

Accuracy of the Aspartic Acid Racemization Technique in Age Estimation of Mammals and the Influence of Body Temperature

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ABSTRACT

The aspartic acid racemization (AAR) technique has been applied for age estimation of humans and other mammals for more than four decades. In this study, eye lenses from 124 animals representing 25 mammalian species were collected and D/L ratios obtained using the AAR technique. The animals were either of known age or had the age estimated by other methods. The purpose of the study was to: a) estimate the accuracy of the AAR technique, and b) examine the effect of body temperature on racemization rates. Samples from four of the 25 species covered the range of ages that is needed to estimate species-specific racemization rates. The sample size from a single species of known age, the pygmy goat (*Capra hircus*, $n = 35$), was also large enough to investigate the accuracy of ages obtained using the AAR technique. The 35 goats were divided into three datasets: all goats ($n = 35$), goats >0.5 yrs old ($n = 26$) and goats >2 yrs old ($n = 19$). Leave-one-out analyses were performed on the three sets of data. Normalized root mean squared errors for the group of goats >0.5 yrs old were found to be the smallest. The higher variation in D/L measurements found for young goats <0.5 yrs can probably be explained by a period of continued postnatal growth of the eye lens. Normalized root mean squared errors from the leave-one-out cross-validation analyses based on goats >0.5 yrs old was for three age groups of the goats: 0.934 yrs for young goats <2 yrs ($n = 16$), 0.102 yrs for adult goats from 2–8 yrs ($n = 15$) and 0.133 yrs for old goats >8 yrs ($n = 4$). Thus, the age of an adult or an old animal can be predicted with approximately 10% accuracy, whereas the age of a young animal is difficult to predict. A goat specific racemization rate, as a $2k_{\text{Asp}}$ value, was

Garde E, Bertelsen MF, Ditlevsen S, Heide-Jørgensen MP, Nielsen NH, Frie AK, Olafsdottir D, Siebert U and Hansen SH (2018) Accuracy of the aspartic acid racemization technique in age estimation of mammals and the influence of body temperature. NAMMCO Scientific Publications, Volume 10. doi: <http://dx.doi.org/10.7557/3.4400>

estimated to $0.0107 \pm 3.8 \times 10^{-4}$ SE ($n = 26$). The $2k_{\text{Asp}}$ values from 12 species, four estimated in this study and another eight published, were used to examine the effect of core body temperature on the rate of racemization. A positive relationship between AAR and temperature was found ($r^2 = 0.321$) but results also suggest that other factors besides temperature are involved in the racemization process in living animals. Based on our results we emphasize that non-species-specific racemization rates should be used with care in AAR age estimation studies and that the period of postnatal growth of the eye lens be considered when estimating species-specific D/L₀ values and ages of young individuals.

INTRODUCTION

The ability to accurately estimate the age of animals and humans is crucial in e.g. studies of life history parameters of wild animal populations, body condition of wildlife, or age at death in forensic science (Ohtani 1995, Ritz-Timme *et al.* 2000, Garde *et al.* 2010, Ohtani and Yamamoto 2010). Counting annual growth layers in teeth is the most widely used method of age estimation for most mammal species, however some animals lack teeth such as the baleen whales (Mysticeti) while others, like the narwhal (*Monodon monoceros*), has a highly specialized tooth structure. Reliable and routinely applicable age estimation methods are therefore needed for these species.

Age estimation using the racemization of aspartic acid in teeth and eye lens nuclei has been a field of research for the past 40 years (Helfman and Bada 1975, Nerini 1983). The aspartic acid racemization (AAR) technique has been thoroughly investigated using humans of known age (Helfman and Bada 1976, Masters *et al.* 1977, Ohtani *et al.* 1995a, Ritz-Timme *et al.* 2003), but only a few studies have been conducted on known-age individuals from non-human mammal species with limited sample sizes as well (Bada *et al.* 1980, Fujii *et al.* 1989, Ohtani *et al.* 1995b). Although it is a promising alternative to traditional age estimation methods, AAR still requires validation from known-age animals as well as a more thorough examination of the racemization rates for different species.

Aspartic acid, as all amino acids except glycine, comes in two different isomeric forms, the laevo- (L) and the dextro- (D) (some amino acids can exist in four different chiral forms, e.g. isoleucine). In living proteins, only the L-form is utilized. However, in metabolically stable proteins the L-form is slowly, and at a constant rate, converted to the D-form, a process called racemization (Duin and Collins 1998). Aspartic acid racemizes the fastest of the amino acids and is as such measurable in living organisms. AAR has been detected in several types of human tissues such as teeth (Helfman and Bada 1976), eye lens nuclei (Masters *et al.* 1977), bone (Ohtani *et al.* 1998), brain (Fisher *et al.* 1992), and elastin (Ritz-Timme *et al.* 2003, Meissner and Ritz-Timme 2010). Tissue from eye lens nuclei is especially suitable for AAR analysis as this is among the most stable in the human body (Masters *et al.*

1977). The accumulation of D-aspartic acid in the proteins of the various tissues has been found to cause significant damage and has been linked to a variety of age-related diseases such as cataracts (Masters *et al.* 1978), Alzheimer's (Fisher *et al.* 1992) and atherosclerosis (Powell *et al.* 1992).

The rate at which L-aspartic acid racemizes to D-aspartic acid differs among tissues depending on the physiochemical properties of the amino residues of aspartic acid and asparagine (Ritz-Timme and Collins 2002). Also, the racemization rate accelerates with increasing temperatures (Bada and Schroeder 1975). It was previously suggested that racemization rates in teeth and eye lens nuclei were similar between mammalian species (Bada *et al.* 1980), however rates have been found to vary in lens nuclei from species such as humans (Masters *et al.* 1977), cetaceans (Nerini 1983, Garde *et al.* 2012, Rosa *et al.* 2013, Nielsen *et al.* 2013) and seals (Garde *et al.* 2010), as well as in teeth from humans (Helfman and Bada 1976) and rats (Ohtani *et al.* 1995b). Inter-species differences in core body temperature have been proposed as the reason for these differences (Ohtani *et al.* 1995b, Garde *et al.* 2007, Rosa *et al.* 2013).

This study is two-fold and focuses on: a) determining the accuracy of the AAR technique in age estimation of mammals of known ages by use of the pygmy goat (*Capra hircus*) as a case species, and b) examining the effect of core body temperature on species-specific racemization rates using animals of known age from zoological gardens and free-ranging animals, where ages were estimated using a traditional ageing method.

MATERIAL AND METHODS

Samples

Eye lenses for age estimation using the AAR technique were collected from 124 animals (25 mammal species; 7 orders) that had been held in captivity (zoos) or were free-ranging at the time of death (Appendix 1). In the period May 2007 – November 2009, eyes from 97 zoo animals (20 species, 6 orders) of approximate known age were collected. The zoo staff retrieved the eyes upon the animal's death and attained the individual ages. The remaining 27 animals were free-ranging (7 species; 3 orders) and had age estimated using other ageing techniques (Appendix 1). These included hooded seal (*Cystophora cristata*), harbour seal (*Phoca vitulina*), grey seal (*Halichoerus grypus*), fin whale (*Balaenoptera physalus*), killer whale (*Orcinus orca*), polar bear (*Ursus maritimus*) and reindeer (*Rangifer tarandus*).

Only a single species, the pygmy goat, constituted a sufficiently large and age-dispersed sample ($n = 36$) to predict the accuracy of the AAR technique for age estimation. Uncertainty regarding the age of one of the goats (ID no.

Z1) led to exclusion of that particular goat in the following analyses. Of the 35 remaining goats, nine goats were <0.5 yrs old, seven were >0.5–1 yrs old, 16 were 2–8 yrs old and four goats were 9–11 yrs old.

To be able to produce species-specific racemization rates, samples covering the range of ages for each species are needed (Garde *et al.* 2010). Such sets of samples were available for two of the known-age zoo species, the red-necked wallaby (*Macropus rufogriseus*, $n = 10$) and the lion (*Panthera leo*, $n = 5$), and two species of the free-ranging marine mammals, the hooded seal ($n = 6$) and the fin whale ($n = 7$) (Appendix 1). Age estimates for the hooded seals were obtained by counting of GLGs in the cementum of lower canine teeth (Mansfield 1991). Results for the fin whales were previously published (Nielsen *et al.* 2013) and are not presented here, but the fin whale specific racemization rate established in Nielsen *et al.* (2013) were used in the analysis of temperature-dependent racemization rates in this study. The difficulty in obtaining large sample sizes from both captive as well as free-ranging animals has resulted in small sample sizes of which the racemization rates for the red-necked wallaby, lion and hooded seal are based. These rates are therefore to be considered preliminary.

Eye lens dissection

Eyes were frozen at -20°C as soon as possible upon collection. In the laboratory, eye lenses were dissected out of the eyes. The outer lens layers were removed by rolling the lens on paper, and any remaining layers were removed under a stereoscope leaving only the lens nucleus for hydrolysis and subsequent High-Performance Liquid Chromatography (HPLC) analysis as described in detail in Garde *et al.* (2007; 2012). Any lenses containing blood or with signs of putrefaction were discarded. The lens nuclei were parted in two – one half was used for analysis and the other half was archived at -20°C .

Hydrolysis and HPLC analysis

Procedures of Zhao and Bada (1995) and Garde *et al.* (2007; 2010) were followed for hydrolysis of samples and analysis by HPLC. Eye lens nuclei were hydrolysed in glass tubes containing 1 ml 6 M HCl for 6 hours at 100°C . Chromatography and data analysis was performed using an Agilent 1100 Series HPLC system (Agilent Technologies, Walbronn, Germany). Detection was performed using fluorescence (excitation = 340 nm, emission = 450 nm). The column was a Zorbax Eclipse XDB-C18, 4.6×150 mm, with particle size $3.5 \mu\text{m}$.

The D/L ratios measured by HPLC were calibrated using the following D/L standards: 0.5/99.5, 1/99, 2/98, 5/95, 10/90, and 15/85, which were run at the beginning and end of each HPLC run. Measured D/L ratios from the eye lens nuclei were recalculated using calibration equations (linear regression) for the

D/L standards. Two standard curves were produced for each HPLC run using D/L standards as described above. Linear regression equations for each run were calculated by regression of theoretical D/L ratios versus the measured D/L ratios from the D/L standards. The coefficient was $r^2 = 0.99$ for each run. Equations with a slope closest to 1 (range: 1.0 – 1.3) from each run were used to recalculate D/L ratios for the samples.

D/L ratios, racemization rates (as $2k_{\text{Asp}}$ values) and age estimates

Individual D/L ratios were generated for each of the 124 animals (Appendix 1). For 26 of the 124 individuals, both left and right eyes were analysed. A paired *t*-test showed no difference between the two eyes ($t = 0.74$, *d.f.* = 25, $P = 0.46$) and an average D/L ratio was used in subsequent analyses.

The pygmy goat

Equations to estimate the age of a goat from an AAR measurement (D/L value) by regressing age on AAR were made following the procedures presented in Garde *et al.* (2012; 2015) and Ohtani and Yamamoto (2011). Three equations were made: the first equation included all goats ($n = 35$), the second excluded nine goats <0.5 yrs old ($n = 26$), and the third further excluded seven goats between >0.5–1 yrs old ($n = 19$). The D/L values from young individuals show higher variation than older individuals (George *et al.* 1999, Garde *et al.* 2007; 2010) and exclusion of the younger groups was done to find the best fit of the regression line. Known ages were regressed against individual ratios of $x = \ln\left[\frac{1 + D/L}{1 - D/L}\right]$ (eq. (1); Garde *et al.* 2015). The ages from the 35 goats were not known exactly and the uncertainty (as standard deviation) differed between goats (Appendix 1) thus weighted regressions were used. Standard deviations for the known ages of each goat were assessed based on information on goat ages from Copenhagen Zoo. A leave-one-out cross-validation analysis was done for all three equations by performing 35, 26 and 19 regressions, one for each goat. Each regression was made on the remaining 34, 25 and 18 goats and the results from this regression were used to predict the age on the one remaining goat. The accuracy of an age estimate was determined by the distance between the predicted and the known age, normalized by the known age. Final prediction equations were made from 35, 26 and 19 goats. Accuracy of the ages was measured by the normalized root mean squared error from the leave-one-out cross-validation analysis, split into three age groups: young (<2 yrs, $n = 16$), adult (2–8 yrs, $n = 15$) and old (>8 yrs, $n = 4$).

A goat specific racemization rate was estimated based on the group of goats with the smallest error ($n = 26$, >0.5 yrs old goats) and estimated following Garde *et al.* (2012). In this bootstrap study, the ages were drawn from uniform distributions on intervals given by the estimated age ± 2 times the standard deviation. The uniform distribution was chosen because ages were often

given as rounded values, and thus, there was no information about where the true age might lie within an interval around the given age. The number of bootstrap replicates used was 10,000. The average estimate of the slope of the regression line corresponds to twice the racemization rate ($2k_{\text{Asp}}$) and the intercept corresponds to twice the $(\text{D/L})_0$ value.

Species-specific racemization rates for the red-necked wallaby, lion and hooded seal

For the red-necked wallaby, lion and hooded seal species-specific racemization rates were made following the procedures in Garde *et al.* (2012; 2015). Standard deviations for the known ages of each wallaby and lion were assessed based on information on individual ages from the zoos. For the hooded seals ($n = 6$) a standard deviation of one year was considered appropriate.

The effect of core body temperature on the rate of racemization

The effect of core body temperature on the rate of racemization was investigated by weighted regression of the four species-specific racemization rates (for pygmy goat, red-necked wallaby, lion and hooded seal) estimated in this study and eight published rates against the species-specific core body temperatures ($^{\circ}\text{C}$). The eight published racemization rates were for humans (Masters *et al.* 1977), narwhals (Garde *et al.* 2012), harp seals (*Pagophilus groenlandicus*, Garde *et al.* 2010), harbour porpoises (*Phocoena phocoena*, Nielsen *et al.* 2013), fin whales (*Balaenoptera physalus*, Nielsen *et al.* 2013), bowhead whales (*Balaena mysticeti*, Rosa *et al.* 2013), beluga whales (*Delphinapterus leucas*, Pleskach *et al.* 2016) and minke whales (*Balaenoptera acutorostrata*, Nielsen *et al.* 2017). Pleskach *et al.* (2016) presented two racemization rates for the beluga whale estimated using two different analytical methods. For comparison reasons, the rate presented here was based on HPLC UV/VIS detection (Pleskach *et al.* 2016), which is the most widely used method to measure aspartic acid racemization.

Core body temperatures instead of eyeball temperatures were chosen for the regression for three main reasons: 1) eye temperatures have been shown to be practically the same as or at least correlate with core body temperatures in several mammal species (Melero *et al.* 2015), 2) in situ measurements of whale eyeball temperatures are difficult if not impossible (Rosa *et al.* 2013), and 3) published eyeball temperatures for all the 12 species used in this study were not available.

RESULTS

Accuracy of goat AAR ages and estimation of a goat specific $2k_{\text{Asp}}$ and $(\text{D/L})_0$ value

The slopes and intercepts of the three equations (weighted regressions, with weights proportional to the inverse of the variance) for the 35 goats, 26 goats >0.5 yrs and 19 goats >2 yrs had standard deviations of 6.9 and 0.7, 4.9 and 0.5, and 6.8 and 0.8, respectively (Table 1). Smallest standard deviations was found for the equation excluding nine goats <0.5 yrs even though this analysis builds on a smaller data set ($n = 26$). This equation was estimated to be:

$$\text{Age} = 98.3 \pm 4.9 \text{ SE} \times \text{AAR} - 5.5 \pm 0.5 \text{ SE} \quad (1)$$

where AAR equals $x = \ln\left[\frac{1 + D/L}{1 - D/L}\right]$ (Fig. 1). The three equations (Table 1) were then used to estimate how well we predict the ages by the normalized root mean squared errors in the leave-one-out cross-validation analyses. The best age predictions were obtained when excluding goats <0.5 yrs old (Eq. 1, Table 1), where errors were found to be 0.49 yrs for all 35 goats, and then 0.93 yrs for the young group (<2 yrs, $n = 16$), 0.10 yrs for the adult group (2–8 yrs, $n = 15$) and 0.13 yrs for the old group (>8 yrs, $n = 4$). Thus, the age of an adult or an old animal can be predicted with approximately 10% accuracy, whereas the age of a young animal is difficult to predict. Note that the adult group is expected to have a lower error since they fall in the middle of the covariate distribution.

A goat specific racemization rate and D/L_0 value was estimated based on the 26 pygmy goats >0.5 yrs old. Known ages were regressed against values of $x = \ln\left[\frac{1 + D/L}{1 - D/L}\right]$ and a bootstrap study was used to determine standard errors (Garde *et al.* 2012). The average estimate of the slope ($2k_{\text{Asp}}$) was $0.0107 \pm 3.8 \times 10^{-4}$ SE with an intercept of 0.0526 ± 0.0022 SE (r^2 between 0.91 and 0.96) (Fig. 2). We estimate the racemization rate to $0.00536 \pm 1.9 \times 10^{-4}$ SE and the $(D/L)_0$ to 0.0263 ± 0.0011 SE.

Estimation of species-specific $2k_{\text{Asp}}$ and $(D/L)_0$ values for the red-necked wallaby, lion, and hooded seal

Species-specific racemization rates and D/L_0 values were estimated for the red-necked wallaby, the lion and the hooded seal by regression of known or estimated ages against values of $x = \ln\left[\frac{1 + D/L}{1 - D/L}\right]$. A bootstrap study was used to determine standard errors (Garde *et al.* 2012).

Table 1. Equations for all goats ($n = 35$), goats > 0.5 yrs old ($n = 26$) and goats >2 yrs old ($n = 19$) and the normalized root mean squared errors (RMSE) from the leave-one-out cross-validation analyses for all 35 goats and split into three groups: young (<2 yrs, $n = 16$), adult (2–8 yrs, $n = 15$) and old (>8 yrs, $n = 4$).

Goats	Equation	RMSE (yrs)
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		All data	Young goats	Adult goats	Old goats
All goats	Age=110.7 ± 6.9 SE x AAR - 7.1 ± 0.7 SE	3.0	4.43	0.145	0.130
Goats > 0.5 yrs	Age=98.3 ± 4.9 SE x AAR - 5.5 ± 0.5 SE	0.493	0.934	0.102	0.113
Goats > 2 yrs	Age=103.1 ± 6.8 SE x AAR - 6.0 ± 0.8 SE	0.112	NA	0.107	0.128

Red-necked wallaby

The average estimate of the slope for the red-necked wallaby ($n = 10$) was $0.00464 \pm 3.6 \times 10^{-4}$ SE with an intercept of 0.061 ± 0.0027 SE (r^2 between 0.61 and 0.95, Fig. 2). The red-necked wallaby specific racemization rate was then estimated to $0.00232 \pm 1.8 \times 10^{-4}$ SE and the $(D/L)_0$ to 0.0305 ± 0.0014 SE.

Lion

The average estimate of the slope for the lion ($n = 5$) was $0.00701 \pm 2.8 \times 10^{-4}$ SE with an intercept of 0.0489 ± 0.0017 SE (r^2 between 0.88 and 0.98, Fig. 2). The lion specific racemization rate was estimated to $0.0035 \pm 1.4 \times 10^{-4}$ SE and the $(D/L)_0$ to $0.0245 \pm 9.0 \times 10^{-4}$ SE.

Hooded seal

The average estimate of the slope for the hooded seal ($n = 6$) was $0.00305 \pm 1.8 \times 10^{-4}$ SE with an intercept of 0.0445 ± 0.0014 SE (r^2 between 0.74 and 0.96, Fig. 2). The hooded seal specific racemization rate was estimated to $0.00152 \pm 9.0 \times 10^{-5}$ SE and the $(D/L)_0$ to $0.0223 \pm 7.0 \times 10^{-4}$ SE.

Correlation of $2k_{Asp}$ values against core body temperatures

The four species-specific $2k_{Asp}$ values estimated in this study plus an additional eight published $2k_{Asp}$ values were regressed against average species-specific core body temperatures ($^{\circ}C$) (Fig. 3; Table 2). A positive relationship was found between $2k_{Asp}$ values and core body temperatures ($r^2 = 0.321$).

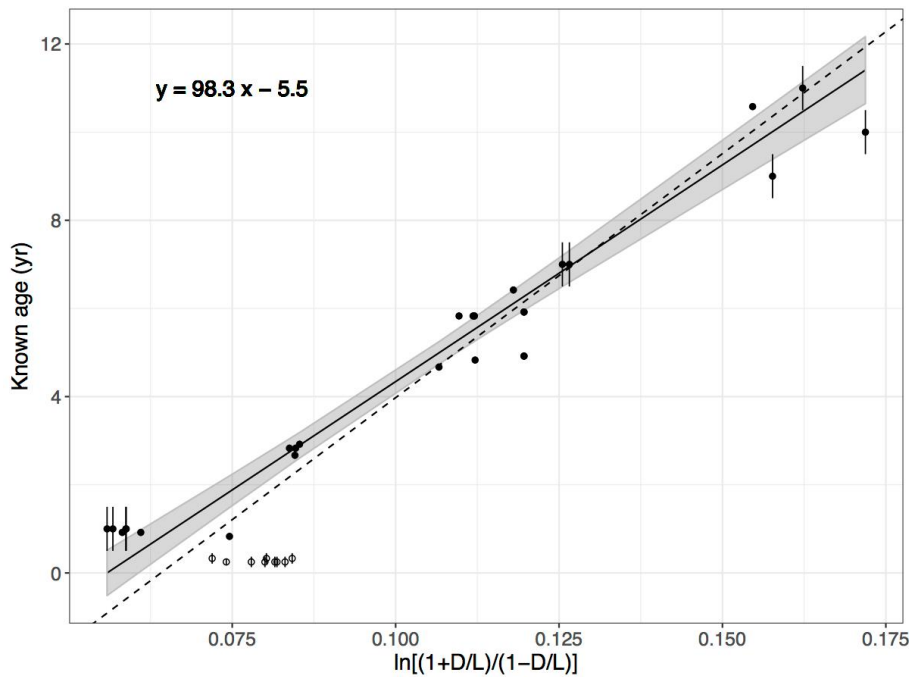


Fig. 1. Weighted linear regression of $x = \ln\left[\frac{1 + D/L}{1 - D/L}\right]$ against known age (yr) for 26 pygmy goats. The dark band is the 95% confidence intervals for the line. The lines through each point are 2 times the standard deviation. Some points have practically no uncertainty and therefore very short lines. The dashed line is the estimation using the entire pygmy goat data set ($n = 35$), and the open circles are the measurements from the youngest goats ($n = 9$) that do not enter in the final analysis.

DISCUSSION

Sampling

Robust estimation of species-specific racemization rates and $(D/L)_0$ values ideally requires samples covering the range of ages including near-term fetuses or postpartum individuals. Large-scale collection of free-ranging known-age animals that grow to old ages is a challenge and inference from captive animals is the most obvious alternative. Collection of samples from captive known-age animals that grow to old ages has, however, proved difficult. Collaboration with the Copenhagen Zoo and Givskud Zoo in Denmark for a 2.5-year period resulted in samples from a range of species but only three species constituted sufficiently large sample sizes covering the range of ages to be used for estimation of species-specific racemization rates. Collection of samples from bovines from a Danish slaughterhouse was also attempted, however, most bovines are put down before the age of 8, which is not representable of longevity of about 20 years for domestic cattle (AnAge Database, September 2017). Collection of other domestic

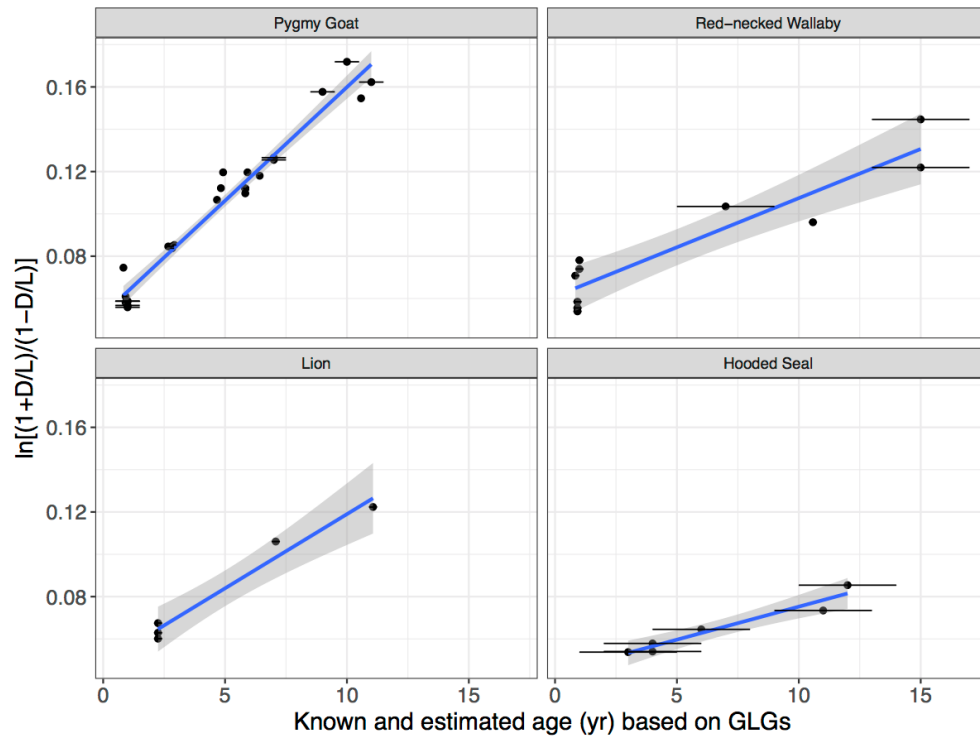


Fig. 2. Regression line from the bootstrap study of D/L ratios against known and estimated ages for the pygmy goat, lion, red-necked wallaby and hooded seal. The slope of the regression line corresponds to twice the racemization rate ($2k_{Asp}$) and the intercept corresponds to twice the $(D/L)_0$ value.

animals e.g. horses, dogs or cats would not only require collaboration with numerous veterinarians but also assembling approvals from the owners, which was considered beyond the scope of this study.

Accuracy of the AAR technique for age estimation of known-age pygmy goats

During the sampling period only a single species, the pygmy goat, provided a large and age-dispersed sample. We found highest accuracy between predicted and known ages for the adult and old goats compared to the young. Our findings demonstrate that the AAR method is more reliable for adult and older pygmy goats, which is considered also to apply for other mammals (Garde *et al.* 2010; 2015). In Garde *et al.* (2010), intra-specific variation in D/L ratios of 12 postpartum harp seals, used to estimate a species-specific (D/L)₀ value, were also observed. Underestimation in AAR ages for young harp seals 1–7 years old was partly explained by this intra-specific variation and error in the (D/L)₀ estimate. Overall, the study involving 113 harp seals found, however, good agreement between the two ageing methods used; AAR in eye lens nuclei and GLGs in teeth.

The variation found in D/L measurements for young pygmy goats as well as for young individuals of other mammalian species could be a result of eye lenses not being fully developed postpartum.

Development of the eye lens postpartum and its implication for AAR age estimation studies

The lens grows rapidly during late embryonic and early postnatal stages by cell division and differentiation (Lovicu and McAvoy, 2005). In humans, the lens grows rapidly in the embryo and during the first postnatal year (Lynnerup *et al.* 2008; 2010, Bebe 2003). A recent study has shown that amino acids are incorporated into the proteins of rat eye lenses during fetal development and at pup age, revealing a postnatal growth of the rat lens. Beyond the age of 25 days synthesis of proteins in the rat eye lenses could no longer be detected and hence, no further growth of the lens was expected beyond this age. Proteins synthesized during fetal development and a few days after birth were also present later in the rat's life (>1 year) (Bechshøft *et al.* 2017). Fris and Midelfart (2007) also found changes in the contents of energy metabolites and amino acids in rat eye lenses postnatal. They suggested that the changes could be caused by the transformation of primary fiber cells to the fetal nucleus – a transformation that occurs during the critical maturation of the rat lens from between day 12 and 16 to approximately 21 days after birth. After the age of approximately 30 days the lens will become resistant and the metabolic activity will diminish. The critical maturation period of the rat lens is related to the atrophy of a supporting vascular bed tissue (tunica vasculosa lentis) (Fris and Midelfart 2007). By the fourth week after birth the tunica

vasculosa lentis has completely regressed and delivery of several compounds to the avascular lens tissue significantly reduced (Lang 1997: Fig. 3 for illustration of the regressing tunica vasculosa lentis). During the postnatal development of the lens, the content of specific amino acids in the lens will decline, among them aspartic acid, which is probably because aspartic acid is used in the synthesis of the lenticular proteins (Heinämäki and Lindfors 1988). The variation in D/L values observed in this study for pygmy goats <0.5 yrs old can probably be explained by a continued postnatal growth of the pygmy goat eye lens.

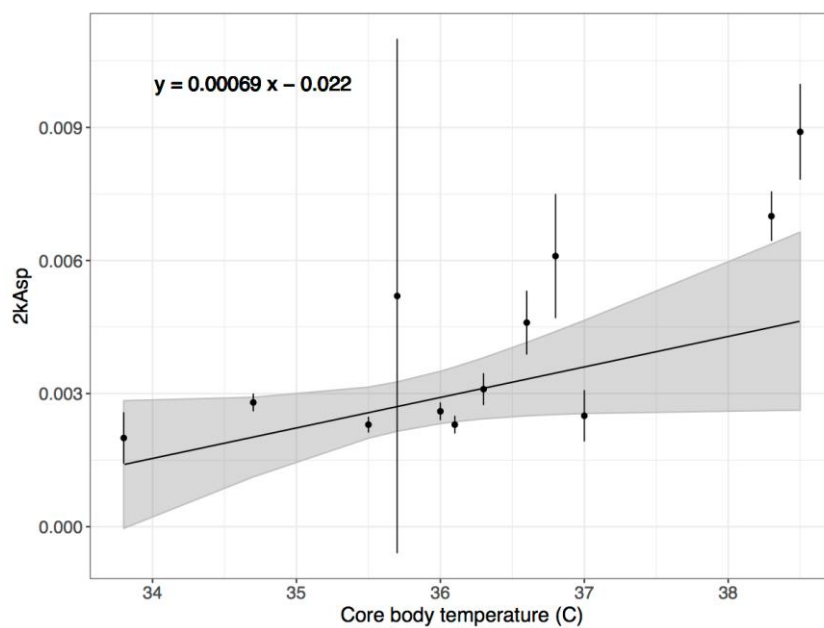


Fig. 3. The $2k_{ASP}$ values estimated in this study for the pygmy goat, red-necked wallaby, lion and hooded seal, and eight published $2k_{ASP}$ values for harp seal, narwhal, harbour porpoise, fin whale, bowhead whale, beluga whale, minke whale and human regressed (weighted regression) against average core body temperatures ($^{\circ}\text{C}$) for each of the 12 species. The specific values are listed in Table 2. Error bars equal \pm SE.

Higher variation in D/L values of young individuals compared to adults and older individuals has also been observed in a range of marine mammals, including the bowhead whale (George *et al.* 1999, Rosa *et al.* 2013), narwhal (Garde *et al.* 2007; 2015), harp seal (Garde *et al.* 2010), and minke whale (Nielsen *et al.* 2017) suggesting that eye lenses of marine mammals are also not fully developed postpartum. At which age the lens will cease growing in different species of marine mammals is, to our knowledge, unknown. The tunica vasculosa lentis, or a similar capillary structure, has been observed surrounding the eye lenses of young narwhals during dissection of the lens

nucleus for AAR age estimation (pers. obs. E. Garde). Continued growth of the eye lens postnatally has significant implications for estimation of species-specific D/L_0 values and the accuracy of age estimation of young marine mammals using the AAR technique. The AAR technique relies on a linear rate of racemization from birth to death of the individual, which is not the case in metabolically active tissues. Investigations in the development of the marine mammal lens as well as establishment of a model taking the early growth pattern into account will be a significant contribution to the continued use of AAR age estimation studies of young marine mammals.

Species-specific racemization rates and the effect of core body temperature

For the red-necked wallaby, lion, and hooded seal, a smaller yet representative sample size was collected. We recognize that the small sample sizes result in less precise estimation of the species-specific racemization rates and $(D/L)_0$ values, but also emphasize the difficulty in obtaining samples from known-age captive animals of especially older age.

We found a positive relationship between the rate of racemization of aspartic acid and core body temperatures ($r^2 = 0.321$, Fig. 3), which is not surprising as it is well known that the racemization rate is a function of temperature (Bada and Schroeder 1975). Higher racemization rates were found for the pygmy goat and lion, which also have the highest core body temperatures ($>38^\circ\text{C}$) of the species presented in Fig. 3. The harbour porpoise racemization rate was the highest of the marine mammals, which is consistent with the higher core body temperature of the harbour porpoise compared to the other marine mammals (Fig. 3). The rate of the beluga, however, seem high considering a relatively low core body temperature of 35.7°C (Melero *et al.* 2015). Also the narwhal, the beluga's closest relative, has a racemization rate considerably lower even though core body temperatures of the two species is almost the same (Fig. 3). The large SE of the beluga racemization rate estimate could explain the difference in rates observed for the beluga and the other marine mammals with similar low core body temperatures. The bowhead whale has the lowest racemization rate of all the marine mammals (Fig. 3) consistent with a low core body temperature. Our findings show that core body temperature indeed is a major driver of AAR, however, considering the differences in racemization rates of species with almost similar core body temperatures, e.g. the harbour porpoise (36.8°C) versus humans (36.8°C), also suggests that other factors besides temperature are involved. These other factors could include protein primary and secondary structures (Ritz-Timme *et al.* 2003), however, no

Table 2. Core body temperature (°C), estimate of $2k_{ASP}$ and $(D/L)_0$ value and associated SE for four species in this study and published values for another eight species. Average core temperatures (°C) are from Teare (2002), except for records of fin whale (Brodie and Paasche 1985), minke whale (Folkow and Blix 1992), harbour porpoise (Desportes *et al.* (2003), bowhead whales (Rosa *et al.* 2013), narwhal (Heide-Jørgensen *et al.* 2014), and beluga (Melero *et al.* 2015).

Species	Latin name	n	Core body temp. (°C)	$2k_{ASP}$	SE	$(D/L)_0$	SE	r^2	Age (yrs)	Reference*
Pygmy goat	<i>Capra hircus</i>	35	38.5	0.0089	5.4×10^{-4}	0.0327	1.3×10^{-3}	0.89 [†]	Known	This study
Red-necked wallaby	<i>Macropus rufogriseus</i>	10	36.6	0.0046	3.6×10^{-4}	0.0305	1.3×10^{-3}	0.87 [†]	Known	This study
Løve	<i>Panthera leo</i>	5	38.3	0.0070	2.8×10^{-4}	0.0244	9.0×10^{-4}	0.97 [†]	Known	This study
Hooded seal	<i>Cystophora cristata</i>	6	36.3	0.0031	1.8×10^{-4}	0.0223	7.0×10^{-4}	0.94 [†]	Known	This study
Human	<i>Homo sapiens</i>	17	37.0	0.0025	2.9×10^{-4}	0.0560	-	0.83	Known	Masters <i>et al.</i> 1977
Harp seal	<i>Pagophilus groenlandicus</i>	25	36.0	0.0026	1.0×10^{-4}	0.0193	4.8×10^{-4}	0.96	GLG	Garde <i>et al.</i> 2010
Harbour porpoise	<i>Phocoena phocoena</i>	17	36.8	0.0061	7.0×10^{-4}	0.0228	1.8×10^{-3}	0.83	GLG	Nielsen <i>et al.</i> 2013
Narwhal	<i>Monodon monoceros</i>	9	35.5	0.0023	8.9×10^{-5}	0.0290	9.3×10^{-4}	0.98	GLG	Garde <i>et al.</i> 2012
Beluga whale	<i>Delphinapterus leucas</i>	11	35.7	0.0052 [§]	2.9×10^{-3}	0.0520 [§]	5.7×10^{-2}	0.25	GLG	Pleskach <i>et al.</i> 2016
Minke whale	<i>Balaenoptera acutorostrata</i>	30	34.7	0.0028	1.0×10^{-4}	0.0194	1.2×10^{-3}	0.96	Ear plugs	Nielsen <i>et al.</i> 2017
Fin whale	<i>Balaenoptera physalus</i>	30	36.1	0.0023	1.0×10^{-4}	0.0282	1.2×10^{-3}	0.91	Ear plugs	Nielsen <i>et al.</i> 2013
Bowhead whale	<i>Balaena mysticetus</i>	29	33.8	0.0020	2.9×10^{-4}	0.0250	1.3×10^{-3}	-	Baleen/ovarian corpora	Rosa <i>et al.</i> 2013

*References refers to $2k_{ASP}$ and $(D/L)_0$ values.

[†]Bootstrap study.

[§] Values for the HPLC-UV/VIS method is referenced here.

additional experiments were done in this study to further elucidate this feature, and a full discussion on this topic is beyond the scope of this paper.

Rosa *et al.* (2013) also found a positive relationship between core body temperature and racemization rates using data from bowhead whales (Rosa *et al.* 2013), fin whales (Nerini 1983) and humans (Masters *et al.* 1977) and based on data from these three species constructed an equation for predicting $2k_{\text{Asp}}$ values, when core body temperature was given. Rosa *et al.* (2013) suggested that age estimates can be obtained using the relationship between the rate of racemization and core body temperatures but also stated that it is better to estimate a rate for the species being studied than to use values from other species. We find that the slope of $2k_{\text{Asp}}$ on core body temperatures in the range from 33.8–38.5°C was considerably greater than shown by Rosa *et al.* (2013) (Fig. 3).

To summarize, the accuracy of the AAR technique for age estimation of mammals was predicted using known age pygmy goats. The technique was found to be reliable for adults and older individuals, whereas it was less accurate for younger individuals, as also found for harp seals (Garde *et al.* 2010) and narwhals (Garde *et al.* 2012). This can probably be explained by a period of continued postnatal growth of the mammalian eye lens. Species-specific racemization rates were estimated for the pygmy goat, red-necked wallaby, lion, and hooded seal and a positive relationship was found by regression of racemization rates against core body temperatures. We found that core body temperature is a major driver of AAR, however, other factors besides temperature are involved in the racemization process in living animals. Based on our results we emphasize that non-species-specific racemization rates should be used with care in AAR age estimation studies, and that the period of postnatal growth of the eye lens be considered when estimating species-specific D/L_0 values and ages of young individuals.

ACKNOWLEDGEMENTS

Thanks to Kim Rasmussen from Givskud Zoo and Svend Tougaard from the Fisheries and Maritime Museum, Esbjerg, for providing eye samples. Also thanks to the editor and two reviewers' for their helpful comments and innovative ideas that have improved the manuscript.

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Appendix 1. Data, including D/L values, age (yrs) and assessed error, for 117 animals (24 species; 7 orders) in this study. Data on the fin whale ($n = 7$) was presented in Nielsen *et al.* (2013) and are not shown here. The assessed errors were based on the specific age information from the zoos. For the hooded seals ($n = 6$) assessed errors of 1 year was anticipated. Samples from the hooded seals were collected during commercial hunting operations. Samples from harbour seals, grey seals, and the single killer whale were from stranded animals. Underlined D/L ratios are an average of D/L ratios from the left and right eye.

^a Cph Zoo: Copenhagen Zoo; DE: Germany; DK: Denmark; NL: the Netherlands; EG: East Greenland; WG: West Greenland; IS: Iceland; NO: Norway.

^b All captive animals from Copenhagen Zoo or Givskud Zoo were of approximate known ages. Age estimates for the seals were estimated by counting of GLGs in the cementum of lower canine teeth. The age estimate for the polar bear from East Greenland (ID No II V) was based on body length and condition and size of teeth. It was taken in subsistence hunting in East Greenland. The stranded killer whale was young, assessed to be less than one year. Samples from the three reindeer from West Greenland were collected as part of a project performed by the Greenland Institute of Natural Resources. Two were near-term fetuses (= 0 years old) and one a calf from the previous season (= 1 years old).

ID no.	Species	Latin name	Order	Provenance ^a	D/L	Known or estimated age (yrs) ^b	Assessed error (yr) on age estimates
Z82	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.07716	10.6	0.04
Z84	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.05594	5.8	0.04
Z81	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.05587	5.8	0.04
Z79	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.05596	5.8	0.04
Z77	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.05602	4.8	0.04
Z80	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.05894	6.4	0.04
Z85	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.02836	1.0	0.25
Z86	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.02792	1.0	0.25
Z87	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.02935	1.0	0.25
Z88	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.02938	1.0	0.25
ICE5A29	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.0548	5.8	0.04
ICDAF2B	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.06267	7.0	0.25
11417	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.02908	0.92	0.04
5276	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.04262	2.9	0.04
13369	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.03049	0.92	0.04
15882	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.05327	4.7	0.04
ICF016D	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.06321	7.0	0.25
43268	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.04185	2.8	0.04
1CEDB1C	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.05975	4.9	0.04
389	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.04227	2.7	0.04
1CDC5E3	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.05975	5.9	0.04
7348	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.04233	2.8	0.04
6955F	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.08096	11.0	0.25
813	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.03727	0.8	0.04
Z1	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	<u>0.07864</u>	8.0	-
Z2	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	<u>0.03595</u>	0.33	0.06
Z3	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	<u>0.04152</u>	0.25	0.06
Z14	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	<u>0.07867</u>	9.0	0.25
Z15	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	<u>0.08573</u>	10.0	0.25
Z16	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.03703	0.3	0.04
Z17	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	<u>0.03894</u>	0.25	0.06
Z18	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	<u>0.04074</u>	0.25	0.06
Z19	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	<u>0.03998</u>	0.25	0.06
Z20	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	<u>0.0409</u>	0.25	0.06
Z21	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	<u>0.04009</u>	0.33	0.06

Z22	Pygmy goat	<i>Capra hircus</i>	Artiodactyla	Cph Zoo	0.04206	0.33	0.06
Z53	Red-necked Wallaby	<i>Macropus rufogriseus</i>	Diprotodontia	Cph Zoo	0.02696	0.9	0.08
Z54	Red-necked Wallaby	<i>Macropus rufogriseus</i>	Diprotodontia	Cph Zoo	0.02776	0.9	0.08
Z58	Red-necked Wallaby	<i>Macropus rufogriseus</i>	Diprotodontia	Cph Zoo	0.02923	0.9	0.08
Z72	Red-necked Wallaby	<i>Macropus rufogriseus</i>	Diprotodontia	Cph Zoo	0.04799	10.6	0.08
K7yr	Red-necked Wallaby	<i>Macropus rufogriseus</i>	Diprotodontia	Cph Zoo	0.0517	7.0	1.0
Z23	Red-necked Wallaby	<i>Macropus rufogriseus</i>	Diprotodontia	Cph Zoo	<u>0.03538</u>	0.8	0.08
Z24	Red-necked Wallaby	<i>Macropus rufogriseus</i>	Diprotodontia	Cph Zoo	<u>0.03696</u>	1.0	0.08
Z25	Red-necked Wallaby	<i>Macropus rufogriseus</i>	Diprotodontia	Cph Zoo	0.06089	15.0	1.0
Z26	Red-necked Wallaby	<i>Macropus rufogriseus</i>	Diprotodontia	Cph Zoo	<u>0.03902</u>	1.0	0.08
Z27	Red-necked Wallaby	<i>Macropus rufogriseus</i>	Diprotodontia	Cph Zoo	<u>0.07219</u>	15.0	1.0
G.Lø	Lion	<i>Panthera leo</i>	Carnivora	Givskud Zoo	0.0611	11.1	0.08
Z75	Lion	<i>Panthera leo</i>	Carnivora	Cph Zoo	0.03373	2.3	0.08
Z76	Lion	<i>Panthera leo</i>	Carnivora	Cph Zoo	0.03144	2.3	0.08
Z78	Lion	<i>Panthera leo</i>	Carnivora	Cph Zoo	0.03005	2.3	0.08
Z89	Lion	<i>Panthera leo</i>	Carnivora	Givskud Zoo	0.05297	7.1	0.08
33/08	Hooded seal	<i>Cystophora cristata</i>	Carnivora	NO	0.04269	12.0	1.0
32/08	Hooded seal	<i>Cystophora cristata</i>	Carnivora	NO	0.02703	4.0	1.0
30/08	Hooded seal	<i>Cystophora cristata</i>	Carnivora	NO	0.03671	11.0	1.0
28-08	Hooded seal	<i>Cystophora cristata</i>	Carnivora	NO	0.02688	3.0	1.0
25/08	Hooded seal	<i>Cystophora cristata</i>	Carnivora	NO	0.03225	6.0	1.0
20-08	Hooded seal	<i>Cystophora cristata</i>	Carnivora	NO	0.02893	4.0	1.0
Z50	Tiger	<i>Panthera tigris</i>	Carnivora	Cph Zoo	0.02051	0.0	-
Z51	Tiger	<i>Panthera tigris</i>	Carnivora	Cph Zoo	0.02235	0.0	-
Z37	Tiger	<i>Panthera tigris</i>	Carnivora	Cph Zoo	0.04404	1.8	-
Z38	Tiger	<i>Panthera tigris</i>	Carnivora	Cph Zoo	0.04357	1.8	-
Z39	Tiger	<i>Panthera tigris</i>	Carnivora	Cph Zoo	<u>0.03335</u>	0.0	-
Z40	Brown bear	<i>Ursus arctos</i>	Carnivora	Cph Zoo	0.04154	1.5	-
Z41	Brown bear	<i>Ursus arctos</i>	Carnivora	Cph Zoo	0.03978	1.5	-
Z45	Caracal	<i>Felis caracal</i>	Carnivora	Cph Zoo	0.05218	1.8	-
G.ulv	Wolf	<i>Canis lupus</i>	Carnivora	Givskud Zoo	0.03168	1.0	-
Z56	Emperor Tamarin	<i>Saguinus imperator</i>	Primates	Cph Zoo	0.0379	0.5	-
Z52	Ring-tailed Lemur	<i>Lemur catta</i>	Primates	Cph Zoo	0.05123	6.0	-
Z43	Ring-tailed Lemur	<i>Lemur catta</i>	Primates	Cph Zoo	<u>0.07977</u>	13.0	-
Z61	Hamadryas baboon	<i>Papio hamadryas</i>	Primates	Cph Zoo	0.04073	1.0	-
Z62	Hamadryas baboon	<i>Papio hamadryas</i>	Primates	Cph Zoo	0.0509	1.2	-
Z63	Hamadryas baboon	<i>Papio hamadryas</i>	Primates	Cph Zoo	0.03513	1.0	-
Z66	Hamadryas baboon	<i>Papio hamadryas</i>	Primates	Cph Zoo	0.05082	2.0	-
Z68	Hamadryas baboon	<i>Papio hamadryas</i>	Primates	Cph Zoo	0.05932	3.0	-
Z69	Hamadryas baboon	<i>Papio hamadryas</i>	Primates	Cph Zoo	0.0466	1.2	-
Z70	Hamadryas baboon	<i>Papio hamadryas</i>	Primates	Cph Zoo	0.05467	2.0	-
Z71	Hamadryas baboon	<i>Papio hamadryas</i>	Primates	Cph Zoo	0.06127	4.0	-
Z73	Hamadryas baboon	<i>Papio hamadryas</i>	Primates	Cph Zoo	0.03993	0.7	-
Z74	Hamadryas baboon	<i>Papio hamadryas</i>	Primates	Cph Zoo	0.04546	0.7	-
Z60	Hamadryas baboon	<i>Papio hamadryas</i>	Primates	Cph Zoo	0.04301	1.0	-
Z64	Hamadryas baboon	<i>Papio hamadryas</i>	Primates	Cph Zoo	0.03398	1.0	-
Z65	Hamadryas baboon	<i>Papio hamadryas</i>	Primates	Cph Zoo	0.04339	1.0	-
Z67	Hamadryas baboon	<i>Papio hamadryas</i>	Primates	Cph Zoo	0.04838	1.5	-
Z28	Patagonian mara	<i>Dolichotis patagonum</i>	Rodentia	Cph Zoo	0.03422	0.3	-
Z31	Patagonian mara	<i>Dolichotis patagonum</i>	Rodentia	Cph Zoo	0.04105	0.1	-
Z32	Patagonian mara	<i>Dolichotis patagonum</i>	Rodentia	Cph Zoo	0.04272	0.7	-
Z33	Patagonian mara	<i>Dolichotis patagonum</i>	Rodentia	Cph Zoo	<u>0.04431</u>	0.4	-
Z47	Domestic Rabbit	<i>Oryctolagus cuniculus</i>	Lagomorpha	Cph Zoo	<u>0.03395</u>	0.6	-
Z42	Muskox	<i>Ovibos moschatus</i>	Artiodactyla	Cph Zoo	0.08126	10.2	-
Z83	Muskox	<i>Ovibos moschatus</i>	Artiodactyla	Cph Zoo	0.06367	7.9	-
M16	Muskox	<i>Ovibos moschatus</i>	Artiodactyla	Cph Zoo	0.10283	16.5	-
Z59	Impala	<i>Aepyceros melampus</i>	Artiodactyla	Cph Zoo	0.03208	1.5	-
Z44	Impala	<i>Aepyceros melampus</i>	Artiodactyla	Cph Zoo	0.04056	1.5	-
Z46	Domestic pig	<i>Sus scrofa f.</i>	Artiodactyla	Cph Zoo	0.05618	4.0	-

G.gnu	Wildebeest	<i>Connochaetes taurinus</i>	Artiodactyla	Givskud Zoo	0.03518	1.1	-
G.ko	Domestic cow	<i>Bos taurus</i>	Artiodactyla	Givskud Zoo	0.04346	4.3	-
Z55	Sable Antelope	<i>Hippotragus niger</i>	Artiodactyla	Cph Zoo	0.02538	0.0	-
Z35	Reindeer	<i>Rangifer tarandus</i>	Artiodactyla	Cph Zoo	0.03552	0.3	-
Z36	Reindeer	<i>Rangifer tarandus</i>	Artiodactyla	Cph Zoo	0.03692	0.3	-
Z57	Reindeer	<i>Rangifer tarandus</i>	Artiodactyla	Cph Zoo	0.0828	13.0	-
AK08-23F	Reindeer	<i>Rangifer tarandus</i>	Artiodactyla	WG	0.03314	0.0	-
AK08-25F	Reindeer	<i>Rangifer tarandus</i>	Artiodactyla	WG	0.03358	0.0	-
AK08-5C	Reindeer	<i>Rangifer tarandus</i>	Artiodactyla	WG	0.04419	1.0	-
I1V	Polar bear	<i>Ursus maritimus</i>	Carnivora	EGL	0.04648	5.0	-
Isb (F)	Polar bear	<i>Ursus maritimus</i>	Carnivora	Cph Zoo	0.16911	35.0	-
Isb (M)	Polar bear	<i>Ursus maritimus</i>	Carnivora	Cph Zoo	0.16277	34.0	-
P.V. 1	Harbour seal	<i>Phoca vitulina</i>	Carnivora	DE	0.02294	0.0	-
P.V. 2	Harbour seal	<i>Phoca vitulina</i>	Carnivora	DE	0.02302	0.0	-
P.V. 3	Harbour seal	<i>Phoca vitulina</i>	Carnivora	DE	0.02308	1.0	-
P.V. 4	Harbour seal	<i>Phoca vitulina</i>	Carnivora	DE	0.03411	6.0	-
P.V. 5	Harbour seal	<i>Phoca vitulina</i>	Carnivora	DE	0.02143	0.0	-
1432	Harbour seal	<i>Phoca vitulina</i>	Carnivora	DK	0.03708	2.7	-
H.g. 1	Grey seal	<i>Halichoerus grypus</i>	Carnivora	NL	0.02204	1.0	-
H.g. 2	Grey seal	<i>Halichoerus grypus</i>	Carnivora	DE	0.02407	1.0	-
1431	Grey seal	<i>Halichoerus grypus</i>	Carnivora	DK	0.03616	0.1	-
C258	Killer whale	<i>Orcinus orca</i>	Cetacea	DK	0.02928	0.0	-