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Possible Involvement of a Sperm-associated Body in the Process of Fertilization in Quail

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ABSTRACT—The present paper describes a novel structure, termed the sperm-associated body, which is found both in the lumen at the oviductal infundibulum and in the vitelline membrane of the ovum in the quail *Coturnix japonica*. The fully developed sperm-associated body, which is about 100 μm long, consisted of two parts; a core of concentric-circular appearance and a cortex of needle-like projections. The outer surface of the body was coated with $CaCO_3$. The body was always accompanied by spermatozoa. About 70 sperm-associated bodies were observed in a single ovum. Electron-microscopically, small numbers of holes were detected in the vitelline membranes of a fertile ovum, and the sperm-associated bodies were always present in these holes. Frequently observed in the vitelline membranes was a disk speculated to be a portion of the inner layer of the membrane partially affected by spermatozoa. However, neither sperm-associated bodies nor spermatozoa were observed there. It was suggested that the sperm-associated bodies assist fertile spermatozoa in binding the inner layer of the vitelline membrane and penetrating it.

Key words: quail, infundibulum, vitelline membrane, sperm-associated body, fertilization

INTRODUCTION

The avian ovum surrounded by the inner layer of the vitelline membrane at ovulation is soon grasped in the funnel of the oviductal infundibulum and given spermatozoa and materials for the outer layer of the vitelline membrane at the posterior of the infundibulum (Burley and Vadehra, 1989; Wishart and Horrocks, 2000). The spermatozoa released from secondary storage sites (Bakst *et al*., 1994) bind to the inner layer of the vitelline membrane (Kuroki and Mori, 1997) and undergo an acrosome reaction presumably induced by ZPC (Pan *et al*., 1999; Takeuchi *et al*., 1999). Okamura and Nishiyama (1978) showed that the fabric of this inner layer dissolved under the tip of the sperm head, resulting in a hole about 9 μm in diameter. It seems likely that the hole was produced by hydrolytic enzymes secreted from the spermatozoon (Langford and Howarth, 1974).

The holes in the inner layer of the vitelline membrane are diagnostic of a successful sperm-egg interaction (Wishart and Horrocks, 2000). Spermatozoa form holes *in vitro* when incubated with an isolated inner layer of vitelline membrane (Birkhead and Fletcher, 1994; Steele *et al*, 1994; Robertson *et al*, 1997), and the frequency of the holes per unit area was positively correlated with the concentration of the spermatozoa (Birkhead and Fletcher, 1994; Robertson *et al*, 1997). In laid eggs in which excess sperm were trapped, the number of holes was correlated also with the number of trapped sperm (Birkhead *et al*., 1994)

Although in aves several spermatozoa can enter a single ovum, a phenomenon known as physiological polyspermy, extremely large numbers of spermatozoa are trapped in the outer layer of the vitelline membrane (Birkhead *et al*., 1994; Wishart, 1997) during the process of fertilization and deposited around the inner layer. This layer prevents further penetration of the inner layer by the spermatozoa *in vitro* (Howarth and Digby, 1973) and appears to have the same function *in vivo* (Baskst and Howarth, 1977). However, it is not clear which factors in the posterior portion of the infundibulum enable fertile spermatozoa to penetrate the vitelline membranes. The present paper describes a novel structure that is supposed to assist with the penetration in quail.

MATERIALS AND METHODS

Japanese quails *Coturnix japonica* were housed individually in cages located in a poultry house, which was illuminated for 16 h each day. They were fed *ad libitum* on a layer's diet. The quails were killed at 12 to 20 weeks of age by cervical dislocation; this pro-

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tocol was approved by an Institutional Animal Care and Use Committee (No.02017). The oviducts were removed from adult females several hours before spawning.

Light Microscopy

For light microscopy, the infundibulum of the oviduct was fixed in Bouin's solution, cut into pieces 1cm long, and embedded in paraffin. Paraffin sections (6 μm) were stained with Alcian blue and eosin or haematoxylin and eosin.

Electron microscopy

For transmission electron microscopy, the egg-envelope and

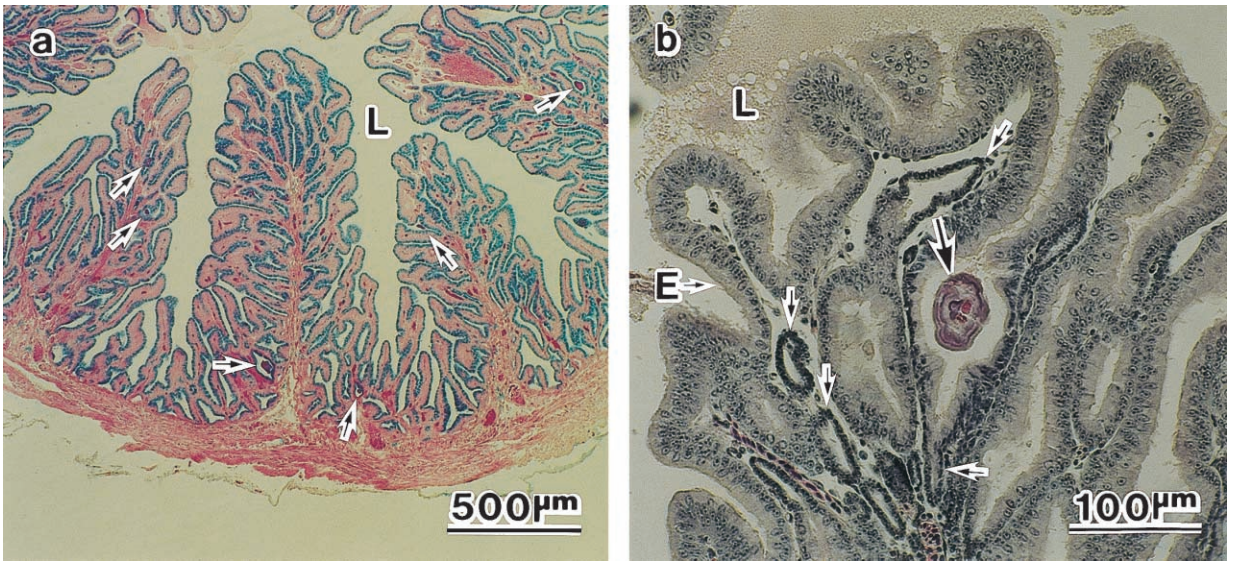

Fig. 1. Light micrographs of the oviduct in the posterior portion of the infundibulum from females several hours before spawning. (a) Cross section showing an elaborate folding of mucosa stained with Alcian blue. Arrows indicate sperm-associated bodies. (b) The mucosa is composed of a luminal epithelium (E) and tubular glands (small arrows) which grow out of the epithelium. The large arrow indicates a sperm-associated body. Stained with Alcian blue-eosin (a) or haematoxylin-eosin (b). L, lumen.

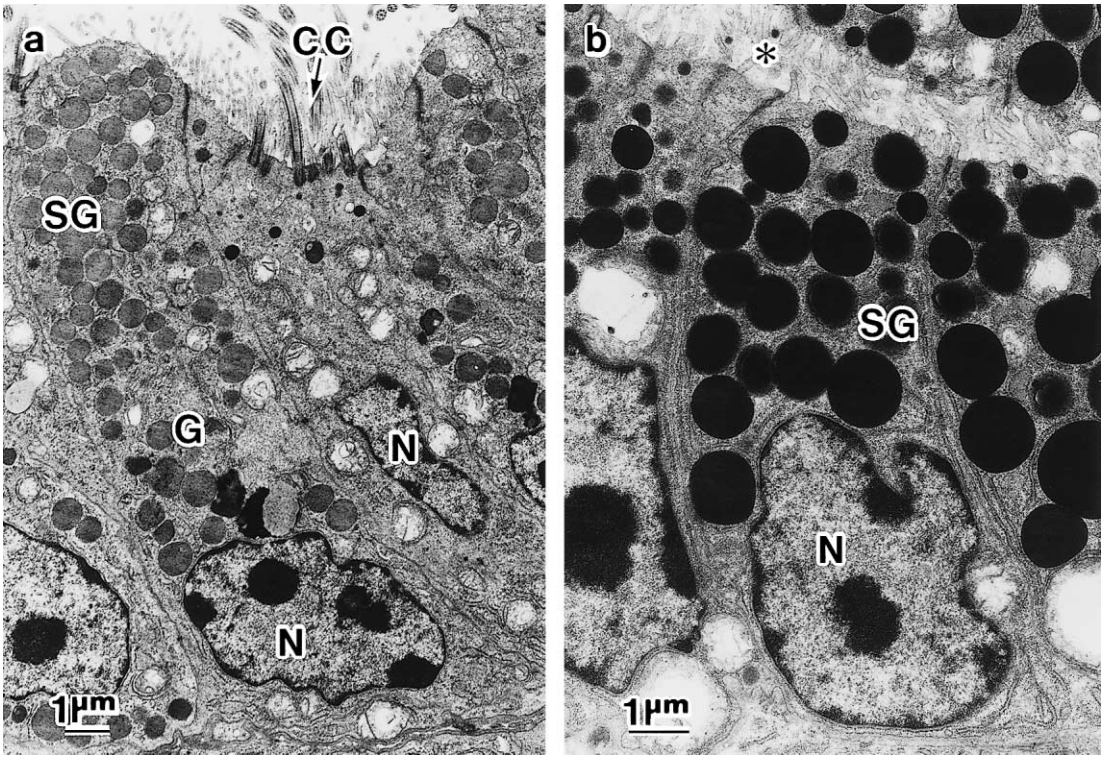

Fig. 2. Electron micrographs of the mucosa at the infundibulum. (a) The luminal epithelium consists of alternately distributed secretory cells and ciliated cells (CC). The apical cytoplasm of secretory cells is filled with electron-lucent secretory granules (SG). (b) The tubular gland consists exclusively of secretory cells possessing electron-dense secretory granules. The asterisk (∗) indicates the glandular canal. G, Golgi complex; N, nucleus.

infundibulum were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), cut into pieces 5 mm long, and kept overnight at 4°C in the same solution. Specimens were then rinsed with the buffer and post-fixed with 1% (w/v) OsO_4 in the same buffer for 3 h at 4°C. After fixation they were dehydrated in acetone and embedded in epoxy resin. Utrathin sections were stained with uranyl acetate and lead citrate and observed with a model H-8000 electron microscope (Hitachi, Japan). Some sections of the egg envelope were stained with toluidin blue and observed with a light microscope.

For scanning electron microscopy, specimens were dehydrated in acetone, dried in a critical point apparatus, the HCP-1 (Hitachi, Japan), coated with a layer of platinum in an IB-3 ion coater (Eiko, Japan) and examined with a model S-4300 electron microscope (Hitachi).

Isolation of sperm-associated bodies

Sperm-associated bodies were isolated as follows: The infundibulum was treated with 50% (v/v) ethanol for 15 min and vigorously shaken in distilled water to remove as much unnecessary tissue as possible. The remaining tissue was then dissolved by treatment with a 1 M NaOH solution overnight at 37°C. Sperm-associated bodies collected in a test tube soon precipitated at the bottom. The precipitate was washed several times with distilled water. The isolated sperm-associated bodies were dried in an oven at 60°C overnight. For the isolation of sperm-associated bodies from vitelline membranes, the vitelline membranes were collected from infertile ova and directly treated with 1 M NaOH. Some sperm-associated bodies were treated with 150 mM EDTA in 0.1 M cacodylate buffer (pH 7.7) for 1 day.

X-ray microanalysis

Isolated sperm-associated bodies were coated with platinum as mentioned above. To demonstrate the elements in the bodies,

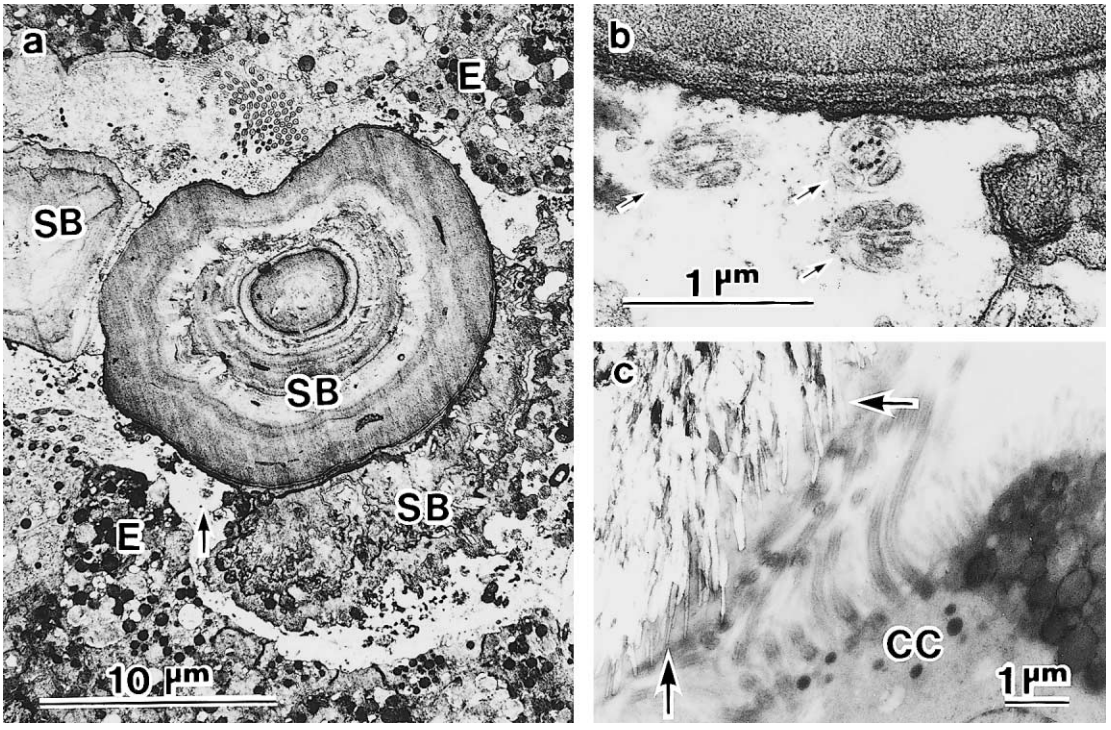

Fig. 3. Electron micrographs of sperm-associated bodies (SB) in the infundibulum. (a) Growing sperm-associated bodies are present in the lumen between the epithelial folds (E). (b) Enlargement of the portion indicated by an arrow in a. Three sections of sperm flagellum are shown (arrows). (c) Trace of a calcified sperm-associated body. The needle-like traces in embedding resin are shown (arrows). CC, ciliated cell.

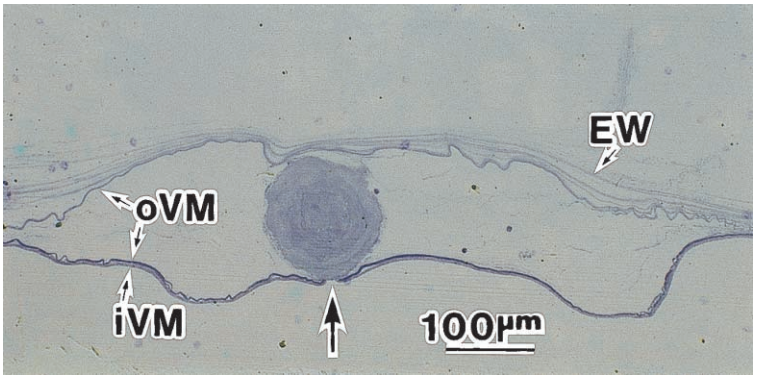

Fig. 4. A cross section of the egg-envelope from a 0-day embryo showing a sperm-associated body on the vitelline membrane. An arrow indicates the sperm-associated body and a hole in the vitelline membrane. Stained with toluidine blue. EW, egg white; iVM and oVM, inner and outer layer of the vitelline membrane, respectively.

the specimens were examined with an energy dispersive X-ray micro analyzer, the EMAX EX-220 (Horiba, Japan), attached to the S-4300 scanning electron microscope.

DAPI stain

For the detection of nuclei, vitelline membranes from 0-day fertile ova were fixed with Carnoy solution and stained in periodic acid schiff (PAS) and then in DAPI (4, 6, diamidino-2-phenylindol) (Waddington *et al*., 1998). They were examined microscopically under UV illumination in the epifluorescent mode.

RESULTS

Sperm-associated bodies in the infundibulum

The quail oviduct from an adult female is about 20 cm long and comprises the infundibulum, magnum, magnum-

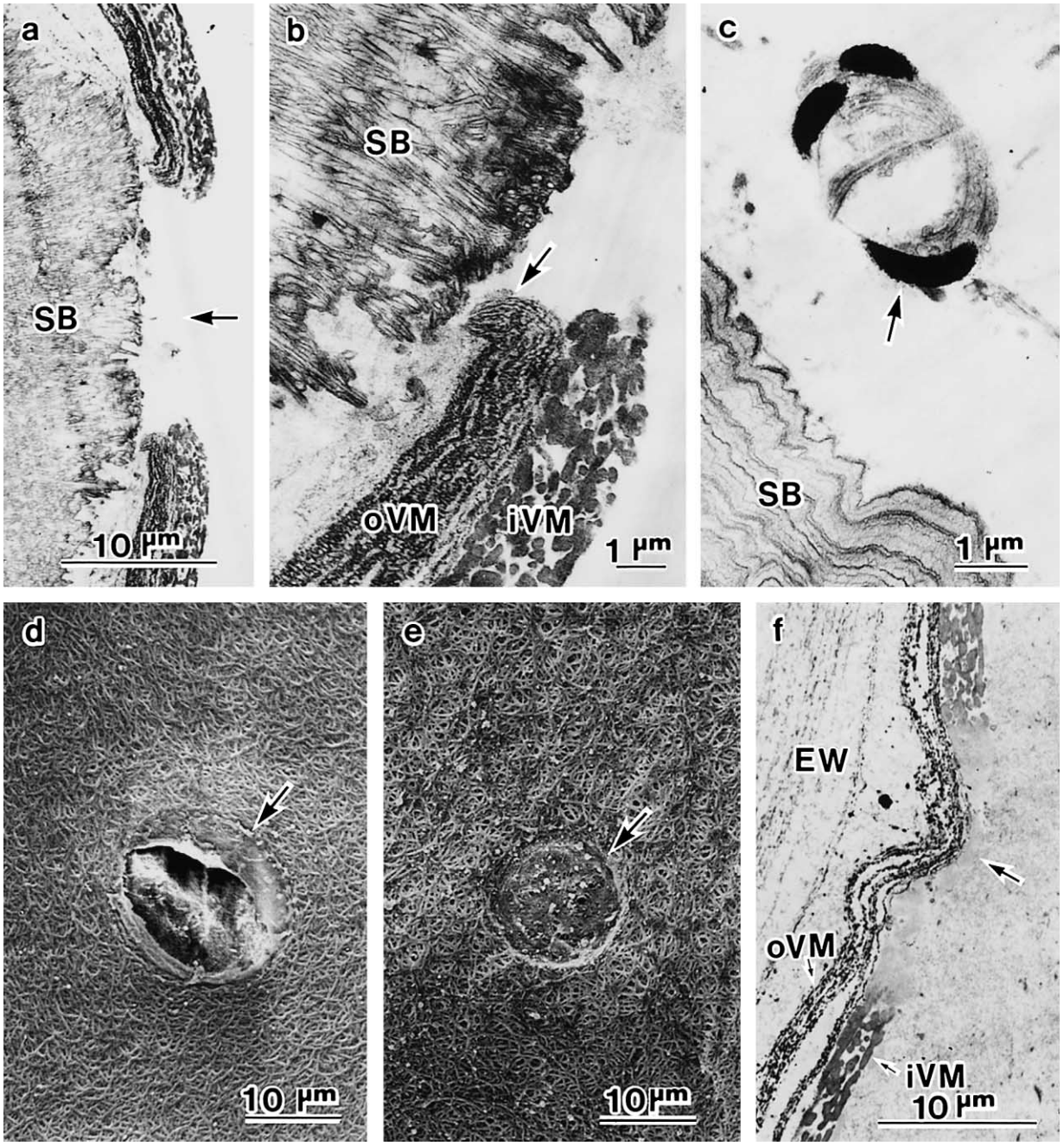

Fig. 5. Electron micrographs of envelopes from 0-day embryos. (a) A section neighbouring that shown in Fig. 4. A sperm-associated body (SB) resides close to a hole (arrow) in the vitelline membrane. (b) At the hole, the cut edge of the inner layer of the vitelline membrane (iVM) is sharp whereas the edge of the outer layer (oVM) bends towards the surface of the sperm-associated body and tapers around it (arrow). A needle-like configuration is apparent at the cortex of the body. (c) Spermatozoa (arrow) accumulated near the sperm-associated body. (d, e) Scanning electron micrographs showing the inner surface of the vitelline membrane. An arrow indicates a complete hole in d, and a partially affected disk in e. (f) A transmission electron micrograph showing the partially affected disk (arrow). EW, egg white.

isthmus junction, isthmus, uterus and vagina (Sultana *et al*., 2003). The infundibulum occupies the anterior-most 14% of the oviduct. Fig. 1 shows cross sections of the posterior portion of the infundibulum. The luminal epithelium, which stains deeply with Alcian blue, exhibits a configuration of primary and secondary folds (Fig. 1a). It consists of alternately distributed cilia cells and granular cells (Fig. 2a). The granular cells contain secretory granules 750 nm in size. At the bottom of the secondary folds of the epithelium, there are tubular outgrowths, the tubular glands, beneath the luminal epithelium (Fig. 1b). The tubular glands consist exclusively of a single type of granular cell, which contains electron-dense, secretory granules 1 μm in size (Fig. 2b).

We found eosin-positive bodies of a concentric-circular appearance at or near the bottom of the luminal epithelium from both mated and non-mated females (Fig. 1). When observed ultrastructurally, these bodies appeared to be either i) concentric and circular with a diameter of up to 50 μm, ii) aggregates surrounding a pre-existing body or forming a new body (Fig. 3a), or iii) covered with radially arranged, needle-like projections. In the third case, the outer surface

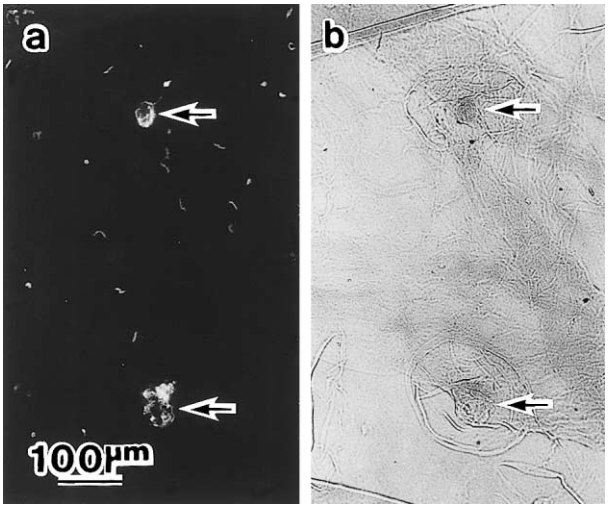

Fig. 6. Light micrographs of the egg-envelope from a 0-day embryo, stained with DAPI (a) and PAS (b). Arrows indicate sperm-associated bodies with spermatozoa. String-like stains and dot stains indicate nuclei of spermatozoa and granulocytes, respectively.

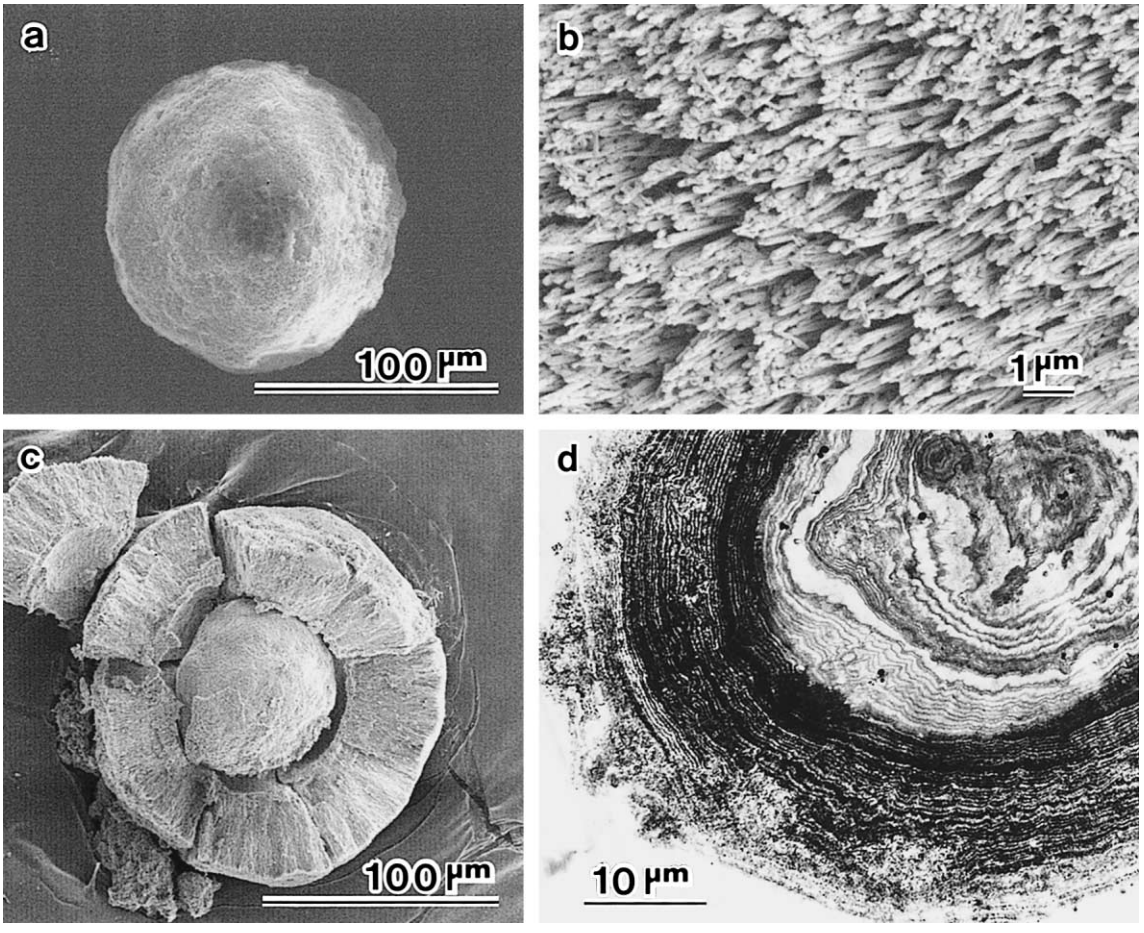

Fig. 7. Electron micrographs of sperm-associated bodies isolated from the oviduct. (a–c) Scanning electron micrographs. The surface of the sperm-associated body is decorated with needle-like projections (a, b). The body is composed of a central core and radial cortex (c). (d) Transmission electron micrograph of a sperm-associated body treated with EDTA. The cortex was removed during the preparation. The core is a concentric arrangement of layers.

of the projections seemed to be coated with minerals, which resisted infiltration by the epoxy resin, and thus only their contour was visualized in electron micrographs (Fig. 3c).

The heads of spermatozoa were often observed in secretory canals of the tubular glands of the infundibulum from mated females (not shown), whereas the flagella were always observed on or around the above-mentioned bodies (Fig. 3b). Hence, we call these bodies sperm-associated bodies.

Sperm-associated bodies in fertile ova

Sperm-associated bodies were found in the space formed in between the outer layer of the vitelline membrane of oviposited ova irrespective of fertility (Fig. 4). In isolated preparations of vitelline membranes, an average number of 70 sperm-associated bodies per ovum was counted, the range of values being 22 to 135 (n=10). On a few occasions, a hole was observed in the cross section of the vitelline membrane at the site where the sperm-associated bodies resided in the fertile ovum (Fig. 4). When observed by transmission electron microscopy, the edge of the inner layer of the vitelline membrane at the hole showed a clear-cut appearance whereas that of the outer layer looked to bend toward the sperm-associated bodies and gradually taper around it(Fig. 5a, b). On most occasions when sperm-associated bodies were observed, both layers of the vitelline membrane remained intact. All three types of sperm-associated bodies were again observed in the outer layer of the vitelline membrane (Fig. 5a, c). The spermatozoa not involved in the fertilization were always observed around the sperm-associated bodies (Fig. 5c).

Under the scanning electron microscope, the innermost

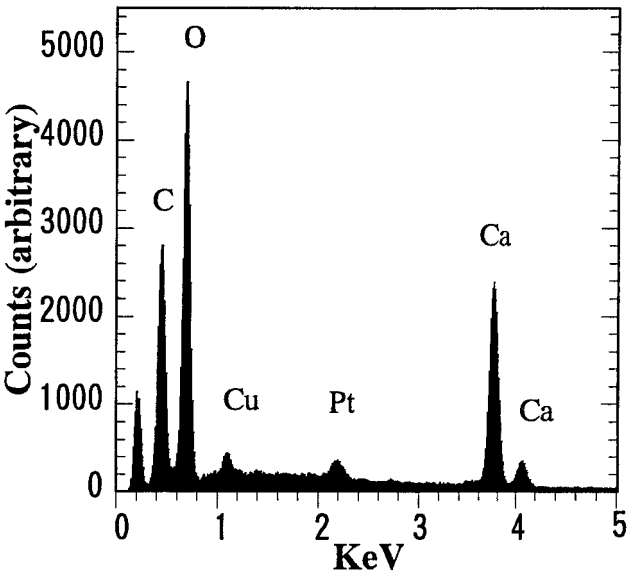

Fig. 8. X-ray microanalysis of a calcified sperm-associated body.

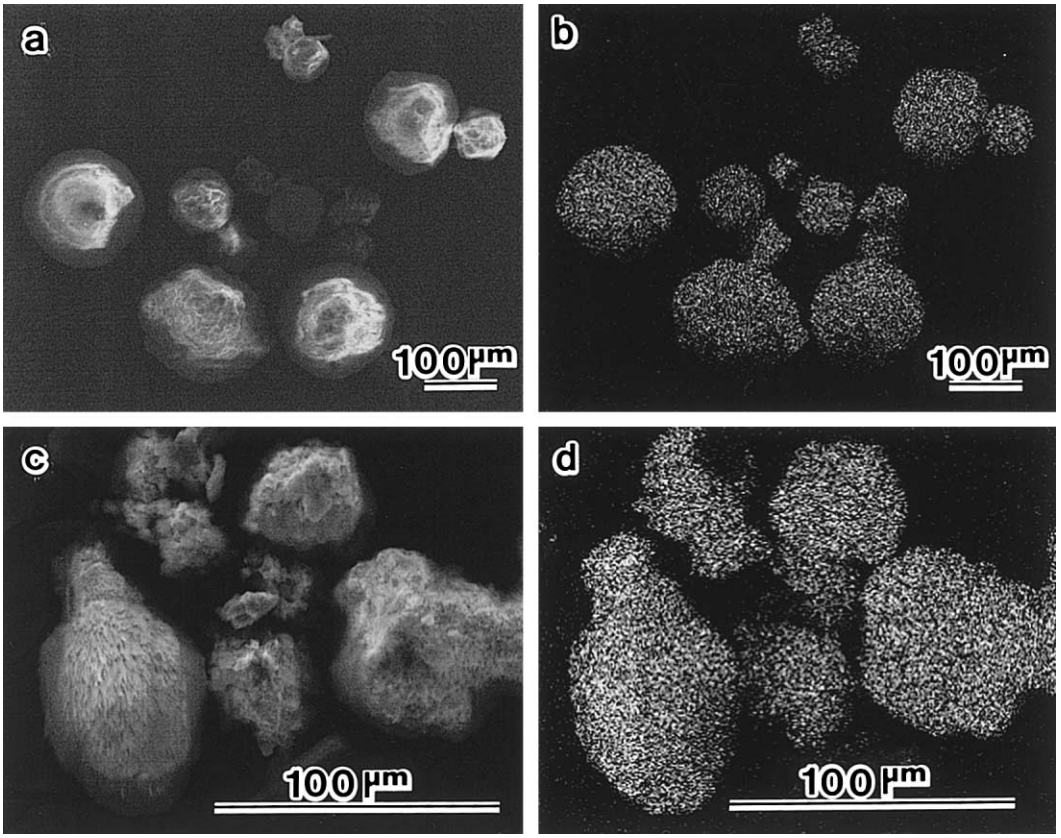

Fig. 9. Mapping of calcium on the sperm-associated bodies isolated from the oviductal infundibulum (a, b) or vitelline membrane of infertile ova (c, d). (a, c) Scanning electron micrographs. (b, d) Calcium mapping.

surface of the vitelline membrane appeared as a meshwork of fibers. Small numbers of holes, the diameters of which were about 15 μm, were observed in a single vitelline membrane (Fig. 5d). However, the configuration often observed was a disk without a meshwork but with a smooth appearance (Fig. 5e). A cross section of the disk showed fibers of the inner vitelline membrane to be dissociated but not completely dissolved and fibers of the outer vitelline membrane to be intact (Fig. 5f). Neither sperm-associated bodies nor spermatozoa were observed at the disk. No such holes and partially affected disks were observed in the vitelline membrane of ova spawned by non-mated females (not shown).

Association of spermatozoa with the bodies

To confirm the relationship between sperm-associated bodies and spermatozoa, we subjected the vitelline membrane of 0-day embryos to DAPI staining (Fig. 6a). In addition to solitary spermatozoa and granulocytes scattered in the vitelline membranes, many spermatozoa were stained on sperm-associated bodies, indicating an intimate relationship. A circle, the rim of the space formed in the outer layer of the vitelline membrane, was always observed around the bodies (Fig. 6b).

X-ray microanalysis of sperm-associated bodies

Fig. 7a shows the largest sperm-associated body isolated from oviducts. It is 100 μm in diameter and has needle-like projections on its outer surface (Fig. 7b). The cracked preparation showed it consisted of two components, a core and cortex (Fig. 7c). After removal of the cortex with EDTA treatment, ultrastructural observation showed that the core was a concentric-circular body (Fig. 7d).

X-ray microanalysis indicated that sperm-associated bodies possess calcium carbonates and a small amount of copper (Fig. 8). All three types contain calcium as shown by calcium mapping (Fig. 9a, b). The same mapping image was obtained for the bodies isolated from infertile ova (Fig. 9c, d).

DISCUSSION

The sperm-associated body, reported in the present study, is a novel structure found both in the lumen at the posterior of the oviductal infundibulum and in the vitelline membrane of embryos in quail. The avian infundibulum is an interesting organ where several events occur, such as (1) the formation of the outer layer of the vitelline membrane (Baskst and Howarth, 1977), (2) the storage of spermatozoa (Fujii and Tamura, 1963; Bakst, 1981; Bakst *et al*., 1994) and (3) fertilization (Olsen and Neher, 1948; Howarth and Digby, 1973; Okamura and Nishiyama, 1978). The production of sperm-associated bodies may be a newly identified function of the infundibulum. However, as yet we do not know whether they are produced by cells at the infundibulum or by other segments of the oviduct and then transported to the infundibulum. They are not, however, of male origin because even the oviducts of non-mated females possess them.

The function of sperm-associated bodies appears to assist the spermatozoa in fertilization. This notion is supported by the finding that sperm-associated bodies (1) have an intimate relationship with the spermatozoa in the infundibulum of mated females, (2) are always present around holes in the vitelline membrane identifiable by electron microscope, and (3) bind with some spermatozoa in the vitelline membrane. It is well recognised that the outer layer of the vitelline membrane blocks excessive sperm penetration of the ovum (Baskst and Howarth, 1977; Wishart and Horrocks, 2000) and traps spermatozoa (Wishart, 1997). Spermatozoa that have bound to the inner layer of the vitelline membrane (Kuroki and Mori, 1997) might be dislodged during the formation of the outer layer. Then, what factor determines which spermatozoon penetrates the vitelline membrane and which does not? We speculate that the sperm-associated bodies bound to the inner layer are not affected by polymerization of the outer vitelline membrane's materials, and hence the spermatozoa bound to them are able to accomplish the fertilization process. About 70 sperm-associated bodies are given to an individual ovum as reported in the present study. This, however, does not mean that all the bodies succeed in their task to assist with sperm penetration. As the sperm-associated bodies are supplied to the ovum together with the materials of the outer layer of the vitelline membrane, they probably need to bind with the inner layer before the establishment of the outer layer to function successfully. Exactly, the present study showed that the outer layer was not present beneath the sperm-associated bodies at the hole but merely covered above them. On the other hand, most of the bodies we observed were present on the outer layer of vitelline membranes with no hole, indicating a failure of penetration by the accompanying spermatozoa.

The penetration of the vitelline membranes by spermatozoa is preceded by an acrosome reaction in spermatozoa that might be induced by an avian ZPC (Takeuchi *et al*., 1999; Pan *et al*., 1999) as in the mouse (Bleil and Wassarman, 1980; 1983; 1988). Although few holes were present in the vitelline membrane of a fertile ovum, many disks were present on the inner layer of the membrane as observed with the scanning electron microscope. The fabric of the inner membrane was apparently dissociated at the disk. The dissociation might be caused by an acrosomal enzyme secreted from the spermatozoa, but further perforation might not be brought about because solitary spermatozoa were expelled as the outer layer of the vitelline membrane formed. Fully developed outer layers were intact but neither spermatozoa nor sperm-associated bodies were observed at the disk.

In chicken, "holes'' were concentrated in the inner layer of the vitelline membrane over the germinal disc in fertile ova (Bramwell and Howarth, 1992), whereas they were distributed at random throughout other regions of the ova (Steele *et al*., 1994). It has been widely reported that the vitelline

membrane contains many spermatozoa and many "holes" (Koyanagi *et al*., 1988; Takeuchi *et al*., 2001). The number of holes was expected to increase when the concentration of spermatozoa used was increased during *in vitro* insemination (Bramwell and Howarth, 1992). However, all counts were made under a light microscope. As far as the present study is concerned, the number of real holes is very small whereas the number of disks is large. In reality, holes might not have been counted by earlier researchers because they were hidden by sperm-associated bodies. A strict comparison is therefore awaited as to the number of holes detected by light- and electron-microscopic observations.

The avian body temperature is 39–40°C. The avian spermatozoa do not move at this temperature but move at 30°C *in vitro* (Ashizawa and Sano, 1990). A specific mixture of EDTA and calcium activates the spermatozoa even at body temperature (Bramwell and Howarth, 1992). As shown in the present study, sperm-associated bodies were coated with $CaCO_3$. Although a quantitative difference in the concentration of calcium could not be detected based on atomic absorption between the sperm-associated bodies from the oviduct and those from the vitelline membranes, the former bodies seemed to be coated with more $CaCO_3$ than the latter because the plastic resin for electron microscopy could not penetrate the mature form of the former. Calcium might be released from the sperm-associated bodies in the vitelline membranes, and this may stimulate the spermatozoa to become active.

Sperm-associated bodies were histologically stained with eosin as shown in the present study. They were also stained with the PAS-stain and could be solubilised only by treatment with the strong acids (Sultana, unpublished). To strengthen our hypothesis on their function, it is necessary to biochemically characterize the sperm-associated bodies and to show an affinity for the spermatozoa.

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REFERENCES

Ashizawa K, Sano R (1990) Effects of temperature on the mobilization and the initiation of motility of spermatozoa in the male reproductive tract of the domestic fowl, *Gallus domesticus*. Comp Biochem Physiol 96A: 297–301

Bakst MR, Howarth BJr (1977) The fine structure of the hen's ovum at ovulation. Biol Reprod 17: 361–369

Bakst MR (1981) Sperm recovery from oviducts of turkeys at known intervals after insemination and oviposition. J Reprod Fertil 62: 159–164

Baskt MR, Wishart G, Brillard JP (1994) Oviductal sperm selection, transport and storage in poultry. Poult Sci Rev 5: 117–143

Birkhead TR, Fletcher F (1994) Numbers of spermatozoa attached to and penetrating perivitelline layers of Japanese quail eggs. The Auk 111: 997–1000

Birkhead TR, Sheldon BC, Fletcher F (1994) A comparative study of sperm-egg interaction in birds. J Reprod Fertil 101: 353–361

Bleil JD, Wassarman PM (1980) Structure and function of the zona pellucida. Dev Biol 76: 185–202

Bleil JD, Wassarman PM (1983) Sperm-egg interactions in the mouse. Dev Biol 95: 317–324

Bleil JD, Wassarman PM (1988) Galactose at the nonreducing terminus of O-linked oligosaccharides of mouse egg zona pellucida glycoprotein ZP3 is essential for the glycoprotein's sperm receptor activity. Proc Natl Acad Sci USA 85: 6778–6782

Bramwell RK, Howarth B (1992) Preferential attachment of cock spermatozoa to the perivitelline layer directly over the germinal disc of the hen's ovum. Biol Reprod 47: 1113–1117

Burley RW, Vadehra DV (1989) The Avian Egg: Chemistry and Biology. John Wiley & Sons, New York

Fujii S, Tamura T (1963) Location of sperms in the oviduct of the domestic fowl with special reference to storage of sperms in the vaginal gland. J Fac Fish Husb Hiroshima Univ 5: 145–163

Howarth B, Digby ST (1973) Evidence for the penetration of the hen's ovum by a trypsin-like acrosomal enzyme. J Reprod Fertil 33: 123–125

Koyanagi F, Masuda S, Nishiyama H (1988) Acrosome reaction of cock spermatozoa incubated with the perivitelline layer of hen's ovum. Poult Sci 67: 1770–1774

Kuroki M, Mori M (1997) Binding of spermatozoa to the perivitelline layer in the presence of protease inhibitor. Poult Sci 76: 748–752

Langford BB, Howarth J (1974) A trypsin-like enzyme in acrosomal extracts of chicken, turkey and quail spermatozoa. Poult Sci 53: 834–837

Okamura F, Nishiyama H (1978) The passage of spermatozoa through the vitelline membrane in the domestic fowl *Gallus gallus*. Cell Tiss Res 188: 497–508

Olsen MW, Neher BH (1948) The site of fertilization in the domestic fowl. J Exp Zool 109: 355–366

Pan J, Mori M, Kono Y, Matsuda T (1999) Expression and regulation of vitelline membrane protein messenger RNA in quail granulosa cells during follicular development. In "Recent Progress in Molecular and Comparative Endocrinology" Ed by HB Kwon, JMP Joss, S Ishii, Chonnam National University, Kwongju (Korea), pp 129–133

Robertson L, Brown H, Staines HJ, Wishart GJ (1997) Characterization and application of an avian *in vitro* spermatozoa. J Reprod Fertil 110: 205–211

Steele MG, Meldrum W, Brillard JP, Wishart GJ (1994) The interaction of avian spermatozoa with the perivitelline layer *in vitro* and *in vivo*. J Reprod Fertil 101: 599–603

Sultana F, Yokoe A, Ito Y, Mao KM, Yoshizaki N (2003) Peri-albumen layer: a novel structure in egg envelopes of an avian egg. J Anat 203: 115–122

Takeuchi Y, Cho R, Iwata Y, Nishimura K, Kato T, Aoki N, Kitajima K, Matsuda T (2001) Morphological and biochemical changes of isolated chicken egg-envelope during sperm penetration. Biol Reprod 64: 822–830

Takeuchi Y, Nishimura K, Aoki N, Adachi T, Sato C, Kitajima K, Matsuda T (1999) A 42-kDa glycoprotein from chicken egg envelope, an avian homolog of the ZPC family glycoproteins in mammalian zona pellucida. Eur J Biochem 260: 736–742

Waddington D, Gribbin C, Sterling RJ, Sang HM, Perry MM (1998) Chronology of events in the first cell cycle of the polyspermic egg of the domestic fowl (*Gallus domesticus*). Int J Dev Biol 42: 625–628

Wishart GJ (1997) Quantitative aspects of sperm. Anim Reprod Sci 48: 81–92

Wishart GJ, Horrocks AJ (2000) Fertilization in birds. In "Fertilization in Protozoa and Metazoan Animals" Ed by JJ Tarin, A Cano, Springer-Verlag, Heidelberg, pp 193–222

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