Use of the hypo-osmotic swelling test and aniline blue staining to improve the evaluation of seasonal sperm variation in native Spanish free-range poultry

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ABSTRACT The season may affect the values of fresh semen variables and therefore influence the success of cryopreservation. The aim of this study was to improve the evaluation of seasonal changes in semen quality in Spanish Black Castellana roosters maintained under natural environmental conditions. Semen was collected from 11 Black Castellana roosters (housed under natural photoperiod and temperature conditions) by massage twice every month for 12 mo. In addition to determining ejaculate volume, sperm concentration, and sperm motility (the classic sperm variables), we used the hypo-osmotic swelling test to examine the membrane integrity of the spermatozoa. Further, morphological abnormalities and acrosome integrity were assessed via aniline blue staining. Semen volume \( P < 0.05 \), sperm concentration \( P < 0.01 \), and the percentage of spermatozoa with an intact acrosome \( P < 0.01 \) were significantly affected by the season of the year. The annual profile of the percentage of spermatozoa showing acrosome integrity followed a trend roughly parallel to annual variations in temperature (Spearman rank correlation \( = 0.77, P < 0.01 \)). According to the hypo-osmotic swelling test, membrane integrity fell in July \( P < 0.05 \) compared with all other months, the month of highest temperatures. Aniline blue staining and the hypo-osmotic swelling test provide an easy and useful means of evaluating sperm abnormalities, including acrosome morphology and membrane integrity, and could be easily introduced into routine avian semen quality assessments. The results show that high semen quality is associated with long day photoperiods. Extreme heat or cold appear to exert a negative influence on sperm quality.

Key words: spermatozoa, season, free-range, fowl, Black Castellana breed

INTRODUCTION

The ex situ conservation of Spanish native chicken breeds, via genetic resource banking (Watson and Holt, 2001), could help guarantee their preservation and is of special importance given the risks of epidemics such as avian influenza. Spermatozoa are the most accessible sex cells, and they are currently the primary type preserved in most banks of this kind (Blesbois et al., 2007). The spermatozoa face a succession of thermal, osmotic, and mechanical stresses during cryopreservation (Bakst and Sexton, 1979; Blesbois and Brillard, 2007). Certainly, fowl spermatozoa appear to be highly susceptible to morphological disruptions, including small increases in their volume, during the freeze-thaw process (Xia et al., 1988; Watson et al., 1992; Blanco et al., 2008) as well as during dilution (Bakst and Sexton, 1979) and storage (Bakst et al., 1991; Bakst and Cecil, 1992). Because the fertility rate of cryopreserved poultry sperm is dramatically lower than that of any domestic mammalian species (Long, 2006), the initial quality of freshly collected sperm needs to be very high (Lake and Stewart, 1978). The season may affect the values of fresh semen variables (Kamar and Badreldin, 1959) and therefore influence the success of cryopreservation. The effect of season is more evident in chicken breeds that are maintained outdoors year-round, and many native chicken breeds included in conservation programs are commonly kept under such conditions. Optimizing the evaluation of the seasonal changes in semen quality might therefore help us make better predictions regarding the freezability of fresh semen samples.

It has been shown that sperm motility, concentration, and semen volume can vary with the season (Kamar and Badreldin, 1959; Saeid and Al-Soudi, 1975). However, the effect of season on acrosome and membrane...
integrity in fowl semen is not well known, even though acrosome integrity is a valuable indicator of fertilizing capacity in mammals (Pursel and Johnson, 1974).

Aniline blue staining allows good visualization of mammalian spermatozoa morphology and of the acrosome. It is habitually used to examine the integrity of the DNA in human spermatozoa (Dadoune et al., 1988), and in some species, seasonal changes in this integrity are detected via alterations in staining intensity (Dadoune et al., 1988; Henkel et al., 2001). However, to our knowledge, aniline blue staining has never been used in the examination of fowl semen. In addition, the hypo-osmotic swelling test has been used to examine membrane integrity in both mammals (Jeyendran et al., 1984) and avian spermatozoa (Malecki et al., 2005). The ability of the sperm tail to swell in the presence of a hypo-osmotic solution is a sign that the transport of water across the membrane is able to take place and therefore that the spermatozoa membrane is functioning normally (Jeyendran et al., 1984).

The aim of this study was to determine if there are seasonal changes in semen quality from the Black Castellana breed, one of the oldest chicken breeds in Spain (Orozco, 1989). This breed is usually reared under free-range conditions, and it is thought that, like other free-range breeds (e.g., Golden Gauloise; Blesbois et al., 2007), it may have retained a pronounced seasonal nature now lost in most commercial breeds.

MATERIALS AND METHODS

Experimental Birds

Eleven Black Castellana roosters, all of which were 1 yr old at the beginning of the experiment, were housed under natural photoperiod and temperature conditions in a 8-m² sand-floor pen with a partial roof cover at the El Encín Research Station (Madrid, Spain, 40°31'N). These birds were raised as part of a genetic resource conservation program started in 1975 (Campo and Orozco, 1982; Campo, 1998). All birds had a constant diet throughout the experimental period. Specifically, the birds were fed a commercial maize (Zea mays)-barley-based feed with a 2.8% fat and 15% CP content.

Sperm Collection and Evaluation

Semen was collected twice monthly (2 wk apart) in 15-mL graduated centrifuge tubes (Sterilin, Bibby Sterilin Ltd., Stone, Staffs, UK) from individual roosters using the massage technique described by Burrows and Quinn (1937). Ejaculate volumes were recorded in these same tubes. The collection period lasted 12 mo (July 2007 to June 2008). Collected semen was immediately diluted 1:1 (vol/vol) at field temperature using a medium (Lake and Ravie, 1984) composed of sodium glutamate (1.92 g), glucose (0.8 g), magnesium acetate 4H₂O (0.08 g), potassium acetate (0.5 g), polyvinylpyr-
blue mixed with 2% acetic acid (pH = 3.5) for 5 min, washed with distilled H2O, and air-dried. Briefly, the staining solution was prepared by adding 5 g of aniline blue (Water blue, Fluka, Buchs, Switzerland) to 100 mL of PBS, filtering, and adjusting the pH to 3.5 with 2% glacial acetic acid (Merck, Darmstadt, Germany). A total of 200 spermatozoa were examined and the percentage of stained sperm heads was calculated. Two classes of staining intensities were distinguished (Henskel et al., 2001): spermatozoa showing strong and very strong aniline blue staining were regarded as aniline blue-positive, whereas those staining only weakly or not at all were regarded as aniline blue-negative.

The sensitivity of aniline blue staining for the identification of abnormal acrosomes was tested by its comparison against fluorescent staining with conjugated peanut (Arachis hypogea) agglutinin-fluorescein isothiocyanate (PNA-FITC; Soler et al., 2005). Tests were performed using double aliquots of 22 fresh or frozen-thawed heterospermic pool samples [freezing was performed according to the dimethyl acetamide straw method described by Blesbois et al. (2007)], and the results obtained with the fluorescence and aniline blue techniques were compared.

**Hypo-Osmotic Swelling Test**

To determine plasma membrane integrity via the hypo-osmotic swelling test (Jeyendran et al., 1984), 25 μL of diluted semen was mixed with 500 μL of a hypo-osmotic solution (100 mOsm/kg) prepared by adding 1 g of sodium citrate to 100 mL of distilled H2O. Given the high metabolic index of avian sperm, the test was also performed at lower incubation temperatures (e.g., laboratory temperature: about 23°C) and over shorter incubation times (15 min) than employed when examining mammalian sperm (Jeyendran et al., 1984). Tests were performed on 20 pooled ejaculates to select the optimum temperature (room temperature vs. 37°C) and time of incubation (15 min vs. 30 min). An incubation period of 30 min at 37°C was finally selected (see Results). The percentages of spermatozoa showing coiled mid-pieces and tail segments were determined after fixing the samples in buffered 2% glutaraldehyde solution at 37°C and examining 200 spermatozoa under a phase-contrast microscope (magnification 1,000×).

Propidium iodide (PI) was used as a fluorochrome for the fluorescence examination of membrane integrity. Propidium iodide is a DNA-specific stain that cannot cross the intact plasma membrane; it therefore allows the identification of viable spermatozoa that exclude the dye (see Soler et al., 2005 for details of the technique). The percentages of spermatozoa showing positive endosmosis in the hypo-osmotic swelling test and showing viability in the fluorescence technique were compared using double aliquots of 28 fresh or frozen-thawed heterospermic pool samples.

**Temperature Data**

Temperature data were recorded in situ at the El Encín Research Station. The data were recorded by the National Meteorological Agency of Spain.

**Statistical Analyses**

Sperm variables with a skewed distribution (as determined by the Shapiro-Wilk’s test: P < 0.05) were arcsine-transformed before statistical analysis.

To optimize the hypo-osmotic swelling test, the effects of different temperatures and incubation times were analyzed via Student t-test (matched pairs) comparisons of the different results obtained. The same test was used to compare the mean sperm variable values for spermatozoa showing positive endosmosis with those that excluded PI, and to compare those of spermatozoa showing acrosome abnormalities via aniline blue staining with those showing PNA-FITC green fluorescence. Pearson correlation coefficients (r) were calculated in each case.

Monthly and seasonal changes in sperm variable values were analyzed by repeated measures ANOVA, following the statistical model \( x_{ijk} = m + A_i + B_j + AB_{ij} + e_{ijk} \), where \( x_{ijk} \) = the measured variable; \( m \) = the overall mean of variable X; \( A_i \) = the effect of month or season (i = 1 to 12 for months; i = 1 to 4 for seasons); \( B_j \) = the effect of each individual animal (j = 11); \( AB_{ij} \) = the interaction between A and B; and \( e_{ijk} \) = the residual (k = 1 to 2 for months, 1 to 6 for season). The different seasons were defined as follows: summer (July to September), autumn (October to December), winter (January to March), and spring (April to June). When a monthly or seasonal effect was significant, mean differences between seasons were compared using Duncan’s multiple range test. The Spearman rank correlation test (rs) was used to assess the significance of correlations between sperm variable values and temperature fluctuations over the year. The results are presented as means ± SEM. Significance was set at \( P < 0.05 \). All calculations were performed using Statistica software for Windows v.5.0. (StatSoft Inc., Tulsa, OK).

**RESULTS**

**Correlation Between the Hypo-Osmotic Swelling Test-Aniline Blue Staining and the Corresponding Fluorescence Staining Techniques**

Heat treatment (incubation at 37°C) did not affect the percentage of spermatozoa showing a coiled tail: 82.56 ± 1.54 at 37°C compared with 77.42 ± 2.75 at laboratory temperature (23°C). Maximum swelling occurred when the spermatozoa were incubated for 30 min (76.33 ± 2.84 for incubation at 37°C for 30 min
compared with 65.72 ± 3.86 at 15 min; \( P < 0.01 \); and 80.00 ± 2.52 vs. 71.06 ± 2.58 for incubation at laboratory temperature for 30 and 15 min, respectively; \( P = 0.06 \). Further, all later experiments were performed at 37°C because this temperature is easier to control than room temperature.

The morphological changes occurring when spermatozoa were subjected to the hypo-osmotic solution were clearly visible by phase-contrast microscopy. Reacting spermatozoa showed a swollen area at the tip of the tail, a hairpin curvature, and swelling of the tail and mid-piece, or, most commonly, a shortened and thickened tail (Figure 1). The mean percentage of viable spermatozoa according to PI staining was 7.9 ± 2.7% lower (\( P < 0.01 \)) than that detected by the hypo-osmotic test. However, these variables showed a strong correlation (\( r = 0.86; P < 0.001 \)).

The percentage of spermatozoa showing no fluorescence in the PNA-FITC test (i.e., with an intact acrosome) correlated well with the percentage of spermatozoa showing normal acrosome morphology as shown by aniline blue staining (\( r = 0.94; P < 0.001 \)). The mean percentage of spermatozoa with an intact acrosome according to fluorescence staining was 12.7 ± 3.2% higher (\( P < 0.01 \)) than that obtained with the aniline blue test (Figure 2).

**Figure 1.** Morphological changes in spermatozoa with positive endosmosis: swelling of the mid-piece (a), swollen area at the tip of the tail (b), swelling of the tail (c), and shortened and thickened tail (d).
Seasonal Changes in Sperm Variable Values

Figures 3, 4, 5, and 6 show the annual variation of monthly mean temperatures and the seasonal changes in sperm variable values throughout the year. The lowest temperatures were recorded in December (mean = 4.2°C; minimum recorded = −1.5°C) and the highest in July (mean = 24.0°C; maximum recorded = 32.8°C).

Both semen volume (P < 0.05) and sperm concentration (P < 0.01) were affected by season (Table 1), with maximum values occurring over the period of maximum day length (winter and spring; Figure 3). However, wide individual variability was seen: volume = 0.05 to 1.0 mL; concentration = 20 × 10⁶ to 3,279 × 10⁶ spermatozoa/mL.

A decrease in the number of motile spermatozoa (P < 0.05) was clearly recorded in December (50.0 ± 5.3% motile spermatozoa), coinciding with the shortest day lengths and lower temperatures (Figure 4). The percentage of spermatozoa with an intact acrosome, as determined by aniline blue staining, was affected (P < 0.01) by the season (Table 1). The annual profile for this variable followed a trend roughly parallel to annual variations in temperature (r = 0.77, P < 0.01; Figure 5). However, the percentage of entire spermatozoa showing aniline blue-positive staining experienced no significant seasonal variation over the experimental period (Table 1).

The sperm abnormalities (Figure 5) seen were coiled tails, bent tails, abnormal heads, mid-piece defects (double mid-pieces, bent mid-pieces, swollen mid-pieces, filiform mid-pieces), and abnormal heads (coiled heads, vacuolated heads, macro- and microheads). The most commonly detected abnormalities were abnormal heads, bent tails, and coiled tails. No abnormality was affected by season (Table 2). Membrane integrity fell significantly in July (the hottest month) compared with all other months (P < 0.05; Figure 6).
DISCUSSION

The results show that aniline blue staining and the hypo-osmotic swelling test are useful in the analysis of fowl semen samples. Sperm swelling under hypo-osmotic conditions implies normal membrane integrity and function because an equilibrium between the fluid compartment within the cell and the external surroundings is established. Susceptibility to hypotonic stress in fowl semen has been evaluated with 50-mOsm NaCl solutions (Blanco et al., 2000) and at osmolarities of <260 mOsm prepared with Beltsville Poultry Semen Extender diluent (plus 12% Ficoll to increase refringence; Blesbois et al., 2008). In the present work, a 100 mOsm/kg sodium citrate solution was used, and the sperm was then fixed in a buffered 2% glutaraldehyde solution at 37°C (Jeyendran et al., 1984). Good sperm visualization was achieved under the phase-contrast microscope, and good correlation was obtained between the number of spermatozoa showing endosmosis and sperm viability as determined by fluorescent PI staining (although the percentage returned in the first test was always higher than that returned by the second). However, this correlation must be interpreted with care because a live spermatozoon may have a nonfunctional membrane and be incapable of adjusting to hypo-osmotic stress yet still be capable of preventing PI from entering.

Avian sperm morphology and viability have been traditionally examined by hematoxylin (Bilgili et al., 1985) or nigrosin-eosin staining (Ernst and Ogasawara, 1970; Clarke et al., 1984; Bilgili et al., 1985). The present study is the first to describe aniline blue staining of fowl sperm. The percentage of spermatozoa showing acrosome integrity according to PNA-FITC staining was always higher than that obtained with aniline blue staining. This may be explained by the fact that some morphologically abnormal acrosomes (as determined by aniline blue staining) may preserve their acrosomal membrane integrity and therefore will not be stained by the fluorochrome. Because the greatest differences between the results obtained with these techniques were recorded in frozen-thawed samples, some interaction between the cryoprotectant (dimethyl acetamide) and the stain (aniline blue) may prevent the correct visualization of some normal acrosomes.

Although the present findings show that aniline blue staining can be successfully used for morphological estimations and to determine acrosome integrity in fowl semen, its value in the discrimination of avian spermatozoa with damaged DNA remains unclear. Much more is known about spermatogenesis in mammals than in birds, and observations made in mammals can only be used as speculative support for what might occur in avian species (Thurston and Korn, 2000). Mammalian sperm DNA is the most tightly packed eukaryotic DNA (Hammadeh et al., 1999). The main sperm maturation event that affects the structure of the chromatin depends on the proper replacement of histones by protamines, the molecules that induce the compact packaging of the DNA via the cross-linking of disulfide bonds (Erenpreiss et al., 2001). Acidic aniline blue staining can be used as a marker of sperm chromatin defects because the chromatin proteins in sperm nuclei with impaired DNA appear to be more accessible to the dye (Auger et al., 1990). Because spermatozoa thus stained indicate the persistence of histones (Dadoune et al., 1988), the proportion of well-condensed sperm heads can be evaluated in smears by the percentage of heads not stained by the dye (Terquem and Dadoune, 1983). Unlike in human sperm (Henkel et al., 2001), seasonal changes were not seen in fowl sperm staining intensity. This may be explained by interspecific differences in the seasonal retention of histones and chromatin packaging (Erenpreiss et al., 2006). The strong selection of spermatozoa that occurs in the avian oviduct (Brillard and Bakst, 1990; Brillard, 1993) may be necessary because of the number carrying impaired DNA.

Like other free-range chickens (Blesbois et al., 2007), the Black Castellana breed retains pronounced seasonality, a trait lost in breeds reared in indoor systems. The present findings show substantial differences in semen production over the year, with maximum volumes coinciding with the longest photoperiod and moderate temperatures, and the lowest volumes coinciding with shorter photoperiods. This pattern is similar to that reported in previous studies in White Leghorn, New Hampshire, and Iraqi breeds maintained at 34°N latitude (Saeid and Al-Soudi, 1975), and in New Hampshire cocks and Rhode Island Red cocks maintained at
35°N latitude (Parker et al., 1942; Parker and McSpadden, 1943). However, the present results disagree with early studies performed at higher latitudes (55°N) with White Leghorns, which showed peak semen volume during November and December, decreasing to a minimum in August (Polge, 1950). Similar observations have been described in Barred Plymouth Rock males maintained at 35°N, in which semen volume increased in November and remained at high levels until March (Wheeler and Andrews, 1943). In Fayoumi cocks, maximum semen volumes were also recorded during times of decreasing day length (October), whereas minimum volumes and concentrations occurred during times of increasing day length (May; Kamar and Badreldin, 1959).

Minimum sperm viability, according to the hypo-osmotic test, was observed in July, the month of highest temperatures. The same has been reported for other breeds (Saied and Al-Soudi, 1975). These results appear to reflect the effect of heat stress and its disturbance of testicular function (Datta et al., 1980). The reduction in the number of motile spermatozoa in December coincided with the shortest photoperiod and lowest temperatures. Because the shortening photoperiod from June until November was not correlated with reduced sperm motility, and given that the lowest temperature was recorded in December (−1.5°C), it would appear that low temperatures have a negative influence on this sperm variable.

The present results provide no evidence of the seasonal prevalence of certain sperm abnormalities, unlike that reported for the turkey (Wakely and Kosin, 1951). Bent and coiled tails are one of the most commonly detected abnormalities in both turkey and chickens, conditions previously thought primarily due to osmotic damage (Yamane, 1972; Bakst, 1980) and long periods of semen storage or incubation (Clarke et al., 1984). However, in the present work, sperm analysis was always performed 45 min after collection, and because the semen was diluted in an isotonic semen extender

Figure 5. Annual changes in sperm variables analyzed by aniline blue staining (% morphological abnormalities, % intact acrosomes, % aniline blue-positive sperm) (mean ± SEM).

Figure 6. Annual changes in sperm membrane integrity (% positive endosmosis) (mean ± SEM).

### Table 1. Sperm characteristics in fresh semen collected from 11 mature Black Castellana roosters over a period of 1 yr

<table>
<thead>
<tr>
<th>Item</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
<th>Spring</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (mL)</td>
<td>0.19a</td>
<td>0.12b</td>
<td>0.20a</td>
<td>0.20c</td>
<td>0.02</td>
</tr>
<tr>
<td>Concentration (10⁶ spermatozoa/mL)</td>
<td>899.44a</td>
<td>713.68b</td>
<td>1,763.83a</td>
<td>1,664.45a</td>
<td>196.91</td>
</tr>
<tr>
<td>Motile sperm (%)</td>
<td>72.16</td>
<td>67.67</td>
<td>72.72</td>
<td>77.61</td>
<td>2.90</td>
</tr>
<tr>
<td>Quality of motility (0 to 5)</td>
<td>3.82</td>
<td>3.75</td>
<td>3.84</td>
<td>3.99</td>
<td>0.14</td>
</tr>
<tr>
<td>Morphological abnormalities (%)</td>
<td>14.32</td>
<td>11.60</td>
<td>10.97</td>
<td>16.88</td>
<td>1.87</td>
</tr>
<tr>
<td>Intact acrosomes (%)</td>
<td>97.67a</td>
<td>95.55b</td>
<td>91.02b</td>
<td>97.38c</td>
<td>1.09</td>
</tr>
<tr>
<td>Aniline blue-positive sperm (%)</td>
<td>67.56</td>
<td>60.06</td>
<td>56.95</td>
<td>48.35</td>
<td>5.71</td>
</tr>
<tr>
<td>Positive endosmosis (%)</td>
<td>68.82</td>
<td>71.39</td>
<td>81.67</td>
<td>81.23</td>
<td>2.91</td>
</tr>
</tbody>
</table>

a, b Different superscripts within rows indicate significant differences (P < 0.05).

1Summer = July to September; autumn = October to December, winter = January to March; spring = April to June.
(343 mOsm/kg), the sperm abnormalities detected cannot be attributed to adverse osmotic conditions or storage (Clarke et al., 1984); they must therefore have been primary abnormalities.

The percentage of spermatozoa with an intact acrosome was affected by the season. Although this percentage was usually close to 99%, a decrease in December to values of <90% was seen, which must reduce the likelihood of successful reproduction. Indeed, only about 1% of the initial sperm population deposited intravaginally reaches the sperm storage tubules (Brillard and Bakst, 1990; Brillard, 1993). The integrity of the acrosome surface may be involved in this intensive selection of sperm in the vagina of the hen.

In summary, the present results show that, at temperate latitude, long days and moderate temperatures favor semen production in the Black Castellana chicken breed. Extreme heat or cold appear to exert a negative influence on sperm quality. Because seasonal changes in the values of sperm variables are influenced by breed and geographic origin (Saeid and Al-Soudi, 1975), acquiring a sound knowledge of the seasonal reproductive activity of each breed of chickens is a prerequisite for the creation of an efficient avian genetic resource bank and the successful use of reproductive technologies. Aniline blue staining and the hypo-osmotic swelling test may be useful in obtaining such knowledge and help estimate semen quality before freezing.

REFERENCES


Table 2. Percentages of different types of sperm abnormality.1,2

<table>
<thead>
<tr>
<th>Item</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
<th>Spring</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bent tails (%)</td>
<td>14.87</td>
<td>19.03</td>
<td>30.97</td>
<td>31.00</td>
<td>2.96</td>
</tr>
<tr>
<td>Coiled tails (%)</td>
<td>35.10</td>
<td>23.87</td>
<td>14.40</td>
<td>21.03</td>
<td>2.58</td>
</tr>
<tr>
<td>Loose normal heads (%)</td>
<td>6.67</td>
<td>19.63</td>
<td>11.30</td>
<td>4.47</td>
<td>2.79</td>
</tr>
<tr>
<td>Abnormal heads (%)</td>
<td>38.07</td>
<td>31.93</td>
<td>31.47</td>
<td>33.43</td>
<td>3.98</td>
</tr>
<tr>
<td>Mid-piece defects (%)</td>
<td>5.30</td>
<td>5.53</td>
<td>11.87</td>
<td>10.07</td>
<td>1.83</td>
</tr>
</tbody>
</table>

1No significant influence (P > 0.05) of season was seen in terms of the percentages of the different types of sperm abnormalities encountered.
2Summer = July to September; autumn = October to December; winter = January to March; spring = April to June.


