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Comparison of semen variables, sperm DNA damage and sperm membrane proteins in two male layer breeder lines

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ABSTRACT

Semen variables are affected by the breed and strain of chicken. The present study was undertaken to compare the semen quality in two lines of adult chickens with particular reference to sperm chromatin condensation, sperm DNA damage and sperm membrane proteins. Semen from a PD3 and White Leghorn control line was collected at 46 and 47 weeks and 55 weeks of age. The semen was evaluated for gross variables and sperm chromatin condensation by aniline blue staining. Sperm DNA damage was assessed by using the comet assay at 47 weeks of age and sperm membrane proteins were assessed at 55 weeks of age. The duration of fertility was studied by inseminating 100 million sperm once into the hens of the same line as well as another line. The eggs were collected after insemination for 15 days and incubated. The eggs were candled on 18th day of incubation for observing embryonic development. The White Leghorn control line had a greater sperm concentration and lesser percentage of morphologically abnormal sperm at the different ages where assessments occurred. There was no difference in sperm chromatin condensation, DNA damage and membrane proteins between the lines. Only low molecular weight protein bands of less than 95 kDa were observed in samples of both lines. The line from which semen was used had no effect on the duration over which fertility was sustained after insemination either when used in the same line or another line. Thus, from the results of the present study it may be concluded that there was a difference in gross semen variables between the lines that were studied, however, the sperm chromatin condensation, DNA damage, membrane proteins and duration over which fertility was sustained after insemination did not differ between the lines.

Keywords: Chicken; DNA damage; Fertility; Semen

1. Introduction

The rooster reproductive potential is known by assessing the semen quality. The semen quality and quantity are affected by the breed and strain of chicken (Omeje and Marire, 1990; Peters et al., 2008; Prieto et al., 2011; Shanmugam et al., 2012). Genetic selection for higher egg production affects semen quality (Shanmugam et al., 2013). The White Leghorn lines selected for higher egg production had poorer semen quality – less semen volume, a lesser sperm concentration and greater seminal plasma lipid peroxidation in comparison with that of the control line. Apoptosis, also referred to as programmed cell death, occurs through a series of

cellular, morphological and biochemical alterations without eliciting an inflammatory response. The comet assay is a simple method to study apoptosis-like DNA fragmentation in the sperm samples, and this method was used to study the effects of chicken sperm cryopreservation (Gliozzi et al., 2011). The comets formed after electrophoresis is usually stained with fluorescent dye and visualized using a fluorescent microscope (Madeddu et al., 2010; Gliozzi et al., 2011). Alternatively, the comets can be silver stained and visualized under a light microscope (Nadin et al., 2001) that can be visually scored or analyzed using computer software (García et al., 2007).

During the course of transport of sperm in the ductus deferens the sperm adsorb proteins from the luminal fluid (Esponda and Bedford, 1985). The sperm surface proteins in chicken sperm were studied and shown to be important in the sperm traversing the vagina to reach sperm storage tubules (Steele and Wishart, 1996).The sperm membrane proteins and role in fertility have been studied in humans (Rajeev and Reddy, 2004) and cattle (Roncoletta et al., 2006). In the literature, no articles reporting sperm membrane proteins in chickens have been found.

There are adverse effects of genetic selection for higher egg production on rooster semen variables such as sperm concentration and sperm viability, however, subcellular changes may occur in sperm as a result of selection for egg production in chickens. The aim of the present study was to assess the rooster semen quality, sperm DNA damage and sperm membrane proteins of chicken lines selected for egg production.

2. Materials and methods

The study was conducted at the experimental poultry farm of the institute located in Hyderabad, India. The roosters of two parent layer breeder lines, the White Leghorn control line and PD3 line (derived from Dahlem Red breed), were used in the experiment. The White Leghorn control line is a random bred pedigreed population without any selection being practiced and the PD3 line is selected for part period egg mass up to 40 weeks of age. The White Leghorn control line produces white eggs and the PD3 line brown eggs. Roosters from the two lines of the same hatching event were randomly selected and reared in individual cages in an open-sided house. The birds were provided with a layer breeder diet of 2600 ME (kcal/kg), 16% crude protein, 1% Ca, and 0.4% available P, and all the birds had free access to feed and water. The semen from the birds was collected at 46, 47 and 55 weeks of age. The experiment was conducted according to the guidelines of the Institutional Animal Ethics Committee.

2.1.Semen collection and evaluation

Semen from the birds was collected by abdominal massage (Burrows and Quinn, 1937) and evaluated for different gross semen variables such as semen volume, sperm concentration, sperm motility, dye reduction assay, live and abnormal sperm. Soon after collection the neat semen was diluted four times (volume/volume) using a high temperature diluent (suitable for storing semen at 20 or 40°C), with a composition of NaCl 0.8 g; TES 1.374 g; 1MNaOH 2.75 mL; glucose 0.6 g, dissolved in 100 mL of double-distilled water and pH adjusted to 7.4, osmotic

pressure to 382 mOsmol/kg water; Chaudhuri and Lake, 1988) and used for further analysis. The volume of the ejaculated semen was assessed by using a 1 ml syringe. The raw semen appearance was visually scored 1 to 5 (McDaniel and Craig, 1959). The percentage of progressively motile sperm was assessed subjectively by placing a drop of diluted semen on a Makler chamber and examining at 20 × magnifications. Sperm concentration was determined in a colorimeter at 540 nm (Taneja and Gowe, 1961). The Tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction test was conducted and absorbance was recorded using a colorimeter (CL 157, Elico Ltd, Hyderabad, India) at 570 nm (Hazary et al., 2001). The samples that had greater metabolically active sperm produce a deep purple colored end product. The MTT dye reduction test may be used as predictor of sperm fertilizing capacity. Percent live sperm was estimated by the differential staining technique using eosin–nigrosin stain (Campbell et al., 1953). The slides were used for estimating the percent abnormal morphological sperm on the basis of observable abnormalities. Sperm were assessed for chromatin condensation by use of aniline blue staining (Park et al., 2011). Briefly, a smear of semen was made on a glass slide and was fixed in 4% formalin for 5 min. The slide was then stained with 5% aniline blue solution in PBS, pH adjusted to 3.5 using 4% acetic acid for 5 min. The slide was rinsed, dried and examined under oil immersion. Immature sperm heads stained dark blue were considered as aniline blue positive while mature sperm with protamine protein stained light blue in color were considered as aniline blue negative. A total of 100 sperm per slide was counted and percent of light blue stained sperm was calculated.

2.2. Duration of fertility after insemination

At 55 weeks of age semen samples from the roosters were collected, pooled line wise and diluted to have 100 million sperm in 0.1 ml semen. This fixed sperm dose from each line was inseminated intra-vaginally at approximately a 2.5cm depth once into the same and other line of hens (20 hens per line) at 1500 h. The eggs were collected from the second day after insemination for15 days to study the duration of fertility. The eggs were marked for line and day of collection, and stored under refrigeration until incubation. The eggs were candled on the 18th day of incubation for observing developing embryos and percent fertility was calculated. Infertile eggs were opened by breaking the shell and contents were assessed for confirmation of fertilization.

2.3.COMET assay

The status of sperm DNA damage was assessed by comet assay. Semen samples of seven birds from each breed collected at 47 weeks of age was analyzed for sperm DNA damage. The neutral comet assay for determining DNA fragmentation was performed as described by Gliozzi et al. (2011) for chicken sperm with a few modifications. Briefly, the semen samples were washed twice by centrifugation in PBS. The sperm (approximately 1×10^6/ml) were mixed with low melting point agarose gel solution (LMPA, 0.8% w/v in PBS) at 37°C and pipetted on a glass slide coated with normal melting point agarose (NMPA, 1.5% w/v in PBS) and was

covered with a cover glass. The slides were placed on an ice pack for 10 min for the agarose to solidify. The cover glass was removed and the third layer of LMPA 0.8% w/v in PBS was applied, and subsequently a cover glass was applied and the sample was cooled on an ice pack. After removing the cover glass the slides were stored in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 (TX), 10 mM DTT, pH 10) at 4°C for 1 h. The slides were subsequently incubated in 5 µg/ml of proteinase K in lysis buffer kept at 37°C for 1 h. After the 1 h incubation the slides were removed, thoroughly rinsed in PBS and equilibrated in a horizontal electrophoresis tank with a freshly prepared electrophoresis neutral solution (TAE, pH 7.3) for 20 min. Electrophoresis of the slides was performed at 16 V and 30 mA for 15 min at 4°C. The slides were then washed in neutralising buffer (0.4 M Tris-HCl, pH 7.5), fixed in methanol and stored until staining.

The slides were silver stained using the procedure described by Nadin et al. (2001). The slides were kept immersed in a fixative solution (15% trichloroacetic acid, 5% zinc sulfate, 5% glycerol) for 10 min. The fixed slides were then washed three times with distilled water and dried overnight at room temperature. Before silver staining the slides were rehydrated for 5 min with distilled water and stained with freshly prepared solution (34 ml of Solution B (0.2% w/v ammonium nitrate, 0.2% w/v silver nitrate, 0.5% w/v tungstosilicic acid, 0.15% v/v formaldehyde, and 5% w/v sodium carbonate) added to 66 ml of Solution A (5% sodium carbonate)). The slides were kept in the solution under constant shaking for 20 min or until the gel became grey or brown. After staining, the slides were washed thrice in distilled water and immersed in a stop solution (1% acetic acid) for 5 min that was followed by washing two times in distilled water, and air drying. The slides were examined under oil immersion of light microscope. Comets (n = 200) per slide were examined randomly and were visually scored by giving a 0 to 4 rating according to the criteria described by García et al. (2004) and arbitrary units (AU) calculated (García et al., 2011).

2.4.Sperm membrane protein

The sperm membrane proteins were studied in the semen sample collected at 55 weeks of age. The sperm membrane protein was isolated as per the procedure described by Selvaraju et al. (2010) with few modifications. The neat semen that was pooled from three birds of each breed was washed thrice (560 *g* for 5 min at 4°C) using TC buffer (40 mM Tris, 2 mM $CaC1_2$, 1 mM PMSF, 0.01% sodium azide, pH 7.3). The sperm were re-suspended in 1 ml of TC buffer containing 0.1% Triton X-100 and incubated for 1 h at 4°C while vortexing every 10 min. After the incubation period, samples were centrifuged at 5600 *g* for 30 min and cellular debris pelleted. The supernatant containing sperm membrane proteins were precipitated with nine volumes of cold ethanol and the tube was centrifuged at 560 *g* for 10 min. The ethanol washing was repeated twice and the precipitate was solubilized in distilled water. A discontinuous SDS-PAGE process was performed to separate the proteins (Laemmli, 1970) and the gel was stained with coomassie brilliant blue.

2.5.Statistical analysis

Statistical analyses to determine differences in semen variables and duration of fertility between the lines were conducted using the Student's t-test (SAS 9.2). The comet scores were analyzed using a nonparametric one-way ANOVA (NPAR1WAY procedure - SAS 9.2). The treatment means were compared at $P< 0.05$. Percent values of sperm motility, live and morphologically abnormal sperm, and aniline blue test were arcsine transformed before analysis of data. The data normality was evaluated using a normal probability plot (CAPABILITY procedure - SAS 9.2).

3. Results

The semen volume and percent of abnormal sperm were greater ($P<0.05$) in roosters of the PD3line whereas the sperm concentration was greater in White Leghorn roosters at both the ages where assessments occurred (Table 1 and 2). The duration of fertility was similar after insemination of the same line of hens or other line of hens (Table 2). No difference in sperm chromatin condensation between the lines was observed (Fig. 1). Sperm comets of different shapes could be observed and scored (Fig. 2). However, there was no difference in the scores between the lines. The SDS-PAGE gel containing sperm membrane proteins were observed at the 95 kDa molecular weight (Fig. 3). No difference in the bands between the lines could be observed.

4. Discussion

The results of difference in semen volume, sperm concentration and percent abnormal sperm between the lines that were studied in the present experiment are similar to other reports where different genotypes/strains have been shown to have varying semen variables (Omeje and Marire, 1990; Peters et al., 2008; Shanmugam et al., 2012). The White Leghorn line in the present experiment is a control population without any genetic selection being applied. The PD3 line that was selected for 40 week part period egg mass had a greater semen volume at both the ages where assessments occurred. This greater semen volume but not sperm concentration that was detected in the present study is similar to that reported by Nestor (1977) where semen volume and sperm concentration was positively associated with a line that was genetically selected for increased egg production. The objective assessment of the sperm metabolic activity using the MTT dye reduction test did not indicate any differences between the lines and the result was similar for sperm motility. The greater percentage of abnormal sperm in the PD3 line may be due to the effect of genetic selection and is similar to the result reported by Shanmugam et al. (2013). The morphological sperm abnormalities observed in the PD3 line were knotted heads, bent heads, bending or knotting in the region of the head mid piece junction and curled tails. In human sperm, a positive relationship between normal sperm morphology and nuclear maturity has been reported (Sellami et al., 2013). There was no difference in the aniline blue staining between the lines and also at the varying weeks of age where assessments occurred in

the present study. The protamines are the most abundant nuclear proteins in mature sperm that replace the histones during spermatogenesis. Protamine deficiency and increased histone remnants in sperm results in premature chromatin condensation, a cause of failure in fertilization and development of embryos (Oliva, 2006). In human sperm, protamines constitute approximately 85% and histones about 15% of nuclear proteins (Carrell et al., 2007). In the present study, the acidic aniline blue light stained sperm indicative of protamine proteins was in the range of 81% to 91% similar to the results from earlier reports. In contrast, the results of the present study are inconsistent with those of Santiago-Moreno et al. (2009) who reported a wide range (4%-100%) in chicken sperm taking up aniline blue stain and it was suggested that this staining method may not be of much use in determining sperm chromatin condensation status. In the present study, there were consistent staining results with acidic aniline blue staining and the difference from the previous report may be due to the different fixation method or line of birds being different in the two studies. From observations in the present study, it is suggested that the acidic aniline blue staining method may be of use in determining sperm chromatin condensation status. However, further information is needed with regards to the protamination process in chicken sperm for the regular application of the aniline blue test in studying sperm chromatin condensation status. It is noted that the present knowledge of sperm protamine proteins are predominantly those obtained from mammalian sperm studies.

A fixed number of sperm use for inseminations in chickens is the standard in studying fertility of chickens. Though after a single insemination with a fixed number of sperm the hen produces fertile eggs for up to 22 days (Goerzen et al., 1996), in the lines used in the present study it was observed that fertile eggs were produced up to 15 days after a single insemination. Hence, the eggs were collected for 15 days to study the duration of fertility. There was no difference in duration in the period over which fertility was sustained between the lines though there was a significant difference in percent abnormal sperm. The sperm that are motile and morphologically normal reach the sperm storage tubules after insemination (Allen and Grigg, 1957). Similarly, Parker et al. (1942) did not detect any correlation between fertility and abnormal sperm percent in chickens. In contrast, there was a negative correlation between sperm defects and fertilizing capacity of cockerel semen that was previously reported (Allen and Champion, 1955).In the present study, the semen from one line was used in another line to observe if there were differences or interactions of sperm surface factors with the cells of the female reproductive tract. The fertility results indicated that there is absence of chicken line specific interactions between sperm and cells of sperm storage tubules.

In the present study, silver staining of comets was conducted with chicken sperm. For visualizing and analyzing the silver stained comets relatively simple equipment needed and the method is reliable (García et al., 2004). No difference in the comet score indicates that there is no difference in the DNA integrity between the lines used in the present study. Thus, it may be inferred that the greater percentage of abnormal sperm encountered in the PD3 line may not be have resulted from nuclear abnormalities. Furthermore, in mammals it has been reported that

immature sperm have greater DNA damage and may have alterations in protamination and chromatin packaging (Sharma et al., 2004). Though there is an association between DNA damage and sperm chromatin protamination and packaging in the present study, there was no significant difference in the selected chicken line when sperm DNA damage was assessed using the Comet assay and sperm protamination assessed by aniline blue staining.

Sperm surface associated proteins are important to traverse the vagina and reach the sperm storage tubules, however, in the absence of these proteins only the ability of sperm to migrate in vagina is limited and other functions such as metabolic activity and fertilization are not affected (Steele and Wishart, 1996). Sperm membrane proteins of molecular weights up to 205 kDa have been reported in bulls (Selvaraju et al., 2010; Karunakaran et al., 2012). Specific sperm membrane proteins are associated with fertility (Rajeev and Reddy, 2004; Karunakaran et al., 2012). Sperm membrane proteins are important for binding of sperm with the zona pellucida (Ashrafzadeh et al., 2013) and also have other functions such as in the movement of calcium ions into sperm. In the present study, proteins of less than 95 kDa were observed with no difference in protein bands between the lines. The sperm membrane proteins analyzed using SDS-PAGE in the present study only provides preliminary results. Further studies on intact isolation of membrane proteins and analysis using mass spectrometry are required.

In conclusion, there is a difference in the semen volume, sperm concentration, live and abnormal sperm in the two lines studied in the present experiment and this may be due to the line characteristics and effect of genetic selection in PD3 line. However, with regard to sub-cellular structures and fertilization function, there is no difference between the lines.

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Fig. 1. Representative image of sperm stained with acidic aniline blue dye; A- Mature sperm heads stained light blue in color were considered as aniline blue negative; B- Immature sperm heads stained dark blue were considered as aniline blue positive

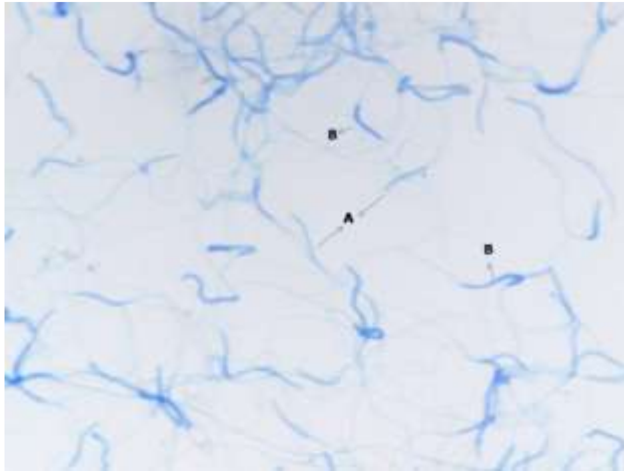

Fig. 2. Sperm comets silver stained showing different degrees of DNA damage; Numbers 0 - 4 are scores assigned to the comets; 0 represents undamaged sperm and 4 represents most extensively damaged sperm

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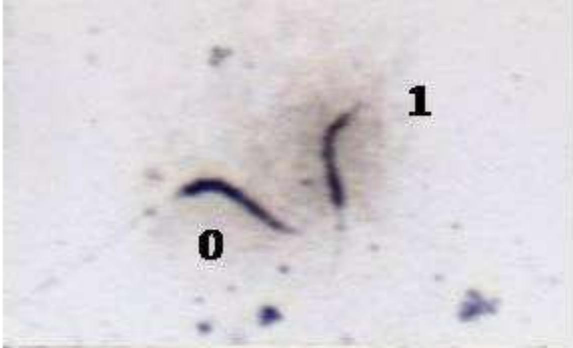

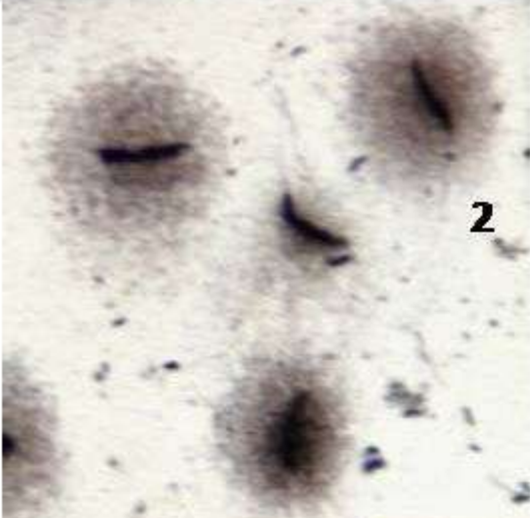

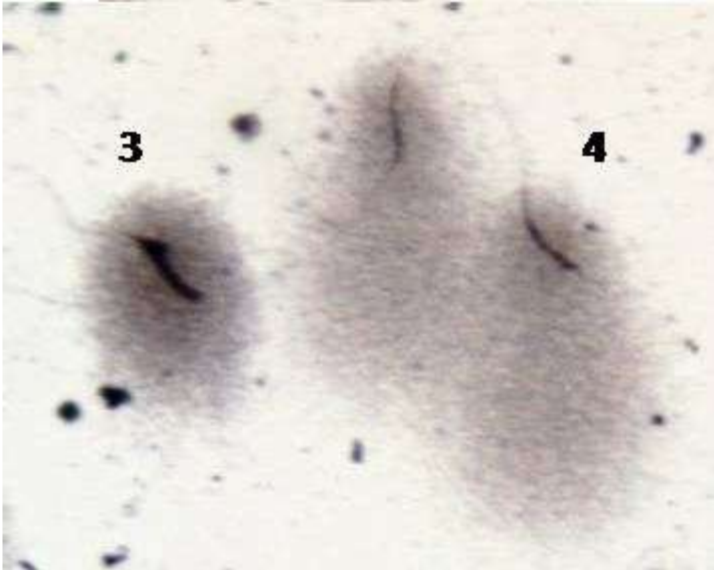

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Fig. 3. Separation of sperm membrane proteins by SDS-PAGE; Lane M -Protein standard markers; PD3 - PD3 line; WL - White Leghorn line

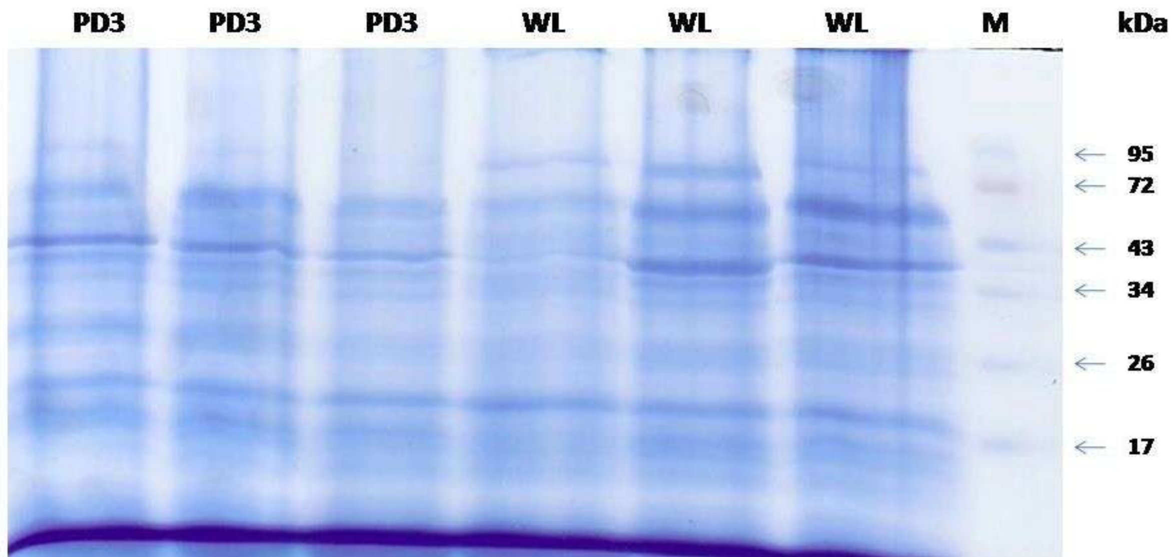

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Table 1

Average (46and 47 weeks) semen variables (mean ± SE) in layer breeder lines

Semen variables	White Leghorn (*n*=18)	PD3 (*n*=18)
Volume (ml)	0.30±0.04[b]	0.40 ±0.03[a]
Sperm motility (%)	58.44±2.83	52.19±2.29
Sperm concentration (million/ µl)	4.47± 0.43[a]	3.38±0.40[b]
MTT dye reduction test (nM of MTT Formazan /min/million sperm)	30.28±0.73	28.95±1.30
Live sperm (%)	91.17±1.25[b]	93.60±0.47[a]
Morphological abnormal sperm (%)	0.76± 0.23[b]	2.88±1.18[a]
Aniline blue negative (%)	83.41 ± 2.43	81.35 ± 3.37
Comet score (AU)	100.06 ± 19.68	110.00 ± 19.65

Means with different superscripts in a row differ ($P < 0.05$)

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Table 2
Semen variables (mean ± SE) at 55 weeks of age in layer breeder lines and duration of fertility after fixed sperm insemination from semen collected at 56 weeks of age

Semen variables	White Leghorn (*n*=13)	PD3 (*n*=14)
Volume (ml)	0.30±0.03[b]	0.40 ±0.04[a]
Sperm motility (%)	63.85±4.05	59.29±3.09
Sperm concentration (million/ µl)	4.61± 0.51[a]	3.84±0.27[b]
MTT dye reduction test (nM of MTT Formazan /min/million sperm)	27.92±1.94	24.38±1.28
Live sperm (%)	89.57±1.14	89.27±1.16
Morphological abnormal sperm (%)	1.17± 0.37[b]	4.24±1.60[a]
Aniline blue negative (%)	88.89 ± 1.48	91.37 ± 0.88
Average duration of fertility (days)		
× White leghorn hens	11.5	11
× PD3 hens	13	14

Means with different superscripts in a row differ ($P< 0.05$)