Effect of season on characteristics of red deer /Cervus elaphus L./ semen collected using modified artificial vagina

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SUMMARY

During three reproductive seasons, 572 ejaculates were collected from five farmed red deer stags using a modified artificial vagina. The ejaculates were evaluated in terms of their quality and quantity by standard procedures applied for domestic animals. The total length of sexual activity was 245 days, from August 4 to April 6. Significant changes in the semen parameters were noted over this period, divided into three stages: pre-mating, mating, post-mating. During mating season the following values of sperm parameters were recorded: 1/ volume of white and yellow fractions - 0.18 and 2.03 ml, respectively; 2/ pH of white and yellow fraction - 6.80-7.41 and 6.65-7.45, respectively; 3/ sperm concentration - 2.27 mln/mm³; 4/ sperm motility - 47% (with 80% of them showing steady progressive motion at moderate speed); 5/ duration of sperm motility - 6 hours 39 minutes, and 6/ low percentage of major and minor sperm defects (<5% and <10%, respectively). In summary, measures of semen quality including fraction volume, pH, sperm

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concentration and sperm motility change gradually during the pre-mating, mating and post-mating seasons of red deer. The period of greatest libido (from the end of September until the end October) coincides with highest semen quality. Reproductive Biology 2004 4 (1): 51-66.

Key words: red deer, semen, seasonality, artificial vagina.

INTRODUCTION

The seasonality of reproduction was examined in many species of the deer family /Cervidae/. The authors used various approaches including direct behavioral observations. The changes in pituitary hormone levels [6, 7, 26, 31, 33] and, in consequence, the changes of androgenic activity of the testes [1, 2, 5, 16, 25] have also been described. In addition, seasonal hormonal profiles were shown to reflect dramatic changes in male reproductive organs, including scrotal circumference, size and weight of testes and accessory glands (macroscopic changes) as well as sperm concentration and testicular histology (microscopic changes) [8, 10, 14, 17, 24, 27, 28, 32, 34].

Deer reproduction seasonality studies are very often based on quantitative and qualitative evaluations of semen. At present, three different methods of semen collection are used; electroejaculation, artificial vagina (AV), and post-mortem epididymal recovery. However other methods like internal AV [9, 18] or a rubber sponge inserted into the vagina [23] are also considered. Semen characteristics can be significantly influenced by the method of collection. Collection of semen using electroejaculation results in ejaculates of high volume due to increased volumes of fluids from accessory glands and epididymis. Higher quality semen can be obtained using an artificial vagina. However, neither electroejaculation nor an AV separate the ejaculate into semen fractions [21]. Since the development of modified AV [11] fractions of ejaculate different in color and consistency can clearly be distinguished and sampled with sufficient precision.

Data concerning seasonal changes of semen are important for better understanding the seasonality of red deer reproduction. Up to now only fragmentary data concerning this issue are available [20, 21, 22]. The general aim of the present study was a biological assessment of red deer
semen collected by using modified artificial vagina. Specifically, the follow-
ing ejaculate characteristics were examined over a three complete periods of sexual activity; volume, pH, sperm concentration, sperm progressive motility, duration of progressive motility, and preliminary assessment of sperm morphology.

MATERIALS AND METHODS

During three reproductive seasons 1995-98 at the Polish Academy of Sciences Research Station in Popielno, 572 ejaculates were collected from five stags at the age of 2-13 years born and raised on the farm. The stags were kept separately in enclosures 2500 m² separated by enclosures of similar area, occupied by hinds. The semen was collected weekly (seasons 1995/96 and 1996/97) and every 10th day (1997/98) from the beginning of August till February/April. The actual end of the collection season was determined by the lack of libido of the individual stag. Ejaculates of each stag were collected twice per day with one-hour interval (collection 1 and collection 2) using an artificial vagina as modified by Gizejewski [11]. Since the development of modified the AV adapted for use in red deer, the white fraction of epididymal origin, and the yellow fraction – secretion of the vesicular glands can be clearly distinguished and sampled with sufficient precision.

The ejaculates were evaluated using standard procedures of semen evaluation of domestic animals. The pH of separated fractions was measured by pH-meter (Hanna Instruments, USA) equipped with microelectrode (HI 1083), adapted to small sample volumes (50 µl). Sperm concentration was determined with a light microscope (x125) using Bürker chamber.

For sperm morphology analysis semen samples were prepared either as air-dried smears or fixed in formol-saline. The red deer semen, both with normal and abnormal spermatozoa, is similar to that of domestic ruminants. For such reason the preliminary morphological evaluation of semen were performed on 200 spermatozoa by methodology developed by Blom [4]. The sperm progressive motion and duration of motility were evaluated using semen diluted in PBS at the ratio of 1 to 50, and assessed using a light microscope (x250) equipped with a heated block (+38°C). The following
scale was used: +++ rapid progressive motion, ++ steady progressive motion at moderate speed, + slow progressive motion. Duration of the progressive motility, until decline of motility below 10%, was also measured. Color of fraction was estimated visually using the Pantone Colour Formal guide as described previously [13]. The volume of the white fraction was measured using calibrated Eppendorf tube to the nearest 0.05 ml and the yellow fraction was measured using graded semen plastic collector to the nearest 0.1 ml.

The data are expressed as mean ± SD (text) and mean ± SEM (figures). Statistical evaluation was performed using SAS statistical software [30]. Analysis of variance was used to assess the effect of season, individual animal or collection number on semen characteristics.

RESULTS AND DISCUSSION

Changes in macroscopic appearance of semen

The period of sexual activity (occurrence of libido) of red deer stags, amounted to 245 days, and lasted from 4 August to 6 April of each year. Macroscopic appearance of ejaculates was closely related to sexual behavior of the stags. Three periods of sexual activity can be distinguished (fig. 1); (A) pre-mating season, (B) mating season and (C) post-mating season as was previously described [12, 13].

The first ejaculates, collected at the beginning of August, were homogenous, with a watery or milky consistency and a grey color (grey fraction, GF). Ejaculates collected during the pre-mating season contained both spermatozoa and secretion of accessory, mainly vesicular, glands. From mid-August, the ejaculates contained two fractions typical of the mating period, the yellow fraction (YF; accessory gland-derived) and the white fraction (WF; of epididymal origin). The yellow fraction was initially pale yellow, and then its intensity grew until it became yellow. The change in color of YF was accompanied by a change in ejaculate consistency, from creamy to then fresh honey, to the dense honey with high viscosity. The white fraction of ejaculates collected in this period had the consistency of milk or cream.
At the end of November, the yellow fraction gradually became thinner, which was accompanied by the loss of its color intensity that turned pale yellow. At the end of December, ejaculates in the form of homogenous grey secretions, were observed again and became more and more watery in January and February. These properties of particular ejaculates fractions were highly repeatable in respective periods of consecutive mating seasons (fig. 1). It was found that the changes were progressive within one season, i.e. the process of ejaculate fractionating or fraction diminishing, once started was not reversed.

**Changes in volume of ejaculates**

The volume of the yellow fraction (fig. 2) was determined only during the mating period (fig.1). Volumes of fractions collected in the beginning of August ranged from 1.2 to 1.5 ml. From the end of August a steady increase in volume was observed with plateau (2.0–3.0 ml) from the middle of Sep-
September to the middle of November. This was followed by a steady decrease reaching volumes of 1.2–1.5 ml by the end of mating period (beginning of December). The yellow fraction volume of the first ejaculate (2.21±1.28 ml; collection no. 1) was larger than the second one (1.82±1.16 ml; collection no. 2). However, average percentage of YF in both ejaculates was constant and amounted to approximately 90% of the total ejaculate volume. Volumes of YF (and total volumes) were significantly affected by season, stag and number of collection (tab. 1).

In contrast to the volume of the YF, no significant changes in volume of the white fraction were observed (tab. 1, fig. 3). The average volume of WF was low, (0.18±0.12 ml; range 0.05–0.55 ml). The volumes of WF of the first and the second ejaculate were 0.20 ml±0.13 ml, and 0.16 ml±0.11 ml, respectively. The relative volume of WF in both ejaculates was constant and amounted to approximately 10 % of the total ejaculate volume.

Volumes of the grey fraction collected in the beginning of sexual activity were highly variable. This variability may can be related to different response to pheromone stimulation of particular stags at the beginning
Tab. 1. Effect of season, stag, and collection number on semen characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>x</th>
<th>±SD</th>
<th>Season</th>
<th>Stag</th>
<th>Collection number&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm concentration WF x 10&lt;sup&gt;6&lt;/sup&gt;/mm&lt;sup&gt;3&lt;/sup&gt;</td>
<td>281</td>
<td>2.27</td>
<td>1.36</td>
<td>***</td>
<td>***</td>
<td>*</td>
</tr>
<tr>
<td>Volume of white fraction ml</td>
<td>281</td>
<td>0.19</td>
<td>0.12</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Volume of yellow fraction ml</td>
<td>260</td>
<td>2.01</td>
<td>1.23</td>
<td>***</td>
<td>***</td>
<td>*</td>
</tr>
<tr>
<td>Volume of ejaculate ml</td>
<td>271</td>
<td>2.14</td>
<td>1.27</td>
<td>***</td>
<td>***</td>
<td>**</td>
</tr>
<tr>
<td>pH of white fraction</td>
<td>129</td>
<td>7.08</td>
<td>0.34</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>pH of yellow fraction</td>
<td>155</td>
<td>6.82</td>
<td>0.30</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

<sup>a</sup> collection number: first or second ejaculate

* p<0.05; ** p<0.01; *** p<0.001; NS p>0.05

Fig. 3. Seasonal changes of mean volumes (ml±SEM) of white fraction (WF); n=330 and grey fraction (GF); n=249, 5 stags, years 1995-98.
of reproductive reason. Due to the disappearance of yellow and white fractions in the post-mating season, the grey fraction constituted 100% of the ejaculate volume at this period (0.34 to 0.55 ml). Similarly to YF and WF, higher volumes of GF were recorded in the first ejaculate than in the second one (0.55±0.31 ml vs. 0.38±0.21 ml).

**Changes in the pH of semen fraction**

Values of pH of the yellow fraction were variable at the beginning of the mating season. By the end of September they stabilized and a steady increase (from 6.65 to 7.45) was observed at the end of season (fig. 4). No differences were found between the pH value of YF of the first and second ejaculates. The pH value of the white fraction was stable, and similarly to YF, an increase ranging from 6.80 to 7.41 was observed at the end of season (fig. 5). No differences were found between the pH value of YF of the first and second ejaculates. The pH of the grey fraction, unlike YF and WF, fluctuated strongly, ranging from 6.07 to 8.69.

![Figure 4](image_url)

**Fig. 4.** Seasonal changes of pH (mean±SEM) of the yellow (YF) fraction; n=190, 5 stags, years 1995-98.
Changes in sperm concentration and total number of spermatozoa

Changes in sperm concentration are shown in fig. 6. The sperm concentration of grey fraction, collected during pre-mating season, was low (0.2–0.45x10^6/mm^3). At the same time, the sperm concentration of the white fractions ranged from 1.14 to 2.27x10^6/mm^3. A distinct increase in sperm concentration of WF was recorded at the end of September and was maintained until the end of October (2.62 to 3.18x10^6/mm^3). A steady decrease was recorded in the beginning of November, reaching about 2x10^6/mm^3 by the end of December. At this time sperm concentration was variable, with unusually high sperm concentration in the middle of December. Sperm concentration of the grey fraction declined steadily from the beginning of December to the end of March. The mean concentration of spermatozoa during mating season was higher in the first ejaculate (2.4x10^6±1.41/mm^3) than in the second one (2.12±1.28/mm^3). Sperm concentration was significantly affected by period of the reproductive season, stag and semen collection (tab. 1).

Changes in the sperm concentration clearly confirmed seasonality of red deer reproduction. The period of maximal libido seems to be accompanied
Fig. 6. Seasonal changes of the sperm concentration (x 10^6/mm^3±SEM) in white fraction (WF; n=328) and gray fraction (GF; n=274), 5 stags, years 1995-98.

by the highest concentrations of spermatozoa (end of September – end of October). In the final period of libido, (end of March and the beginning of April), a total of six watery azoospermic ejaculates were collected. Similar, azoospermic ejaculates were obtained by Krzywiński and Jaczewski [22] in May and June and by Haigh et al. [16] in mid-June and at the beginning July from the Rocky Mountains wapiti (Cervus elaphus nelsoni). However, these ejaculates were obtained by electroejaculation when stags already lacked libido.

The changes in the total number of spermatozoa in ejaculates collected during the mating season were similar to the changes in sperm concentration during this period (fig. 7). The highest total number of spermatozoa was recorded at the period of the greatest libido, from the end of September to the end October, ranging from 569.4x10^6 to 793.9x10^6. Assuming that an insemination dose for artificial insemination should contain 20x10^6 motile spermatozoa (and 70% motility) this translates to 20–30 doses per ejaculate.

Changes in the progressive motility of spermatozoa and its duration

Two periods with differing sperm motility could be distinguished in the semen samples (fig. 8). A steady increase in motility was observed from the
middle of August (40%) to the end of October (60%). This was followed by a dramatic decrease in motility to below 20% by the end of March. High variability of sperm motility was observed starting in late December. It is interesting that elevated pH levels were also recorded during this period.

Fig. 7. Seasonal changes of the total number of spermatozoa (x 10⁶±SEM) in the white fraction (WF; n=322), 5 stags, years 1995-98.

Fig. 8. Seasonal changes of spermatozoa progressive motility (% ±SEM; n=100), 4 stags, season 1995/96.
Red deer semen

( fig. 5 ). These results may suggest that composition of the grey fraction is not optimal for supporting sperm motility. During mating season, 47±24% of spermatozoa exhibited progressive movement and 80% of them were characterized by steady progressive motion at moderate speed (++). The increased progressive motility of sperm was usually recorded in ejaculates collected at the top of the mating season, and those with higher sperm concentrations (2.73 to 4.19×10⁶/mm³).

The average duration of sperm motility during mating season was 6 hours 39 minutes (± 2h 23min), and was nearly twice as long as during the pre- and post-mating season (3 h 28 min±1h 12 min). The duration of sperm motility fluctuated greatly during all periods of reproductive activity.

Morphological changes of spermatozoa

The morphological defects of red deer spermatozoa are shown in fig. 9. A preliminary evaluation indicated that the prevalence of morphological sperm abnormalities was associated with the stages of reproductive period (data not shown). The lowest percentage of major defects of sperm (< 5%), was recorded during the mating season whereas a higher percentage (>10%) was recorded during the pre- and post-mating season. The number of minor sperm abnormalities was the lowest during the period of maximal libido (September/October) and ranged from 6 to 12%. This was followed by an increase of these defects (>25%). Seasonal increase in the number of sperm with altered morphology during the pre-mating and post-mating periods agrees with the findings concerning sperm morphology of red deer [19, 22], Rocky Mountains wapiti [16] and fallow deer [14, 15].

Among the most commonly encountered defects are spermatozoa with distal droplets (fig. 9d). This defect is characteristic for epididymal semen. It was shown, that epididymal fluids collected post mortem during mating season from red dear [8, 10], contained high incidence (71.2% to 80.3%) of spermatozoa with droplets in the distal position. Distal droplets are classified as a minor defect and are lost during the movement of spermatozoa along the spermatic duct [3, 29].
In summary, measures of semen quality including fraction volume, pH, sperm concentration and sperm motility change gradually during the pre-mating, mating and post-mating seasons of red deer. The period of greatest libido (from the end of September until the end October) coincides with highest semen quality.

Fig. 9. Normal and abnormal spermatozoa of the red deer.
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