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**The biology of mammalian spermatozoa in the oviduct**

**Smith, Todd Timothy, Ph.D.**

**University of Hawaii, 1990**

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**THE BIOLOGY OF MAMMALIAN SPERMATOZOA  
IN THE OVIDUCT**

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE  
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE  
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BY

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## ABSTRACT

The oviduct occupies a unique position in mammalian reproduction as the site of sperm transport, the final maturation of sperm and egg, fertilization, and the initial development of the embryo. This dissertation examines the factors that control the number, distribution and physiological state of spermatozoa in the oviduct. The golden hamster (Mesocricetus auratus) was used as the animal model. The following summarizes my findings. The uterotubal junction restricts the passage of homologous and heterologous spermatozoa into the oviduct, furthermore, sperm motility is essential for efficient passage. After mating, spermatozoa rapidly enter the oviductal isthmus where they are stored. When mating occurs shortly after the onset of estrus, spermatozoa are stored for at least 8 h until near the time of ovulation. When mating occurs during ovulation, spermatozoa are stored for a minimum of 3 h. Spermatozoa stored in the isthmus during the preovulatory period do not become fully capacitated until near the time of ovulation. When mating occurs during ovulation, spermatozoa require a minimum of 3 h in the isthmus to become fully capacitated. Although many thousands of spermatozoa are stored in the isthmus, only a relatively small percentage of these spermatozoa survive. The spermatozoa that do survive attach to the oviductal mucosa during storage. Later, due to physiological changes in the sperm head plasma membrane that accompany capacitation, a small number of these spermatozoa detach from the mucosa and ascend to the ampulla to fertilize the eggs.

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## PREFACE

Some of the material contained in this dissertation was published or is currently submitted for publication. All relevant text and figures have been reproduced with the express permission of the publishers (see Appendix). Material in Chapter 1 was published under the title "Quantitative Comparison of the Passage of Homologous and Heterologous Spermatozoa Through the Uterotubal Junction of the Golden Hamster" (Smith, TT., Koyanagi, F., Yanagimachi, R.) in Gamete Research 19:227-234. Material in Chapter 2 was published under the title "Distribution and Number of Spermatozoa in the Oviduct of the Golden Hamster after Natural Mating and Artificial Insemination" (Smith, TT., Koyanagi, F., Yanagimachi, R.) in Biology of Reproduction, 37:225-234. Material in Chapter 3 was published under the title "Capacitation status of hamster spermatozoa in the oviduct at various times after mating" (Smith, TT. and Yanagimachi, R.) in Journal of Reproduction and Fertility, 86:255-261. Material in Chapter 4 was published under the title "The Viability of Hamster Spermatozoa Stored in the Hamster Oviduct: The Importance of Sperm-Epithelium Contact for Survival" (Smith, TT. and Yanagimachi, R.) in Biology of Reproduction, 42:450-457. Material in Chapter 5 has been submitted for publication under the title "Attachment and release of spermatozoa from the caudal isthmus of the hamster oviduct" (Smith, TT. and Yanagimachi, R.) to the Journal of Reproduction and Fertility.

With respect to the multiple authorship of these publications, I, as senior author of the publications was responsible for designing and conducting the experiments, interpreting the results and writing the manuscripts. The contributions of the co-authors include technical assistance (F. Koyanagi) and assistance in the preparation of the manuscripts (R. Yanagimachi).

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## INTRODUCTION

The oviduct occupies a unique position in mammalian reproduction as the site of sperm transport, the final maturation of sperm and egg, fertilization and the initial development of the embryo. The diversity of these functions makes the oviduct one of the most interesting systems to investigate. This dissertation examines the factors that control the number, distribution and the physiological state of spermatozoa in the oviduct.

Beginning about twenty-five years ago, with the advent of *in vitro* techniques to explore gamete biology, the study of gametes *in vivo* has been largely ignored. Although our knowledge of gamete biology has been greatly advanced by the use of *in vitro* techniques, the data obtained from these studies must be interpreted with caution as they may or may not represent the true picture of what is occurring *in vivo*. The purpose of the studies presented here is to integrate knowledge obtained from *in vitro* studies with newly developed techniques to study the biology of spermatozoa in the oviduct.

With respect to spermatozoa, the current view of the oviduct is a contradictory one. On one hand, the oviduct is thought to provide the optimal environment for sperm storage and capacitation prior to fertilization. On the other hand, the oviduct severely restricts the number of spermatozoa which reach the site of fertilization. How does the oviduct perform these

seemingly opposing functions? The answer is not yet clear. Indeed, from the following quotation taken from a review article on the oviduct written by Eddy and Pauerstein in 1980, it is clear that our state of knowledge about the various functions of the oviduct was limited.

"After more than half a century of clinical interest in female infertility, we are unable to assess accurately a single physiological function of the Fallopian tube."

During the intervening ten years since this article was written, very little information has been added to our knowledge of the functions of the oviduct. It is my hope that the studies included in this dissertation will contribute to our knowledge of the oviduct and its functions.

We owe the first accurate anatomical description of the oviduct to Gabriel Fallopius who described it as "...fleshy through its red color and its end is torn and ragged like the fringe of a well worn garment..." in his book "Observationes anatomica" written in 1561. To recognize his contribution, the human oviduct bears his name and is referred to in human anatomy texts as the Fallopian tube. In other species the oviduct has been called the salpinx, uterine tube or just tube. This has led to some confusion in terminology. To simplify the situation, the term oviduct is now used uniformly in the literature to describe this structure in all mammalian species.

Anatomically, the oviduct begins at the uterotubal junction. This juncture plays a role of varying importance, depending on species, in restricting sperm

access to the oviduct. In animals such as the rabbit, cow, and human, semen is deposited in the vagina at coitus. In these mammals, both the cervix and the uterotubal junction act to reduce the number of spermatozoa entering the oviduct (Bedford, 1970; Overstreet, 1977). In other animals like the golden hamster, rat, and pig, millions of spermatozoa are deposited directly into the uterus during coitus (Chang and Shaefer, 1957; Hunter, 1975). In these species, the uterotubal junction (UTJ) is the first major barrier that spermatozoa encounter. The UTJ reduces the number of spermatozoa that enter the oviduct by several orders of magnitude (Hunter, 1975). How the UTJ controls the passage of spermatozoa is not well understood, but several possibilities have been suggested, including sperm motility and sperm morphology (for review, see Hafez and Black, 1969). Factors controlling sperm transport through the hamster UTJ will be explored in Chapter 1.

Although several thousand spermatozoa enter the lower segments of the hamster oviduct shortly after mating (Yanagimachi and Chang, 1963), only a very small fraction of them ascend to the ampulla to participate in fertilization (Cummins and Yanagimachi, 1982). This is not unique to the hamster. In both uterine and vaginal semen depositors, the oviductal isthmus serves as a sperm reservoir during the preovulatory period (Hunter, 1975). A rapid phase of sperm transport has been reported for the hamster (Yamanaka and Solderwall, 1960), cow (VanDemark and Moeller, 1954), and rabbit (Overstreet and Cooper, 1978a) in which a vanguard population of

spermatozoa are rapidly transported to the ampulla within minutes of ejaculation. However, at least for the rabbit, these vanguard spermatozoa were dead and did not participate in fertilization (Overstreet and Cooper, 1978a). The spermatozoa which did participate in fertilization were among the spermatozoa stored in the isthmus during the preovulatory period (Overstreet and Cooper, 1978b). Therefore, the oviductal isthmus appears to play an important role in the storage of spermatozoa during the preovulatory period in a variety of species. An investigation into sperm transport and storage in the hamster oviduct constitutes Chapter 2 of this dissertation.

In addition to being a site of sperm storage, the lower oviduct has been implicated as a major site of sperm capacitation (Yanagimachi, 1988). Very little is currently known about how sperm capacitation progresses within the oviduct. Even the simple questions of where and when capacitation is completed in the oviduct are presently unclear. These questions will be addressed in Chapter 3.

Although many spermatozoa enter the hamster oviduct within one hour after mating (Yanagimachi and Chang, 1963), we do not know what proportion of these spermatozoa are viable at the time of ovulation. Previous studies on the viability of spermatozoa in the female tract have been concerned with the fertilizable life of spermatozoa, that is, how long can spermatozoa remain in the female tract and still be capable of fertilizing an egg (for review, see Austin, 1975). Values ranging from 12 hours for mouse

spermatozoa (McGaughey et al., 1968) to 126 hours for ferret spermatozoa (Chang, 1965) have been reported. Such studies reveal the maximum functional life of spermatozoa in the female tract, but give little information about the physiological condition of the general population of spermatozoa in the female tract at various times after mating. The viability of spermatozoa stored in the oviduct during the preovulatory period will be discussed in Chapter 4.

How spermatozoa are sequestered in the lower isthmus during the preovulatory period is currently unknown. Depressed sperm motility in the isthmus has been suggested as a possible mechanism for limited sperm ascent in the rabbit (Cooper et al., 1979). Immotile spermatozoa were recovered from rabbit oviducts (Overstreet and Cooper, 1975) and their immotile state was reversed under certain specific in vitro conditions (Burkman et al., 1984). Sperm attachment to the isthmic epithelium has been observed in the rabbit (Overstreet and Cooper, 1975), pig (Flechon and Hunter, 1981; Hunter et al., 1987), and mouse (Suarez, 1987) and has been suggested to play an important role in sequestering the spermatozoa in the lower oviduct. Coordinated oviductal contractions during the periovulatory period (Battalia and Yanagimachi, 1979) and hyperactivated sperm motility (Suarez and Osman, 1987) both have been suggested as possible mechanisms for the release of spermatozoa from the isthmus near the time of ovulation. Chapter

5 examines attachment and release of spermatozoa from the caudal isthmus of the oviduct.

Although the studies included in this dissertation address several questions with regard to the biology of spermatozoa in the oviduct, much remains to be studied in this largely neglected, yet very important, area of reproduction.

# CHAPTER 1

## A Quantitative Comparison of the Passage of Homologous and Heterologous Spermatozoa Through the Uterotubal Junction of the Golden Hamster

### Introduction

Mammals may be arbitrarily divided into two groups according to the site of semen deposition at coitus. For the first group, which includes the rabbit, cow, and human, semen is deposited in the vagina at coitus. In these mammals, both the cervix and uterotubal junction (UTJ) act to reduce the number of spermatozoa entering the oviduct (Bedford, 1970; Overstreet, 1977). In the second group, semen is deposited directly into the uterus at coitus. The golden hamster, pig, and dog belong to this group. In these animals, millions of spermatozoa are deposited in the uterus at coitus, but the number of spermatozoa reaching the oviducts is reduced by several orders of magnitude (Hunter, 1975; Smith *et al.*, 1987). In another study I showed that following artificial insemination of female hamsters with  $4 \times 10^7$  epididymal spermatozoa, only a maximum mean of  $6 \times 10^3$  spermatozoa entered each oviduct. The UTJ must therefore act as a major barrier to sperm ascent following coitus and artificial insemination in these animals. The mechanisms by which the UTJ restricts the number of ascending spermatozoa

are not completely understood, but several possibilities have been suggested (for review, see Hafez and Black, 1969).

To date, all studies on the passage of foreign and immotile spermatozoa through the UTJ into the oviduct following artificial insemination have been accomplished by using one of the following three techniques. The first, used by Leonard and Perlman (1949), Howe and Black (1963), and Marcus (1965), was to excise the oviduct following artificial insemination and express its contents onto a microscope slide. This method was adequate to determine the mere presence or absence of spermatozoa, but it does not yield quantitative data. Quantitative data are necessary to make a direct comparison between the ability of homologous and heterologous spermatozoa, or motile and immotile spermatozoa, to pass through the UTJ into the oviduct.

Gaddum-Rosse (1981) used a novel approach to the question of sperm transport through the UTJ by using a preparation which allowed the direct observation of spermatozoa as they emerged from the severed end of the intramural isthmus. Although quantitative results were obtained using this preparation, the number of spermatozoa emerging 30-60 min after mating was low compared to the number recovered from the oviduct at 45 min by Shalgi and Kracier (1978). This suggests that the preparation used by Gaddum-Rosse may not have been physiological. The third technique used has been to flush the excised oviduct with a simple physiologically balanced salt solution following insemination with foreign or immotile spermatozoa and to then

count the spermatozoa in the resulting sperm suspension (Phillips and Andrews, 1937; Coggins and Baker, 1968; Baker and Degen, 1972; Viring, 1981). This method also yields quantitative results, but the data obtained by this method must be interpreted with caution because, when a detergent is not added to the salt solution used for flushing, many spermatozoa remain stuck in the epithelial crypts and cannot be recovered (Overstreet and Cooper, 1978 a, b). Since as many as half the spermatozoa in the hamster oviduct were found in these crypts (Smith *et al.*, 1987), the use of this technique can lead to a significant underestimation of the number of spermatozoa present in the oviduct.

Also, with the exception of Viring (1981), none of the above studies determined the number of spermatozoa present in the intramural isthmus of the oviduct. In these studies, the oviduct was excised at its external junction with the uterus, leaving the intramural isthmus behind. The spermatozoa in this segment were not recovered in either uterine or oviductal flushings and hence, were not considered. Since the number of foreign and immotile spermatozoa passing through the UTJ may be greatly reduced, and their advance into the oviduct restricted to the intramural isthmus, it is, therefore, of critical importance to examine this segment of the oviduct. At the present time, the only way to accurately determine the number of spermatozoa present in the intramural isthmus and other segments of the oviduct is to serially

section the entire oviduct (from infundibulum to UTJ) and count the spermatozoa in the resulting sections.

## **Materials and Methods**

### Animals

The female golden hamsters used in this study were raised from weaning in an air conditioned room with a reversed light cycle (light 17:00-7:00 h; dark, 7:00-17:00 h). Mature females (3-4 months old) raised under this light regime ovulated between 13:00 and 15:00 h of every fourth day. Every evening, each female was checked by the method of Orsini (1961) for a post ovulatory vaginal discharge. Females exhibiting this discharge were designated to be in the evening of day 1 of the cycle.

### Sperm collection and insemination

The spermatozoa of the hamster, rat, mouse, guinea pig, and rabbit were obtained from caudae epididymides. Spermatozoa were suspended in Tyrode's solution containing 0.1% bovine serum albumin (TA) (Cummins and Yanagimachi, 1982). Immotile (dead) hamster spermatozoa were prepared by either treating the sperm suspension (in TA) with heat (65°C) for 30 min or suspending the spermatozoa first in distilled water for 20 min, then resuspending them in TA after centrifugation. The final concentration of spermatozoa in all suspensions used in this study was approximately  $1 \times 10^8$  cells/ml.

Insemination was performed around 08:00 h of day 1 by anesthetizing an estrous female with ether, exposing the uteri through a mid-ventral incision and injecting 0.2 ml of sperm suspension ( $2 \times 10^7$  spermatozoa) into each uteri through a 26 gauge needle attached to a 1 ml syringe. Using this method, the number of spermatozoa inseminated was within the range of the number of spermatozoa found in the uteri of hamsters mated for 1 h (Chang and Sheaffer, 1957). I found that ether anesthesia had no effect on sperm transport into the oviduct (Smith *et al.*, 1987).

#### Histology and sperm counting

All females were killed by cervical dislocation under ether anesthesia at 5 h after insemination (about the time of ovulation). Oviducts were fixed in situ for 10 min by injection of 10 ml of warm (37°C) A.F.A. fixative (25% ethanol, 10% formalin, 10% acetic acid, 55% water) (Orsini, 1962) into the dorsal peritoneal cavity immediately after cervical dislocation. Oviducts were then removed with the adjacent ovary, fat pad, and the uppermost 5 mm of uterus attached and then immersed in fresh A.F.A. fixative for 24 hours at room temperature. After fixation, oviducts were trimmed of the ovary and fat pad, dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin. No attempt was made to straighten the oviducts prior to sectioning. Each oviduct was then serially sectioned (10  $\mu\text{m}$ ) from the infundibulum to UTJ. Sections were stained with Schiff's reagent and counter stained with Fast Green F.C.F. (Smith *et al.*, 1987).

The hamster oviduct was considered to consist of four segments: 1) uterotubal junction and intramural isthmus, the segment which makes 1 loop in the wall of the uterus; 2) caudal isthmus, the first 5-6 loops outside the uterus; 3) cephalic isthmus, the next 3-4 loops; 4) ampulla the last 3 loops (for detailed criteria defining segments, see Chapter 2). Individual spermatozoa were counted in each serial section and scored according to the oviductal segment in which they were found. Three females (i.e. six oviducts) were used for each determination. Since no significant difference (paired t-test,  $p > 0.05$ ) was found between the number of spermatozoa entering the right and left oviducts, data from both right and left oviducts were combined.

#### Sperm viability in the uterus

In addition to determining the number and distribution of spermatozoa in the oviduct, the motility of spermatozoa in the uterus at 1 and 5 h after insemination was also determined. For each experimental group, four additional females were inseminated and killed by cervical dislocation under ether anesthesia at 1 h (2 females) or 5 h (2 females) after insemination. The uterus from each female was removed, flushed with 0.4 ml TA (37°C) into a watchglass and the motility of the spermatozoa in the flushings was examined immediately.

## Results

Table 1 summarizes the number and distribution of homologous and heterologous (foreign) spermatozoa in the various segments of the hamster oviduct at 5 h after insemination. It can be seen that in all cases heterologous spermatozoa passed through the UTJ within 5 h after insemination, but in much smaller numbers compared to the number of hamster (homologous)spermatozoa found in the oviduct at that time. Among the foreign spermatozoa, rabbit spermatozoa were the most efficient in passing through the UTJ and mouse spermatozoa were the least efficient. Immotile (dead) hamster spermatozoa passed through the UTJ into the oviduct, but only in very small numbers.

At 5 h after insemination, the majority of hamster spermatozoa were found in the caudal isthmus (Table 1) as previously reported by Smith *et al.* (1987). The majority of foreign spermatozoa were found in the intramural segment of the oviduct. This was particularly true for the guinea pig spermatozoa which were found exclusively in this segment. The few immotile and foreign spermatozoa found in the caudal isthmus were typically located in the first ascending loop of this segment. This was in contrast to hamster spermatozoa which were motile at the time of insemination. These spermatozoa were distributed throughout the caudal isthmus and had advanced into the cephalic isthmus by this time.

**Table 1. Number  $\pm$  s.d., (range), and distribution of hamster and foreign spermatozoa in the various segments of the hamster oviduct at 5h after insemination. Three females were used for each determination.**

Spermatozoa of	Mean $\pm$ s.d. (range) of spermatozoa in			
	Uterotubal junction & Intramural Isthmus	Caudal Isthmus	Cephalic Isthmus	Ampulla
Hamster	920 $\pm$ 923 (316 - 2773)	2951 $\pm$ 388 (2356 - 3521)	14 $\pm$ 17 (3 - 46)	0
Rabbit	287 $\pm$ 226 (0 - 545)	30 $\pm$ 28 (0 - 66)	0	0
Rat	184 $\pm$ 143 (10 - 386)	15 $\pm$ 29 (0 - 73)	0	0
Guinea Pig	104 $\pm$ 105 (21 - 256)	0	0	0
Mouse	30 $\pm$ 38 (3 - 99)	9 $\pm$ 9 (0 - 22)	0	0
Hamster Immotile <sup>a</sup>	6 $\pm$ 4 (0 - 12)	9 $\pm$ 19 (0 - 67)	0	0

<sup>a</sup> Six females were used in this group, three were inseminated with spermatozoa immobilized (killed) by heating, the other three with spermatozoa immobilized (killed) with distilled water. Since the results obtained were essentially the same, data for the two groups were combined.

There was a considerable decrease in the motility of foreign and homologous spermatozoa recovered from the uterus at 1 h after insemination (1-30% motile) compared to prior to insemination (60-90% motile), with the notable exception of guinea pig spermatozoa, of which the majority (about 70%) were still actively motile at 1 h after insemination. By 5 h after insemination, all spermatozoa recovered from the uterus, foreign and homologous, were immotile and presumably dead except for a few (about 1%) weakly motile rat spermatozoa which were presumably dying.

#### **Discussion**

I found that foreign and immotile spermatozoa could pass through the UTJ and advance into the oviduct. This finding is in agreement with previous studies of this type (Yochem, 1929; Phillips and Andrews, 1937; Howe and Black, 1963; Marcus, 1965; Coggins and Baker, 1968; Bedford, 1967). I observed a considerable difference between both motile and immotile, and foreign and homologous spermatozoa, with respect to their number and distribution in the oviduct, demonstrating that the hamster UTJ functions as a selective barrier to immotile and foreign spermatozoa.

The nature of this selective process is not clear. In the present study, the shape of the sperm head did not appear to play a significant role in the passage of spermatozoa through the UTJ. Our data suggests that rat and mouse spermatozoa with "hooked" heads like hamster spermatozoa did not

demonstrate any consistent advantage over rabbit and guinea pig spermatozoa with "round" heads in passing through the UTJ. There is evidence to suggest that the size of the sperm head may effect the passage of spermatozoa through the UTJ. Mortimer (1977) suggested that the rabbit UTJ may select against diploid rabbit spermatozoa and since diploid spermatozoa have larger heads than their haploid counterparts, selection may be occurring on the basis of head size. In the present study, guinea pig spermatozoa, which have relatively large heads compared to the spermatozoa of the other species used, passed through the UTJ only in very small numbers and did not advance beyond the initial portion of the intramural segment. Their relatively large head size may have contributed to their reduced number and restricted advance in the hamster oviduct. In addition, the majority of mature guinea pig spermatozoa are in highly regular head-to-head agglutinated groups (rouleaux) both in vivo (Martan and Shepherd, 1973) and in vitro (Yanagimachi, 1972). An alternative explanation for the low number of guinea pig spermatozoa observed in the hamster oviduct is that these groups are too large to pass through the hamster UTJ. The guinea pig spermatozoa observed in the hamster oviduct were found either singly or in small randomly associated groups and did not appear to be in the highly ordered rouleaux formation. This suggests that guinea pig spermatozoa passed through the hamster UTJ singly and then randomly associated again in the oviduct. It

should be pointed out, however, that the seemingly random appearance of the groups may have been an artifact of fixation.

The number and distribution of foreign spermatozoa in the hamster oviduct does not appear to be related simply to their viability in the hamster uterus. Although the greatest number of spermatozoa in the oviduct were found following insemination with hamster spermatozoa, the motility of hamster spermatozoa in the uterus had decreased from about 60% immediately prior to insemination to about 10% within 1 h after insemination. On the other hand, guinea pig spermatozoa which were incapable of progressing beyond the initial portion of the intramural segment, exhibited good motility (about 70%) when recovered from the hamster uterus 1 h after insemination. These results can be related to those of Howe and Black (1963), who found that although human spermatozoa survived as well as rabbit spermatozoa in the rabbit uterus, human spermatozoa did not enter the rabbit oviduct in large numbers. Therefore, in the present study, sperm viability alone does not explain why the spermatozoa of some species were present in the hamster oviduct in larger numbers than those of other species. My observation that the majority of hamster spermatozoa recovered from the uterus at 1 h after insemination were immotile (presumably dead or dying) taken together with the notion that sperm motility is important for the passage of spermatozoa through the UTJ, suggests that under these conditions, most live hamster spermatozoa enter the oviduct within 1 h after artificial insemination. This is in agreement with my

previous findings that 75% of the maximum mean number of hamster spermatozoa found in the oviduct following artificial insemination were present by 1 h after insemination (Smith et al., 1987).

All the spermatozoa recovered from the uterus at 5 h after insemination, including hamster spermatozoa, were immotile except for a very few weakly motile rat spermatozoa. The reduced viability of uterine spermatozoa at 5 h after insemination suggests that the hamster uterine environment is hostile to the epididymal spermatozoa of any species, including hamster.

My results indicate that sperm motility strongly influences the passage of spermatozoa through the UTJ. I found that immotile spermatozoa were passively transported through the UTJ into the oviduct, but only in very small numbers, implying that the inherent motility of spermatozoa plays a pivotal role at this juncture. My results are in agreement with VanDemark and Moeller (1951), Baker and Degen (1972) and Viring (1981) who also found a small number of immotile spermatozoa were transported into the oviduct. Gaddum-Rosse (1981) did not observe the emergence of any immotile spermatozoa from the severed end of the intramural isthmus of the rat. However, in the Gaddum-Rosse study, the extramural portion of the oviduct had been removed and it is possible that the removal of these segments of the oviduct eliminates the driving force which is necessary for the passive transport of immotile spermatozoa through the UTJ and intramural segment. The passive transport of spermatozoa through the UTJ and intramural isthmus

is difficult to explain, but may have been due to spermatozoa having been sucked through the UTJ into the oviduct during the relaxation phase of oviductal contractions in a bellows-like fashion. Since the majority of the spermatozoa in the oviduct following insemination with immotile (dead) spermatozoa were found wedged into furrows along the wall of the intramural segment, it is possible that after entering the oviduct and becoming wedged, they remained in the oviduct and were not pushed back out into the uterus during subsequent oviductal contractions.

Although immotile (dead) hamster spermatozoa were passively transported to the first ascending loop of the caudal isthmus by 5 h after insemination, motile (live) hamster spermatozoa had advanced to the cephalic isthmus in significant numbers by this time. These results indicate that the adovarian oviductal contractions which begin about this time (just prior to ovulation) (Battalia and Yanagimachi, 1979) were not sufficient to propel immotile spermatozoa to the cephalic isthmus. This was also found to be true of foreign spermatozoa. A possible explanation for the retarded advance of foreign spermatozoa is that the luminal environment of the intramural and caudal isthmus was hostile to foreign spermatozoa. They may have died there, or, if they remained alive, they could not undergo the necessary physiological changes (e.g. hyperactivation) which would facilitate their advance to the cephalic isthmus and ampulla. It would appear then, that the

intramural and caudal isthmus act as a second barrier to the passage of foreign spermatozoa in the hamster oviduct.

## CHAPTER 2

### Distribution and Number of Spermatozoa in the Oviduct of the Golden Hamster after Natural Mating and Artificial Insemination

#### Introduction

In mammals, it is in the ampullary region of the oviduct where spermatozoa and eggs meet and fertilized eggs (zygotes) begin to develop. At coitus, millions of spermatozoa are deposited in the lower part of the female genital (i.e., vagina or uterus), but the cervix, utero-tubal junction and/or the lower part of the oviduct, each act as a "barrier" or "sieve" to sequentially reduce the number of spermatozoa which reach the upper regions of the tract, ensuring that only a small number of spermatozoa reach the ampulla (for reviews, see Blandau, 1969, 1973; Bedford, 1970; Hunter 1973, 1980; Ahlgren *et al.*, 1975; Mortimer, 1978, 1983; Polge, 1978; Harper, 1982; Hawk, 1983; Overstreet, 1983).

Before spermatozoa become capable of fertilizing eggs, they must undergo the process of capacitation. The site where capacitation is initiated and completed may vary from species to species. In species where semen is deposited "directly" into the uterus during coitus (e.g., many rodents, dog, and pig), sperm capacitation may begin in the uterus or soon after the spermatozoa enter the oviduct. For species in which semen is deposited in the

vagina at coitus (e.g., rabbit, cow, and human), capacitation may begin in the vagina or as spermatozoa pass through the cervix.

This laboratory and many others have used the golden hamster as a model for the analysis of fertilization mechanisms in mammals. Although a great deal of information has been obtained concerning sperm capacitation in vitro (for references, see Bavister, 1980; Yanagimachi, 1981; Meizel, 1984), the questions of where and how capacitation occurs in vivo remains to be answered. As a preliminary to addressing these questions, it is necessary to know how spermatozoa are distributed in the female genital tract before and during fertilization. Several previous studies have reported the distribution of hamster spermatozoa in the female genital tract (Strauss, 1956; Chang and Sheaffer, 1957; Yamanaka and Soderwall, 1960; Yanagimachi and Chang, 1963), but the methods used in these studies were not sufficiently quantitative.

In general, three methods have been used to study the transport or distribution of mammalian spermatozoa in the female genital tract. The first has been to flush the contents of different segments of the tract with a physiologically balanced salt solution at various times after mating or artificial insemination and to count the number of spermatozoa collected. Although several studies have used this technique (e.g., Braden, 1953), this technique tends to underestimate the number of spermatozoa present in the tract because many spermatozoa remain in the epithelial crypts of the tract and

cannot be easily flushed out (Thibault, 1973). This problem can be overcome by using detergent in the flushing medium which acts to dislodge spermatozoa from the epithelial crypts (Overstreet and Cooper, 1978a, b). However, the exact location of spermatozoa within the female genital tract cannot be determined using this technique.

Another approach has been to place a ligature at a specified place of the tract at various times after mating to observe what effect this had on the number of eggs which were later fertilized. This technique was first used to examine sperm transport in rabbits (Adams, 1956) and has more recently been employed to examine sperm transport in the ewe and sow (Hunter *et al.*, 1980; Hunter, 1981; Hunter and Nichol, 1983; Hunter, 1984). The main focus of these studies was to determine when enough spermatozoa had reached the oviduct to effect complete fertilization. However, this technique also has a limitation in that the number and specific position of spermatozoa can only be inferred.

Lastly, with respect to the number and distribution of spermatozoa in the tract, more direct results can be obtained by examining serial sections of the tract. Using this method, Morricard and Bossu (1951) counted the number of spermatozoa in the ampullary segment of the rat oviduct around the time of fertilization. Yanagimachi and Chang (1963) examined the distribution of spermatozoa in serial sections of the hamster oviduct, but limited their observations to one hour after mating. Thibault *et al.* (1975) serially

sectioned the entire length of the bovine oviduct and counted the spermatozoa in the various segments. Considering the length of the bovine oviduct (about 14 cm), this was an admittedly laborious task, but worthwhile in that they obtained a very detailed picture of sperm number and distribution at different times after mating.

Compared to the cow oviduct, the hamster oviduct is relatively short (about 4 cm; Battalia and Yanagimachi, 1979) and follows an extremely tortuous path in situ. Because of these anatomical features, I have chosen to make practical use of the serial section technique to examine the number and distribution of spermatozoa in the hamster oviduct at various times after mating and artificial insemination.

## **Materials and Methods**

### **Animals**

Mature golden hamster females (7-10 weeks old) and males (12-20 weeks old) were used in the study. Animals were raised and kept in an air conditioned room under controlled light conditions (light, 05:00-19:00 h; dark, 19:00-05:00 h). Under this regime, mature females came into estrus by 1800 h of every fourth day and ovulated between 01:00 and 03:00 h of the following day. Females were checked, each morning for four consecutive days, for the presence of a post-ovulatory vaginal discharge (Orsini, 1961). The day of the post-ovulatory discharge (the day of ovulation) was designated as Day

1 of the estrous cycle. After determining their cycle, females were individually housed in small cages (20 X 26 X 15 cm). Females exhibited estrus on the evening of Day 4. Males used for mating were housed individually in large cages (30 X 35 X 17 cm). Those used for collection of spermatozoa for artificial insemination were raised together and housed in groups of up to six in each large cage.

#### Mating

Each estrus female was placed in the cage of a male of proven fertility which had been rested for a least two days prior to each experiment. The onset of intromissions (which normally takes place within 1 min after the introduction of the female into the male's cage) was considered to be the onset of mating. The female remained in the male's cage for 30-60 min until a vaginal smear confirmed that at least one ejaculation had occurred. The female was then returned to its previous cage. A group of 18 females was mated at 20:00 h of Day 4 (5-7 h prior to ovulation). Another group of 9 females was mated at 0200 h of Day 1 (during the time of ovulation).

#### Artificial insemination

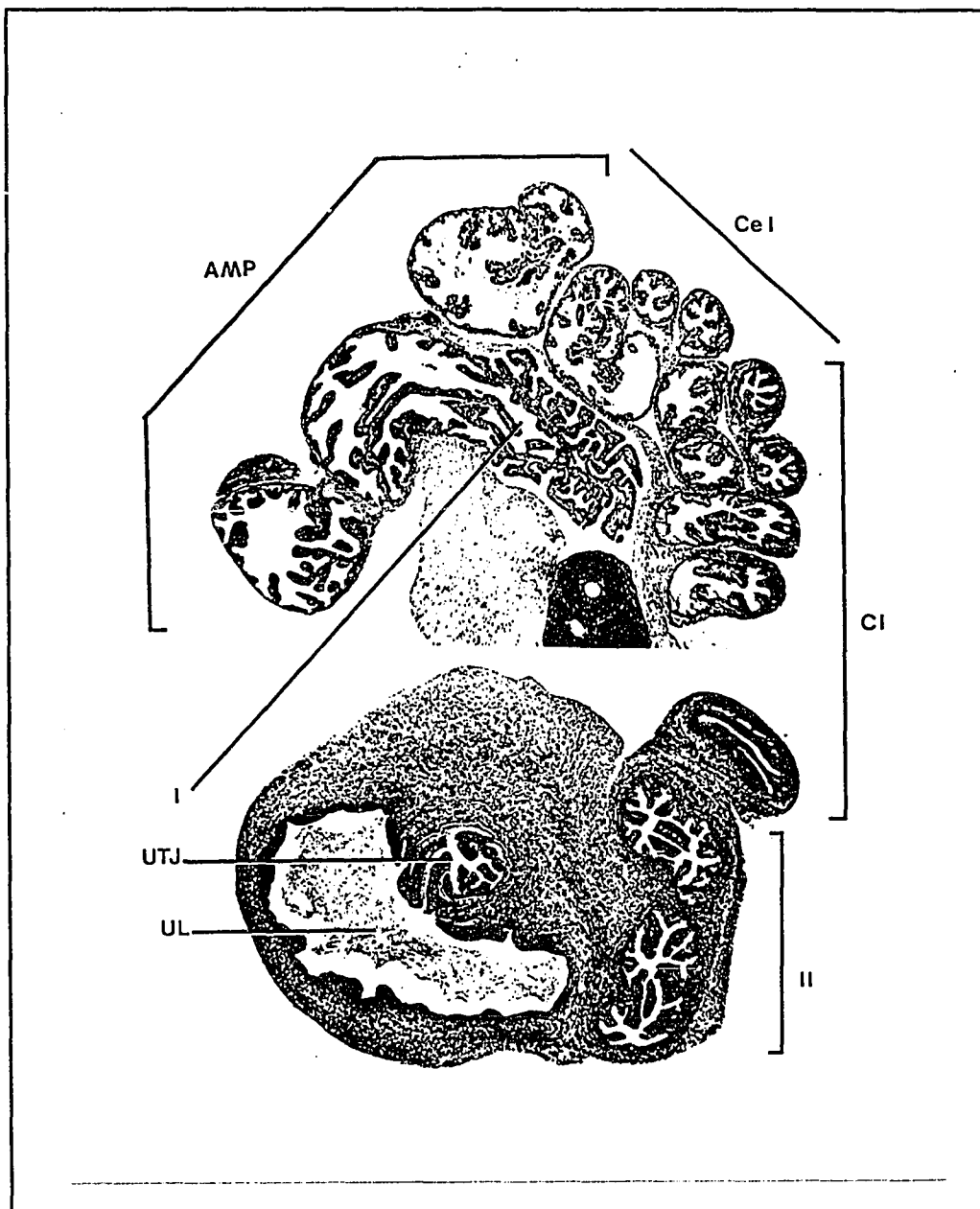
Spermatozoa were collected from caudae epididymides and suspended in 0.9% NaCl at a concentration of approximately  $1.7 \times 10^8$  cells/ml. A group of 21 females was artificially inseminated between 20:00 and 21:00 h of Day 4 (5-7 h prior to ovulation). This was done by exposing uteri under ether

anesthesia and injecting 0.2 ml of fresh sperm suspension into each uterus through a 26 gauge needle attached to a tuberculin syringe.

#### Histology and sperm counting

At various times after mating or artificial insemination, females were anesthetized with ether. Ten to 15 ml of A.F.A. fixative (25% ethanol, 10% formalin, 10% glacial acetic acid, 55% distilled water; Orsini, 1962), prewarmed to 37°C, was injected into the dorsal peritoneal cavity to fix the genital tract in situ. Ten minutes later, oviducts with adjoining ovaries and uteri were excised and immersed in fresh A.F.A. fixative for 6 to 24 h at room temperature. After fixation, the oviducts were trimmed of excess tissue, dehydrated in graded alcohols, cleared with xylene, and embedded in paraffin. Each oviduct was serially sectioned (10 $\mu$ ) beginning at the ovarian end to avoid contamination of subsequent sections with uterine spermatozoa. Sections were hydrolyzed with 1 N HCl at 60°C for 6 min, stained with Schiff's reagent and counterstained with Fast Green F.C.F., with the exception of the sections from a female mated prior to ovulation which were stained with hematoxylin and eosin for photography (Figures 1 and 2) (sperm tails do not stain well with Fast Green F.C.F.).

Figure 1 shows cross sections of the upper and lower portions of the hamster oviduct. Although morphologically, the oviduct makes a more or less continuous transition from the utero-tubal junction to the infundibulum, distinct segments are apparent. For the purpose of the present study, I



**Figure 1.** Upper and lower regions of the hamster oviduct. UL, Uterine Lumen; UTJ, Uterotubal junction; II, Intramural Isthmus; CI, Caudal Isthmus, CeI, Cephalic Isthmus, AMP, Ampulla; I, Infundibulum. [10 $\mu$ m, H&E, X30]

arbitrarily divided the oviduct into four segments: (1) utero-tubal junction and the intramural isthmus, which makes a single loop within the wall of the

uterus; (2) caudal isthmus, the portion composed of the first four or five loops of the oviduct outside the uterine wall; (3) cephalic isthmus, the next three or four loops having slightly larger outside diameter and lumen than the preceding loops; (4) ampulla, the last two or three loops having a thin wall and much larger outside diameter and lumen than the preceding loops.

Individual spermatozoa were counted in each serial section and scored according to the segment of the oviduct in which they were found. Those found in the cumulus oophorus, on and in eggs, and in the infundibulum were recorded as ampullary spermatozoa.

An egg was recorded as fertilized when a spermatozoon was seen on/in the surface of the vitellus, or a decondensing sperm nucleus or both sperm and egg pronuclei was/were discernible within the vitellus. Resumption of meiotic division was taken as another indication of fertilization.

## Results

Within experimental groups, the number of spermatozoa found in each oviduct varied greatly from animal to animal. In tables 2-5, the range has been given to show this variation among animals. For each time period, there was no significant difference ( $p > 0.05$ ) between the number of spermatozoa found in right and left oviducts (paired t-test).

In all three experimental groups, most spermatozoa were found in the lower segments of the oviduct throughout the period of observation. More



**Figure 2.** A small group of spermatozoa wedged into an epithelial crypt of the caudal isthmus [10 $\mu$ m, H&E, X30].

than half of the spermatozoa in the lower segments of the oviduct had formed small groups (Figure 2). The spermatozoa in the cephalic isthmus were usually observed singly and were located towards the center of the lumen. Spermatozoa in the ampulla were found most commonly within the egg-cumulus oophorus complex.

Table 2 shows the number of spermatozoa found in various segments of the oviduct of females mated 5 to 7 h prior to ovulation. At 1 h after the onset of mating (1 h post coitus or 1 h p.c.), a large number of spermatozoa was observed in the lower two segments of the oviduct. By 3 h p.c., the

**Table 2 Mean number, (range) and % distribution of spermatozoa in various segments of the oviduct of females mated from 20:00 to 21:00 h Day 4 (5-7 hours prior to ovulation). Six oviducts were used for each determination.**

REPRODUCTIVE STAGE	HOURS POST COITUS	SEGMENTS OF THE OVIDUCT				
		Utero-tubal Junction & Intramural Isthmus	Caudal Isthmus	Cephalic Isthmus	Ampulla	Total
Preovulatory	1	4278 (1310-7839) 68%	1977 (506-3871) 32%	0.2 (0 - 1) <1%	0.2 (0 - 1) <1%	6255 (1816-9398) 100%
	3	2288 (937-4164) 27%	6150 (2784-9610) 73%	0.0 (0) 0%	0.0 (0) 0%	8438 (3721-10729) 100%
Periovulatory	5	3896 (31-6794) 25%	12005 (206-20210) 75%	3.5 (1-12) <1%	0.2 (0-1) <1%	15905 (249-26311) 100%
Postovulatory	7	3500 (105-7505) 18%	15434 (4536-28479) 81%	7.0 (2-14) <1%	0.3 (0-1) <1%	18941 (4655-34395) 100%
	9	4300 (377-11556) 28%	11202 (8503-13250) 72%	31.0 (2-70) <1%	2.5 (1-6) <1%	15536 (8913-24809) 100%
	11	2283 (3-5911) 23%	7484 (63-10050) 76%	22.5 (1-44) <1%	14.8 (3-33) <1%	9804 (79-18157) 100%

majority of spermatozoa (73%) had moved to the caudal isthmus. From this time onward, the percentage of spermatozoa found in the caudal isthmus remained more or less constant (72% - 81%). The total number of spermatozoa in the oviduct continued to increase to a maximum at 7 h p.c., but after this time, the total declined. In this group, a single spermatozoon was found in the infundibulum of one oviduct at 1 h p.c.. No spermatozoa were found in the periovarian space. Although a single spermatozoon was found in the ampullae of some females at 5 and 7 h p.c., it was not until 9 h p.c. that spermatozoa reached the ampulla in all females. By 11 h p.c., a significant number (3 - 33) of spermatozoa had arrived at the ampulla and fertilization was nearing completion (Figure 3).

Table 3 shows the number of spermatozoa in the oviduct of females mated during the ovulatory period. As with the animals mated prior to ovulation, a considerable number of spermatozoa was found in the lower portions of the oviduct at 1 h p.c. A larger proportion of spermatozoa was found in the caudal isthmus at an earlier time in this group (50% at 1 h p.c.; cf. Table 3) when compared to animals mated early in estrus (32% at 1 h p.c.; cf. Table 2). From this, it would appear that spermatozoa were being transported to the caudal isthmus at a faster rate in animals mated during ovulation as compared to animals mated several hours before ovulation. In animals mated near ovulation, two spermatozoa were found in the infundibulum of one oviduct at 1 h p.c.. A few more spermatozoa (1-3) were found in the

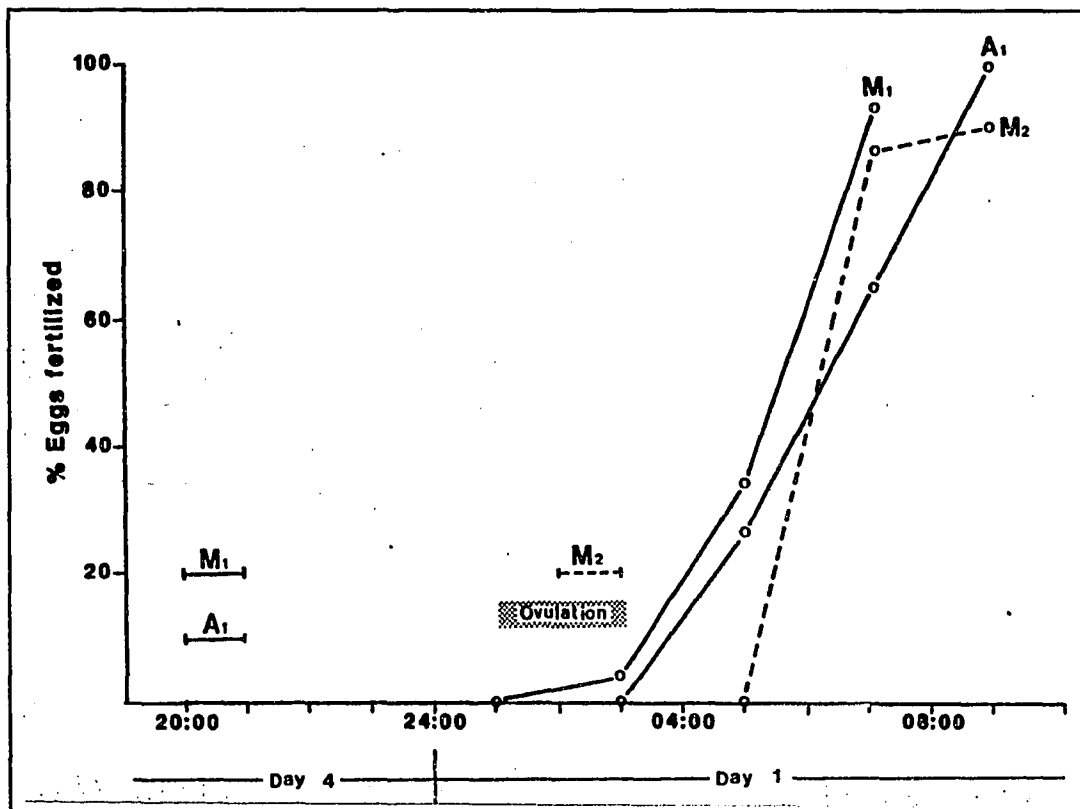


Figure 3. Fertilization rates following mating and artificial insemination. M<sub>1</sub>, early mating; M<sub>2</sub>, late mating; AI, artificial insemination.

ampulla at 3 h p.c.. The number of spermatozoa in the ampulla had increased markedly (2-243) by 5 h p.c.. At this time, the eggs had reached the lower ampulla and fertilization was nearly complete (Figure 3).

The results of artificial insemination performed 5 to 7 h before ovulation are summarized in Table 4. The total number of spermatozoa entering the oviduct following artificial insemination was notably lower than the number following natural mating. The total number of spermatozoa in the oviduct increased until 3 h after insemination and then slowly declined for the remainder of the observation period. When the percentage of spermatozoa in

**Table 3 Mean number, (range) and % distribution of spermatozoa in various segments of the oviduct of females mated from 02:00 to 03:00 h Day 1 (during the time of ovulation). Six oviducts were used for each determination.**

REPRODUCTIVE STAGE	HOURS POST COITUS	SEGMENTS OF THE OVIDUCT				
		Utero-tubal Junction & Intramural Isthmus	Caudal Isthmus	Cephalic Isthmus	Ampulla	Total
Periovulatory	1	3699 (1178-8214) 50%	3701 (1945-4913) 50%	2.3 (1-5) <1%	0.5 (0-2) <1%	7403 (3941-13134) 100%
	3	4822 (3850-5638) 40%	7329 (4536-28479) 60%	16.5 (1-51) <1%	1.2 (1-3) <1%	12169 (8529-15615) 100%
Postovulatory	5	7843 (126-10683) 38%	12975 (3211-20287) 62%	63.3 (2-269) <1%	58.5 (2-243) <1%	20940 (3482-35295) 100%

**Table 4 Mean number, (range) and % distribution of spermatozoa in various segments of the oviduct of females artificially inseminated (AI) from 20:00 to 21:00 h Day 4 (5-7 hours before ovulation). Six oviducts were used for each determination.**

REPRODUCTIVE STAGE	HOURS POST AI	SEGMENTS OF THE OVIDUCT				
		UTJ & Intramural Isthmus	Caudal Isthmus	Cephalic Isthmus	Ampulla	Total
Preovulatory	1	3317 (2039-5932) 73%	1207 (598-1807) 27%	0.0 (0) 0%	0.0 (0) 0%	4524 (2983-7255) 100%
	3	4536 (2138-5607) 75%	1523 (532-2537) 25%	0.0 (0) 0%	0.0 (0) 0%	6059 (3291-7466) 100%
Periovulatory	5	976 (205-2141) 29%	2349 (842-4255) 70%	2.0 (0-5) <1%	2.3 (0-14) <1%	3329 (1202-6414) 100%
Postovulatory	7	980 (58-4901) 36%	1700 (1052-3375) 63%	23.8 (5-65) <1%	0.5 (0-1) <1%	2705 (1167-8288) 100%
	9	145 (0-488) 14%	883 (65-1961) 85%	8.8 (0-26) 1%	3.5 (0-10) <1%	1040 (73-2023) 100%
	11	83 (26-208) 21%	284 (87-505) 71%	14.3 (4-22) 4%	15.2 (1-32) 4%	397 (155-631) 100%
	13	36 (12-71) 13%	158 (28-413) 56%	31.8 (5-83) 11%	55.3 (22-82) 20%	281 (83-649) 100%

the segments of the oviduct of this group are compared to percentages found following mating prior to ovulation (Table 2), it can be seen that, although the number of spermatozoa in the oviduct is smaller following artificial insemination, the distribution of spermatozoa is very similar in the two groups. The overall rate of sperm transport to the ampulla was about the same in artificially inseminated animals as that found in animals naturally mated during the same time period (5-7 h prior to ovulation). The first spermatozoon arrived at the ampulla 5 h after insemination, when ovulation is about to begin. From this time on, the number of spermatozoa in the ampulla increased. It should be noted that although the total number of spermatozoa entering the oviduct in this group was relatively small, all eggs were fertilized (Fig. 3).

### Discussion

The present study revealed that there is a large variation in the total number of spermatozoa transported into the hamster oviduct. Two factors may have been responsible for the variation encountered. Firstly, there is a wide range in the number of spermatozoa ejaculated by different males and by an individual male on different occasions (Chang and Sheaffer, 1957). Compounding this problem, the number of ejaculations which occurred during the 30 to 60 min mating period was not controlled in these experiments. The major concern was that at least one ejaculation had occurred. Variations in

the total number of ejaculations may have had a marked effect on the number of spermatozoa observed later in the oviduct. To eliminate this large variation in future experiments, the number of ejaculations during the mating period could be determined and controlled using the behavioral criteria described by Bunnell *et al.* (1977).

Although care was taken to artificially inseminate each female with a number of spermatozoa comparable to the number ejaculated during one hour of natural mating (Chang and Sheaffer, 1957), the number of spermatozoa transported into the oviduct following artificial insemination was considerably less than the number transported into the oviduct following natural mating (compare Tables 2 and 4). This could have been due to several factors. Coital stimulus and semen components were absent under the conditions of artificial insemination. These factors may play a role in inducing uterine contractions that facilitate the entry of spermatozoa into the oviduct. Another possible explanation is that 0.9% NaCl was used to suspend the spermatozoa for artificial insemination. This may not be the best medium to support sperm motility. Perhaps more spermatozoa might have entered the oviduct following artificial insemination if a medium containing energy sources had been used.

During sperm transport in the hamster oviduct, the first segment which spermatozoa encounter after negotiating the uterotubal junction is the intramural isthmus. Spermatozoa do not appear to reside in this segment for extended periods, but, instead, move directly to the caudal isthmus. This is

particularly clear in animals mated several hours prior to ovulation. From 3 h p.c. onward, the vast majority of spermatozoa had moved to the caudal isthmus.

I observed that in animals mated during ovulation, spermatozoa moved through the intramural isthmus to the caudal isthmus at a faster rate than in animals mated several hours before ovulation. Although I did not measure the lumen diameter directly, the faster sperm transport to the caudal isthmus at the time of ovulation could have been due to an increase in the lumen diameter of the intramural isthmus at this time, thereby facilitating the movement of spermatozoa through this segment to the caudal isthmus.

Yanagimachi and Chang (1963) observed large numbers of spermatozoa in the intramural and caudal isthmus at 1 h p.c.. I found, in all three groups, that the majority of spermatozoa were in the caudal isthmus from 3 h p.c. or 5 h post insemination onward. It would appear then, that the caudal isthmus is acting as a reservoir for spermatozoa during the period from mating to ovulation. This phenomenon is not unique to the hamster and has been found in other species (Hunter, 1973, 1980, 1984, 1985).

Of the large number of spermatozoa in the caudal isthmus, I observed that more than half of these spermatozoa had formed groups of five or more. These groups were generally found wedged into the folds of the oviduct wall, lying in close association to the oviduct epithelium. From our fixed material, it was impossible to tell if this association was of a permanent nature.

However, on different occasions, I observed groups of living spermatozoa through the wall of a freshly excised oviduct. These groups of spermatozoa appeared to become stuck to the oviduct wall for a time, break free and swim for some distance, then become stuck again (unpublished observations). At present, the nature of this association with the oviduct epithelium is unknown. However, it is tempting to speculate that during their passage through the caudal isthmus, spermatozoa may interact in some way with the epithelium of that region. This interaction may represent part of the *in vivo* capacitation process.

Yanagimachi and Mahi (1976) observed that guinea-pig spermatozoa remained in the lower isthmus before ovulation and were transported to the ampulla at the time of ovulation. This has also been reported in the mouse (Zamboni, 1972), rat (Shalgi and Kraicer, 1978), rabbit (Overstreet and Cooper, 1978b), sow (Hunter, 1981), and ewe (Hunter and Nichol, 1983). In agreement with these findings, I also observed, in animals mated or artificially inseminated prior to ovulation, that spermatozoa did not advance to the ampulla in significant numbers until about the time of ovulation.

For the hamster, it is not well understood what triggers the movement of spermatozoa from the caudal isthmus to the ampulla at the time of ovulation. Battalia and Yanagimachi (1979) observed coordinated propulsive movements of the hamster oviduct during the periovulatory period that were absent at other times of the estrous cycle. It may be that spermatozoa remain in the

lower isthmus until the coordinated movement of the oviduct propels them towards the ampulla. This hypothesis is consistent with our observation of animals mated or inseminated 5 to 7 h prior to ovulation. This hypothesis does not, however, explain our observation that most spermatozoa remained in the caudal isthmus for a minimum of three hours in animals mated at the time of ovulation; during the period when these coordinated propulsive movements take place. This could partially be explained by my observation that many spermatozoa were wedged into the epithelial crypts of the oviduct and probably would not have been easily displaced by muscular contractions. However, I also observed many spermatozoa free in the lumen which, presumably, could have been easily transported to the ampulla at this time. It is possible that coordinated muscular movements of the oviduct alone are insufficient to transport spermatozoa from the isthmus to the ampulla. Perhaps the spermatozoa themselves must undergo some physiological changes (e.g. initiation of hyperactivated motility) in the isthmus before advancing to the ampulla.

In females mated several hours before ovulation, spermatozoa remained in the lower isthmus for 7 h, until the time of ovulation (Table 2). These spermatozoa could have become capacitated at any time during this 7 h period. In females mated during ovulation, spermatozoa reached the lower isthmus by 1 h after the onset of mating and remained there for only 2 more hours before ascending to the ampulla to effect fertilization (Table 3). These

spermatozoa must have completed capacitation during the 3 h period from the onset mating until the beginning of fertilization. Therefore, in addition to acting as a sperm reservoir, the lower isthmus may also be acting as a site for sperm capacitation in vivo and this 3 h period may represent the minimum capacitation time for hamster spermatozoa in vivo.

Few spermatozoa were found in the cephalic isthmus even when fertilization was in progress in the ampulla. The spermatozoa leaving the caudal isthmus must be transported very rapidly to the ampulla. Either an increase in forward progressive motility of spermatozoa and/or an increase in the propulsive movement of the oviduct could be responsible for this. In all experimental groups, the number of spermatozoa observed in the ampulla during the early stage of fertilization was very small. This has been previously observed in the mouse (Zamboni, 1972), rat (Shalgi and Kraicer, 1978), and hamster (Cummins and Yanagimachi, 1982). I observed that many spermatozoa continued to arrive to the ampulla even after fertilization was complete. These late arriving spermatozoa may function to break up the cumulus oophorus to facilitate the passage of the eggs into the more constricted lumen of the oviductal isthmus.

I did find a very small number of spermatozoa in infundibulum at 1 h p.c. in naturally mated animals, however, this was the exception rather than the rule. Because these spermatozoa were found in the infundibulum, I thought that these spermatozoa may represent "stragglers" from a larger number of

spermatozoa transported into the periovarian space during the 1 hour preceding our first observation. In order to clarify this, I examined the periovarian space of 4 oviducts from females which had been sacrificed at 1 h p.c.. No spermatozoa were found in the periovarian space. The spermatozoa found in the infundibulum at 1 h p.c. could have been from contamination which occurred during sectioning or staining, but this seems unlikely as they were found within the narrow lumen of the infundibulum and the probability of these spermatozoa being deposited there by chance seems small. Therefore, the very few spermatozoa found in the infundibulum of females at 1 h p.c. must represent rapidly transported spermatozoa. Evidence for rapid transport of spermatozoa in the hamster oviduct has been reported in the past. According to Yamanaka and Soderwall (1960) spermatozoa can be recovered from the "ampullary segment" of the hamster genital tract within 2 min after coitus. However, upon careful reading of their description, it has become clear that the term "ampullary segment" these authors used, referred to the entire oviduct, not the ampulla per se. Rabbit spermatozoa which were transported to the ampulla during the "rapid phase" of transport were found to be dead and cleared to the periovarian space before fertilization begins (Overstreet and Cooper 1978a). Since our data is based on fixed material, it is impossible to ascertain the condition of the spermatozoa found in the infundibulum at 1 h p.c.. But, since eggs were not fertilized until they

reached the mid or lower ampulla, it would seem reasonable to infer that these spermatozoa, even if they were alive, do not participate in fertilization.

## CHAPTER 3

### Capacitation Status of Hamster Spermatozoa in the Oviduct at Various Times after Mating

#### Introduction

In all eutherian mammals studied to date, spermatozoa must reside in the female reproductive tract for some time before they become capable of fertilizing eggs; a process called "capacitation" (Chang, 1951; Austin, 1951) (for review, see Yanagimachi, 1988). The site within the female tract where capacitation begins may vary depending on species. For those species in which semen enters the uterus upon ejaculation (e.g. most laboratory rodents and the pig), capacitation may begin in the uterus or shortly after spermatozoa enter the oviduct. When a female hamster mates shortly after the onset of estrus, thousands of spermatozoa enter the lower segments (intramural and caudal isthmus) of the oviduct within 1 h of mating (Yanagimachi and Chang, 1963; Smith *et al.*, 1987). These spermatozoa do not advance beyond the lower segments in significant numbers until the time of ovulation, when a relative few advance to the ampulla to effect fertilization (Smith *et al.*, 1987). This implicates the lower segments of the oviduct as the site for sperm capacitation in the hamster. The question arises as to when these spermatozoa become capacitated. At least two possibilities exist. Either

these spermatozoa become capacitated shortly after entering the isthmus or they remain uncapacitated until ovulation commences.

In a previous study (Smith *et al.*, 1987) I reported that when female hamsters were mated near the time of ovulation, the vast majority of oviductal spermatozoa remained in the isthmus for a minimum of 3 h before advancing to the ampulla to affect fertilization. This may represent the minimum capacitation time for hamster spermatozoa *in vivo*. The time required for capacitation *in vivo* may depend on the time of mating. It is possible that sperm capacitation requires less time when mating occurs after ovulation than when mating occurs shortly after the onset of estrus. The purpose of this study was to determine when the spermatozoa in the isthmus become fully capacitated and whether the time required for sperm capacitation is influenced by the time of mating.

## Materials and Methods

### Animals

The mature female golden hamsters (7-10 weeks old) used in this study were born and raised to weaning in an air conditioned room with controlled light conditions (light, 05:00-19:00 h; dark, 19:00-05:00 h). At weaning, up to 6 females were housed together in a large cage (30 X 35 X 17 cm) and transferred to a cabinet with reversed light conditions (light, 17:00-07:00 h; dark, 07:00-17:00 h) and allowed to mature for at least 4 weeks. Under this

light regimen, mature females come into estrus by 06:30 h of every 4th day, ovulate between 12:30 and 13:30 h and exhibit a postovulatory vaginal discharge by 18:00 h of the same day. After reaching maturity, females were checked in the evening for 4 consecutive days for the presence of a postovulatory discharge (Orsini, 1961). The day of the postovulatory discharge (the day of ovulation) was designated Day 1 of the estrous cycle. After determination of the estrous cycle, each female was then housed individually in a smaller cage (20 X 26 X 15 cm). The mature males (16-24 weeks old) of proven fertility used for mating were housed as sibling pairs in large cages (30 X 35 X 17 cm). Males used in this study were raised and kept in an air conditioned room with the regular light regimen (light, 05:00-19:00 h, dark, 19:00-05:00 h).

#### Collection of eggs

Mature females were each injected intramuscularly with 20-30 IU pregnant mare serum gonadotropin (PMSG) (Calbiochem, La Jolla, CA) in the afternoon or evening of Day 1 followed by 20-25 IU human chorionic gonadotropin (hCG) (Ayerst, New York, NY) 50-56 h later. Oviducts were removed 18 h after hCG injection and a cumulus mass containing 20 - 30 eggs was recovered by flushing each oviduct with about 100  $\mu$ l m-TALP-7 medium (pH 7.4) (Uto *et al.*, 1988). Cumuli were transferred to medium m-TALP-7 containing 0.1% bovine testicular hyaluronidase (ICN Biochemicals, Cleveland, OH; 300 U.S.P. units/mg) to dissolve the cumulus matrix surrounding the

eggs. The cumulus-free eggs were rinsed twice in medium m-TALP-7 and kept at 38°C in medium m-TALP-7 supplemented with 20% heat-treated (60°C, 30 min) hamster serum for less than 30 min before insemination. Serum was collected from males and from females in all stages of the oestrous cycle and pooled before heat treatment. Since the serum raised the pH of the medium, the pH was adjusted to 7.4 by adding a small quantity of 0.1 M HCl. It was important to keep the medium under mineral oil (Squibb & Sons, Princeton, NJ) to maintain this slightly alkaline pH. The pH of this medium, when covered with mineral oil, was stable (7.4 - 7.5) under pure air (38°C) for up to 8 h.

### Mating

An estrous female (Day 1) was introduced into the large cage of a pair of sibling males at 07:00 h (5.5 h before the onset of ovulation) or 14:00 h (shortly after ovulation was completed). In this chapter, these will be referred to as early mating and late mating respectively. Mating (intromissions) began within 2-5 min. Each female was allowed to remain in the males' cage until just before sperm collection.

### Collection of spermatozoa and insemination of eggs

At various times (1-8 h) after the onset of mating, females were killed by inhalation of 100% CO<sub>2</sub> (about 45 s) and immediately placed in a 38°C operating chamber. All subsequent steps were carried out at 38°C; all instruments, equipment, media and mineral oil were warmed to 38°C before

use. Oviducts were removed from females with care being taken to include the first loop of the extramural oviduct which adheres to the outside of the uterine apex. Each oviduct was rinsed in medium m-TALP-7, blotted on filter paper to remove any blood and then placed in a watchglass containing mineral oil. A 30 gauge needle attached to a 50  $\mu$ l glass syringe was inserted into the infundibulum and 20  $\mu$ l medium m-TALP-7 with 20% hamster serum was gently flushed through the oviduct. Immediately after flushing, the oviduct was transferred to another watchglass containing mineral oil. About 3 min later, an additional 20  $\mu$ l of m-TALP-7 with 20% serum was flushed through the oviduct. When spermatozoa were to be recovered from the oviducts of ovulated females, each isolated oviduct was cut at the ampullary-isthmic junction. The needle was inserted into the cephalic isthmus and only the isthmic region was flushed. This ensured that the recovered spermatozoa had not previously come into contact with the cumulus oophorus or eggs. The 1st and 2nd flushes of each oviduct were examined and the flush containing the greater number of motile spermatozoa was used for insemination.

Insemination was performed by introducing 20-30 cumulus-free eggs into the oviductal flush under mineral oil in the watchglass. Incubation was carried out at 38°C. At 30 min intervals, 5-10 eggs were removed from the watchglass and examined for evidence of fertilization. Eggs were considered fertilized when a sperm head was seen to have begun decondensation in the ooplasm. When sperm-egg fusion was suspected, but decondensation was not

yet apparent, the cortical granules of the ooplasm were examined (Austin, 1956). If the exocytosis of cortical granules was evident, the egg was considered fertilized. In addition, the acrosomal status of motile spermatozoa on the zona pellucida was determined at 30 min after insemination. Spermatozoa were scored as having intact, modified or reacted acrosomes (Uto et al., 1988).

A group of eggs were inseminated with ejaculated spermatozoa which had never resided in the oviduct. Semen was collected from the uteri of females 20-30 min after the onset of mating (unlike the rat uterus, the hamster uterus contains very little uterine fluid during the estrous period, and so the spermatozoa collected from the uterus shortly after mating were in almost pure seminal plasma). Using a Pasteur pipette, about 20  $\mu$ l of the semen were carefully placed at the bottom of a short test tube (15 x 50 mm) containing 2 ml of medium m-TALP-7 with 20% hamster serum (38°C) to allow the actively motile spermatozoa to swim up into the medium. After 1-2 min, the upper 30  $\mu$ l of the medium were removed and the concentration of spermatozoa was adjusted to approximately  $1 \times 10^5$  cells/ml. Twenty microliters of this sperm suspension were placed in a watchglass and covered with mineral oil. Insemination and examination of the eggs were performed as already described.

### Examination of eggs fertilized in vivo

Females were mated either shortly after the onset of estrus (5.5-6.5 h before ovulation) or shortly after ovulation was completed. At various times (0.5-7.5 h) after the end of ovulation, eggs were recovered by flushing the oviducts. The cumulus cells were removed and the eggs were examined for evidence of fertilization as previously described.

### **Results**

Many (65-94%) of the spermatozoa recovered in the 1st oviductal flush were immotile regardless of the time of recovery. Most spermatozoa were recovered as large agglutinated masses and did not regain their motility in the medium m-TALP-7S. In general, the 2nd flush contained a higher proportion of motile spermatozoa (10-25%), but the absolute number of spermatozoa was considerably less than that in the 1st flush. The 2nd flush was usually used for insemination. The initial concentration of motile spermatozoa at insemination ranged from  $2-6 \times 10^3$  cells/ml (45-120 spermatozoa/20  $\mu$ l). When medium m-TALP-7 was used without serum supplement, most of the motile spermatozoa flushed from the oviduct died within 30 min, indicating the fragile nature of these spermatozoa. When medium m-TALP-7 was supplemented with 20% serum, a higher proportion of recovered spermatozoa remained alive during subsequent in vitro incubation. A small proportion of the spermatozoa (about 1-2%) recovered from the oviduct between 4 and 8 h

after early mating exhibited hyperactivated motility (Yanagimachi, 1981). A similar proportion of spermatozoa exhibited hyperactivated motility in the medium when they were recovered from the oviduct 4 h after late mating. Because of the relatively low motile sperm:egg ratio (less than 10:1), not all eggs were fertilized. However, most eggs had at least one spermatozoon attached within 5 min of insemination. At 30 min after insemination, 80-90% of the motile spermatozoa on the zonae pellucidae were acrosome-reacted when spermatozoa were recovered 8 h after early mating or 4 h after late mating. None of the spermatozoa underwent the acrosome reaction on the zonae within 30 min when they were recovered from the oviduct before these times.

Figure 4(a) shows the minimum time required for spermatozoa to fertilize eggs *in vitro* after being recovered from the oviduct following early mating. The spermatozoa that were recovered from the isthmus at 1 h after mating spent at least 2 more hours in the medium (on zonae) before fertilizing the eggs. The interval between insemination and the first eggs fertilized was 1.5 and 1 h when spermatozoa were recovered from the isthmus at 2 and 4-6 h after mating respectively. The spermatozoa collected from the isthmus 8 h after mating could fertilize eggs almost immediately (within 30 min). Spermatozoa required less time *in vitro* to fertilize eggs when animals were mated immediately after ovulation (late mating) (Figure 4b).

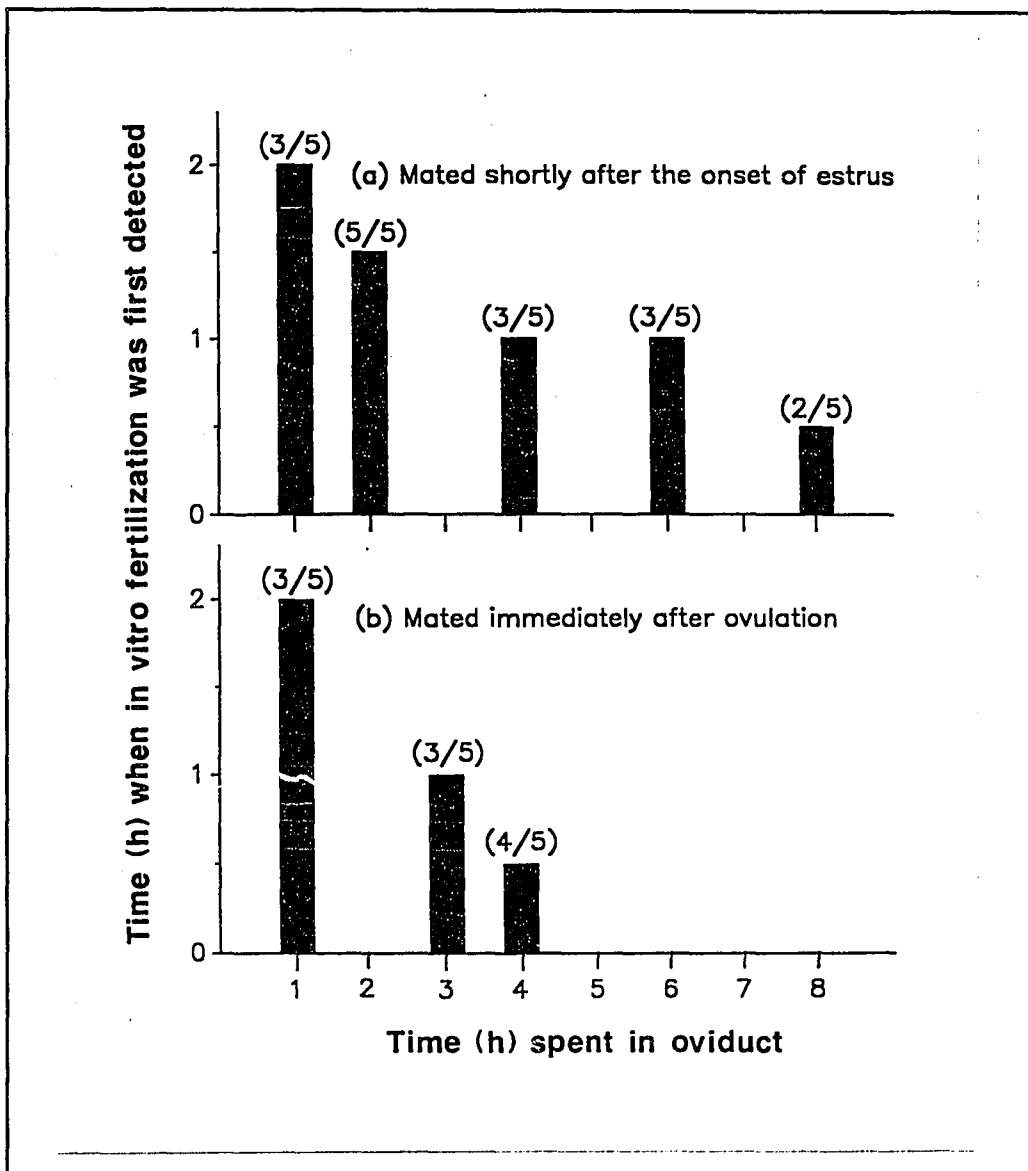


Figure 4. Minimum time spermatozoa recovered from the isthmus spend *in vitro* before fertilizing eggs. (n/5) the number of times in 5 trials that fertilization has occurred by the time indicated by the bar.

Freshly ejaculated spermatozoa (recovered from the uterus 20-30 min after the onset of mating) were not consistently able to fertilize eggs under the *in vitro* conditions used in this study. Although the initial percentage of motile spermatozoa was high (>95%), only about 50% were still motile and a few

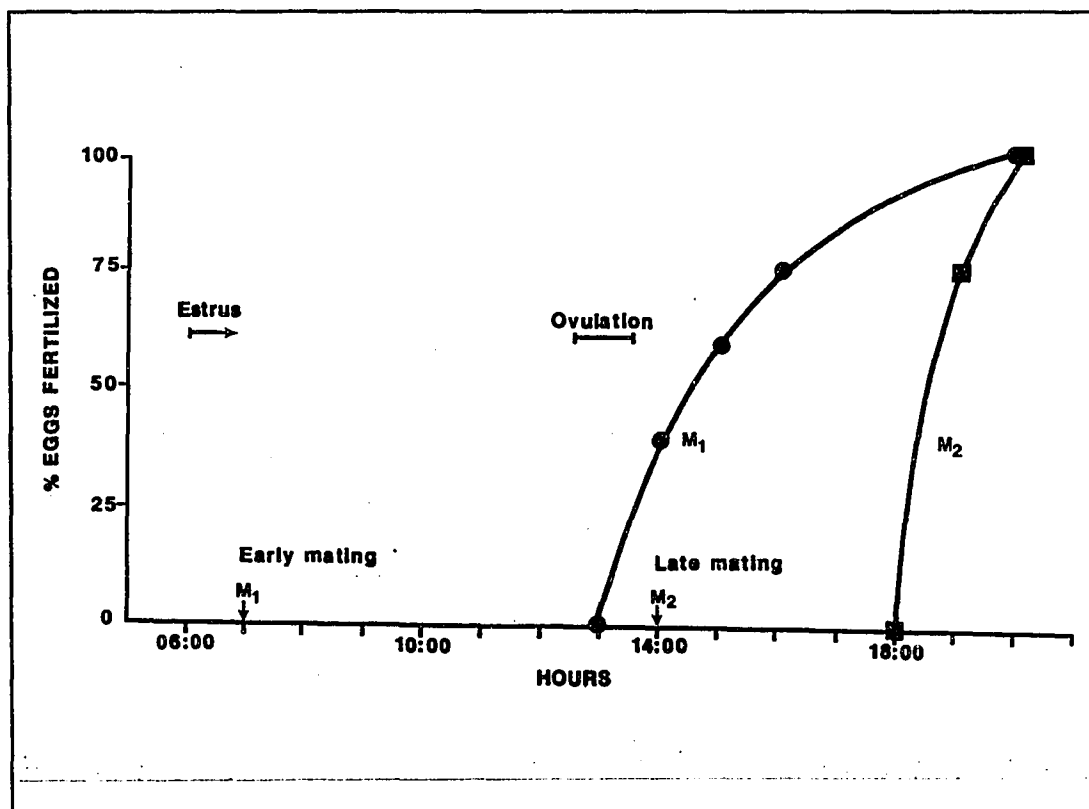


Figure 5. The progression of fertilization in females mated shortly after the onset of estrus (07:00) or immediately after ovulation (14:00) (n = 30-50 eggs/point)

(<1%) were hyperactivated at 3 h after the start of incubation. At 5 h after insemination, <5% of the free swimming spermatozoa were motile. At this time, only about 20% of the spermatozoa on the zonae pellucidae were motile, the remainder being immotile and presumably dead. In spite of the fact that most of the motile spermatozoa on the zonae were acrosome reacted at 5 h after insemination, zona penetration and subsequent fertilization had occurred only rarely by this time (3/54 eggs in 5 trials).

Figure 5 shows the progression of in vivo fertilization following early and late matings. In females mated soon after the onset of estrus (early mating),

fertilization began at 7 h and was completed by 13 h after mating. In the females mated immediately after ovulation (late mating), fertilization began sometime between 4 and 5 h after mating and was completed within the next 2 h.

## Discussion

A large proportion of immotile spermatozoa were recovered from the oviduct regardless of the time of mating and sperm recovery. This does not appear to be an artefact of flushing. Many immotile spermatozoa were observed through the wall of the caudal isthmus of an uncompressed preparation of excised oviduct illuminated with strong transmitted light. Large numbers of immotile spermatozoa were also observed in the contents of the isthmus which had been released directly into mineral oil on a slide glass (unpublished observations). The presence of immotile spermatozoa in the isthmus is not unique to the hamster. Immotile spermatozoa were seen in oviductal flushings of the rabbit isthmus (Overstreet and Cooper, 1975; Burkman *et al.*, 1984) and through the wall of the mouse isthmus (Suarez, 1987). The immotile hamster spermatozoa recovered from the isthmus did not regain their motility. Apparently they were dead or dying while they were in the isthmus. Since hamster spermatozoa must be actively motile to pass through the uterotubal junction and reach the isthmus (Smith *et al.*, 1988), these spermatozoa must have died after reaching the isthmus. The fragility of the isthmic spermatozoa was evident when medium m-TALP-7 without serum was used as the flushing medium; many or all of initially motile spermatozoa became

motionless (presumably dead) within 30 min. Hamster spermatozoa collected from the cauda epididymidis could survive well in medium m-TALP-7 without serum (Uto *et al.*, 1988). This was also true for hamster spermatozoa recovered from semen (unpublished observations). Therefore, it appears that spermatozoa become unadaptable to this serum-free medium once they have been exposed to the oviduct milieu. Some serum components added to the medium seem to rescue flushed isthmic spermatozoa that would otherwise die in serum-free medium.

Capacitation was assessed by determining how quickly flushed isthmic spermatozoa could fertilize eggs *in vitro*. If the spermatozoa penetrated the eggs immediately or at least within 30 min after sperm-egg contact, they must have been fully capacitated before they were flushed. On the contrary, if spermatozoa penetrated the eggs only after they had been in the medium for some time, they must have been only partly capacitated. The results obtained indicate that when females are mated shortly after the onset of oestrus (early mating), capacitation proceeds steadily during the first 4 h and then stays at a plateau until it is completed some time between 6 and 8 h after mating (Figure 4a). Since the females in this group ovulated about 6 h after mating, we may conclude that the completion of capacitation (at least for some spermatozoa) is more or less synchronous with the appearance of eggs in the oviduct. The plateau in capacitation status which occurs between 4 and 6 h after mating could be due to a temporal arrest of capacitation until the time of ovulation. Alternatively, some essential factor for sperm capacitation may be missing from the oviductal milieu

until about the time of ovulation. It is possible that the epithelium of the oviduct is triggered to produce the missing factor by the change in the estrogen/progesterone ratio which occurs near the time of ovulation. Hunter (1987a) has suggested that for species with a prolonged period between mating and ovulation (e.g. cow and pig), the timing of sperm capacitation in the oviduct is controlled by locally-transmitted hormonal influences from Graafian follicles. In the hamster, the time between mating and ovulation is relatively short, but it is probable that the timing of sperm capacitation is controlled in a similar manner. When females were mated at about the time of ovulation (late mating), capacitation was completed at least in some spermatozoa by 4 h after mating (Figure 4b). This means that capacitation proceeds twice as fast following late mating compared to early mating. The plateau in capacitation status observed following early mating was absent in this group of females. It would appear that the postovulatory oviduct milieu provides a more efficient environment for sperm capacitation.

The time when isthmic spermatozoa begin to become fully capacitated following early and late mating (as determined by *in vitro* fertilization) was coincident with the time of initiation of *in vivo* fertilization (compare Figures 4 and 5). It would appear that, as soon as isthmic spermatozoa become fully capacitated (6-8 h following early mating and 4 h following late mating), they begin to advance to the ampulla to affect fertilization.

The medium used (m-TALP-7 with 20% serum) did not consistently support fertilization by spermatozoa obtained from semen. These spermatozoa could undergo the acrosome reaction on the zonae pellucidae of the eggs, but seldom penetrated the zonae. On the other hand, the spermatozoa collected from the isthmus penetrated the zonae consistently. These facts suggest that spermatozoa change their characteristics soon after entering the oviduct. This change could represent the first step of capacitation in vivo.

Spermatozoa which are about to fertilize eggs in the ampulla are hyperactivated and ready to undergo the acrosome reaction (Cummins and Yanagimachi, 1982). A small proportion of spermatozoa recovered from the isthmus 4 h following early mating displayed hyperactive movement when they were flushed out of the isthmus. We are unable to say whether these spermatozoa were hyperactivated within the isthmus, but they certainly had the potential for hyperactivation. Hyperactivated movement of mouse spermatozoa was observed in the isthmus 1-2 h after mating (Suarez and Osman, 1987). At least in the hamster and mouse, the vigorous whiplash-like beatings of the tail, characteristic of this type of movement, may assist the spermatozoon in changing direction and escaping from the epithelial pockets of the isthmus and thereby assist it in its advance towards the ampulla.

All motile spermatozoa recovered from the isthmus had apparently intact acrosomes. They were able to attach to the zonae pellucidae, but were unable to undergo the acrosome reaction within 30 min except for those spermatozoa that had resided in the isthmus for 8 h (after early mating) or 4 h (after late mating).

These data also support the contention that capacitation is completed faster in females mated shortly after ovulation than in those mated shortly after the onset of estrus.

A minimum capacitation time of 3-4 h for hamster spermatozoa following mating after ovulation was determined by indirect methods in previous studies (Strauss, 1956; Yanagimachi, 1966; Smith *et al.*, 1987). The result of this study provides direct evidence to support this. However, depending on when mating occurs, the minimum time may not be the actual time required for capacitation. Indeed, as Hunter (1987b) has pointed out, for species in which mating occurs many hours before ovulation, there would be little value for spermatozoa to become capacitated within a few hours after ejaculation since capacitated spermatozoa are fragile, short-lived cells and would be non-functional by the time of ovulation. From the data presented here, it appears that the oviduct is capable of controlling the timing of sperm capacitation in order to synchronize the physiological state of the gametes and thereby maximize fertilization.

## CHAPTER 4

### The Viability of Hamster Spermatozoa Stored in the Isthmus of the Oviduct:

#### The Importance of Sperm-Epithelium Contact for Survival

##### Introduction

The mammalian oviduct holds a unique position in the reproductive process. It is within the oviduct that fertilization and the initial stages of development occur. It is also the site where spermatozoa await the arrival of the eggs. When a female hamster mates shortly after the onset of estrus, thousands of spermatozoa enter the lower oviduct (isthmus) within one hour of mating (Yanagimachi and Chang, 1963; Smith *et al.*, 1987). The spermatozoa remain in the isthmus for 6-8 h, until near the time of ovulation, when a very small fraction of them ascend to the upper oviduct (ampulla) to affect fertilization (Smith *et al.*, 1987). In the ampulla, the ratio of spermatozoa to eggs is less than 1:1 until at least half of the eggs are fertilized (Cummins and Yanagimachi, 1982). Therefore, with regard to spermatozoa, the isthmus performs two functions: to act as (1) a site for sperm storage during the preovulatory period and (2) a sieve to decrease the number of spermatozoa reaching the site of fertilization. How the isthmus performs these functions is not fully understood (for a comprehensive review of the physiology of the oviduct, see Hunter, 1988).

Storage of spermatozoa in the isthmus is not unique to the hamster. Studies in sheep, pigs and cows have demonstrated that the lower isthmus acts as a functional sperm reservoir during the preovulatory period (Hunter et al., 1982; Hunter 1984; Hunter and Wilmut, 1984). Several mechanisms have been proposed to explain how the oviduct acts to sequester spermatozoa during the preovulatory period. Localized constriction of the isthmus (Suarez, 1987), slightly decreased temperature of the lower isthmus (Hunter and Nichol, 1986), the presence of viscous mucus in the isthmus (Jansen, 1978) and sperm attachment to the mucosal surface (Cooper et al., 1979; ; Smith et al., 1987; Suarez, 1987) have all been suggested as possible mechanisms. Sperm motility in the oviduct may also play a pivotal role (for review, see Katz et al., 1989). In a previous study (Smith and Yanagimachi, 1989) we recovered immotile spermatozoa from the oviduct of the mated hamster. Since active motility of hamster spermatozoa is required for efficient passage through the uterotubal junction (Smith et al., 1988), these spermatozoa must have become immotile after entering the oviduct. Immotile spermatozoa have been observed through the wall of the mouse isthmus (Suarez, 1987) and recovered from the rabbit isthmus (Overstreet and Cooper, 1975). Depressed motility of spermatozoa in the lower oviduct has been suggested as a major factor cause of limited sperm ascent in the rabbit (Cooper et al., 1979) and has been shown to be reversible under specific in vitro conditions (Burkman et al., 1984). Unlike rabbit spermatozoa, immotile hamster spermatozoa recovered from the isthmus do not regain motility in a variety of media in vitro (Smith and

Yanagimachi, 1989) suggesting that the hamster oviduct is less hospitable toward spermatozoa than the rabbit oviduct.

Classical studies on sperm viability in the female tract have been primarily concerned with the "fertilizable life" of spermatozoa of various species (for review, see Austin, 1975). These studies employed the method of inseminating females at various times prior to ovulation to determine how long spermatozoa could remain in the female tract and still be able to fertilize. Values ranging from 12 h for mouse spermatozoa (McGaughey *et al.*, 1968) to 126 h for ferret spermatozoa (Chang, 1965) have been obtained. The results of these studies demonstrated the maximum functional life of spermatozoa in the female tract, but do not give much information about the viability of the general sperm population following natural mating. The purpose of the present study was to examine what proportion of the spermatozoa stored in the isthmus survive until fertilization takes place.

## **Materials and Methods**

### **Animals**

The female golden hamsters used in this study were born and raised to weaning in an air conditioned room with regular light conditions (light 05:00-19:00 h). At weaning, up to 6 females were housed together in a cage (30 X 35 X 17 cm) and placed in a ventilated cabinet having a reversed light regimen (light 17:00-07:00 h). Females matured under these conditions ovulated between 13:00 and 14:30 h of every 4th day and display a postovulatory vaginal discharge by 18:00 h of the

same day. Females (8-14 weeks old) were checked in the evening for 4 consecutive days for the presence of a postovulatory discharge (Orsini, 1961). The day of the discharge was designated Day 1 of the cycle. After determination of their cycle, each female was individually housed in a smaller cage (20 X 26 X 15 cm). Mature males (18-30 weeks old) used in this study were housed as sibling pairs in large cages (30 X 35 X 17 cm) and maintained under the regular light regimen (light 05:00-19:00 h).

#### Mating, oviduct removal and sperm recovery

An estrus female was placed in a cage containing a pair of sibling males at 0800 h of Day 1 (about 5 to 6.5 h before ovulation). Mating (intromissions) began within 3-5 min. Females were allowed to reside in the male's cage until just prior to oviduct collection. Females were killed at various times after the onset of mating by cervical dislocation under ether anesthesia. The extramural portion of the oviduct was removed with care being taken to include the initial segment of the caudal isthmus which adheres to the uterus, then blotted on filter paper to remove any blood and placed in a watchglass. To recover spermatozoa, oviducts were flushed with medium (see below) after inserting a blunt 30 gauge needle attached to a 100  $\mu$ l syringe into the infundibulum. When spermatozoa were to be recovered from oviducts after ovulation, the ampullary-isthmic junction was cut and the needle was inserted into the cephalic isthmus and only the isthmus was flushed to avoid contamination of the flush with eggs and their investments. No attempt was made to recover spermatozoa from the intramural portion of the oviduct.

#### Selective removal of spermatozoa from the oviduct

At 2 h after mating, oviducts were removed and immediately placed in separate watchglasses containing warm (37°C) modified Tyrode's medium, m-TALP-7 (Uto *et al.*, 1988), supplemented with 20% heat inactivated hamster serum (m-TALP-7S). The addition of serum to m-TALP-7 has been previously shown to sustain the viability of spermatozoa recovered from the oviduct (Smith and Yanagimachi, 1989). One oviduct from each female was flushed once with 20  $\mu$ l m-TALP-7S while the other was flushed twice with 20  $\mu$ l of m-TALP-7S (total 40  $\mu$ l), waiting 3-4 min between flushes. Oviducts from another group of mated females were not flushed and served as controls.

#### Direct observation of spermatozoa through the oviduct wall

Either immediately after removal from females (control) or after flushing, oviducts were cut at the ampullary-isthmic junction. The isthmus was placed on a warm microscope slide, slightly compressed under a coverglass supported by four pillars of Vaseline/paraffin mixture, bathed with warm m-TALP-7S and placed on a microscope stage maintained at 37°C by an air curtain incubator (Sage Instruments, White Plains, NY). Spermatozoa in the region of the 1st loop of the caudal isthmus were observed through the semi-transparent wall of the oviduct using strong transmitted light and regular optics (x 400). Observations were made within the 3-5 min immediately following removal.

#### Determination of the number of spermatozoa recovered from the oviduct

At various times (2-8 h) after the onset of mating, oviducts were removed from females and placed in watchglasses. Each oviduct was flushed first with 20 $\mu$ l of m-TALP-7S containing 2% trypan blue (Sigma, St. Louis, MO) and then left for 3-4 min before being flushed with another 20 $\mu$ l of m-TALP-7S without trypan blue. Each oviduct was then flushed a 3rd time with 50 $\mu$ l of m-TALP-7S containing 0.5% Triton X-100 (Sigma, St. Louis, MO) to remove spermatozoa that had firmly attached to the mucosal surface. The first two flushes were collected in separate watchglasses and each diluted with 20 $\mu$ l of distilled water. After dilution, each flush was transferred to a 400 $\mu$ l microcentrifuge tube (Beckman, Fullerton, CA). The microcentrifuge tube was placed in a 100 ml cold water bath containing a sonicating probe (Biosonik, Bronwill Scientific, Rochester, NY) and gently sonicated for 10 s to break up the agglutinated masses of spermatozoa in the flush. The sperm suspension was drawn in and out of a large bore pipette 2-3 times and a drop was placed on a hemocytometer. The spermatozoa were counted and the number of spermatozoa recovered in each of the first two flushes was estimated. The 3rd flush was collected on a microscope slide and immediately covered with a coverslip. The 3rd flush contained much fewer spermatozoa than the other flushes, so the entire suspension was examined and all the spermatozoa in that flush were counted. After being flushed three times, oviducts were fixed in A.F.A. fixative (Orsini, 1962) for 24 h, dehydrated, embedded in paraffin, serially sectioned (10 $\mu$ m) and stained with Schiff's reagent and Fast Green F.C.F. The spermatozoa remaining in the oviduct were counted as described by Smith *et al.* (1987). Due

to the length of time required for the counting procedure and the necessity for sonication to obtain an accurate count, no attempt was made to determine the viability of spermatozoa recovered in these flushes (see next section).

#### Examination of the viability of spermatozoa recovered from the oviduct

At various times (2-8 h) after the onset of mating, females were killed and immediately placed in a 38°C chamber. All subsequent steps were carried out at 38°C; all instruments, glassware, media and mineral oil were prewarmed to 38°C. Oviducts were removed from the females and placed under mineral oil (Sqibb and Sons, Princetown, NJ). Each oviduct was flushed three times using the same volumes and media described in the preceding section. After each flush, the medium was drawn up from around the oviduct using a small bore pipette placed on a microscope slide and covered with a coverslip. After each flush, the oviduct was blotted on filter paper before it was transferred to a watchglass containing fresh mineral oil. The recovered flushes were immediately examined under the microscope (x 400) and about 100 spermatozoa were scored as either dead (stained) or alive (unstained). Trypan blue stained only dead spermatozoa; live acrosome-reacted hamster spermatozoa did not take up the trypan blue stain. The 1st flush typically contained agglutinated masses of dead spermatozoa which were not considered in live/dead scoring to avoid bias in sampling. The staining of dead spermatozoa was stable and did not fade even when recovered in m-TALP-7S containing Triton X-100. Therefore, even though all spermatozoa were killed by

Triton-containing medium during the 3rd flush, we could still determine which spermatozoa were alive or dead prior to flushing.

## Results

When the caudal isthmus of the oviduct was isolated at 2 h after the onset of mating and examined using a strong transmitted light, spermatozoa were seen free in the lumen, attached to the mucosal surface of the wall and clustered in epithelial crypts. Spermatozoa in the lumen were either weakly motile (a few) or immotile (most). Most immotile spermatozoa appeared as agglutinated masses aggregated with the cell debris in the oviductal lumen. Most of the spermatozoa associated with the wall or in the crypts exhibited flagellar movement. The spermatozoa attached to the mucosal surface of the wall rocked back and forth on the rostral surface of their head and on rare occasion broke free to swim a short distance before attaching again. The crypts usually had a tuft of ciliated cells at the base and these cilia appeared to "massage" the heads of the spermatozoa in the crypts.

Sequential flushing of the oviduct removed spermatozoa selectively. The 1st flush removed the spermatozoa that were free in the lumen and those loosely attached to the mucosal surface of the wall. During the first few minutes after the 1st flush, some of the spermatozoa that had remained attached to the wall mucosa became detached and began to swim freely in the lumen. The 2nd flush removed these spermatozoa and any that had become loose during the time between the

two flushes. The spermatozoa that remained in the oviduct after two flushes were found clustered in the epithelial crypts or firmly attached to the wall mucosa. These spermatozoa exhibited vigorous flagellar movement. The 3rd flush removed most, but not all, of these spermatozoa.

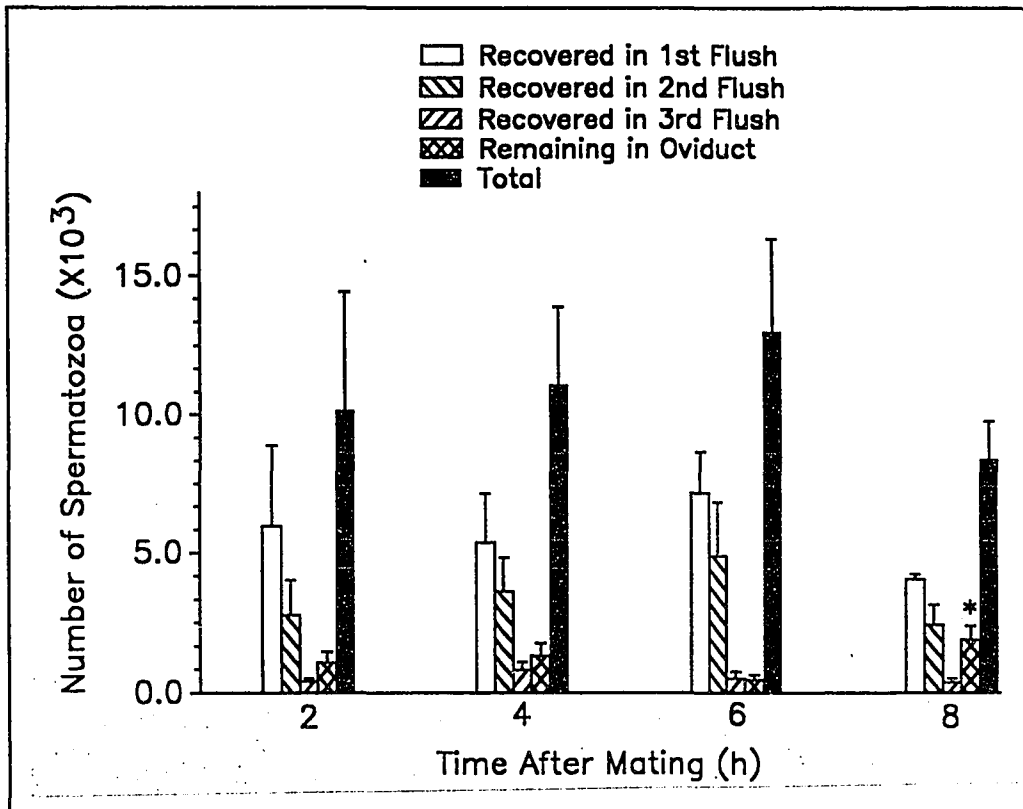


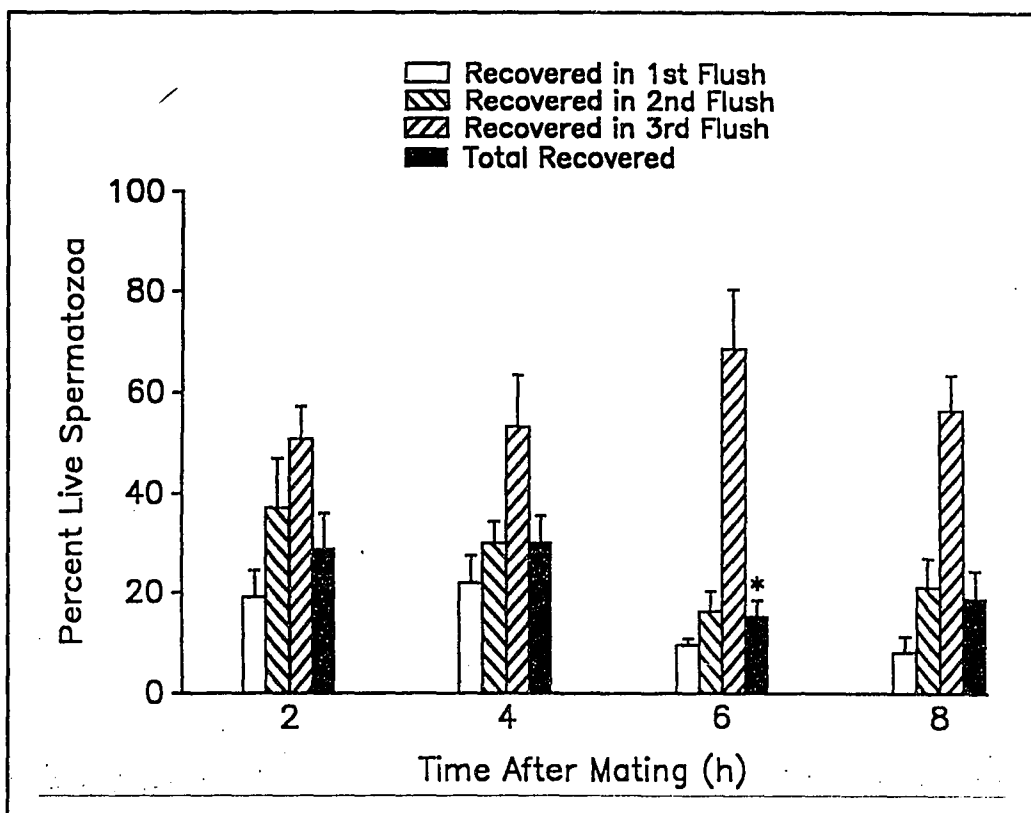
Figure 6. Number (mean  $\pm$  s.e.m., n=6) of spermatozoa recovered from the oviduct and remaining in the oviduct after flushing at various times after mating.

Figure 6 shows the number of spermatozoa recovered in each of the three flushes and the number of spermatozoa remaining in the oviduct after the 3rd flush. The total represents the sum of the spermatozoa recovered by flushing and those counted in serial sections. The proportion of the total spermatozoa recovered in each of the three flushes remained constant regardless of the time after mating.

The highest proportion of spermatozoa (48-58%) were always recovered in the 1st flush, the next highest (27-37%) were recovered in the 2nd and the smallest (4-7%) in the 3rd flush. The proportion of spermatozoa remaining in the oviduct after three flushes represents 3-22% of the total spermatozoa in the isthmus.

Although there appears to be an increase in the total number of spermatozoa in the isthmus until 6 h after mating followed by a decrease at 8 h after mating, there was no significant change (t-test,  $P > 0.05$ ) in the number of spermatozoa in the isthmus during this period. Therefore, it appears from the data that the majority of spermatozoa which enter the oviduct during the preovulatory period do so by 2 h after the onset of mating. There was a significant increase (t-test,  $p < 0.05$ ) in the number of spermatozoa remaining in the isthmus after three flushes at 8 h when compared to that at 6 h after mating.

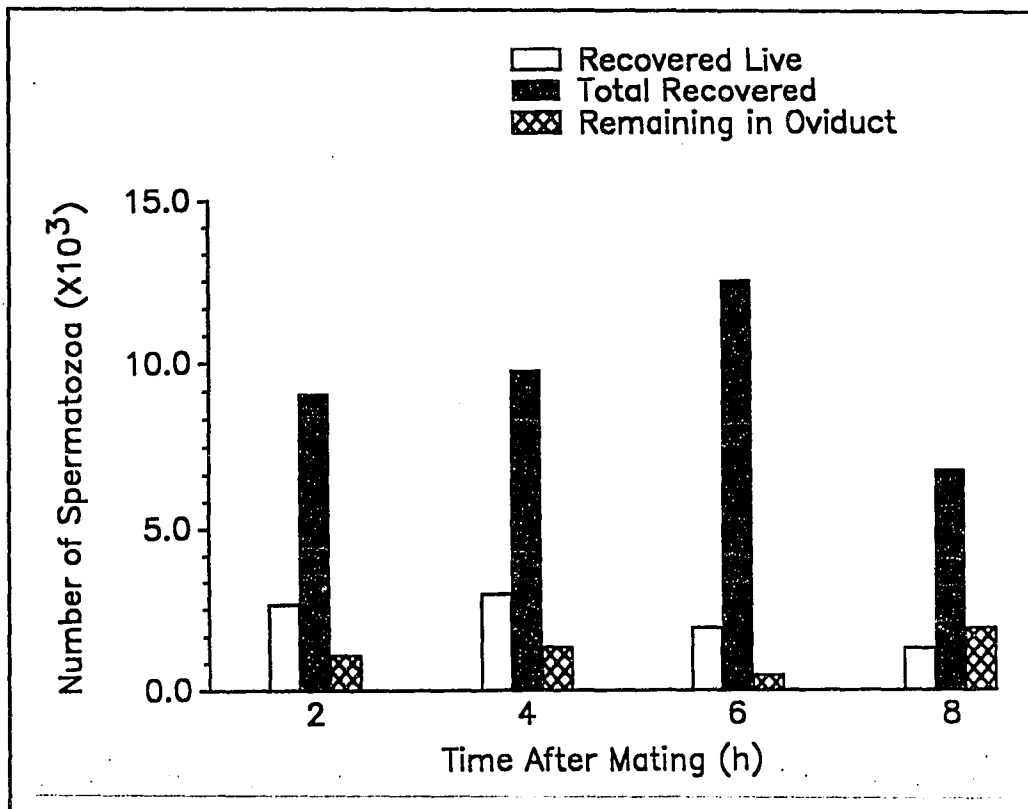
Figure 7 shows the percentage of live spermatozoa recovered in each of the three flushes and the mean percentage of live spermatozoa (mean number of live spermatozoa / mean number of spermatozoa X 100%) flushed from the isthmus at various times after mating. The 1st flush recovered the smallest percentage of live spermatozoa (2-22%), the 2nd flush 16-37% and the 3rd flush recovered the highest (51-69%) regardless of the time after mating. There was no significant difference (t-test,  $p > 0.05$ ) between the mean percentage of live spermatozoa recovered from the isthmus at 2, 4 and 8 h after mating. However, there was a significant decrease (t-test,  $p < 0.05$ ) in the mean percentage of live spermatozoa recovered from the isthmus at 6 h after mating (during ovulation) compared to



**Figure 7.** Percentage (mean  $\pm$  s.e.m., n=6) of live spermatozoa recovered from the oviduct at various times after mating. Significant ( $p < 0.05$ ) decrease in percentage of live spermatozoa recovered (\*).

previous times.

Figure 8 shows the mean number of live spermatozoa in the isthmus at various times after mating (mean number of spermatozoa recovered X mean % live spermatozoa recovered). About 3,000 live spermatozoa were recovered at 2 h after mating which decreased to about 1,500 live spermatozoa recovered at 8 h after mating (onset of fertilization). The number of live spermatozoa recovered underestimates the actual number of live spermatozoa in the isthmus by not taking into account the live spermatozoa that remained in the isthmus after three flushes. Based on our direct observations that the vast majority of spermatozoa remaining



**Figure 8.** Mean number of live spermatozoa recovered from the oviduct at various times after mating.

in the isthmus after two flushes, including those in the crypts, exhibited vigorous flagellar movement and that the highest proportion of live spermatozoa were recovered in the 3rd flush, most of the spermatozoa which remained in the crypts after three flushes were probably alive.

### Discussion

The hamster oviduct can be partitioned into three compartments: the lumen, which varies in diameter with the rhythmic contractions of the myosalpinx, the mucosal surface of the wall, which has longitudinal folds that project into the

lumen and the crypts, which extend to depth of about one third the length of the hamster spermatozoon and usually have a small number of ciliated cells at the base. I observed three distinct subpopulations of spermatozoa having different characteristics within the three compartment of the isthmus. Those found in the crypts formed small clusters and a very high proportion of these exhibited flagellar movement. The majority of those attached to the mucosal surface of the wall were unclustered and exhibited moderate flagellar movement. Conversely, the spermatozoa found in the lumen were either weakly motile (a few) or immotile (most). The oviduct has traditionally been divided in terms of its length with different functions assigned to different segments; isthmus for sperm storage, ampulla for fertilization. My observations of subpopulations of spermatozoa in the isthmus suggest the need for a functional division of this segment into three compartments (microenvironments): the lumen, the mucosal surface of the wall and the crypts.

In a previous study (Smith *et al.*, 1987), I found that spermatozoa enter the hamster oviduct shortly after mating and remain in the lower isthmus until the time of ovulation. In that study, I reported that no spermatozoa were found in the ovarian bursa at 1 h after mating and found little evidence for a rapid phase of sperm transport like that reported for the rabbit (Overstreet and Cooper, 1978a). In a later study (Smith *et al.*, 1988), I found that motility is required for the efficient passage of hamster spermatozoa through the uterotubal junction into the oviduct and, following artificial insemination with dead spermatozoa, very few

spermatozoa passed into the oviduct. Therefore, the dead spermatozoa recovered from the oviduct in the present study must have been motile (viable) at the time they entered the oviduct and have died at some point after their arrival in the oviduct. There was no significant increase in the number of spermatozoa recovered from the oviduct after my initial observation at 2 h after the onset of mating (Figure 6), so it seems valid to assume that I was looking at the same population of spermatozoa throughout the preovulatory period with a possibility of some limited exchange occurring through the uterotubal junction.

Although there was little exchange of spermatozoa through the uterotubal junction or between segments of the oviduct, a certain amount of exchange between the sperm subpopulations would be expected under the dynamic conditions within the isthmus. For example, a spermatozoon may become detached from the mucosa and enter the lumen, swim a short distance, and become attached to the mucosa again. We did observe this kind of sperm behavior occasionally in oviducts excised at 2 h after the onset of mating. However, it was rather rare, which indicates there was little exchange between the subpopulations. In addition, the relative proportion of the total spermatozoa recovered in each flush was consistent regardless of the time of recovery (Figure 6). This would also indicate that little overall exchange between the subpopulations occurred during the observation period. Therefore, the sequential flushing technique employed in this study appears to be a valid approach to study the subpopulations of spermatozoa in the isthmus.

When I initially viewed spermatozoa through the oviduct wall, I observed agglutinated masses of immotile spermatozoa aggregated with cell debris in the lumen. This is probably what Suarez (1987) described as "columns" of immotile spermatozoa in short segments of the mouse isthmus. Suarez suggested the possible presence of a molecule in certain segments of the isthmus that suppressed motility by increasing the viscoelasticity of the oviductal fluid. I did not find any evidence to support this, because spermatozoa recovered from the isthmus did not regain motility in any of the media used (Smith and Yanagimachi, 1989) despite extensive dilution of the viscous oviductal fluid. After flushing the oviduct with medium containing trypan blue, the vast majority of immotile spermatozoa were agglutinated and darkly stained (dead).

The first subpopulation of spermatozoa were recovered from the lumen in the 1st flush (about half the spermatozoa in the oviduct). Although the highest number of spermatozoa were recovered in this flush, it invariably had the lowest percentage of live spermatozoa. Very little is currently known about factors that effect survival of spermatozoa in the isthmus, but at least two factors may have contributed to the death of such a large number of spermatozoa in the lumen. Starting about 12 h before ovulation, the lumen of the hamster isthmus begins to accumulate "cell debris" (unpublished observation), presumably derived from the sloughing off of the mucosal epithelium. By estrus, this debris may provide a formidable barrier in which some spermatozoa become permanently trapped and die. As more spermatozoa encounter the debris and become entrapped, the masses become larger

and appear to act as plugs in the lumen. The coordinated contractions of the myosalpinx which occur near the time of ovulation (Battalia and Yanagimachi, 1979) do not move these sperm masses to the ampulla (Smith *et al.*, 1987) and so, at least in the hamster, this may represent one of the mechanisms which prevents the majority of spermatozoa from ascending to the ampulla.

The second factor which may have contributed to the high mortality among the spermatozoa in the lumen is a shortage of energy sources. Any metabolic substrates found in oviductal fluid must diffuse from the blood or be actively secreted by the mucosal epithelium. With many thousands of actively motile spermatozoa in the isthmus during the preovulatory period, the available substrates may become depleted. The farther from the source (mucosal surface), the lower the concentration of an energy source. Therefore, those spermatozoa in the center of the lumen would be exposed to the lowest concentration of energy sources and would presumably die first, while those which remain in close proximity to the mucosal surface would fare better.

The second subpopulation of spermatozoa were those attached to the mucosal surface. When observed through the wall of an unflushed isthmus at 2 h after the onset of mating, the majority of these spermatozoa exhibited flagellar activity and rocked back and forth on the rostral surface of their head. I observed a few spermatozoa break free of the mucosa and enter the lumen, which indicates that attachment to the mucosa, at least for some spermatozoa, was not permanent. Most of the spermatozoa attached to the mucosa were recovered in the 2nd flush.

However, some spermatozoa remained attached to the mucosa even after the 2nd flush. This suggests that some spermatozoa were more strongly attached to the mucosa. The nature of this attachment is not clear (i.e. chemical or physical). When spermatozoa were viewed through the wall of the oviduct, they appeared to be adhering to a thin layer of mucus-like material on the apical surface of the epithelial cells. Mucus secretions have been suggested to play a role in sperm adhesion in the rabbit oviduct (Cooper et al., 1979). However, it may not have been a stronger attachment per se (i.e. chemical attachment) which kept spermatozoa from being flushed out of the isthmus. When spermatozoa were viewed through the wall of an actively contracting excised oviduct, the spermatozoa were not swept away by the oviductal fluid which passed over them, but instead, held their position on the mucosa by "digging in" the point of their hooked head. Some spermatozoa may have resisted flushing in a similar manner (i.e. physical attachment).

The higher percentage of viable spermatozoa in the 2nd flush suggests that the mucosa surface protects the spermatozoa from the apparently hostile environment of the lumen. How the mucosa may perform this function is not clear. It may be that sperm attachment alone is sufficient to improve viability. Indeed, Pollard et al. (1989) showed that bovine spermatozoa attached to cultured oviductal epithelium retain their motility and fertilizing capacity significantly longer than free swimming spermatozoa in the same medium. However, they also found that cultured tracheal epithelium was less effective in this regard than cultured oviductal

epithelium. This suggests a unique interaction between spermatozoa and oviduct possibly mediated through specialized secretions of the oviduct epithelium.

The third subpopulation of spermatozoa were found in the crypts of the isthmus. Spermatozoa in the crypts formed clusters and appeared to attach to both ciliated and non-ciliated cells. The third flush recovered most, but not all, of these spermatozoa. The difficulty encountered in removing the spermatozoa from the crypts may have been due to a strong attachment to the ciliated cells at the base of the crypt. Strong attachment of spermatozoa to the ciliated cells of the rabbit oviduct was reported (Motta and Van Blerkom (1975) and Cooper *et al.* (1979). Alternatively, due to their distance away from the lumen, the spermatozoa in the crypts may have resisted flushing because they were not exposed to the hydrodynamic force of the flush and hence remained clustered in the crypts. The resistance to recovery by flushing appears to reach a maximum near the onset of fertilization (8 h after the onset of mating) as illustrated by the increase in the number of spermatozoa remaining in the oviduct after three flushes at that time (Figure 6). Why sperm attachment to the epithelium should be strongest at this time is not clear.

The high percentage of viable spermatozoa recovered in the 3rd flush indicates that the crypts provide the most hospitable microenvironment for spermatozoa in the isthmus. This may be due to a locally high concentration of energy sources in the crypts or simply because these spermatozoa are the farthest away from the apparently hostile environment of the lumen. It appears likely that the crypts act

as storage sites that ensure a viable population of spermatozoa in the isthmus during the preovulatory period.

In the hamster, where semen passes rapidly into the uterus at ejaculation, the crypts of the oviductal isthmus may be performing the same function as the cervical crypts in species where semen is deposited in the vagina (e.g. rabbit, human). I observed several characteristics for spermatozoa in the hamster oviductal crypts that were similar to those that have been reported for spermatozoa in the cervical crypts of the rabbit. Spermatozoa sequestered from the lumen in the crypts of the rabbit cervix could not be easily removed by flushing, were grouped in clusters (head-to-head aggregates), exhibited flagellar movement and were observed adhering to the ciliated cells of the endocervical epithelium (Overstreet, et al., 1978b).

It is not yet clear if hamster spermatozoa are capable of leaving the crypts to ascend to the ampulla to affect fertilization. Hyperactivated motility (Yanagimachi, 1981) associated with capacitation may provide a way for the spermatozoa to leave the crypts. In the ampulla, hyperactivated hamster spermatozoa are characterized by large amplitude, whiplash-like flagellar undulations (Katz and Yanagimachi, 1980). Suarez and Osman (1987) observed spermatozoa in the isthmus of the mouse oviduct which appeared to be hyperactivated. Although not observed in vivo, hamster spermatozoa recovered from the isthmus exhibited hyperactivated motility in the flushing medium (Smith and Yanagimachi, 1989). A spermatozoon which becomes hyperactivated within a crypt may be able to break its attachment

to the epithelium and escape into the lumen. Although a possible mechanism, additional studies will be required to determine if hyperactivated motility plays a part in the release of spermatozoa from the crypts.

I was surprised to find that only about 3,000 of the 9,000 spermatozoa recovered from the isthmus were alive at 2 h after mating. There was a significant decrease in the number of viable spermatozoa recovered from the isthmus at 6 h after mating. This overall decrease was due to the decrease in the percentage of viable spermatozoa recovered in the 1st and 2nd flushes (Figure 7), that is, spermatozoa from the lumen and mucosal surface of the wall. By 2 h after ovulation (8 h after the onset of mating) when fertilization begins, only about 1,500 of the spermatozoa recovered from the isthmus were alive (Figure 8). Since more spermatozoa remained in the crypts after flushing at this time, the actual number of live spermatozoa was most probably higher than that estimated by flushing. Since motility (viability) is required for efficient passage of spermatozoa through the uterotubal junction, our data indicates that the majority of spermatozoa that enter the isthmus die before ovulation. Nevertheless, the relatively small proportion of spermatozoa that survive in the isthmus following mating shortly after the onset of estrus is sufficient to fertilize a high percentage of the eggs.

## CHAPTER 5

### Attachment and Release of Spermatozoa from the Caudal Isthmus of the Hamster Oviduct

#### Introduction

When a female hamster mates shortly after the onset of oestrus, thousands of spermatozoa quickly enter the isthmic region of the oviduct (Yanagimachi & Chang, 1963; Smith *et al.*, 1987). These spermatozoa remain in the isthmus for nearly eight hours until about the time of ovulation when a relatively small number of them ascend to the ampulla to participate in fertilization (Smith *et al.*, 1987). Sperm storage in the isthmus also occurs when females mate during ovulation, but the storage period is shorter (Smith *et al.*, 1987). Sperm storage in the oviductal isthmus during the preovulatory period is not unique to the hamster. It also occurs in guinea pigs (Yanagimachi & Mahi, 1976), rabbits (Overstreet & Cooper, 1978), sheep (Hunter *et al.*, 1982), pigs (Hunter, 1984), cows (Hunter & Wilmut, 1984), and mice (Suarez, 1987). Little is known about how the spermatozoa are sequestered in the isthmus. Several mechanisms have been suggested, including localized constriction of the isthmus (Suarez, 1987), the trapping of spermatozoa in viscous isthmic mucus (Jansen, 1978), depressed sperm motility in the isthmus (Overstreet & Cooper, 1975; Suarez, 1987), and sperm attachment to the mucosal surface (Cooper *et al.*, 1979; Flechon & Hunter, 1981; Smith *et al.*, 1987; Suarez, 1987).

When we removed hamster spermatozoa selectively from the lumen and mucosal surface of the oviductal isthmus by flushing, we found the percentage of live spermatozoa was much higher among spermatozoa attached to the mucosal surface than those free in the lumen (Smith & Yanagimachi, 1990), implicating the importance of the mucosal surface in maintaining sperm viability during storage. However, we did not determine whether the spermatozoa attached to the isthmic mucosa are capable of detaching and ascending to the ampulla to fertilize eggs. The present study was initiated to answer this question and to determine if the degree of sperm attachment is altered by temporal changes in the mucosal surface and/or the capacitation status of the spermatozoa.

## **Materials and Methods**

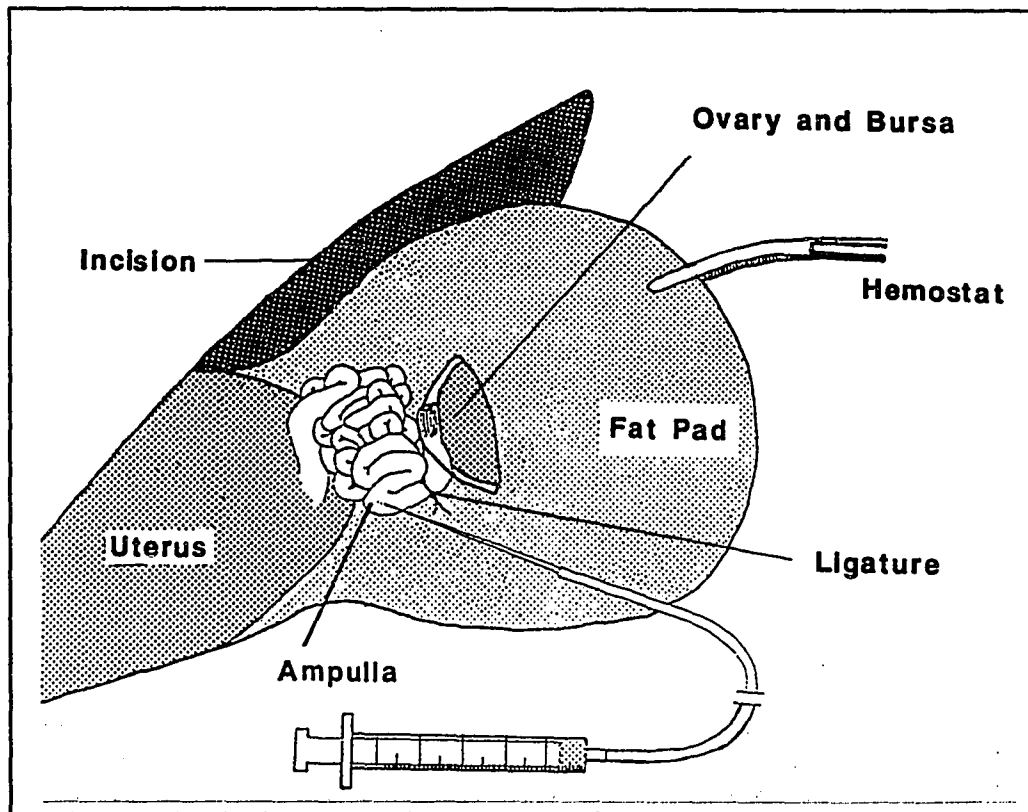
### **Animals and mating**

The female golden hamsters used in this study were raised to weaning under regular light conditions (light 05:00 to 19:00 h). At weaning, females were transferred to a cabinet with a reversed light cycle (light 17:00 to 07:00 h) and housed in groups of up to 6 in large cages (15 X 27 X 43 cm) until maturity. Under reversed light conditions, mature females (2-4 months old) exhibited behavioral oestrus by 06:30 h and ovulated between 12:30 and 14:00 h of every 4th day. Mature females were checked every day at 08:00 h for the presence of transparent mucus in the vagina indicating that the female was in oestrus (Orsini, 1961). An oestrous female was then placed in a large cage containing a pair of

mature (4-8 months old) sibling males of proven fertility. Mating began within 5 min. The males used in this study were maintained under regular light conditions (light 05:00 to 19:00 h).

#### Flushing of the oviduct in situ

We previously developed a technique to selectively remove spermatozoa from surgically isolated oviducts (Smith & Yanagimachi, 1990). For the present study, we modified this technique to allow selective removal of spermatozoa from the isthmic lumen in situ. At 3 or 6 h after the onset of mating, females were removed from the male's cage, anesthetized with ether and the right oviduct was exposed through a 1 cm flank incision. The first loop of the ampulla was ligated and pierced with a micropipette attached to a 100  $\mu$ l glass syringe mounted in a microinjector (Figure 9). When an oviduct was flushed before ovulation (at 11:00 h, i.e. 3 h after mating), the tip of the micropipette was inserted through the wall of the ampulla. When an oviduct was flushed during ovulation (at 14:00 h, i.e. 6 h after mating), the tip was inserted through the wall of the cephalic isthmus to leave the egg-cumulus complex undisturbed. The medium used for flushing was Ham's F-12 (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 1.2 mM calcium lactate



**Figure 9.** Procedure for flushing the hamster oviduct *in situ*. The 1st loop of the ampulla is ligated with a suture. A medium-filled micropipette (50  $\mu$ l capillary tube drawn to an 80  $\mu$ m tip and ground to a beveled 20° point) is inserted into the ampulla or cephalic isthmus (see text). The pipette is connected to 30 cm of Silastic tubing that is in turn connected to a 100  $\mu$ l Hamilton syringe mounted in a microinjector. During flushing, the medium and apparatus are kept warm (approx. 37°C) under an infrared lamp.

(Mann Research Laboratories, New York, NY, USA), 0.5 mM hypotaurine (Sigma), 59  $\mu$ g/ml gentamicin sulfate (Sigma) and 12 mg/ml bovine serum albumin (Fr. V, Calbiochem, San Diego, CA, USA). The pH of the medium was 7.3 when equilibrated with 5% CO<sub>2</sub> in air. The volume of medium used for flushing was 30, 90 or 180  $\mu$ l. In the case of 180  $\mu$ l, the oviduct was flushed twice with 90  $\mu$ l, waiting 3 min between flushes. Our preliminary experiments showed that a slower rate of flushing (25  $\mu$ l/min) did not remove as many spermatozoa as a faster rate

(6 $\mu$ l/sec), so the faster rate of flushing was used in this study. Immediately after flushing, the ligature was removed from the ampulla.

To prevent spermatozoa in the uterus (including those carried down to the uterus by flushing) from entering the flushed oviduct, 0.2 ml of saline (0.9% NaCl) was introduced into the right uterine lumen through a no. 20 gauge needle attached to a 1 ml tuberculin syringe. The diluted uterine spermatozoa were drawn into the syringe and discarded. The uterine lumen was then filled with distilled water (0.2 ml) and left for 1-2 min to kill any remaining spermatozoa. The distilled water was withdrawn and the uterine lumen was rinsed with saline. The uterine apex (intramural isthmus) was then ligated with a 6-0 suture (Dermalon, Davis and Geck, Manati, Puerto Rico) to prevent any spermatozoa that remained in the uterotubal junction from entering the isthmus. The oviduct was returned to the peritoneal cavity and the incision closed. The left side of the genital tract of each female was left untouched and served as the control. The entire operation took about 10 minutes. As an additional control, sham operations were performed on 3 females. Sham operations were performed in a manner identical to the flushing procedure outlined above except that oviducts were not flushed.

#### Examination of eggs after flushing in situ

At about 20 h after flushing the oviducts, females were euthanized with 100% CO<sub>2</sub> and their oviducts were removed. The right (experimental) and left (control) oviducts were placed in separate watchglasses and flushed with 0.2 ml of F-12 medium. Recovered eggs were mounted between a slide and coverslip and

examined for evidence of fertilization (the presence of a sperm tail in the ooplasm) and development.

Determination of the number of spermatozoa remaining in the oviduct after flushing

To interpret the results of the above fertilization experiments, it was necessary to determine the number of spermatozoa that remained attached to the isthmic mucosa after flushing. To accomplish this, right oviducts were flushed in situ with 30, 90 or 180  $\mu$ l of medium at 3 h after the onset of mating as previously described. Immediately after flushing, both right and left oviducts were fixed with A.F.A. fixative (Orsini, 1962), serially sectioned (10  $\mu$ m) and stained to determine the number of spermatozoa in the caudal isthmus (Smith et al., 1987). Three females were used for each experiment. Due to the large degree of variability in the number of spermatozoa found in the caudal isthmus of different oviducts, the percentage, rather than the absolute number, of spermatozoa remaining in the caudal isthmus was used for comparison. The percentage of spermatozoa remaining in the caudal isthmus after flushing was calculated from: [number of sperm in the flushed caudal isthmus / number of sperm in the unflushed caudal isthmus] X 100. This calculation is based on the assumption that equal numbers of spermatozoa enter the right (flushed) and left (control) oviducts. Although there is variation in the number of spermatozoa found in the right and left oviducts of an individual female and between individuals, neither side consistently contains a larger number of spermatozoa (Smith et al., 1987). In a previous study

(Smith & Yanagimachi, 1990), we showed that the number of spermatozoa attached to the mucosa of the caudal isthmus remains constant during the pre- and peri-ovulatory periods. Therefore, in this case, using the percentage of spermatozoa remaining in the caudal isthmus is a valid means of comparison.

Introduction of uncapacitated and capacitated spermatozoa into the oviducts of unmated females

To determine whether sperm attachment to the oviductal mucosa is influenced by the capacitation status of the spermatozoa, suspensions of uncapacitated and capacitated spermatozoa were introduced into the oviduct and the spermatozoa were assessed for their ability to attach to the mucosa. Uncapacitated and capacitated spermatozoa were prepared as follows. A highly motile suspension of cauda epididymal spermatozoa were prepared by a "swim-up" method (Uto *et al.*, 1988) in a modified Tyrode's medium, m-TALP (Yanagimachi, 1982). This medium contained 0.5 mM hypotaurine instead of taurine. An aliquot (0.3 ml) of sperm suspension ( $1$  to  $2 \times 10^6$  cells/ml) was incubated under mineral oil (Squibb and Sons, Princeton, NJ, USA) for 0 to 3 h (Yanagimachi, 1982). Spermatozoa incubated for 3 h were considered "capacitated". More than 90% of these spermatozoa exhibited hyperactivated motility, but the incidence of spontaneous acrosome reaction was less than 1% as assessed by phase contrast microscopy. Spermatozoa used immediately after "swim-up" (0% hyperactivated, 0% acrosome reacted) were considered "uncapacitated".

Oviducts were isolated from mature unmated females at 11:00 h (about 2-3 h before ovulation), placed in a prewarmed (37°) watchglass and 50  $\mu$ l of either capacitated or uncapacitated sperm suspension was passed through each oviduct. After introducing sperm suspensions, some oviducts were immediately transferred into prewarmed medium and, 15 min later, each oviduct was flushed with 50  $\mu$ l of warm m-TALP to remove any unbound or loosely bound spermatozoa. These oviducts were fixed, serially sectioned, and the number of spermatozoa bound to the oviductal mucosa was counted. Other oviducts were mounted between a slide and coverslip, slightly compressed and examined for the behavior of spermatozoa within the oviduct by viewing them through the oviductal wall (Smith & Yanagimachi, 1990).

## Results

### Fertilization following flushing of the oviduct in situ

Regardless of the volume of medium used for flushing (30, 90, or 180  $\mu$ l), a high percentage of the eggs recovered from flushed oviducts were fertilized (Table 5), indicating the spermatozoa that remained attached to the isthmic mucosa during flushing were capable of later detaching and ascending to the ampulla to fertilize eggs. Similarly, a high percentage of eggs recovered from sham operated oviducts were fertilized. However, the percentage of two-celled eggs recovered from flushed and sham operated oviducts were lower than in control (unflushed) oviducts (Table 5).

#### Number of spermatozoa remaining in the isthmus after flushing in situ

Preliminary experiments (data not shown) showed that a faster rate of flushing (6  $\mu\text{l}/\text{sec}$ ) was more effective in removing spermatozoa from the isthmus than a slower rate (25  $\mu\text{l}/\text{min}$ ). However, neither the volume of flushing medium (30, 90, 180  $\mu\text{l}$ ) nor the time of flushing (3 or 6 h after mating) had a significant effect on the percentage of spermatozoa remaining in the caudal isthmus (Table 6).

#### Binding of uncapacitated and capacitated spermatozoa to the oviductal mucosa

There was a striking difference between uncapacitated and capacitated spermatozoa with respect to their ability to attach to the oviductal mucosa.

Capacitated spermatozoa were virtually unable to attach, whereas many uncapacitated spermatozoa attached firmly to the mucosa (Table 7).

Interestingly, a significant number of uncapacitated spermatozoa attached to the mucosa of both the ampulla and isthmus. Examination of live spermatozoa through the oviductal wall revealed that capacitated (hyperactivated) spermatozoa swam freely within the lumen and did not attach to the mucosa when they encountered the wall of the oviduct, while uncapacitated spermatozoa swam freely only until they encountered the mucosa and then became attached. On occasion attached, uncapacitated spermatozoa detached from the mucosa and swam for a short distance in the lumen before becoming attached again, indicating that some uncapacitated spermatozoa do not attach firmly to the mucosa upon their first contact.

**Table 5 Percentage of eggs fertilized after flushing oviduct *in situ* with various volumes of medium. Eggs were examined 20 h after flushing (i.e. 18 h after ovulation).**

Volume of flushing medium ( $\mu$ l)	No. of oviducts	No. of eggs examined	% Eggs fertilized	% Fertilized eggs at	
				Pronuclear stage	2 cell stage
30	5	33	91	53	47
0 (control)	5	25	100	26	74
90	5	18	100	50	50
0 (control)	5	31	100	7	93
180	5	28	87	64	36
0 (control)	5	25	100	16	84
0 (Sham surgery)	3	19	93	53	37
0 (control)	3	20	100	10	90

**Table 6 Mean percentage ( $\pm$  s.d.) of spermatozoa remaining in the caudal isthmus (CI) after flushing the oviduct in situ at different times after mating or with different volumes of medium.**

<b>Time of flushing</b>	<b>Volume (<math>\mu</math>l) of medium</b>	<b>No. of oviducts flushed <u>in situ</u></b>	<b>% spermatozoa remaining in CI</b>
<b>3 h after mating</b>	30	3	43 $\pm$ 6.3
	90	3	36 $\pm$ 0.7
	180	3	40 $\pm$ 3.9
<b>6 h after mating</b>	30	3	32 $\pm$ 11.4

**Table 7 Mean number ( $\pm$  s.d.) of spermatozoa attached to the oviduct mucosa after flushing with uncapacitated and capacitated sperm suspensions ( $1-2 \times 10^6$  cells/ml)**

<b>Type of sperm suspension</b>	<b>Segment of the oviduct</b>	
	<b>Isthmus</b>	<b>Ampulla</b>
<b>Uncapacitated</b>	105 $\pm$ 67.2	148 $\pm$ 90.3
<b>Capacitated</b>	4 $\pm$ 2.5	2 $\pm$ 2.3

## **Discussion**

Sperm storage in the oviductal isthmus is not unique to the hamster and several storage mechanisms have been suggested. However, most of these proposed mechanisms do not seem to be involved in the hamster. For example, in the mouse localized constriction of the isthmus was observed (Nilsson & Reinius, 1969) and this was considered as a possible mechanism for immobilizing the spermatozoa in the isthmus (Suarez, 1987). Although localized constrictions do occur in the hamster isthmus during the preovulatory period (Battalia & Yanagimachi, 1979), they are transient in nature and appear to be responsible for mixing the luminal contents rather than immobilizing spermatozoa.

Jansen (1978) suggested that a viscous mucus present in the isthmus of oestrous rabbits inhibits sperm passage through the isthmus. The oviduct of the oestrous hamster does not contain any distinct mucus (Smith & Yanagimachi, unpublished observations), but it does contain cell debris similar to that observed in the mouse oviduct (Suarez, 1987), presumably derived from apocrine secretion of the mucosa (Jansen, 1978). The role of this luminal debris in sperm transport is not clear, but it may act as a "plug" to inhibit sperm ascent (Smith & Yanagimachi, 1990). However, this debris is still present in the lumen near the time of ovulation when spermatozoa begin

to ascend to the ampulla (Smith & Yanagimachi, unpublished observations), so it does not act as an absolute barrier to sperm transport.

In the rabbit, the motility of spermatozoa in the isthmus is depressed (Overstreet & Cooper, 1975) and this has been suggested as the major cause of limited sperm ascent in this species (Cooper *et al.*, 1979). When we examined hamster spermatozoa through wall of the oviduct, we also observed many immotile (dead) spermatozoa in the isthmic lumen (Smith & Yanagimachi, 1990). At the same time, we observed many motile spermatozoa attached to the isthmic mucosa. This mucosal attachment appears to be the major factor responsible for the temporal storage of fertilizing spermatozoa in the isthmus.

In a previous study (Smith and Yanagimachi, 1989), we collected fertile spermatozoa from the isthmus of mated hamsters, but we were not certain whether these spermatozoa had been bound to the mucosa. Some of them may have been recovered from the lumen and others from the mucosa. In the present study (Table 5), we clearly demonstrated that the spermatozoa which were firmly attached to the isthmic mucosa (those resistant to flushing) were capable of detaching, ascending to the ampulla and participating in fertilization. We cannot negate the possibility that under normal *in vivo* conditions spermatozoa that never attach to the isthmic mucosa can become capacitated and fertilize the eggs. However, the fact that the majority of viable spermatozoa were attached to the oviductal mucosa (Smith &

Yanagimachi, 1990) taken together with the results of the present study, it appears more likely that the majority of eggs are fertilized by spermatozoa that attach to the isthmic mucosa.

Contact between spermatozoa and the mucosa is known to be beneficial for sperm survival both *in vitro* (Pollard *et al.*, 1989) and *in vivo* (Smith & Yanagimachi, 1990) and may be necessary for capacitation. Sperm attachment to the mucosa is not permanent. Otherwise, the spermatozoa that resisted flushing by remaining attached to the mucosa would not have been able to later fertilize the eggs. Since the number of spermatozoa that reach the ampulla is very low, even after all the eggs are fertilized (Cummins & Yanagimachi, 1982; Smith *et al.*, 1987), only a very small fraction of the thousands of spermatozoa stored in the isthmus must be released from the mucosa before and during fertilization. One of the experiments of the current study demonstrated that the sperm-holding capacity of the isthmic mucosa remained virtually unchanged before and during ovulation. Therefore, it must be a change in sperm characteristics that is responsible for sperm release from the isthmic mucosa. Sperm capacitation appears to be involved in this change, as capacitated spermatozoa lose their affinity for the oviductal mucosa. It was interesting that uncapacitated spermatozoa were capable of firmly attaching to both the isthmic and ampullary mucosa, suggesting that there is no major difference in the mucosae of these regions with respect to sperm attachment. Why then, do we not see spermatozoa attached to the mucosa of the ampulla

shortly after mating? Perhaps all the live spermatozoa are confined to the isthmus and there is no mechanism to transport the spermatozoa from the isthmus to the ampulla until about the time of ovulation.

In summary, it seems likely that spermatozoa remain attached to the isthmic mucosa until they become capacitated. It is only then that they are able to leave the isthmus and ascend to the ampulla. Capacitated, hyperactivated spermatozoa do not attach to the ampulla. Indeed, there would be no advantage for capacitated spermatozoa to have a high affinity for the ampullary mucosa when unfertilized eggs are present nearby. The fact that a very small fraction of the isthmic spermatozoa ever leave the isthmus suggests that only a very small number of spermatozoa complete capacitation in the isthmus.

## CONCLUSIONS

When studying any system in vivo, there are many complicating factors that cannot always be resolved. In my opinion, it is better to take a holistic approach to the problem, rather than expect to see a direct effect of variable "a" on variable "b", as in the case of in vitro studies. For the biology of spermatozoa in the oviduct, the system is complex and the whole is greater than the sum of the parts. Keeping this in mind, the following is a summary of the main conclusions of the dissertation:

1. In the hamster, the uterotubal junction restricts the passage of homologous and heterologous spermatozoa into the oviduct.
2. Sperm motility is essential for the efficient passage of hamster spermatozoa through the uterotubal junction.
3. When mating occurs shortly after the onset of estrus, spermatozoa are stored in the caudal isthmus of the oviduct until near the time of ovulation when a few ascend to the ampulla to participate in fertilization.
4. The period of sperm storage in the caudal isthmus is shorter and sperm transport to the ampulla is faster when mating occurs near the time of ovulation.

5. Spermatozoa stored in the caudal isthmus during the preovulatory period do not become fully capacitated until near the time of ovulation.
6. Sperm capacitation is faster when mating occurs near the time of ovulation.
7. Only a relatively small percentage of the spermatozoa stored in the caudal isthmus during the preovulatory period are viable at the time of ovulation.
8. Sperm attachment to the isthmic mucosa is beneficial in maintaining sperm viability during storage.
9. Spermatozoa attached to the mucosa during storage are capable of later detaching and ascending to the ampulla to effect fertilization.
10. Sperm release from the isthmic mucosa is probably due to the physiological changes in the head plasma membrane resulting from sperm capacitation.

These facts indicate the importance of the sperm-mucosa relationship in the oviductal isthmus. This relationship is certainly involved in maintaining sperm viability during storage and may be necessary for sperm capacitation. Future studies will be concentrated on the elucidation of the nature (i.e. physical, biochemical, and molecular) of the sperm-mucosa relationship and its role in the fertilization process..

## EPILOGUE

When I began working with Dr. Yanagimachi in 1985, I asked him for a few papers that would introduce me to his area of research in reproductive biology, a brand new field to me. He handed me five bound volumes of research papers entitled "Publications of Yanagimachi's Group". There were over 175 papers in those volumes. After reading several, I began to feel as though all the "good" topics in sperm biology had already been explored. Now, of course, I realize just how naive I was. Although thousands of papers have been published in the field of sperm biology during the last twenty-five years, by far the vast majority of these studies used *in vitro* techniques. When I began the research for this dissertation, I was surprised to find that very little was known about the biology of spermatozoa in the female genital tract. Clearly, I had my work cut out for me.

This dissertation was composed of five chapters, each of which dealt with a particular aspect of sperm biology *in vivo*. The first two chapters determined when, where and how many spermatozoa are in the oviduct, a necessary preliminary to examining their biology, and the last three chapters explored various specific aspects of sperm physiology in the oviduct, namely their capacitation status, viability and their relationship with the oviductal mucosa. The results obtained from these studies explain some of the fundamental

aspects of sperm biology in vivo. The methods I used to answer these questions were not glamorous. On the contrary, they were traditional and admittedly rather tedious. No state-of-the-art molecular biological techniques were used here. In the absense of basic knowlege of the system, it would be putting the cart before the horse. The results of this dissertation are a start of a foundation of knowledge about the biology of spermatozoa in the oviduct upon which the superstructure of my future studies in this area will be built. In that, I am satisfied. However, much remains to be done in this important, but largely neglected, field of reproductive biology.

#### LITERATURE CITED

- Adams CE, 1956. A study of fertilization in the rabbit: The effect of post coital ligation of the Fallopian tube or uterine horn. *J Endocrinol* 13:296-308
- Ahlgren M, Bostrom K, Malmqvist R, 1975. Sperm transport and survival in women with special reference to the Fallopian tube. In: Hafez ESE, Thibault CG (eds.), *The Biology of Spermatozoa*, Karger, Basal: INSERM Int. Symp., Nouzilly, pp. 63-73
- Austin CR, 1951. Observations on the penetration of the sperm into the mammalian egg. *Aust J Sci Res (B)* 4:581-596
- Austin CR, 1956. Cortical granules in hamster eggs. *Exp Cell Res* 10:553-540
- Austin CR, 1975. Sperm fertility, viability and persistence in the female tract. *J Reprod Fertil*, suppl., 22:75-89
- Baker RD, Degen AA, 1972. Transport of live and dead boar spermatozoa within the reproductive tract of gilts. *J Reprod Fertil* 28:369-377
- Battalia DE, Yanagimachi R, 1979. Enhanced and coordinated movement of the hamster oviduct during the periovulatory period. *J Reprod Fertil* 56:515-20
- Bavister BD, 1980. Recent progress in the study of early events in mammalian fertilization. *Dev Growth and Differ* 22:385-402
- Bedford JM, 1967. Fertile life of rabbit spermatozoa in rat uterus. *Nature* 213:1097-1099
- Bedford MJ, 1970. The saga of mammalian sperm from ejaculation to syngamy. In: Gibian H, Plotz EJ (eds.), *Mammalian Reproduction*, New York: Springer Verlag, pp. 124-182
- Blandau RJ, 1969. Gamete transport-comparative aspects. In: Hafez ESE, Blandau RJ (eds.), *The Mammalian Oviduct*, Chicago: University of Chicago Press, pp. 129-162

- Blandau RJ, 1973. Gamete transport in the female mammal. In: Greep RO, Astwood EB, Geiger SR (eds.), Handbook of Physiology, Sect. 7, vol. II, part 2, Washington D.C.: American Physiological Society, pp. 153-163
- Braden AWH, 1953. Distribution of sperms in the genital tract of the female rabbit after coitus. Aust J Biol Sci 6:693-705
- Bunnell BN, Boland BD, Dewsbury DA, 1977. Copulatory behavior of golden hamsters (*Mesocricetus auratus*). Behavior 61:180-206
- Burkman LJ, Overstreet JW, Katz DF, 1984. A possible role for potassium and pyruvate in the modulation of sperm motility in the rabbit oviductal isthmus. J Reprod Fertil 71:367-376
- Chang MC, 1951. Fertilizing capacity of spermatozoa deposited in the Fallopian tubes. Nature 168:997-998.
- Chang MC, 1965. Fertilizing life of ferret sperm in the female tract. J Exp Zool 158:87-100
- Chang MC, Sheaffer D, 1957. Number of spermatozoa ejaculated at copulation, transported into the female tract, and present in the male tract of the golden hamster. J Heredity 48:107-109
- Coggins EG, Baker RD, 1968. Survival and transport of foreign spermatozoa in the genital tract of the rabbit. Proc 6 Int Cong Animal Reprod Artif Insem 1:47-50
- Cooper GW, Overstreet JW, Katz DF, 1979. The motility of rabbit spermatozoa recovered from the female reproductive tract. Gamete Res 2:35-42
- Cummins JM, Yanagimachi R, 1982. Sperm-egg ratios and the site of the acrosome reaction during in vivo fertilization in the hamster. Gamete Res 5:239-256
- Eddy CA, Pauerstein CJ, 1980. Anatomy and physiology of the Fallopian tube. Clin Obstet Gynecol 23:1177-1193
- Fallopian G, 1561. Observationes anatomicae, Venice.
- Flechon JE, Hunter, RHF, 1981. Distribution of spermatozoa in the utero-tubal junction and the isthmus of pigs, and their relationship to the luminal

- epithelium after mating. A scanning electron microscope study. *Tissue Cell* 13:127-139
- Gaddum-Rosse P, 1981. Some observations on sperm transport through the uterotubal junction of the rat. *Am J Anat* 160:333-341
- Hafez ESE, Black DL, 1969. The mammalian uterotubal junction. In: Hafez ESE, Blandau RJ (eds.), *The Mammalian Oviduct*, Chicago: University of Chicago Press, pp. 85-126
- Harper MJK, 1982. Sperm and egg transport. In: Austin CR, Short RV (eds.) *Reproduction in Mammals*, New York: Cambridge University Press, pp. 102-127
- Hawk HW, 1983. Sperm survival and transport in the female reproductive tract. *J Dairy Sci* 66:2645-2660
- Howe GR, Black DL, 1963. Migration of rat and foreign spermatozoa through the utero-tubal junction of the oestrous rat. *J Reprod Fertil* 5:95-100
- Hunter RHF, 1973. Transport, migration and survival of spermatozoa in the female genital tract: species with intra-uterine deposition of semen. In: Hafez ESE, Thibault C (eds.), *Sperm Transport, Survival and Fertilizing Ability*, Paris: INSERM., pp. 309-342
- Hunter RHF, 1975. Transport, migration and survival of spermatozoa in the female genital tract: species with intra-uterine deposition of semen. In: Hafez ESE, Thibault, C (eds.), *The Biology of Spermatozoa*, Basal: Karger-Basel, pp. 145-55
- Hunter RHF, 1980. Mating, sperm transport in the female genital tract, and artificial insemination. In: Hunter RHF (ed.), *Physiology and Technology of Reproduction in Female Domestic Animals*, New York: Academic Press pp. 104-144
- Hunter RHF, 1981. Sperm transport and reservoirs in the pig oviduct in relation to the time of ovulation. *J Reprod Fertil* 63:109-17
- Hunter RHF, 1984. Pre-ovulatory arrest and peri-ovulatory redistribution of competent spermatozoa in the isthmus of the pig oviduct. *J Reprod Fertil* 72:203-211

- Hunter RHF, 1985. Experimental studies of sperm transport in sheep, cows, and pigs. *Vet Rec* 16:188
- Hunter RHF, 1987a. Peri-ovulatory physiology of the oviduct, with special reference to progression, storage, and capacitation of spermatozoa. In: Mohri H (ed), *New Horizons in Sperm Cell Research*, New York: Breach Scientific Publishers, pp. 31-45
- Hunter RHF, 1987b. The timing of capacitation in mammalian spermatozoa - a reinterpretation. *Res Reprod* 19:3-4.
- Hunter RHF, 1988. *The Fallopian Tubes. Their role in Fertility and Infertility*, New York: Springer-Verlag.
- Hunter RHF, Nichol R, 1983. Transport of spermatozoa in the sheep oviduct: Preovulatory sequestering of cells in the caudal isthmus. *J Exp Zool* 228:121-28
- Hunter RHF, Nichol R, 1986. A preovulatory temperature gradient between the isthmus and ampulla of pig oviducts during the phase of sperm storage. *J Reprod Fertil* 77:599-606
- Hunter RHF, Wilmut I, 1984. Sperm transport in the cow: peri-ovulatory redistribution of viable cells within the oviduct. *Reprod Nutr Develop* 24:597-608
- Hunter RHF, Barwise L, King R, 1982. Sperm transport, storage and release in the sheep oviduct in relation to the time of ovulation. *Br vet J* 138:225-232
- Hunter RHF, Flechon B, Flechon JE, 1987. Pre- and peri-ovulatory distribution of viable spermatozoa in the pig oviduct: A scanning electron microscope study. *Tiss Cell* 19:423-436
- Hunter RHF, Nichol R, Crabtree SM, 1980. Transport of spermatozoa in the ewe: timing of the establishment of a functional population in the oviduct. *Reprod Nutr Dev* 20:1869-75
- Jansen RPS, 1978. Fallopian tube isthmic mucous and ovum transport. *Science* 201:349-351
- Katz DF, Yanagimachi R, 1980. Movement characteristics of hamster spermatozoa within the oviduct. *Biol Reprod* 22:759-764.

- Katz, DF, Drobnis EZ, Overstreet J, 1989. Factors regulating mammalian sperm migration through the female reproductive tract and oocyte vestments. *Gamete Res* 22:443-469
- Leonard SL, Perlman PL 1949. Conditions effecting the passage of spermatozoa through the utero-tubal junction of the rat. *Anat Rec* 104:89-102
- Marcus SL, 1965. The passage of rat and foreign spermatozoa through the utero-tubal junction of the rat. *Am J Obstet Gynec* 91:985-989
- Martan J, Shepherd B, 1973. Spermatozoa in rouleaux in the female guinea pig genital tract. *Anat Rec* 175:625-630
- McGaughey RW, Martson JH, Chang MC, 1968. Fertilizing life of mouse spermatozoa in the female tract. *J Reprod Fertil* 16:147-150
- Meizel S, 1984. The importance of hydrolytic enzymes to an exocytotic event, the mammalian sperm acrosome reaction. *Biol Rev* 59:125-57
- Moricard R, Bossu J, 1951. Arrival of fertilizing sperm at the follicular cell of the secondary oocyte. *Fertil Steril* 2:260-66
- Mortimer D, 1977. The survival and transport to the site of fertilization of diploid rabbit spermatozoa. *J Reprod Fertil* 51:99-104
- Mortimer D, 1978. Selectivity of sperm transport in the female genital tract. In: Cohen J, Hendry WF (eds.), *Spermatozoa, Antibodies and Infertility*, Oxford: Blackwell Scientific Publications, pp. 37-54
- Mortimer D, 1983. Sperm transport in the human female reproductive tract. In: Finn CA (ed.), *Oxford Review of Reproductive Biology*, vol. 5, Oxford: Oxford University Press, pp. 30-61
- Motta P, Van Blerkom J, 1975. A scanning electron microscopic study of rabbit spermatozoa in the female reproductive tract following coitus. *Cell Tiss Res* 163:29-44
- Nilsson O, Reinius S, 1969. Light and electron microscopic structure of the oviduct. In: Hafez ESE, Blandau RJ, (eds.), *The Mammalian Oviduct*, Chicago:University of Chicago Press, pp. 57-83

- Orsini MW, 1961. The external vaginal phenomena characterizing the stages of the estrus cycle, pregnancy, pseudopregnancy, lactation, and the anestrus hamster, Mesocricetus auratus Waterhouse. Proc Anim Care Panel 11:193-206
- Orsini MW, 1962. Technique of preparation, study and photography of benzyl-benzoate cleared material for embryological studies. J Reprod Fertil 3:283-287
- Overstreet JW, 1977. Sperm transport and selection in the female genital tract. In: Johnson MH (ed.), Development in Mammals, New York: North Holland Publishing, vol 2, pp. 31-65
- Overstreet JW, 1983. Transport of gametes in the reproductive tract of the female mammal. In: Hartman JF (ed.), Mechanisms and Control of Animal Fertilization, New York: Academic Press, pp. 499-543
- Overstreet JW, Cooper GW, 1975. Reduced sperm motility in the isthmus of the rabbit oviduct. Nature 258:718-719
- Overstreet JW, Cooper GW, 1978a. Sperm transport in the reproductive tract of the rabbit: I. The rapid transit phase of transport. Biol Reprod 19:101-114
- Overstreet JW, Cooper GW, 1978b. Sperm transport in the reproductive tract of the rabbit: II. The sustained phase of transport. Biol Reprod 19:115-132
- Phillips RW, Andrews FN, 1937. The speed of travel of ram spermatozoa. Anat Rec 68:127-132
- Polge C, 1978. Fertilization in the pig and horse. J Reprod Fertil 54:461-470
- Pollard JW, Plante C, King WA, Hansen PJ, Suarez SS, Betteridge KJ, 1990. Sperm fertilizing capacity is maintained by binding to oviductal epithelial cells. Biol Reprod, submitted
- Shalgi R, Kraicer PF, 1978. Timing of sperm transport, sperm penetration and cleavage in the rat. J Exp Zool 204:353-360
- Smith TT, Koyanagi F, Yanagimachi R, 1987. Distribution and number of spermatozoa in the oviduct of the golden hamster after natural mating and artificial insemination. Biol Reprod 37:225-234

- Smith TT, Koyanagi F, Yanagimachi R, 1988. Quantitative comparison of the passage of homologous and heterologous spermatozoa through the uterotubal junction of the golden hamster. *Gamete Res* 19:227-234
- Smith TT, Yanagimachi R, 1989. Capacitation status of hamster spermatozoa in the oviduct at various times after mating. *J Reprod Fertil* 86:255-261
- Smith TT, Yanagimachi R, 1990. The viability of hamster spermatozoa stored in the isthmus of the oviduct: The importance of sperm-epithelium contact for sperm survival. *Biol Reprod* 42:450-457
- Strauss F, 1956. Time and place of fertilization in the golden hamster. *J Embryol exp Morph* 4:42-56
- Suarez SS, 1987. Sperm transport and motility in the mouse oviduct: Observations in situ. *Biol Reprod* 36:203-210
- Suarez SS, Osman RA, 1987. Initiation of hyperactivated flagellar bending in mouse sperm within the female genital tract. *Biol Reprod* 36:1191-1198
- Thibault C, 1973. Sperm transport and storage in vertebrates. *J Reprod Fertil, suppl.*, 18:39-53
- Thibault C, Gerard M, Heyman Y, 1975. Transport and survival of spermatozoa in cattle. In: Hafez ESE, Thibault C (eds.), *The biology of Spermatozoa*, Basal: Karger, pp. 156-65
- Uto N, Yoshimatsu N, Lopata A, Yanagimachi R, 1988. Zona-induced acrosome reaction of hamster spermatozoa. *J exp Zool* 248:113-120
- VanDemark NL, Moeller AN, 1951. Speed of spermatozoan transport in reproductive tract of estrus cow. *Am J Physiol* 165:674-679
- Viring S, 1981. Distribution of live and dead spermatozoa in the genital tract of gilts at different times after insemination. *Acta Vet Scand* 21:587-597
- Yamanaka HS, Soderwall AL, 1960. Transport of spermatozoa through the female genital tract of hamsters. *Fertil Steril* 11:470-474
- Yanagimachi R, 1966. Time and process of sperm penetration into hamster ova in vivo and in vitro. *J Reprod Fertil* 11:359-370

- Yanagimachi R, 1972. Fertilization of guinea pig eggs in vitro. *Anat Rec* 174:9-19
- Yanagimachi R, 1981. Mechanisms of fertilization in mammals. In: Mastroianni L, Biggers JD, (eds.), *Fertilization and Embryonic Development In Vitro*, New York: Plenum Press, pp. 81-182
- Yanagimachi R, 1982. In vitro sperm capacitation and fertilization of golden hamster eggs in a chemically define medium. In: Hafez ESE, Semm K, (eds.), *In Vitro Fertilization and Embryo Transfer*, Lancaster UK:MTP Press, pp. 65-76
- Yanagimachi R, 1988. Mammalian fertilization. In: Knobil E, Neil J (eds.), *The Physiology of Reproduction*, New York: Raven Press, pp. 135-185
- Yanagimachi R, Chang MC, 1963. Sperm ascent through the oviduct of the hamster and rabbit in relation to the time of ovulation. *J Reprod Fertil* 6:413-420
- Yanagimachi R, Mahi CA, 1976. The sperm acrosome reaction and fertilization in the guinea-pig: a study in vivo. *J Reprod Fertil* 46:49-54
- Yochem DE, 1929. Spermatozoon life in the female reproductive tract of the guinea pig and rat. *Biol Bull* 56:274-297
- Zamboni L, 1972. Fertilization in the mouse. In: Moghissi KS, Hafez ESE (eds.), *Biology of Mammalian Fertilization and Implantation*, Springfield: Charles C Thomas, pp. 213-262

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John A. Burns School of Medicine  
Department of Anatomy and Reproductive Biology  
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## University of Hawaii at Manoa

John A. Burns School of Medicine  
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1960 East-West Road • Honolulu, Hawaii 96822  
February 20, 1990

Dr. Fuller W. Bazer, Ph.D.  
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