Selectivity in the transport of spermatozoa to oviductal reservoirs in the menstruating fruit bat, *Carollia perspicillata*

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Abstract

To better document the timing of ovulation and fertilization, female reproductive tracts were collected every 12 h from captive-bred fruit bats (*Carollia perspicillata*) on days 1–3 postcoitum and examined histologically. This also permitted observations on sperm transport, storage, and disposition. As the animals had previously been sexually segregated, most had been cycling and possessed menstrual uteri at the time of collection. Menstruation is periovulatory in this species. A widespread, headfirst orientation of spermatozoa to the uterine mucosa was observed in specimens apparently collected soon after insemination. Thereafter, however, this relationship was limited in most cases to the area around the entrance of each uterotubal junction (UTJ). A small number of spermatozoa also colonized the UTJs, which functioned as temporary sperm reservoirs on days 1–2. Although *C. perspicillata* is monovular, no consistent differences were observed between the two oviducts in the pattern of sperm storage and release. Very few sperm were ever observed in the isthmus or ampulla (the site of fertilization). Menstrual debris (including fine particulate matter) and leukocytes present in the uterine cavity in most tracts did not gain access to the UTJ with the spermatozoa. Smooth muscle and abundant elastic fibers in the wall of the intramural UTJ, as well as receptors on its luminal epithelial cells, may play roles in the selective transport of spermatozoa to the fertilization site. While some spermatozoa are phagocytosed in the uterine lumen or by epithelial cells in the UTJ, the fate of most is probably expulsion into the vagina.


Introduction

The short-tailed fruit bat, *Carollia perspicillata*, which is very common in forested regions of the lowland tropics of the New World, readily adapts to captivity in a research setting. It is of value as a model organism for the large mammalian order Chiroptera and exhibits many reproductive and developmental similarities to humans. These include being monovular, a simplex uterus, true menstruation, interstitial implantation of the blastocyst, highly invasive trophoblast, and a discoidal hemochorial placenta. Furthermore, during implantation and gastrulation, its embryo exhibits more morphological similarities to those of humans than those of laboratory rodents (Rasweiler et al. 2009).

In order to characterize more precisely ovulation, fertilization, and early embryonic development in *C. perspicillata*, females were bred in captivity. Reproductive tracts were then collected at 12-h intervals over the first 3 days postcoitum (p.c.) and examined histologically. This also permitted the observations, reported here, on sperm transport, storage, and disposition in the female tract.

These processes, which have rarely been examined *in situ* in monovular mammals, are of interest in *C. perspicillata* for several reasons. When maintained in sexual isolation prior to being bred, most females in our colony ovulate spontaneously and run non-pregnant cycles with a functional luteal phase. The cycles are terminated by endometrial desquamation and bleeding (i.e. true menstruation). In contrast to primates, however, menstruation generally occurs during the periovulatory period (Rasweiler & de Bonilla 1992). This means that an abundance of blood, cellular debris, and particulate material is generally present in the uterine lumina of cycling *C. perspicillata* during the period when spermatozoa are being transported into the oviducts. Our studies suggest that the menstrual uteri of newly mated animals are not especially favorable sites for even the short-term storage of spermatozoa. There is, however, an area immediately around the entrance to each uterotubal junction (UTJ) that appears to promote the longer survival of a small population of spermatozoa and their entry into the UTJ. Previous studies indicate that this region may perform similar functions in dogs (England et al. 2006). Transport into the UTJ is also highly selective, as it involves only small number of spermatozoa, but with no menstrual debris or leukocytes.
Results

Breeding success

For the purpose of this study and several unrelated studies, 67 females housed in groups of 10–15 were placed with single, stud males. Sixty-one of the females (91.0%) bred within 30 days. Most (28/33; 84.8%) of the reproductive tracts removed from newly inseminated females for this study appeared normal when examined histologically. These fell into the following categories: 1) two females were non-pregnant with tracts inappropriate for their postcoital timings; 2) five females possessed normal tracts with large Graafian follicles; 3) one female (CW 26) possessed a normal tract, but her Graafian follicle exhibited premature early luteinization; 4) three postovulatory females carried normal secondary oocytes in their oviducts; 5) one postovulatory female carried an unfertilized ovum in the uterus and 6) twenty-one postovulatory females carried ova in the process of being fertilized or normal, early embryos. One of the latter was unusual, however, in also carrying an older embryo (an implanted blastocyst).

Of the 33 mated females, only 5 bore vaginal plugs, and all of these were found on day 1 p.c. Thus, checking for vaginal plugs once per day is not a reliable means of monitoring female C. perspicillata for mating activity.

Anatomy of the uterus and oviducts

The uterus of C. perspicillata is externally simplex. Internally, its anatomy varies depending upon the recent reproductive history of the female. If substantial postovulatory development of the endometrium has occurred, it has two short internal cornua with endometrial glands, interposed between the main uterine cavity and the UTJ (Fig. 1). These have been termed ‘intramural uterine cornua’ (IUCs). During pregnancy, the single blastocyst normally implants within the IUC on the side of ovulation or just outside of it (i.e. from the main uterine cavity; Rasweiler & Badwaik 1999). In this study, IUCs were observed in most (26/33) uteri. They were short or absent in seven uteri that possessed late menstrual and/or early proliferative endometria.

Each oviduct of C. perspicillata is composed of an infundibulum, ampulla, isthmus, and UTJ (Fig. 1). The UTJ consists of intramural and extramural segments. During the periovulatory period and the first part of the tubal journey of the embryo, secretory cells in the ampulla and isthmus, and to a variable extent in the infundibulum, appear unusually vacuolated due to massive accumulations of glycogen. Among mammals, similar secretory cells have only been observed in closely related, noctilionoid bats (de Bonilla & Rasweiler 1992; NN Rasweiler & NK Badwaik, unpublished observations).

Distinctive characteristics of each oviductal segment during the periovulatory period are as follows (Fig. 2): infundibulum – prominent mucosal folds; highest proportion of ciliated to secretory cells; thin tunica muscularis; few and fine elastic fibers in the lamina propria and adjacent portion of the muscularis. Ampulla – prominent mucosal folds; abundant, highly stimulated (vacuolated) secretory cells; fewer ciliated cells; thin tunica muscularis;

Figure 1 Diagram depicting major uterine and oviductal regions in the short-tailed fruit bat, Carollia perspicillata.
Timing of ovulation and fertilization

Three of five females examined on the morning of day 1 p.c. were preovulatory, while ovulation had been completed in most females ($n=4/5$) examined on the evening of that day. Fertilization had commenced in most females ($n=6/10$) examined on the evening of day 1 or the morning of day 2 p.c. Exceptional females were still preovulatory ($n=1$) or had unfertilized oviductal ova ($n=1$) on the morning of day 3 p.c.

Regional abundance of spermatozoa

In most animals ($14/21; 66.7\%$) examined on days 1 and 2 p.c., spermatozoa were moderately ($++$) to very ($+++\$) abundant in the uterine lumina (Table 1). With the exception of one bat ($1/10$), sperm were present only in low number ($+$) or absent from the uterus on day 3 p.c. The density of spermatozoa was affected, in part, by the amount of fluid in the uterine lumen. The uteri of many pre- and recently postovulatory females ($n=17/23; 73.9\%$) exhibited some dilatation with fluid, while this was not the case in any of females ($n=0/8$) carrying cleaving embryos (Table 1).

Uterine spermatozoa were most abundant on the morning of days 1 and 3 p.c. in three females that had apparently been very recently inseminated. Two of these (CW 17 and CL 99) possessed a regressing corpus luteum (CL) and a menstrual uterus (Fig. 5A). CW 17 (day 1) had also recently ovulated and carried an ovum being fertilized (Fig. 5B). CL 99 (day 3) carried a large

Figure 2 Sections of different oviductal regions in a short-tailed fruit bat (CR 2), Carollia perspicillata, processed on the evening of day 3 p.c. These sections were stained with Masson’s trichrome procedure, plus Weigert’s resorcin-fuchsin.

(A) Section of the intramural segment of the UTJ. Purple-stained elastic fibers are abundant in the lamina propria (LP) and immediately adjacent parts of the thick tunica muscularis (TM). (B) Section of the extramural segment of the UTJ. Elastic fibers (at arrows) are still abundant in the lamina propria (LP) and immediately adjacent tunica muscularis (TM), but less prominent than in the intramural segment. Many of the oviductal secretory cells (S) also appear more vacuolated here than in the intramural segment. (C) Section of the oviductal isthmus. Elastic fibers are still abundant in the lamina propria (LP) and immediately adjacent tunica muscularis (TM), but appear less prominent than in the UTJ. The oviductal secretory cells (S) also appear much more vacuolated here than in the extramural segment of the UTJ. C, ciliated cell. (D) Section of the oviductal ampulla. Elastic fibers are still present in the lamina propria (LP) and immediately adjacent tunica muscularis (TM), but appear much less numerous and conspicuous because of their thinness. The oviductal secretory cells (S) are highly vacuolated, while the tunica muscularis (TM) is thin. C, ciliated cell; scale bars 25 $\mu$m (A–D).
preovulatory follicle with an ovum in the first meiotic division. The last female (CW 31, day 1) lacked a regressing CL, but possessed a preovulatory follicle (with its ovum in the first meiotic division) and a shallow, proliferative endometrium. Uterine spermatozoa in all three females were associated with abundant, flocculent, extracellular material. The latter is assumed to be of seminal plasma origin because intact spermatozoa were abundant in these three uteri, and similar material was not obvious in the other 30 uteri. In the case of the two menstruating animals, some erythrocytes and menstrual debris were intermingled with the spermatozoa as well.

The cervices of these three females contained very few spermatozoa and only traces of precipitated extracellular material. These observations indicate that ejaculation must be intruterine in _C. perspicillata_.

The menstrual females (CW 17 and CL 99) were also unusual in having large number of spermatozoa oriented with their heads toward the widely denuded endometrial surfaces and clumps of desquamated endometrial tissue (Fig. 5A). This orientation, in the absence of possible epithelial binding, indicates that the sperm must have been viable. The greatest density of spermatozoa in these uteri was at their fundic ends. Although spermatozoa were also abundant in the uterine lumen of the preovulatory female (CW 31) with the shallow, proliferative endometrium, fewer (but still many) were oriented toward the endometrial surface. In that animal, the mucosa was covered by a luminal epithelium.

In these females, much smaller number of spermatozoa were observed in the UTJ (Table 1). Although very few spermatozoa were present in the UTJ of one menstruating female (CW 17), her newly ovulated egg was nevertheless in the process of being fertilized (Fig. 5B). No menstrual debris or seminal plasma components were evident in the UTJs of these bats.

Sperm orientation relative to the uterine mucosal surface varied between the animals (n=15) examined on days 1 or the morning of day 2 p.c. (Figs 4 and 6–8). In 2/15 bats (one menstrual and one proliferative endometrium), many spermatozoa exhibited a headfirst orientation toward the mucosal surface in much of the uterine cavity. In 7/15 bats (five menstrual and two late menstrual/early proliferative), a headfirst orientation was evident only around the entrance to the UTJ. In 3/15 bats (one menstrual and two proliferative), this orientation was observed immediately around the entrance to the UTJ and also in their IUCs. In 3/15 bats (three menstrual), no clustering or regular orientation of spermatozoa was observed in any part of the uterine cavity.

In one of these females (CR 16, day 1 p.c.), significant regrowth of the endometrium had occurred, resulting in the development of relatively long IUCs for that day. Spermatozoa were abundant and oriented headfirst toward the luminal epithelium within these IUCs (Fig. 7), as well as being congregated around the entrances to the UTJs. No spermatozoa in the main uterine cavity exhibited a similar orientation. That cavity was unusual, however, in containing a large amount of apparently fresh blood. This must have been the result of some type of uterine hemorrhagic event, as the endometrium of CR 16 was proliferative and not menstrual. A few blood cells were also present within the IUCs.

The parallel alignment and orientation of sperm around the UTJ entrances (Figs 4, 6 and 8) in most uteri collected on day 1 and the morning of day 2 p.c.
suggest a temporary binding to specialized populations of luminal epithelial cells. Some sperm clustering was also frequently observed in immediately adjacent luminal areas. Generally, when spermatozoa were aligned or clustered in this way, the adjacent luminal epithelial cells appeared healthy (Figs 4 and 6–8). Spermatozoa exhibited no binding to shrunken luminal epithelial cells that presumably were destined for menstrual sloughing.

On the evening of day 2 p.c. and at both sampling times on day 3 p.c., spermatozoa were no longer clustered or oriented around the entrances to the UTJs in most bats (n=15/16). The one exception was a preovulatory female (CL 99, day 3 p.c.) that exhibited a widespread, headfirst orientation of spermatozoa to her uterine mucosal surface (as mentioned above).

In most (11/12) of the menstrual animals examined on days 1 and 2 p.c., spermatozoa were no longer clustered or oriented around the entrances to the UTJs in most bats (n=15/16). The one exception was a preovulatory female (CL 99, day 3 p.c.) that exhibited a widespread, headfirst orientation of spermatozoa to her uterine mucosal surface (as mentioned above).

In most (11/12) of the menstrual animals examined on days 1 and 2 p.c., spermatozoa were intermingled with desquamated endometrial tissue and blood in the uterine lumen. Spermatozoa had also penetrated the superficial endometrial stroma to a limited extent where the epithelium had been lost (Fig. 5A). With the exceptions mentioned above, none of the luminal sperm exhibited a headfirst orientation to the endometrial surface. All of these non-oriented spermatozoa must therefore have been immotile or seriously deficient in motility.

Spermatozoa were present as well in some of the endometrial glands on days 1 and 2 p.c. By day 3 p.c., the number of such spermatozoa had been substantially reduced, except in the case of 2/10 females that had ovulated late (CW 32) or seemed destined to do so (CL 99). Our observations indicate that the endometrial glands do not function as significant, temporary sperm reservoirs in C. perspicillata. If viable/motile sperm were stored and then released from the glands, at least some should have been observed with their heads oriented toward the uterine mucosal surface. This was not observed on days 1 and 2, with the limited exceptions already mentioned.

On day 1 and the morning of day 2 p.c., a small number of spermatozoa were generally evident within the intramural and, to a lesser extent, the extramural segments of the UTJ (Table 1 and Fig. 9). Although many spermatozoa in the UTJs had heads in contact with the luminal epithelium, they were generally not lined up in parallel, with a headfirst orientation. Instead, many were positioned with their heads flat against the epithelium. Spermatozoa were observed only very rarely in the isthmus and ampulla – the site of fertilization – on days 1–3 p.c.

When counts were made of spermatozoa in the intramural and extramural segments, higher number was present in the former in 30/31 bats. Furthermore, no consistent pattern was observed when sperm counts were compared between the two intramural segments in individual animals. In 14 animals, the higher number was in the oviduct ipsilateral to the ovulating ovary or
ovary with the Graafian follicle, while in 13 animals, the higher number was in the contralateral oviduct (Table 1). There also was no correlation with the presence or absence of a unilateral oviductal reaction. In most cases (26/33), the ipsilateral oviduct was more stimulated (i.e. exhibiting greater epithelial hypertrophy and secretory cell vacuolation) than the contralateral oviduct. However, in five animals, both the oviducts appeared equally stimulated, and in two animals (non-periovulatory and not included in Table 1), neither oviduct was stimulated. When differential stimulation of the oviducts was evident, a larger number of sperm were counted in the more stimulated/ipsilateral oviduct of 12 bats and in the less stimulated/contralateral oviduct of 10 bats. No spermatozoa were evident in either oviduct of the remaining four bats. When both oviducts were equally stimulated, more sperm were counted in the ipsilateral oviduct in two bats and more in the contralateral oviduct in three bats. Spermatozoa were observed too infrequently in the isthmus and ampulla to draw any conclusions about differences in their number in those segments. By day 3 p.c., spermatozoa had been almost completely cleared from the UTJs in most animals (8/10; 80%; Table 1).

No menstrual debris and only a few (one to several) erythrocytes were ever identified in the UTJ. In most cases, the erythrocytes appeared to have been artificially displaced during slide coverslipping, because some were scattered abnormally elsewhere on the same slides. In one exceptional female (CR 16) with a proliferative endometrium, however, the uterine lumen contained abundant blood. A small amount of this had been naturally transported into one UTJ, as it could be identified in the same region in adjacent serial sections.

Closure of the intramural segment of the UTJ to menstrual debris, larger number of spermatozoa often typical of the uterine lumen, leukocytes and seminal plasma components generally began at its caudal end (Figs 4 and 6). This had a thick muscularis (like the entire UTJ), but was also the site of a prominent, three-dimensional meshwork of elastin (Fig. 3). Some of the elastin in this meshwork took the form of amorphous masses, rather than slender fibrils. Although fine fibrils were commonly observed in connective tissues of the reproductive tract, the only other large accumulations of elastin occurred in the walls of major arteries and veins.

Fate of excess spermatozoa

On days 1–3 p.c., mononuclear cells and neutrophils were commonly observed in the uterine lumen, but only a small portion of the spermatozoa had been phagocytosed. As spermatozoa were observed in vaginal aspirates for several days after the onset of estrus, their expulsion in that direction also occurs. It seems unlikely that many spermatozoa pass into the peritoneal cavity, as very few were ever observed in the isthmus, ampulla, and infundibulum.

A small number of spermatozoa were taken up by epithelial cells of the intramural UTJ in at least 11/21 females examined on days 1–2 p.c. (Fig. 10). In one animal examined on the morning of day 1 p.c., sperm heads were also observed in epithelial cells at the entrance to the UTJ and adjacent areas of endometrium. By day 3 p.c., however, intraepithelial sperm were recognizable in the intramural segment of the UTJ in only 2/10 females. Most females (n=9/10) examined on that day exhibited scattered cellular debris (e.g. pycnotic
nuclei and intensely stained chromatin masses) within the epithelium of those segments, probably derived from spermatozoa. Similar debris has been observed within uterine and oviductal epithelial cells in another bat species known to exhibit the phagocytosis of sperm by those maternal elements (Rasweiler 1987). Phagocytes containing spermatozoa were never observed in the UTJ.

**Conception during a preexisting pregnancy**

One female (CW 21) in this study had bred within 24 h of being placed with a male. As such matings result in conceptions less frequently (Rasweiler & Badwaik 1996), this female was allowed to stay with the male, and daily vaginal aspirates were continued. On days 17 and 18 p.c., she exhibited new sperm-positive aspirates. When killed on the evening of day 18 p.c., she was found to be carrying an implanted blastocyst at the mouth of her right IUC (Fig. 11A and B). Although this blastocyst appeared normal (cf. Badwaik et al. 1997, Rasweiler et al. 2002), an unusual dilatation of the IUC was evident beneath the implantation site. The associated CL was in shallow except luteal (ovary/egg) Endometrium (Table 1), none were present in her right UTJ. The female had been processed on day 18 p.c., commencing on day 2 p.c. from the second estrus. No spermatozoa had been able to enter the UTJ on the side of the uterus where the blastocyst had implanted.

Table 1 Distribution and abundance of spermatozoa in the female reproductive tract of Carollia perspicillata in relation to ovulatory status, fertilization or embryonic development, and endometrial condition.

<table>
<thead>
<tr>
<th>Time of collection</th>
<th>Reproductive status (ovary/egg)</th>
<th>Uterine condition</th>
<th>Abundance of spermatozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Endometrium</td>
<td>Luminal dilatation</td>
</tr>
<tr>
<td>Day 1 p.c. (morning)</td>
<td>CW 1 Preovul.</td>
<td>Menstrual</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CW 17 Postovul./fertilization had commenced</td>
<td>Menstrual</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CW 25 Postovul./oocyte</td>
<td>Menstrual</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CW 31 Preovul.</td>
<td>Proliferative</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CP 40 Preovul.</td>
<td>Menstrual</td>
<td>+</td>
</tr>
<tr>
<td>Day 1 p.c. (evening)</td>
<td>CR 16 Postovul./pronuclear</td>
<td>Proliferative</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CP 17 Postovul./pronuclear</td>
<td>Menstrual</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CP 18 Postovul./oocyte</td>
<td>Menstrual</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CW 24 Postovul./fertilization had commenced</td>
<td>Menstrual</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CW 34 Preovul.</td>
<td>Late menstrual/ early proliferative</td>
<td>+</td>
</tr>
<tr>
<td>Day 2 p.c. (morning)</td>
<td>CP 13 Postovul./pronuclear</td>
<td>Menstrual</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CW 26 Preovul.</td>
<td>Late menstrual</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CW 27 Postovul./abnormalb</td>
<td>Proliferative</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CW 28 Postovul./pronuclear</td>
<td>Menstrual</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CB 32 Postovul./fertilization had commenced</td>
<td>Menstrual</td>
<td>+</td>
</tr>
<tr>
<td>Day 2 p.c. (evening)</td>
<td>CW 12 Postovul./cleaving embryo</td>
<td>Menstrual</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CW 18 Postovul./pronuclear</td>
<td>Proliferative</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CP 19 Postovul./pronuclear</td>
<td>Menstrual</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CW 21 Postovul./pronuclear/+ implanted blastc</td>
<td>Shallow except luteal around blastocyst</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CW 23 Postovul./pronuclear</td>
<td>Menstrual</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CY 63 Postovul./pronuclear</td>
<td>Menstrual</td>
<td>–</td>
</tr>
<tr>
<td>Day 3 p.c. (morning)</td>
<td>CW 8 Postovul./cleaving embryo</td>
<td>Menstrual</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CW 16 Postovul./cleaving embryo</td>
<td>Menstrual</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CR 21 Postovul./pronuclear</td>
<td>Menstrual</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CW 32 Postovul./oocyte</td>
<td>Proliferative</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CL 99 Preovul.</td>
<td>Menstrual</td>
<td>+</td>
</tr>
<tr>
<td>Day 3 p.c. (evening)</td>
<td>CR 2 Postovul./cleaving embryo</td>
<td>Menstrual</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CW 2 Postovul./cleaving embryo</td>
<td>Menstrual</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CW 6 Postovul./cleaving embryo</td>
<td>Menstrual</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CW 7 Postovul./cleaving embryo</td>
<td>Menstrual</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CW 9 Postovul./cleaving embryo</td>
<td>Late menstrual/ early proliferative</td>
<td>–</td>
</tr>
</tbody>
</table>

*Number of spermatozoa counted near the center of the indicated oviductal segments that were ipsilateral (I) or contralateral (C) to the ovary containing the preovulatory or newly ruptured follicle. The counts were made in luminal regions of fixed length (238 μm). bThe newly ovulated egg in bat CW 27 had been transported into the main uterine cavity. cBat CW 21 carried a pronuclear stage egg plus an implanted blastocyst that had been conceived during a prior estrus. The female had been processed on day 18 p.c. counted from the first estrus and day 2 p.c. from the second estrus. dNo spermatozoa had been able to enter the UTJ on the side of the uterus where the blastocyst had implanted.

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Discussion

The number and condition of spermatozoa in the tracts examined for this study presumably were determined to some extent by the recent breeding history of the animals. Unfortunately, it was not feasible to closely monitor copulatory activity by either sex because the bats were housed in groups, their cages had darkened roosting boxes (where the animals retreated when humans were present), and the females had long cycles with limited periods of estrus. Most females that bred, failed to establish an ongoing pregnancy, and then bred again had cycles of 19–27 days. In most cases, periods of sperm-positive vaginal aspirates varied from 1 to 5 days, but the actual duration of estrus was not determined (JJ Rasweiler & NK Badwaik 1996, unpublished observations). Although stud males may have sometimes been incapable of inseminating (or re-inseminating) all estrous females in their cages, the breeding results in the colony have generally been good, as observed here and elsewhere (Rasweiler & Badwaik 1996).

Other investigators have described temporary sperm reservoirs in the isthmic region of the oviducts of a variety of common mammals (Zamboni 1972, Yamagimachi & Mahi 1976, Overstreet & Cooper 1978, Hunter et al. 1982, Hunter 1984, Hunter & Wilmut 1984, Smith et al. 1987, Suarez 1987). Sperm are thought to bind temporarily to the epithelium within these reservoirs and then be gradually released to ascend in small number to the site of fertilization (Suarez 2008). In some insectivores (according to genus), crypts in the oviductal isthmus or ampulla seem to perform the functions of temporary sperm storage or sequestration of excess number and regulated release (Bedford et al. 1997a, 1997b, 1999, Bedford 2004b). Sperm storage in some bat species can be prolonged and occurs in the uterus, oviduct, and/or UTJ according to species (Krishna & Dominic 1978, Racey 1979, Racey et al. 1987, Uchida & Mori 1987).

Most temporary sperm storage in female *C. perspicillata* appears to take place around the entrances to and within the UTJs. Clustered and oriented spermatozoa were observed around the entrances to the UTJs in most animals on day 1 and the morning of day 2 p.c. Spermatozoa were also moderately abundant within the UTJs (particularly the intramural segments) of most bats on days 1 and 2 p.c. By day 3 p.c., however, sperm number in the UTJs had dropped markedly except for a couple of late-ovulating animals.

As the most caudal oviductal segments in *C. perspicillata* are morphologically and physiologically distinct from the isthmus, these are probably best referred to as the intramural and extramural segments of the UTJ (terminology respectively of Nilsson & Reinius (1969) and Suarez (1987)). The reservoirs established there would appear to be the origin of the very small number of spermatozoa that pass upward into the isthmus and ampulla – the normal site of fertilization in this species. This presumably serves to limit the possibility of polyspermic fertilization.

Entrance into the UTJ in *C. perspicillata* is clearly selective, as only limited number of spermatozoa gain access. Furthermore, many animals in this study were menstruating when colonization of the reservoirs occurred. Yet, no menstrual debris was transported in with the spermatozoa. This raises a question as to whether the caudal ends of the UTJs ever become significantly patent during the periovulatory period. In mice, there is evidence that the colliculi tubarii become

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**Figure 6** (A) Section of uterine fundus from a short-tailed fruit bat (CW 34), *Carollia perspicillata*, processed on the evening of day 1 p.c. The section was stained with hematoxylin and eosin. This uterus was in a late menstrual to early proliferative state, with much blood, extensively dissolved menstrual debris, and leukocytes in its lumen (UC). Many spermatozoa (arrowhead) are oriented headfirst toward the luminal epithelium at the mouth of each UTJ (only one is shown), but this orientation is not observed elsewhere in the main uterine cavity. Spermatozoa within the UTJ are not so regularly oriented. The space between the spermatozoa or menstrual debris and the luminal epithelium of the main uterine cavity (UC) is a shrinkage artifact. Note that no menstrual debris or leukocytes are evident within the UTJ. (B) Higher power view of oriented spermatozoa immediately outside of the UTJ. Scale bars = 50 μm (A) and 25 μm (B).
briefly patent after copulation, as some bacteria and exfoliated cells of vaginal origin also gain entry. This is thought to permit colonization of the oviductal reservoirs with spermatozoa (Zamboni 1972, Suarez 1987).

It has long been recognized that sperm transport into these reservoirs is selective in a variety of mammals (Leonard & Perlman 1949, Hafez & Black 1969, Gaddum-Rosse 1981, Shalgi et al. 1992). Studies of several mutant strains of mice have established that certain epitopes must be exposed on the surface of their spermatozoa, if the latter are to enter successfully into the UTJ (Nakanishi et al. 2004, Suarez & Pacey 2006, Yamaguchi et al. 2009). In these cases, the mutant spermatozoa that were unable to enter appeared otherwise normal with respect to motility and morphology.

In C. perspicillata, a temporary attachment to epithelial cells at and around the entrance to the UTJ may be a first step in the selective passage of spermatozoa into the oviduct. In mice, the movement of sperm within the oviduct toward the site of fertilization has been found to consist of a series of detachments and reattachments (DeMott & Suarez 1992).

A headfirst orientation of spermatozoa toward the endometrial surface was observed widely in the uterine cavity of only a few C. perspicillata. These included females that had apparently been recently inseminated and one with a proliferative uterus, in which relatively well-developed IUCs seemed to have created a protected environment for the spermatozoa. Much of this, with the exception of the IUCs and the area immediately outside of the UTJ, may be non-specific (i.e. not receptor mediated). In the case of two menstrual uteri, sperm had become oriented to both epithelial – covered and denuded endometrial surfaces, as well as to clumps of desquamated tissue. When Racey (1975) placed a droplet of semen collected from a bat uterus in paraffin oil, spermatozoa soon became oriented in the same way at the oil–semen interface.

In most menstrual uteri, only a small portion of the spermatozoa were highly oriented relative to luminal epithelial cells on and around the entrance to the UTJ. None of the abundant spermatozoa in the body of the same uteri exhibited a similar orientation to the endometrial surface (whether denuded or not). This suggests that none of these latter spermatozoa were motile. This may have been attributable to limitations on their viability in the uterus, as well as their exposure to menstrual blood. Exposure to blood serum has long been known to induce acrosomal breakdown, and to immobilize and kill spermatozoa (Walsh 1925, Chang 1947, Johnson 1968, Bedford 1970, Russo & Metz 1974, Hancock 1976, Suarez & Oliphant 1982).

Although a few females with proliferative uteri were examined, these did not provide insights into the uterine longevity of C. perspicillata spermatozoa in the absence of blood. One of these bats had apparently been recently inseminated, and another contained abundant blood in her main uterine cavity. In the remaining females, a headfirst orientation of spermatozoa was observed only around the UTJs and within the IUCs, or was absent. The interval since the last insemination in these animals could have been as long as 36–72 h, however.

Assuming that a substantial exposure to blood serum can harm spermatozoa in C. perspicillata, the question arises as to how sufficient spermatozoa successfully traverse menstrual uteri to fertilize eggs. The present observations suggest that this may be accomplished in several ways: 1) many viable sperm appear to be...
deposited by intrauterine ejaculation very close to the UTJs. 2) Seminal plasma associated with those sperm may provide temporary protection from spermotoxic factors in the menstrual blood. 3) A close association of spermatozoa with epithelial cells around the entrance to the UTJ may be protective. Several studies have indicated that antisperm antibodies attach preferentially in the acrosomal region. Furthermore, the ultrastructural lesions induced by antibody and complement, at least in rabbits and guinea pigs, seem focused upon this region (Beck et al. 1962, Symons 1967, Bedford 1970, Russo & Metz 1974, Le Bouteiller et al. 1975). 4) A small population of viable spermatozoa may be transported rapidly into the protected confines of the UTJ. 5) Finally, fresh spermatozoa may be introduced by re-inseminations. Vaginal plugs are rarely found in captive-mated *C. perspicillata*, but new plugs have occasionally been observed on days 1 and 2 in already mated females (JJ Rasweiler IV & NK Badwaik, unpublished observations).

Aside from having an important role in producing vaginal plugs (in those species that form them), or in simply providing a vehicle for the spermatozoa, the adaptive significance of male sex accessory gland secretions in eutherian mammals has not been well elucidated (Bedford 2004). In bats exhibiting peri-ovulatory menstruation (e.g. *C. perspicillata* and some of its close relatives; Rasweiler & Badwaik 2000), seminal plasma components might play an essential role in temporarily protecting at least some spermatozoa from exposure to menstrual blood. The spermotoxicity of blood serum is complement dependent (Chang 1947), and seminal plasma has been shown to inhibit complement activation (Johnson 1968, Russo & Metz 1974, Suarez & Oliphant 1982).

In contrast to some of the uterine spermatozoa, those within the UTJs of *C. perspicillata* were never observed clustered with the tips of their heads oriented toward the oviductal epithelium. This indicates a difference in the nature of the putative binding, but its basis and significance remain unclear.

Sperm phagocytosis by oviductal and/or uterine epithelial cells has now been observed in a variety of mammals (Austin 1960, Rasweiler 1987). Interestingly, in *C. perspicillata*, this was restricted to epithelial cells either within or immediately outside of the UTJ. This raises the possibility that binding to receptors on those cells may predispose some spermatozoa to phagocytosis instead of release.

During the periovulatory period in dogs, spermatozoa bind to the epithelium of the glandular mound that surrounds the entrance to the UTJ, as well as in uterine crypts and glands. This has been viewed as evidence that these sites, as well as the UTJ, may function as temporary sperm reservoirs (Rijsselaere et al. 2004, England et al. 2006). It has also been suggested that endometrial glands, crypts, and clefts in cows and dogs may sequester excess spermatozoa, thereby reducing the number that can reach the ampulla and enhancing the probability of monospermic fertilization (Hunter 1995, Rijsselaere et al. 2004, England et al. 2006).

The passage of only a few spermatozoa and no menstrual debris into the UTJ of *C. perspicillata* points to the presence of a selective sphincter at its entrance. Components responsible for this probably include the thick muscularis of the UTJ, the well-developed elastin meshwork in its wall, and the narrowness of its lumen. A similar concentration of elastin in the UTJ has not been reported for other species (Hafez & Black 1969, Beck & Boots 1974), but this deserves further study.

Figure 8 Sections of uterine fundus from a short-tailed fruit bat (CP 18), *Carollia perspicillata*, processed on the evening of day 1 p.c. These had been stained with hematoxylin and eosin. (A) Spermatozoa (between arrowheads) were clustered immediately outside of each UTJ, and many were aligned with a headfirst orientation toward the epithelium around its entrance (not evident in this section). Spermatozoa did not exhibit a similar orientation toward the remaining menstrual mucosa, which was denuded (*) or covered by a degenerative epithelium (**). EN, endometrium. (B) Higher power view of oriented spermatozoa (arrowheads) clustered immediately outside of the UTJ. Scale bars = 50 µm (A) and 25 µm (B).
Given proximity of the usual implantation site in *C. perspicillata* to the UTJ (Rasweiler & Badwaik 1999, Oliveira et al. 2000), contractions of the oviductal musculature and dilatation of the sphincter might also play central roles in regulating embryo transport to the proper site.

A case was documented in this study of *C. perspicillata* ovulating and conceiving again during a preexisting pregnancy. This cannot be dismissed as just a rare anomaly because a similar case, involving two blastocysts of different ages being carried by a female, was reported previously (de Bonilla & Rasweiler 1974). Furthermore, another captive-bred *C. perspicillata* was found to be carrying an implanting blastocyst and an oviductal secondary oocyte. In that case, however, the female had not been housed with a male at the time of the second ovulation. Therefore, the possibility of a second conception was precluded (Rasweiler & Badwaik 1996).

These observations are of interest because *C. perspicillata* have well-developed CLs during implantation, and females exhibit significantly elevated plasma progesterone levels than the baseline levels found in ovariectomized or day 1 p.c. animals (as determined by a progesterone RIA validated for the species; J. J. Rasweiler IV & V. J. Nacharaju, unpublished observations). This suggests that rising progesterone levels do not always inhibit ovulation or interfere with receptivity and normal sperm transport in the female tract of *C. perspicillata*.

Although most *C. perspicillata* exhibited preferential stimulation of the oviduct ipsilateral to the ovary containing the Graafian follicle or newly ruptured follicle, sperm entry into or release from this oviduct was not consistently affected. These observations, as well as the occurrence of a normal conception during a preexisting pregnancy, do not support the suggestion that periovulatory progesterone released from Graafian follicles and delivered locally controls sperm storage and transport (Hunter 2008), at least in this bat. There is substantial evidence that capacitation-associated changes in sperm (e.g. the shedding of extrinsic proteins and hyperactivation) are actually associated with their release from oviductal reservoirs in a number of species (Suarez 2008).

This study has revealed several novel aspects of sperm transport and storage in *C. perspicillata*. As menstruation in this species is periovulatory, spermatozoa introduced into mated, cycling females usually have to traverse uteri containing abundant menstrual debris in order to reach the normal fertilization site. Although breeding success was good, such uteri do not appear to be particularly favorable sites for more than the brief storage and passage sperm. Areas immediately around and within the UTJ seem to function as the principal sites for temporary sperm storage in this species. Entrance into the UTJ was restricted to small number of sperm, and very few of these were found to be released into the isthmus or ampulla (site of fertilization). Although *C. perspicillata* will occasionally ovulate again during existing early pregnancies, the associated endocrine milieu does not preclude successful sperm transport and fertilization. Finally, no evidence was observed of a local ovarian effect upon sperm transport and storage, although this species exhibits preferential stimulation of the oviduct on the side of ovulation.

**Materials and Methods**

**Source of animals**

All animals used in this study were born and raised in a laboratory colony. They were derived from animals that had been collected on the West Indian island of Trinidad. The captive colony was maintained in centralized animal facilities...
at the Weill Medical College of Cornell University and subsequently at the State University of New York Downstate Medical Center in accordance with the ‘Principles of Laboratory Animal Care’ (NIH publication no. 86-23).

Animal maintenance

The bats were kept in rooms with a controlled light cycle (12 h light:12 h darkness), and the dark phase was set to commence at 1600 or 1500 h at the respective institutions. The temperature was maintained between 24 and 27 °C. The bats were housed in bipartite cages with darkened roosting boxes, large enough to permit the animals to fly. Prior to being bred, the females were housed in groups of 10–15 animals. The males were housed in groups of 8–12 animals or, more recently, singly (because of a propensity for some postpubertal males to harass and injure others).

The bats were routinely fed a fruit-based diet prepared from readily available canned and powdered components (peach or apricot nectar, pureed canned peaches, ground monkey chow, dibasic calcium phosphate, corn oil, an emulsifier, and a multivitamin preparation). This diet was occasionally supplemented with small amounts of sliced apple, banana, or melon as treats. The animals were fed every night, and the diet was served cold, no more than 60–90 min before the room lights went off, to minimize microbial growth. Water was provided ad libitum in chicken waterers (Rasweiler & Badwaik 1996, Rasweiler et al. 2009).

Animal marking

For identification purposes, the animals were fitted with bead chain necklaces carrying numbered, colored plastic bird bands (Rasweiler & Badwaik 1996). The second letter in the numbering system indicates the band color.

Animal breeding and timing of reproductive stages

For breeding purposes, a single male with prominent testes was housed with each group of 10–15 females. This is in accord with the known propensity of *C. perspicillata* to organize into harems consisting of one male and multiple females (Fleming 1988). On subsequent mornings, between 0530 and 0830 h, a small quantity of distilled water was aspirated in the vagina of each female with a microeyedropper (Rasweiler et al. 2009). The aspirate was then spread on a slide, dried, and examined for spermatozoa. The first day on which spermatozoa were observed in an aspirate was considered to be day 1 p.c. Mating activity by each female could have been initiated up to 24 h earlier. Daily vaginal aspirates were collected once per day until a mated bat was killed. As the females were always housed with the stud male until the time of killing, re-inseminations of the same female were possible.

Histological procedures

All of the bats were killed between 0900 and 1000 or 2100 and 2200 h by administering an i.p. injection of sodium pentobarbital at a dosage of ~90 mg/kg body weight. During dissection, the reproductive tract was retained as one unit above approximately the level of the vaginal–cervical junction. All of the sectioned tracts included ovaries, oviducts, uterus, and the cervix. Manipulation of the tract during severance of its mesenteric attachments and transfer to fixative were done by holding the urinary bladder with a fine forceps. There was no squeezing or manipulation of the uterus or oviducts. The tracts were fixed in Zenker's fluid for 8–10.5 h, immersed in 2.5% aqueous potassium dichromate for an additional 2 h, washed overnight in running tap water, dehydrated through graded ethyl alcohols, cleared overnight in warm cedar wood oil (37 °C) followed by Histosol (National Diagnostics, Atlanta, GA, USA), and embedded in paraffin wax. The tracts were then serially sectioned in a frontal plane at 6 μm. The histological sections were stained with hematoxylin and eosin or Weigert's resorcin-fuchsin for elastic fibers (Clifford & Taylor 1973) followed by Masson's trichrome procedure (modified from Humason 1972). Powdered Weigert's resorcin-fuchsin stain (#1A 294) was obtained from Chroma Gesellschaft Schmid GmbH & Co. (48161 Münster, Germany). In the version of
Masson’s procedure used, mordanting in iron alum was omitted, and the nuclei were stained with stabilized iron chloride hematoxylin (Lillie 1965). Also, when the sections were washed in running water after being stained with acid fuchsin and ponceau de xylidine, the wash time and water temperature (23°C) were precisely controlled in order to regulate cytoplasmic staining.

Quantification of regional sperm abundance

Sperm concentrations in the main uterine cavity frequently exhibited marked regional variations even within the same animal and were also affected by the degree of uterine dilatation with fluid. Comparisons between animals were therefore made by subjectively categorizing the overall abundance of spermatozoa in each uterine cavity as high (+++), moderate (++), or low (+). No effort was made to quantify differences in uterine volume because these had not been anticipated. Furthermore, efforts to collect the fluid would have been disruptive to morphological preservation, particularly during the menstrual process (which was a focus of the original study). Instead, the degree of luminal dilatation was assessed histologically.

Sperm counts for the UTJ were made manually on regions of fixed length (238 μm, as determined by a calibrated eyepiece reticle) near the middle of each intramural and extramural segment. Because the lumina of these segments were narrow and contained epithelial folds, regions selected for counts were as close to the mid-frontal plane as possible (to maximize luminal area). These were done for both UTJs in each bat, i.e. ipsilateral or contralateral to the preovulatory follicle or the newly ruptured follicle. Counts were not made on multiple sections for each side and the differences then tested for significance. This would have necessitated counts away from the mid-frontal plane, thereby introducing another major source of variability. Counts also were not compared for different groups of females because it was unclear how to define the groups. Bats differed with respect to ovarian and uterine status, as well as to the development of the egg or embryo. In addition, sperm counts presumably would have been affected by the time elapsed since the last insemination and by the abundance of spermatozoa in previous ejaculates; however, these parameters were unknown.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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