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<https://doi.org/10.5109/23757>

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出版情報 : 九州大学大学院農学研究院紀要. 27 (1/2), pp.47-53, 1982-10. Kyushu University  
バージョン :  
権利関係 :

## Sperm Storage in the Oviduct of the Japanese Greater Horseshoe Bat, *Rhinolophus ferrumequinum nippon*

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(Received June 5, 1982)

In the Japanese greater horseshoe bat, *Rhinolophus ferrumequinum nippon*, copulation took place in mid- or late October and ovulation was induced early in April. The principal site of sperm storage was the caudal isthmus of the oviduct, lined with non-ciliated epithelium, although moderate or small numbers of spermatozoa were found also in the intramural part of the uterotubal junction. The intact spermatozoa in contact with the microvilli of epithelial cells in folds in the storage sites appeared normal, while some degenerated spermatozoa were eliminated by extensive epithelial engulfment throughout the period of storage; leucocytic phagocytosis rarely occurred and was confined to the period just before or after ovulation.

### INTRODUCTION

Matthews (1937) reported that in the British greater horseshoe bat, *Rhinolophus ferrumequinum insulanus* and lesser horseshoe bat, *R. hipposideros minutus*, spermatozoa inseminated in the autumn are stored in the Fallopian tube, uterus and vagina of the female until spring when the single ovum discharged from the right ovary only is fertilized by a spermatozoon stored in the upper genital tract for at least five months. Racey (1975, 1979), however, provided histological evidence that in over-wintering *R. ferrumequinum* the spermatozoa found in the uterus and vaginal plug are dead, and that the site of sperm storage is the oviduct. The present study was made to clarify where spermatozoa are stored in the oviduct of the Japanese greater horseshoe bat, *R. f. nippon* and to investigate the status of spermatozoa stored there between copulation in autumn and ovulation in spring. In later publications we will discuss the fate of spermatozoa stored in the female reproductive tract with reference to structure and role of the vaginal plug, ovulation and fertilization, etc.

### MATERIALS AND METHODS

Total 20 adult female Japanese greater horseshoe bats (*R. f. nippon*) were collected at caves in the Fukuoka, Kumamoto and Yamaguchi Prefectures during

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the period from immediately before copulation (mid-October before hibernation) to just before ovulation (early in April at the end of hibernation) in 1969-1980 (cf. Table 1). Out of 10 bats captured in April, gonadotropic hormones were administered at once to 7 in 'order to obtain data on fertilization succeeding induced ovulation as follows ; about 48 h after subcutaneous injection of 5 units PMSG (Teikoku-zōki Pharmaceutical Co., Ltd.), 2 units HCG (Mochida Pharmaceutical Co., Ltd.) were also administered subcutaneously. About 24 h later the bats were perfused through the dorsal aorta with 3 % glutaraldehyde in 0.2 M-phosphate buffer (pH 7.4) under ether anesthesia, or killed by decapitation.

The reproductive organs were removed and then the tissues were promptly placed in cold 3 % glutaraldehyde in 0.2 M-phosphate buffer (pH 7.4) for 4 h. After being thoroughly rinsed with the same buffer, the tissues were post-fixed with 1.3 % osmium tetroxide in the same buffer, dehydrated with acetone and embedded in Epon 812. Thick sections (1.5  $\mu\text{m}$ ) were stained with 0.5 % toluidine blue and examined with a light microscope. Thin sections ( $\sim 60$  nm) for electron microscopy were doubly stained with uranyl and lead acetate and examined with an Hitachi HS-9 electron microscope (75 kV). But, the reproductive organs fixed with 10 % formalin from Bats C-9, G-2 and 6 were embedded in Paraffin and stained with Harris' hematoxylin and eosin.

## RESULTS

*R. f. nippon* enters into hibernation early in November and arouses on about 10 April. As shown in Table 1, Bats C 1 and 2 collected on 13 October were not yet inseminated, and out of 6 bats on 17 and 24 October, Bats C-3 and 11 had not yet mated but Bats C-4, 5, 12 and 13 were thought to have mated recently; on the other hand, all the adult females taken during hibernation had copulated. Intact spermatozoa were always found in the caudal isthmus of the oviduct and/or the intramural part of the uterotubal junction (UTJ) until ovulation, and although some spermatozoa underwent degenerative changes and death in the storage sites. The spermatozoa in these two states are described separately.

### Morphology of surviving spermatozoa in the storage sites

In *R. f. nippon*, the UTJ was divided into two parts, the intramural part and colliculus tubaricus. The former was characterized by a tube-like lumen with some small mucosal folds but was devoid of deep diverticulum. The latter was a moderate-sized projection of the oviduct into the uterine lumen, whose epithelium varied but represented a continuous layer of the oviductal epithelium in its interior and a continuous part of the endometrial epithelium covering the projection into the uterine lumen (Pl. I, Fig. 1). The oviductal epithelium consisted almost entirely of non-ciliated epithelial cells except for the ampulla.

In the bats just after insemination, large or moderate 'numbers of intact spermatozoa were stored in the caudal isthmus or intramural part of the UTJ

**Table 1.** Sperm storage in the oviduct and occurrence of ovulation in the Japanese greater horseshoe bat.

Date of capture	Bat	Spermatozoa stored in :		Occurrence of ovulation
		Intramural part (UTJ)	Caudal isthmus	
Before hibernation				
Oct. 13, 1969	C-1*		—	
Oct. 13, 1969	C-2*	—		
Oct. 17, 1980	C-11*	—	—	—
Oct. 17, 1980	C-12	+	⦿	—
Oct. 17, 1980	C-13		⦿	
Oct. 24, 1970	C-3*	—		
Oct. 24, 1970	C-4	⦿		
Oct. 24, 1970	C-5	⦿	+	—
In hibernation				
Jan. 9, 1978	C-6	⦿	⦿	—
Jan. 22, 1980	C-7	+	⦿	—
Apr. 2, 1980	C-8	—	⦿	—
Apr. 2, 1980	C-9	?	⦿	—
Apr. 2, 1980	C-10	+	⦿	—
Apr. 2, 1980	<b>G-1</b>	+	⦿	—
Apr. 2, 1980	G-2	⦿	⦿	
Apr. 2, 1980	G-3	+	⦿	+
Apr. 2, 1980	G-4	⦿	⦿	+
Apr. 3, 1980	G-5	⦿	?	+
Apr. 5, 1980	G-6	⦿	⦿	—
Apr. 5, 1980	G-7	+	⦿	

The C series represents non-treated group and the G series hormone-injected group with 5 units PMSG and 2 units HCG, except G-5 administered with only PMSG. The asterisk (\*) indicates non-copulated bats. The symbols of —, +, ⦿ and ⦿ in the sites of sperm storage show absence of sperm, presence of small, moderate and large numbers of spermatozoa, respectively.

with a few in colliculus tubaricus; but in bats taken from hibernation the majority of spermatozoa were found in the caudal isthmus, and this situation persisted consistently until immediately after ovulation in spring. The spermatozoa appeared to be clustered with their heads orientated in parallel towards the oviductal epithelial cells, chiefly among the mucosal folds of the caudal isthmus of the principal storage site (Pl. I, Fig. 2). A close association between the intact spermatozoa and the microvilli of the epithelial cells was established. No morphological modifications of the spermatozoa were recognized as well as epididymal spermatozoa of the copulatory season (Pl. II, Fig. 3 and inset); spermatozoa remaining free in the lumen were seldom found. Consequently, to judge from their structural integrity, most of the spermatozoa stored in the caudal isthmus seemed to be alive, also in the intramural part. These observations were repeated throughout hibernation.

#### Death of spermatozoa in the storage sites

Even in bats which had not ovulated, some spermatozoa in the storage sites (both the caudal isthmus and the UTJ) degenerated and were finally engulfed by the epithelial cells (cf. Pl. I, Fig. 2). Principal pieces scrolled by the cytoplasmic pseudopodia on the luminal surface were frequently observed (Pl. III, Fig. 4), together with sperm heads and tails engulfed by the epithe-

lial cells (Pl. III, Fig. 5).

In the recently ovulated bats, the great majority of the spermatozoa found in the oviduct underwent remarkable degenerative changes such as swelling of the space between the plasma membrane and the mitochondrial or fibrous sheath, and breakdown of the axial filament complex with heightening of electron-density (Pl. III, Fig. 6). Phagocytosis of degenerated spermatozoa by leucocytes rarely occurred in the storage sites of the bats and only just before or after spring ovulation (Pl. III, Fig. 7). No leucocytic infiltration was recognized in the bats just after copulation in autumn and during hibernation.

## DISCUSSION

### Sperm storage and its mode

Since the first discovery of the distended uterus with live spermatozoa during winter in *Pipistrellus pipistrellus* (Pagenstecher, 1859), the reproductive biology of bats has aroused interest in the variety of sites and modes of sperm storage (Racey, 1975, 1979). The uterus has been regarded as a storage site by many investigators (Racey, 1975), but in *P. abramus* only the spermatozoa with heads orientated towards the uterine epithelium are motile, and the remainder occupying the great part of the uterine lumen eventually die (Hiraiwa and Uchida, 1955). Electron microscopic observations in *P. pipistrellus* reveals contact of the uterine epithelial cells with the cell membrane of the spermatozoa (Racey and Potts, 1970), and similar phenomena are reported light microscopically also in *Scotophilus heathi* (Krishna and Dominic, 1978). It is therefore deduced that in most vespertilionine bats so far examined, the uterus may play an important role as a storage site for the spermatozoa in contact with the uterine epithelium, but not for the spermatozoa far from it.

In some vespertilionine bats, however, such as *Chalinolobus gouldii* (Kitchener, 1975) and *Myotis daubentoni* (Racey, 1975) spermatozoa are stored in the uterotubal junction (UTJ), and in a miniopterine bat, *Miniopterus schreibersii fuliginosus* the storage site is strictly limited to the UTJ where intact spermatozoa are found in diverticula formed by the mucosal folds (Mōri and Uchida, 1980). In *Tylonycteris pachypus* and *T. robustula*, spermatozoa are stored in the oviduct and concentrated in indentations of the epithelial cells throughout the junctura and isthmus with a few in the ampulla; the plasma membrane of the sperm head is closely applied to that of the epithelial cells (Racey et al., 1975). In both *R. ferrumequinum* and *R. hipposideros*, and *Myotis nattereri*, intact spermatozoa are found in the oviduct (Racey, 1975), and the present study has demonstrated that the principal site of sperm storage in *R. f. nippon* is the caudal isthmus, where a great number of intact spermatozoa were arranged with their heads associated closely with the microvilli of the non-ciliated epithelial cells, although the intramural part of the UTJ also contained moderate or small numbers of them.

The present study thus provides further evidence that spermatozoa, wherever they are stored in the female reproductive tract, have close relation-

ships with the microvilli or indentations of the epithelial cells in the mucosal folds. Such a mutual association strongly suggests that the epithelial cells actively provide the spermatozoa with nutrients for their prolonged survival (Racey and Potts, 1970). For example, glycogen in the uterine epithelium of *P. abramus* (Nakano, 1928) and *P. pipistrellus* (Racey, 1975), and fructose in the uterine plasma of *P. pipistrellus* (Racey, 1975) and *Myotis lucifugus* and *M. velifer* (Crichton *et al.*, 1981).

### Engulfment of spermatozoa by the oviducal epithelium

Numerous spermatozoa are observed with the light microscope within the uterine mucosa of elasmobranch fish, domestic fowl, bats (*Rousettus amplexicaudatus* and *Pipistrellus stenopterus*) and the rabbit, within the uterine submucosa of *R. amplexicaudatus* and within the oviducal mucosa of the mouse (Kohlbrugge, 1910, 1913). Vojtíšková (1956) had serious doubts concerning the validity of the above observations including her own earlier data because spermatozoa can be transferred from the lumen into the epithelium by the microtome knife. However, the occurrence of many spermatozoon heads are found in the isthmus mucosa of *R. fewumequinum* and *P. pipistrellus* (Austin and Bishop, 1959), and similar finding in the rat, rabbit, hedgehog, mole and stoat (Austin, 1959, 1960) was considered by these authors to be a real rather than an artefactual phenomenon.

The mechanism by which spermatozoa came to be within oviducal cells remained a matter for speculation until Mbri and Uchida (1974) presented ultrastructural evidence that in *P. abramus* spermatozoa do not enter into the oviducal epithelial cells by their own motion but undergo phagocytosis by them, and finally are digested by lysosomes. Chakraborty and Nelson (1975) also have electron microscopically reported in the mouse that phagocytosis of spermatozoa occurs mainly in the epithelial lining of the caudal isthmus. The present study has demonstrated engulfment of spermatozoa in *R. f. nippon* by the epithelium of both caudal isthmus and the UTJ.

### ACKNOWLEDGEMENTS

We thank Mr. S. Arai of our Laboratory and Dr. T. Kuramoto of the Akiyoshi-dai Museum of Natural History for their kind help in collecting specimens, Professor E. W. Jameson, Jr., University of California for comments on the manuscript and Dr. P. A. Racey, University of Aberdeen for advice and encouragement in the course of this study.

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### Explanation of Plates I-III

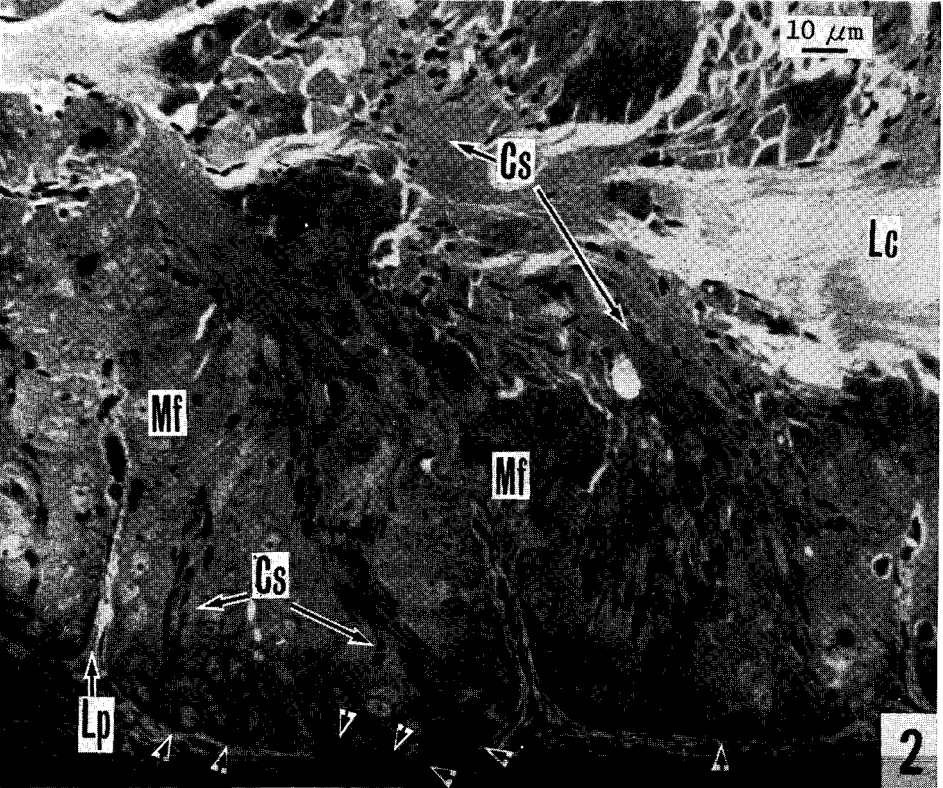
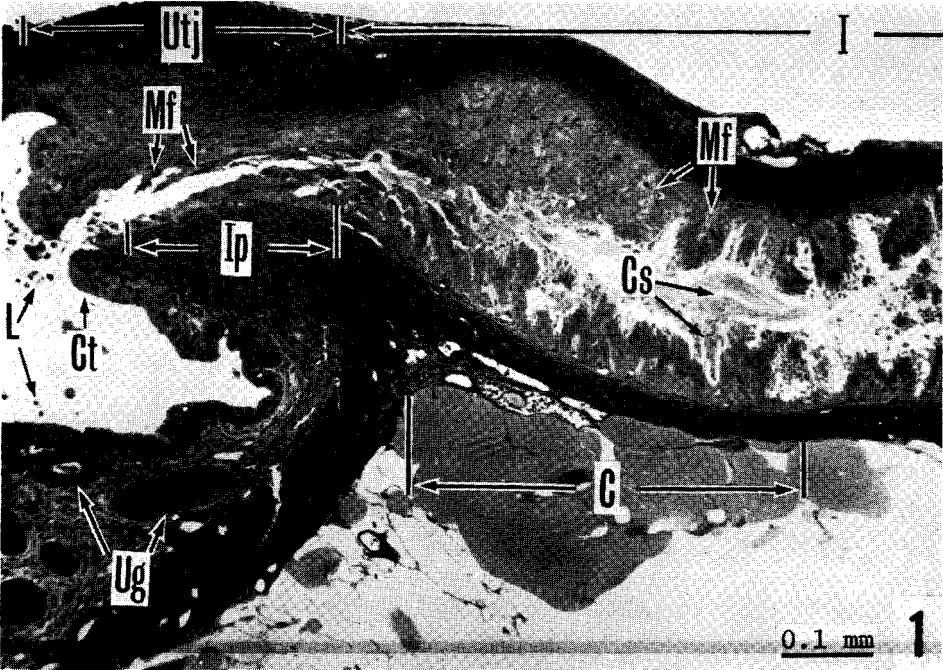
**Abbreviations:** A, acrosome ; C, caudal isthmus ; Cs, clump of spermatozoa ; Ct, colliculus tubaricus; Ec, epithelial cell; Es, engulfed spermatozoon ; G, Golgi complex; I, isthmus ; **Ip**, intramural part; L, leucocyte ; Lc, lumen of caudal isthmus; Lp, lamina propria; M, mitochondrion; Mf, Mucosal fold; Mi, microvilli; N, nucleus; P, pseudopodium; Sn, sperm nucleus ; Spm, sperm plasma membrane ; Ug, uterine gland ; Utj, uterotubal junction.



### Plate I

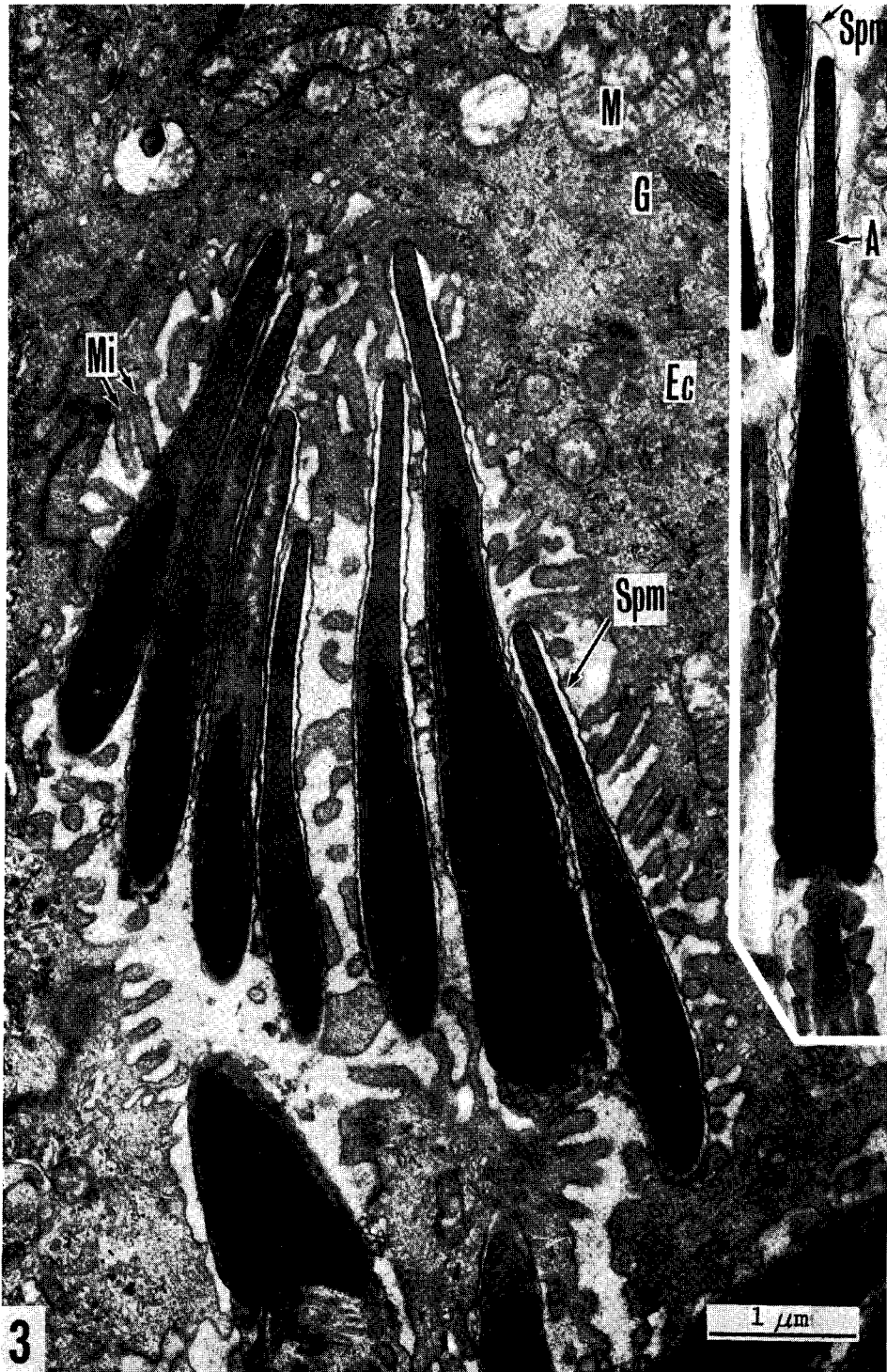
Fig. 1. Longitudinal section showing many clumps of spermatozoa recognized as dark streams among the well developed mucosal folds of the caudal isthmus in hibernating Bat C-7.

Fig. 2. Spermatozoa in the caudal isthmus of Bat C-7, clustering with their heads orientated towards the epithelial cells. Several spermatozoa within the epithelium (arrow-heads) also are visible.



## Plate II

Fig. 3. Electron micrograph showing intact spermatozoa in close contact with microvilli of the epithelial cells in the caudal isthmus of Bat C-7. Compare them with live epididymal spermatozoa (inset).



### Plate III

**Fig. 4.** Electron micrograph showing a typical early stage in engulfment of sperm tail by the epithelial pseudopodia of the caudal isthmus in preovulatory Bat C-10. Principal piece of an abnormal spermatozoon with a triple tail (arrow) about to be scrolled by pseudopodia is also seen on the luminal surface.

**Fig. 5.** Electron micrograph of engulfed spermatozoa within epithelial cells of the caudal isthmus in Bat C-7.

**Fig. 6.** Electron micrograph showing cross sections of a clump of sperm tails in the caudal isthmus of freshly ovulated Bat G-4, showing their remarkable degenerating changes. Engulfed sperm tails (lower left) within an epithelial cell and abnormal spermatozoa with multiple tails (arrows) are also visible.

**Fig. 7.** Electron micrograph showing a polymorphonuclear leucocyte engulfing two sperm heads in the lumen of the caudal isthmus in Bat G-4. A sperm tail is engulfed within an epithelial cell.

