

## TRANSPORT OF LIVE AND DEAD BOAR SPERMATOZOA WITHIN THE REPRODUCTIVE TRACT OF GILTS

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**Summary.** Twenty-four gilts were inseminated with live spermatozoa, dead spermatozoa (killed by rapid freezing and thawing) or a mixture containing an equal number of live and dead spermatozoa. Before insemination, the live spermatozoa were stained with fluoresceine isothiocyanate. The females were killed 8 hr after insemination and spermatozoa were recovered from their reproductive tracts. Significantly more spermatozoa were recovered from the oviducts of gilts inseminated with live spermatozoa than from oviducts of gilts inseminated with either dead spermatozoa or the mixture. In gilts inseminated with the mixture, 54, 73, 71 and 100% of the spermatozoa recovered from the posterior half of the uterine horns, anterior half of the uterine horns, tubal flushings and the surface of ova, respectively, showed fluorescence.

In a second experiment, the left oviducts and right uterine horns were cannulated in eight gilts before cervically inseminating under anaesthesia with equal numbers of live-stained and dead spermatozoa. Fluid was collected from eight uterine and three tubal cannulae, the flow beginning within  $1\frac{1}{2}$  and  $4\frac{1}{2}$  min, respectively, after the onset of insemination. Both the volume of fluid collected and the concentration of spermatozoa in the fluid decreased with time after insemination, whereas the percentage of fluorescent spermatozoa increased. Dead spermatozoa were transported up the reproductive tract, but their transport was less efficient than that of live spermatozoa. Mechanisms of sperm transport are discussed.

### INTRODUCTION

The rôle of sperm motility in the transport of spermatozoa through the female genital tract is not clear. Reports have been made that only live spermatozoa pass through the cervix of ewes (Dauzier, 1953) and rabbits (Noyes, Adams & Walton, 1958), and the uterotubal junction of rats (Leonard & Perlman, 1949) and ewes (Dauzier, 1955). By contrast, dead spermatozoa were reported to be

transported through the cervix of ewes (Mattner & Braden, 1963) and through the uterotubal junction of rats (Howe & Black, 1963), ewes (Mattner & Braden, 1963) and cows (VanDemark & Moeller, 1951).

In pigs, a large volume of semen passes directly into the uterus during mating or insemination. Within 2 hr, most of the semen has disappeared from the uterus (Du Mesnil du Buisson & Dauzier, 1955b; Rigby, 1964; First, Short, Peters & Stratman, 1968) and only a very small proportion of the spermatozoa can be recovered from the oviduct (Rodolfo, 1934; Burger, 1952; Du Mesnil du Buisson & Dauzier, 1955a; First *et al.*, 1968). Nevertheless, dead spermatozoa are transported through the uterotubal junction into the oviduct (First *et al.*, 1968; Leidl, 1968).

The report that boar spermatozoa can be stained with fluoresceine isothiocyanate without adversely influencing sperm transport and fertility (Mellish & Baker, 1970) provides, for the first time, an effective way to compare directly the transport of two populations of spermatozoa within females. In the present study, this labelling technique has been used to compare the transport of live and dead spermatozoa within the genital tract of gilts.

## MATERIALS AND METHODS

### *Collection of semen and staining of spermatozoa*

A Yorkshire boar of known fertility provided semen for the present study. The semen was collected in an insulated plastic bottle after the boar mounted a 'dummy' sow. The gelatinous fraction of the semen was removed by straining through gauze. A sample of each ejaculate was examined for the percentage of motile spermatozoa, rate of motility and concentration of spermatozoa.

Each ejaculate was divided into two equal subsamples. Fifty micrograms of fluoresceine isothiocyanate (FITC) were dissolved in 0.05 ml modified Illini-Variable Temperature diluent (IVT, Bennett & O'Hagan, 1964) and added to one subsample per ml of semen. The mixture of semen and stain was stirred gently for 20 to 30 min at room temperature. A small sample was then examined for motility and fluorescence. The intensity of fluorescence was recorded as none, slight, moderate or high with a reflected light microscope ( $\times 400$ ) equipped with a mercury vapour lamp, exciter filter BG12 and barrier filters 53 and 44. During the staining period, the remaining subsample was quick-frozen in ethyl alcohol at  $-55^{\circ}\text{C}$ . After the semen had completely solidified, it was thawed by immersing in a hot water-bath and stirred in the bath until it reached room temperature. A small aliquot was then examined for sperm motility. After either freezing or staining, the semen was centrifuged for 10 min at 1500 g. The supernatant was removed and the spermatozoa were resuspended in the required volumes by adding IVT.

*Experiment 1.* Eight groups of three prepuberal crossbred gilts weighing 80 to 90 kg were injected with 1000 i.u. PMSG followed 48 to 60 hr later by 500 i.u. HCG to induce ovulation approximately 42 hr after HCG (Baker, Mellish & Segal, 1969). All gilts were inseminated 42 hr after the HCG injection with 100 ml of IVT containing  $18$  to  $32 \times 10^9$  spermatozoa. Within each group, the gilts were inseminated with equal numbers of spermatozoa. One gilt was inseminated

with live-stained spermatozoa, one with dead spermatozoa and one with a mixture containing an equal number of live and dead spermatozoa.

The gilts were slaughtered 8 hr after insemination. Their reproductive tracts were removed and the number of follicles and recent ovulation points were counted. The oviducts were removed by cutting at the uterotubal junction and the length of each uterine horn was measured. Each horn was then cut into two equal lengths, care being taken to prevent loss of fluid from the lumen, and each section was flushed twice with 15 ml IVT. The concentration of the spermatozoa was determined by counting a sample on a Neubauer haemocytometer. A sample of 100 spermatozoa was examined under ultraviolet light for fluorescence.

Each oviduct was flushed twice with 10 ml saline, and the recovered ova were mounted and examined microscopically. Spermatozoa attached to or embedded in the zona pellucida were counted and examined for fluorescence. The ova were then fixed in 25% acetic alcohol for approximately 48 hr, stained with 0.5% orcein in 45% acetic acid, and examined for evidence of sperm penetration or pronuclear development. The flushing fluid from each oviduct was examined directly or centrifuged to concentrate spermatozoa for counting.

Data on recovery of spermatozoa from the oviducts were transformed to square roots ( $\sqrt{X+1}$ ; Snedecor, 1956) before analysis because the data were not randomly distributed. The transformed data and 'uterine data' were analysed by conventional analysis of variance.

*Experiment 2.* Eight prepuberal crossbred gilts weighing 80 to 90 kg were treated with PMSG and HCG as in Exp. 1 and subjected to laparotomy 38 to 40 hr after HCG while under anaesthesia induced by pentobarbitone and maintained by Fluothane (Ayerst Lab). A polyvinyl chloride (PVC) cannula (0.86 mm i.d.  $\times$  1.52 mm o.d.) was inserted into the left oviduct through the fimbria, passed down the oviduct to the ampullary-isthmic junction and ligated in position. A second PVC cannula (3.5 mm i.d.  $\times$  6 mm o.d.) was inserted through a small incision near the uterotubal junction and passed into the lumen of the right uterine horn. A ligature was placed around the uterine horn and cannula to prevent semen loss through the incision or up the oviduct. The free ends of the cannulae were passed through the dorsal wall or out of the mid-ventral incision. The cannulae were cut to the desired length (30 to 60 cm) and attached to a graduated (15-ml) test tube. Before insemination, the midventral incision was closed. The anaesthetized gilts were cervically inseminated with 100 ml IVT which contained equal numbers of live-stained and dead spermatozoa.

The time between insemination and fluid first entering the collecting tube was recorded. The collecting tubes were changed at 15-min intervals and, at the end of 1 hr, the midventral incision was reopened. A blunt 18-gauge needle was passed through the wall of the right uterine horn and into the cannula. The cannula was flushed with 10 to 20 ml saline. A blunt 22-gauge needle was passed through the left oviduct, into the fixed end of the small cannula and flushed with 10 ml saline.

The fluids collected from the cannulae or voided through the vulva during insemination and the collection period were examined for sperm concentration

using a haemocytometer. Where possible, at least 100 spermatozoa from each sample were examined for fluorescence.

## RESULTS

Both the proportion of motile spermatozoa and the rate of motility decreased as a result of stirring the mixture of semen and stain at room temperature and centrifuging to remove the excess stain. Nevertheless, all spermatozoa examined were moderately to highly fluorescent, and 60 to 75% showed progressive motility at the time of insemination.

The method of quick-freezing and thawing semen employed in this study completely immobilized all the spermatozoa and they were presumed to be dead. It was noted that these spermatozoa tended to clump after thawing.

### *Experiment 1*

The type of spermatozoa inseminated had no significant effect on the number of spermatozoa recovered from either the posterior or anterior halves of the uterus (Table 1). The large variability observed in the numbers of spermatozoa recovered appeared to result in part from differences in size of the uterus.

TABLE 1  
RECOVERY OF SPERMATOZOA FROM UTERINE HORNS OF GILTS 8 HR  
AFTER INSEMINATION

<i>Spermatozoa inseminated</i>	<i>No. of females inseminated</i>	<i>Spermatozoa (<math>\times 10^6</math>) recovered from uterine horns</i>		
		<i>Posterior half</i>	<i>Anterior half</i>	<i>Total</i>
Live (FITC)	8	$8 \pm 3$	$14 \pm 5$	$21 \pm 7$
Mixture				
50% live		$7 \pm 4$	$4 \pm 2$	$11 \pm 5$
50% dead		$6 \pm 3$	$2 \pm 1$	$8 \pm 4$
Total	8	$13 \pm 7$	$6 \pm 3$	$19 \pm 10$
Dead (frozen)	8	$21 \pm 12$	$14 \pm 8$	$31 \pm 19$

Values are combined means + standard errors of both the right and left uterine horns.

Although there was a decrease in the fluorescent intensity of spermatozoa recovered from gilts inseminated with the stained spermatozoa, moderate levels of fluorescence were observed on 98 to 100% of the spermatozoa. Within the gilts inseminated with the mixture of live-stained and dead spermatozoa, 54% of the spermatozoa recovered from the posterior half of the uterine horns were fluorescent, compared with 73% recovered from the anterior half of the horns.

All twenty-four gilts had ovulated (overall mean of sixteen ovulations) at slaughter 50 hr after HCG (Table 2). Four to forty-three (mean of 14.6) ova were recovered per gilt to give an 89% recovery rate as judged by the number of ovulation points.

Spermatozoa were recovered from the oviducts of all eight gilts inseminated with live-stained spermatozoa (Table 2). Seven gilts had one or more fluorescent

spermatozoa embedded well into the zonae pellucidae of 55% of the ova, and 53% of the ova contained a fertilizing spermatozoon or were in the early pronuclear stage of development. By contrast, tubal spermatozoa and fertilized ova were recovered from only three of the gilts inseminated with the mixture of live-stained and dead spermatozoa. Fluorescence was observed on all spermatozoa associated with the ova recovered from gilts inseminated with the mixture.

Four of the seven gilts inseminated with dead spermatozoa had spermatozoa in their oviducts. Data from one gilt was not included in the analysis. The ovaries of this gilt contained evidence of seven recent ovulations, though eight fully mature corpora lutea were present. Large quantities of spermatozoa were recovered from the uterus ( $1.3 \times 10^8$ ) and oviducts ( $4.8 \times 10^4$ ), and an estimated 200 spermatozoa were attached to the surface rather than embedded in the zonae of three ova recovered from the left oviduct. A number of spermatozoa were attached to the zonae by the mid-piece or tail. Nevertheless, they were not dislodged by vigorous physical agitation. There were no spermatozoa on the ova recovered from the right oviduct of this gilt or from the oviducts of the

TABLE 2  
RECOVERY OF SPERMATOOA AND OVA FROM OVIDUCTS OF GILTS  
8 HR AFTER INSEMINATION AND OVULATION

	Live (FITC)	Mixture (live + dead)	Dead (frozen)
Gilts inseminated (No.)	8	8	7†
Gilts ovulated (No.)	8	8	7
Ovulation per gilt ( $\bar{x}$ )	20	13	18
Ova recovered per gilt ( $\bar{x}$ )	18	11	15
Gilts with sperm. in oviduct (No.)	8	5	4
Sperm. in fluid (No. $\times 10^3$ )	9.1*	3.6	1.8
Gilts with sperm. on ova (No.)	7	3	0
Sperm. on ova per gilt (No.)	416*	109	0
Gilts with fertilized ova (No.)	7	3	0
Fertilized ova per gilt (%)	47	25	0

\* Significantly greater ( $P < 0.05$ ) than mixture and dead sperm treatments.

† Data from cycling (luteal phase) gilt excluded.

other gilts inseminated with dead spermatozoa. None of the ova recovered from females inseminated with dead spermatozoa were fertilized.

### Experiment 2

The mean total number and percentage of fluorescent spermatozoa inseminated in this experiment were  $21 \times 10^9$  (range 19 to  $24 \times 10^9$ ) and 48.6 (range 46 to 52), respectively. A mean of 17 ml (range 5 to 50 ml) of semen was voided from the vulva during the insemination and collection period. The concentration of spermatozoa in the fluid lost from the vulva was similar to that in the inseminate in all cases. One gilt which lost 50 ml (50%) of the inseminate from the vulva within 15 min of the onset of insemination showed very poor sperm transport in that 0.2 ml and 0 ml of fluid were collected from the uterine and tubal cannulae, respectively. The remaining seven gilts all lost less than 20 ml of the inseminate from the vulva and, in four of these gilts, it

was noted that fluid flowed from the uterine cannulae in a pulsating manner.

Fluid was collected from all eight uterine cannulae (Table 3). The first fluid began flowing  $1\frac{1}{2}$  min and the last 22 min after the insemination had begun. There appeared to be a positive relationship between the onset of flow from the uterine cannulae and the total number of spermatozoa recovered in that the earlier fluid started to flow, the more fluid collected. A mean of 22 ml (range 9.8 to 37.2 ml) of fluid which began flowing within 5 min of the onset of insemination was collected from five uterine cannulae. By contrast, a mean of only 1.2 ml (range 0.2 to 1.8 ml) which began flowing more than 5 min after insemination had begun was collected from the remaining three uterine cannulae. Within gilts, the volume of fluid and the concentration of spermatozoa in the fluid decreased with increasing time after insemination.

TABLE 3  
RECOVERY OF SPERMATOZOA FROM CANNULAE IN THE RIGHT UTERINE HORNS  
AND LEFT OVIDUCTS OF EIGHT GILTS

	Time after insemination (min)				In cannula
	15	30	45	60	
No. of uterine samples	6	5	4	2	8
Volume of fluid (ml)	5.9	3.8	1.8	0.5	—
Sperm concentration ( $\times 10^6$ /ml)	62	28	15	11	—
Total sperm. ( $\times 10^6$ )	364	107	28	6	43
Fluorescent sperm. (%)	49	58	77	68	65
No. of tubal samples	2	2	0	0	4
Volume of fluid (ml)	0.22	0.15	0	0	—
Total sperm. ( $\times 10^3$ )	540	374	0	0	197
Fluorescent sperm. (%)	60	66	—	—	75

Within any one female, the percentage of fluorescent spermatozoa recovered from the uterine cannulae increased with the time elapsing after insemination. The high mean percentage of fluorescent spermatozoa recovered 45 min after insemination (Table 3) resulted from differences in flow rate and percentage fluorescence between females rather than a marked increase in percentage fluorescence within gilts.

As might be expected, fewer and much smaller samples of fluid were collected from the tubal cannulae. The first fluid began dripping from the cannula 4.5 min, the second 7 min and the third 20 min after insemination had begun. The remaining five tubal cannulae did not drip. However, two of these five cannulae contained fluid at the end of the collection period and spermatozoa were observed in the fluid flushed through one of them. In all cases, the percentage of fluorescent spermatozoa recovered from the tubal cannula was higher than that recovered from the contralateral uterine cannula.

## DISCUSSION

Results of the present study demonstrate that both live and dead spermatozoa were rapidly transported through the uterine horns to the uterotubal junction.

These observations are in agreement with reports that seminal plasma and spermatozoa arrive at the uterotubal junction within a short time after mating (Du Mesnil du Buisson & Dauzier, 1955b; Mann, Polge & Rowson, 1956). However, Pitkjanen (1958) reported that boar spermatozoa require a mean of 41.6 min to reach a cannula in the uterine horns near the uterotubal junction in unanaesthetized sows which had been inseminated with 150 ml semen. Her observation that the sows discharged through the vulva more than 100 ml of the semen during or soon after insemination might account for at least part of the difference between these investigations.

The findings that dead spermatozoa were transported, that fluid from five of eight uterine cannulae began flowing during the insemination and that fluid often flowed from the uterine cannulae in a pulsating manner indicate that the main forces involved in transporting the spermatozoa through the uteri were pressures exerted during insemination together with uterine contractions. Observations during cannulation immediately before insemination confirmed that the uteri were turgid and undergoing contraction in the gilts under halothane anaesthesia. However, these observations do not exclude the possibility that the halothane reduced sperm transport by decreasing uterine contractions (Lightfoot & Restall, 1971).

Recovery of tubal fluid containing dead and FITC-stained spermatozoa 4½ to 15 min and 6 to 15 min after the onset of insemination support findings by Burger (1952) and First *et al.* (1968) that boar spermatozoa reach the upper half of the oviduct within 15 min of mating or insemination. There was no evidence in this study or in that of First *et al.* (1968) that dead spermatozoa were transported into the oviducts any less rapidly than motile spermatozoa. However, the finding that significantly fewer spermatozoa were recovered from the oviducts of gilts inseminated with dead spermatozoa than from oviducts of gilts inseminated with live spermatozoa are at variance with results in sows reported by First *et al.* (1968). Consistently fewer dead than live spermatozoa were recovered from oviducts when gilts were inseminated with equal numbers of both. Failure to detect a single non-fluorescent spermatozoon on the ova recovered from gilts inseminated with the mixture and the recovery of dead spermatozoa 'adherent to' the zonae of ova recovered from one oviduct of the 'luteal phase' gilt indicate that although motility is not essential for transport up the tract, it is important in sperm attachment and penetration of the zona pellucida.

Several reports have supported the concept that the population of spermatozoa found in the oviduct at any one time is in transit and does not reflect the total number transported up the oviduct after an insemination. Asplund (1952) found that radio-opaque material frequently passed from the uterus into the abdominal cavity in women undergoing hystero-grams, and Horne & Thibault (1962) observed live spermatozoa in peritoneal fluid from five of fourteen women at laparotomy performed 24 hr *post coitum*. Mattner & Braden (1963) found that the numbers of spermatozoa recovered from occluded oviducts were far in excess of those found in the patent oviduct of sheep. In the present study, the numbers of spermatozoa recovered from the tubal cannulae were considerably greater than those recovered from oviducts in previous reports (Burger,

1952; Du Mesnil du Buisson & Dauzier, 1955a; Rigby, 1966; Leidl, 1968; First *et al.*, 1968; Baker, Dziuk & Norton, 1968) or in Exp. 1.

The mechanism whereby dead spermatozoa are transported through the uterotubal junction is not well understood. Presumably fluid containing both dead and live spermatozoa is forced through the uterotubal junction by uterine contractions. This assumption is in keeping with the finding of Baker *et al.* (1968) that, when adequate volumes of semen are inseminated, the number of spermatozoa that pass into the oviduct is related to the concentration of spermatozoa in the semen. In this study, the concentration of spermatozoa in fluid collected from the uterine cannulae decreased with time following insemination. The concentration of spermatozoa collected from uterine cannulae in sows decreased less rapidly following natural mating than following artificial insemination (Pitkjanen, 1958). The approximate tenfold difference in concentration of spermatozoa between the uterine and tubal fluids in this study suggests that the uterine fluid was diluted by tubal secretions or that some of the spermatozoa were removed as the fluid passed through the uterotubal junction and up the isthmus of the oviduct.

The consistent reduction in the percentage of dead spermatozoa recovered with increasing time within gilts inseminated with equal numbers of live and dead spermatozoa probably resulted from the removal of the dead spermatozoa from the fluid in the lumen of the tract. The dead spermatozoa may be adhering to the uterine mucosa, since a high proportion of the dead spermatozoa were not voided through the vulva or engulfed by leucocytes. Histological examination of the uteri of sows after mating demonstrated that boar spermatozoa do adhere to uterine cilia, glandular tubules and surface epithelium (Lovell & Getty, 1968). In addition, dead bull spermatozoa selectively stuck to the surface of glass beads (Bangham & Hancock, 1955) and, in the present study, the freezing and thawing noticeably increased the incidence of sperm clumping. Therefore, it seems plausible to suggest that the increased stickiness of the spermatozoa after their death facilitates their removal from the luminal fluid. If this suggestion can be supported experimentally, it might help to account for the numerous unsuccessful attempts to impregnate gilts inseminated with frozen-thawed boar semen (review by Polge, Salamon & Wilmut, 1970).

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