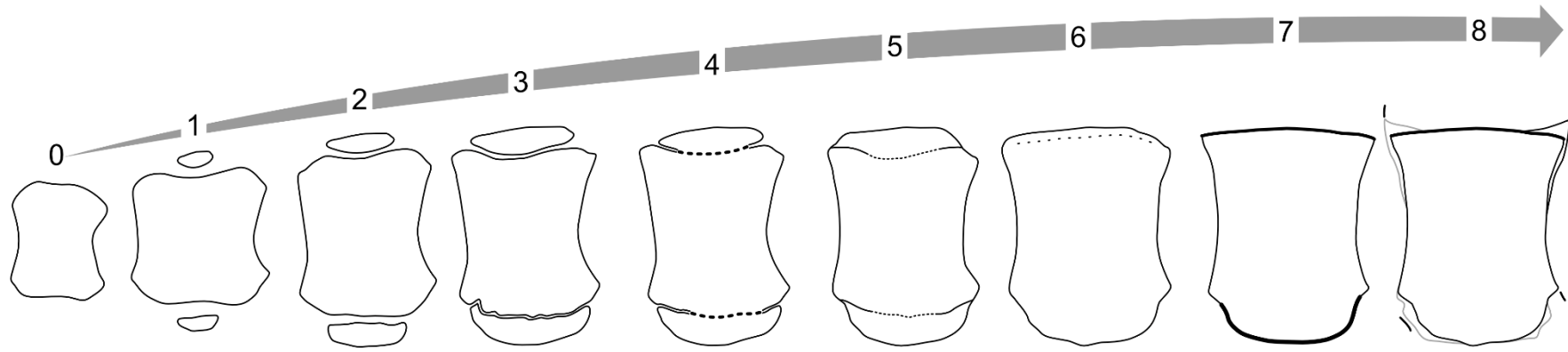


Figure A3.2. Long bone scoring scheme, reprinted from Barratclough, Sanz-Requena et al., (2019) and illustrated by Veronica Candejas. **Score –1 (not shown):** No primary ossification centre visible. **Score 0:** The primary centre is present, but no secondary ossification centre is visible. **Score 1:** An epiphyseal ossification centre is present but only occupies <50% of the width of the adjacent metaphysis. **Score 2:** The secondary ossification centre is well established, ranging from 50–100% of the metaphyseal width. The physis is evident as a distinct radiolucent space, while the secondary centre appears less mature and irregular in depth across its width. Maturation progresses in a midline-to-abaxial direction. **Score 3:** Thinning of the radiolucent physis but no evidence of fusion of the metaphysis and the secondary ossification centre. Mineral density increases in the subchondral bone on both sides of the physis. Late stage 2 can resemble early stage 3; the distinction lies in whether physis width is irregular (stage 2) or uniform (stage 3). **Score 4:** Osseous bridges begin to form; in the radius and ulna this starts midline and proceeds abaxially. The abaxial margins remain open, and both early bridge formation and >90% bridging qualify as stage 4. **Score 5:** Complete closure is present, accompanied by a faint hyperdense “ghost” physal line or epiphyseal scar spanning at least 50% of the plate. The epiphyseal plate may not reach the full diaphyseal length, but closure is complete. **Score 6:** The physal line is remodelled, with <50% to no evidence of the hypermineralised transverse remnant, and the epiphyseal plate extends across the full diaphyseal length. **Score 7:** Complete fusion occurs with no evidence of a previous ghost physal plate and flattening of the physal surfaces with accentuated pointed edges. **Score 8:** Arthritic changes are visible, including osteophytes, osteolysis, articular calcification, and in some cases fusion of metacarpals or phalanges.

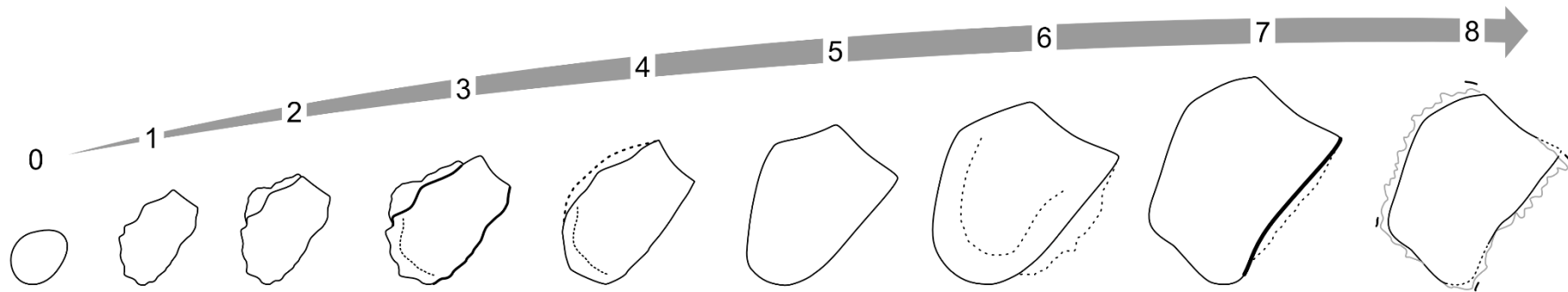


Figure A3.3. Delta bone scoring scheme, reprinted from Barratclough, Sanz-Requena et al. (2019) and illustrated by Veronica Candejas. **Stage -1:** Not present. **Stage 0:** Very small oval shape, undefined delta shape. **Stage 1:** Axial surface starts to be linear, consistent with a delta shape. **Stage 2:** One flat surface. Areas of slight irregular perimetral surface typically on the proximal side with a degree of adjacent mineralisation which can oppose the epiphyseal surface but not necessarily show definitive osseous bridge formation. Bone density is clearly reduced in peripheral new bone in comparison to the primary ossification centre. **Stage 3:** Increased mineralisation of adjacent proximal cartilaginous surface, consolidation may not be present. **Stage 4:** Area increases in consolidation and mineralisation, slightly less dense than the body of the delta bone. A defined hypermineralised physal plate line is present. The abaxial aspect can show the initiation of the new straight lateral border rather than a horseshoe shape. **Stage 5:** Density of the secondary ossification centre is similar to primary bone and initial mineralisation is present on the distal surface, with the proximal side showing a defined straight lateral border rather than a horseshoe shape. Individual variation may result in non-linear borders; therefore, emphasis should be placed on the secondary ossification centres on the proximal and distal surfaces. **Stage 6:** Smooth proximal surface with matching density to the primary ossification centre, distal surface still shows some incongruent mineralisation. Physal line is still visible, denoted by a dotted line. **Stage 7:** Smooth proximal surface with complete consolidation of the secondary ossification centre on both sides and dissolution of the physal line. **Stage 8:** Degenerative changes present.

Appendix 4

Radiographic assessment of bone maturation: A tool for age estimation in common dolphins (*Delphinus delphis*)

1. Extended methods

To ensure a transparent and structurally consistent comparison between skeletal and methylation-based age estimation approaches, additional model variants were fitted. The originally published epigenetic clock (Hanninger et al., 2025) was developed using a pooled-sex framework, whereas the primary polynomial bone ageing models were fitted separately for females and males. To account for this structural difference and avoid confounding model comparison by sex stratification, we implemented two complementary analyses.

First, the epigenetic clock was refitted independently for females and males using the ‘*restricted*’ subset (excluding individuals with an age prediction error >6 years) and analytical procedures described in Hanninger et al. (2025). Second, a pooled-sex polynomial regression model was fitted to the combined male and female dataset following the same procedures outlined in the main methods for skeletal ageing (excluding individuals with an age prediction error > 6 years). These additional analyses were conducted solely to harmonise model structure and to evaluate whether differences in predictive performance could be attributed to sex stratification rather than to the underlying ageing approach.

1.1. Sex-specific refitting of epigenetic clock (Hanninger et al., 2025)

To evaluate whether sex-specific stratification influences predictive performance, the epigenetic clock originally described in Hanninger et al. (2025) was refitted independently for females and males using the ‘*restricted*’ subset (excluding individuals with an age prediction error > 6 years). The statistical framework, including elastic net regression and leave-one-out cross-validation, followed the procedures described in Hanninger et al. (2025).

The sole structural modification was that model fitting was conducted independently within each sex rather than on the pooled dataset. In contrast to the original pooled model (Hanninger et al., 2025), which applied a single mean age at attainment of sexual maturity (ASM) across

both sexes (8.15 years), the sex-specific refitting incorporated sex-specific average ASM values (female ASM: 7.5 years; male ASM: 8.8 years; Palmer et al., 2022, 2023).

1.2. Bone ageing models fitted to combined sexes

For completeness, both the polynomial regression and the CAP model were also conducted on the combined male and female dataset. The modelling approach followed the procedures outlined in the main methods section. Outlier exclusion criteria were identical to those applied in the sex-specific analyses; individuals with an age prediction error > 6 years in the model fitted to the full dataset were excluded. No additional model modifications were implemented.

2. Extended results

2.1. Sex-specific refitting of epigenetic clock (Hanninger et al., 2025)

Sex-specific refitting of the epigenetic clock in females ($n = 36$) yielded a Pearson correlation of $r = 0.89$ ($p = 1.95 \times 10^{-13}$) and an R^2 of 0.80 (Figure A4.1A). The regression slope of predicted on observed age was 0.62 with an intercept of 2.63 years. Mean absolute error was 2.72 years, and the median absolute error (MAE) was 2.31 years. Two individuals exhibited absolute prediction errors exceeding 6 years, with a maximum absolute error of 8.8 years.

Sex-specific refitting of the epigenetic clock in males ($n = 27$) yielded a Pearson correlation of $r = 0.80$ ($p = 5.45 \times 10^{-7}$) and an R^2 of 0.64 (Figure A4.1B). The regression slope of predicted on observed age was 0.42 with an intercept of 3.18 years. Mean absolute error was 3.35 years, and the median absolute error (MAE) was 2.79 years. Three individuals exhibited absolute prediction errors exceeding 6 years, including one exceeding 10 years, with a maximum absolute error of 15.14 years.

2.2. Bone ageing models fitted to combined sexes

2.2.1. Polynomial regression

The pooled polynomial bone ageing model (combined sexes) yielded a Spearman correlation of $\rho = 0.94$ and an R^2 of 0.87 from the regression of predicted on observed age (Figure A4.2A). The regression slope was 0.88 with an intercept of 0.92 years. The mean absolute error was 1.75 years (SD = 1.42 years), with a median absolute error (MAE) of 1.32 years. Two

individuals exhibited absolute prediction errors exceeding 6 years, and no individuals exceeded 10 years. The maximum absolute error was 6.45 years. The formula for the pooled polynomial regression is given as follows, where y denotes predicted age (years) and x the total bone score:

$$y \text{ (pooled)} = -0.969052 + 0.0687288 x + 0.0011129 x^2$$

2.2.2. CAP model

For the pooled CAP model, the first canonical axis (CAP1) showed a canonical correlation of $\delta = 0.91$, corresponding to a squared canonical correlation (δ^2) of 0.83. CAP-derived fitted values for individuals of known ages showed a mean absolute error of 1.90 years (median = 1.56 years; SD = 1.57 years), with a Spearman correlation of $\rho = 0.92$ (Figure A4.2B). Linear regression yielded a slope of 0.88, an intercept of 0.84, and an R^2 of 0.85. Two individuals exhibited absolute errors larger than 6 years, with a maximum absolute error of 8.21 years.

3. Extended discussion

3.1. Sex-specific refitting of epigenetic clock (Hanninger et al., 2025)

To ensure a valid comparison between bone ageing and epigenetic ageing, we refitted the epigenetic clock published by Hanninger et al. (2025) independently for females and males. Refitting the model by sex did not improve predictive performance. Correlation coefficients declined relative to the pooled model (MAE = 1.80, $r = 0.91$, $R^2 = 0.82$; Hanninger et al., 2025), and mean absolute error increased in both sexes (females: MAE = 2.31, $r = 0.89$, $R^2 = 0.80$; males: MAE = 2.79, $r = 0.80$, $R^2 = 0.64$). These findings indicate that sex-specific refitting did not enhance age prediction accuracy within this population. The absence of improvement is consistent with prior evaluations of chronological DNA methylation clocks in humans, where sex-specific stratification has not consistently resulted in improved predictive performance (Carlsen et al., 2023; Sala et al., 2024).

In our modelling framework, epigenetic clock development required a training sample of approximately 70 individuals to achieve stable predictive performance (Mayne et al., 2021). Both sex-specific subsets fell well below this threshold. High-dimensional penalized regression approaches, such as elastic net, are sensitive to reductions in training size when the number of predictors greatly exceeds the number of samples, and the reduced sample sizes under

stratification likely contributed to the observed decline in model performance (Mayne et al., 2021). Taken together, these results demonstrate that sex-specific refitting does not improve model performance within this study population. Accordingly, there is no empirical support for stratifying the epigenetic clock by sex under the present sample sizes.

3.2. Bone ageing models fitted to combined sexes

When fitted to the combined dataset, the polynomial bone ageing model demonstrated stronger predictive performance than the pooled epigenetic clock. The skeletal model achieved a Spearman correlation of $\rho = 0.94$ and an R^2 of 0.87, with a median absolute error (MAE) of 1.32 years. The CAP model achieved similar results with a MAE of 1.56 years, a Spearman correlation of $\rho = 0.92$ and an R^2 of 0.85. In comparison, the pooled epigenetic model yielded an MAE of 1.80 years, $r = 0.91$, and $R^2 = 0.82$ (Hanninger et al., 2025). Absolute prediction error was therefore lower in the skeletal model, and extreme errors were less frequent. These results indicate that, within this study population and under cross-validated conditions, radiographic bone maturation provides more precise chronological age estimates than the current methylation-based approach.

4. Supplementary tables

Table A4.1: Comparison of polynomial regression model performance for female common dolphins (*Delphinus delphis*) for using the total bone score calculated as the sum across all bone locations; differences between methods relate to the handling of the bones ‘D2P2’ and ‘D3P2’: *separate epiphyses* — proximal and distal epiphyses of these bones were scored separately; *combined score (average)* — each bone represented by the mean of proximal and distal epiphysis scores; *combined score (max)* — each bone represented by the higher of its proximal and distal epiphysis scores; *combined score (min)* — each bone represented by the lower of its proximal and distal epiphysis scores. The table reports absolute error distributions (SD of AE, Mean AE, Median AE) and predictive strength (Spearman correlation ρ) for each model. Due to bilateral asymmetry between left and right flipper, models were generated for the lower (‘BSL’), higher (‘BSH’) and average bone score (‘ABS’) of both pectoral flippers, as well as the left (‘Left’) and right flipper (‘Right’) individually. Values are presented for the model calibrated using the full dataset.

Separate epiphyses					
Metric	BSL	BSH	ABS	Left	Right
SD of AE	2.304426503	2.31692293	2.309711131	2.318220879	2.303496597
Mean AE	2.056128628	2.03443985	2.045139225	2.031725173	2.058469638
Median AE	1.384441195	1.392257652	1.380808347	1.393343285	1.37950293
Correlation ρ	0.912009911	0.911563645	0.911563645	0.911494831	0.911426016
Combined score (average)					
Metric	BSL	BSH	ABS	Left	Right
SD of AE	2.27222026	2.285164404	2.277467568	2.286876297	2.270586275
Mean AE	2.02850359	2.005279078	2.01679937	2.002029134	2.031667297
Median AE	1.38475681	1.35634075	1.354663124	1.369583993	1.382807018
Correlation ρ	0.912654231	0.912700107	0.912791859	0.912700107	0.912080783

Table A4.1: Comparison of polynomial regression model performance for female common dolphins (*Delphinus delphis*), continued.

Metric	Combined score (max)				
	BSL	BSH	ABS	Left	Right
SD of AE	2.273700499	2.28801265	2.279550371	2.289931714	2.271852855
Mean AE	2.024175081	1.999372273	2.011617007	1.995726769	2.027714
Median AE	1.384114947	1.331029115	1.350514552	1.375987718	1.360803092
Correlation ρ	0.913352824	0.913077566	0.913742773	0.9133987	0.912458235
Metric	Combined score (min)				
	BSL	BSH	ABS	Left	Right
SD of AE	2.271221478	2.282767565	2.275866168	2.28430509	2.269812542
Mean AE	2.032709613	2.011138276	2.021863857	2.008233523	2.035499785
Median AE	1.35122163	1.327043601	1.331230933	1.343365293	1.350051885
Correlation ρ	0.909741114	0.910268687	0.910268687	0.910268687	0.909741114

Table A4.2: Comparison of polynomial regression model performance for male common dolphins (*Delphinus delphis*) for using the total bone score calculated as the sum across all bone locations; differences between methods relate to the handling of the bones ‘D2P2’ and ‘D3P2’: *separate epiphyses* — proximal and distal epiphyses of these bones were scored separately; *combined score (average)* — each bone represented by the mean of proximal and distal epiphysis scores; *combined score (max)* — each bone represented by the higher of its proximal and distal epiphysis scores; *combined score (min)* — each bone represented by the lower of its proximal and distal epiphysis scores. The table reports absolute error distributions (SD of AE, Mean AE, Median AE) and predictive strength (Spearman correlation ρ). Due to bilateral asymmetry between left and right flipper, models were generated for the lower (‘BSL’), higher (‘BSH’) and average bone score (‘ABS’) of both pectoral flippers, as well as the left (‘Left’) and right flipper (‘Right’) individually. Values are presented for the model calibrated using the full dataset.

Separate epiphyses					
Metric	BSL	BSH	ABS	Left	Right
SD of AE	2.245175653	2.193492687	2.217891651	2.245561395	2.192899137
Mean AE	2.140668162	2.111756121	2.126465922	2.14028755	2.11230438
Median AE	1.404106133	1.387175281	1.381826617	1.399306545	1.400619043
Correlation ρ	0.941810777	0.943384396	0.942358123	0.942734423	0.941502895
Combined score (average)					
Metric	BSL	BSH	ABS	Left	Right
SD of AE	2.255090054	2.213887874	2.23349049	2.25684191	2.212126767
Mean AE	2.156476639	2.132520864	2.144821647	2.154219884	2.134741974
Median AE	1.395988273	1.407394249	1.38857795	1.364783817	1.401119946
Correlation ρ	0.941023968	0.942118659	0.942221286	0.942289704	0.941331849

Table A4.2: Comparison of polynomial regression model performance for male common dolphins (*Delphinus delphis*), continued.

Metric	Combined score (max)				
	BSL	BSH	ABS	Left	Right
SD of AE	2.255507061	2.209047971	2.230968893	2.257536542	2.20699857
Mean AE	2.152772143	2.124930736	2.139269899	2.149945008	2.127724889
Median AE	1.395501583	1.393517868	1.374373473	1.352211772	1.398486689
Correlation ρ	0.942358123	0.943863323	0.943555441	0.943623859	0.942118659

Metric	Combined score (min)				
	BSL	BSH	ABS	Left	Right
SD of AE	2.254670811	2.219025591	2.236184689	2.256193361	2.217195708
Mean AE	2.160316464	2.140031988	2.150337253	2.158573378	2.142051411
Median AE	1.385035199	1.421344065	1.398018656	1.377086	1.40268362
Correlation ρ	0.940750295	0.941844986	0.941810777	0.942016031	0.941058177

Table A4.3: Polynomial regression models describing the relationship between total pectoral flipper bone score and chronological age in common dolphins (*Delphinus delphis*). For each model, two aspects vary: (1) the treatment of the second phalanges of digits II and III ('D2P2' and 'D3P2'), and (2) the handling of bilateral differences between left and right flippers. With respect to 'D2P2' and 'D3P2', four scoring approaches are shown: separate epiphyses, where the proximal and distal epiphyses are scored independently and each epiphysis contributes separately to the total score; combined score (average), where each bone is represented by the mean of its proximal and distal epiphysis scores; combined score (max), where each bone is represented by the higher of its two epiphysis scores; and combined score (min), where each bone is represented by the lower of its two epiphysis scores. Bilateral asymmetry is addressed using five variants: 'BSL', based on the lower-scoring flipper; 'BSH', based on the higher-scoring flipper; 'ABS', based on the average of left and right flipper scores; and separate models using the left ('Left') and right ('Right') flippers individually. The table lists the resulting second-order polynomial equations of the form $y = \beta_0 + \beta_1x + \beta_2x^2$, where x is the total bone score and y is predicted age (years). These equations were calculated based on the full data set.

Model	Females	Males
Separate epiphyses		
'BSL'	$y = -0.7376 + 0.036396 \times x + 0.00117899 \times x^2$	$y = 0.158557 + 0.0271375 \times x + 0.00105291 \times x^2$
'BSH'	$y = -0.829114 + 0.0379345 \times x + 0.00117051 \times x^2$	$y = 0.146565 + 0.0268819 \times x + 0.00105153 \times x^2$
'ABS'	$y = -0.780987 + 0.0370384 \times x + 0.0011759 \times x^2$	$y = 0.155889 + 0.02681 \times x + 0.00105394 \times x^2$
'Left'	$y = -0.761154 + 0.0361074 \times x + 0.00118189 \times x^2$	$y = 0.121697 + 0.0279779 \times x + 0.0010484 \times x^2$
'Right'	$y = -0.801872 + 0.0380927 \times x + 0.00116854 \times x^2$	$y = 0.182992 + 0.0260534 \times x + 0.00105596 \times x^2$
Combined score average		
'BSL'	$y = -1.00156 + 0.0471288 \times x + 0.00144368 \times x^2$	$y = 0.0528455 + 0.0334955 \times x + 0.00130271 \times x^2$
'BSH'	$y = -1.07108 + 0.0480113 \times x + 0.00143955 \times x^2$	$y = 0.0507911 + 0.0328258 \times x + 0.00130549 \times x^2$
'ABS'	$y = -1.03375 + 0.0474111 \times x + 0.00144324 \times x^2$	$y = 0.053803 + 0.0330274 \times x + 0.00130539 \times x^2$
'Left'	$y = -0.99657 + 0.04576 \times x + 0.00145529 \times x^2$	$y = 0.0223684 + 0.0343353 \times x + 0.0012974 \times x^2$
'Right'	$y = -1.07363 + 0.0492808 \times x + 0.00142874 \times x^2$	$y = 0.0808683 + 0.0319989 \times x + 0.0013107 \times x^2$

Table A4.3: Polynomial regression models describing the relationship between total pectoral flipper bone score and chronological age in common dolphins (*Delphinus delphis*), continued.

Model	Females	Males
Combined score (max)		
'BSL'	$y = -1.03169 + 0.0474985 \times x + 0.00144261 \times x^2$	$y = 0.0451948 + 0.0331951 \times x + 0.00130663 \times x^2$
'BSH'	$y = -1.08317 + 0.0476272 \times x + 0.00144443 \times x^2$	$y = 0.0444677 + 0.0325156 \times x + 0.00130866 \times x^2$
'ABS'	$y = -1.05451 + 0.0473807 \times x + 0.00144539 \times x^2$	$y = 0.0475891 + 0.0326732 \times x + 0.00130939 \times x^2$
'Left'	$y = -1.01363 + 0.0455433 \times x + 0.0014589 \times x^2$	$y = 0.0157955 + 0.0340519 \times x + 0.00130103 \times x^2$
'Right'	$y = -1.09958 + 0.0495122 \times x + 0.0014589 \times x^2$	$y = 0.0735636 + 0.0316692 \times x + 0.00131418 \times x^2$
Combined score (min)		
'BSL'	$y = -0.970026 + 0.0467022 \times x + 0.00144517 \times x^2$	$y = 0.0613129 + 0.0337571 \times x + 0.00129908 \times x^2$
'BSH'	$y = -1.05726 + 0.0483327 \times x + 0.0014351 \times x^2$	$y = 0.0574401 + 0.0331287 \times x + 0.00130233 \times x^2$
'ABS'	$y = -1.0115 + 0.0473849 \times x + 0.0014415 \times x^2$	$y = 0.0606674 + 0.0333535 \times x + 0.00130158 \times x^2$
'Left'	$y = -0.977924 + 0.0459177 \times x + 0.00145206 \times x^2$	$y = 0.0297298 + 0.034582 \times x + 0.00129405 \times x^2$
'Right'	$y = -1.04608 + 0.0489863 \times x + 0.00142926 \times x^2$	$y = 0.0885223 + 0.0323195 \times x + 0.00130724 \times x^2$

5. Supplementary figures

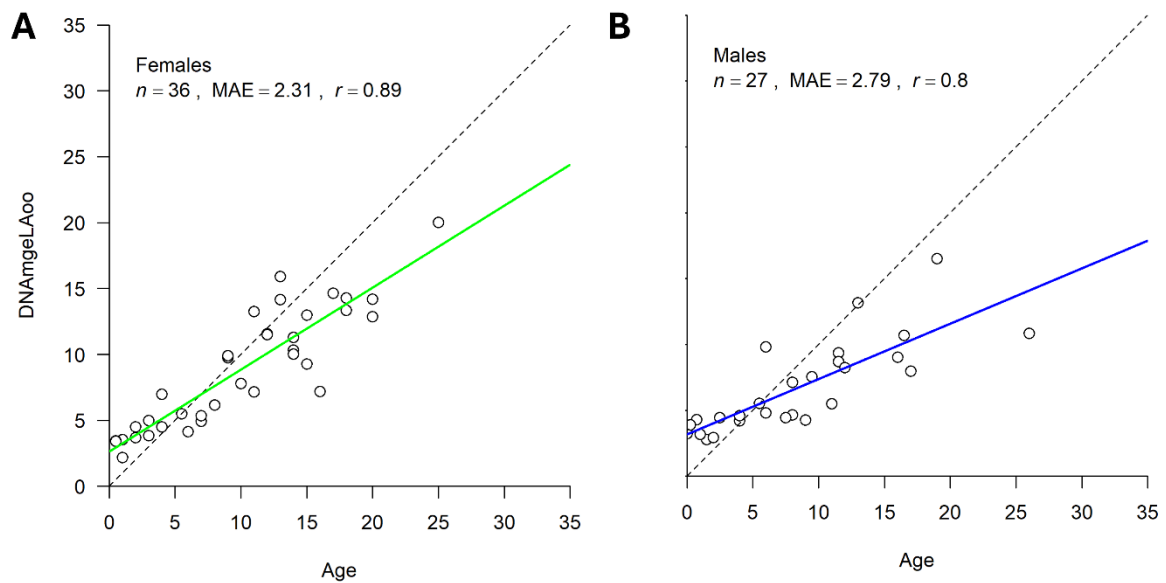


Figure A4.1: Sex-specific refitting of the epigenetic age model originally published by Hanninger et al. (2025) using the restricted subset of common dolphins (*Delphinus delphis*). The model was refitted separately for **A**) females and **B**) males to evaluate whether sex-specific stratification improves predictive performance relative to the pooled model. DNAmAgeLOO (leave-one-out cross-validated predictions) is plotted against chronological age. Solid lines represent linear regression fits; dashed lines indicate the 1:1 relationship. Sample size (n), mean absolute error (MAE), and Pearson correlation coefficient (r) are shown for each sex.

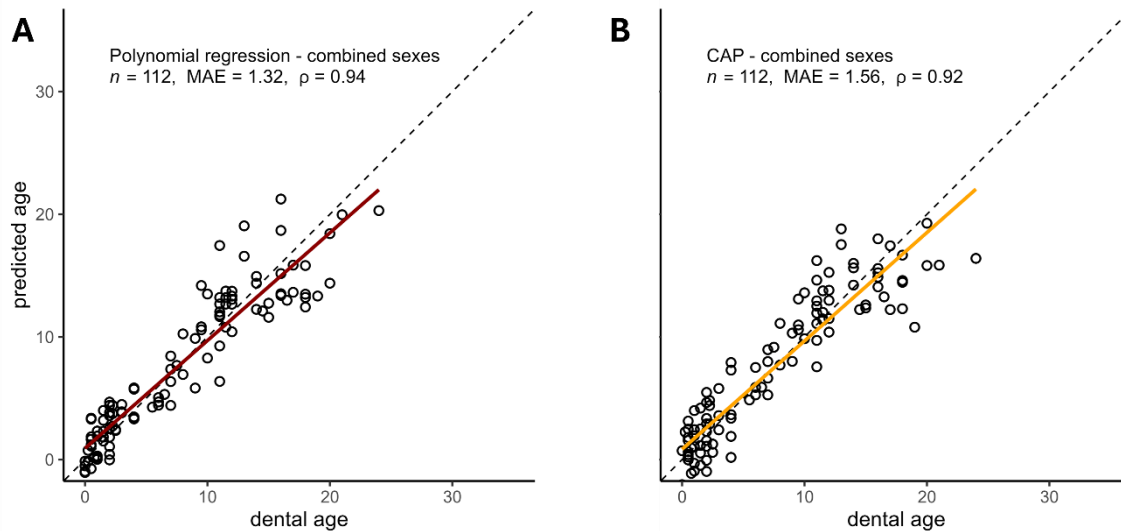


Figure A4.2: Relationship between dental age and predicted age for common dolphins (*Delphinus delphis*). **A)** Pooled polynomial bone-ageing model (combined sexes; outliers excluded). Predicted ages were generated via leave-one-out cross-validation (LOOCV) of a second-degree polynomial regression fitted to ossification scores derived from radiographic assessment of the right pectoral flipper. **B)** LOOCV-predicted ages from the CAP model. The solid red (polynomial regression) and orange (CAP) lines represent linear regressions of predicted on observed age, and the dashed line indicates the 1:1 relationship. Model performance metrics (sample size, n ; median absolute error, MAE; and Spearman's rank correlation, ρ) are provided within each panel.

Appendix 5

Radiographic assessment of bone maturation: A tool to estimate sexual maturity in New Zealand common dolphins (*Delphinus delphis*)?

1. Supplementary tables

Table A5.1: Diagnostics of Bayesian logistic regression models for the median bone score at attainment of sexual maturity in common dolphins (*Delphinus delphis*). The ‘Right’ models used the total ossification score of the right flipper as the predictor variable (x) in the Bayesian logistic regression of sexual maturity status. The total bone score (‘Right’) represented each dolphin’s overall degree of skeletal development and was used to estimate the probability of sexual maturity as a function of ossification, yielding the midpoint (mp) and slope (w) parameters of the maturity curve. Posterior medians and 95% credible intervals (CrI) are shown for mp and w. Pareto-k diagnostics indicate the proportion of observations with high influence on the PSIS-LOO approximation (bad: $k > 0.7$; very bad: $k > 1$). Truncation refers to restricting the analysis to the bone-score range corresponding to the observed transition zone from immature to mature individuals, thereby excluding extreme scores with probabilities near 0 or 1 that caused unstable importance weights. The ϵ -adjustment adds a small misclassification term ($\epsilon \leq 0.1$) to the likelihood, allowing for a minor probability that any maturity label could be erroneous; this regularises the model and stabilises PSIS diagnostics. For both females and males, results are shown for models fitted to the full dataset (without truncation or ϵ -adjustment = NoTr/No ϵ) and for the truncated dataset (without ϵ -adjustment = Tr/No ϵ).

Diagnostics	‘Right’ females		‘Right’ males	
	NoTr/No ϵ	Tr/No ϵ	NoTr/No ϵ	Tr/No ϵ
Model configuration				
n (immature / mature)	41 / 31	16 / 20	38 / 25	19 / 16
mp (Estimate $\pm$ 95 % CrI)	74.82 (72.53 – 77.00)	74.84 (72.64 – 76.97)	68.16 (63.55 – 72.63)	68.16 (63.55–72.63)
w (Estimate $\pm$ 95 % CrI)	0.85 (0.51 – 1.24)	0.85 (0.51 – 1.21)	0.32 (0.18 – 0.49)	0.32 (0.18 – 0.49)
Pareto-k diagnostics	“bad”: $n = 6$ (8.3%)	“bad”: $n = 5$ (13.9 %)	“bad”: $n = 13$ (20.5 %)	-
	“very bad”: $n = 41$ (56.9 %)	“very bad”: $n = 10$ (27.8 %)	“very bad”: $n = 11$ (17.5 %)	-