Influence of constant long days on ejaculate parameters of rabbits reared under natural environment conditions of Mediterranean area

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Abstract

Since rabbit bucks are usually housed under constant long daylight in artificial insemination (AI) centers, the main purpose of this study was to investigate whether constant long day influenced ejaculate parameters of rabbits housed in AI centers in the Spanish Mediterranean area. The study was carried out in Murcia, Spain (37° N). Twenty commercial hybrid male rabbits, aged between 14 and 15 weeks, were randomly allotted to two groups and housed under either natural day length (\(n=10\), ND) or a constant 16-h daylight exposure of 16 h (\(n=10\), CLD). Other management conditions, such as air temperature or reproductive handling, were identical for both groups. Two successive ejaculates were collected twice weekly from every male, and the first one was used to monitor ejaculate characteristics. Measurement of semen production, in terms of ejaculate and semen volume, sperm concentration and total sperm per ejaculate, and sperm quality, in terms of motility index, viability, morphology and acrosome integrity, was assessed in 783 ejaculates collected during 15 months (from October to December). No differences (\(P<0.05\)) in either semen production or sperm quality were shown among ejaculates collected from rabbits housed under ND and CLD conditions. A limited influence of season was observed (\(P<0.01\)); semen volume and motility index were highest and lowest, respectively, during summer. The increase of air temperature and humidity index (THI) had a significant detrimental effect (\(P<0.01\)) on both sperm production and quality parameters with a lag of 6 and 3 weeks, respectively. On the basis of these findings, annual variations of semen production and sperm quality in male rabbits seems more related to THI than to daylight length under conditions of AI management in the Mediterranean area of Spain.

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Keywords: Rabbit; Semen; Long day; Air temperature; Mediterranean area

1. Introduction

Artificial insemination (AI) in rabbits is practised in many of the European countries, especially in the Mediterranean area, where rabbit production for
consumption is of importance. In France, Italy and Spain, AI is an ordinary technique in the breeding programs of commercial meat rabbit production. Optimising semen production is of great importance for breeding programmes, especially when AI is used. Therefore, collection of ejaculates with a high number of spermatozoa of excellent quality is the most important goal for rabbit AI centers. Unfortunately, ejaculate parameters vary during the life of the rabbits. Both semen production and sperm quality can differ depending on individual characteristics and environmental and management factors. Accurate knowledge of these factors may help to improve the efficiency of rabbit AI centers.

The influence of environmental conditions on reproductive performance of domestic rabbits has been reported (Hudson et al., 1994; Marai et al., 2002). Environment can exert its influence by variations in daylight length, air temperature and relative humidity. The influence of these environmental factors on ejaculate characteristics of male rabbits has been studied under experimental conditions and, normally, in an independent way using environmental control chambers (Finzi et al., 1995; Theau-Clément et al., 1995). In general, reproductive performance of male rabbits is enhanced under long-day conditions and disturbed with high temperature. However, the above experiments do not provide a holistic view about the influence of variations of natural environmental factors on the ejaculate parameters of rabbits living under AI center conditions.

Semen production and sperm quality are not constant during the year in ejaculates collected from rabbits living under natural environmental conditions (Marai et al., 2002). The magnitude of the variations, as well as the time of the year in which they occur, vary between geographic latitudes. Although information is available on ejaculate parameters of rabbits living in the Mediterranean area (Lavara et al., 2000; Nizza et al., 2003), systematic studies evaluating the effect of day length and/or air temperature on ejaculate parameters of rabbits housed under AI center conditions are not available. Since rabbit bucks are usually housed under constant long daylight in AI centers, the main purpose of this study was to investigate whether constant long daylight influences the ejaculate parameters of rabbits housed in AI centers of the Spanish Mediterranean area. In addition, this study presents observations of annual variations in the more common ejaculate parameters of rabbits living under AI center and natural environment conditions of the Spanish Mediterranean area.

2. Materials and methods

2.1. Location and climatic data

The study was conducted on the experimental farm of the University of Murcia located in Murcia province in the southeast of Spain (37° 59′N, 1° 08′W). Natural day length varies from 14 h and 54 min of light at the summer solstice to 9 h and 30 min of light at the winter solstice.

The climatic data were continuously recorded using a hydrothermograph for a period of the 17 months from August to December. The mean daily air temperature and relative humidity were calculated as the average of four recordings (11 am and 01, 03 and 05 pm). The temperature–humidity index (THI) was computed using the formula reported by Marai et al. (2002) for rabbits:

$$THI = \frac{db^\circ C - [(0.31 - 0.31(RH/100))/(db^\circ C - 14.4)]}{db^\circ C}$$

where $db^\circ C$ is dry bulb temperature, and RH is the relative humidity expressed in percentage.

2.2. Animals and management

Twenty commercial hybrid male rabbits (aged between 14 and 15 weeks) originating from the same herd were used in the experiment. In the experimental farm, male rabbits were randomly divided into two groups of 10 males each. The first group served as the control, and the males were housed in a room with windows exposed to the natural day length (natural day length group [ND]). The second group of male rabbits were housed in a room with windows exposed to a natural day length with 200 lux of supplementary light for 16 h daily (constant long-day group [CLD]). The supplementary light was turned on at 6 a.m. and turned off at 10 p.m. by means of an electric clock. Male rabbits were acclimatized for 12 weeks to their respective experimental light regimes before the start...
of the experiment. All rabbits from both groups were kept in individual flat deck cages and daily fed with 180 g of a commercial pelleted diet and had free access to water.

### 2.3. Ejaculate collection and evaluation

Ejaculates were collected using an artificial vagina following the procedure described by Boussit (1994). The experiment was carried out for a period of 15 months from the first week of October to the last week of December. Rabbits were subjected to regular, twice weekly collection sessions. In each collection session, two successive ejaculates were obtained. One ejaculate per week from each male (first ejaculate collected) was used for this experiment.

### 2.4. Measurement of semen production

Semen production was assessed in terms of ejaculate and gel-free semen volume, sperm concentration and total number of spermatozoa per ejaculate. Immediately after collection, the ejaculate volume (semen and gel fraction together) was assessed from the graduated collection test tube. After removing the gel fraction, semen volume was also assessed. Sperm concentration was evaluated in a hemocytometer after extending (1:400, v/v) an aliquot of semen with 0.3% formaldehyde in phosphate-buffered saline. The number of total spermatozoa per ejaculate was calculated multiplying semen volume by sperm concentration.

### 2.5. Evaluation of sperm quality

Evaluation of spermatozoa quality included sperm motility index and the percentages of spermatozoa with normal plasma membrane integrity, sperm morphology and acrosome integrity. Before the evaluation, semen was diluted (1:8 ratio) in a Tris-buffered extender (Roca et al., 2000) and incubated for 30 min in a warm water bath at 30 °C. To determine sperm motility index, the percentage of motile spermatozoa (PMS) and the quality of sperm movement (QSM) were evaluated from three samples of the diluted spermatozoa placed under a coverslip in the centre of a prewarmed (37 °C) slide and transferred to a heated microscope stage set at 37 °C. The evaluation was subjectively assessed using phase contrast microscopy (×200 magnification). The PMS was recorded on a five multiple scale, and QSM was determined using an arbitrary scale of 0 to 5 where 0 is absence of movement and 5 is when all motile spermatozoa are showing progressive motility. The motility index (MI) was computed using the following formula:

\[
MI = \frac{\text{PMS} \times \text{QSM}}{5}
\]

The percentage of spermatozoa with normal plasma membrane integrity (PMI) was evaluated with a fluorescence microscope (magnification ×400) using a standard fluorescein filter set (Nikkon, Japan) after staining the diluted spermatozoa with 6-carboxyfluorescein diacetate (Sigma, St. Louis, MO, USA), as described previously by Harrison and Vickers (1990). The proportions of spermatozoa with normal morphology (SNM) and acrosomal integrity (normal apical ridge [NAR]) were measured by viewing wet mounts of diluted semen fixed in buffered 2% glutaraldehyde solution (Pursel and Johnson, 1974) under a phase contrast microscope at the magnification of 1000×. Morphologic abnormalities included head, midpiece (excluding distal cytoplasmic droplets) and tail defects. Two hundred spermatozoa were counted from each preparation of PMI, SNM and NAR.

### 2.6. Statistical analyses

From the 20 rabbits at the beginning of the experiment, three failed on semen production, and two died before the end of the study. These rabbits were removed from the study to avoid confounding the fluctuations in semen parameters due to rabbit’s replacement. Thus, data were analysed from seven rabbits on ND and from eight rabbits on CLD. The total number of ejaculates collected was 900 (420 and 480 ejaculates from ND and CLD rabbits, respectively), from them, 117 were discarded due to urine contamination. The total number of ejaculates fully evaluated was 783 (383 and 400 ejaculates from ND and CLD rabbits, respectively).

To determine seasonal changes in ejaculate parameters, recorded data from the 15 months were divided into five seasonal periods of 3 months each: autumn-1 (October, November and December), winter (January,
February and March), spring (April, May and June), summer (July, August and September) and autumn (October, November and December).

A mixed model analysis, taking into account the variation among animals and the covariation within their records, according to a repeated measures design (PROC MIXED, SAS Institute, Cary, NC), was used to analyze the data. The variables of the ejaculates were analysed according to the following model:

\[ Y_{ijkl} = \text{LT}_i + S_j + \beta \text{THI}_k + a_{il} + e_{ijkl} \]

- \( \text{LT}_i \) is the light treatment effect, with two levels (natural daylight length or constant long day);
- \( S_j \) is the season effect, with five levels (one level each 3 months);
- \( \text{THI}_k \) is the temperature–humidity index used as a covariate, \( k \) being the number of weeks lapsed between the THI record and the semen collection;
- \( \beta \) is the partial regression coefficient;
- \( a_{il} \) is the random permanent effect of the ilth animal, common to all the records of the same animal; each animal was maintained in the same light treatment throughout the experiment; and
- \( e \) is the residual of the model.

The lags between changes in THI and ejaculate parameters were fitted to each variable. THI records from 0 to 7 weeks before semen collection were used to assess its influence on the variables of the ejaculates. The least squares means option was used to compare different means and differences were considered to be significant at \( P<0.05 \).

3. Results

3.1. Climatic conditions

No differences (\( P>0.05 \)) were found in air temperature and humidity between ND and CLD rooms. THI monthly means of the 15 months in which the experiment was carried out are shown in Fig. 1. The THI data indicated a prevalence towards absence of heat stress conditions overall the study time frame with occasional periods of moderate heat stress during the months of July and August.

3.2. Semen production

Animal was a significant (\( P<0.001 \)) source of variation accounting for 43.3%, 43.7%, 26.4% and 15.8% of the total variance in ejaculate volume, semen volume, sperm concentration and total spermatozoa per ejaculate, respectively.

No significant differences (\( P>0.05 \)) between rabbits housed in ND or CLD rooms were found for any of the semen production parameters evaluated (Table 1).

Semen volume was influenced (\( P<0.001 \)) by season, being higher in summer than in autumn, winter or spring. Total ejaculate volume, sperm

![Fig. 1. Evolution of the temperature–humidity index (■) during 2 months before the beginning and throughout the experimental period (■). Horizontal lines indicate the limits of heat stress degrees for small mammals according to Marai et al. (2002); dotted line (absence of heat stress, <27.8 °C); dashed line (moderate heat stress, 27.8–28.9 °C); solid line (severe heat stress, 28.9–30 °C) and above the solid line (very severe heat stress, >30 °C).]
concentration and total number of spermatozoa per ejaculate did not differ significantly ($P > 0.05$) among seasons (Table 1).

Changes in THI during the year did not affect ($P > 0.05$) neither total ejaculate nor semen volume so the results are presented without the covariate for both variables. However, sperm concentration and total number of spermatozoa per ejaculate appeared to follow changes in THI with a lag of 6 weeks (Table 1).

The increase in THI had a significant ($P < 0.01$) detrimental effect 6 weeks later on sperm concentration and total number of spermatozoa per ejaculate (Fig. 2).

### 3.3. Sperm quality

Animal was a significant ($P < 0.001$) source of variation accounting for 16.1%, 32.5%, 47.2% and

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**Table 1**

Least squares means (±S.E.) and significance for semen production parameters as influenced by day length, season and temperature–humidity index (THI)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ejaculate volume (ml)</th>
<th>Semen volume (ml)</th>
<th>Sperm concentration ($\times 10^6$ cells/ml)</th>
<th>Total spermatozoa ($\times 10^6$ cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daylight length</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ND</td>
<td>0.92±0.16</td>
<td>0.64±0.08</td>
<td>493.88±42.29</td>
<td>323.68±29.53</td>
</tr>
<tr>
<td>CLD</td>
<td>1.31±0.15</td>
<td>0.83±0.08</td>
<td>420.38±39.68</td>
<td>331.05±27.77</td>
</tr>
<tr>
<td>Season</td>
<td>NS</td>
<td>***</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Autumn</td>
<td>1.12±0.12</td>
<td>0.76±0.06</td>
<td>447.92±37.22</td>
<td>336.61±29.88</td>
</tr>
<tr>
<td>Winter</td>
<td>1.14±0.11</td>
<td>0.74±0.06</td>
<td>476.36±32.31</td>
<td>338.42±24.31</td>
</tr>
<tr>
<td>Spring</td>
<td>1.09±0.11</td>
<td>0.76±0.06</td>
<td>465.71±31.49</td>
<td>340.81±23.33</td>
</tr>
<tr>
<td>Summer</td>
<td>1.05±0.12</td>
<td>0.81±0.06</td>
<td>439.24±32.64</td>
<td>346.35±24.69</td>
</tr>
<tr>
<td>Autumn</td>
<td>1.18±0.12</td>
<td>0.75±0.06</td>
<td>456.32±31.55</td>
<td>331.64±23.41</td>
</tr>
<tr>
<td>THI</td>
<td>NS</td>
<td>NS</td>
<td>***</td>
<td>**</td>
</tr>
</tbody>
</table>

Results summarize data from 783 ejaculates collected from 15 rabbit bucks.

ND—natural day length; CLD—constant long day.

NS—not significant; **$P < 0.01$; ***$P < 0.001$. Means within the same column with different superscripts are significantly different ($P < 0.05$).

$^1$Regression coefficient.
39.5% of the total variance in motility index, plasma membrane integrity, acrosome integrity and spermatozoa with normal morphology, respectively.

Similarly to semen production parameters, no significant differences ($P < 0.05$) between ejaculates collected from rabbits housed in ND or CLD rooms were noticed for any of the sperm quality parameters evaluated (Table 2).

Season only affected ($P < 0.01$) sperm motility index. As shown in Table 2, the lowest motility index was recorded in summer, increasing gradually until reaching the highest values during spring. The percentages of spermatozoa with intact plasma membrane, acrosome integrity and spermatozoa with normal morphology did not differ ($P > 0.05$) among seasons.

Changes in THI during the year affected ($P < 0.05$) the four sperm quality parameters evaluated. An increase in THI affected adversely sperm motility index and the percentages of spermatozoa with normal morphology, with intact plasma membrane and intact acrosome with a lag of 3 weeks (Fig. 3).

### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Motility index</th>
<th>Plasma membrane integrity (%)</th>
<th>Acrosome integrity (%)</th>
<th>Sperm with normal morphology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daylight length</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ND</td>
<td>66.02±1.61</td>
<td>88.63±1.19</td>
<td>89.21±1.67</td>
<td>92.12±1.73</td>
</tr>
<tr>
<td>CLD</td>
<td>66.43±1.51</td>
<td>90.12±1.12</td>
<td>90.70±1.56</td>
<td>89.01±1.62</td>
</tr>
<tr>
<td>Season **</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Autumn $a,b$</td>
<td>65.67±1.66</td>
<td>89.78±1.30</td>
<td>88.91±1.30</td>
<td>90.55±1.33</td>
</tr>
<tr>
<td>Winter $a,b$</td>
<td>66.03±1.33</td>
<td>89.29±0.94</td>
<td>90.05±1.20</td>
<td>91.08±1.24</td>
</tr>
<tr>
<td>Spring</td>
<td>66.88±1.26</td>
<td>89.14±0.89</td>
<td>90.48±1.18</td>
<td>91.18±1.22</td>
</tr>
<tr>
<td>Summer</td>
<td>63.12±1.51 $b$</td>
<td>90.06±0.97</td>
<td>90.56±1.25</td>
<td>89.49±1.28</td>
</tr>
<tr>
<td>Autumn</td>
<td>64.41±1.26 $a,b$</td>
<td>89.45±0.89</td>
<td>89.76±1.18</td>
<td>90.53±1.22</td>
</tr>
<tr>
<td>THI **</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>**</td>
</tr>
<tr>
<td>(−0.47±0.15) $f$</td>
<td>(−0.42±0.07)</td>
<td>(−0.48±0.08)</td>
<td>(−0.28±0.07)</td>
<td></td>
</tr>
</tbody>
</table>

Results summarize data from 783 ejaculates collected from 15 rabbit bucks.
ND—natural day length; CLD—constant long day.
NS—not significant; **$P<0.01$; ***$P<0.001$. Means within the same column with different superscripts are significantly different ($P<0.05$).

Changes in THI during the year affected ($P < 0.05$) the four sperm quality parameters evaluated. An increase in THI affected adversely sperm motility index and the percentages of spermatozoa with normal morphology, with intact plasma membrane and intact acrosome with a lag of 3 weeks (Fig. 3).

### 4. Discussion

Traditionally, bucks in AI Centers are placed under constant long daylight, from 12 to 16 h light daily (Nizza et al., 2003), the same duration of lighting being recommended for rearing breeding females. Accordingly, this management procedure implies that long days exert a favorable influence on semen production and sperm quality. Nevertheless, experiments relating to the influence of the length of illumination on the quantity and quality of spermatozoa have so far been inconclusive. The literature on this topic is limited to studies in which rabbits were exposed to long or short days for short periods of time. Under these experimental conditions, the effect of lighting on ejaculate characteristics of rabbits is evident. Exposure of rabbits to long days (16 h light:8 h dark) improved the quantity and quality of spermatozoa present in the ejaculates in comparison to those collected in rabbits exposed to short days (8 h light:16 h dark; Theau-Clémente et al., 1995). However, these experiments do not provide a holistic view of how supplementary lighting can influence the ejaculate parameters of rabbits housed in AI centers. The present study used data of ejaculates collected during 15 months from rabbits maintained under standard AI conditions (reproductive management, health status and nutrition). Under these conditions, neither semen production nor sperm quality parameters differ between ejaculates collected from rabbits housed under natural day length or long days. These results indicate that artificial light supplementation does not improve the ejaculate characteristics of rabbits under AI center conditions in the Mediterra-
ean area. This suggests that annual photoperiod variations in the Mediterranean area are not sufficiently wide to induce changes in semen production and sperm quality in rabbits.

Seasonal variations in ejaculate parameters are well documented in male rabbits (Mathur et al., 1989), being the seasonal pattern and the importance of the seasonality variable according to environmental and management conditions (for a review, see Marai et al., 2002). In our study, a limited influence of natural season was shown. Only semen volume and motility index were influenced by season, showing the highest and lowest values during summer, respectively. Since total sperm outputs did not vary, it is clear that the variation in semen volume is related to the change in the volume of seminal plasma. Moreover, as the ejaculate volume (gel-free semen and gel fraction together) either varies, the seasonal variation in gel-
free semen volume is related to changes in gel volume, which was lowest during summer. This could indicate that, during summer, there is a minor gelatinization of seminal plasma, probably due to the adverse effect of high ambient temperature. The fact that motility index was lowest during summer also suggests that the seasonal variations could be associated with changes in the environmental temperature. The seasonality of ejaculate parameters in the rabbit is particularly attributed to the adverse effect of an increase in environmental temperature, becoming more pronounced when the relative humidity is high (Marai et al., 2002). Such relationship can be measured as the heat stress using the temperature–humidity index (THI; LPHSI, 1990).

It has been demonstrated that rabbits are highly sensitive to heat stress (Franci et al., 1996). The adverse effect of the heat stress on ejaculate parameters of rabbits has been also studied. The daily exposure of rabbits in a climatic chamber with high ambient temperature (30 °C) and humidity (70%) for 21 h during 60 days increased the number of abnormal spermatozoa (Finzi et al., 1995). Although the above experiment provides information of the unfavourable effect of high temperature and humidity on the male rabbit reproductive performance, there is little information available from data collected under commercial AI conditions. Under field conditions, ambient temperature and relative humidity vary from day to day and ameliorate during the night. As a result, the response of the rabbits could be different from those observed under experimental conditions using climatic chambers. It has been demonstrated that rabbit body temperature is at least temporarily increased when the environmental temperature exceeds 28 °C (Amici et al., 1995). Environmental conditions implicating heat stress as a causative factor in reduced reproductive performance of rabbits can begin to occur when the THI is ≥27.8 °C, and a severe heat stress may be found when the THIs up to 28.9 °C are reached (Marai et al., 2002). In the present study, THI monthly means up to 28 °C were observed during summer which, according to THI indices, would be classified as moderate heat stress conditions. Our results indicate that most ejaculate parameters are significantly influenced by THI. These results confirm that male rabbits are very sensitive to THI stress since ejaculates showed not only a significant reduction in the number of spermatozoa but also a lost of quality related to an increase of the THI index. However, as the extent of damage is directly related to the intensity and duration of stress, the deleterious effects of elevated THI on ejaculate parameters were of a temporary nature. The reduction in sperm production and quality occurs almost exclusively during a short period of time at the end of summer and the beginning of autumn. The adverse effects of THI stress were not immediate. The reduction in sperm production and sperm quality paralleled maximum THI but was delayed by 6 and 3 weeks, respectively. These findings are consistent with previous observations in the same Mediterranean area where Lavara et al. (2000) observed that the increase in air temperature during August negatively affected the sperm production of ejaculates collected 2 months later. Similar findings were reported by Mathur et al. (1989) who demonstrated that semen characteristics of Soviet Angora rabbits in a semiarid zone of a subtropical region of India have significant seasonal variations which are attributable to high ambient temperatures and humidity of summer season, showing that poor semen characteristics were extended during the 6 weeks after the end of summer season. According to the duration of spermatogenesis and epididymal transit in rabbits (Swierstra and Foote, 1965), the time lapsed from THI stress to the occurrence of ejaculate deviation indicates that spermatogenesis but not the epididymal transit was negatively affected. Therefore, sperm being formed in the testicle are very sensitive to heat and readily damaged, whereas those that have moved out of the testicle are much more resistant. Epididymal transit time in rabbits has been estimated to last for 8–10 days (Swierstra and Foote, 1965), so epididymal function in the rabbit is probably not affected by the high THI of the Mediterranean area.

Finally, it is important to point out that a marked variation among bucks was shown in all ejaculate parameters evaluated in agreement with the results reported by Theau-Clémente et al. (1995). The cause of these differences, which contributed a substantial portion of the total experimental variation, is not currently known. The differences may have a genetic source. The bucks used in the present study were not previously preselected for their ejaculate parameters,
indicating that this should be considered to select the most suitable AI bucks sires.

In conclusion, our results suggest that ejaculate parameters of male rabbits living in the Spanish Mediterranean area are only moderately affected by season, and that the exposure of the rabbits to long days does not seem to improve either sperm production or quality. These findings suggest that, in this geographic latitude, heat stress is the major environmental factor responsible for annual variations in ejaculate parameters. From a practical standpoint, it seems more important to develop strategies to alleviate negative effects of heat stress than to use artificial long days to improve semen production and quality in male rabbits maintained under AI conditions in the Mediterranean area.

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