Regional influences of the Fallopian tubes on the rate of boar sperm capacitation in surgically inseminated gilts

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Aliquots of ejaculated boar semen containing known numbers of spermatozoa were deposited into the caudal isthmus or rostral ampulla of the Fallopian tubes of gilts at, or immediately after, ovulation to assess regional influences on the rate of capacitation. Eggs were recovered during a second intervention 4, 5, 6 or 7 h after surgical insemination and were examined by phase-contrast microscopy. Results were obtained from ten animals in each of the 4-, 5- and 6-h groups and from eight animals in the 7-h group. With two exceptions, fertilized eggs were not recovered until 6 h after insemination into the isthmus, the proportion (45.6%) being significantly greater than the corresponding figure (1.4%) for ampullary insemination ($P < 0.001$). Similarly, the proportion of fertilized eggs recovered 7 h after insemination into the isthmus (58.7%) was significantly greater than after ampullary insemination (21.9%; $P < 0.01$). Numbers of spermatozoa associated with the zona pellucida remained low in all these instances, with mean figures per egg ranging from 0.3 to 3.8. Insemination into the isthmus gave a 1–2 h advantage in fertilization compared with insemination into the ampulla. Although relative rates of sperm cell progression to the site of fertilization may have contributed to this, there is strong evidence that rates of capacitation differ significantly in the respective portions of the Fallopian tube. Therefore, attention was focused on: (1) the viscous glycoprotein secretion in the caudal isthmus acting to remove seminal plasma from the sperm surface; and (2) the phase of sperm head binding to the isthmus epithelium. Gradients in local endocrine modulation by the adjacent ovary offer one explanation for the functional specialization of different regions of the Fallopian tubes.

Introduction

Definitions of the process of capacitation appear frequently in the literature, even though details of the underlying cellular modifications are not clear. Definitions usually concern the period of residence in the female tract before spermatozoa can attach to and penetrate the zona pellucida of recently ovulated eggs (Austin, 1951; Chang, 1951). An alternative definition refers to changes in the sperm cell that precede and permit a coordinated acrosome reaction (Bedford, 1970; Chang and Hunter, 1975). Both approaches focus principally on the sperm cell rather than on interactions between spermatozoa and the female tract. Furthermore, recent studies have moved largely to *in vitro* experimentation in an attempt to characterize molecular modifications in spermatozoa completing the process of capacitation. As an alternative to the *in vitro* approach the technique of surgical insemination of quantified sperm populations into particular portions of the genital tract has been used. Such studies take into account the following: (1)

the coordinating role of the female tract in sperm surface modifications (Yanagimachi, 1994); (2) a local influence of ovarian endocrine programming on specific regions of the Fallopian tube epithelium (Hunter *et al.*, 1983; Hunter, 1995a); and (3) the intimate endosalpingeal–sperm head interactions in the hours before ovulation (Racey *et al.*, 1975; Fléchon and Hunter, 1981; Hunter *et al.*, 1987, 1991; Suarez, 1987; Smith and Yanagimachi, 1990, 1991; Pollard *et al.*, 1991). Furthermore, the *in vivo* approach recognizes that completion of the process of capacitation is a periovulatory event (Hunter, 1987a,b; Smith and Yanagimachi, 1989). These studies suggest that at least the final stages of capacitation are programmed by ovarian cues, almost certainly of an endocrine nature, although they are modulated by the endosalpinx.

Previous studies have demonstrated regional variations in the capacitating potential of the female reproductive tract, suggesting that there are synergistic influences between the uterus and Fallopian tubes in the capacitation of spermatozoa (Adams and Chang, 1962; Bedford, 1968, 1969; Hunter, 1969; Hunter and Hall, 1974). In the present study, surgical insemination was used to examine regional influences on the rate of capacitation in boar spermatozoa exposed only to the Fallopian tubes. The ampullary and isthmus regions of the pig

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Fallopian tube differ in morphology (Andersen, 1927; Hafez and Blandau, 1969), epithelial folding and organization of surface organelles (Stalheim *et al.*, 1975; Wu *et al.*, 1976; Fléchon and Hunter, 1981), vascular arcade and lymphatic bed (Andersen, 1927; Hunter, 1988), luminal and surface temperatures (Hunter and Nichol, 1986; Hunter *et al.*, 1997), and the composition of luminal fluids (Hunter, 1990; Nichol *et al.*, 1992, 1997). Therefore, it is reasonable to anticipate that different rates of capacitation will occur in the different regions. In the present study, observation was made of the ability of spermatozoa to penetrate the egg investments and prompt activation of the secondary oocytes. In a parallel study (Hunter *et al.*, unpublished), different regions of the pig endosalpinx were examined for the presence of particular classes of mRNA that encode macromolecules involved in capacitation. The overall premise was that the more precisely defined the physiological circumstances under which capacitation occurs in the Fallopian tubes, the more meaningful and rigorous the corresponding molecular interpretation would be.

Materials and Methods

Animals

Pigs were obtained from the University of Göttingen experimental farm, Relliehausen, at approximately 5.5–7.0 months of age and close to the onset of puberty. They were crossbred Large White × Landrace and weighed between 80 and 112 kg. The animals were housed in indoor pens at the institute in groups of four–six. Natural daylight was supplemented by artificial light during the winter months. Animals were bedded on straw, fed a standard commercial diet of pelleted concentrates, and had free access to drinking water. None of the animals had previously been subjected to any form of insemination or other experimental procedure.

Ovarian stimulation

A well-established technique of inducing ovulation at a predetermined time was used in these experiments (Holtz, 1996). Each animal received a single subcutaneous injection of 1000 iu pregnant mares' serum gonadotrophin (PMSG) (Intergonan; Intervet, Toenisvorst) followed 78–82 h later by a single injection of 500 iu hCG (Ovogest; Intervet, Toenisvorst). Ovulation was expected approximately 41–42 h after the hCG injection, a response that was verified at surgery. Vulval swelling and reddening occurred after the PMSG injection, usually in conjunction with oestrous behaviour and standing oestrus.

Semen samples

Freshly collected semen from boars of proven fertility (used as commercial artificial insemination sires) was

provided by the Veterinary Institute, University of Göttingen. Aliquots of whole semen, filtered free of the gelatinous bulbourethral secretion, were transferred to the operating theatre in a Dewar flask prewarmed to a temperature of 35°C. Pasteur glass pipettes, microscope slides and coverslips were used at 37°C to examine and evaluate microdroplets of the sperm suspension by phase-contrast microscopy for progressive motility. They were scored on a subjective scale of 1–5; 5 representing the best motility. Only those samples scoring at least 3 in this assessment were used in the experiment. After suitable dilution ($\times 100$), the sperm concentration was estimated in a haemocytometer slide (Neubauer ruling). The range in concentration of the inseminated samples was $2.21\text{--}3.87 \times 10^8$ cells ml^{-1} .

Surgical intervention and insemination

Animals were starved for 12 h before surgery and then given a 5 ml intramuscular injection of the preoperative sedative Stresnil (Azaperone, 40 mg ml^{-1} ; Janssen, Neuss) followed 15 min later by intravenous administration of 10 ml Hypnodil (Metomidathydrochloride, 50 mg ml^{-1} ; Janssen, Neuss). This intravenous anaesthetic was supplemented as required to maintain full surgical relaxation.

The reproductive organs were exposed during a mid-ventral laparotomy and the ovaries were examined briefly for the presence of recent ovulations. Individual Fallopian tubes were then withdrawn for surgical insemination with an absolute minimum of handling. On one side of the tract, 0.02 ml of the sperm suspension was introduced into the caudal tip (0.5 cm) of the isthmus using a polished (round-tipped) 2.2 cm $\times$ 20 or 21 gauge needle attached to a 1.0 ml sterile disposable syringe (Sabre; Gillette Surgical, Isleworth). The needle was inserted into the lumen of the isthmus by puncturing the adjacent uterine wall and carefully negotiating through the swollen processes of the utero-tubal junction. Both needle and syringe were prewarmed to 37°C before removal of the sperm suspension from the Dewar flask. The suspension was introduced into the caudal isthmus with minimal disturbance of the luminal contents. This side of the tract was identified with a fine silk ligature placed in the wall of the corresponding uterine horn.

The contralateral side of the tract was inseminated into the rostral portion of the ampulla. The same syringe and needle, wiped clean with a sterile surgical gauze, was used to deposit 0.02 ml of the same sperm suspension to a depth of 1–2 cm into the ampulla. The lumen of the ampulla was accessed by displacing the fimbriated extremity and introducing the needle without tearing the epithelium. Immediately after this insemination, the fimbriated folds were replaced around the ovary, the reproductive tract was restored to the abdomen, and the incision closed in three layers with continuous sutures. These surgical manipulations routinely occupied less than 20 min. Aseptic procedures were followed throughout. Animals were left to regain consciousness in a straw-bedded pen adjoining the operating theatre.

Recovery of eggs and closure of abdomen

Animals were tranquillized and anaesthetized as described earlier 15–20 min before egg recovery at 4, 5, 6 or 7 h after insemination. The reproductive tract was exposed and the number of recent ovulations was noted. After transection at the utero–tubal junction, the caudal 4–5 cm of each isthmus was liberated from the supporting mesosalpinx by blunt dissection using intermittent puncture sites made with the outside edges of a pair of fine scissors in the mesentery immediately adjoining the isthmus, followed by controlled digital tearing (Hunter and Dziuk, 1968). Haemostats were briefly applied to the cut edge of the mesosalpinx, and all surface blood was absorbed with surgical gauze. The freed end of the isthmus was introduced into the neck of a 100 ml glass cylinder, and the dissected portion of the tube supported on surgical gauze. Each Fallopian tube was flushed from the ovarian end with 40 ml of PBS (290 mOsmol) or physiological saline at 37°C using a disposable hypodermic syringe and a polished (blunted) 4.5 cm × 14 gauge needle introduced into the rostral portion of the ampulla. These flushings were decanted into sterile tissue culture dishes of 9 cm diameter, and then searched systematically for eggs under a Wild stereo-zoom dissecting microscope.

The ovaries were checked a second time for the number of ovulations, after which the reproductive tract was cleansed of surface blood, moistened with physiological saline, and restored to the abdomen. The incision was closed in three layers: a continuous suture of No. 3 Mersilk (Ethicon Ltd, Edinburgh) was used in the peritoneum and interrupted sutures of No. 6 Mersilk (Ethicon Ltd, Edinburgh) were used to close the body wall and skin. All animals recovered from surgery without incident.

Mounting and examination of eggs

Whole mounts were made of the recovered eggs between slide and coverslip supported on pillars of a mixture of paraffin wax and petroleum jelly (Chang, 1952). After a detailed examination for spermatozoa on or in the zona pellucida, eggs were fixed for 24–48 h in 25% acetic alcohol (1:3 glacial acetic acid:ethanol), immersed briefly in 100% ethanol, and stained with 1% orcein in 45% acetic acid. This final step, performed under a phase-contrast microscope, induced a gradual swelling and then protracted dissolution of the zona pellucida, enabling an accurate count to be made of the number of surface-associated spermatozoa or those with heads embedded in the zona substance. Nuclear structures in the stained preparations were subsequently examined and recorded. Eggs activated by a spermatozoon in the vitellus are referred to as fertilized.

Experimental design and statistical analysis

Aliquots of the same sperm suspension were introduced into either the caudal isthmus or rostral ampulla of Fallopian tubes during, or immediately after, ovulation. The side of the

reproductive tract for each insemination was chosen in an arbitrary manner. Results were analysed by the chi-squared test.

Results

Animals not included in analysis

Sixty-two animals were allocated to the experiment but 24 of these failed to give results that could be included in the analysis. They were found at surgery to be unsuitable for the experiment: 11 were already in the luteal phase of the oestrous cycle with well-developed corpora lutea and had reached puberty before the start of treatment; six had ovulated in only one ovary, thereby preventing a comparison between the two insemination treatments; three had haemorrhagic cystic follicles (13–20 mm diameter) as a response to PMSG; three had not ovulated at the time of second laparotomy; and in one animal no eggs were recovered from the flushed tubes.

Ovulation and egg recovery

As noted at laparotomy, 30 of the 38 animals (78.9%) included in the results had just completed ovulation at the time of surgical insemination; the remaining eight animals were in the middle of ovulation during the first intervention. Ovulation was complete by the second intervention.

The mean number of ovulations in these animals was 12.7, 13.2, 14.0 and 11.1 for the 4-, 5-, 6- and 7-h groups, respectively (Table 1). The proportion of eggs recovered varied from 87.6 to 93.6%, the overall mean being 90.2% (Table 1).

Number of zona spermatozoa

Phase-contrast examination of whole-mount preparations failed to show whether binding of spermatozoa to the zona pellucida had occurred, as distinct from simply surface contact. However, in the results presented below, initial binding or penetration seem probable since spermatozoa were not displaced by the fixation and staining procedures.

In the 4-h group, three instances of insemination into the isthmus yielded eggs with zona spermatozoa, but spermatozoa were not seen on or in the zona pellucida of eggs after ampullary insemination (Table 2). Among the 5-h group, five of the isthmic inseminations yielded zona spermatozoa and four of these five animals also yielded zona spermatozoa after ampullary insemination. Within the 6-h group, seven of the isthmic inseminations resulted in zona spermatozoa compared with four of the ampullary inseminations. In the 7-h group, five of the eight inseminations into the isthmus resulted in zona spermatozoa and five ampullary inseminations also gave zona spermatozoa.

The proportion of eggs possessing zona spermatozoa showed an increasing trend with interval from insemination

Table 1. The number of gilts yielding eggs from both Fallopian tubes, the total number of ovulations and the proportion of eggs recovered at four different intervals after surgical insemination

Interval from surgical insemination to egg recovery (h)	Number of animals	Number of ovulations	Eggs recovered	
			Number	Percentage
4	10	127	113	88.9
5	10	132	118	89.4
6	10	140	131	93.6
7	8	89	78	87.6
Total	38	488	440	90.2

Table 2. The number of eggs with spermatozoa on or in the zona pellucida at four intervals after surgical insemination into the caudal isthmus or the rostral ampulla of the Fallopian tubes of gilts at ovulation

Interval from surgical insemination to egg recovery (h)	Insemination into isthmus					Insemination into ampulla				
	Number of tubes yielding eggs with zona spermatozoa	Number of eggs examined	Number of eggs with spermatozoa	Number of spermatozoa per egg		Number of tubes yielding eggs with zona spermatozoa	Number of eggs examined	Number of eggs with spermatozoa	Number of spermatozoa per egg	
				Range	Mean				Range	Mean
4	3	55	15 ^a	3–9	3.8	0	58	0 ^b	0	0
5	5	59	14 ^a	1–4	0.6	4	59	7 ^b	1–3	0.3
6	7	57	35 ^a	1–6	1.5	4	74	6 ^b	1–2	1.2
7	5	46	29 ^c	1–6	2.6	5	32	12 ^d	1–3	1.0
Total	20	217	93	1–9	—	13	223	25	0–3	—

Statistical significance was assessed by the chi-squared test.

Values with superscripts a and b are significantly different, $P < 0.001$.

Values with superscripts c and d are significantly different, $P < 0.05$.

until recovery, especially in the ampullary group, although the mean number of zona spermatozoa was lower after insemination into the ampulla (Table 2).

There was no detectable correlation between the number of spermatozoa inseminated and the number of zona spermatozoa in the corresponding Fallopian tubes, or with the proportion of eggs activated in each insemination group.

Activation and development of eggs

The proportion of eggs activated by a penetrated spermatozoon, referred to here as fertilized, was analysed according to the interval between insemination and egg recovery (Table 3).

Only one fertilized egg was recovered from each of the groups examined 4 and 5 h after isthmic insemination compared with 26 fertilized eggs in the group examined 6 h after a comparable insemination ($P < 0.001$). The proportion of fertilized eggs recovered 4 and 5 h after isthmic insemination (1.8 and 1.7%, respectively) was similar to the proportion of fertilized eggs recovered 6 h after ampullary insemination (1.4%). However, the latter figure is significantly different ($P < 0.001$) from the corresponding proportion (45.6%) of fertilized eggs recovered 6 h after isthmic insemination. The proportion of fertilized eggs

recovered 7 h after isthmic insemination (58.7%) was significantly greater than the proportion recovered 7 h after ampullary insemination (21.9%; $P < 0.01$). These results show that insemination of ejaculated spermatozoa into the isthmus gives a 1–2 h advantage in the timing of fertilization compared with insemination into the ampulla.

Eggs activated by a fertilizing spermatozoon invariably had chromosomes arranged at late anaphase or, more frequently, telophase on the second meiotic spindle. Such eggs always contained an enlarged sperm head with detached mid-piece and at least a proportion of discernible flagellum. Well-formed pronuclei were distinguishable in nine eggs recovered 7 h after isthmic insemination, but were not observed in the ampullary insemination group. Polyspermic penetration of the vitellus was not detected in this study, but two primary oocytes were recovered. These had a large peripheral germinal vesicle, but no attached or penetrated spermatozoa.

Discussion

The most important finding of this study is that when identical aliquots of the same boar semen sample are inseminated into the Fallopian tubes at, or immediately after, ovulation, spermatozoa introduced into the caudal tip of the

Table 3. The proportion of fertilized eggs recovered at four intervals after surgical insemination into the caudal isthmus or rostral ampulla of the Fallopian tubes of gilts at ovulation

Interval from surgical insemination to egg recovery (h)	Number of animals	Insemination into caudal isthmus			Insemination into rostral ampulla		
		Number of eggs collected	Number of fertilized eggs	Percentage	Number of eggs collected	Number of fertilized eggs	Percentage
4	10	55	1	1.8 ^a	58	0	0 ^a
5	10	59	1	1.7 ^a	59	0	0 ^a
6	10	57	26	45.6 ^b	74	1	1.4 ^a
7	8	46	27	58.7 ^{bc}	32	7	21.9 ^{bd}
Total	38	217	55	25.3	223	8	3.6

Statistical significance was assessed by the chi-squared test.

Values with superscripts a and b are significantly different, $P < 0.001$.

Values with superscripts c and d are significantly different, $P < 0.01$.

isthmus are able to penetrate and activate oocytes 1–2 h earlier than those introduced into the rostral ampulla of the contralateral tube. Although the relative rates of sperm progression to the site of fertilization could be a factor contributing to this difference, there is evidence to suggest that rates of capacitation differ significantly in the respective portions of the Fallopian tubes.

Introduction of sperm suspensions into the Fallopian tube ampulla permits fertilization in various species of mammal. Indeed, this was the original approach used in classical studies of capacitation in rabbits (Chang, 1951; Noyes, 1953) and is also known to be effective in pigs (Polge, 1978). In addition, insemination by the intraperitoneal route has been successful in many mammals (rabbit: Dautier and Thibault, 1956; Hadek, 1958; Rowlands, 1958; Adams, 1969; guinea-pig: Rowlands, 1957; cow: Skjerven, 1955; pig: Hunter, 1978), and can lead to 70% fertilization in pigs if performed close to ovulation (Hunter, 1978). We would expect the ampullary insemination in the present study to be successful because transport systems in the isthmus and ampulla function in opposing directions at, and shortly after, ovulation to displace male and female gametes towards the site of fertilization (Hafez and Blandau, 1969; Hunter, 1977). Although the rate of movement of competent spermatozoa down the ampulla has not been determined, the movement of newly ovulated or transplanted eggs to the site of fertilization requires 6–15 min in cats and rabbits (Harper, 1961a,b; Blandau, 1969) and no more than 30–45 min in pigs (Oxenreider and Day, 1965; Hunter, 1974). Therefore, it is unlikely that motile spermatozoa would require a period of hours to complete the same movement, especially if introduced directly into the ampulla. Indeed, in this study small numbers of spermatozoa were associated with some eggs 5 h after insemination.

The temporal advantage gained by boar spermatozoa inseminated into the caudal tip of the isthmus may be partly explained by the viscous glycoprotein secretion that occludes this portion of the duct in oestrous animals before ovulation (Fléchon and Hunter, 1981; Hunter *et al.*, 1987; Hunter, 1995a,b). One function of this viscous plug in mated animals

is to prevent the entry of uterine fluid and suspended polymorphonuclear leucocytes into the Fallopian tubes. Before the progressive destabilization that permits a coordinated acrosome reaction can occur, seminal plasma is displaced from the sperm plasmalemma (Bedford, 1970; Chang and Hunter, 1975). Under physiological conditions, the oedematous polypoid processes at the utero-tubal junction exclude the bulk of seminal plasma from the Fallopian tubes (Du Mesnil du Buisson and Dautier, 1955; Hunter *et al.*, 1972; Hunter, 1973), even though constituents of the male secretions can still be detected in the tubes of artificially inseminated animals (Einarsson *et al.*, 1980). Subsequent interactions with the viscous glycoprotein ('mucus plug') are a fine-tuning system involved with preovulatory sperm storage in the isthmus (Hunter, 1981, 1984) and preparation for a periovulatory acrosome reaction (Hunter, 1987a,b). The much larger lumen of the ampulla is not occluded by similar viscous material, so a rapid 'filtering' of spermatozoa from the male secretions would not be anticipated.

As is the case for laboratory species such as mice (Suarez, 1987) and hamsters (Smith and Yanagimachi, 1990, 1991), the heads of boar spermatozoa become intimately engaged with the epithelium of the isthmus during the preovulatory interval (Fléchon and Hunter, 1981; Hunter *et al.*, 1987) or *in vitro* (Suarez *et al.*, 1991). This is referred to as sperm adhesion or sperm binding and it makes an important contribution to the formation of the sperm reservoir and the establishment of a sperm gradient along the isthmus to the site of fertilization. Indeed, the sperm gradient itself may enable more subtle molecular and ionic interactions between limited numbers of spermatozoa and progressive regions of the endosalpinx. In addition, an intimate relationship between the sperm head and the genital tract may stimulate the synthesis of new classes of macromolecules by epithelial cells (Ellington *et al.*, 1993) and, directly or indirectly, permit further modification and remodelling of the sperm head surface membranes. The possible involvement of locally high progesterone concentrations, Ca^{2+} ion transfer, or even catecholamines on such spermatozoa bound in the isthmus

has been considered in some detail (Hunter *et al.*, 1983; Hunter, 1995a, 1996, 1997). As a result of sperm head-epithelial binding in the isthmus, spermatozoa may gain temporal advantages in completion of capacitation compared with spermatozoa introduced directly into the ampulla. Lectin binding sites differ between the two regions of the duct epithelium (Raychoudhury *et al.*, 1993), even though spermatozoa can bind in the ampulla.

Chronological aspects of sperm capacitation during passage along the female tract or within particular portions of the tract deserve attention. Way *et al.* (1997) reported more effective capacitation of bull spermatozoa *in vitro* when incubated in isthmus fluids compared with ampullary fluids, as judged by subsequent spermatozoon-egg binding. Similarly, more effective induction of the acrosome reaction was reported with isthmus fluid (Grippe *et al.*, 1995). Although Thibault (1959) and Hancock (1961) both suggested that boar spermatozoa require 5–6 h for capacitation *in vivo*, Hunter and Dziuk (1968) demonstrated that peri- or postovulatory intracervical insemination enabled pig eggs to be penetrated and activated within 3 h. In a subsequent analysis of regional contributions to the process of capacitation, Hunter and Hall (1974) found that the uterus and Fallopian tubes acted synergistically to promote capacitation of boar spermatozoa within 3 h of mating. In contrast, when freshly ejaculated boar spermatozoa were introduced directly into the isthmus, capacitation required 5–6 h, as endorsed by the current study. This 2-h period of retardation was shown to be due to the presence of whole seminal plasma deposited in the Fallopian tubes at insemination. This finding was later highlighted using suspensions of epididymal spermatozoa (Hunter *et al.*, 1978). Boar epididymal plasma prepared from the caudal region of the duct retarded capacitation of sperm suspensions from the corpus epididymidis by approximately 2 h when it accompanied surgical insemination directly into the isthmus. This delay was considered to be the period of time required to remove male secretions from the lumen of the isthmus by absorption, dilution and/or metabolism before subtle modification of the sperm surface that would allow the completion of capacitation and membrane destabilization could occur.

In conclusion, the present work suggests that the isthmus is more effective in 'neutralizing' seminal plasma components than the ampulla. This is perhaps surprising, considering the extensive undulations of the ampullary mucosa and epithelium, and its prominent vascular bed in oestrous animals, but may reflect the complex lymphatic network bordering the caudal isthmus (Andersen, 1927). However, significant questions remain as to: (1) precisely what rate-limits the number of spermatozoa completing capacitation at any one time (Hunter, 1995a); (2) how such consistent timing is achieved between animals and between experiments; and (3) the location of this timing programme. Does it lie principally in the sperm cell or is it generated by specific interactions between spermatozoa, the endosalpinx and the luminal fluids? Our current molecular approach (B. Brenig *et al.*, unpublished) to the regional synthesis of macromolecules should clarify these questions.

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