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# Fine Structure of the Storage Micropocket of Spermatozoa in the Ovary of the Guppy *Poecilia reticulata*

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**ABSTRACT**—To investigate the internal fertilization of the guppy *Poecilia reticulata*, the present electron microscopic observations were focused on the morphology of the sperm storage site in females. In the ovary of the mature female guppy, many spermatozoa were found in a synaptic knob-shaped micropocket (SSP) as the sperm storage (probably sperm entry) site on the follicle surface which was the expanded blind alley of a small tract extending from the ovarian cavity. Oocytes in the developmental stage of oil droplet formation already showed the attachment of the terminal end of the small tract opening into the ovarian cavity. The lateral wall of the tract attaching to the follicle surface consisted of epithelial cells fast jointed with tight junctions and desmosomes. The thick lateral wall of SSP was constructed with complex epithelial cell layers, and the terminal bottom was comprised of a single layer of epithelial cells on the surface of the follicular layer, which consisted of a very thin thecal cell layer, basement membrane, and granulosa cell layer. The vitellus was enclosed by the follicular layer and thin chorion, in which the micropyle was absent. In fully-grown oocytes, the germinal vesicle containing comparative short chromosomes did not always locate in the vicinity of the storage SSP of spermatozoa.

**Key words:** guppy, *Poecilia reticulata*, sperm storage, ovarian follicle, viviparity

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## INTRODUCTION

Fertilization in fishes has mostly been investigated on eggs in oviparous fishes in which it occurs externally, making observation relatively easy. However, fertilization in viviparous fishes, on the other hand, remains to be clarified, because it is difficult to observe under *in vivo* conditions. In viviparous fishes, the internal fertilization is confirmed only by the presence of spermatozoa in the ovarian cavity of the female (Burns *et al.*, 1995). No investigators have reported the precise observation of the internal fertilization process even in *Poecilia reticulata* which has been extensively used for analysis of reproductive phenomena. The most important point of mature oocytes for fertilization is to meet internally the spermatozoon. The ovary of the guppy forms a “seminal receptacle” (Jalabert and Billard, 1969) localized at the antero-dorsal region in ovarian cavity and a “delle” (Takano, unpublished; cf. Nagahama, 1985) as a localized expansion extending from the ovarian cavity to the follicle, in which viable spermatozoa are stored for a relatively long period. In oviparous fishes, the spermatozoon penetrates the ooplasm through the micropyle, which is a small pore existing in the

chorion (egg envelope). The micropyle is opened by detaching the micropylar cell together with the whole follicular cell layers from the chorion at the time of ovulation (Nakashima and Iwamatsu, 1989). So far, such a formation of the micropyle has not been reported in eggs in viviparous fishes, in which follicular cell layers are not removed before fertilization.

In oviparous oocytes, the germinal vesicle moves towards and locates in the cortical cytoplasm in the vicinity of the micropyle at the animal pole, as yolk formation proceeds during oogenesis. Thus, in large telolecithian fish eggs, the sperm nucleus successfully penetrating through the micropyle associates with the egg nucleus at the time of fertilization. However, the relationship between the nucleus and the polarity (sperm entry site) of oocytes remains also to be investigated in viviparous fishes. The present study is the aim to observe preliminarily the ultrastructure of the sperm storage pocket, probably as sperm entry site, and to examine the relationship between the oocyte nucleus and the sperm entry site.

## MATERIALS AND METHODS

Ovaries of the adult guppy (*Poecilia reticulata*), a kind gift from the World's Medaka Aquarium in Nagoya Higashiyama Zoo, were removed from the body cavity into saline for *Oryzias* oocytes

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(Iwamatsu *et al.*, 1984). Ovarian follicles including oocytes were isolated by excising with a pair of scalpels under a binocular dissecting microscope. Before fixation, various follicle diameters were each measured, along with the bottom of micropockets (SSP), and germinal vesicles (GV), in addition to the distance between SSP and GV. Then, they were immediately prefixed in a modified Karnovsky's fixative containing 0.1% picric acid (pH 7.3; Ito and Karnovsky, 1968) for 4–5 hr at room temperature. After thorough rinsing in 0.1 M cacodylate buffer (pH 7.3) at 0–4°C, the samples were postfixed in 1% OsO<sub>4</sub> in the same buffer on ice for 1–2 hr. The fixed samples were dehydrated in a graded ethanol series, and then embedded in Spurr's low viscosity epoxy resin (Taab, Reading, UK). For an ordinary light microscopy, besides live samples, 0.5µm-thick sections were stained with 0.5% toluidine blue in 1% sodium borate. Ultrathin sections, silver to gold, were double stained with uranyl acetate and lead citrate, and observed with an electron microscope JEM-1010 (JEOL Ltd., Tokyo, Japan) at 80 kV.

To examine the state of the nucleus, usually large oocytes over 900µm in diameter were fixed in 5% glutaraldehyde in 0.1M phosphate buffer (pH 7.3), and stained with Hoechst 33258 (Sigma) for 10 min. After washing in the same buffer, a small piece of the cortical cytoplasm including the GV was excised in the buffer, mounted onto the clean slide, and observed with an epifluorescent microscope (Olympus Kogyo, Tokyo, Japan).

In an attempt to know how spermatozoa reach the SSP to be stored, using a micropipet we introduced a small amount of carbon particles dissolved in saline to females through the genital pore into the ovarian cavity of adult, and observed histologically their distribution in the ovary.

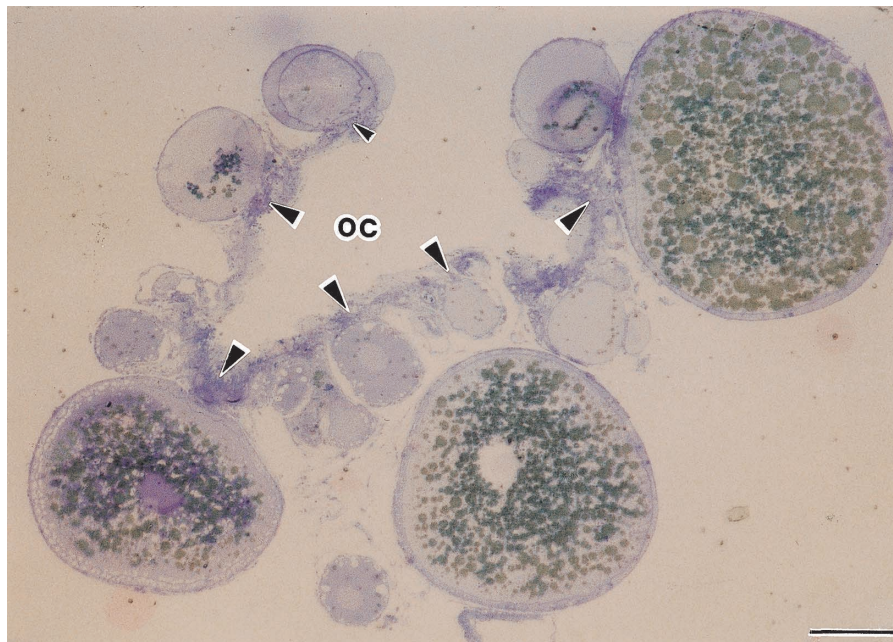
The developmental stages of the oocytes in the present study were in part presented according to the descriptions of Takano (1964).

## RESULTS AND DISCUSSION

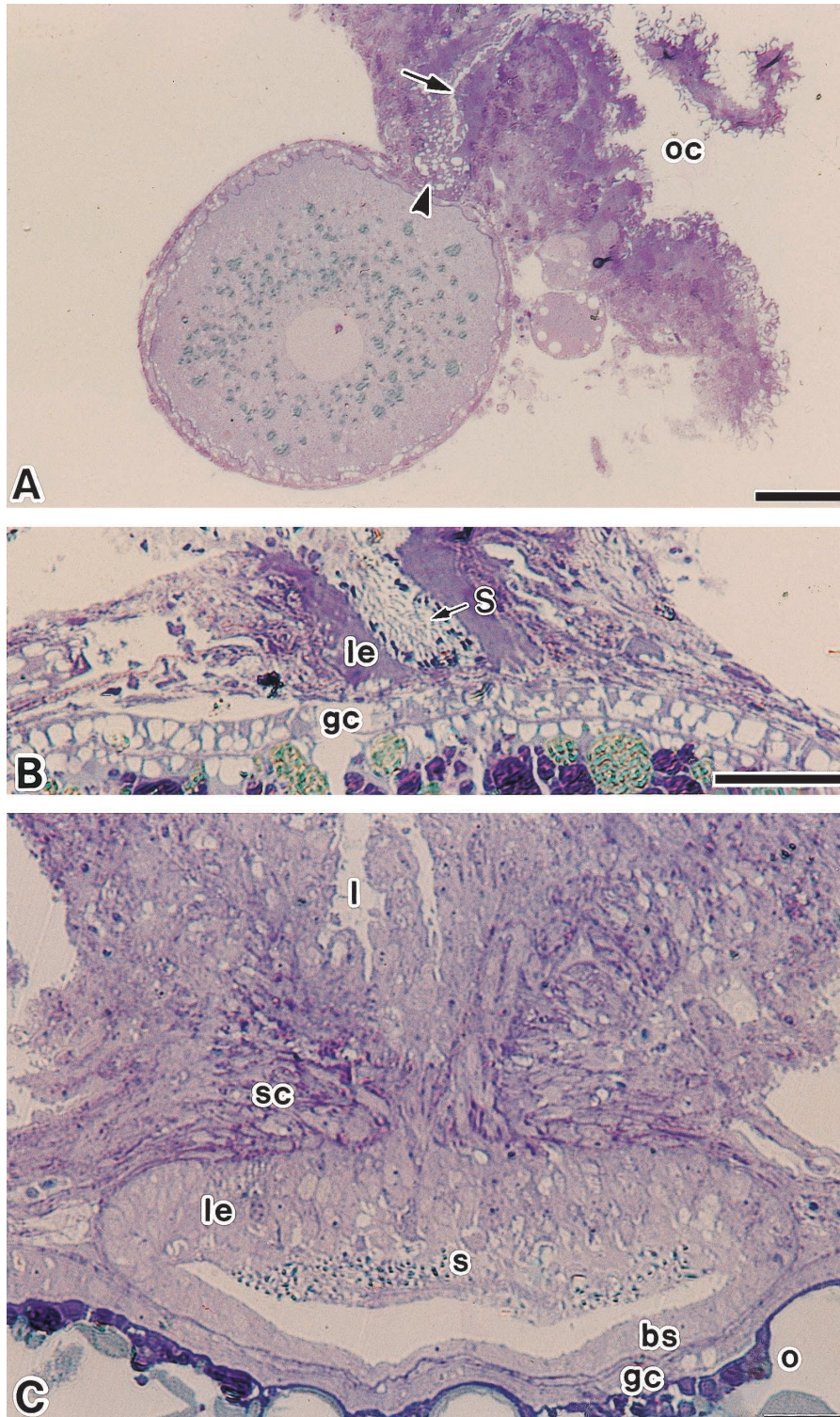
### Morphology of the micropocket as the sperm storage

In the ovary of adult females, the ovarian follicles with developing oocytes of various size were observed to con-

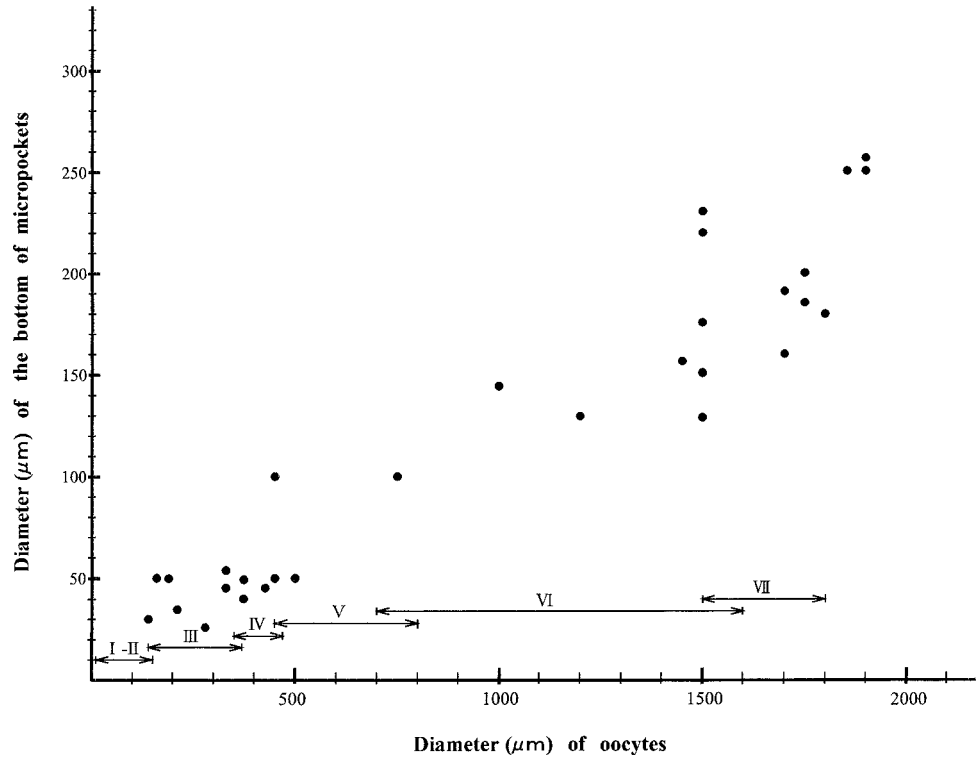
nect with the ovarian cavity through a small tract (Fig. 1). The distinct tract consisting of ovarian epithelium was recognizable, when the chorion (egg envelope) began to be formed on the surface of the oocyte in the stage of oil droplet formation. The terminal region of the tract extending from the ovarian cavity to the follicle became expanded on the follicular surface (Fig. 2A-C). Numerous spermatozoa were packed within the expanded terminal region of the tract attaching to the surface of the fully-grown follicle (Fig. 2C), which is here called the sperm storage micropocket (SSP). The size of the bottom of the SSP increased dependently on the development of oocytes (Fig. 3). The SSP consisted of a single thin layer of epithelial cells in the bottom and thick layers of epithelial cells in the lateral side, and progressively developed as the diameter of the developing follicles increased (Figs. 2, 3). The SSP in small follicles 250µm in diameter was narrow, and few spermatozoa were found (Figs. 2A, 4). The tight junctions and desmosomes were frequently observed on the lumen side of epithelial cells of the short tract (Figs. 4–6). Seemingly, it appears as if the epithelial cells of the tract fast jointed with these junctions to prevent spermatozoa from slipping through them. The follicle layer was constructed by the thecal cell layer as the outermost layer, basement membrane, and granulosa cell layer: collagen fibers were abundant around the thecal cells (Figs. 5, 7). The chorion was formed by accumulating an electron-dense material among numerous microvilli of the oocyte (Fig. 5). In the follicle 1.2 mm in diameter, epithelial cells of the lateral wall of the short tract remained unchanged, but the terminal end (bottom side) of the SSP came to exhibit a very thin single layer of the epithelial cells on the follicle (Fig. 7). The SSP on the follicle 1.5 mm in diameter exhibited a



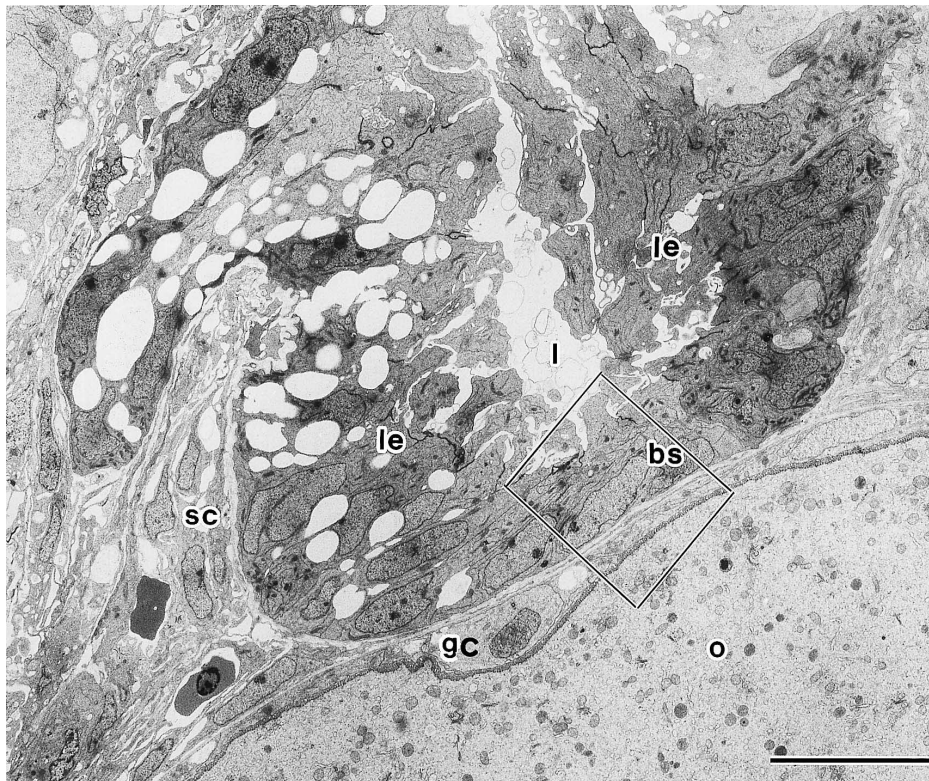
**Fig. 1.** Guppy follicles various in size in the dissected ovary. Micropockets with short tracts (arrowheads) extending from the ovarian cavity (oc) were seen in each follicle. Bar, 55µm.



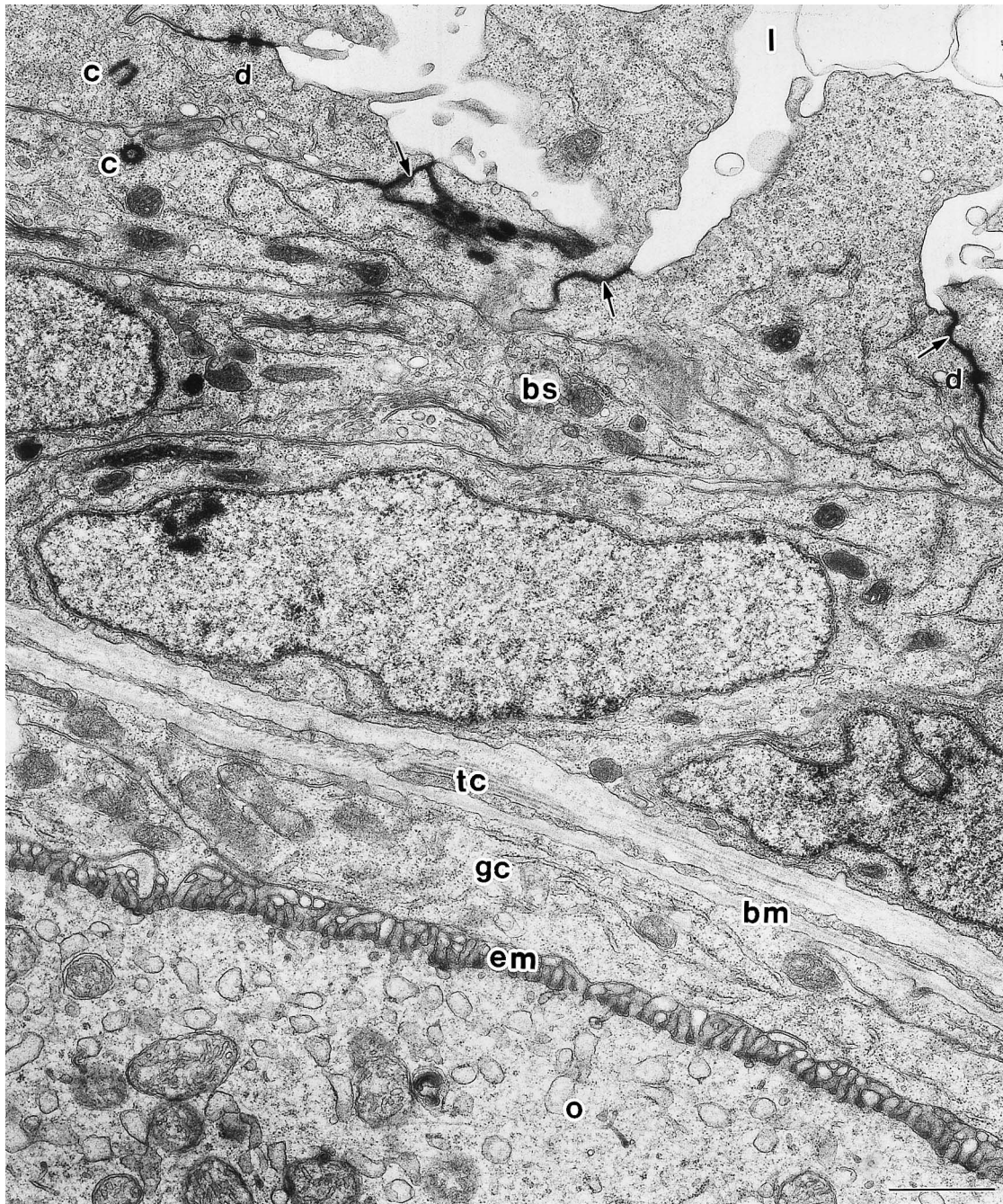
**Fig. 2.** Micrographs of micropockets (SSPs) on the surface of follicles of different size. A: A small follicle 250 $\mu$ m in diameter showing the SSP (arrowhead) as the terminal end of short tract (arrow) with a thick lateral wall extending from the ovarian cavity (oc). Bar, 65 $\mu$ m. B: An SSP with the lateral wall of the thick epithelial layer (le) exhibits many spermatozoa (s) in a follicle 550 $\mu$ m in diameter. gc, granulosa cell layer. Bar, 40 $\mu$ m. C: An SSP like a synapse in shape on the surface of a large follicle 1.5 mm in diameter. bs, bottom cell layer of the SSP; gc, granulosa cell layer; l, lumen of the tract; le, lateral wall epithelium; o, oocyte; s, spermatozoa; sc, supporting cells. Bar, 30 $\mu$ m.



**Fig. 3.** Relationship between diameters of oocytes and the bottom of micropockets during oogenesis. I-VII: Developmental stages of oocytes in *P. reticulata* according to Takano (1964). I, chromatin-nucleolus stage; II, peri-nucleolus stage; III, oil drop stage; IV, primary yolk stage; V, secondary yolk stage; VI, tertiary yolk stage; VII, maturation stage.



**Fig. 4.** Electron microscopic photograph of the micropocket (SSP) on a young follicle 250 $\mu$ m in diameter. The bottom (bs) of the SSP is comparatively thick, and the lateral wall epithelium(le) is distinct from the supporting cells (sc). gc, granulose cell layer; l, lumen of the SSP; o, oocyte. Bar, 10 $\mu$ m.



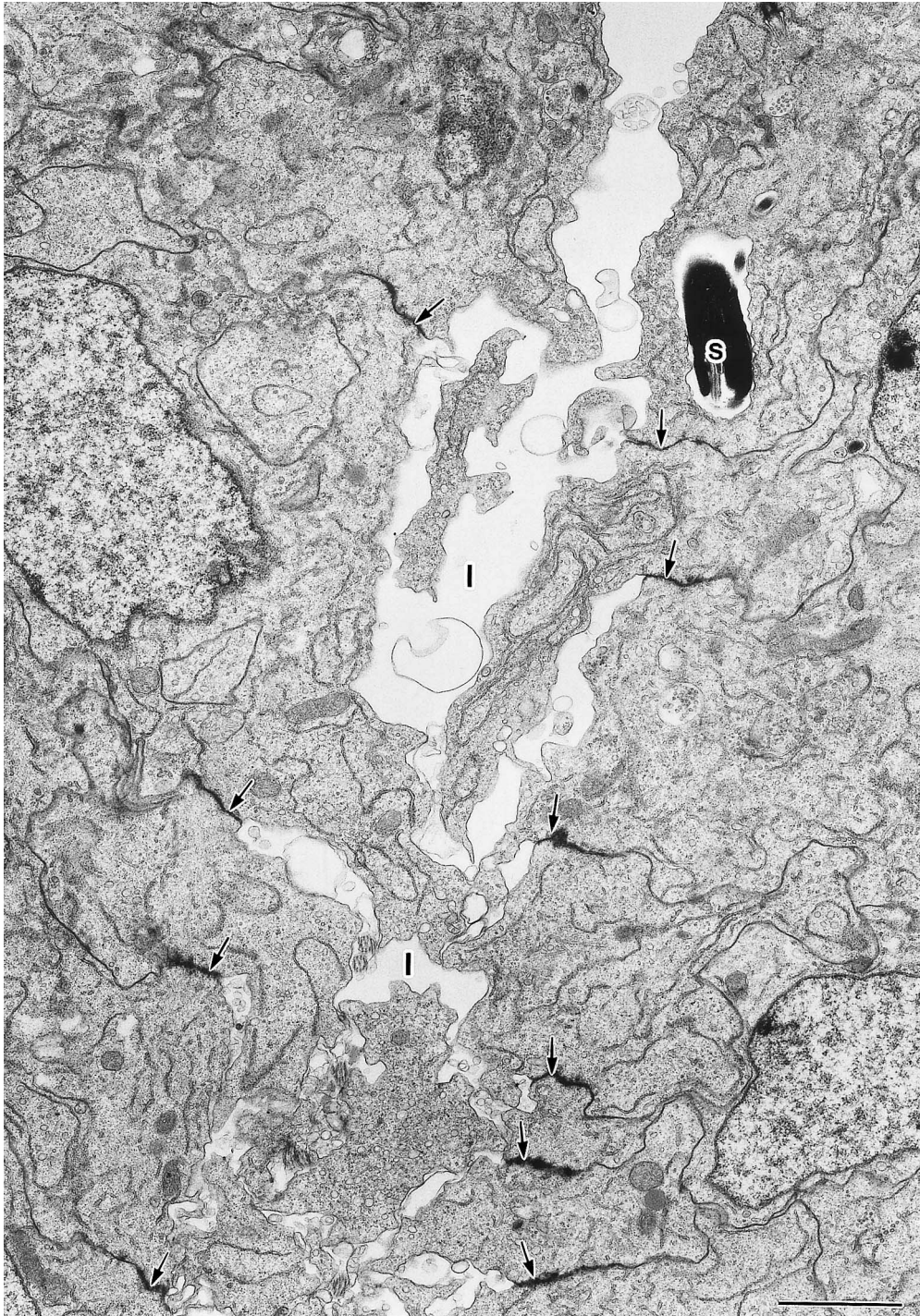
**Fig. 5.** Magnification of boxed area in Fig. 4. Thick epithelial cells at the bottom side of the SSP were tightly connected with one another with desmosomes (d) and tight junctions (arrows). bm, basement membrane; bs, epithelial cells of the SSP bottom; c, centrioles; em, oocyte surface; gc, granulosa cell layer; l, lumen of the SSP; o, oocyte; tc, thecal cell layer. Bar, 1mm.

compact sperm mass within its large chamber (Fig. 8). Most spermatozoa brought their heads lacking acrosome into contact with thick epithelial cells of the lateral wall. In the follicle at the more advanced stage of 1.8 mm in diameter, it was seen that spermatozoa in the SSP oriented their heads towards the bottom surface consisting of one very thin layer of epithelial cells (Fig. 9). However, there was found no special structure for sperm entry.

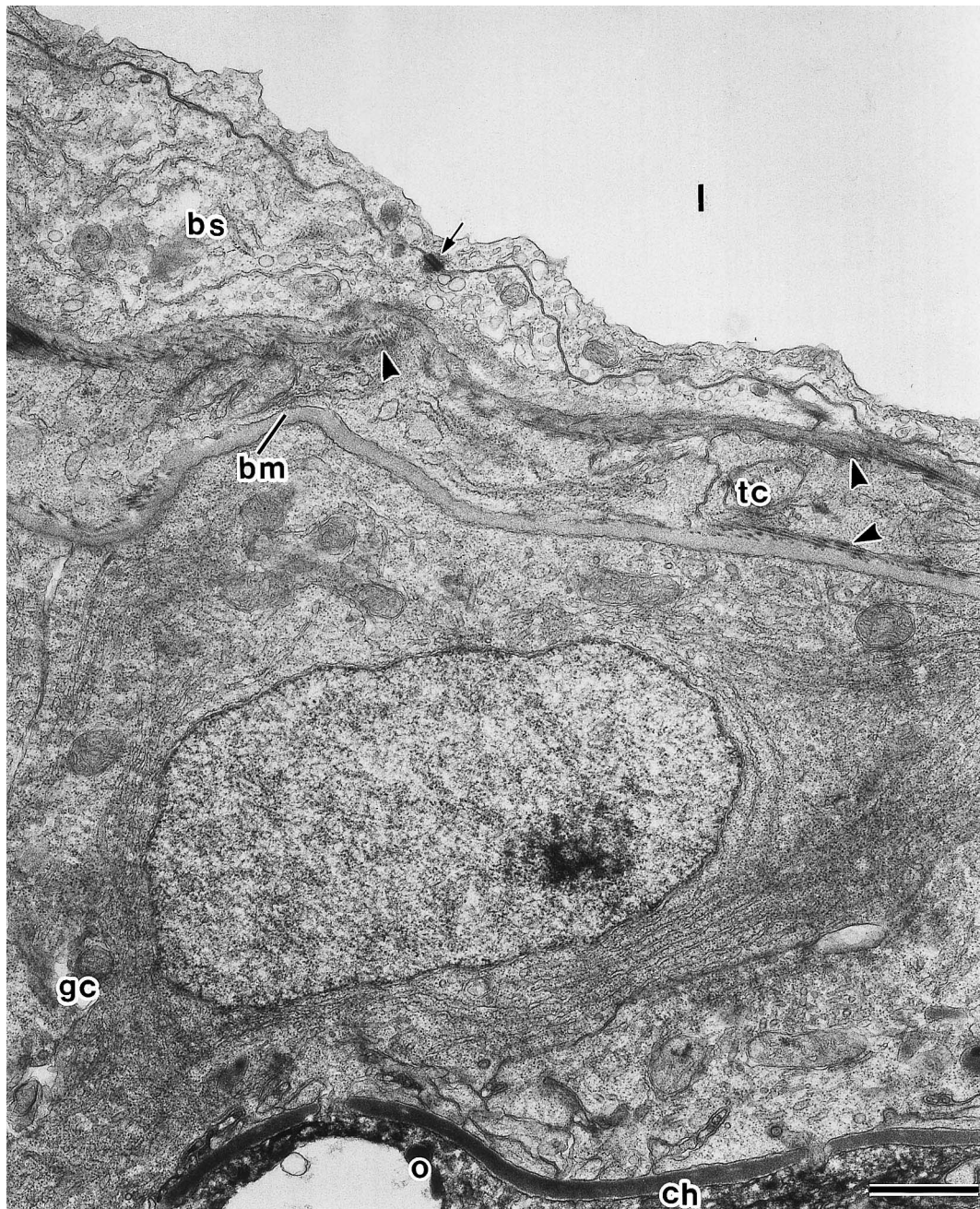
The follicle layer comprised the thecal cell layer, base-

ment membrane and granulosa cell layer. The chorion as a single layer of the electron dense material corresponds morphologically to the outermost layer of the chorion in *Oryzias* eggs. The thickness of the thin chorion with many canals was about 0.2  $\mu\text{m}$ .

As described above, the SSPs did not display any remarkable changes in the morphology of the cellular components among those on the fully-grown follicles. The micropyle or micropyle-like structure was not found in the chorion



**Fig. 6.** Epithelial cells in the lateral wall of the tract extending from the ovarian cavity. Epithelial cells in the lateral wall of the tract exhibit the tight junctions (arrows) on the side of the tract lumen (l). s, spermatozoon. Bar, 2 $\mu$ m.



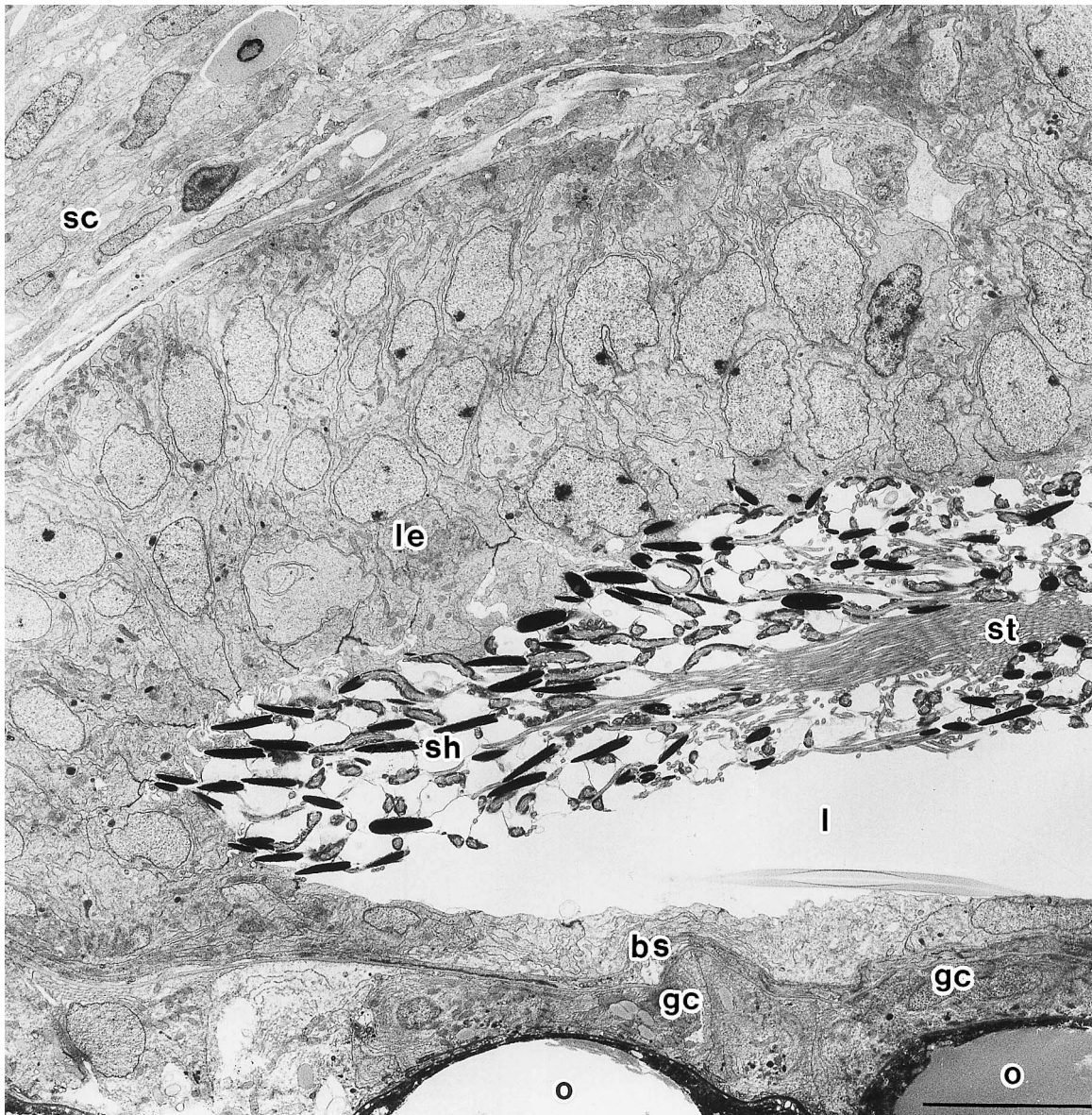
**Fig. 7.** Bottom region of micropocket (SSP) in a large follicle 1.2 mm in diameter. Desmosome (arrow) is seen between the epithelial cells of SSP bottom (bs). Arrowheads indicate collagen fibers on both sides of a thecal cell layer (tc). bm, basement membrane; ch, chorion; gc, granulosa cell; l, lumen of the SSP; o, ooplasm. Bar, 1  $\mu$ m.

of developing oocytes in the guppy. These facts suggest that the SSP bottom, follicular layer and the chorion near the germinal vesicle (GV) in the cortical cytoplasm must be perforated by the spermatozoon at the time of fertilization. Unfortunately, the present study failed to observe any spermatozoa penetrating the ooplasm through these cellular constructions.

#### **Distance between germinal vesicle (GV) and the sperm storage micropocket (SSP) on the follicle, and diameter of GV during oogenesis**

In intact samples, the GV began to be observed among oil droplets in the cortical cytoplasm of the transparent oocytes from stage VI onwards, whose diameters were more than 900  $\mu$ m (Fig. 10A–C). The SSP portion of the tract extending from the ovarian cavity was also detectable in





**Fig. 8.** Micropocket (SSP) region on a large follicle 1.5 mm in diameter. Spermatozoa with heads (sh) oriented to the lateral wall side of epithelial cell (le) in the lumen (l) of the SSP. bs, epithelial cell of the SSP bottom; gc, granulosa cell layer; o, oil droplet of the oocyte; sc, supporting cells; st, sperm tail. Bar, 10 $\mu$ m.

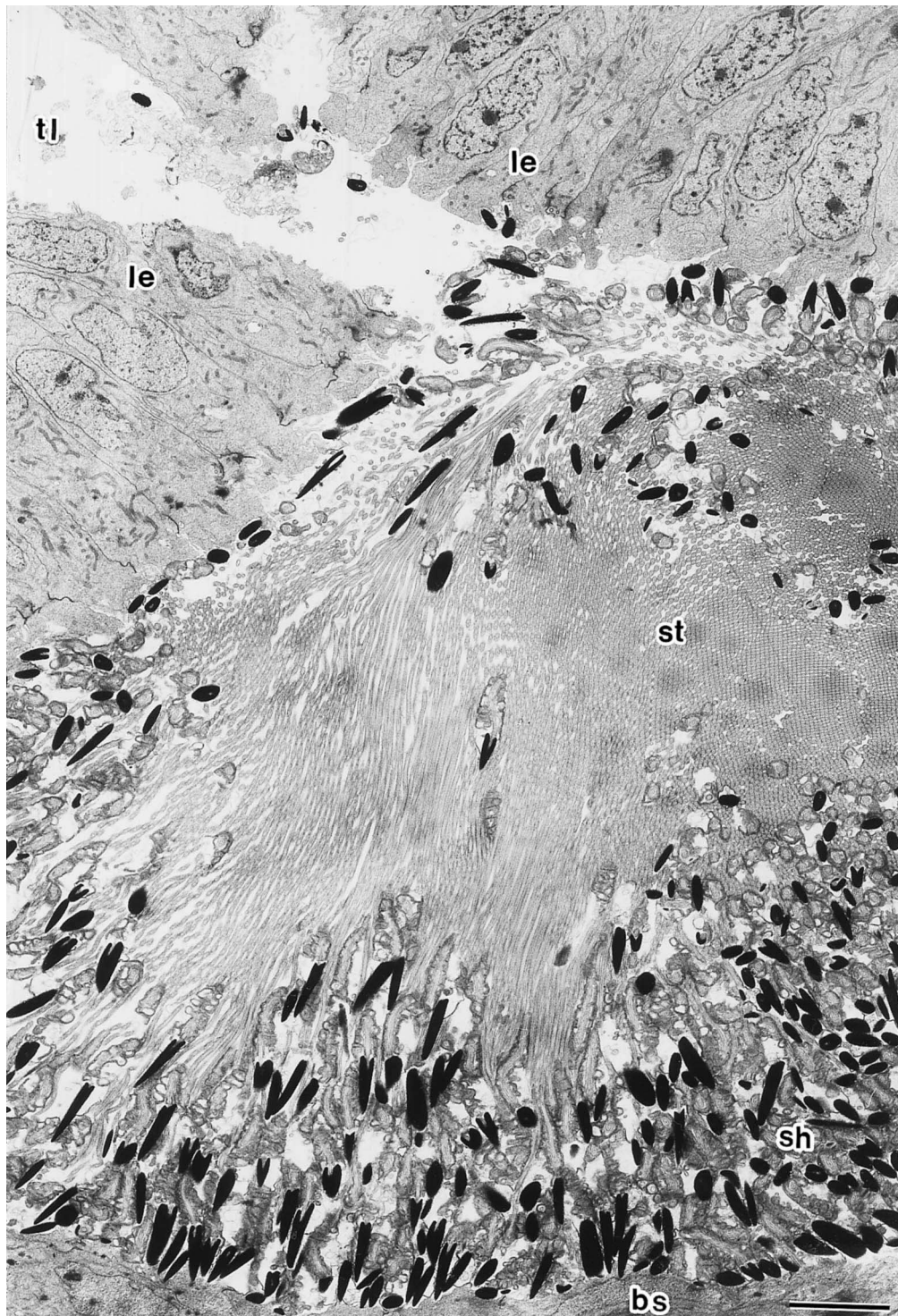
each follicle (Fig. 10A, C). During this period, the morphological change in the GV was not recognized to depend on the size of the oocytes (Fig. 11). In such GVs unchanged in morphology, shortened chromosomes were recognized but not Lamp-brush chromosomes (Fig. 10D).

On the other hand, the position of GV in the oocyte did not always correlate with that of the SSP (Fig. 11). Thus, the SSP does not always locate close to the GV in oocytes various in size. In connection with this, it has been reported that if the nucleus was experimentally shifted from the animal pole region into the cytoplasm of the vegetal hemisphere of the oocyte by centrifugation, karyogamy does not occur in *O. latipes* (Iwamatsu and Mori, 1968). From these observa-

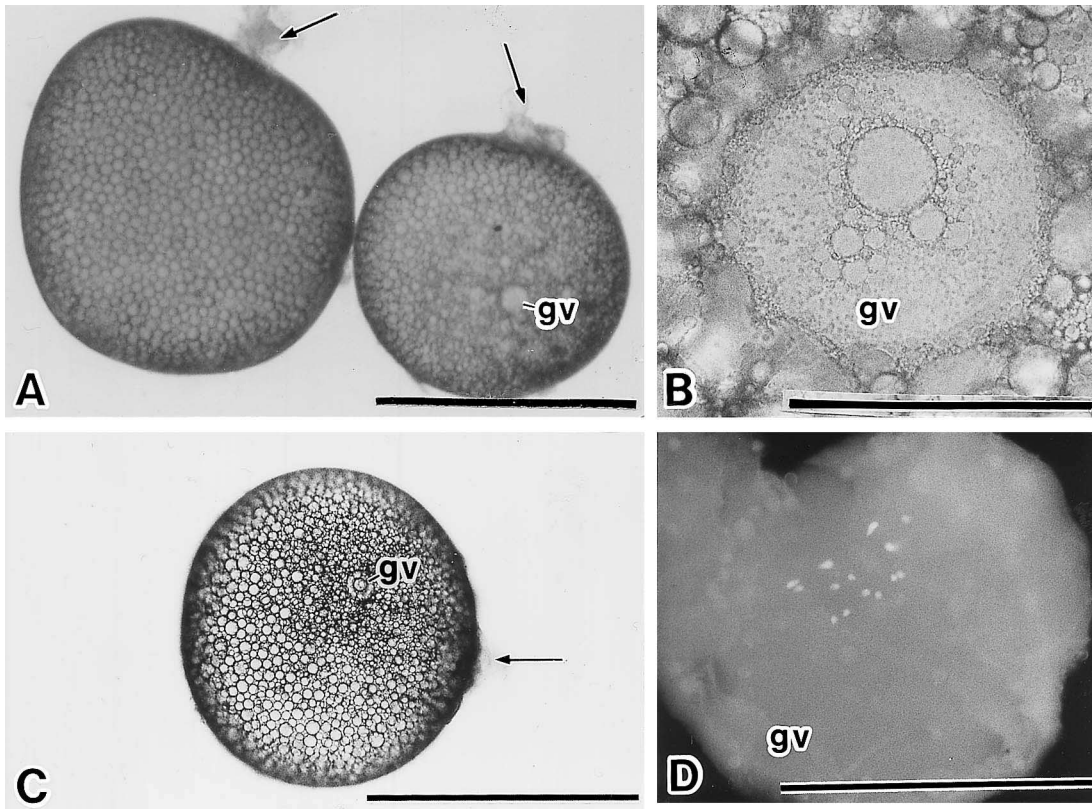
tional results, it may be inferred that the female nucleus to fuse with the sperm nucleus becomes located in the vicinity of the SSP at the time of fertilization probably by rotation of the oocyte, as seen in *Oryzias* oocytes (Iwamatsu, 1994).

#### **Distribution of carbon particles within the ovary**

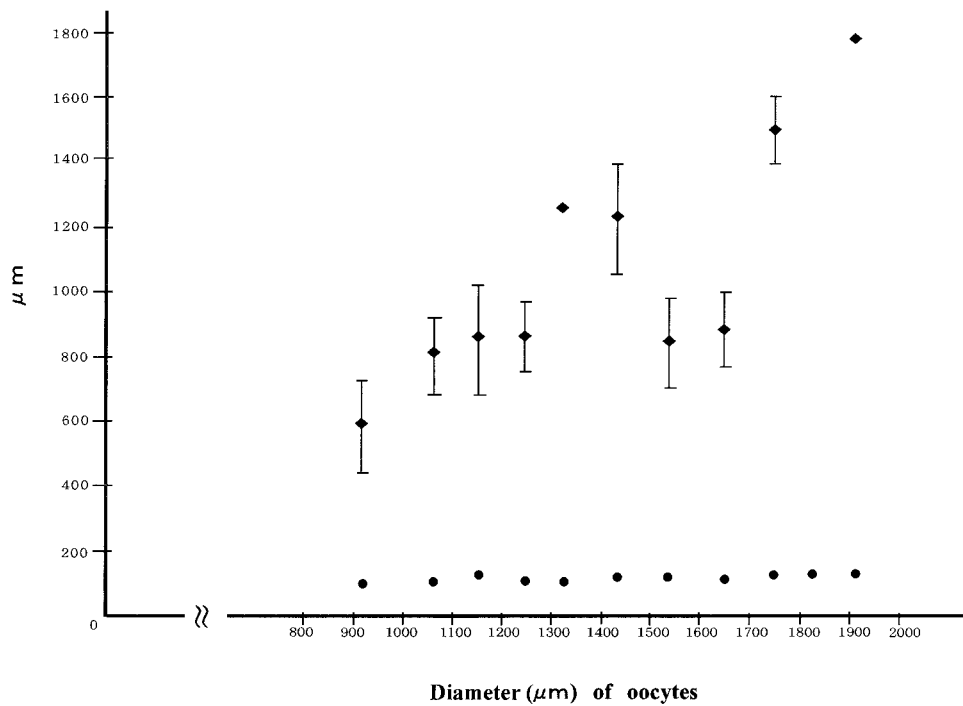
When carbon particles were introduced into the ovarian cavity through the genital pore in adult female fishes, they were observed in the SSP on the oocyte follicle in some fishes but not all (data not shown). This does not necessarily seem to show the active migration of spermatozoa into the bottom chamber of the SSP.



**Fig. 9.** Spermatozoa packed in the SSP on a large follicle 1.8 mm in diameter. The SSP is filled by numerous spermatozoa with their heads (sh) arrayed towards the side of SSP bottom (bs). The lumen of tract (tl) consisting of thick epithelial cells (le) contains only a few spermatozoa. sh, sperm head; st, sperm tail. Bar, 5 $\mu$ m.



**Fig. 10.** Germinal vesicle (GV) in guppy oocytes. A: Mature oocyte and growing oocyte. Arrows indicate sperm storage micropockets (SSPs). Bar, 1.7 mm. B: Intact GV in a fully-grown oocyte. Bar, 100 $\mu$ m. C: Fully-grown oocyte. gv, germinal vesicle; arrow, SSP. Bar, 1.7 mm. D: Chromosomes in the germinal vesicle (gv) of a fully-grown oocyte. Bar, 100 $\mu$ m.



**Fig. 11.** Relationship between diameters of oocytes and germinal vesicles (GV) during oogenesis, and distance from GV to SSP on each follicle. Solid circles indicate diameter ( $\mu$ m) of the GV. Solid diamonds indicate distance ( $\mu$ m) between GV and SSP, with vertical bars showing the standard deviation.

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