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Difficulties with the choice of proper method for determination the levels of serum of 17β-estradiol (E2) in European bison males

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Abstract: The study objective was to compare two methods used to determine serum levels of 17β-estradiol (E2) in European bison *Bison bonasus* (Linnaeus, 1758) males: the radioimmunological method (RIA) and the immunoenzymatic method (ELISA). The study material included sera from 101 European bison males aged 2 months – 19 years, which were culled in the autumn and winter periods (in the post-rutting season), in the years 1993–2002, in the Białowieża Primeval Forest. E2 levels were determined using the two methods (RIA and ELISA) in the same animals. The following age groups were distinguished: class I – calves under the age of 1 year, class II –juveniles under the age of 2 years, class III –juveniles under the age of 3, class IV – adult males aged 4–5 years, class V – adult males aged 6–12 years, class VI-old bulls over the age of 12.

Using the RIA method to determine serum concentrations of E2 in European bison males, all the values obtained were below the bottom limit for this method. However, the use of the ELISA method revealed serum levels of E2 in all the 101 males. The highest median serum levels of E2 were noted in the youngest European bison from age classes I and II, whereas the lowest in adult bulls (age class V), actively participating in reproduction. However, in the 4–5 -year-old animals (age class IV), sexually mature, but not involved in reproduction due to a lower status in the herd, the median serum level of E2 was nearly twice as high as in the class V bulls. The remaining European bison specimens (juveniles aged 3 years and old bulls over 12) had almost identical median serum levels of E2, the values falling in the middle, between the highest and the lowest median levels observed in the other animals. We found great individual differences in the serum levels of E2 in the respective animals in all the age classes studied.

Key words: European bison, 17β-estradiol, ELISA, RIA

Introduction

Studies conducted in the second half of the 1990s showed the role of estrogens in spermatogenesis regulation as well as in sexual behavior, and its effect on the sexual dimorphism. In sexually immature males, estrogens are produced mainly in Sertoli cells, whereas in sexually mature animals, Sertoli cells, Leydig cells and spermatogenic cells play this role. Estrogens include estrone (E1), 17β-estradiol (E2) and estriol (E3). Unlike other steroid hormones, the estrogenic hormones can be produced only from androgens. Estrone originating from androstendion is the major estrogen. 17β-estradiol is the most active estrogen.

Gill (1999) compared the serum levels of E2 in young and adult European bison bulls, as well as E2 concentrations observed in healthy young and adult bulls as opposed to those affected by *posthitis/balanoposthitis*.

Other authors have investigated serum estrogen levels mainly in horses (Ganjam, Kenney 1975; Roser, Hughes 1992; Inoue *et al.* 1993; Raeside, Christie 1997; Stewart, Roser 1998; Hoffman, Landeck 1999; Lemazieur *et al.* 2001) and in other ungulate species (*Odocoileus virginianus*) (Bubenik *et al.* 2005) or in domestic cattle (Weathersbee, Lodge 1976; Eiler, Graves 1977).

The current study objective was to compare two methods used for the determination of serum levels of E2 in European bison males: the radioimmunological method (RIA) and the immunoenzymatic method (ELISA).

Materials and Methods

The material used for analysis included serum samples obtained from 101 European bison males aged 2 months – 19 years, culled in autumn and winter seasons (in the post-rutting period) (Krasiński, Raczyński 1967), in the years 1993–2002, within the Białowieża Primeval Forest. The animals, which were culled to reduce the population number, were chosen for elimination for various reasons, including diseases, especially *posthitis/balanoposthitis*, poor condition, exterior defects, injuries of various origin, aggression towards people or senile age (Krasińska, Krasiński 2007). The age of bison from the free-ranging population was determined by Krasiński (BNP) according to a sequence of deciduous tooth eruption and exchange from deciduous into permanent dentition (Węgrzyn, Serwatka 1984). The bison were shot in the morning hours. Whole blood was collected from the femoral artery (*post mortem*) and then sera were centrifuged in the Mammal Research Institute PAS in Białowieża. The serum samples were frozen and stored at –20°C.

The study animals were divided into age classes: class I – calves up to the age of 1 year, class II – juveniles under the age of 2 years, class III – juveniles under the age of 3, class IV – adult males aged 4–5 years, class V – adult males aged 6–12 years, class VI – old bulls over the age of 12 (Table 1). The division into 6 age classes resulted from our earlier study (Czykier *et al.* 1999; Czykier, Krasińska 2004), which revealed morphological differences in the course of spermatogenesis in seminiferous tubules of the bison testes between these classes.

The serum levels of E2 in European bison males were determined using two methods: the radioimmunological method (RIA) No 68633 (Orion Diagnostica, Espoo, Finland) with a sensitivity of 10 pg/ml and the immunoenzymatic method (ELISA) No RE 52041 (Immuno-Biological Laboratories, Hamburg, Germany) with a sensitivity of 15 pg/ml. Serum E2 levels in the same European bison males were determined by RIA in the Laboratory of the

Table 1. Median serum concentrations of E2 in European bison specimens in various age classes. Class I-calves up to one year old, class II – juveniles up to 2 years old, class III – juveniles up to 3 years old, class IV – adult male 4 and 5 years old, class V-adult male 6 and 12 years old, class VI – old bulls above 12 years old. ns-non significant difference, n-number of individuals

Age class	n	Min	Q_1	Me	Q_3	Max	p
I	42	1.02	46.88	103.18	290.70	1186.00	ns
II	19	4.62	36.18	106.90	170.82	1800.00	ns
III	7	3.27	23.00	65.00	180.00	510.00	ns
IV	13	14.14	17.66	47.26	86.00	485.90	ns
V	12	12.44	15.38	26.72	132.50	317.75	ns
VI	8	9.73	22.47	61.00	98.00	966.00	ns

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For statistical analysis, a nonparametric Kruskal-Wallis ANOVA test by ranks was used to compare quantitative variables without normal distribution with post-hoc test of multiple comparisons of mean ranks for all samples, in the case of many groups. The values were considered statistically significant at $p < 0.05$. The Statistica 8.0 package, StatSoft, was applied for calculations.

Results

All the serum levels of E2 obtained in the current study using the RIA method were below the bottom limit for this method. However, the use of the ELISA method revealed serum levels of E2 in all the 101 study males. The highest medians of serum E2 were noted in the youngest European bison from age classes I and II (Table 1), whereas the lowest in adult bulls (age class V), taking active part in reproduction (Table 1). However, in the 4–5 year-old animals (age class IV), sexually mature but not engaged in reproduction due to a lower status in the herd, the median of serum E2 level was nearly twice as high as in the age class V bulls. The remaining European bison specimens (juveniles aged 3 years and old bulls over 12) had almost identical medians of serum E2, the values the values falling in the middle between the highest and the lowest medians observed in the other animals (Table 1).

No statistically significant differences were found between the 6 age classes studied with regard to the distribution of the median levels of E2 in the serum of European bison.

In the present study, we observed great individual differences in the serum levels of E2 in the respective animals from all the age classes. Most dispersed values were found in the animals from age classes I, II and VI, whereas the lowest dispersion was noted in age class V (Table 1).

Discussion

We found the median serum concentrations of E2 in young European bison (age classes I, II and III) to be lower than the mean values reported by Gill from young bulls (1999). In our study, the median serum concentrations of E2 in young bulls from age class IV and old males over 12 are close to the mean values noted by Gill in bulls (1999). However, we observed the median serum level of E2 in adult bulls from age class V to be nearly by 50% lower as compared to the mean level of E2 in the sera of European bison bulls described by Gill (1999). These discrepancies may result from different statistical approaches used in the two studies. In his monograph, Gill (1999) used the mean values of E2, whereas our study, due to a lack of normal distribution of quantitative variables, was based on the median values.

The median serum concentration of E2 obtained in our study (ELISA) in adult European bison from age class V is similar to the values reported by other authors in stallions (Stewart, Roser 1998; Hoffman, Landeck 1999), bulls (Weathersbee, Lodge 1976) and wild animals: white-tailed deer (Bubenik *et al.* 2005). However, Roser and Hughes (1992) report that adult horses have lower mean serum levels of E2 as compared to adult European bison. According to literature data, prepubertal male horses have lower mean serum concentrations of E2 (Stewart, Roser 1998; Lemazieur *et al.* 2001) than the median serum concentration of E2 in prepubertal male European bison from age classes I and II.

The median serum concentrations of E2 (ELISA) in European bison males in all the age classes are largely affected by substantial differentiation of individual serum levels of E2, especially the maximum and high values shown by some of the animals. High individual differentiation of serum levels of E2 in European bison specimens was described by Gill (1999). However, the wide range of serum levels is not only associated with E2, but has also been reported for total testosterone (TT) (Gill 1999, Czykier in press) and free testosterone (FT) (Czykier, Krasińska 2006; Czykier 2008) in European bison.

In our study, the RIA method used to determine the serum levels of E2 in European bison males yielded 100% of results below the bottom limit for this method. In addition to the determination of serum E2 in males, we measured this hormone in a European bison cow (using RIA) to obtain 15pg/ml. We were not able to explain why using the RIA method to determine E2 concentrations in European bison males all the results were below the bottom limit. Perhaps, this was due to a laboratory or human error, or the method is

not sensitive enough to determine E2 in European bison males, although its bottom limit is lower than in ELISA. Therefore, we decided not to employ the RIA method for the determination of E2 in the serum of European bison males.

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Problem wyboru metody oznaczania stężeń 17β-estradiolu (E2) w surowicy samców żubra

Streszczenie: Celem obecnego badania jest porównanie dwóch metod służących do oznaczania stężeń 17β-estradiolu (E2) w surowicy samców żubra *Bison bonasus* (Linnaeus, 1758) : metody immunoenzymatycznej (ELISA) oraz metody radioimmunologicznej (RIA). Do badań użyto surowice 101 samców żubrów w wieku od 2 miesięcy do 19 lat, pobrane od zwierząt odstrzelonych w sezonach jesienno-zimowych (po zakończeniu okresu rujowego) w latach 1993–2002 na terenie Puszczy Białowieskiej. Stężenia E2 oznaczano w surowicy samców żubra u tych samych zwierząt dwiema metodami: metodą radioimmunologiczną (RIA) oraz metodą immunoenzymatyczną (ELISA). Zwierzęta należały do następujących klas wiekowych: klasa I-cielęta do 1 roku, klasa II-młodzież w wieku do 2 lat, klasa III-młode samce w wieku do 3 lat, klasa IV-dorosłe samce w wieku 4–5 lat, klasa V-dorosłe samce w wieku 6–12 lat, klasa VI-stare samce powyżej 12 roku. Po zastosowaniu metody RIA do oznaczania stężenia E2 w surowicy żubrów otrzymaliśmy wszystkie wyniki poniżej dolnej granicy normy, natomiast po zastosowaniu metody ELISA uzyskaliśmy wyniki stężeń E2 w surowicy u wszystkich 101 badanych samców. Posługując się metodą ELISA stwierdziliśmy najwyższe wartości mediany stężenia E2 w surowicy najmłodszych żubrów z I i II klasy wiekowej, natomiast najniższe wartości mediany stężenia E2 w surowicy obserwowaliśmy u dorosłych byków (V klasa wiekowa), biorących aktywny udział w rozrodzie. Natomiast zwierzęta w wieku 4–5 lat (IV klasa wiekowa), dojrzałe płciowo, ale nie uczestniczące w rozrodzie z racji niższej pozycji w hierarchii stada, miały niemal dwukrotnie wyższą medianę stężenia E2 w surowicy w stosunku do byków z V klasy wiekowej. U pozostałych żubrów (młodzież w wieku 3 lat i stare byki po 12 roku) stwierdziliśmy niemal identyczne mediany stężeń E2 w surowicy, przy czym ich wartości mieściły się pośrodku między wartościami mediany najwyższymi, a najniższymi obserwowanymi u pozostałych zwierząt. W naszym obecnym badaniu obserwowaliśmy duże różnice indywidualne w stężeniach E2 w surowicy poszczególnych zwierząt ze wszystkich klas wiekowych.