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Research

Segregation of spermatozoa within sperm storage tubules of fowl and turkey hens

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In avian species, spermatozoa reside in the oviduct for prolonged periods in specialized structures known as sperm storage tubules, but little is known about the relative distribution of spermatozoa in these tubules after successive inseminations by different males. The staining efficacies of various fluorescent dyes for fowl and turkey spermatozoa were evaluated to investigate one proposed mechanism of sperm competition. Hens were then inseminated at different intervals with stained and unstained spermatozoa to observe the spatial distribution of spermatozoa within the storage tubules. Several novel fluorescent lipophilic tracers that successfully stain mammalian spermatozoa either did not stain fowl or turkey spermatozoa, or greatly impaired sperm motility. In contrast, Hoechst 33342 readily stained sperm nuclei (fowl: 25 nmol l^{-1}; turkey: 77 nmol l^{-1}) within 4 h without inhibiting sperm motility, or affecting fertility or the hatching ability of the eggs. Hens were tandemly inseminated with equal numbers of stained or unstained spermatozoa at 24 h intervals and were killed 24 h after the final insemination to study sperm entry and storage within the tubules. Oviductal mucosa containing sperm storage tubules was removed, and individual tubules were classified as containing stained spermatozoa, unstained spermatozoa, a mixture of stained and unstained spermatozoa, or as not containing spermatozoa. Results from the present study indicate that spermatozoa from two different inseminations generally segregate into different storage tubules in both fowl and turkey hens. Storage tubules containing mixed populations of spermatozoa were found in only 4% of fowl and 12% of turkey storage tubules examined. Thus, the mechanism of last-male precedence does not appear to be due to the stratification of spermatozoa within the tubules.

Introduction

A significant feature of avian reproductive physiology is the ability of hens to store spermatozoa for prolonged periods. This is due to the presence of sperm storage tubules (SST), which are epithelial invaginations located mainly in the uterovaginal junction of the oviduct, but also present in smaller quantities in the infundibulum (Van Krey *et al.*, 1966). Spermatozoa transferred to the vagina by copulation or artificial insemination (AI) move through the vagina and ascend to the SST in the uterovaginal junction mucosa. Here spermatozoa enter the SST for variable periods, depending on the species, reproductive status and age of the female. Stored spermatozoa are released from the SST over time when the female is producing eggs to ensure that spermatozoa are present at the site of fertilization (Bakst *et al.*, 1994). The mechanisms surrounding sperm selection before their entry into the SST, as well as their release from the SST, are not well characterized, but it is known that only motile and morphologically normal spermatozoa enter the SST (Allen and Grigg, 1958). Maximum storage capacity of spermatozoa in the SST is reached within 2 days of AI in turkeys (Brillard and Bakst, 1990; Brillard, 1993) and within 1 day in fowl hens (Brillard, 1993). Quantification of the number of spermatozoa stored within the SST originally relied on histological procedures, but more recently has relied on moderate grinding (Brillard and Bakst, 1990), collagenase digestion (Brillard, 1993) or homogenization of the SST (McLean and Froman, 1996).

Radiolabels and fluorescent stains have been used to improve visualization of spermatozoa within the SST. Hoechst 33342 (*bis*benzamide) is a fluorescent dye that binds to the DNA of live cells, and is nontoxic and non-mutagenic at low concentrations before exposure to UV radiation (Latt and Stetten, 1976; Durand and Olive, 1982). Bakst (1994) first reported the use of Hoechst 33342 to label turkey spermatozoa for improved visualization of spermatozoa stored within the SST and infundibulum. These qualitative studies described the differential rates at which spermatozoa filled SST in turkey hens before and after egg laying. Bakst (1994) found a slight decrease in egg fertility and in the hatching ability of eggs after a dose of 90 nmol Hoechst 33342 l^{-1}. McDaniel *et al.* (1997) used Hoechst-

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labelled spermatozoa to quantify the storage capacity of the SST after homogenization of uterovaginal junction tissue. They used 116 nmol Hoechst 33342 l^{-1} to label fowl spermatozoa and no observations were reported of the SST containing spermatozoa before homogenization.

The application of fluorescent dyes for staining spermatozoa has also been used to study competitive fertilization. Paternity patterns in many species have demonstrated a last-male mating advantage, in which spermatozoa from the last male fertilizes most eggs during the first few days after the last insemination. This phenomenon has been termed 'last-male sperm precedence' (Birkhead and Møller, 1992). Several authors suggested that last-male sperm precedence in fowl was a result of sperm stratification within the SST (Burke and Ogasawara, 1969; Compton *et al.*, 1978; Christensen, 1981). Van Krey *et al.* (1981) used radio-labelled spermatozoa to study the mechanism of last-male sperm precedence in fowl hens. They performed inseminations at 5 h intervals using [^{3}H]thymidine-labelled and -unlabelled spermatozoa, and found both labelled and unlabelled spermatozoa within the SST. Last-male precedence has not been observed when inseminations occurred at intervals of less than 4 h.

Competitive fertilization has also been studied in many mammals, including rabbits (Overstreet and Adams, 1971; Parrish and Foote, 1985), bulls (Davis *et al.*, 1987) and humans (Blazak *et al.*, 1981). The fluorochromes used in these studies, fluorescein isothiocyanate and tetramethyl-rhodamine, label the proteins on spermatozoa, but also tend to reduce sperm motility (Mellish and Baker, 1970; Davis *et al.*, 1987). Alternatively, newer lipophilic fluorescent dyes permeate the plasmalemma of mammalian spermatozoa without affecting sperm motility (Youakim *et al.*, 1994; Miller *et al.*, 1998). It is not known how these lipophilic fluorochromes affect the motility of spermatozoa, fertilizing capacity and embryo mortality in poultry.

The aim of the present study was to evaluate one of the proposed mechanisms of sperm competition, the concept of last-male precedence, in fowl and turkey hens. Through the use of tandem inseminations with stained and unstained spermatozoa, respectively, the study was intended to provide an insight into the possible stratification of spermatozoa in the SST in relation to last-male precedence. It has been suggested that the last spermatozoa to be transferred into the oviduct overlay spermatozoa already residing in the SST and, therefore, are the first to be released and subsequently transported to the site of fertilization (Compton *et al.*, 1978). However, the biological basis of last-male precedence with respect to sperm stratification in the SST has been questioned (Birkhead *et al.*, 1995).

Materials and Methods

Animals

ISA Brown (Hubbard-ISA, Lyon) fowl hens and males (*Gallus domesticus*), aged 30–40 weeks, and Large White BUTA (British United Turkeys of America, Lewisburg, WV) breeder turkeys (*Meleagris gallopavo*), aged 30–45 weeks, were used in this study. Experiments involving fowl and turkeys were conducted at INRA (Nouzilly) and at ARS (USDA, Beltsville, MD), respectively. Birds were maintained in environmentally controlled houses on a 14 h light:10 h dark photoperiod and were housed individually either in cages (fowls and turkey hens) or in groups of 8–10 in pens (male turkeys). Feed and water were provided *ad libitum*. Semen was collected by abdominal massage (Burrows and Quinn, 1937) and spermatozoa were stained before inseminations as described below. Hens were killed 24 h after the second AI was performed by injection of 2 ml 6% (w/v) sodium pentobarbital into the wing vein and exsanguination (fowl hens), or were killed by cervical dislocation (turkey hens).

Staining of spermatozoa and sperm motility estimates

Semen samples from eight to ten male fowl were pooled. Spermatozoa were labelled by diluting 1 ml semen with 2 ml Lake's diluent (Lake and Ravie, 1982) and adding 50 µl of 1 mg Hoechst 33342 ml^{-1} (Molecular Probes, Eugene, OR). The diluted fowl semen was placed on an orbital shaker at 120 r.p.m. for 4 h at 4°C before use. Semen from seven male turkeys was pooled and spermatozoa were labelled by diluting 1 ml semen with 3 ml Beltsville poultry semen extender (BPSE; Continental Plastics, Delvan, WI) and adding 20 µl of 10 mg Hoechst 33342 ml^{-1}. The diluted turkey semen was placed on an orbital shaker at 120 r.p.m. for 4 h at 22°C to stain the sperm nuclei.

For the different lipophilic dyes, DiQ, $DiOC_{16}$, DiI, DiI-SP and CellTracker™ Orange (all obtained from Molecular Probes, Eugene, OR), staining of spermatozoa was performed according to manufacturer's recommendations at concentrations ranging from 0.5 µmol l^{-1} to 50.0 µmol l^{-1}. After each incubation, 100% of spermatozoa were stained with Hoechst 33342. For the remaining stains, a minimum of 100 spermatozoa in duplicate was evaluated at $\times$ 40 using dual fluorescence and phase contrast microscopy.

Sperm motility estimates were performed in duplicate by the same person for both the fowl and turkey spermatozoa, using the swirling method. Approximately 20 µl of diluted stained and unstained semen was placed on a slide and viewed at $\times$ 40 using dual fluorescence and phase contrast microscopy.

Fertility

A total of 30 fowl hens or 30 turkey hens were inseminated once with 200×10^6 stained ($n = 15$ hens) or unstained ($n = 15$ hens) spermatozoa to establish that staining with Hoechst 33342 did not affect the fertilizing ability of spermatozoa. Eggs were incubated and candled after 7 days (fowl) or 10 days (turkey) of incubation. Eggs considered as infertile were opened and evaluated by stereomicroscopy to assess possible early embryo mortality. Duration of fertility was determined as the number of days from the day after insemination to oviposition of the last fertilized egg (Romanoff and Romanoff, 1963).

Table 1. Efficacy of different fluorescent stains for labelling spermatozoa from several species

Stain	Concentration	Percentage of stained cells (%)[a]	Percentage of sperm motility[b]	Species	Reference
DiQ	2.5 μmol l^{-1}	nr	nr	Mouse	Youakim *et al.*, 1994
	75.0 μmol l^{-1}	66	43	Bull	Miller *et al.*, 1998
	20.0–50.0 μmol l^{-1}	10–40	30	Fowl	Present study
$DiOC_{16}$	75.0 μmol l^{-1}	73	46	Bull	Miller *et al.*, 1998
	20.0–50.0 μmol l^{-1}	10–30	20	Fowl	Present study
DiI	9.0 μmol l^{-1}	< 10	< 10	Fowl	Present study
DiI-SP	11.5 μmol l^{-1}	< 10	< 10	Fowl	Present study
CellTracker™ Orange	0.5 μmol l^{-1}	< 10	< 10	Fowl	Present study
Hoechst 33342	7.6 nmol l^{-1}	nr	100	Horse	Thomas and Ball, 1996
	7.6 nmol l^{-1}	100	100	Bull	Johnson *et al.*, 1987
	116.0 nmol l^{-1}	nr	nr	Fowl	McDaniel *et al.*, 1997
	25.0 nmol l^{-1}	100	100	Fowl	Present study
	90.0 nmol l^{-1}	100	100	Turkey	Bakst, 1994
	77.0 nmol l^{-1}	100	100	Turkey	Present study

[a]Staining was performed according to manufacturer's recommendations or as indicated in the reference.
[b]Sperm motility was estimated by the swirling technique or as indicated in the reference.
nr: not reported.

AI procedures

Tandem inseminations were performed at 24 h intervals in fowl hens using 200×10^6 spermatozoa for each insemination. In half of the experimental hens, the first AI was performed using stained spermatozoa and the second AI was performed using unstained spermatozoa. The other half of the experimental hens was inseminated in the reverse order (first with unstained spermatozoa and then with stained spermatozoa). The same procedure was followed for turkey hens, except that 275×10^6 stained or unstained spermatozoa were used in the AIs.

Tissue preparation and microscopy

Uterovaginal junction mucosa containing SST was removed and folds containing the SST were separated from the underlying muscularis mucosa. Small pieces (approximately 2 mm^2) of mucosa containing SST were spread on a glass slide, moistened with 0.9% (w/v) saline and covered with a glass coverslip. Fluorescent microscopy (Axioplan 2 Zeiss microscope) was performed at × 40 using a DIC filter. Individual SSTs ($n = 50$–100 per hen) were classified as containing stained spermatozoa, unstained spermatozoa, mixed (both stained and unstained) spermatozoa or not containing spermatozoa. Dual transmitted light and fluorescent images were acquired with a kappa image capture system (Fisher Bioblock Scientific, Illkirch) or with a Zeiss LSM 410 laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY) using a C-apo × 40 water immersion objective (1.2 NA) at a zoom factor of two. Bright field transmitted light images were obtained by illumination of the samples with the 633 nm line of a helium-neon laser and captured with the transmitted light detector. Hoechst 33342-labelled nuclear chromatin was excited with the 351 nm line of an argon ion laser, and the emitted light was passed through a 397 nm long-pass filter. The individual optical sections were pseudo-coloured and digitally recombined into a single composite image using LSM software (Carl Zeiss Inc.).

Statistical analysis

Data were reported as mean ± standard error. The fertility data were analysed by a paired *t* test using the Prism program (GraphPad Software, Inc., San Diego, CA). The sperm stratification data were analysed by the maximum likelihood method for the analysis of generalized logits in Proc Catmod (SAS/STAT®, Version 8; SAS Institute, Inc., Cary, NC). A P value of < 0.05 was considered significant.

Results

Several fluorescent lipophilic tracers that successfully stain mammalian sperm membranes (DiQ, $DiOC_{16}$, DiI, DiI-SP) either did not stain fowl or turkey spermatozoa, or stained the spermatozoa, but greatly impaired sperm motility (Table 1). Staining was unsuccessful when DMSO (dimethylsulphoxide), DMF (dimethylformamide) or 100% ethanol was used as a solvent or when a probe that is cleaved into a

Table 2. Effect of insemination with Hoechst 33342-labelled spermatozoa on egg production, fertility and the hatching ability of eggs in fowl and turkey hens

	Fowl hen		Turkey hen	
	Unstained spermatozoa	Stained spermatozoa	Unstained spermatozoa	Stained spermatozoa
Number of hens	30	30	30	30
Number of eggs laid	255	259	236	240
Embryo mortality < 7 days	5	4	5	1
Embryo mortality 7–18 days	1	1	1	0
Embryo mortality > 18 days	7	4	0	1
Number of eggs hatched	112	130	40	37
Maximum duration of fertility (days)	18	20	31	31

Concentration of Hoechst 33342: fowl: 25 nmol l^{-1}; turkey: 77 nmol l^{-1}.

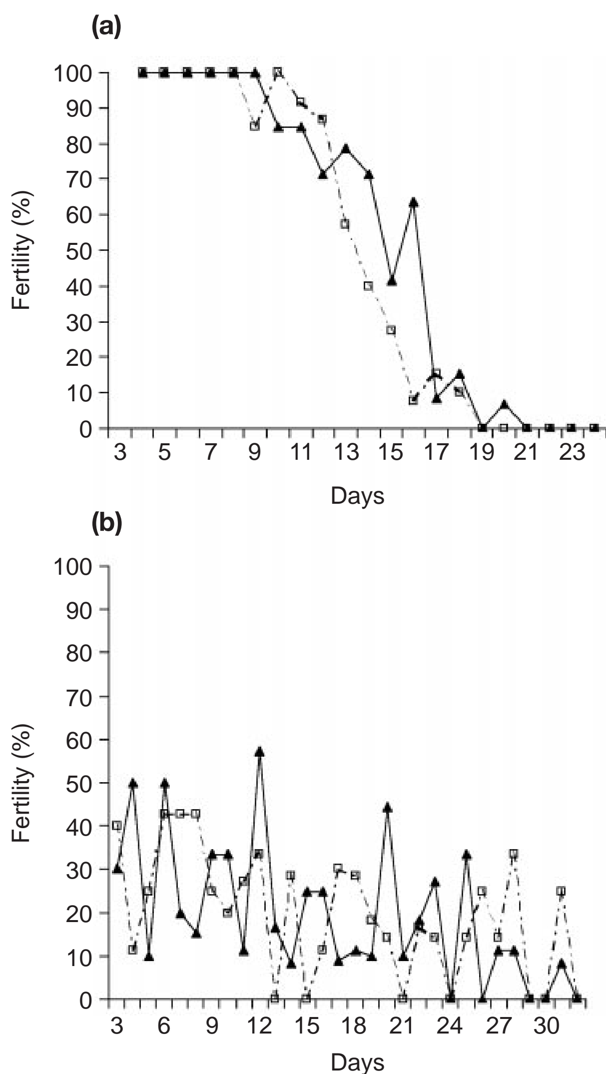

Fig. 1. Fertility of (a) fowl hens (*Gallus domesticus*) and (b) turkey hens (*Meleagris gallopavo*) after one insemination with 200×10^6 unstained (□, control) spermatozoa or Hoechst 33342-stained (▲) spermatozoa (25 nmol l^{-1}), $n = 30$ per group.

fluorescent product inside cells (CellTracker™ Orange) was used. Of the several dyes tested, only Hoechst 33342 stained fowl and turkey sperm nuclei without affecting sperm motility. There were no significant differences within fowl and turkey hens inseminated with stained or unstained semen in the percentage of fertility, number of eggs laid, embryo mortality or the number of eggs hatched (Fig. 1; Table 2).

Spermatozoa from tandem inseminations generally segregated into different SST, regardless of species. In SST that contained mixed (both stained and unstained) spermatozoa, the spermatozoa were distributed randomly, and not layered or stratified into discrete populations (Fig. 2). SST containing mixed populations of spermatozoa (both stained and unstained) were found in approximately 4% of the fowl SST and in approximately 12% of the turkey SST examined, and the order of insemination had no significant effect on this parameter (Table 3).

The order of insemination did not significantly affect the percentages of stained or unstained spermatozoa occupying the SST 24 h after insemination in either the fowl or turkey hens (Table 3). Most of the fowl SST observed contained stained spermatozoa, regardless of the order of insemination. In turkey hens, most of the SST observed either contained stained spermatozoa or did not contain spermatozoa, depending on the order of insemination; however, this difference was not significant. When comparisons were made between the two species, fowl hens were significantly more likely to have SST that contained spermatozoa (either stained or unstained) than turkeys, regardless of the order of insemination. However, turkeys were more likely to have SST that contained mixed (both stained and unstained) spermatozoa than were fowls, regardless of the order of insemination.

Discussion

This study indicates that many of the available fluorescent lipophilic dyes that have been used to label somatic cells (Honig and Hume, 1989; Ledley *et al.*, 1992; Baker *et al.*,

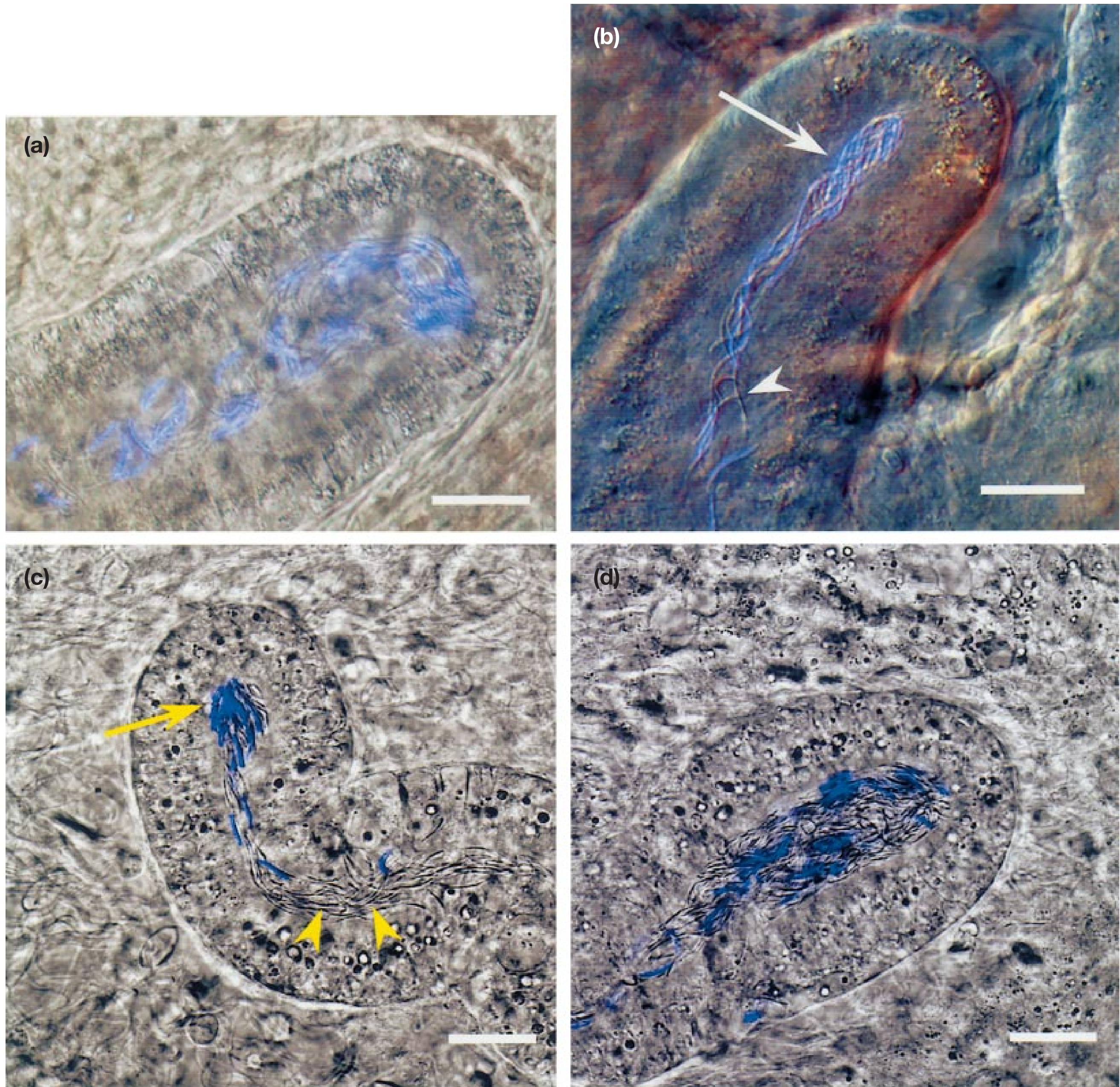

Fig. 2. Photomicrographs of sperm storage tubules containing mixed (both Hoechst 33342-stained and unstained) populations of spermatozoa from (a,b) fowl hens (*Gallus domesticus*) and (c,d) turkey hens (*Meleagris gallopavo*). Arrows indicate Hoechst 33342-stained spermatozoa, arrowheads designate unstained spermatozoa. Scale bars represent 25 μm.

1997; Vercelli *et al*., 2000) were either ineffective in staining or damaging to fowl and turkey spermatozoa. In addition, many lipophilic dyes used to label mammalian spermatozoa (Youakim *et al*., 1994; Miller *et al*., 1998) were inadequate for fowl and turkey spermatozoa. For the purposes of the present study, successful labelling was considered as the incorporation of the fluorescent lipid analogues present in the dyes into or through the sperm membranes without affecting sperm motility. The actual mechanisms involved in the labelling process are not known, although it has been established that poultry spermatozoa differ from mammalian spermatozoa both in glycolipid content and in phospholipid saturation (Parks and Lynch, 1992). Thus, the unique biophysical composition of cell membranes in these species may affect the penetration and binding of lipophilic fluorescent dyes and, therefore, may explain the differential staining capacity between mammalian and poultry spermatozoa.

Despite the lack of two validated staining procedures to label spermatozoa adequately in a differential manner,

Table 3. Distribution of stained and unstained spermatozoa in sperm storage tubules (SST) of fowl and turkey hens after tandem inseminations

	Fowl hens ($n = 7$)		Turkey hens ($n = 6$)	
	Stained–unstained	Unstained–stained	Stained–unstained	Unstained–stained
Percentage of SST containing:				
Stained spermatozoa	59.8 ± 6.0	48.0 ± 11.3	27.6 ± 7.5	42.9 ± 11.0
Unstained spermatozoa	13.7 ± 3.7	19.3 ± 4.0	19.8 ± 6.4	11.7 ± 2.5
No spermatozoa	21.4 ± 2.8	30.3 ± 10.4	36.5 ± 7.8	35.6 ± 9.8
Mixed spermatozoa	5.1 ± 2.8	2.5 ± 1.2	16.1 ± 5.4	9.8 ± 8.6

tandem inseminations with stained (Hoechst 33342) and unstained spermatozoa proved sufficient to determine the effects of staining on hen fertility and also to study competitive fertilization and last-male precedence. Bakst (1994) and McDaniel *et al.* (1997) reported that use of Hoechst 33342-stained spermatozoa resulted in a decrease in the hatching ability of eggs. However, both of these studies used a higher concentration of Hoechst 33342 (90 nmol l^{-1} and 116 nmol l^{-1}, respectively) compared with the present study in which 25 nmol l^{-1} in fowl and 77 nmol l^{-1} in turkeys were used. Higher concentrations of Hoechst 33342 probably contributed to the increased incidence of embryo mortality and to the transfer of fluorescent dye from stained resident spermatozoa to unstained resident spermatozoa and surrounding SST epithelium (Bakst, 1994). In the present study, considerable effort was made to find the minimum concentration of Hoechst 33342 that would successfully label all spermatozoa, but that would not leach into the lumen of SST or affect sperm motility and subsequent egg fertility. No evidence of transfer of Hoechst 33342 between stained and unstained spermatozoa residing in the SST was observed. In addition, no staining of SST epithelial cell nuclei was observed up to 4 days after insemination (data not shown). When higher concentrations of Hoechst 33342 were used to stain spermatozoa used for insemination, SST epithelial cell nuclei were often fluorescent (Bakst, 1998). However, in the present study, fluorescent SST epithelial nuclei were not observed, which strongly supports the assertion that there was no leakage of the Hoechst 33342-labelled spermatozoa within the SST.

There were no significant differences between the control (unstained) spermatozoa and the Hoechst 33342-stained spermatozoa in fowl or turkey hen fertility, number of eggs laid, embryo mortality or number of hatched eggs. In addition, pilot studies were conducted in which the number of spermatozoa trapped in the outer perivitelline layer was determined. This value is positively correlated with the number of spermatozoa residing in the SST (Brillard and Bakst, 1990), with numbers of spermatozoa inseminated (Wishart, 1987) and with flock fertility (Staines *et al.*, 1998). There was no significant difference in the number of perivitelline spermatozoa between hens that had been inseminated with unstained spermatozoa only and hens inseminated with stained spermatozoa only (data not shown). This finding indicates that Hoechst 33342 at the concentrations used in the present study did not affect the ability of spermatozoa to reach and enter the SST, or to reach the site of fertilization.

Compared with fowl, higher percentages of turkey SST did not contain spermatozoa after tandem inseminations. Although highly variable, this finding may be a consequence of the larger population of SST within the turkey (approximately 24 000 SST; Goodrich-Smith and Marquez, 1978) compared with that in fowl (approximately 3000 SST; Brillard *et al.*, 1998). Hens inseminated before the onset of egg production have significantly more SST that contained spermatozoa, and more SST that are filled to maximum capacity than do hens inseminated after the onset of egg production (McIntyre *et al.*, 1982; Brillard and Bakst, 1990; Bakst, 1994). There may be some type of activation of the SST, which occurs just before egg production, at which time most of the SSTs are able to store spermatozoa. After the onset of egg production, hormonal changes (Brillard *et al.*, 1987) or distention of the oviduct associated with egg laying (Bakst, 1994) may interfere with sperm storage in the SST. Events associated with oviposition and ovulation have been shown to influence the rate of sperm release from the SST in fowl hens (Bushman *et al.*, 1985). In addition, certain folds of the uterovaginal junction examined in the present study in both fowl and turkey contained SST that did not contain spermatozoa, or contained either all stained or all unstained spermatozoa. This pattern was observed repeatedly in different regions of the same uterovaginal junction and, therefore, it is unlikely that AI was the cause. Alternatively, this finding may imply the presence of channels in the oviduct through which the spermatozoa travel to the SST and avoid becoming trapped in mucus sheets (for a review of oviductal sperm transport mechanisms, see Bakst *et al.*, 1994).

The present study is the first to distinguish spermatozoa from two different inseminations within the same SST. Although Van Krey *et al.* (1981) described stratification of labelled and unlabelled spermatozoa in the SST, the light micrographs provided were poor. The question of sperm competition, that is, which spermatozoon does actually fertilize the ovum, has been hampered by the inability to visualize directly what is occurring within the SST with

regards to sperm uptake, storage and displacement. The idea of stratification of ejaculates was suggested by Compton *et al*. (1978) as an explanation of last-male sperm precedence ('first in-last out'). Subsequently, Lessells and Birkhead (1990) composed three different mathematical models of sperm competition in an attempt to describe sperm precedence: sperm stratification, passive sperm loss and sperm displacement. These models were tested further by Birkhead and Biggins (1997) and it was determined that passive sperm loss was the most likely explanation for sperm precedence. Likewise, results from the present study and those of Birkhead *et al.* (1995), do not support the stratification model of last-male sperm precedence. However, observations in the present study show that spermatozoa segregate into different SST, or occasionally mix within the same SST. Furthermore, the order of insemination did not significantly affect the percentages of different populations of spermatozoa (in the present study, stained or unstained) within the SST. However, the number of spermatozoa within the SST was not quantified and, therefore, evaluations regarding passive sperm loss or sperm displacement could not be made in this study.

More tubules contained stained rather than unstained spermatozoa, in both the fowl and the turkey. This was an unexpected result that is difficult to interpret. It is possible that visualization of unstained spermatozoa was hindered by the fluorescence of the stained spermatozoa; however, this seems unlikely. It is also possible that the staining process affects sperm entry or exit from the SST. The specific mechanisms of filling and emptying of the SST are unknown, but are affected by sperm motility, the age of the hen and the onset of egg production. In the present study there were no obvious differences in sperm quality, as the stained and unstained spermatozoa both came from the same pool of semen, and the same group of males was used for each insemination. In addition, no differences were observed in fertilization parameters or in the hatching ability of eggs. The significance of the finding that more tubules contained stained than unstained spermatozoa is worthy of further study.

The present study has shown that lipophilic dyes that stain mammalian spermatozoa without inhibiting sperm motility do not perform in the same manner with poultry spermatozoa, emphasizing the role of species differences in sperm membrane composition. As first shown by Bakst (1994), Hoechst 33342 can be used successfully to label turkey spermatozoa to improve understanding of the dynamics of oviductal sperm transport and storage. In the present study, this procedure was modified for use with both fowl and turkey spermatozoa, and concentrations of stain were used that did not affect fertility or transfer of the label to adjacent unstained spermatozoa or tissue.

In conclusion, results from sperm competition studies reported here indicate that spermatozoa generally segregated into different SST in both fowl and turkey hens after tandem inseminations; sperm stratification within the SSTs was not observed. SST containing mixed populations of both stained and unstained spermatozoa were found in 4% of fowl SST and in 12% of turkey SST examined. Thus, the mechanism of last-male precedence does not appear to be due to the stratification of spermatozoa within the SST of fowl or turkey hens.

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