Development of a novel fluorescence technique for quantifying the total number of spermatozoa stored in the uterovaginal junction of hens

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A technique was developed to determine the total number of spermatozoa stored in the uterovaginal junction of hens. After insemination of spermatozoa treated with the nuclear fluorescent dye *bisbenzimidazole*, oviductal tissue was collected from hens and homogenized. Samples of homogenate were dried, and the number of spermatozoa mm^{-2} was determined with the use of a fluorescence microscope. When spermatozoa were added to excised uterovaginal junction tissue before homogenization, results indicated a 1:1 linear relationship between actual numbers of spermatozoa added to the tissue and calculated numbers of spermatozoa added to the tissue. This new technique was used to show that insemination of hens with 25, 50 or 100×10^6 spermatozoa resulted in a linear increase in the number of spermatozoa stored in the uterovaginal junction. Insemination of hens with 328×10^6 spermatozoa produced no increase in uterovaginal junction storage of spermatozoa over insemination with 100×10^6 spermatozoa. At the maximum sperm storage tubule filling dose of 100×10^6 spermatozoa, only 0.22% of the spermatozoa inseminated were found in the uterovaginal junction 24 h after insemination. Treatment of spermatozoa with *bisbenzimidazole* had no detrimental effects on fertility or penetration rates when compared with untreated (control) spermatozoa. However, when spermatozoa were treated with *bisbenzimidazole*, hatchability of fertile eggs was reduced. In conclusion, this new fluorescence technique appears to be valuable in determining the total number of spermatozoa stored in the uterovaginal junction of hens.

Introduction

In research on avian fertility it is often necessary to consider sperm–oviduct interactions such as oviductal storage of spermatozoa. The domesticated hen is capable of storing spermatozoa in storage tubules (SST) located in the uterovaginal junction (UVJ) (for review see Bakst *et al.*, 1994).

Van Krey *et al.* (1971) made histological sections of the uterovaginal junction to determine the number of SST that were full, partially full, or devoid of spermatozoa. However, this technique does not reveal the total number of spermatozoa stored in the uterovaginal junction of the hen. In addition, this technique is very laborious; and during sectioning and histological processing, spermatozoa may be shed from the SST resulting in erroneous estimations.

Brillard and Bakst (1990) have illustrated that brief homogenization of the oviduct of the turkey hen will yield spermatozoa that may be quantified with a haemocytometer. This technique, however, was not tested to determine whether the total number of spermatozoa in the uterovaginal junction of the

chicken hen could be determined. Some difficulties which may arise when using this procedure to estimate storage of spermatozoa are improper filling of the haemocytometer due to oviductal tissue, problems associated with distinguishing spermatozoa from homogenized oviductal tissue, and short time for sample processing necessitated by enzymatic degradation of spermatozoa.

Bakst (1994) inseminated turkey hens with *bisbenzimidazole*-treated spermatozoa to improve visualization of spermatozoa within the SST. *Bisbenzimidazole* is a fluorescent dye that binds to the DNA of living cells and fluoresces bright blue at the proper excitatory wavelength (Latt and Stetten, 1976). Bakst (1994) used fluorescence and differential interference contrast microscopy to determine the number of SST that were full, partially full, or empty. Again, this technique does not reveal the total number of spermatozoa residing in the uterovaginal junction.

The aim of the present work was to develop a fluorescence technique for quantifying the total number of spermatozoa stored in the uterovaginal junction of the hen and to determine whether this technique would measure the number of spermatozoa in the uterovaginal junction accurately. Hens were inseminated with increasing doses of spermatozoa to determine whether this technique would reveal significant differences in

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Fig. 1. Photomicrograph illustrating rooster spermatozoa recovered from the uterovaginal junction after homogenization. Spermatozoa were incubated with the fluorescent dye, *bisbenzimide*, before insemination. The crescent-shaped nuclei of the spermatozoa (s) fluoresce bright blue when exposed to light with a 355 nm wavelength, whereas oviductal tissue (t) appears white. Scale bar represents 20 μm .

the number of spermatozoa stored in the uterovaginal junction. The effect of *bisbenzimide* on spermatozoa storage within the hen's oviduct was assessed by measuring fertility and sperm penetration of ova.

Materials and Methods

Basic technique

Semen was collected from 12 50-week-old, broiler breeder males (*Gallus domesticus*) and diluted 1:1 with minimum essential medium (Howarth, 1981). Diluted semen was incubated with *bisbenzimide* (Hoechst 33342) using a technique similar to that used by Bakst (1994) for turkey semen. Semen was further diluted with two parts of minimum essential medium containing 113 μg *bisbenzimide* ml^{-1} . The diluted spermatozoa were placed on an Adams Nutator (Clay Adams, Parsippany, NJ) and rotated at 24 r.p.m. for 2 h at room temperature.

Thirty-week-old, virgin White Leghorn hens were maintained in 16 h of light day^{-1} (lights on at 03:00 h; lights off at 19:00 h). Laying hens were inseminated with *bisbenzimide*-treated spermatozoa at 14:00 h and killed by cervical dislocation 24 h after insemination. The abdominal cavity was opened, and a segment of the oviduct from the uterus to the vagina was removed. Connective tissue surrounding the uterovaginal junction was removed with the use of fine scissors and forceps so that the oviductal section was straight. A longitudinal cut was made in the oviduct to expose the lumen and SST. The area containing the SST was excised with the use of a dissecting microscope, and placed in a 20 ml scintillation

vial with 0.5 ml of 0.85% (w/v) NaCl. The tissue from the uterovaginal junction was homogenized in the vial at 10 000 r.p.m. for 20 s. After homogenization, a 20 μl sample of the homogenate was placed on a microscope slide and dried for 5 min under forced air at 55°C.

The number of spermatozoa in five separate 0.08 mm^2 areas of each 20 μl drop (average size = 67 mm^2) was determined with a fluorescence microscope set at an excitatory wavelength of 355 nm. Nuclei of the *bisbenzimide*-treated spermatozoa fluoresce bright blue at this wavelength of light (Fig. 1). The five areas counted included one count at the top, bottom, left, right, and centre of each drop. The number of spermatozoa in the entire uterovaginal junction was determined by the following formula: $(\text{spermatozoa } \text{mm}^{-2})(\text{total area of drop} \div 20 \mu\text{l})$ (total volume of homogenate).

Recovery of spermatozoa

Increasing concentrations of spermatozoa were added to the excised uterovaginal junction tissue to determine whether this technique could accurately measure the number of spermatozoa residing in the oviduct. The uterovaginal junction tissue was obtained from seven hens as described previously. Each of these seven oviductal sections was placed in 20 ml scintillation vials with 0.5 ml 0.85% (w/v) NaCl containing one of the following amounts of *bisbenzimide*-treated spermatozoa: 9600, 96 000, 480 000, 960 000, 1 440 000, 1 920 000, and 2 400 000. Successive dilution was used to obtain these concentrations. The concentration of spermatozoa in the original semen sample was determined using the packed cell volume method of Maeza and Buss (1976).

After addition of spermatozoa to the oviductal tissue, the mixture was homogenized and the number of spermatozoa in each sample determined using the previously described fluorescence method. Data were analysed with linear regression of calculated spermatozoa dose over actual spermatozoa addition dose (Steel and Torrie, 1980). Standard regression produced a linear equation with an intercept not significantly different from zero; therefore, the NOINT (no intercept) option of PROC REG was used to obtain the regression equation (SAS Institute, 1985).

Insemination dose and storage of spermatozoa

It was reasoned that there must be an insemination dose at which maximum filling of the SST would occur, and that insemination of hens with less than this maximum filling dose should reveal a dosage effect with decreasing spermatozoa storage. Two experiments were conducted to analyse these two concepts.

Experiment 1: maximum filling dose. Semen was incubated with bisbenzimidazole, and the concentration of spermatozoa was determined using the packed cell volume method. At 14:00 h, five hens were inseminated with 100×10^6 spermatozoa, and four hens were inseminated with 328×10^6 spermatozoa. Twenty-four hours after insemination, hens were killed, their oviducts were removed, and the number of spermatozoa per uterovaginal junction was determined as described previously. Data were subjected to an ANOVA using a completely randomized design (Steel and Torrie, 1980).

Experiment 2: effect of dosage. On two separate occasions hens were inseminated with 25, 50 and 100×10^6 bisbenzimidazole-treated spermatozoa per 50 μ l at 14:00 h. Sixty hens were used (10 hens per dose per time). Birds were individually caged in replicates of five hens each, such that there were two replicates for each insemination dose. Twenty-four hours after insemination, hens were randomly killed in replicate, and the total number of spermatozoa per uterovaginal junction was determined. The percentage of spermatozoa inseminated that were subsequently stored in the uterovaginal junction was calculated by dividing the total number of spermatozoa per uterovaginal junction by the number of spermatozoa inseminated and multiplying the result by 100.

Linear regression and ANOVA were used to determine the relationship of insemination dose with spermatozoa storage and percentage of spermatozoa stored. The ANOVA design was a generalized, randomized complete block design with blocks being the two separate times of insemination. Values were transformed by taking the square root of the value plus 0.5 to normalize total sperm storage data. Means were separated using Student–Newman–Keul's sequential range test (Steel and Torrie, 1980).

Effects of bisbenzimidazole on fertility and penetration of ova

One hundred and twenty 50-week-old Arbor Acre hens were individually caged and divided into 12 groups of 10 hens each. Hens and roosters were maintained on 16 h of light

Fig. 2. Recovery of chicken spermatozoa after homogenization of uterovaginal junction tissue in which seven dilutions of spermatozoa ranging from 9600 to 2 400 000 spermatozoa per 0.5 ml had been added. The line indicates the linear regression of the calculated number of spermatozoa added over the actual number of spermatozoa added ($y = 1.02x$, $P < 0.0001$, standard error of the slope = 0.094, $r^2 = 0.95$).

day⁻¹ (lights on at 05:00 h; lights off at 21:00 h). Semen was collected from 12 roosters, pooled, and divided into two portions. One portion of semen was incubated with bisbenzimidazole and the other remained unstained. Six groups of hens were inseminated with bisbenzimidazole-treated spermatozoa, while the other six groups were inseminated with semen that had not been exposed to bisbenzimidazole. All hens were inseminated at 14:00 h with 100×10^6 spermatozoa. Eggs were collected daily for 8 days after insemination and stored in an egg cooler at 13.3°C. Egg production per hen-day averaged 67% over the 8 days after insemination, resulting in approximately seven eggs per replicate per day.

The eggs from three of the bisbenzimidazole groups and three of the control groups were used for fertility and hatchability measurements. The eggs from the remaining groups were used for analysis of penetration of ova by spermatozoa. Candling fertility was determined at 10 days of incubation. All unhatched eggs were opened to determine true fertility.

Penetration of ova by spermatozoa was determined using the method of Bramwell *et al.* (1995). The perivitelline layer from oviposited eggs was removed, fixed with 20% formalin, and stained with Schiff's reagent. The number of sperm penetration holes was counted in a 1.35 mm² area surrounding the germinal disc. Data were subjected to an ANOVA, using a split-plot design in time (day after insemination). Interaction means were separated with Student–Newman–Keul's sequential range test (Steel and Torrie, 1980).

Results

Recovery of spermatozoa

A positive linear relationship was found between the calculated number of spermatozoa in the uterovaginal junction and the actual number of spermatozoa added to the oviductal tissue (Fig. 2). With a slope of 1.02 ± 0.094 ($P < 0.0001$), the line

Fig. 3. Relationship between insemination dose and sperm storage in the uterovaginal junction of the chicken's oviduct. In Expt 1 ($\bullet$ = results of individual hens; $*$ = treatment means), five hens were inseminated with 100×10^6 bisbenzimidazole-treated spermatozoa, and four hens were inseminated with 328×10^6 bisbenzimidazole-treated spermatozoa. In Expt 2 ($\blacksquare$ = results of individual hens; $+$ = treatment means), ten hens were inseminated with 25, 50 or 100×10^6 bisbenzimidazole-treated spermatozoa on two separate occasions (two groups of five hens each per dose per time). The line indicates the linear relationship between insemination dose and mean number of spermatozoa stored in the uterovaginal junction during Expt 2 ($y = 2654x - 49\,280$, $P < 0.008$, $r^2 = 0.9998$). ^aMeans for Expt 1 were not significantly different ($P > 0.9$, $SEM = 6.8 \times 10^4$). ^{xy}Means for Expt 2 with different superscripts are significantly different ($P < 0.0006$ for square root transformed data, nontransformed $SEM = 22 \times 10^4$). Means for Expt 2 represent four groups of five hens each ($n = 4$).

Fig. 4. Relationship between insemination dose and the percentage of spermatozoa stored in the uterovaginal junction within the chicken hen's oviduct. Ten hens were inseminated with either 25, 50 or 100×10^6 bisbenzimidazole-treated spermatozoa on two separate occasions (two replicates of five hens each per dose per time). Means with different superscripts are significantly different ($P < 0.036$, $SEM = 0.0296$). Values represent the mean of four groups of five hens each ($n = 4$).

approximately 216 000 spermatozoa from the uterovaginal junction (Fig. 3). This value is very close to the 226 000 spermatozoa recovered from the uterovaginal junction when hens were inseminated with 100×10^6 spermatozoa in Expt 1. In addition the coefficient of variation for the five different areas counted on each slide was only 6.25%. In addition, the coefficient of variation between two samples from each of 15 hens was only 6.32%. However, like most fertility characteristics, the variability between individual hens within a treatment was large as is demonstrated in Fig. 3. Analysis of the treatment means revealed that insemination of hens with 25, 50, and 100×10^6 spermatozoa gave a near perfect positive linear relationship between insemination dose and the number of spermatozoa stored in the uterovaginal junction. In addition, the number of spermatozoa residing in the uterovaginal junction was significantly different at each insemination dose when data were analysed using ANOVA.

The relationship between insemination dose and the percentage of spermatozoa inseminated that were subsequently stored in the uterovaginal junction is shown (Fig. 4). Mean separation procedures reveal that when hens were inseminated with 100×10^6 spermatozoa, the percentage of spermatozoa stored was greater than when hens were inseminated with 25×10^6 spermatozoa. The ANOVA results also show that the percentage of spermatozoa stored when hens were inseminated with 50×10^6 spermatozoa was similar to the percentage of spermatozoa stored when hens were inseminated with 25 or 100×10^6 spermatozoa.

Effects of bisbenzimidazole on fertility and penetration of ova

Fertility and the number of spermatozoa penetrating the perivitelline layer were similar when hens were inseminated with control or bisbenzimidazole-treated spermatozoa (Fig. 5). However, the hatchability of fertile eggs was reduced by

indicates a 1:1 relationship between actual spermatozoa addition and calculated spermatozoa addition. The coefficient of determination (r^2) indicated that 95% of the variation in calculating the number of spermatozoa in the uterovaginal junction was due to the number of spermatozoa added to the tissue. On average, 94% of the spermatozoa added to the tissue was recovered after homogenization, as indicated by the short distance of the points from the regression line. The only point not close to the line was at the dose of 2 400 000 spermatozoa.

At the lowest addition dose of 9 600 spermatozoa, only one spermatozoon was counted in all five 0.08 mm^2 areas observed. The calculated addition dose for this single spermatozoon was 11 200 spermatozoa. Therefore, the detection limit of this technique is approximately 11 200 spermatozoa per uterovaginal junction.

Insemination dose and storage of spermatozoa

Experiment 1: maximum filling dose. When hens were inseminated with 328×10^6 spermatozoa, the number of spermatozoa residing in the uterovaginal junction was not greater than when hens were inseminated with 100×10^6 spermatozoa (Fig. 3).

Experiment 2: effect of dosage. Insemination of hens with 100×10^6 spermatozoa in Expt 2 resulted in the recovery of

Fig. 5. Penetration of ova, fertility, and hatchability of fertile chicken eggs resulting from insemination with spermatozoa incubated with (▨) or without (□) bisbenzimid. Values are expressed as means $\pm$ SEM. Results for hatchability with different letters are significantly different ($P < 0.009$). Penetration ($P > 0.94$) and candling fertility ($P > 0.14$) were similar for control and bisbenzimid-treated spermatozoa. Values represent the replicate means of the second to the eighth day after insemination of three replicates of ten hens each ($n = 21$).

Fig. 6. Penetration of ova for each day after insemination when chicken hens were inseminated with control (●) or bisbenzimid-treated (■) spermatozoa (treatment group by day after insemination interaction, $P < 0.07$, SEM = 1.24). Values represent the means of three replicates of ten hens each ($n = 3$). Interaction means with different superscripts differ significantly when analysed with Student–Newman–Keul's sequential range test ($P < 0.05$).

9% when hens were inseminated with bisbenzimid-treated spermatozoa when compared with insemination with control spermatozoa.

A significant interaction of treatment by day after insemination was noted for ova penetration (Fig. 6). At every day after insemination, penetration was similar between the two treatment groups. However, from day 2 to day 3 after insemination, there was a significant increase in penetration in hens inseminated with bisbenzimid-treated spermatozoa. No statistical increase in penetration was seen from day 2 to day 3 after insemination in hens inseminated with control spermatozoa. From day 4 to day 5 after insemination, penetration signifi-

cantly decreased in hens inseminated with control spermatozoa; the decrease was not significant in hens inseminated with bisbenzimid-treated spermatozoa.

Discussion

The results presented here indicate that this new fluorescent technique is reliable and capable of measuring as few as 11 200 spermatozoa per uterovaginal junction with an average sperm recovery of 94%. Preliminary work by Brillard and Bakst (1990) indicated that 86% of turkey spermatozoa added to diluent is recovered following homogenization. However, their spermatozoa recovery technique did not include addition of oviductal tissue before homogenization. In addition, Brillard and Bakst (1990) did not indicate the numbers of spermatozoa that were added to the diluent before homogenization. It is possible that oviductal tissue as well as sperm concentration may influence the number of spermatozoa that are homogenized beyond recognition. Indeed, in the current study, sperm recovery did vary with the concentration of spermatozoa added to the oviductal tissue samples.

The present study shows that maximum storage of spermatozoa in the uterovaginal junction occurs with an insemination dose of approximately 100×10^6 spermatozoa. Brillard and McDaniel (1986) have shown that fertility of dwarf broiler breeders does not improve with single insemination doses higher than 125×10^6 spermatozoa, thus indicating a maximum sperm storage for a single insemination. Compton and Van Krey (1979) found no difference in the number of SST containing spermatozoa when hens were inseminated with 0.023 or 0.047 ml of semen and killed 24 h later. Assuming that 1 ml of semen contains 3.5×10^9 spermatozoa (Johnson, 1986), Compton and Van Krey (1979) inseminated 81 and 165×10^6 spermatozoa. Therefore, the insemination doses of Compton and Van Krey (1979) indicate maximum sperm storage when hens are inseminated with approximately 100×10^6 spermatozoa.

In the current study, insemination doses of less than 100×10^6 spermatozoa resulted in a decrease in the absolute number of spermatozoa stored in the uterovaginal junction. Brillard (1992) has shown that a smaller percentage of SST contain spermatozoa when hens are inseminated with 60×10^6 spermatozoa when compared with insemination with 240×10^6 spermatozoa. When Bramwell *et al.* (1995) inseminated hens with 25 or 50×10^6 spermatozoa, penetration of ova was significantly reduced compared with insemination of hens with 100×10^6 spermatozoa. The number of spermatozoa stored will directly affect the number of spermatozoa available to penetrate the ovum; thus, the results of Bramwell *et al.* (1995) may also indicate a reduction in spermatozoa storage at insemination doses lower than 100×10^6 spermatozoa. Indeed, Brillard and Bakst (1990) and Brillard and Antoine (1990) have shown a strong positive correlation between spermatozoa trapped on the perivitelline layer (Wishart, 1987) and spermatozoa retained in the uterovaginal junction of the turkey and chicken hen, respectively.

As in the study by Compton and Van Krey (1979), the hens in the present study were killed 24 h after insemination when the amount of spermatozoa stored in the SST was determined.

Brillard (1992) has shown that the number of chicken SST containing spermatozoa reaches a maximum at 1–2 days after insemination. Pierson *et al.* (1988) found that, in broiler breeder hens, the number of SST containing spermatozoa reaches a maximum 24 h after insemination. Similar results have been obtained with White Leghorn hens (Schindler *et al.*, 1967). When hens were inseminated with bisbenzimidazole-treated spermatozoa in the present study, penetration of ova was greatest in eggs laid 3 days after insemination. An egg laid 3 days after insemination would have been exposed to spermatozoa approximately 24 h earlier, 2 days after insemination. The hens in the current study were killed at 14:00 h on day 1 after insemination; thus, the egg laid on the second day after insemination was usually already past the site of fertilization and beginning calcification within the uterus. Therefore, spermatozoa within the SST at the time of death could have fertilized the ovum which would have been laid 3 days after insemination. Therefore, in the present study the SST were probably at their maximum fill-rate for a single insemination.

When hens were inseminated with 25, 50, 100, and 328×10^6 spermatozoa, approximately 0.07, 0.16, 0.22, and 0.06% of the spermatozoa inseminated were subsequently stored in the uterovaginal junction, respectively. The increasing percentage of spermatozoa stored with increasing insemination dose (up to 328×10^6 spermatozoa) may involve selection of spermatozoa within the vagina (for review see Bakst *et al.*, 1994). Perhaps, the close proximity of spermatozoa at higher concentrations results in a greater percentage entering the SST. Head-to-head agglutination is seen once chicken spermatozoa enter the SST (Van Krey *et al.*, 1981). Apparently, only a few selected spermatozoa enter the uterovaginal junction and SST. Brillard (1993) suggested that about 0.9% of inseminated spermatozoa are stored in the SST of the broiler breeder hen. In addition, Brillard and Bakst (1990) estimated that less than 1% of spermatozoa inseminated into turkey hens was stored in the SST when hens were inseminated with 200×10^6 spermatozoa. It is understandable that such a small percentage of spermatozoa is stored in the SST, considering approximately 87% of inseminated spermatozoa are evacuated from the turkey hen's cloaca within 1 h of artificial insemination (Howarth, 1971).

Unlike previous studies that evaluated oviductal storage of spermatozoa, the current work involved treating spermatozoa with bisbenzimidazole. There is a lack of information concerning the effects of bisbenzimidazole on chicken fertility. Therefore, it was questioned whether treatment of spermatozoa with this fluorescent dye would alter subsequent oviductal spermatozoa storage. However, treatment of broiler breeder spermatozoa with bisbenzimidazole does not appear to reduce oviductal storage of spermatozoa. Hens inseminated with bisbenzimidazole-treated spermatozoa laid eggs with fertility and penetration rates comparable to those of hens inseminated with untreated (control) spermatozoa. In addition, penetration rates and fertility were similar between the two treatment groups during each of 8 days after insemination. However, hatchability of fertile eggs was depressed when hens were inseminated with bisbenzimidazole-treated spermatozoa. As bisbenzimidazole binds to DNA within the sperm nucleus, it is possible that genetic alteration of the spermatozoon may have affected embryonic development. Bakst (1994) found that, in turkeys, insemination with bisbenzimidazole-treated spermatozoa produced fewer fertile

eggs than did insemination with control spermatozoa; and, although a reduction was seen in the hatchability of fertile eggs, the difference was not statistically significant. It is possible that turkey spermatozoa are more sensitive to treatment with bisbenzimidazole than chicken spermatozoa are.

This new fluorescent spermatozoa technique provides many advantages over previous methods for estimating oviductal storage of spermatozoa. Tedious histological work is not required with this new technique as is required with the technique of Van Krey *et al.* (1971). Because the complete uterovaginal junction is used with this technique, the entire number of spermatozoa residing in the lumen of the uterovaginal junction and stored in the SST is determined. Unlike the technique of Brillard and Bakst (1990) for determining oviductal sperm storage in turkey hens, this new technique eliminates the use of a haemocytometer which may clog with oviductal tissue resulting in improper filling. Staining of spermatozoa with bisbenzimidazole eliminates the likelihood of recording oviductal tissue as spermatozoa. In addition, with the fluorescent spermatozoa technique, homogenate samples are dried and may be analysed at a later time.

The primary disadvantage with this new technique is that hens must be killed to obtain oviductal tissue. Therefore, repeated measurements of spermatozoa storage over time cannot be conducted on the same animal. It is possible that bisbenzimidazole may leach out of spermatozoa that are stored in the oviduct for an extended period of time. Therefore, this technique may be of only limited use in some studies involving long-term oviductal storage of spermatozoa. However, in the present study, no decrease in spermatozoa fluorescence, as determined both visually and with a fluorometer, was observed after 48 h of incubation at 39°C in a dye-free diluent (minimum essential medium). Even in investigations of long-term sperm storage, this new technique may be valuable in demonstrating relative differences in oviductal sperm storage between treatments. Recently, data were presented in abstract form where McDaniel *et al.* (1995) used this novel fluorescence technique to reveal that sperm storage in the uterovaginal junction is decreased when the male broiler breeder is heat-stressed before semen collection and artificial insemination. Therefore, the results of this study show that this new fluorescence technique could be very useful in determining the total number of spermatozoa residing in the uterovaginal junction.

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