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Sperm variables as predictors of fertility in Black Castellana roosters; use in the selection of sperm donors for genome resource banking purposes

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Abstract

Semen was collected from 10 Black Castellana roosters and the classic sperm variables (ejaculate volume, sperm concentration and sperm motility) examined. In addition, the hypo-osmotic swelling test was used to investigate sperm cell membrane integrity, and acidic aniline blue staining used to screen for morphological abnormalities (including acrosome integrity) and to examine the condensation status of the chromatin. The latter was also examined by Gram staining. Large and small semen volumes were associated high and low sperm concentrations respectively (R^2=0.04, P<0.05). The percentage of motile spermatozoa correlated strongly with the percentage of sperm cells showing an intact acrosome (R^2=0.13, P<0.001) and with the percentage of morphologically normal spermatozoa (R^2=0.04, P<0.05). The percentage of Gram positive spermatozoa was positively correlated with semen appearance (R^2=0.12, P<0.05), sperm cell concentration (R^2=0.13, P<0.05), and with the sperm motility variables studied (R^2=0.14, P<0.05 for percentage mobility, and R^2=0.12, P<0.05 for quality of movement). Only three of the 10 roosters, all with fertilisation potentials of 80-90%, were considered potential sperm donors for genome resource banking purposes. The remaining birds were all of low fertility (≤ 50%); in fact, some produced semen volumes too small to perform fertility tests. Semen volume and membrane integrity were found to be the best variables for predicting the fertilisation potential of rooster ejaculates.

Additional key words: aniline blue, artificial insemination, fertility, Gram staining, semen quality

Resumen

Variables espermáticas predictoras de fertilidad en gallos de raza Negra Castellana; uso en la selección de donantes en bancos de recursos genéticos

Se utilizó semen de 10 gallos de raza Negra Castellana, en el que se realizó un espermiograma clásico (volumen eyaculado, concentración espermática, motilidad). Además, se usó el test de endósmosis para evaluar la integridad de membrana plasmática, y la tinción de azul de anilina para analizar morfoanomalías (incluyendo la integridad del acrosoma) y el estatus de condensación de la cromatina, el cual también se evaluó mediante la tinción de Gram. Los resultados muestran que el volumen seminal estaba correlacionado con la concentración espermática (R^2=0,04; P<0,05). El porcentaje de espermatozoides mótiles mostró una correlación con el porcentaje de espermatozoides con acrosomas intactos (R^2=0,13; P<0,001) y con el porcentaje de espermatozoides con morfología normal (R^2=0,04; P<0,05). El porcentaje de células espermáticas Gram positivas estuvo correlacionado con el aspecto seminal (R^2=0,12; P<0,05), la concentración espermática (R^2=0,13; P<0.05) y variables de motilidad (R^2=0,14; P<0,05 y R^2=0,12; P<0,05, para el porcentaje de células mótiles y calidad del movimiento, respectivamente). Sólo tres de los 10 gallos estudiados mostraron altas tasas de fertilidad del 80-90%, y fueron considerados como los únicos donantes potenciales de semen en bancos de recursos genéticos. El resto de los gallos tuvieron baja fertilidad (≤50%) o dieron volúmenes de semen demasiado bajos como para poder realizar el correspondiente test de fertilidad. El volumen seminal y la integridad de membrana fueron las variables espermáticas que mejor predecían la capacidad fertilizante de los eyaculados.

Palabras clave adicionales: azul de anilina, calidad seminal, fertilidad, inseminación artificial, tinción de Gram.

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Introduction

The *ex situ* conservation of autochthonous chicken breeds via genome resource banking (Blesbois *et al*., 2007) could help to guarantee their preservation, and should be seen as a priority complementary activity to classic *in situ* conservation programs (Campo and Orozco, 1982; Campo, 1998). The conservation of bird oocytes and embryos is not possible owing to the characteristics of the megalecithal egg. Further, the cryopreservation of avian embryonic cells is costly and the results achieved with them often leave much to be desired (Petite, 2006). Spermatozoa are therefore the primary cell types preserved in most emerging genome resource banking projects (Blesbois *et al.*, 2007). Since the fertilisation capacity of cryopreserved poultry sperm is dramatically lower than that of any domestic mammalian species (Long, 2006), fresh semen of high quality is required; the succession of thermal, osmotic and mechanical stresses suffered during cryopreservation (Blesbois and Brillard, 2007) renders low quality sperm ineffective. Since sperm quality variables and the capacity to survive cryopreservation show strong within-breed variability (Nishiyama, 1961; Blesbois *et al*., 2007), *in vitro* semen evaluation for the selection of the best semen donors is necessary; only the most fertile roosters should be used as donors in genome resource banking projects. Spermiograms routinely include the assessment of semen volume, semen appearance (colour), sperm concentration and sperm motility (Brown and Graham, 1971; Blesbois *et al*., 2002). However, for genome banking purposes, the fertilisation potential of fresh semen samples should also be taken into account. The examination of quantitative and qualitative variables such as the semen volume, sperm cell concentration, sperm motility, sperm viability, morphological abnormalities and acrosome integrity could provide a valuable way of predicting fertilisation potential (Pursel and Johnson, 1974; Chalah *et al.*, 1999).

The plasma membrane status of sperm cells is of the utmost importance given its role in cell-cell interactions, *e.g.,* between spermatozoa and the epithelium of the female genital tract, the cells of the sperm storage tubules (Briskie, 1996), and oocytes (Robertson *et al*., 1998). The swelling of the sperm tail in the presence of a hypo-osmotic solution is a sign that the transport of water across the membrane is occurring normally; it is therefore indicative of good membrane integrity and normal functioning (Jeyendran *et al*., 1984). This test therefore offers a means of assessing membrane integrity in fowl sperm.

It is also reported that the chromatin integrity of spermatozoa is related to its fertilisation potential (Erenpreiss *et al*., 2006; Aitken and De Luliis, 2007). Gram staining (Radicioni *et al*., 1996; Mantas *et al*., 2006) and acidic aniline blue staining (Terquem and Dadoune, 1983; Dadoune *et al.*, 1988; Auger *et al*., 1990) have been used to evaluate chromatin condensation status in human spermatozoa. The latter stain indicates the persistence of histones (Dadoune *et al*., 1988); it can therefore be used to determine which cells have well-condensed chromatin (Terquem and Dadoune, 1983).

The Black Castellana breed is one of the oldest free-range chicken breeds in Spain, and was once commonly kept on farms for egg production (Orozco, 1989). This breed has been the subject of a genetic resources conservation program since 1975 (Campo and Orozco, 1982; Campo, 1998), and has recently been included in a genome banking program. In the present work, acidic aniline blue staining, Gram staining, and the hypo-osmotic test were used to complement the use of classic spermiograms for selecting potential semen donors among Black Castellana roosters for genome banking purposes. The aims of this work were: i) to determine the relationships among the studied sperm variables, and ii) to examine the relationships between these variables and fertilisation potential.

Material and methods

Birds

Ten Black Castellana roosters, all one year old at the beginning of the experiment, were randomly selected for study at the El Encín research station (Alcalá de Henares, Spain; 40º 31'N). Animals were housed in an 8 m^2 sand-floor pen with a partial roof cover, and under natural photoperiod and temperature conditions. All birds were fed commercial maize/barley-based feed with a 2.8% fat and a 15% crude protein content.

Sperm collection and assessment

Semen was collected in 15 mL centrifuge tubes (Falcon™) using the massage technique of Burrows and Quinn (1937). This requires the "milking" of birds by gripping the base of their copulatory organ after its

being made to protrude by mild stimulation. Semen was immediately diluted 1:1 (v:v) at ambient temperature using a medium containing sodium glutamate (1.92 g), glucose (0.8 g), magnesium acetate ($4H_2O$; 0.08 g), potassium acetate (0.5 g), polyvinylpyrrolidone (M_r 10,000; 0.3 g) and 100 mL H_2O (pH 7.08, osmolality 343 mOsm kg^{-1}). Diluted semen was immediately refrigerated at 5ºC and transported to the laboratory. All semen was examined within 45 min of collection, recording the ejaculate volume and semen appearance (colour), and sperm concentration, motility, morphological abnormalities and acrosome and membrane integrities.

The volume of the ejaculate was recorded in the graduated tube in which it was collected. The semen appearance was given a numerical value: 0 (transparent), 1 (grey-yellow), 2 (white), 3 (cream-white).

Sperm concentrations were calculated by determining the absorbance (at 540 nm) of light by the semen using a spectrophotometer (Spectronic 20, Bausch and Lomb Co., USA). Sperm motility was assessed by placing a small droplet of each sample, previously diluted to 1:20 (v:v) in the medium described above, on a warmed (37ºC) glass slide. The percentage of motile spermatozoa and the quality of motility were evaluated subjectively using a phase-contrast microscope (Zeiss, Germany) at 1000x. The quality of motility was recorded on a scale from 0 (lowest) to 5 (highest).

Morphological abnormalities and acrosome integrity were assessed by acidic aniline blue staining. A drop of 10 µL of each diluted semen sample was spread on a glass slide and allowed to dry. These smears were then fixed at room temperature in buffered 2% glutaraldehyde in PBS for 30 min and air-dried. The slides were then stained with 5% aqueous aniline blue mixed with 2% acetic acid (pH=3.5) for 5 min, washed with distilled water, and air-dried once more. Briefly, the staining solution was prepared by adding 5 g of aniline blue (Water Blue, Fluka, USA) to 100 mL PBS, filtering, and adjusting to a pH of 3.5 with a solution of 2% glacial acetic acid (Merck, Germany). Sperm morphological abnormalities were assessed by counting 200 cells under oil immersion at a magnification of 1000x using bright field illumination. The different types of sperm abnormality were recorded using the method of Wakely and Kosin (1951). The percentage of sperm cells with an intact acrosome was determined by examining 200 spermatozoa under a phase-contrast microscope at 1000x. Spermatozoa classified as not showing acrosome integrity were those with a hooked, swollen, thinned or no acrosome (Wakely and Kosin, 1951).

Acidic aniline blue staining was also used to examine the chromatin condensation status of the collected sperm cells. Two hundred cells were examined and the sperm heads classified (Henkel *et al.*, 2001) as either being strongly/very strongly stained (aniline blue-positive), or weakly/not stained (aniline blue-negative) (Fig. 1).

Plasma membrane integrity was assessed by the hypo-osmotic swelling test (Jeyendran *et al.*, 1984). This consisted of mixing 25 µL of diluted semen with 500 µL of a hypo-osmotic solution (100 mOsm kg^{-1}) prepared by adding 1 g of sodium citrate to 100 mL of distilled water. Semen aliquots were incubated at 37ºC for 30 min in this solution. The percentage of spermatozoa showing a coiling mid piece or flagellum was assessed by examining 200 spermatozoa in samples fixed in buffered 2% glutaraldehyde solution at 37ºC under a phase-contrast microscope (magnification 1000x).

For Gram staining, one drop of semen was smeared onto a microscope slide at room temperature, air dried and flamed. The classic Gram staining technique was followed, using the Gram-Hucker DC kit (Panreac Quimica SA, Spain). Briefly, after flame fixation, crystal violet was employed as a primary stain, followed by Lugol's solution (mordant). Acetone alcohol was used

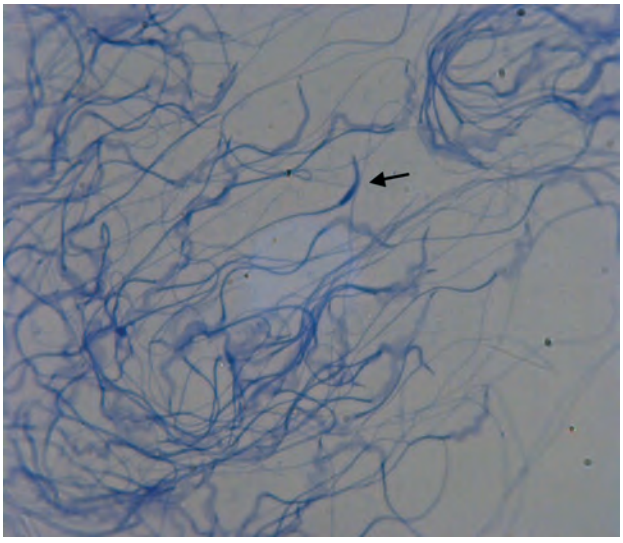

Figure 1. Rooster spermatozoa stained with acidic aniline blue to reveal sperm cell morphology, acrosome integrity, and chromatin condensation status. Two classes of staining intensities were distinguished: strong and very strong staining (aniline blue-positive; spermatozoa marked with the narrow), and no/weak staining (aniline blue-negative; the remaining spermatozoa). Magnification = 1000x.

as a contrast agent, and finally safranin solution as a counter-stain. The preparations were examined at 1000x using a Motic Images Advanced 3.0 image analyser (Micro-Optic Industrial Co., Spain). Two hundred cells were examined for the calculation of the percentage of stained cells.

Artificial insemination

Artificial insemination was performed according to the method described by Burrows and Quinn (1939). Briefly, semen was collected and diluted at 1:1 (v:v) in the medium previously described and maintained at 5ºC until use (always within 45 min of collection). The vaginal orifice was made to protrude by applying pressure to the abdomen (Yamane *et al*., 1966) and a 1 mL tuberculin syringe containing 0.1 mL of diluted semen inserted as far as possible without difficulty (usually about 2-3 cm). Constant, light pressure was maintained on the syringe while the pressure on the hen's abdomen was released, allowing the vaginal orifice to return to its normal position. All inseminations were performed between 12:00 and 14:00 h.

Experimental design

Semen was collected from the 10 randomly selected roosters once every two weeks for 6 months (January - June 2008, *i.e.,* during a time of increasing photoperiod, which is favourable to sperm production in roosters); thus, 14 ejaculates per male were studied. The ejaculate volume and its appearance, the concentration, motility, membrane integrity and acrosome integrity of the sperm cells, and the morphological abnormalities they showed were examined on each occasion. The chromatin condensation status was analysed by acidic aniline blue and Gram staining.

In June 2008, two rounds of artificial insemination were undertaken, three days apart, but only using semen from roosters providing an ejaculate volume of at least 0.2 mL. The number of hens inseminated by each rooster (1-16) depended on the semen volume collected. All hens were isolated from males for at least during 21 days before artificial insemination. All eggs laid on post-insemination days 4-6 (inclusive) were collected, opened and classified as either fertilized or non-fertilized by macroscopic examination of the germinal region of the yolk.

Statistical analysis

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

Pearson correlation coefficients were calculated to examine the correlations among the sperm variables and between these and the fertilisation potentials obtained.

Principal component analysis (PCA) was performed on 10 sperm variables: semen appearance, ejaculate volume, sperm concentration, percentage motility, quality of motility and percentage membrane integrity, the percentage of spermatozoa with normal acrosomes, the percentage with normal morphology, the percentage of spermatozoa not stained with aniline blue, and the percentage of Gram positive spermatozoa. The Pearson correlation coefficient between fertilisation potential and the PCA-generated axis was then determined.

The differences in sperm variables between roosters showing high and low fertilisation potentials (values ≥75% were considered to reflect high fertility, and <75% to reflect low fertility) were analysed by ANOVA following the model $x_{ijk}=m + A_i + B_j + AB_{ij} + e_{ijk}$, where x_{ijk}=the measured variable, m=the overall mean value of the variable X, A_i=the effect of fertility level (i=1-2), B_j=the effect of each individual animal (j=5), AB_{ij}=the interaction between A and B, and e_{ijk}=the residual (k=1-14). Differences in the mean values for sperm variables of high and low fertility roosters were analysed using the F-test.

Significance was set at $P<0.05$. All calculations were performed using StatSoft Inc.'s Statistica software for Windows v.5.0.

Results

Semen appearance correlated strongly with sperm concentration ($R^2=0.32$, $P<0.001$); maximum concentrations were seen when the semen was milk-white in colour. Large and small semen volumes correlated with high and low semen concentrations respectively ($R^2=0.04$, $P<0.05$). The percentage of motile spermatozoa and the quality of movement were also correlated ($R^2=0.31$, $P<0.001$). Further, the percentage of motile spermatozoa correlated strongly with the percentage of spermatozoa with an intact acrosome ($R^2=0.13$, $P<0.001$), and with the percentage of morphologically normal spermatozoa ($R^2=0.04$, $P<0.05$). The quality of movement correlated with the sperm concentration

(R^2=0.27, P<0.01). The percentage of aniline blue-stained sperm heads was not correlated with any of the studied semen variables. In contrast, the Gram staining behaviour of the spermatozoa (Fig. 2) correlated with both quantitative and qualitative sperm variables, for example, with semen appearance (R^2=0.12, P<0.05), sperm concentration (R^2=0.13, P<0.05), the percentage of motile spermatozoa (R^2=0.14, P<0.05), and the quality of sperm cell movement (R^2=0.12, P<0.05). The spermatozoa that responded to the hypo-osmotic swelling test conditions showed a swollen area at the tip of the tail, a hairpin curvature and swelling of the tail and mid-piece, or a shortened and thickened tail. However, the percentage of responding spermatozoa was not correlated with any of the studied semen variables.

Principle components analysis produced an axis that explained 43.5% of the variance (corresponding Eigenvalue 3.48). Semen appearance, semen volume, sperm concentration and acrosome integrity showed high loadings (0.70-0.91) with respect to this axis.

Five males were excluded from the statistical analysis of fertility since they produced either no semen or just 0.10 mL of semen on the days when inseminations were performed. Throughout the experimental period, four of these five males produced maximum semen volumes of 0.10-0.20 mL; those of the remaining rooster ranged from 0-0.05 mL. A total of 45 hens were inseminated with the semen obtained from the remaining five males.

Macroscopic examinations of the germinal region of the eggs allowed the fertility of each male to be determined. Three roosters were thus considered highly fertile (fertilisation potential 81-100%) and two as of low fertility (fertilisation potential 33-50%) (Table 1).

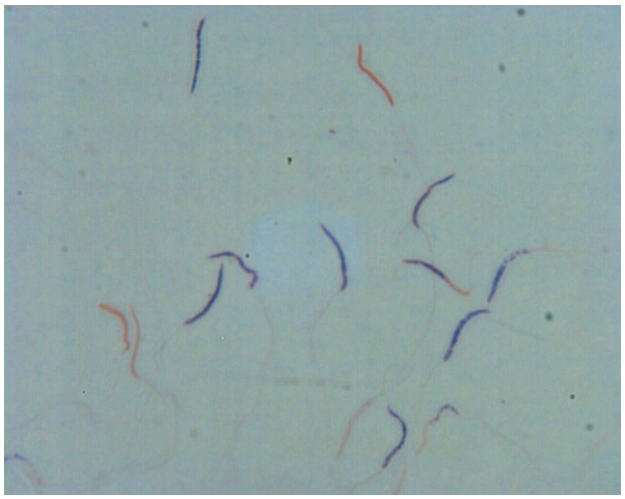

Figure 2. Gram staining of sperm cells to reveal chromatin condensation status. Gram positive spermatozoa were stained blue; Gram negative spermatozoa stained red. Magnification = 1000x.

Pearson correlation analysis revealed no correlations between the sperm variables measured - nor the axis generated by PCA - with fertilisation potential. However, ANOVA showed that the males of higher fertility had larger semen volumes than the low fertility roosters (mean ± SEM; 0.40 ± 0.03 *vs* 0.21 ± 0.02 mL, P<0.001). Further, the highly fertile roosters showed more consistent percentages of viable spermatozoa; positive endosmosis in their semen samples was always greater than 70% (Table 1). In addition, the variability in membrane integrity over the experimental periods was significantly greater in the low fertility birds (P<0.05, *F*=2.37). A low fertility male showed the lowest percentage of normal spermatozoa seen during the sampling period (rooster 831: 53%), while another showed the lowest sperm concentration (rooster 500: $700 \cdot 10^6$ spermatozoa mL^{-1}).

Discussion

Only three of the initial 10 birds (30%) were considered suitable semen donors for genome banking purposes. Five males provided insufficient semen to inseminate many hens, while two males that did provide sufficient semen volume were of low fertility (fertilisation potential ≤50%). ANOVA showed the semen volume and the percentage of spermatozoa showing membrane integrity (according to the hypo-osmotic swelling test) were the most suitable criteria for donor selection.

Following a single intravaginal insemination, a maximum of approximately 1% of the initial population of deposited spermatozoa reaches the sperm storage tubules in chickens (Brillard, 2003). Most spermatozoa deposited within 1-3 hours prior to or just after oviposition are eliminated by the vaginal contractions involved in the latter process (Brillard *et al.*, 1987; Brillard and Bakst, 1990). Hence, it is necessary to select the best semen donor birds if their sperm is to have the best chances of fertilizing any eggs.

The pre-requisite for possible donor selection in this study was semen volume: a rooster should be able to provide an ejaculate of at least a minimum required size. Blesbois *et al*. (2007) first selected only males that provided ejaculates with a minimum volume of 250 µL and a minimum concentration of $3 \cdot 10^9$ spermatozoa mL^{-1}. Among these they then selected those whose sperm

Table 1. Mean and ranges of sperm characteristics in fresh semen collected over six months (January - June 2008) from the five Black Castellana roosters with the minimum volume ejaculates required

Id	Vol[1]	Ap[2]	Concent[3]	Mot[4]	Score[5]	Nar[6]	Host[6]	SN[7]	Gram+[8]	AB-[9]	Fert[10]
523	0.31	2.46	2167.00	72.27	3.95	97.70	78.09	88.82	88.25	49.18	100*
	(0.1-1)	(2-3)	(1020-4640)	(35-90)	(3.5-4.5)	(94-100)	(70-94)	(77-98)	(86-96)	(4-100)	(8/8)
605	0.62	2.46	2155.20	71.5	4.3	94.5	82.2	89.2	89.75	48	81.25*
	(0.4-1)	(1-3)	(1600-3160)	(10-90)	(3.0-5.0)	(74-100)	(70-94)	(78-96)	(85-100)	(1-95)	(13/16)
567	0.28	2.00	2170.82	79.55	4.05	96.73	80.27	86.82	89.60	60.64	85.71*
	(0.1-0.4)	(1-3)	(800-3720)	(50-90)	(3-5)	(93-100)	(71-95)	(75-99)	(76-99)	(8-99)	(6/7)
831	0.16	2.50	2766.36	74.09	4.09	94.09	70	82.73	87.80	44.09	50
	(0.05-0.4)	(1-3)	(1320-4260)	(55-85)	(2-5)	(82-100)	(48-89)	(53-99)	(77-93)	(8-100)	(4/8)
500	0.15	1.23	1026.91	85.45	4.41	96.55	85.09	89.45	86.20	32.09	33.33
	(0.05-0.5)	(0-3)	(700-2297)	(65-95)	(4-5)	(91-100)	(58-94)	(80-95)	(75-94)	(6-57)	(2/6)

[1] Semen volume (mL); [2] semen appearance (score, 0-3); [3] sperm concentration (x10^6 spermatozoa mL^{-1}); [4] motile spermatozoa (%); [5] quality of sperm movement (score, 0-4); [6] spermatozoa with normal acrosome (%); [6] spermatozoa with plasma membrane integrity according hypo-osmotic swelling test (%); [7] spermatozoa with normal morphology (%); [8] spermatozoa with Gram positive staining (%); [9] spermatozoa regarded as aniline blue-negative (%); [10] fertilisation potential (% eggs showing germinal disc in the yolk respect to total eggs). Asterisks indicate highly fertile roosters.

showed the best combination of motility and viability. Selection was undertaken by measuring the mean values for three ejaculates per male. In the present work a total of 14 ejaculates per male were studied. This allowed the identification of roosters providing non-homogeneous data for the sperm variables analysed.

The low fertility roosters showed variable rates of membrane integrity over the experimental period, ranging from 48 to 94%. This might be explained in two ways. First, the environment (wind, rain, temperature, etc.) may affect spermatogenesis differently in different birds since all were maintained in a sand-floor pen with a partial roof cover and under natural photoperiod and temperature conditions (Kamar and Badreldin, 1959; Saeid and Al-Soudi, 1975). However, social dominance might also explain this variation (Cornwallis and Birkhead, 2007); a bird's social status can change quickly, perhaps affecting certain sperm variables (Pizzari *et al*., 2007). Acquiring a more subordinate status may be accompanied with social stress leading to a drop in spermatogenesis.

Motility is usually used as a criterion for donor selection in roosters (Blesbois *et al*., 2007). Certainly, progressive motility and fertility are correlated in turkeys (Brown and Graham, 1971), although early studies reported no such correlation (McCartney, 1956). In the present study, the overall percentage of motile spermatozoa and the quality of movement remained high in all birds, and therefore did not appear as a valid criterion for male selection.

Gram staining appears to provide a measure of sperm quality in humans (Radicioni *et al*., 1996; Mantas *et al.*, 2006), but to our knowledge this is the first report of its use in any bird species. Unfortunately, the interpretation of the reaction (positive/negative) seen in the sperm of the present roosters is not clear; therefore, Gram staining cannot yet be recommended as a means of determining chromatin condensation status. Fischer (1953) indicated that the fixation method may affect the Gram response. For example, the presence of oxidizing agents or acids (air during, picric acid) can cause a Gram negative response in spermatozoa, but Gram negative behaviour can be changed to Gram positive behaviour in the presence of reducing agents or alkalis (Fischer, 1953). Thus, variations in the pH of the seminal fluid might affect the Gram response. Gram staining in microbiology is used to identify the differences in the cell wall physical properties of Gram positive and Gram negative bacteria (Beveridge, 2001). The porosity of the bacteria walls is responsible for retention of the dye, and thus a relationship between membrane integrity and Gram reaction should be expected. However, the present results revealed no correlation between a Gram positive reaction and the percentage of spermatozoa showing a positive response to hypo-osmotic shock; the same was reported in studies on human spermatozoa (Radicioni *et al*., 1996). Gram staining in fowl sperm is therefore not dependent on the state of the membrane. Chromatin condensation status has been analysed by Gram staining

in humans (Radicioni *et al*., 1996; Mantas *et al.*, 2006), and it was shown that the percentage of Gram positive spermatozoa was related to the percentage of spermatozoa showing chromatin integrity. In this regard, it has been hypothesized that normal nuclear condensation prevents the loss of the crystal violet-Lugol's solution complex (Radicioni *et al.*, 1996). However, the Gram reaction is apparently associated with the presence of sulphidril (SH) groups and disulphide bridges (Fischer, 1953), and it is well known that equilibrium among disulphide bridges, free SH groups and zinc-bound SH groups is important for both sperm chromatin condensation and sperm nuclear decondensation during egg penetration.

A strong positive correlation between aniline blue staining (chromatin condensation status), which has been used to analyse sperm chromatin integrity in human spermatozoa (Dadoune *et al*., 1988), and Gram staining has been reported (Mantas *et al.*, 2006), but the present results suggested no such relationship to exist. Although further research is needed to examine the relationships between the Gram reaction and chromatin condensation status, the fact that the number of Gram positive spermatozoa correlated with sperm concentration and sperm motility suggests that it might be useful in the routine analysis of *in vitro* sperm quality.

The presence in smears of human semen of a distinct class of morphologically normal spermatozoa whose nuclei are partially or totally stained by acidic aniline blue suggest the persistence of lysine rich proteins related to a disturbance of chromatin condensation (Terquem and Dadoune, 1983). The percentage of spermatozoa taking up the aniline blue stain varied within each bird (from 4 to 100%) over the experimental period. Although the present findings show that aniline blue can be used for morphological examinations and the determination of acrosome integrity in fowl semen, they also show it may not be of such great use for determining the chromatin condensation status.

In conclusion, together with the classical sperm variables, the hypo-osmotic swelling test, Gram staining and aniline blue staining appear to be useful tools for selecting potential semen donors for genome resource banking purposes. The suitability of staining methods (either aniline blue or Gram staining) for evaluating chromatin condensation status in fowls is less clear; more studies are needed. In addition to *in vitro* semen analysis, the evaluation of *in vivo* fertilisation potential is necessary if only males of optimum breeding potential are to be selected.

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