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Comparisons of sperm storage tubule distribution and number in 4 strains of mature broiler breeders and in turkey hens before and after the onset of photostimulation¹

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ABSTRACT The biological basis of sustained fertility in broiler and turkey hens is their capacity to store sperm in the oviductal sperm storage tubules (SST) located in the uterovaginal junction. The objectives of this study were to determine if the numbers of SST varied between 4 strains of broiler breeders and determine the number of SST in the turkey before (less than 9 d of photostimulation) and after (up to 22 d of photostimulation and laying) photostimulation. No statistical differences were observed in SST numbers in the 4 strains of broilers examined or in turkey hens before and after the onset of egg production. The mean

numbers of SST for broilers and turkeys were 4,893 and 30,566, respectively. We conclude that any differences between the fertility of the 4 broiler breeder strains examined cannot be explained by differences in SST numbers. However, differences in the duration of fertility between broilers and turkeys are, in part, related to their respective numbers of number of SST. Furthermore, we conclude that turkey SST are morphologically differentiated and functional before the onset of photostimulation and while the oviduct is morphologically undeveloped.

Key words: avian, oviductal sperm storage, poultry, duration of fertility

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INTRODUCTION

In poultry, sustained fertility is dependent on the hen's capacity to store and release sperm from the sperm storage tubules (SST) over the course of successive daily ovulatory cycles. The SST are tubular invaginations of the uterovaginal junction's (UVJ) surface epithelium. However, unlike the UVJ, which is lined by a pseudostratified layer of ciliated and nonciliated secretory cells, as well as ciliated and nonciliated secretory cells, the SST epithelium is simple columnar, nonciliated, and nonsecretory.

Recently, Birkhead and Brillard (2007) summarized the major barriers to fertilization in birds after sperm transfer (either by artificial insemination or natural mating) into the cloaca or vaginal orifice. These barriers

begin immediately after sperm transfer and include the following phenomena: 1) full or partial failure of sperm to be transported to the SST, 2) failure of sperm to enter or egress from the SST, 3) failure of sperm to reach the site of fertilization at the infundibulum, 4) failure of sperm to penetrate the perivitelline layer (PL) of the ovum, and 5) failure of the sperm to locate or fuse, or both, with the female pronucleus (i.e., absence of syngamy).

Considering the list above, full or partial failure of sperm to be transported to the SST is, in part, influenced by sperm mobility (Froman et al., 1997) and the vagina's capacity to orchestrate an intense sperm selection process (Bakst et al., 1994). The vagina's sperm selection mechanism significantly reduces the percentage of sperm deposited in the distal vagina from entering the SST. Only 2% of the turkey (Brillard and Bakst, 1990) and 1% of the chicken (Brillard, 1993) sperm inseminated were recovered from the respective SST within 24 h of insemination.

Notwithstanding, this relatively small number of sperm entering the SST was positively correlated with

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numbers of sperm embedded in the outer PL and the duration of fertility (Brillard and Bakst, 1990). Bramwell et al. (1996) monitored rates of sperm penetration of the inner PL over the course of egg production in broiler breeder flocks. Their data revealed that a subpopulation of hens (10 to 20%) have reduced PL sperm penetration values regardless of insemination dose or time in egg production. Bramwell (personal communication) hypothesized that poor fertility in broiler flocks could be attributed to this same subpopulation of hens. Fewer inner PL sperm holes and the corresponding lower fertility could be a result of any one or a combination of the first 4 barriers to fertilization cited above. However, when isolating the UVJ folds containing SST, Bakst (unpublished data) noticed differences in the number of SST within strains of both chicken and turkeys. Logically, one would expect that with a greater number of SST and, presumably, a greater number of sperm reaching the site of fertilization, there would be a corresponding longer fertile period. This has yet to be proven.

Three studies reported the number of SST for several different avian species. Goodrich-Smith and Marquez (1978) estimated between 20,000 and 24,000 SST for the turkey breeders, whereas Birkhead and Hunter (1990) observed 1,000 to 2,000 SST in finches. Birkhead and Moller (1992) conducted a comprehensive survey of SST numbers in 11 domestic and nondomestic bird species including the chicken (domestic chicken, *Gallus domesticus*) and turkey (domesticated turkey, *Meleagris gallopavo*). They reported that the mean numbers of SST were 13,533 and 20,000 for chicken and turkey, respectively.

We report here on the mean numbers of SST, mucosal folds lining the UVJ, and the mean length (mm) of the UVJ mucosal folds with SST in 4 lines of commercial broiler breeder hens of known fertility. [The cumulative commercial fertility for the 4 strains used in this study through 40 wk of age was 96% and through 65 wk of age was 95% (breeder manuals).] In addition, we also report on the number of SST in turkey hens from 0 to 9, 14 to 15, and 21 to 22 d after the onset of photostimulation. The implications of our observations are discussed in the context of sustained fertility and breeding behavior.

MATERIALS AND METHODS

Broilers

Two hundred broiler breeder pullets from each strain (T0, T1, T3, and T8) were obtained from a commercial hatchery and reared into egg production according to industry standards. Hens were photostimulated (16L:8D) at 21 wk of age. At 33 to 38 wk, 6 hens per group were randomly selected and killed.

After exposing the ovary and oviduct, the number of ovarian yellow yolk follicles greater than 10 mm in diameter was recorded. To isolate the uterus and va-

gina, the paired segments were removed as 1 piece and the vagina was dissected free of connective tissue. The UVJ and vaginal mucosae were then exteriorized and the number of mucosal folds at the central region of the UVJ was counted under a stereomicroscope. Six mucosal folds circumferentially equally spaced in the UVJ luminal mucosa were isolated without the underlying muscularis mucosa, placed on a stereomicroscope, and the length of the UVJ fold containing SST was measured to the nearest millimeter. Each fold was further trimmed to only include the mucosa with SST. (Throughout the remainder of this paper, reference to the UVJ folds will imply only the folds containing SST, unless otherwise stated.) The UVJ folds were placed in 1 mM EDTA in PBS and incubated 15 min at 38°C on a rotary shaker (100 rpm). After decanting the EDTA solution, folds were rinsed in PBS and transferred to a dissecting dish (Petri dish with a 3-mm layer of dental wax lining its base) containing fresh PBS. Folds were stretched and pinned with the ciliated surface down to maximize unobstructed viewing of the SST. After further removal of connective tissue, the PBS was decanted off and neutral-buffered formalin (NBF) was added. After 1 h at ambient temperature, the NBF was replaced with PBS and folds were placed on glass slides, cilia side down, and covered with 24 × 40 mm coverslips. The coverslip was gently pressed to squash the UVJ folds. The edges of the coverslip were sealed with nail polish and when dried, each slide was examined using phase contrast microscopy. Quantification of the SST was done by either counting directly off a real-time image or from anterior to posterior serial sequences of digitized images of the SST folds.

Turkeys

Hybrid Converter (Kitchener, CA) turkey hens were raised according to Hybrid's guidelines and photostimulated (14L:10D) at 31 wk of age. At intervals of 2 to 9 d, 14 to 15 d, and 21 to 22 d (treatment groups) after the onset of photostimulation, 6 hens were killed and their oviducts exteriorized. The same technique was used in the preparation of the turkey's UVJ folds to count SST, except the folds were not fixed and were counted within 24 h. The number of SST per fold was determined from anterior to posterior serial sequences of digitized images of the SST fold. For histology, turkey UVJ folds were fixed in NBF and processed for routine paraffin embedding, sectioned at 5 to 7 μm, and stained with hematoxylin.

Statistical Analysis

For broilers, the variables, which included the number of yellow yolk follicles, number of UVJ mucosal folds, number of SST per fold, total number of SST in UVJ, and the length of UVJ fold containing SST, were analyzed separately as 1-factor linear models using PROC MIXED (SAS Institute, 2008) with broiler

strains (T0, T1, T3, and T8) as the treatment. The assumptions of the models were checked and ANOVA, means, SEM, and mean comparisons were determined. The mean comparisons were made with Sidak-adjusted *P*-values so that the experimentwise error was 0.05.

For turkeys, the variables, which included the number of yellow yolk follicles, number of UVJ mucosal folds, number of SST per fold, total number of SST in UVJ, and the length of UVJ fold containing SST, were analyzed separately as 1-factor linear models using PROC MIXED (SAS Institute, 2008) with the days postlighting groups as the treatment. The assumptions of the models were checked. For the number of UVJ mucosal folds and the number of SST per fold, the values were log-transformed to meet the normality assumption. When needed, the variance-grouping technique was used to correct for variance heterogeneity. The ANOVA, means, SEM, and mean comparisons were determined. Because the ANOVA tests were not statistically significant for the number of UVJ mucosal folds and number of SST per fold, the means and SEM are presented for the nontransformed values. Sidak-adjusted *P*-values were used for the mean comparisons to hold the experimentwise error rate at 0.05.

RESULTS

Comparative Morphology of the SST

The SST in the proximal region of the UVJ folds in broiler hens were conspicuously short, some no more than 40 μm in length (Figure 1A). Short SST in the turkey UVJ were similar to those of the broiler as well as to the short SST reported previously (Bakst and Vinyard, 2002). Histologically, the epithelia forming the short SST in both species were more cuboidal than columnar and lacked the extensive lipid content of the columnar epithelia lining the SST in the middle and distal regions of the UVJ.

Within the middle region of the broiler's UVJ folds (Figures 1B and C), the SST were up to 700 μm long and straight to curvy. In contrast, the turkey SST in the middle region were up to 900 μm in length and were characterized by their pleomorphic morphology consisting of straight, convoluted, or coiled SST (Bakst, 1987; Bakst and Vinyard, 2002). The SST in the distal region of the UVJ folds possessed characteristics of the middle region SST but were less densely distributed, with their presence gradually dissipating in the vagina (Figure 1D).

Interestingly, a few squash preparations revealed microfolds on the surface mucosa leading to the orifice of the SST in both broilers and turkeys. The microfolds were narrow furrows up to 800 μm long consisting of tightly apposed ciliated epithelial cells (Figure 1E). The SST orifice was bounded by ciliated cells that had a puckered appearance when viewed under the stereomicroscope. In turkeys, the SST orifice was also fully sur-

rounded by ciliated cells. However, histological sections revealed that the border of ciliated cells lining the SST orifice was occasionally interrupted by an exteriorized extension of the SST epithelium that merged abruptly with the UVJ surface epithelium in the lumen of the UVJ (Figure 1F). In histological sections, microfolds were observed primarily on the lateral surfaces of the primary and secondary mucosal UVJ folds.

Ovarian Yellow Yolk Follicle Numbers and UVJ-SST Observations

Broiler and turkey data for this section are summarized in Tables 1 and 2, respectively. Of the traits listed in Table 1, only the length of the UVJ folds with SST showed statistical variation between broiler hen strains. Broiler hen strain T0 was significantly longer (26.6 mm) than T3 (17.1 mm) and neither were different from strains T1 or T8 (Table 1). With turkeys, the length of the UVJ folds in hens with less than 10 d of photostimulation was significantly shorter than the other 2 groups (Table 2). All broiler strains possessed 5 to 6 ovarian yellow yolk follicles greater than 10 mm in diameter with no statistical variation between lines. The number of yellow yolk follicles was significantly different (Table 2) between each turkey treatment group (days postlighting). The number of yellow yolk follicles in turkey hens at 21 to 22 d of postphotostimulation (hens in this group were in egg production) was twice that observed in broilers.

The number of mucosal folds comprising the UVJ mucosae in the broiler ranged from 16 to 18 folds. The number of mucosal folds between the turkey treatment groups approached significance ($P = 0.07$) and ranged from 21 to 28 folds, with the most immature group (less than 10 d of photostimulation) having fewer folds than the other more sexually mature groups.

There were no significant differences in the total numbers of SST in hens observed between the broiler lines or in the turkey treatment groups (Tables 1 and 2). Interestingly, 2 turkey hens examined after 2 d of photostimulation (included in the less than 10 d group) had no yellow yolk follicles. However, their UVJ-SST folds averaged 14 mm in length and possessed an average of 18,400 SST. The mean numbers of SST were 4,893 in broilers and 30,566 in turkeys.

DISCUSSION

Our objectives were 2-fold: first, determine the number of SST in 4 strains of broilers in egg production, and second, determine the number of SST in turkey hens from the onset of photostimulation to the onset of egg production. The absence of significant differences between the numbers of SST observed and the time interval periods after photostimulation (less than 10, 14 to 15, and 21 to 22 d) was suggested in previous work.

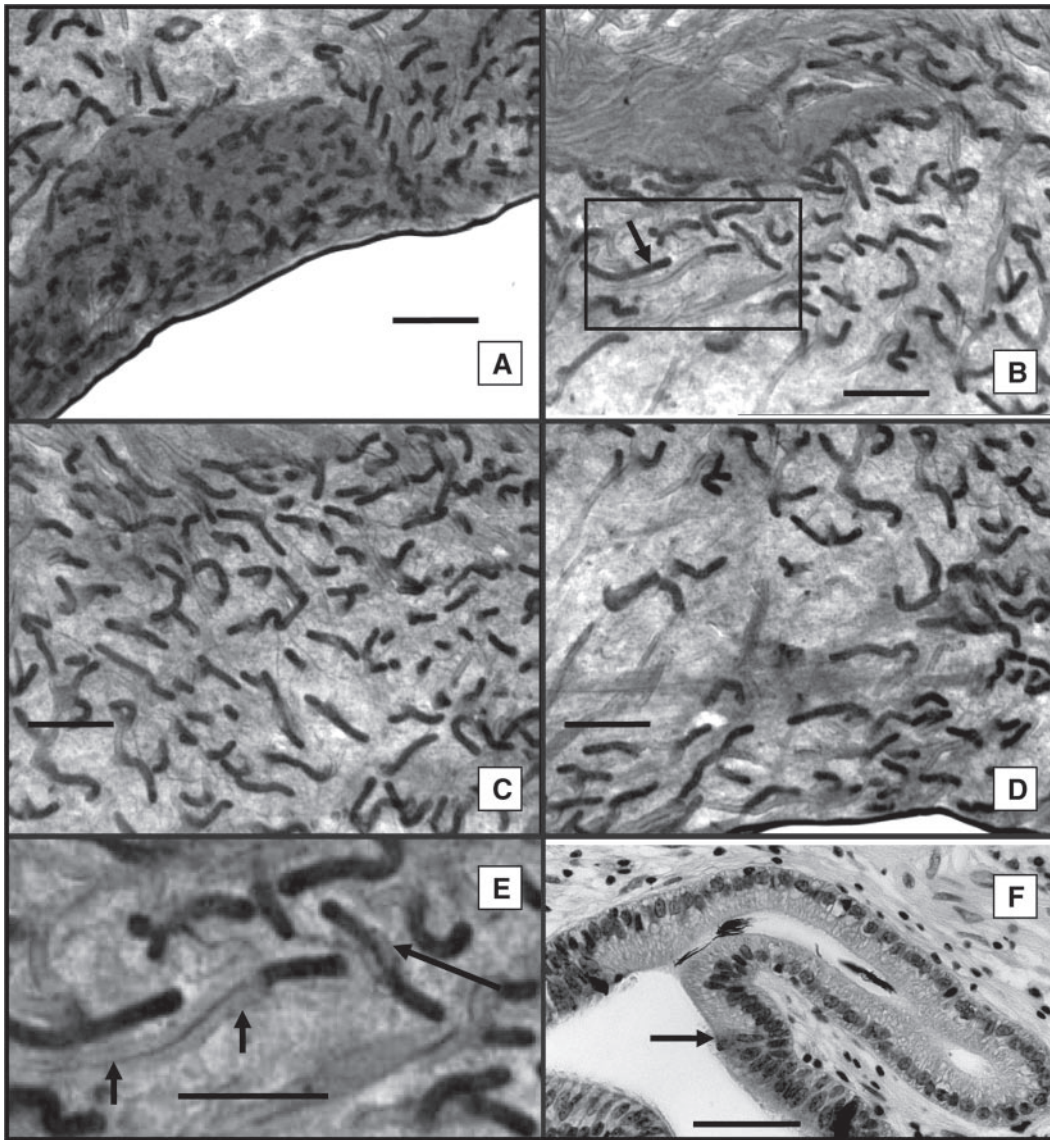


Figure 1. Sperm storage tubules (SST) in broiler breeders and turkeys. A) In this squash preparation of a broiler's proximal region of the uterovaginal junction (UVJ) folds, the SST were conspicuously short, some no more than 40 μm in length. Bar = 500 μm . B and C) In these squash preparations, SST in the middle regions of the broiler's UVJ folds varied in length, with some reaching 700 μm in length (arrow in panel B), and exhibited a straight or curved morphology. Microfurrows (highlighted within box in panel B and at increased magnification in panel E) are also observed leading to the SST orifices. Bar = 500 μm . D) Broiler hen SST in the distal region of the UVJ folds in this squash preparation possessed characteristics of the middle region SST but were less densely distributed. Bar = 500 μm . E) Higher magnification of the box in panel B, which clearly shows a microfurrow leading to the SST orifices (short arrows). An oval, puckered, slit-like opening is the common orifice for 2 SST (long arrow). Bar = 300 μm . F) The extension of turkey's SST epithelium into the UVJ lumen and the abrupt transition to the ciliated epithelium of the UVJ lumen (arrow) is readily apparent in this histological preparation. Sperm are observed within the SST. Bar = 50 μm .

Bakst (1988) inseminated turkey hens (33 wk of age) on the day of photostimulation and observed an average of 67% fertility 5 wk after the onset of photostimulation. Furthermore, the mean weights of the ovary and oviduct at the onset of photostimulation were 14.8 and 28.1 g, respectively. Yet, by 5 wk after the onset of photostimulation, the mean weights of the ovary and oviduct were 151.5 and 114.4 g, respectively (Bakst, 1988). The observations in the present study support the conclusion by Bakst (1988) that "...morphologically differentiated SSTs are functional in 33-week-old hens regardless of the status of the ovary."

Interestingly, Holm and Ridderstrale (2002) observed in Japanese quail that the initial differentiation of the SST coincided with the differentiation of the maginal and uterine tubular glands. They further observed sperm in the SST of hens with large yolk-filled follicles but before the onset of egg production. Although Holm and Ridderstrale (2002) suggested that SST may be functional before the maturation of the ovary and oviduct, the turkey uniquely has the capacity to store sperm in differentiated and functional SST before the onset of yellow yolk formation. Therefore, we agree with the conclusion of Holm and Ridderstrale (2002) that ei-

Table 1. Broiler variables, which included the number of yellow yolk follicles (YF), number of uterovaginal junction (UVJ) mucosal folds, number of sperm storage tubules (SST) per fold, total number of SST per hen, and length of UVJ fold containing SST, analyzed separately as 1-factor linear models using PROC MIXED (SAS Institute, 2008) with the broiler strain (strains T0, T1, T3, and T8) as the treatment¹

Item	df	YF		Number of folds		SST per fold		SST per hen		Fold length	
		F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value
ANOVA	3	0.41	0.7450	0.26	0.8567	0.20	0.8962	0.33	0.8057	3.00	0.0562
Strain		YF (n)		Number of folds (n)		SST per fold (n)		SST per hen (n)		Fold length (mm)	
T0		6.3 (±0.7)		16.4 (±1.8)		258.6 (±80.4)		4,172.0 (±1,247.6)		26.6 ^a (±2.3)	
T1		5.5 (±0.2)		17.5 (±0.7)		306.7 (±64.0)		5,255.3 (±1,028.8)		20.7 ^{ab} (±1.5)	
T3		5.5 (±0.7)		17.7 (±1.9)		238.5 (±67.0)		4,521.7 (±1,358.8)		17.1 ^b (±2.3)	
T8		5.7 (±0.7)		18.2 (±0.7)		249.6 (±65.5)		5,624.7 (±809.9)		21.5 ^{ab} (±2.6)	

^{a,b}Treatment means with different superscripts are different at the 0.05 significance level.

¹The ANOVA results and means and mean comparisons are given, with the SEM in parentheses. Sidak-adjusted *P*-values were used for the comparisons to hold the experimentwise error rate at 0.05.

ther the circulating levels of estrogen or progesterone, or both, before the onset of ovarian maturation or some other factors are sufficient to induce SST differentiation in the absence of an ovary with yellow-yolked follicles.

The question raised by these observations is why are functional SST present in the immature oviduct? Bakst (1988) reported that the mean day for the onset of hen courtship behavior (squatting) and the onset of egg production was 7 and 17 d, respectively, after the onset of photostimulation. Thus, from a behavioral perspective, some hens were exhibiting courtship behavior and were presumably receptive to male attempts at copulation in the first week of photostimulation. Intervals of varying lengths of time between copulation and first ovulation are common in Aves. Holm and Ridderstrale (2002) described this phenomenon in Japanese quail. Hatch (1983), noting the reproductive benefits of oviductal sperm storage, described the phenomenon of delayed fertilization in feral birds. He applied the concept of delayed fertilization to those birds that copulate frequently before the first ovulation, separate to forage, and then return to the nest days or weeks later to begin egg production. Delayed fertilization serves another purpose as well. Bakst and Bird (1987) suggested that the interval between the onset of mating behavior and

the first ovulation, which can be days or weeks, permits the accumulation of a population of selected fit sperm in the SST, which then populate the site of fertilization just before the first and successive ovulations.

In both species, although not statistically significant, there was variation in mucosal fold numbers. This variation between the turkey treatment groups, which approached significance (*P* = 0.07), could be a result of continued maturation of the oviduct and, in both species, a result of some mucosal folds randomly bifurcating while other folds are gradually merging with or emerging from the UVJ mucosal floor. Interestingly, turkey hens with the shortest UVJ folds (photostimulated less than 10 d) had comparable SST numbers to the longest UVJ folds (21 to 22 d of photostimulation). With no significant differences in the number of SST between the turkey hens groups, we speculate that the number of SST in the UVJ folds was established before the onset of photostimulation.

The fertile period, the number of consecutive days that hens lay fertilized eggs, is about 2 to 3 wk in chickens (Beaumont et al., 1992) and 10 to 15 wk in turkeys (Christensen and Bagley, 1989). A factor contributing to this difference in sustained fertility is our observation that turkeys (30,566 SST) have 6.2 times the num-

Table 2. Turkey variables, which included the number of yellow yolk follicles (YF), number of uterovaginal junction (UVJ) mucosal folds, number of sperm storage tubules (SST) per fold, total number of SST per hen, and length of UVJ fold containing SST, analyzed separately as 1-factor linear models using PROC MIXED (SAS Institute, 2008) with the days postlighting groups (less than 10, 14 to 15, and 21 to 22 d) as the treatment¹

Item	df	YF		Number of folds		SST per fold		SST per hen		Fold length	
		F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value
ANOVA	2	25.83	<0.0001	3.64	0.0672	1.12	0.3600	0.04	0.9580	7.20	0.0128
Days postlighting		YF (n)		Number of folds (n)		SST per fold (n)		SST per hen (n)		Fold length (mm)	
<10		2.0 ^c (±1.4)		20.8 (±1.4)		1,508.2 (±249.1)		31,628.6 (±6,184.2)		16.5 ^b (±2.0)	
14 to 15		6.5 ^b (±1.1)		21.5 (±0.9)		1,362.4 (±70.4)		29,091.8 (±1,225.4)		24.8 ^a (±1.8)	
21 to 22		13.5 ^a (±0.8)		27.7 (±2.2)		1,120.7 (±244.1)		30,978.9 (±7,362.4)		31.0 ^a (±3.9)	

^{a-c}Treatment means within a column with different superscripts are different at the 0.05 significance level.

¹The ANOVA results and means and mean comparisons are given, with the SEM in parentheses. Sidak-adjusted *P*-values were used for the comparisons to hold the experimentwise error rate at 0.05.

ber of SST than broilers (4,893 SST). Not only is the number of SST greater in turkeys, but the length of the turkey SST is greater than that of the broiler (Birkhead and Moller, 1992). Furthermore, the maximum number of sperm recovered from turkey SST was 4.1×10^6 (Brillard and Bakst, 1990), representing 1.6% of the sperm number (250×10^6) inseminated, whereas the maximum number of sperm recovered from broiler SST was 2.2×10^6 , 0.9% of the sperm number (250×10^6) inseminated (Brillard, 1993). Thus, the difference in the length of the fertile period between broilers and turkeys is predominantly due to the increased numbers of SST and the subsequent increase in sperm storage capacity of the turkey compared with the broiler. Also contributing to the relatively shorter fertile period of broilers was that sperm release from their SST is much faster than turkeys based on quantification of PL sperm holes, sites where sperm hydrolyzed the PL overlying the germinal disc (Wishart, 1985).

In their comprehensive study on anatomical characteristics of SST relative to body size, sperm length, and SST distribution frequency in several domestic and nondomestic birds, Birkhead and Moller (1992) suggested the following: the number of SST ranged from a low of 500 SST (budgerigar, *Melopsittacus roseogrisea*) to a maximum of 20,000 SST [commercial turkey; Goodrich-Smith and Marquez (1978)], larger birds tended to have more SST than smaller birds, duration of sperm storage was not significantly related to SST numbers or SST volume (number of SST \times length of SST), and the number and length of the SST were positively correlated with the number of sperm in its respective ejaculate and length of the spermatozoa, respectively. Birkhead and Moller (1992) elected not to discuss the proposed lack of correlation between SST number and duration of fertility for lack of data on the number of active SST, those capable of sperm storage. However, such data are now available for turkeys if only limited to the period before and after the onset of egg production. Bakst (1994) observed that 94% of the SST contained sperm (active SST) in turkey hens inseminated and examined before the onset of egg production. In contrast, hens inseminated initially after the onset of lay and then examined revealed that 78% of the SST contained sperm. Thus, in turkeys, approximately all of the SST were active, receptive to sperm, just before the onset of egg production. It is not known if there are periods in the reproductive cycle of other avian species that SST activity is maximized. Interestingly, the maximum filling of the turkey SST after artificial insemination before the onset of egg production corresponds to the same period when Large White turkey hens "...display an intense desire to mate..." (Carte and Leighton, 1969).

The above observations and correlations of Birkhead and Moller (1992) were supported by their collective observations of 11 avian species (albeit only 1 or 2 specimens were examined for most species) and discussed in the context of an evolutionary-behavioral perspective.

Of the correlations discussed by Birkhead and Moller (1992), some were valid when the broiler is compared with the turkey: the larger bird did have a greater number of SST than the smaller bird and the number of SST was positively correlated with the number of sperm in the ejaculate. However, other conclusions by Birkhead and Moller (1992) were not valid when simply comparing the broiler and turkey. For example, when one compares the broiler to the turkey, the duration of fertility is positively correlated to the number of SST in the UVJ. Possible confounding factors leading to the differing conclusions could be that we observed more SST in the turkey ($\sim 30,600$) than reported by Goodrich-Smith and Marquez (1978; 20,000) and fewer SST in broilers ($\sim 4,900$) than those reported for chickens (strain not mentioned; 13,533) by Birkhead and Moller (1992).

To summarize, no statistical differences were observed in SST numbers in the 4 strains of broilers examined or in turkey hens before and after the onset of photostimulation. Thus, possible differences in fertility or the duration of fertility between the broiler strains examined cannot be attributed to their SST numbers. We conclude that for the broiler and turkey, the duration of fertility is, in strong part, directly related to the number of SST in their respective UVJ folds. Furthermore, we conclude that turkey SST are fully differentiated and functional before the onset of photostimulation. Whether these conclusions are applicable to nondomestic birds needs to be determined.

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