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IMPROVING THE METHOD OF LENS MASS PREPARATION FOR AGE ASSESSMENT IN THE EUROPEAN BROWN HARE (*LEPUS EUROPAEUS*)

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Research was conducted on the European hare (*Lepus europaeus* Pall.), one of the most numerous and important small game species both in Europe and in the Republic of Serbia. The aim of the research was to examine the possibility of modifying the standard method, which is a standard in the Republic of Serbia, for determining the age of hares based on the mass of their lenses in order to shorten its duration. Additionally, the goal was to investigate whether there was a difference in the age structure determined by the standard and modified methods. For the purpose of processing and analyzing samples, a total of 410 hare heads were collected from hunting grounds in Central Serbia and Vojvodina, and only heads with intact lenses of both eyes (399) were included in the analysis, resulting in the examination of 798 lenses. It was determined that there was no statistically significant difference in the lens mass between the standard and modified methods, both for age categories of hares up to one year and over one year. High correlation coefficients were found, indicating a strong relationship between the lens mass obtained by the standard and modified methods within both age categories. Furthermore, the real growth rates of hares determined by the standard and modified methods were equal.

Key words: Brown hare, age determination, eye lens, age structure, real growth rate.

INTRODUCTION

The European brown hare is probably the most important game animal in Europe throughout its historical distribution (Tsokana *et al*. 2020). Thanks to its high ecological plasticity, the European hare inhabits diverse habitats, with flat landscapes being the most suitable for its survival (Beuković *et al.* 2009), making it a characteristic species of European agricultural areas (Panek 2018). However, despite its large distribution range, influenced by numerous

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factors, the hare population is increasingly being suppressed both in Europe (Schmidt *et al*. 2004, Smith *et al.* 2005, Hacklander 2012, Ujhegyi *et al*. 2021) and in Serbia (Ristić *et al*. 2021). The abundance of hares is influenced by natural factors such as climate, diseases, and predators, as well as anthropogenic factors including agricultural activities, traffic, and hunting (Pelorosso *et al*. 2008, Beuković *et al*. 2013). One of the most significant factors is the intensification of agricultural production (Petrovan 2011, Johan & Arnold 2021), which has resulted in changes in living conditions, habitat loss, reduced food availability (Edwards *et al*. 2000), and increased use of fertilizers and pesticides (Tscharntke *et al*. 2005).

The reduction in the population size of the European hare is a significant problem today. Considering that certain factors cannot be directly influenced, efforts should be focused on those aspects that breeders can directly or indirectly address, primarily through the improvement of management practices (Popović *et al*. 2013). The foundation of sustainable management lies in understanding and continuously monitoring the population dynamics through its structural elements, which include population density, sex and age structure, fertility (growth), and losses (Pintur *et al*. 2006, Nikolandić & Degmenčić 2007). Šelmić and Đaković (1997) emphasize that population abundance of small game species is of utmost importance in game management. However, for management purposes, not only quantity but also the quality (age structure) of the population is crucial. The age structure of the hare population is an important indicator of growth and is used in planning the degree of hare utilization in hunting grounds (Beuković *et al*. 2013, Đorđević *et al*. 2014). As hares have a fast generation turnover, the age structure significantly affects the reproductive and vital capacity of the population, thus influencing its size. Additionally, Ludwig and Dapson (1977) highlight the importance of understanding population dynamics through knowledge of the age structure. Determining the age structure of a population can be a powerful mechanism for understanding the impact of hunting pressure and habitat management on the survival of young and old hares, as well as the overall population dynamics (Petrovan 2011). The significance of the age structure lies in its ability to determine the actual growth of hares, specifically the ratio between young and old individuals, which forms the basis for planning harvest dynamics.

Therefore, in the management of the hare population, it is necessary to determine the age of harvested individuals (Bray *et al*. 2002, Mallia *et al*. 2009) in order to determine the age structure of the observed population. There are several methods for determining the age of hares, which are based on specific parameters such as: ossification of the protuberance (Stroh's sign) on the lower ulna (Stroh 1931, Wight & Conaway 1962, Flis & Rataj 2019), assessment of the flexibility of the lacrimal processus (Suchentrunk *et al*. 1991), annual growth lines in the mandible (Ohtaishi *et al*. 1976, Frylestam & von Schantz

1977, Frylestam 1980, Iason 1989), estimation based on body mass (Flis *et al*. 2021), and lens mass (Rieck 1962, Andersen & Jensen 1972, Suchentrunk *et al*. 1991, Stankevičiūtė 2011, Beuković *et al*. 2013).

The main drawback of most methods is that they cannot accurately determine the age but can only be used to differentiate between young and old individuals (Suchentrunk *et al*. 1991, Kauhala & Soveri 2001, Loyd 2009, Beuković *et al*. 2013). However, based on the lens mass, the age of hares can be determined more accurately, which represents a significant advantage compared to other methods (Ristić *et al*. 2013). As stated by Šijački and Đaković (1997), hares can be classified into 6 classes based on the lens mass:

- I age class, of up to 100 mg – hares up to 3 months;
- II age class, of 100 to 200 mg – hares aged 3- 6 months;
- III age class, of 200 to 280 mg – hares aged 6 -12 months;
- IV age class, from 280 to 310 mg – hares aged 1 to 2 years;
- V age class, of 310 to 370 mg – hares aged 2-3 years;
- VI age class, over 370 mg – hares over 3 years of age.

The determination of European hare age in the territory of the Republic of Serbia, specifically in the Autonomous Province of Vojvodina, has been conducted annually since 1967 (Beuković *et al*. 2012). The main reason for age determination is to calculate the age structure of the population in a particular hunting ground, based on which recommendations regarding further hunting, whether it should be conducted or not, are issued. The age determination is based on the mass of dried lenses, following the Lord's method (1959), which has been adjusted by Šijački and Đaković (1997). Although this method is used in various countries (Hungary, Czech Republic), its greatest application has been recorded in Vojvodina (Ristić *et al*. 2016). The aforementioned method has been used in numerous studies in the Republic of Serbia (Šijački & Đaković 1997, Beuković *et al*. 2012, Beuković *et al*. 2013, Popović *et al*. 2015, Ristić *et al*. 2016), as well as in Croatia (Pintur *et al*. 2006). The method based on Šijački and Đaković (1997) involves preserving eye lenses in a 10% formalin solution for 72 hours, followed by a drying process of 72 hours at 37°C. This requires 6 days for processing and analysis. Indeed, the main issue with the implementation of this method is its duration. In the Republic of Serbia, hare hunting is conducted from October 15th to December 15th (Regulation on the proclamation of protected wildlife species hunting season, 2021), usually organized on Sundays. Since hunting is conducted on Sundays, using the described method, the final results would be ready on Saturday. Consequently, providing recommendations to hunting area users (whether to follow the hunting plan, adjust it, or suspend hunting) for the upcoming week would not be timely, potentially causing the next week's hunt to be missed. Consequently, this approach reduces the number of hunting weeks during the hare hunting season by half.

The aim of this study was to investigate the possibility of modifying the standard method for determining the age of hares in order to shorten its duration. Additionally, the study aimed to examine whether there are differences in the age structure determined by the standard and modified methods.

MATERIAL AND METHODS

Determining the age structure of hares was primarily conducted using the Lord's method (1959), which was further modified by Šijački and Đaković (1997) based on the mass of the eye lenses. However, since the aim was to investigate the possibility of reducing the duration of the standard method, which is a standard in the Republic of Serbia, the eye lenses underwent various additional treatments.

Hares were harvested during the hunting seasons of 2020/2021 and 2021/2022 in accordance with the Wildlife and Hunting Act (2010). Samples, specifically the heads of harvested hares, were collected from various locations in the Republic of Serbia and delivered to the Physiology Laboratory at the Institute of Animal Science, Faculty of Agriculture, University of Belgrade. The collection and analysis of samples were conducted during the hare hunting season, specifically in late October (last week) and throughout the month of November.

During both hunting seasons, a total of 410 hare heads were submitted from hunting grounds in Central Serbia and Vojvodina for the purpose of sample processing and analysis. However, not all samples could be analyzed. Only heads with both intact eye lenses (399) were included in the analysis, resulting in a total of 798 eye lenses analyzed. There were two main reasons for the exclusion of certain samples. Firstly, in some cases, the entire eye of the hare head was damaged by gunshot, making it impossible to extract and analyze the eye lenses. Secondly, for some samples, the extracted eye lenses were not compact, posing a challenge for further processing and analysis.

After the samples, i.e., the hare heads, were delivered to the laboratory, they underwent processing. One eyeball was extracted from each head, and the eye lenses were then isolated for further processing using the standard (reference) method. The extracted eye lenses were rinsed with water and transferred to labeled test tubes containing a 10% solution of formalin. The treatment, i.e., fixation of the eye lenses in this solution, lasted for 72 hours. Afterward, the eye lenses were dried in an oven at a temperature of 37°C for another 72 hours. Once the drying process was complete, the eye lenses were measured using an analytical balance with a precision of ±1 mg.

After removing one eye lens and completing its full processing using the reference method, all heads that had an undamaged second eyeball (with intact lens) were subjected to various treatments and procedures. Three different procedures were used for processing the eye lenses, involving modifications in the method and duration of formalin fixation for two procedures, while the drying method was changed for the third procedure.

The first procedure involved reducing the duration of formalin fixation for the eye lenses. In this procedure, after removal and rinsing, the eye lenses were also fixed in a 10% formalin solution, but for a duration of 48 hours. The subsequent steps were identical to the reference method, including rinsing the lenses again and drying them in a drying oven at 37°C for 72 hours. Finally, the mass of the eye lenses was measured using an analytical balance with an accuracy of ±1 mg, based on which the hares were classified into the aforementioned age classes. The largest number of samples (303 eye lenses) was processed using this treatment.

A smaller number of samples were treated with **second** procedure, which involved reducing the drying duration by increasing the drying temperature. The first part of the process, namely fixation, was identical to the reference method. However, after fixation, the drying of the eye lenses was carried out in a drying oven at a temperature of 48°C for a duration of 48 hours. The measurement and classification of hares into age classes were performed in the same manner as in the reference method. This procedure was applied to 65 samples.

The **third** procedure involved changing the formalin concentration and reducing the fixation duration. In the heads with intact second ocular lenses, after rinsing, the lens was fixed in a lower concentration solution, specifically a 6% formalin solution, for a shortened fixation time of 48 hours. Subsequently, the drying of ocular lenses followed the same process as in the reference method, with drying at a temperature of 37°C for 72 hours. The further analysis procedure was carried out in the same manner as in the reference method. A total of 31 ocular lenses were processed using this method.

Differences in sample sizes arose due to an unequal number of harvested individuals per hunting week. The research began in 2020 with a limited number of samples, resulting in the application of the first modified procedure to all samples. In the following year, with an expected increase in samples, all samples submitted in the first week were again processed using the first modified method, considering the previously limited number of samples. However, with a smaller number of samples in the following week, all were processed using the second modified procedure. In the last week, all samples were processed using the third procedure, adhering to the experimental plan and applying all three planned methods.

After processing all the samples, basic statistical parameters of the ocular lens mass obtained using both the standard and modified methods were determined. Then, an assessment of the impact of the tested factors on the variation of ocular lens mass was performed using the General Linear Model in the statistical software SAS 9.3 (SAS Institute Inc., 2002-2010).

Afterwards, the correlation between the ocular lens mass obtained using the standard and modified methods was examined using the Pearson correlation coefficient. The strength of the relationship was interpreted based on a rough approximation of the correlation magnitude according to Petz (2004):

- 0.00-0.20 (negligible correlation)
- 0.20-0.40 (weak correlation)
- 0.40-0.70 (moderate correlation)
- 0.70-1.00 (strong correlation).

At the end, based on the determined ocular lens mass using the standard and modified methods, hares were classified into 6 age classes as follows: 1) <100 mg, hares aged up to 3 months; 2) 100–200 mg, hares aged 3-6 months; 3) 200–280 mg, hares aged from 6 months to 1 year; 4) 280–310 mg, hares aged 1–2 years; 5) 310–370 mg, hares aged 2–3 years; 6) >370 mg, hares older than 3 years. The age structure determined by the reference method served as the control group, and it was compared to the age structure obtained by the modified methods.

RESULTS

The procedure involved reducing the duration of fixation in 10% formalin from 72 hours to 48 hours

Based on the measured masses of eye lenses, the average mass of samples in the age category under one year, processed by the standard method, was 216.18 mg. The minimum value was 126.00 mg, while the maximum value was 277.00 mg. Using the modified method, a slightly higher average mass of samples in the under one-year category was determined, with it being 220.70 mg, ranging from 127.00 mg to 279.00 mg. The difference in the mass of eye lenses determined by the standard and modified methods for this category averaged 4.68 mg, ranging from 0.00 mg to 11.00 mg.

Similar to the category up to one-year, and in the category over one year, the average mass determined by the standard method was lower, at 367.40 mg, ranging from 282.00 mg to 469.00 mg. The average mass for the category over one year determined by the modified method was 376.35 mg, ranging from 280.00 mg to 477.00 mg. The difference in mass between the eye lenses determined by these two methods averaged 9.11 mg, ranging from 0.00 to 77.00 mg.

Through statistical analysis, it has been determined that there is no statistically significant difference ($p = 0.26$) in the mass of eye lenses for the category of hares up to one year, depending on whether the standard or modified method is applied, as shown in Table 1. Furthermore, a very high degree of correlation ($r_f = 0.997$; $p < 0.0001$) was observed between the masses of eye lenses of hares in the category up to one year obtained using the standard and modified methods.

Similar to the category of hares up to one year, it has also been determined within the category of hares over one year that there is no statistically significant difference ($p = 0.10$) between the masses of eye lenses obtained using the standard and modified methods, as also depicted in Table 1. Furthermore, a very high degree of correlation ($r_f = 0.993$; $p < 0.0001$) has been established between the masses of eye lenses of hares in the category over one year obtained using the standard and modified methods.

Since it is necessary to have knowledge of age structure and real growth in population management of hares, an analysis of these two parameters was

Table 1. Influence of ocular lens fixation method on their mass.

Characteristic	Age class	SS	df	MS	F	p	R^2
Lens mass (mg)	Up to 1 year	1653.78	1	1653.78	1.25	0.26	0.00388
	Over 1 year	5656.63	1	5656.63	2.71	0.10	0.00957

SS – sum of squares, df – degrees of freedom, MS – mean square, R^2 – coefficient of determination, Statistical significance (p): ns – not significant

Table 2. Age structure and real growth rate of hares for the year 2020 determined using the standard and applied modified method.

Dry lens weight, mg	Age	Number of samples in the method		Real growth rate (%)	
		Standard	Modified	Standard	Modified
< 100	up to 3 months	0	0	0.00	0.00
100–200	3 to 6 months	9	8	20.93	18.61
200–280	6 to 12 months	18	19	41.87	44.19
Up to 1 year in total		27	27	62.80	62.80
280–310	1 to 2 years	4	3	9.30	6.97
310–370	2 to 3 years	6	6	13.95	13.95
>370	over 3 years	6	7	13.95	16.28
Over 1 year in total		16	16	37.20	37.20
Total		43	43	100.00	100.00

also conducted, comparing the results obtained using these two methods, separately for each year (Tables 2 & 3).

Analysis of 520 eye lenses from 260 individuals, collected and processed in 2021 (Table 3), revealed a lower actual growth compared to the one determined in 2020. However, similar to the previous case, the actual growth determined by both the standard and modified applied method did not differ and amounted to 52.31%.

Table 3. Age structure and real growth rate of hares for the year 2021 determined by standard and applied modified method.

Dry lens weight, mg	Age	Number of samples in the method		Real growth rate (%)	
		Standard	Modified	Standard	Modified
< 100	up to 3 months	0	0	0.00	0.00
100–200	3 to 6 months	48	40	18.46	15.39
200–280	6 to 12 months	88	96	33.85	36.92
Up to 1 year in total		136	136	52.31	52.31
280–310	1 to 2 years	13	8	5.00	3.08
310–370	2 to 3 years	60	44	23.08	16.92
>370	over 3 years	51	72	19.61	27.69
Over 1 year in total		124	124	47.69	47.69
Total		260	260	100.00	100.00

The procedure for reducing the drying time from 72 hours to 48 hours is by increasing the drying temperature to 48°C

By analyzing 130 eye lenses taken from 65 individuals, the basic statistical parameters of lens mass were determined for the categories up to one year and over one year using both the standard and modified methods (the second approach). The average mass of the analyzed samples in the category up to one year obtained with the standard method was 226.06 mg, with variations ranging from 139.00 mg to 278.00 mg. In comparison to the standard method, the modified method revealed a slightly lower average lens mass (223.03 mg), with a minimum of 148.00 mg and a maximum of 278.00 mg. The average difference in lens mass determined by these two methods for the up to one year category was 6.62 mg, with a range of 1.00 mg to 17.00 mg.

For the over one-year category, a slightly higher average mass of eye lenses was also determined with the standard method, amounting to 361.84 mg. It ranged from 283.00 mg to 439.00 mg. Using the modified method, the average mass was found to be 353.97 mg, ranging from 281.00 mg to 434.00 mg. The average difference in mass for the over one-year category determined by these methods was 11.16 mg, varying from 1.00 to 39.00 mg.

Statistical analysis of the processed samples revealed that the drying method did not have a statistically significant impact ($p = 0.75$) on the variation in lens mass within the category of hares up to one year (Table 4). The correlation between the masses obtained by the standard and modified methods within this group was very high, measuring $r_f = 0.983$.

A similar situation was observed within the category of hares over one year, where it was also determined that there was no statistically significant difference ($p = 0.43$) in the mass of eye lenses processed by these two methods (Table 4). In this case as well, a very strong relationship ($r_f = 0.981$; $p < 0.0001$) was found between the masses of eye lenses determined by the standard and the presented modified method

When it comes to the actual increase in lens mass determined by the standard and modified methods, it was found to be equal in both cases, amounting to 52.31% (Table 5).

Table 4. Influence of lens drying method on their mass.

Characteristic	Age class	SS	df	MS	F	p	R^2
Lens mass (mg)	Up to 1 year	156.01	1	156.01	0.10	0.75	0.00158
	Over 1 year	960.26	1	960.26	0.63	0.43	0.01038

SS – sum of squares, df – degrees of freedom, MS – mean square, R^2 – coefficient of determination, Statistical significance (p): ns – not significant

Table 5. Age structure and real growth rate of hares in the year 2021 determined by the standard and applied modified method.

Dry lens weight, mg	Age	Number of samples in the method		Real growth rate (%)	
		Standard	Modified	Standard	Modified
< 100	up to 3 months	0	0	0.00	0.00
100–200	3 to 6 months	10	8	15.39	12.31
200–280	6 to 12 months	24	26	36.92	40.00
Up to 1 year in total		34	34	52.31	52.31
280–310	1 to 2 years	4	2	6.15	3.08
310–370	2 to 3 years	14	18	21.54	27.69
>370	over 3 years	13	11	20.00	16.92
Over 1 year in total		31	31	47.69	47.69
Total		65	65	100.00	100.00

The procedure involved shortening the duration of formalin fixation to 48 hours and using a lower concentration (6%) of formalin compared to the standard method

The average mass of eye lenses for the category of hares up to one year, processed by the standard method, was 225.00 mg, with a minimum mass of 170.00 mg and a maximum of 273.00 mg. On the other hand, the average mass of eye lenses for the same category, processed using the applied modified method, was slightly lower (215.40 mg), ranging from 160.00 mg to 257.00 mg. The average difference in lens mass for the up to one-year category determined by these two methods was 10.67 mg, with variation between 4.00 mg and 16.00 mg.

Similarly, in the category of hares over one year, the standard method revealed a slightly higher average mass (385.37 mg), with a range from 170.00 mg to 273.00 mg. The mass determined using the modified method was 375.25 mg, with a range from 297.00 mg to 477.00 mg. The difference in lens mass determined by these two methods for the over one-year category averaged 13.37 mg, with variations between 4.00 mg and 28.00 mg.

Table 6. Influence of lens fixation method on their mass.

Characteristic	Age class	SS	df	MS	F	p	R^2
Lens mass (mg)	Up to 1 year	691.20	1	691.2	0.82	0.37	0.0284
	Over 1 year	820.12	1	820.12	0.25	0.62	0.00813

SS – sum of squares, df – degrees of freedom, MS – mean square, R^2 – coefficient of determination, Statistical significance (p): ns – not significant

Table 7. Age structure and real growth rate of hares for the year 2021 determined by the standard and applied modified method.

Dry lens weight, mg	Age	Number of samples in the method		Real growth rate (%)	
		Standard	Modified	Standard	Modified
< 100	up to 3 months	0	0	0.00	0.00
100–200	3 to 6 months	3	5	9.68	16.13
200–280	6 to 12 months	12	10	38.71	32.26
Up to 1 year in total		15	15	48.39	48.39
280–310	1 to 2 years	2	2	6.45	6.45
310–370	2 to 3 years	6	6	19.35	19.35
>370	over 3 years	8	8	25.81	25.81
Over 1 year in total		16	16	51.61	51.61
Total		31	31	100.00	100.00

Similar to the previous methods, a statistical analysis was conducted on the masses of eye lenses obtained using the standard and the applied modified methods. It was determined that there is no statistically significant difference ($p = 0.37$) in the mass of eye lenses within the category of hares up to one year, depending on the method used (Table 6). Furthermore, a high correlation coefficient ($r_f = 0.980$; $p < 0.0001$) was observed between the masses of eye lenses obtained using the standard and the applied modified method.

A statistically significant difference ($p = 0.62$) in the mass of eye lenses obtained by the standard and the applied modified method was not observed in hares older than one year. The relationship between the masses of eye lenses determined by these two methods was very strong, as indicated by a high correlation coefficient ($r_f = 0.984$; $p < 0.0001$).

The real growth of hares determined by the standard and applied modified method is shown in Table 7. The values of the real growth determined by these two methods did not differ and amounted to 48.39%.

DISCUSSION

It has been determined that there is no statistically significant difference in the mass of eye lenses processed using the standard and the applied modified methods in both age categories. Therefore, with the application of the first and third modified methods, the time required for obtaining the analyses is 5 days, with the fixation duration in formalin being 48 hours. In most of the available studies that dealt with determining the age of hares, the fixation time was longer and ranged from 3 days (Yamada *et al*. 1990, Pintur *et al*. 2006), one week (Caboń-

Raczyńska & Raczyński 1972), one month (Keith and Cary 1979), two months (Vaquerizas *et al*. 2021), to 5 months (Andersen & Jensen 1979). Additionally, the third modified method involved the use of a 6% formalin solution, while the formalin concentration in the aforementioned studies was higher (10%).

The duration of the second modified method is also 5 days, with the aim of shortening the drying time by one day by increasing the drying temperature from 37°C to 48°C. Therefore, the drying duration within the second modified method is 48 hours. This time is also shorter compared to most available studies on hare age determination. Drying time ranged from 3 days at 80°C (Yamada *et al*. 1990) and 3 days at 37°C (Pintur *et al*. 2006), 4 days at 90°C (Andersen & Jensen 1979), 5 days at 80°C (Keith and Cary 1979), all the way to 14 days at 85°C (Vaquerizas *et al*. 2021). On the other hand, in the study conducted by Flis *et al.* (2019), the drying time was shorter (24 hours at a temperature of 100°C), following the methodology of Méres *et al*. (2013).

The possibility of applying the modified methods is also supported by the very high correlation coefficients. By using these methods, the most common problem encountered in practice would be solved, which is the untimely acquisition of final results, especially in assessing real growth rate and providing recommendations for the next week's hunting. In the case of positive results regarding real growth, missing the upcoming hunting week would be prevented, allowing for its successful implementation.

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