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DOI: https://doi.org/10.1007/s10344-010-0390-0

Efficient age determination: how freezing affects eye lens weight of the small rodent species *Arvicola terrestris*

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Abstract

Age determination of animals by measuring the weight of their eye lenses is a widely-used method in wildlife biology. In general, it is recommended to prepare lenses immediately after trapping to avoid errors in the age estimation due to decomposition of lens tissue. However, in many field studies, large numbers of animals need to be trapped over long periods of time, in huge areas and by many different field workers. Therefore, the immediate preparation of eye lenses imposes a considerable logistic constraint that could be avoided by prior freezing of trapped animals. To assess the impact of freezing, lens weights of frozen and unfrozen eyes of 114 *Arvicola terrestris* were compared pair-wise. The frozen lenses weighed at average 3.3% (95% CI: 2.4 – 4.1%) more than the unfrozen ones from the same animals. Freezing time, weight of lenses and mean temperature of the trapping day as an indicator of decomposition speed did not affect the freezing-induced weight increase. Age estimates based on weights of unfrozen lenses varied between 24 and 445 days. Estimates based on frozen lenses were systematically higher. Applying a constant correction factor of 1.033^{-1} for the weight of frozen lenses corrects this overestimation of age. We conclude that age determination with frozen lenses of small rodents can yield valid age estimates if a correction factor for freezing is applied. Thus, age determination can be organized much more efficiently in field studies, which is highly advantageous for many ecological, agricultural and epidemiological research projects.

Key words

age estimation, Arvicola terrestris, calibration, eye lens mass, population dynamic, voles

Introduction

Population dynamics of many rodent species are complex with interfering annual and perennial cycles (Krebs 1996). There is still a debate to what extent different extrinsic forces such as seasonality, food availability, predation and climatic changes (Hanski et al. 2001; Hörnfeldt et al. 2005; Tkadlec and Zejda 1998) and intrinsic factors such as population structure, social stress and maternal effects (Boonstra 1994; Inchausti and Ginzburg 1998; Oli and Dobson 2001) contribute to these cycles which are accompanied by considerable shifts in the age structure of these species (Cerqueira et al. 2006; Janova et al. 2003; Norrdahl and Korpimaki 2002). Efficient and precise methods for age determination are therefore valuable tools to investigate processes that affect the population dynamics of such species.

In wildlife biology, many different methods are used to determine the relative or absolute age of animals (Morris 1972). A common method is the age determination by measuring the weight of formalin-fixed and dried eye lenses (Morris 1972). Lens size increases steadily in a curvilinear manner by continuous proliferation of new lens fibres with a mass that is closely related to its age (Lord 1959; Tanikawa 1993). Although lens weight can be affected by other factors like gender (Janova et al. 2007) and season (Martinet and Spitz 1971; Pokrovskij 1971), the impact of these factors is far less pronounced than their impact on other

age related parameters as size and body weight (Augusteyn 2008; Friend 1967). Therefore the weight of dried eye lenses is considered as a good marker for the absolute age if calibrated with animals of known age (Morris 1972).

Different authors recommend to prepare eye lenses immediately after trapping to avoid decomposition processes that could affect the age estimation (Friend 1967; Montgomery 1963; Rongstad 1966). However, in many field studies, large numbers of animals need to be trapped over long periods of time, in huge areas and by many different field workers who might neither have the training nor the facilities for the immediate preparation of eye lenses. Therefore, prior freezing of animals would increase the efficiency of material collection. However, conflicting results have been published documenting an impact of freezing on eye lens weight (Broekhuizen 1971; Montgomery 1963; Pelton 1970) or no effect (Friend 1967; Kauhala and Soveri 2001; Longhurst 1964; Millar and Iverson 1976).

The water vole *Arvicola terrestris* is a very abundant rodent species in Western and Central Europe (Görner and Hackethal 1988) and an important prey species (Weber et al. 2002). It is one of the most important agricultural pests (Morilhat et al. 2007). Furthermore, *A. terrestris* is an important intermediate host for the fox tapeworm *Echinococcus multilocularis* which causes alveolar echinococcosis, a severe human liver disease (Eckert and Deplazes 2004; Eckert et al. 2001). An efficient method for assessing the age structure in *A. terrestris* populations over time and space would contribute to investigations addressing different ecological, agricultural and epidemiological questions. In this study, we therefore analysed whether and to what extent freezing affects eye lens weights of this rodent species.

Methods

From January 2007 to August 2008, a total of 281 *A. terrestris* were trapped in the periphery of the city of Zurich (Switzerland), in the framework of a rodent control program in agriculture. Field workers used unbaited Topcat traps (Topcat GmBH, L'Auberson; Switzerland) and tongue traps (Hauptner Instrumente GmbH, Dietlikon, Switzerland) which were inserted into vole galleries in grassland areas. All rodents were brought to the laboratory directly after trapping and were not frozen before dissection.

To analyse the effect of freezing on eye lens weight, eyes of 131 animals were removed and fixed in formalin (10%) for 4 weeks, one of each pair directly after trapping and the others after a defined period in a chest freezer at constant temperature of -20ºC (sample A). All eyes of the remaining 150 animals were fixed without prior freezing (sample B). After the fixation, eyes were slit open and the lenses were removed by applying light pressure. Lenses were cleaned from remaining tissue, carefully dried with a soft paper towel, put into open vials and air-dried at +80°C in a hybridisation oven for 48 hours. To minimize the exposure to atmospheric moisture, individual lenses were removed separately from the oven just before weighting with a microbalance (AT260 Delta Range, Mettler Toledo, Greifensee, Switzerland). A subset of eight lens pairs was repeatedly weighed during a drying period of 168 hours (immediately, after 1, 2, 4, 8, 24, 28, 32, 48, 52, 120, 124, 128, 144, 148, 152, 168 hours) to identify the optimal drying time. Highly asymmetric lens pairs (deviation from mean lens weight ratio > 1.5 interquartile ranges) were excluded from our analyses. Weights of frozen and unfrozen lenses were compared by a paired t-test. The weight ratio of each frozen lens to its unfrozen counterpart was used to assess the impact of lens weight,

freezing time, day temperature (as an indicator for the decomposition speed after trapping) and gender on a possible freezing effect by performing multivariate linear regression analyses.

To calculate absolute ages, we used results from two studies (Boujard 1982; Morel 1981), where the relationship between absolute age and lens weight was calculated based on the data of *A. terrestris* individuals of known age. According to these studies, we use the equation $x=e^{\frac{y-h}{m}}$, where *x* is the age in months, *y* the weight of a single eye lens in mg, *m* the slope and *h* the axis intercept. The authors calculated population-specific values for *m* and *h* for different populations in Switzerland (Morel 1981) and the adjacent French Jura mountains (Boujard 1982). However, these values were based on small sample sizes (sample sizes: 12-116 animals, median 33 animals) and might rather reflect random variations than population-specific differences, which are unlikely according to other studies (Augusteyn 2007, 2008). Therefore, we considered taking the mean parameter values h = 1.858 and m = 1.202 as best approximation for estimating the absolute age.

All statistical tests were performed using SPSS software vs. 17.0.

Results

The mean weight of the 8 lens pairs that were oven-dried was 68% higher before drying than the final weight after 168 hours drying time. During the first day of the drying process, the relative weight dropped sharply. After 48 hours the mean weight of the lenses did not differ significantly from their final weight (mean

deviation 0.3%, 95%-CI -0.4-1.0%). Therefore, all other lenses were dried for 48 hours.

The weight ratios of frozen to unfrozen lenses (sample A) showed a similar variation (SD 0.147) as the weight ratios of unfrozen lens pairs (sample B, SD 0.186, levene's test of homogeneity of variance: $F = 0.67$, $p = 0.41$). After removing all outliers (n= 17), 114 lens pairs remained for the pair-wise comparison of frozen and unfrozen lenses (sample A). The weight ratio of small lens pairs (frozen lens ≤ 3 mg) was higher (SD 0.049) than the ratio of larger lens pairs (frozen lens > 4 mg, SD 0.028, $F = 11.5$, $p = 0.001$). The frozen lenses weighed on average 3.3% more (95% CI: 2.4 – 4.1%) than the unfrozen ones of the same animals ($t = -8.12$, df 113, $p < 0.0001$). Multivariate linear regression models were constructed to predict the freezing-induced weight increase by freezing time (range: 27 to 251 days), mean day temperature (-3.3ºC to 19.4ºC), lens weight (1.6 to 5.1 mg) and gender. Models were built with all possible combinations of the independent variables, but no model revealed a significant influence of any of these factors.

Age estimates of the investigated animals based on weights of unfrozen lenses varied between 24 and 445 days. A comparison with the frozen lenses revealed that freezing causes an overestimation of age, e.g. by 39 days for one year old animals. Considering the average freezing-induced weight increase by applying a constant factor of 1.033^{-1} for the weight of frozen lenses corrects this overestimation (Fig. 1).

Discussion

The freezing of lenses resulted in moderate but significant higher weights which is in contradiction to other studies reporting either no (Friend 1967; Kauhala and Soveri 2001; Longhurst 1964; Millar and Iverson 1976) or a weight-reducing effect (Broekhuizen 1971; Montgomery 1963; Pelton 1970) of freezing. However, weight differences between frozen and unfrozen lenses in all these studies were moderate. Furthermore small sample sizes (Friend 1967; Millar and Iverson 1976) or a less sensitive approach of comparing groups of frozen and unfrozen animals instead of applying pair-wise comparisons (Kauhala and Soveri 2001) were used. A reason for the lower weights of frozen lenses found in some studies could be that much larger animals like e.g. racoons (Montgomery 1963) or leporid species (Broekhuizen 1971; Pelton 1970) were investigated. Such species need more time to thaw and therefore decomposition processes could progress further as compared to voles where the eyes can be removed and put into formalin directly after taking the animals out of the freezer. However, to avoid any effects of decomposition, we recommend freezing voles as fast as possible after trapping. The unexpected increase of eye lens weights after freezing could be caused by increased incorporation of formaldehyde into freezing induced lesions providing a larger surface. The ratio of frozen to unfrozen lenses showed a higher variation for small eye lens pairs, which can be explained by the weighting precision of 0.1 mg. Therefore, the use of more sensitive balances could increase the precision of age estimates, especially for young animals. Further improvements could be achieved by calibration studies with animals of known age that investigate the potential effects of gender and season on lens growth rates as shown for Microtus by other studies (Janova et al. 2007; Martinet and Spitz 1971; Pokrovskij 1971)

The high amount of outliers (13% strongly asymmetric lenses) was probably caused by slight lens ruptures during the dissecting procedure (Friend1967), by injuries due to the subterraneous living of the species and by an asymmetric eye development in some individuals. The comparison with eye pairs of which neither of the two lenses were frozen (sample B) revealed that freezing did not affect lens weight variance and gives evidence that the observed asymmetries were not affected by the freezing procedure.

The freezing induced increase of lens weights was not affected by the weight of the lenses, the duration the lenses were stored in the freezer or the mean air temperature at the trapping day, the latter being regarded as indicator for speed of decomposition. Therefore, we suggest that the age of *A. terrestris* can be determined based on frozen lenses with the same precision as with unfrozen lenses by applying a constant correction factor of 1.033^{-1}. It can be expected that similar correction factors can be calculated for other small rodents and, as consequence, that age determination can be organized much more efficiently for population dynamic studies of small rodents that depend on analysing high number of animals.

Ethical standards

The authors declare that the study comply with the current laws of the country in which they were performed.

Competing interests

The authors declare that they have no conflict of interest.

Acknowledgements

We are grateful to Marianne Fritzsche from Grün Stadt Zürich and to Andreas Weidmann, Simon Franz and all other field workers that delivered the trapped rodents. We thank Alexander Mathis for valuable comments on the manuscript and acknowledge the support of our research activities by the Swiss Federal Veterinary Office (Nr. 1.07.04). This work represents part of the dissertation of Pierre Burlet, veterinarian.

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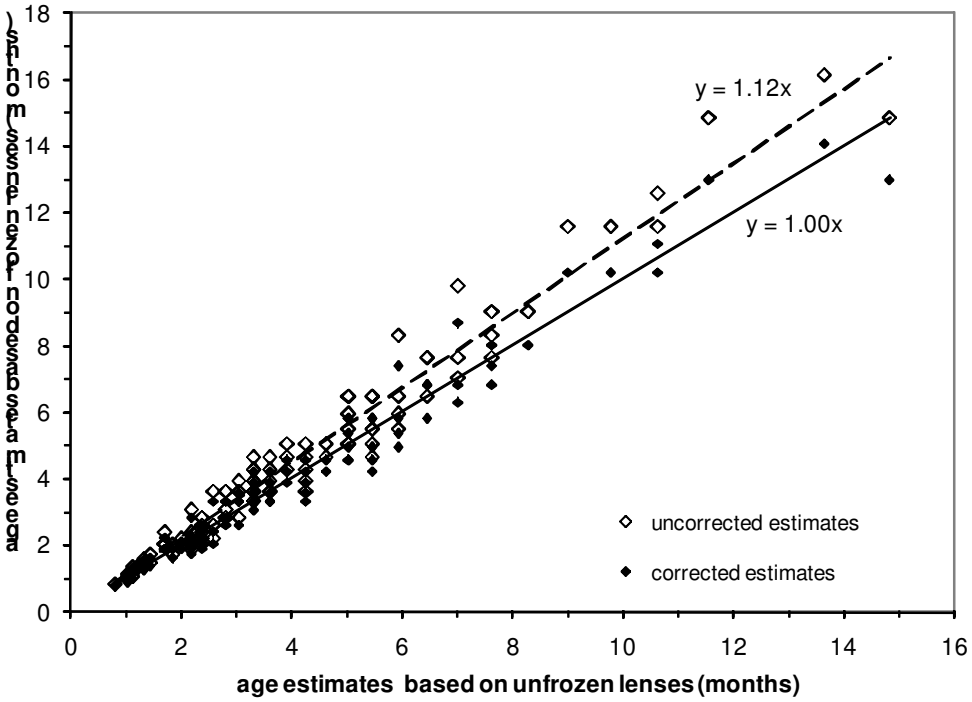
Fig. 1 Absolute age estimates for *Arvicola terrestris* and linear regressions calculated with unfrozen lenses and their frozen counterpart (n = 114). Blank diamonds and dashed line: age estimates of frozen lenses are calculated without applying a correction factor; solid diamonds and solid line: age estimates of frozen lenses were calculated after dividing the weight of the frozen lens by the factor 1.033.


y = 1.12x
y = 1.00x
uncorrected estimates
corrected estimates
0
2
4
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8
10
12
14
16
18
age estimates based on unfrozen lenses (months)