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Application of a novel molecular method to age free-living wild Bechstein's bats

Running title

Using DNA methylation to age bats

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Abstract

The age profile of populations fundamentally affects their conservation status. Yet age is frequently difficult to assess in wild animals. Here, we assessed the use of DNA methylation of homologous genes to establish the age structure of a rare and elusive wild mammal: the Bechstein's bat (*Myotis bechsteinii*). We collected 62 wing punches from individuals whose ages were known as a result of a long-term banding study. DNA methylation was measured at seven CpG sites from three genes which have previously shown age-associated changes in humans and laboratory mice. All CpG sites from the tested genes showed a significant relationship between DNA methylation and age, both individually and in combination (multiple linear regression $R^2=0.58$, $p<0.001$). Despite slight approximation around estimates, the approach is sufficiently precise to place animals into practically useful age cohorts. This method is of considerable practical benefit as it can reliably age individual bats. It is also much faster than traditional capture-mark-recapture techniques, with the potential to collect information on the age structure of an entire colony from a single sampling session to better inform conservation actions for Bechstein's bats. By identifying three genes where DNA methylation correlates with age across distantly related species, this study also suggests that the technique can potentially be applied across a wide range of mammals.

Keywords

Mammals, Wildlife Management, Epigenetics, DNA Methylation, Conservation, Chiroptera

Introduction

Information on the age of individuals in wildlife populations is essential to establish the structure of populations (Dunshea et al., 2011; Oli & Dobson, 2003). Such information can help predict the impact of habitat, climate change or hunting pressure on population viability estimates (Botsford, Holland, Samhour, White, & Hastings, 2011; Sand et al., 2012; Tella, Rojas, Carrete, & Hiraldo, 2013). However, estimating age through marking (e.g. using tags and rings) and recapture can be challenging and can have negative impacts on animal welfare (Nelson, 2002). In addition, recaptures throughout the lifetime of the animals are required to gather the necessary information, making the process time-consuming and difficult to execute for rare species (Brunet-Rossini, Wilkinson, Kunz, & Parsons, 2009). Non-invasive approaches to age wildlife, such as the unique identification of individuals via markings (e.g. cetaceans, tigers) (Mizroch, Beard, & Lynde, 1990; Speed, Meekan, & Bradshaw, 2007), are suitable only for a limited number of species. Molecular tools for producing reliable age estimates have, by contrast, received little attention. Most research has focused on understanding the biological process of ageing, for example through studies of telomere shortening (e.g. Turbill, Ruf, Smith, & Bieber, 2013), rather than on the development of routinely applicable techniques to estimate chronological age.

The biological process of ageing combines both programmed and environmental processes (Jung & Pfeifer, 2015; Petkovich et al., 2017). The presence or absence of methyl groups at the C5 position of cytosines followed by guanines ('CpG sites') has an important role in the control of gene expression; as changes in levels of methylation at CpG sites are associated with alterations in gene transcription rates (Hannum et al., 2013; Horvath, 2013). The availability of multiple loci where DNA

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methylation is linearly associated with age and the possibility of using multiple tissues (e.g. blood, skin) means that DNA methylation assessment is now a powerful tool employed in human forensic science (e.g. Bekaert, Kamalandua, Zapico, Van de Voorde, & Decorte, 2015; Goel, Karir, & Garg, 2017; Horvath, 2013; Zbieć-Piekarska et al., 2015). The measure of DNA hypo- and hypermethylation of specific sites has been used as an age predictor on humans (*Homo sapiens*) (Christensen et al., 2009; Gröniger et al., 2010; Horvath, 2013) and mice (*Mus musculus*) (Maegawa et al., 2010; Stubbs et al., 2017). However, this approach had not been attempted for the purpose of studying wild animals until Polanowski, Robbins, Chandler, and Jarman (2014) developed an age assay for humpback whales (*Megaptera novaengliae*) by measuring DNA methylation from three CpG sites from different genes. Their results were far more accurate than previously developed techniques using telomere length (Dennis, 2006; Olsen, Bérubé, Robbins, & Palsbøll, 2012; Olsen, Robbins, Bérubé, Rew, & Palsbøll, 2014).

Bats live substantially longer than other mammals of similar size and metabolic rate (Wilkinson & South, 2002). Certain species, such as Brandt's bat (*Myotis brandtii*), have been recorded surviving in the wild for over four decades (Gaisler, Hanák, Hanzal, & Jarský, 2003). The long lifespan of bats is thought to have evolved from a lower risk of extrinsic mortality due to the evolution of flight along with the use of roosting and hibernation sites (Munshi-South & Wilkinson, 2010). Age estimates of bat populations are particularly important for their conservation, because their long lifespan often results in a delayed response to recent changes in their habitat. Up until now, such estimates depended solely on long-term ringing studies (Gaisler et al., 2003; Wilkinson & South, 2002). Whilst a variety of additional methods give some indication of whether an animal is a juvenile, such as analysing linear growth of

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bones (Kunz & Hood, 2000), epiphyseal-diaphyseal fusion (De Paz, 1986), chin spots (Richardson, 1994), body mass and pelage coloration (Cheng & Lee, 2002), these are helpful for only the first few months of life. For adult bats, tooth wear and incremental dentin may give an indication of age (Batulevicius, Pauziene, & Pauza, 2001; Storz, Bhat, & Kunz, 2000) but examination is invasive and the results are imprecise, so the methods are not widely adopted.

In this study, we examined the potential of measuring DNA methylation to age a rare woodland bat, *M. bechsteinii*. To test our method we used a population where the ages of all bats were known from a long-term banding study. Our finding of genes in which the level of methylation relates to age opens the possibility of using molecular approaches to deliver estimates of age in bats.

Materials and methods

Sample collection

Wing tissue samples were collected from *M. bechsteinii* using 3 mm wing biopsy punches (Stiefel Laboratories, Wooburn Green, UK) and stored in absolute ethanol at -4°C. Genomic DNA was extracted from the biopsy punches with the DNeasy blood & tissue kit (Qiagen) and samples were eluted in 120 µl of buffer AE. We used 60 female samples collected from Brackett's Coppice (Dorset; 50.860456, -2.6918909), a maternal colony monitored since 1999. We also included two juveniles which were found dead in bat boxes. Biopsy punches were stored in ethanol after being found and were included in the study as DNA methylation is chemically stable and can be measured on ancient DNA (Briggs et al., 2009; Llamas et al., 2012). No male samples were collected as they tend to lead solitary lives and are very rarely observed after they are weaned, while females maintain tight social bonds

throughout their lives (Kerth & König, 1999). The bats included in this study represent an even distribution of ages ranging from 0 to 14 years old. Eight droppings were also collected from individuals of known age from the same colony and DNA was extracted using a QIAmp DNA Stool Mini Kit (QIAGEN) following the protocol recommended by Puechmaile, Mathy, and Petit (2007).

PCR and pyrosequencing

Genes with age-related epigenetic changes in humans, mice and humpback whales were identified through literature searches (Supplementary 1). Candidate 5' regulatory region sequences were taken from GenBank and used as queries for BLASTN (Altschul, Gish, Miller, Myers, & Lipman, 1990) searches of Chiroptera sequences in GenBank and BLAT searches of the Little brown bat (*Myotis lucifugus*) genome (Cunningham et al., 2014). Primer sequences were then matched to the Bechstein's bat genome which was sequenced using 100 bp paired end sequencing on an Illumina HiSeq 2500 (EBI access N°: PRJEB23351).

PCR and pyrosequencing assays were designed using the PyroMark Assay design software (Qiagen, Hilden, Germany). The target sequences analysed comprised CpG sites in genes known to undergo age-related epigenetic changes in other species (Koch et al., 2011; Polanowski et al., 2014; Weidner et al., 2014). Template preparation and pyrosequencing was carried out as described by Tost and Gut (2007). Genomic DNA was treated with sodium bisulfite using the EZ-96 DNA Methylation-Gold Kit (Zymo Research, CA, USA) according to the manufacturer's standard protocol. Samples were then incubated twice for 5 minutes in 15 µl and 10 µl of M-Elution buffer to form a final solution of 25 µl. Water negative controls were run to verify the absence of DNA contamination. Bisulfite-PCR amplification was

performed using the primers in Table 1. Water controls were included to confirm the absence of DNA contamination, and unmodified DNA samples from wing punches were included during primer optimisation to confirm primer specificity for bisulfite-modified DNA.

Amplification reactions consisted of 3 μ l of 5x HOT FIREPol® Blend Master Mix, 0.75 μ l each (0.075 μ M) of forward and reverse primers, 1 μ l of bisulfite converted template DNA and 9.5 μ l of RNase-free water. PCR conditions were 15 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C and a final extension step of 10 min at 72 °C. Before pyrosequencing, all samples were diluted in 15 μ l of water. Pyrosequencing was performed on a PYROMARK 24 Pyrosequencing System (Qiagen). The PYROMARK Q24 software gave percentage methylation values for each CpG site and we used eight duplicates to test for pyrosequencing precision.

Data analysis

All statistical analysis was undertaken using R studio (RCoreTeam, 2016; RStudio, 2012). All CpG sites reported in this study have previously been shown to undergo linear age-associated methylation changes (Supplementary 1). Therefore, we maintained a linear analysis throughout the study and assumed any non-linearity as an artefact of sample size. The differences in percentage methylation between bats of varying ages was initially explored using univariate linear regression in methylation percentages for each CpG site separately. All individuals with missing data were omitted from the analysis. All CpG sites showing a significant relationship with age were considered for developing the multiple linear regression and we included the interactions between sites from the same gene as neighbouring CpG sites tend to be highly correlated in terms of methylation. This model was then used

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as an epigenetic age assay for *M. bechsteinii*. The predicted age of each bat was plotted against their known age to test the consistency of the model. In addition, we assessed the practical utility of the assay in classifying individuals into three meaningful age cohorts linked to the species reproductive status (Fleischer, Gampe, Scheuerlein, & Kerth, 2017); young bats with low fertility (0-3 years); mature bats with high fertility (4-8 years); and old bats with decreasing fertility (≥ 9 years) as these can provide valuable information on population viability (Mallet, Zouros, Gartner-Kepkay, Freeman, & Dickie, 1985). The predictive power in achieving the correct classification was assessed by calculating kappa values using CIA 2.0.0 (Trevor Bryant, University of Southampton, Southampton, UK) where values close to 1 indicate a good predictive power.

We assessed the precision of the age estimate assay by performing a Leave One Out Cross Validation (LOOCV) analysis as performed by Polanowski et al. (2014). Here, the multiple linear regression was tested by using all wing samples but one (N-1) to estimate the individual's age; the predicted age was then plotted against the known age of the individual.

Results

From the 13 sequences, each identified from different genes containing CpG sites known to undergo age-associated CpG methylation changes in other species (Supplementary 1), we successfully designed assays and amplified the regulatory region of three genes for *M. bechsteinii* (TET2, GRIA2, and ASPA; Table 1). Seven CpG sites from these genes were included in the final model to estimate age. Of the 62 wing samples 58 successfully amplified for all three assays. The four individuals

with incomplete data were excluded from further analysis to avoid instability in the regression models due to missing data.

All seven CpG sites tested in this study showed a significant relationship with the age of *M. bechsteinii* (Fig. 1). For sites from the TET2 and GRIA2 genes, increasing age was associated with increased methylation, whereas the reverse was true for ASPA (Fig. 1g). The average difference in percentage methylation between duplicates was 3.9 and did not affect the results of the multiple linear regression model (Supplementary 2). The multiple linear regression included all CpG sites, whilst taking into account interactions between sites from the same gene. This full model explained 58% of age variation ($R^2 = 0.576$, $p < 0.001$) and the overall precision of the predictions was 2.08 years by calculating the root mean-square error (Fig. 2). Our model slightly overestimated the age of young bats whilst underestimating the older individuals. However, the split of our data in three categorical age groups (Fig. 2b) showed that the developed age prediction model could effectively differentiate all age classes with very little overlap (one-way Anova: $F = 39.6$, $p < 0.001$) and a kappa value of 0.646 (0.467-0.824) which indicates a good level of prediction for practical purposes.

The leave-one-out cross validation analysis (LOOCV) was performed to provide an unbiased estimate of the accuracy of the Bechstein's bat age estimates. The overall precision of the LOOCV was estimated at 1.52 years by calculating the standard deviation of the mean difference between known and estimated ages (Fig. 3). Methylation levels of the eight droppings tested fell outside the range of the epigenetics age assay which was designed for wing punches. Methylation values in droppings appeared to vary more between individuals than wing punches (TET2 (A) for droppings ranged from 1 to 96% compared to 29 to 71% for wing punches).

Discussion

This is the first epigenetic age assay developed for monitoring bats, a taxon which forms a third of all mammalian species, and only the third designed for use on a wild species after humpback whales (Polanowski et al., 2014) and wolves (*Canis lupus*) (Thompson, vonHoldt, Horvath, & Pellegrini, 2017). The age prediction model developed explained 64% of variance and predicted age from wing punches with a standard deviation of 1.52 years. Although the accuracy of this assay is lower than human epigenetic age assays which have gradually tested a greater number of CpGs to improve model accuracy, this method is of considerable practical value in being able to precisely age *M. bechsteinii* and give indications on population trends. For example, populations lacking juveniles might indicate recent poor breeding success which could subsequently lead to a delayed population decline. This information could then be used to assess the impact of recent environmental changes in the environment (e.g. felling of large roosting trees, weather conditions) on breeding success or increased mortality (Fleischer et al., 2017).

The Bechstein's bat age assay provides a novel tool offering necessary insight on the age structure of bat colonies, but will require further development and validation prior to widespread use. The elusive nature of bats makes the collection of known age samples time-consuming and labour intensive. The oldest bat in this study was 14 years. Yet evidence suggests that individual *M. bechsteinii* can live for more than 20 years (Dietz, Nill, & von Helversen, 2009). It may therefore be possible to further improve this epigenetics age assay by including older bats. Additionally, the sampling of females from a single colony could also potentially bias results as females within a colony may have been subjected to similar environmental stresses which can sometimes impact levels of methylation (Teschendorff, West, & Beck,

2013). Therefore, other colonies or solitary males may show slight differences in DNA methylation. However, it is reasonable to assume that such differences would be minor and would have little effect on our results as males and females, for example, show very similar trends in DNA methylation in most studies (e.g. Polanowski et al., 2014; Zbieć-Piekarska et al., 2015).

Although we only tested a few bat droppings, we detected important variations in methylation levels which would not allow us to accurately estimate the age of bats. A separate dropping age estimate assay is required as changes in DNA methylation are often tissue specific (Christensen et al., 2009). The development of such an assay would also need to consider all points mentioned above whilst using a larger sample size. Indeed, unlike tissue samples from wings, droppings may be more indicative of biological age and show high levels of instability, as these would comprise cells from the intestinal tract (Jones & Laird, 1999; Maegawa et al., 2010). Additionally, the quality and quantity of DNA along with a higher risk of contamination from droppings could also affect results, as these vary significantly more than wing punches (Puechmaille et al., 2007).

This study demonstrates that useful and rapid age estimates can be derived from an epigenetic assay (Fig. 4). Our methods provide sufficient sensitivity to confidently estimate the age of Bechstein's bats ranging from 0 to 14 years old at a cost of approximately 15 GBP per sample. Such techniques could be used to inform about the age structure of bat colonies and further improve their conservation. The use of three genes applicable for age assays across distantly related species, such as humans, whales, mice and bats, suggests the potential for a widespread use of these techniques for mammal conservation in the future.

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Authors' contributions

C.M. and H. S. undertook the long term ringing study. F.M., C.M. and P.W. collected the samples. F.M., P.H., H.S., E.D. and P.W. conceived and designed the methodology. E.D., A.S. & J.B. provided access and supervision to the facilities for the lab work which was undertaken by P.W. The data was analysed by P.W. and the writing of the manuscript was also led by P.W. All authors contributed critically to the drafts and gave final approval for publication.

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Data accessibility

All R scripts and CpG methylation data has been deposited in the DRYAD database archive for this study (<https://doi.org/10.5061/dryad.rn0198d>). The three *Myotis bechsteinii* samples used in this study have also been deposited in GenBank (MF322927, MF322926 and MF322925).

Figures and tables

Table 1: PCR and sequencing primers of the three analysed assays along with the GenBank reference sequences and previous studies analysing age associated changes in DNA methylation for each assay.

Gene (Accession number)	References	Primer name	Sequence (5' to 3')	T _m (°C)
TET2 (MF322927)	Polanowski et al. (2014), Grönniger et al. (2010)	TET2_Koch_F1b	Biotin-GAAATTTTGGTTTTT TTATAATAGAGGT	57
		TET2_Koch_R1	CCAAAAAATTTCTCAATA ACTCTACTT	59
		TET2_Koch_Seq1	TTCTCAATAACTCTACTCT	44
GRIA2 (MF322926)	Polanowski et al. (2014), Koch et al. (2011), Chakrabarti, Bandyopadhyay, and Poddar (2001)	GRIA2_F1	GTGTATGGGAGGGTGTGA ATATTTTAA	61
		GRIA2_R1b	Biotin-AACAAAAAATTCCTA TTCCCAAATCC	61
		GRIA2_Seq1	GTTGAATATTTAAGTTTTGG GATTAT	47
ASPA (MF322925)	Bekaert et al. (2015), Weidner et al. (2014)	ASPA_F1	GAGTTAATAGGAGTATTTTGG GTAAAGTA	58
		ASPA_R1B	Biotin-AAATAATTTTACCTCCA ATCCTATTCT	58
		ASPA_Seq1	GGAGTATTTTGGTTAAGTAT	44

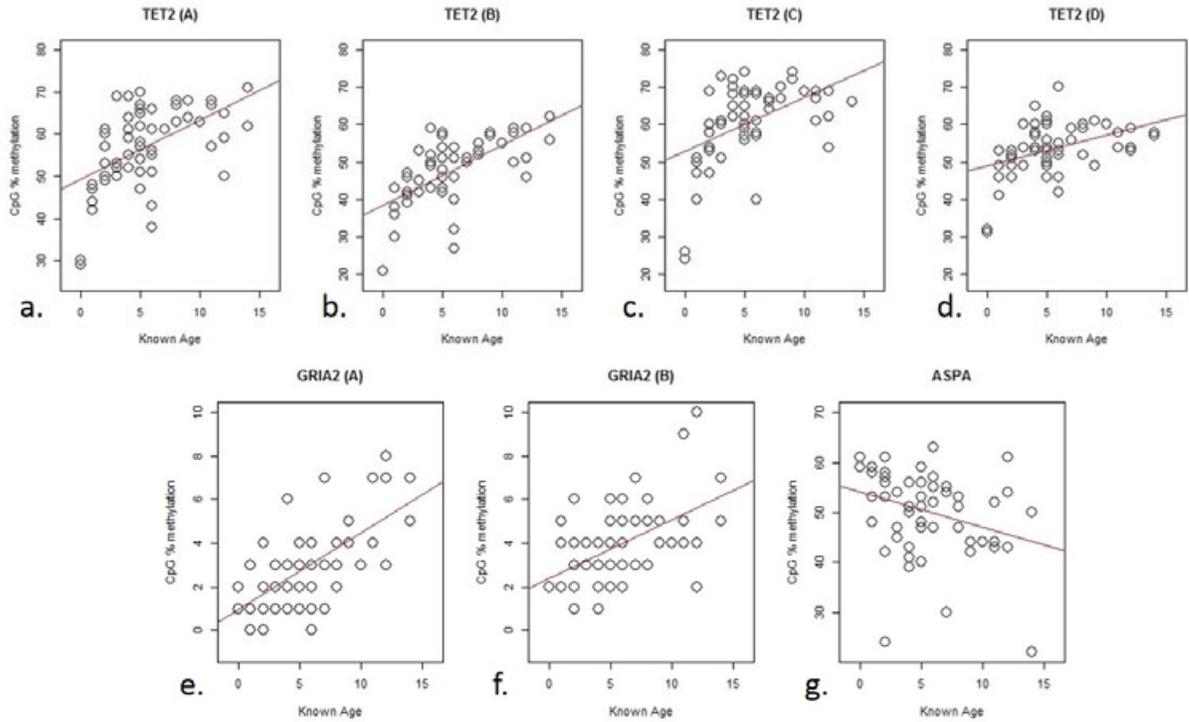


Figure 1: Percentage methylation versus age for seven markers validated in three different genes. TET2 (A): $R^2=0.263$, $p < 0.001$; TET2 (B): $R^2=0.384$, $p < 0.001$; TET2 (C): $R^2=0.221$, $p < 0.001$; TET2 (D): $R^2=0.168$, $p < 0.001$; GRIA2 (A): $R^2=0.419$, $p < 0.001$; GRIA2 (B): $R^2=0.256$, $p < 0.001$; ASPA: $R^2=0.0711$, $p = 0.0241$.

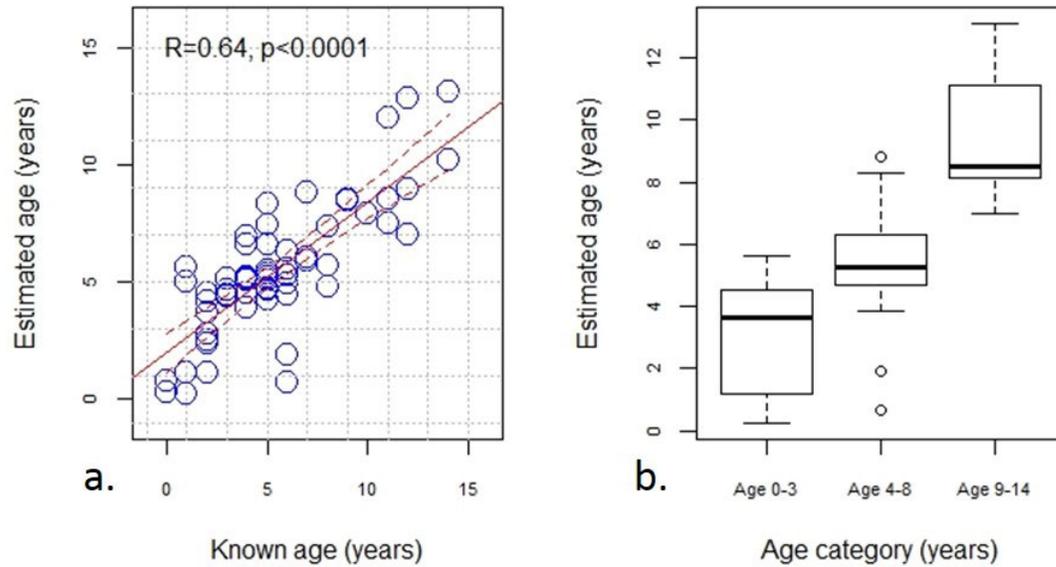


Figure 2: a) Multiple linear regression for the predicted age *M. bechsteinii* from measurement of CpG methylation at seven CpG sites with 95% confidence limits b) boxplots representing the known age of bats in three distinct categories of known age (0-3 years old, 4-8 years old, 9-14 years old).

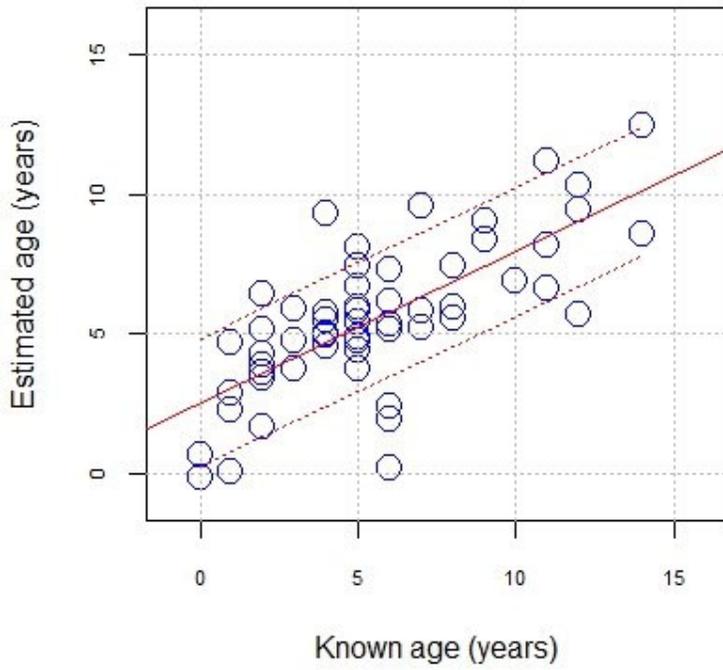


Figure 3: Results of ‘Leave One Out Cross Validation’ (LOOCV) analysis for the wing punches. The estimated ages of every bat when the predictive model is based on data for the other $N = 57$ bats are plotted with together with 95% confidence limits.

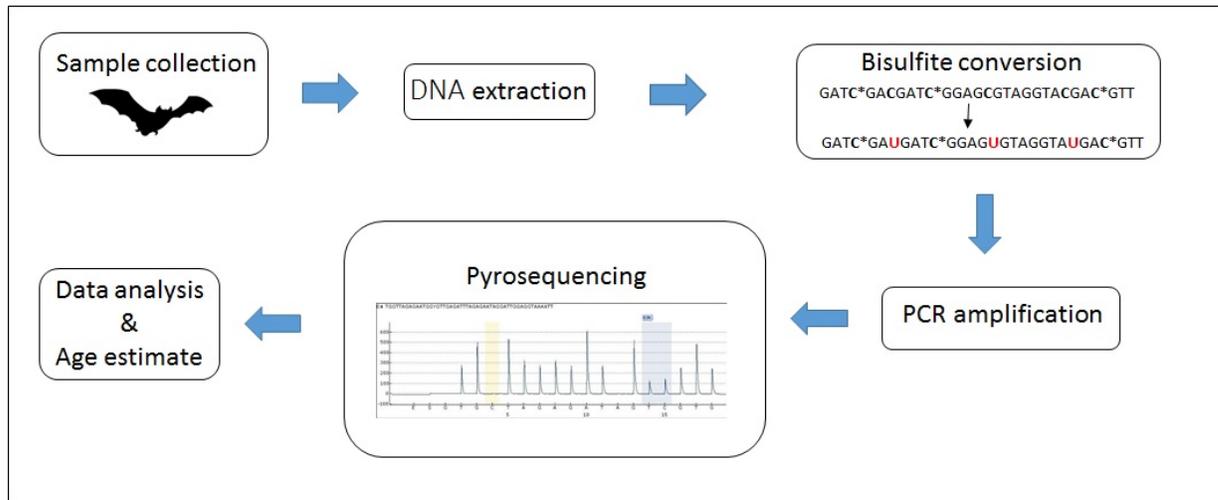


Figure 4: Summary of the key steps for estimating the age of bats by measuring DNA methylation. Methylated cytosines are represented by *.