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NUMBERS AND DISTRIBUTION OF SPERM IN THE UTEROVAGINAL SPERM STORAGE TUBULES OF THE ZEBRA FINCH[1]

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Abstract. The number and distribution of sperm in the sperm storage tubules (SSTs) in the uterovaginal junction (UVJ) of the Zebra Finch (*Poephila guttata*) oviduct was examined under three copulation patterns: single insemination, multiple insemination by the same male, and multiple insemination by two males in sequence. The proportion of SSTs containing sperm varied markedly between birds (range = 22–99%), and was positively correlated with the mean number of sperm per SST. All SSTs were apparently 'active' and capable of storing sperm. Multiply mated females had higher sperm loads than those mated once, with one exception. The maximum number of sperm in the UVJ of a single bird was 62,000; the minimum, 540. Within birds, sperm were distributed nonrandomly between SSTs. In all birds most sperm were located at the distal end of SSTs in a single clump. There was no evidence that sperm from separate ejaculates from either the same or different males remained stratified within SSTs. The degree of stratification was low and was determined by the number of sperm in the UVJ. It seems unlikely that stratification of sperm within SSTs accounts for last male sperm precedence in the Zebra Finch.

Key words: *Zebra Finch;* Poephila guttata; *sperm; sperm storage tubule; sperm competition.*

INTRODUCTION

The females of several bird species are known to store sperm following copulation, prior to using it to fertilize their eggs (Howarth 1974, Birkhead 1988). The primary storage site is at the uterovaginal junction (UVJ), where sperm are held in sperm storage tubules (SSTs) (Bakst 1987). The SSTs of domestic chickens and turkeys have been studied extensively, but the way in which sperm enter, are maintained, and leave the SSTs is poorly understood (Zavaleta and Ogasawara 1987). Sperm storage tubules are present in all nondomesticated species examined to date (Hatch 1983, Shugart 1988), but the manner in which they store sperm has not previously been examined.

The fact that birds store sperm prior to fertilization may predispose them to sperm competition (Birkhead 1988). If a female bird is inseminated by more than one male, the sperm from the last mating usually has precedence over those introduced earlier (Birkhead 1988, Birkhead et al. 1988a). This pattern, referred to as last male sperm precedence, is thought to occur because sperm from successive inseminations remain stratified within the SSTs, with a last in-first out system operating (Compton et al. 1978, Van Krey et al. 1981).

In the Zebra Finch (*Poephila guttata*), the only passerine in which sperm storage and sperm competition have been investigated, females can store sperm for a median duration of 10 days (maximally 13 days; Birkhead et al. 1989). Extra-pair copulation occurs among wild Zebra Finches and can result in extra-pair paternity (Birkhead et al. 1988b; Birkhead et al., unpubl.). Our experiments have shown that when two males copulate with a female during a single breeding cycle, last male sperm precedence occurs (Birkhead et al. 1988a, 1989). Elsewhere we have presented a quantitative description of the numbers and gross structure of SSTs in the Zebra Finch (Birkhead and Hunter 1990a). In the present paper we describe the numbers and distribution of spermatozoa in Zebra Finch SSTs. The manner in which sperm are distributed within and between SSTs may help us understand the way that sperm are stored and the way in which sperm from different males compete to fertilize eggs. The aim of this and our other studies is to understand the mechanisms involved in sperm storage and sperm precedence.

METHODS

Zebra Finches used in this study were domesticated birds bred at Sheffield University from a

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population established in 1985. Birds were maintained on a L:D regime of 14:10 at 20°C, and provided with food and water ad libitum. Under these conditions Zebra Finches are capable of breeding throughout the year.

We examined the UVJ region of eight Zebra Finch oviducts: birds 1, 2, and 3 received a single insemination, birds 4, 5, and 6 received multiple inseminations from the same male, and birds 7 and 8 received multiple inseminations from two different males in succession. The sample sizes were small to minimize the number of birds sacrificed. All females had reproduced at least once before but had not been with a male for 6 months prior to the present study. Prior to being used in the present study males were maintained in all-male flocks in an aviary and had bred at least once before. No male had been with a female for 4 weeks prior to the present study. Birds of both sexes were in reproductive condition when the study was carried out.

During a breeding cycle a female Zebra Finch typically copulates with her partner about 12 times. Defining day 0 as the day on which the first egg of the clutch is laid, most copulations occur during days −5 to 0. A single insemination is sufficient to fertilize the entire clutch of six eggs (Birkhead et al. 1988a, 1989). In the present study we compared the SSTs of females copulating (and being inseminated) once with a fertile male, with birds which had copulated several times. The birds copulating only once (birds 1, 2, and 3) were initially paired to a vasectomized male (to stimulate a normal reproductive cycle, but allowing us to control the number of matings: see Birkhead et al. 1989) and after 4 days allowed to copulate once with an intact male. The UVJs of these birds were examined 4, 18, and 24 hr after their single insemination, on day −4 of their cycle (estimated from follicle size: T. R. Birkhead, unpubl.). The females copulating several times were paired to intact males and their behavior video-taped continuously for 3 to 7 days. We then examined the tapes to determine the total number of behaviorally successful copulations (i.e., those with cloacal contact). Bird 4 copulated 14 times over 7 days and was examined on day −1, 30 hr after the last copulation. Bird 5 copulated 12 times over 7 days, and was examined on day −3, 23 hr after the last copulation. Bird 6 copulated six times over 3 days and was examined on day −3, 26 hr after the last copulation.

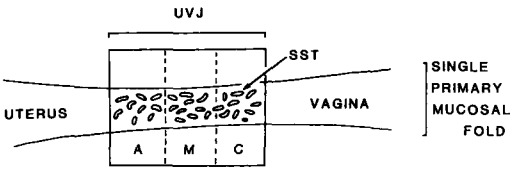

FIGURE 1. Diagram to show the manner in which a single, primary mucosal fold was divided into three regions: anterior, mid, and caudal (see text, and also Fig. 4a) in the Zebra Finch.

Birds were sacrificed, the oviducts removed, and material prepared as described elsewhere (Birkhead and Hunter 1990a). Briefly, the lamina propria of individual, primary mucosal folds from the UVJ was removed and examined microscopically, unfixed, in phosphate buffered saline (PBS). Under medium power (×100 magnification) we recorded the structure of the SSTs, that is, the number of branches of each SST, and under high power (×400 magnification), the contents of each branch (see Fig. 4). Although the SSTs of poultry have been examined in detail, they have been studied using standard histological sections and there have been no quantitative accounts of the numbers and distribution of sperm in SSTs using fresh, unfixed material. The Zebra Finch is ideal for this type of study because its UVJ mucosal tissue is transparent and the relatively low numbers of sperm in individual SSTs means that they can be easily counted in unfixed, unstained preparations.

For each bird we examined five, randomly selected, primary mucosal folds. We divided the portion of each fold containing SSTs, transversely into three equal sized areas. This was done by simply marking the zones on the cover slip of the preparation with a waterproof pen. The zones were referred to as anterior, mid, and caudal (Fig. 1): we examined 10 SSTs in each. Thus we obtained information from a total of 50 SSTs for each zone and a total of 150 SSTs for each bird. The following information was recorded for each SST branch: the total number of sperm, the distribution of the sperm in the SST lumen, and the orientation of the sperm heads. We were particularly interested in whether sperm were stratified within the lumen of SSTs, and to estimate the extent of stratification we used the following system. Sperm storage tubule branches were divided into those containing less than 10 sperm, or 10 or more sperm. For the former we recorded whether the sperm were at the distal end of the

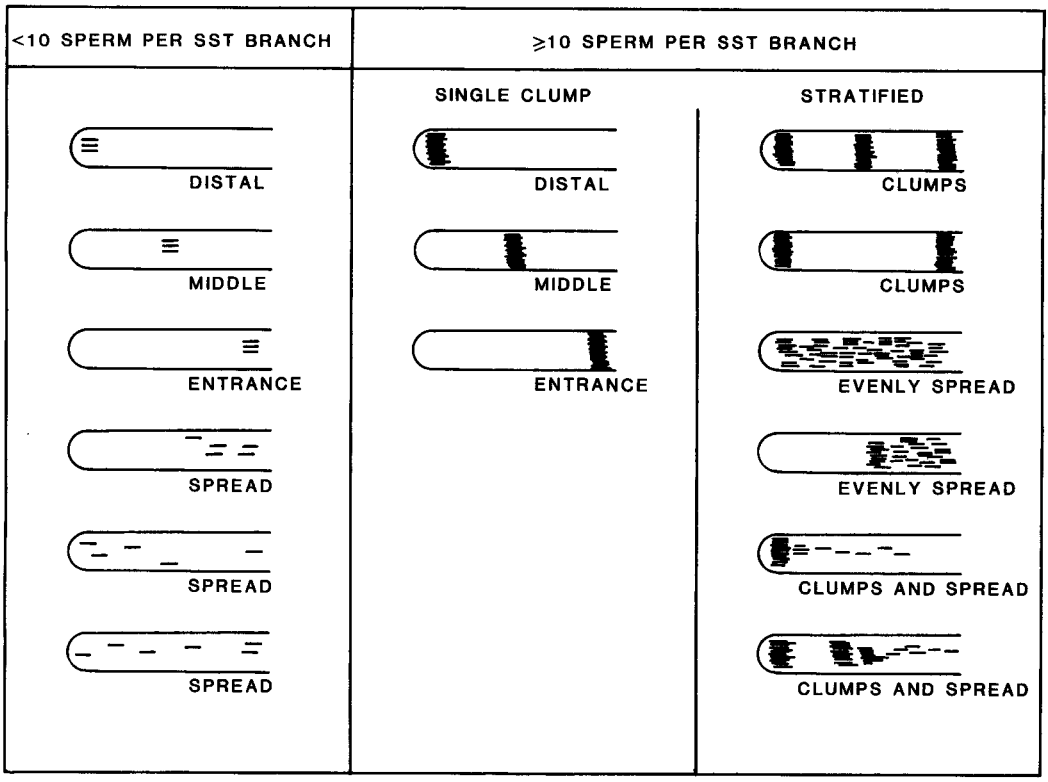

FIGURE 2. Diagram to show the way in which the distribution of sperm within sperm storage tubule (SST) branches was classified in the Zebra Finch.

tubule branch, in the middle, near the entrance, or spread along the lumen (Fig. 2). Tubule branches with 10 or more sperm were divided into those where the sperm formed a single clump and those where it did not. For SSTs with single clumps of sperm we recorded its positions: distal, middle or entrance (as above). SST branches which contained sperm not in a single clump were classed as stratified and were divided into three categories: more than one clump, evenly distributed between two or more regions (e.g., between distal and middle, or distal and entrance), and mixture of clumped and spread. These different categories are illustrated diagrammatically in Figure 2.

To address the question of whether last male sperm precedence occurs through sperm stratification in SSTs, we simulated the pattern of copulation which occurred during our sperm competition experiments in which last male sperm precedence occurred (Birkhead et al. 1988a) by mating females 7 and 8 sequentially with two different males. Previous studies have demonstrated that last male sperm precedence occurs only when inseminations are separated by four or more hours (Compton et al. 1978, Birkhead 1988, Birkhead and Hunter 1990b). Since sperm numbers in the Zebra Finch oviduct were generally low (see below), birds 7 and 8 were implanted with 17β estradiol to increase the number of copulations they performed within a specific time period (see Clayton and Prove, in press). Bird 7 copulated 10 times; four times with one male over 24 hr and six times with another 22 hr later. Bird 8 copulated 10 times: eight times with one male (over 5 days) and twice with another male 7 hr later. We then compared stratification in birds 1–6 with birds 7 and 8.

RESULTS

SST STRUCTURE

In all birds most SSTs were either unbranched (69%) or comprised two branches (21%): tubules

TABLE 1. Numbers of sperm storage tubules (SSTs) with one, two, or more branches in three regions of the uterovaginal junction of the Zebra Finch. Values are mean percentages derived from birds 1 to 6 (see text).

	No. of SST branches								
	1			2			>2		
Zone	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range
Anterior	23.70	9.01	7.7–32.7	8.53	5.85	1.3–19.2	2.13	2.44	0–6.7
Mid	22.42	7.13	12.5–32.0	9.97	3.28	4.7–12.5	2.95	3.48	0–8.3
Caudal	22.48	6.04	16.0–32.7	8.62	1.71	7.3–10.7	3.23	2.91	0–6.7
Total	68.6	21.05	57.4–97.4	27.17	8.67	13.3–40.0	8.31	8.67	0–21.7

Note: Three-way G-test: Zone × number of branches: $\chi^2 = 3.3$, df = 4, ns. Bird × number of branches: $\chi^2 = 178.5$, df = 10, $P < 0.001$. Bird, zone, and number of branches interaction: $\chi^2 = 31.1$, df = 20, ns.

with three or more branches were relatively rare. The number of branches per SST did not differ significantly between different regions of the UVJ within birds, but did differ significantly between birds (Table 1).

SPERM DISTRIBUTION WITHIN THE UVJ

The proportion of SSTs containing sperm differed markedly between birds, ranging from 22 to 99%. Singly mated birds had both the lowest values and the highest value (Table 2). Mean numbers of sperm per SST varied from less than one, to over 40 (Table 2). We used three-way G-tests (Sokal and Rohlf 1981) to examine differences between three variables: birds, UVJ regions, and sperm numbers (classifying SSTs into those containing 0, 1–10, 11–20, and 21 or more sperm: Fig. 3). Separate G-tests were performed on singly and multiply mated females. For singly mated birds the number of sperm did not differ significantly betweeen UVJ regions ($\chi^2 = 4.3$, df = 6, ns), but there was a marked difference between birds ($\chi^2 = 424.2$, df = 6, $P < 0.001$), due mainly to bird 3 which contained a much larger number of sperm (Fig. 3). There was a weak interaction between the three variables ($\chi^2 = 23.0$, df = 12, $P < 0.05$). In multiply mated females sperm numbers differed significantly between UVJ regions within birds ($\chi^2 = 21.7$, df = 6, $P < 0.01$). However, the significant interaction between sperm numbers, UVJ region and bird ($\chi^2 = 26.2$, df = 12, $P < 0.02$), means that there was no consistent pattern between birds in this respect. As with females mated singly, sperm numbers differed significantly between birds ($\chi^2 = 33.5$, df = 6, $P < 0.001$). No overall comparison between singly and multiply mated females has been attempted because of the very high sperm numbers in bird 3 and low numbers in bird 1 (see Table 2).

SPERM NUMBERS

Total sperm loads within SSTs for each bird were estimated by multiplying the mean number of SSTs per bird; 1,499 (coefficient of variation 11.6%; Birkhead and Hunter 1990a) with the mean number of sperm per SST for each bird. Values spanned two orders of magnitude, from 540 to 62,310 (Table 2). There was a significant correlation between the mean number of sperm per SST and the percentage (arcsine transformed) of SSTs containing sperm ($r = 0.986$, df = 4, $P < 0.001$).

SPERM DISTRIBUTION BETWEEN SSTs

We examined the distribution of sperm between SSTs within birds using variance:mean ratios (Elliot 1971). For all birds the distribution of sperm was significantly aggregated (Table 3). To check that these nonrandom distributions did not arise simply because some SSTs were

TABLE 2. Mean numbers of spermatozoa per sperm storage tubule (SST), percentage of SSTs with sperm, and estimated total numbers of sperm in the uterovaginal junction SSTs of the Zebra Finch.

Bird	Sperm per SST[1]		% SSTs with sperm	Sperm load in all SSTs[2]
	$\bar{x}$	SD		
Single insemination				
Bird 1	0.36	0.91	22	540[3]
Bird 2	3.17	5.59	69	4,752
Bird 3	41.54	31.86	99	62,310
Multiple insemination				
Bird 4	7.20	6.12	92	9,929
Bird 5	4.05	5.55	70	4,250
Bird 6	3.76	4.62	81	5,636

[1] Data include empty SSTs.
[2] Calculated from mean number of sperm per SST × 1,499 (mean number of SSTs per bird; see text).
[3] Low numbers of sperm in this bird could have been due to the relatively short interval between insemination and dissection (see text).

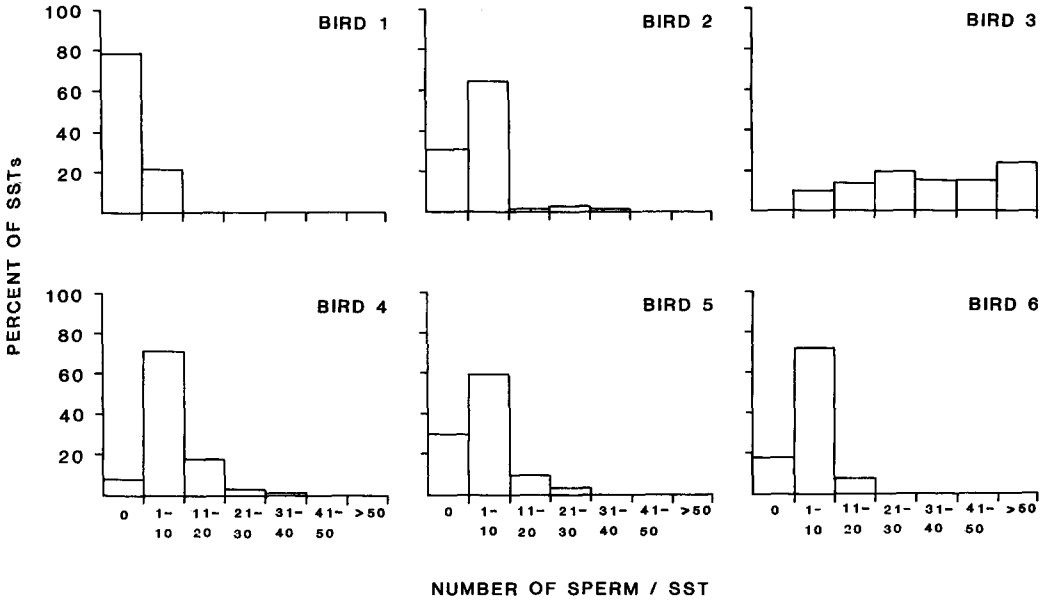

FIGURE 3. Frequency distribution of sperm numbers in all sperm storage tubules (SSTs) for birds 1 to 6. All distributions are significantly aggregated (see text and Tables 2 and 3).

"switched off" (see below), we repeated the analysis but excluded all SSTs without sperm. The result of this second analysis again showed significant aggregated distributions, except for bird 1 in which sperm numbers were extremely low (Table 2).

Most studies of chickens and poultry (using fixed, stained tissue) have described sperm lying in tight, agglutinated bundles within the SSTs (Zavaleta and Ogasawara 1987). While this was true in the Zebra Finch, we also observed some sperm spiralling along their longitudinal axes (as described by Humphreys 1972), undulating, or apparently "vibrating." In some cases sperm within SSTs were still actively moving 24 hr after the previous insemination.

LOCATION OF SPERM IN SSTs

In all birds the majority of sperm was located at the distal end of the tubule (Table 4), with their heads orientated towards the distal end (99.9%). Figure 4 shows the orientation and distribution of sperm within sperm storage tubules. The proportion of SST endings containing 10 or more sperm varied from 0% to 79% between birds (Table 4).

TABLE 3. Nonrandom distributions of spermatozoa within Zebra Finch sperm storage tubules (SSTs). Sample sizes are 150 for all SSTs, and for SSTs with sperm, 32,104,119,139,106, and 121 for birds 1–6, respectively.

Bird	All SSTs			SSTs with sperm		
	V:M[1]	d	*P*	V:M	d	*P*
Single insemination						
Bird 1	2.30	8.95	<0.001	1.06	0.18	ns
Bird 2	9.86	36.97	<0.001	8.27	26.90	<0.001
Bird 3	24.43	60.86	<0.001	24.08	59.99	<0.001
Multiple insemination						
Bird 4	5.20	22.10	<0.001	4.65	19.18	<0.001
Bird 5	7.61	30.33	<0.001	5.92	20.73	<0.001
Bird 6	5.68	23.84	<0.001	4.77	18.31	<0.001

[1] Variance mean ratio, from which the statistic d is calculated (see Elliot 1971).

SPERM IN MUTIPLY MATED FEMALES

Birds 7 and 8 had relatively high mean numbers of sperm per SST (14.65 ± 14.07 SD, and 21.83 ± 17.67), percentages of SSTs containing sperm (87% and 93%) and total sperm loads (21,960 and 32,733, respectively). As in the other birds, sperm were distributed in an aggregated manner between SSTs (variance : mean ratios [and d statistic] including SSTs with no sperm: 13.51 [46.22], 14.3 [48.0], and excluding empty SSTs: 11.2 [37.8] and 12.5 [42.3]; all d values $P < 0.001$). Given the high number of copulations these birds experienced, these results are not unexpected. However, none of the values for birds 7 and 8 were as high as in bird 3, which mated only once.

Both bird 7 and 8 had relatively high proportions of SSTs containing 10 or more sperm (23% and 44%, respectively). They also had relatively high proportions of SSTs in which sperm were stratified (14% and 22%, respectively), but again these values did not exceed those of bird 3, or bird 5 (a multiply mated female) (Table 4). Overall, our results indicate that the extent of stratification of sperm within SSTs is determined mainly by the total number of sperm in the UVJ rather than by the interval between successive inseminations. The correlations between log sperm numbers and the percentage (arcsine) of SSTs with more than 10 sperm, or with stratified sperm for all birds (birds 1 to 8), were highly significant ($r = 0.901$, df = 6, $P < 0.01$; $r = 0.908$, df = 6, $P < 0.01$, respectively).

DISCUSSION

Our main results are that the proportion of SSTs containing sperm varied markedly between birds and was positively correlated with the mean number of sperm per tubule. The greatest number of sperm in the UVJ of one bird was estimated as 62,000. Within birds sperm were highly aggregated. Only birds with high sperm loads had SSTs in which sperm showed any stratification.

DISTRIBUTION OF SPERM WITHIN THE UVJ

Verma and Cherms (1965) postulated that in turkeys the SSTs might fill sequentially from the caudal (i.e., vaginal) end of the oviduct. If this were true then we might have expected birds with low sperm loads to have proportionately more SSTs in the vaginal region containing sperm, but this was not the case.

Studies of chickens (*Gallus*) and turkeys (*Me-*

TABLE 4. Distribution of spermatozoa within sperm storage tubule (SST) branches of the Zebra Finch. Values are percentages of SST branches, and exclude SST branches without spermatozoa.

Copulation pattern Bird no.	SST branches (*n*)	<10 sperm per SST branch				≥10 sperm per SST branch						% SSTs with ≥10 sperm
						Single clump			>1 clump			
		Anterior	Mid	Caudal	Spread	Anterior	Mid	Caudal	Clumped	Evenly spread	Clumped and spread	
Single copulation												
Bird 1	126	83.3	4.0	0	5.5	0.8	0	0	0.8	4.0	1.6	7.1
Bird 2	37	86.5	5.4	0	8.1	0	0	0	0	0	0	0
Bird 3	223	19.7	0	0	0.9	39.0	0.9	0.4	10.3	10.8	17.9	79.0
Multiple copulation												
Bird 4	121	52.9	14.9	3.3	12.4	11.6	2.5	0	0	2.5	0	16.5
Bird 5	144	55.5	8.3	0.7	10.4	19.4	0	0	2.1	2.8	0.7	25.0
Bird 6	143	79.0	1.4	0	10.5	7.0	0	0	0.7	1.4	0	9.0
Two males multiple copulation												
Bird 7	171	68.4	7.6	1.1	0	8.8	0	0	0	6.4	7.6	22.8
Bird 8	184	50.5	2.7	1.1	1.6	22.2	0	0	0	9.9	11.9	44.0

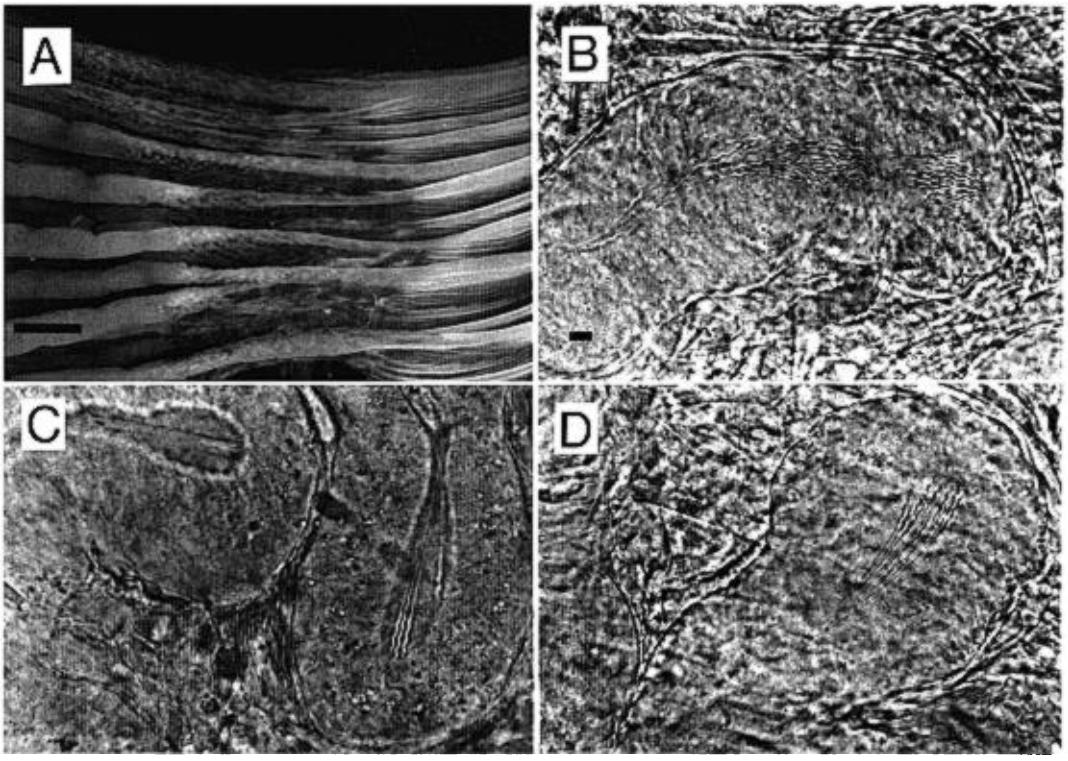

FIGURE 4. A. Reproductive tract of a female Zebra Finch showing the uterovaginal junction with sperm storage tubules (SST) appearing as small granules. Part of the uterus is visible to the left, and part of the vagina to the right. The scale bar = 1 mm. B.–D. Distal end of single SST, all at the same magnification: the scale bar in left hand corner of B = 10 μ. B provides an example of clumping with one group of sperm at the distal end and another group of sperm behind it. C shows a SST containing four sperm: the lumen of the SST is clearly visible. D shows a single group of 12 sperm at the distal end of the SST: the tails of these sperm are clearly visible.

leagris) have shown that relatively low proportions of SSTs contain sperm after insemination, indicating either that extreme aggregation of sperm occurs, or that not all SSTs are active at any one time (Bakst 1989). The proportion of sperm-containing SSTs in chickens range from 25% to 60% (Burke et al. 1969, Van Krey et al. 1971; Compton and Van Krey 1979; Pierson et al. 1988; Bushman et al. 1985;), and from 42% to 72% in turkeys (Verma and Cherms 1965, Van Krey et al. 1974, McIntyre and Christensen 1983). These values are notably lower than those we report here. However, the techniques used in the poultry studies differed from ours in that the former used histological sections of UVJ material and then examined SST sections to record the abundance or presence of sperm. Since this method results in SSTs being sectioned in various planes, it probably underestimates the proportion of SSTs containing sperm. Nonetheless, it seems likely that not all chicken and turkey SSTs contain sperm. Moreover, our examination of another galliform, the Japanese Quail (*Coturnix japonica*), using the technique used on Zebra Finches, showed that, even after multiple inseminations, about 50% of all SSTs remain empty, while others appear to be completely full.

Our results clearly show that sperm are not distributed randomly between SSTs. This effect did not occur because some SSTs were inactive: excluding empty SSTs from our analysis still gave aggregated distributions. The fact that in chickens and turkeys many SST sections are empty while others contain high numbers of sperm also suggests aggregated distributions. Our observations of Japanese Quail also show aggregated

sperm distributions of sperm between SSTs (unpubl. results). How these patterns arise is unknown, but several possibilities exist: (1) Sperm may travel in clumps or bundles: our observations of Zebra Finch ejaculates indicate that this sometimes occurs, and similar observations have been made on ejaculates of canaries, *Serinus canaria* (Humphreys 1972). (2) Some SSTs may be more active than others in attracting sperm, or (3) incoming sperm may be attracted to sperm already present in SSTs, or to SSTs containing sperm.

SPERM NUMBERS IN SINGLY AND MULTIPLY MATED FEMALES

Since Zebra Finches copulate about 12 times on average for each clutch of eggs, we expected sperm numbers to be higher in those females which had copulated several times compared with those copulating only once. With one exception this was true. However, bird 3 contained more sperm than any of the multiply mated females. There are several possible explanations for this: (1) There are large differences between males in the numbers of sperm in a single ejaculate. This is certainly true in some poultry (Parker et al. 1942) and in budgerigars (*Melopsittacus undulatus*) (J. Samour, pers. comm.). We are currently investigating this in the Zebra Finch, and our results so far indicate that intermale differences in sperm numbers ejaculated are similar to those recorded in the present study, so this seems the most likely explanation for the variation in our data. Other possibilities include (2) that females differ in the proportion of sperm they sequester in the SSTs, and (3) sperm numbers present in SSTs vary with time from the last insemination (Wishart 1987).

The only other species in which UVJ sperm numbers have been estimated is the domestic turkey. Any comparison with the Zebra Finch must be made with caution because the biology and husbandry of the two species differ dramatically (see Bakst 1989). Turkeys have about 20,000 SSTs (Goodrich-Smith and Marquez 1978), and can store a total of about 4,000,000 sperm (J. Brillard and M. R. Bakst, pers. comm.).

STRATIFICATION OF SPERM WITHIN SSTs

Sperm competition studies of Zebra Finches have shown that the last male to inseminate the female before egg laying fertilizes the majority of eggs (Birkhead 1988a). Compton et al. (1978) suggested that last male sperm precedence in chickens occurred because successive inseminations remained stratified within the SSTs, and that sperm from the last insemination are first to leave the SSTs. This hypothesis is consistent with much of what is known about sperm competition in chickens: (1) Some SSTs are more or less completely filled with sperm, so stratification could occur. (2) Last male sperm precedence occurs only if successive inseminations take place 4 hr or more apart. If inseminations take place less than 4 hr apart paternity is proportional to the numbers of sperm from each male (Martin et al. 1974). It has been assumed that this occurs because instead of remaining stratified, the sperm from different inseminations mix before entering the SSTs (See Warren and Gish 1943, Cheng et al. 1983). (3) Since not all SSTs appear to be active in chickens (and turkeys: see above), it is feasible that the sperm from the last mating could enter most or all SSTs already containing sperm and thereby overlie most sperm present from previous inseminations.

However, there is no direct evidence that last male sperm precedence occurs through stratification of sperm within the SSTs for any bird species. Moreover, one set of observations is inconsistent with the stratification hypothesis: in a situation where two artificial inseminations have taken place, as in poultry studies, one might expect that after several days of egg laying the sperm from the last male would be more or less completely utilized, and that sperm from the earlier mating might start to fertilize eggs. However, despite the apparently exponential decline in sperm numbers from SSTs (Wishart 1987), there is no evidence for such an effect (Lodge et al. 1971, Compton et al. 1978). A theoretical model of sperm competition in the chicken (Lessells and Birkhead, in press) also shows that this lack of a long-term decline in precedence is inconsistent with stratification as a mechanism of sperm competition.

The results from the present study are consistent with the idea that sperm precedence in the Zebra Finch occurs through some means other than stratification.

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