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Fertilization Process in the Arrow Worm *Spadella cephaloptera* (Chaetognatha).

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ABSTRACT—To reveal the fertilization process of arrow worms, oocytes from fixed specimens of *Spadella cephaloptera* were isolated and observed by staining fluorescent nucleic acid dyes. Fully grown oocytes with a germinal vesicle (GV oocytes) are associated with an accessory fertilization cell (AFC), which is easily removed from the oocyte during isolation. Oocytes after germinal vesicle breakdown (GVBD oocytes) do not have an AFC and are at the metaphase of the first meiosis. In most cases GVBD oocytes contain a small bright spot that is regarded as a condensed sperm chromatin. In rare cases the sperm chromatin in GVBD oocytes has the form of a thin-thread (20 μ m long) which corresponds to the sperm nucleus immediately after fertilization. Therefore, fertilization occurs at the first meiotic metaphase after disappearance of the AFC. Numerous zygotes from a single ovary have an hourglass shape, indicative of passage of ovulating eggs into the oviducal complex. Thus, ovulation occurs after fertilization in *S. cephaloptera* as reported in the genus *Sagitta*.

INTRODUCTION

Chaetognaths have internal fertilization. Sperm entering the female gonopores after mating are stored in the oviducal complex. This is situated laterally in the elongate ovary and has a double-walled structure: an outer “cellular sheath” encloses a central “syncytium”. Sperm are present in the syncytium and zygotes enter a membrane-bound lumen in the syncytium (Stevens, 1910; Ghirardelli, 1968; Shinn, 1992, 1994). Chaetognaths show a unique pattern of fertilization; sperm pass through accessory fertilization cells (AFCs) in order to reach oocytes attached to the outside of the oviducal complex (cf. Grassi, 1883; Stevens, 1910; Ghirardelli, 1968; Shinn, 1994). The AFCs are differentiated from oviducal cells and are serially arranged along the medial side of the oviducal complex. The AFCs occur in pairs, the members of which are called AFC1 and AFC2; the latter attaches to a single differentiating oocyte. Sperm reach the oocyte through a complexly shaped “fertilization canal”.

The most recent work on the fertilization of chaetognaths was a series of ultrastructural studies done by Shinn (1992, 1994, 1997) using *Sagitta hispida*. He found that the fertilization canal is normally occluded by a close-fitting cytoplasmic extension of AFC2 and that the cytoplasmic process disappears from the fertilization canal at fertilization, opening the pathway for sperm (Shinn, 1994, 1997). He showed the profile of sperm in an egg, demonstrating that fertilization occurs before egg enters the oviducal complex (Shinn, 1994). In ad-

dition, he substantiated classical reports that ovulation occurs immediately after fertilization.

Studies of this unique fertilization process are important not only for chaetognath biology but also for developmental biology in general. So far, the morphological features of the AFCs have been examined by in vivo observations and by sectional views. In the present study of chaetognath fertilization I have examined the existence of the AFCs and the stage of egg to be fertilized using wholemount preparations stained with fluorescent nucleic acid dyes to reveal the status of egg and sperm nuclei. For this study I have used a benthic chaetognath *Spadella cephaloptera* which has relatively transparent oocytes and is easily kept in the laboratory.

MATERIALS AND METHODS

Adult specimens of *S. cephaloptera* were collected at a depth of 3–5 m in the *Posidonia oceanica* bed facing Lacco Ameno (Island of Ischia, Gulf of Naples) and kept in a constant temperature room at 17°C with a 12 hr LD cycle. About 20 arrow worms were placed in a glass bowl (20 cm in diameter) containing sea water, which was changed every week. Methods for the laboratory culture of this species are described in Goto (1995).

The living specimens containing various stages of oocytes were observed under a compound microscope equipped with Nomarski optics (Zeiss Axiophot or Nikon Optiphot). These specimens were fixed with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 0.4 M sucrose for 1 h and kept in the same buffer. The oocytes were isolated in phosphate-buffered saline (PBS) by dissection, stained with Hoechst 33342 dye (5–10 μ M), mounted in a PBS-glycerin mixture (1:1), and observed under an epifluorescence microscope (Zeiss Axiophot or Nikon Optiphot). 812 oocytes from 145 specimens were examined. Sperm and the oviducal complex were also examined by the same method.

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The oocytes after germinal vesicle breakdown (GVBD oocytes) were also examined by immunofluorescence staining for microtubules. The specimens containing GVBD oocytes were fixed with 80% cold ethanol for 30 min. After washing in PBS, the GVBD oocytes were isolated and kept in PBS containing 0.1% Triton (PBS-T) for 12 hr, followed by incubation with 2% bovine serum albumin (BSA) for 12 hr. The oocytes were then incubated with a monoclonal anti- α -tubulin antibody (Amersham) diluted at 1:1000 for 12 hr, rinsed with PBS, and incubated with a secondary antiserum (FITC-conjugated goat anti-mouse IgG, Cappel) diluted at 1:500 for 12 hr. After washing in PBS, they were stained with DAPI (2-5 μ M) for chromosome observation, mounted in PBS-glycerin, and examined under the microscope with appropriate filters. Photographs were taken on Kodak Tri X film or Fijichrom Provia film.

RESULTS

In sexually mature arrow worms, an ovary is located on each side of the intestine and growing oocytes in the ovaries are arranged in a single column. Fig. 1 shows body regions of *S. cephaloptera* provided with oocytes. The oocytes typically show one of two states. Oocytes commonly contain a large germinal vesicle (GV oocyte) as shown in Fig. 1a. A spheroidal "seminal pouch" is located near the female gonopore and a column of sperm in the syncytium of the oviducal complex extends anteriorly along the GV oocytes. Alternatively, oocytes may lack a germinal vesicle as a result of germinal vesicle breakdown (GVBD oocyte) as shown in Fig. 1b. Without observing GVBD and ovulation in live specimens, it is difficult to distinguish whether GVBD oocytes are still in the ovarian space, or have been ovulated into the oviducal complex. The GVBD oocytes move into the oviducal complex and are stored there until being laid. As described later, in specimens kept in mass culture, most of GVBD oocytes are already fertilized and do not develop further until being laid. In this species,

fertilized eggs remain in the oviducal complex for several hours after ovulation. The germinal vesicle moves to the medial side of the oocyte just before GVBD (Fig. 1c).

In living *S. cephaloptera* with growing oocytes, a circular structure can be seen between the oviducal complex and each oocyte (Fig. 2a). This structure is also evident in isolated preparations of the oviducal complex (Fig. 2b). Staining with Hoechst dye reveals that the arrangement of nuclei in the structure looks like a rosette; it consists of single central nucleus and several peripheral nuclei (Fig. 2c). The former corresponds to the nucleus of accessory fertilization cell 1 (AFC1) and the latter is of supporting oviducal sheath cells.

Various stages of isolated oocytes stained with Hoechst dye are shown in Fig. 3. A fluorescent spot is detected in fully grown GV oocytes (Fig. 3a). This is the nucleus of accessory fertilization cell 2 (AFC2). AFC2 is located at an opening in the egg coat and is attached to the oocyte surface (Fig. 3b,c). However, 65% of fully grown oocytes examined ($n=439$) in the present study do not show attachment of AFC2 (Table 1). A sperm nucleus has never been detected at this stage, indicating that fertilization has not yet occurred.

In living preparations, GVBD oocytes develop when isolated in sea water by dissection (Goto, unpublished), indicating that they are fertilized at this stage. GVBD oocytes are at the metaphase of the first meiosis (Fig. 3d-f). Immunostaining for microtubules reveals the formation of spindle in GVBD oocytes (Fig. 3g). It is evident that the haploid chromosome number is 9 (inset of Fig. 3d).

In rare cases (6% of GVBD oocytes examined, $n=373$, Table 1), a sperm nucleus, having the form of a thin-thread is observed in the cytoplasm (Fig. 3d). The length of a sperm nucleus is the same as that of these thin threads. Fig. 4 shows a single sperm observed under Nomarski optics (Fig. 4a) and

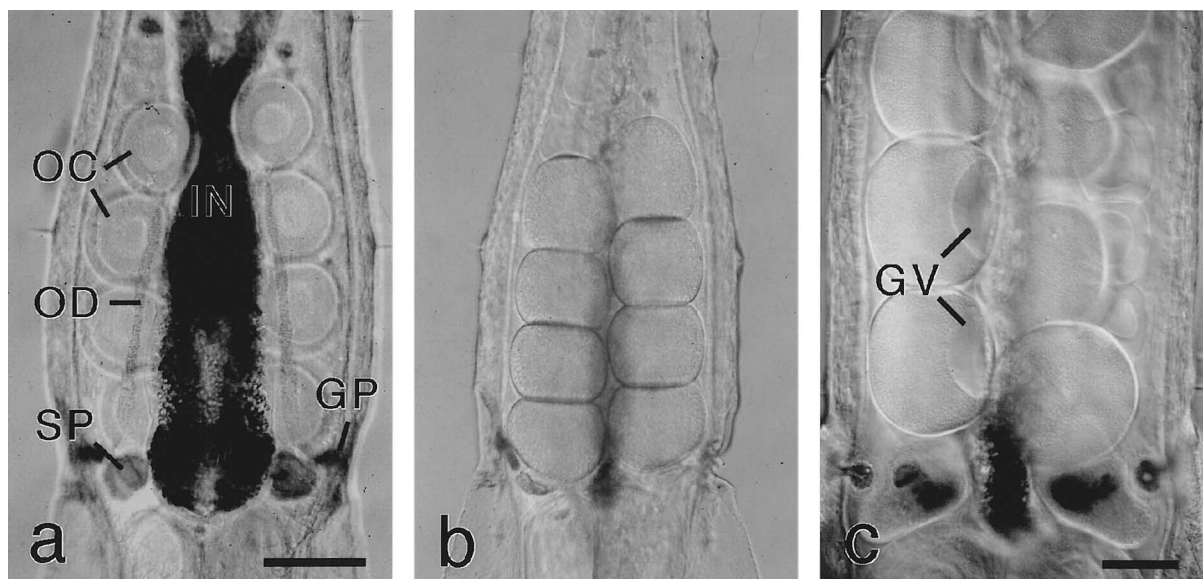


Fig. 1. Trunk regions of living specimens of *Spadella cephaloptera*. (a) Fully grown oocytes in the ovaries. GP, female gonopore; IN, intestine; OC, oocytes; OD, oviducal complex; SP, sperm pouch. (b) Oocytes after germinal vesicle breakdown (GVBD). These oocytes are fertilized and are ovulated. (c) Oocytes just before GVBD. GV, germinal vesicle. Bars = 200 μ m in a, b; 100 μ m in c.

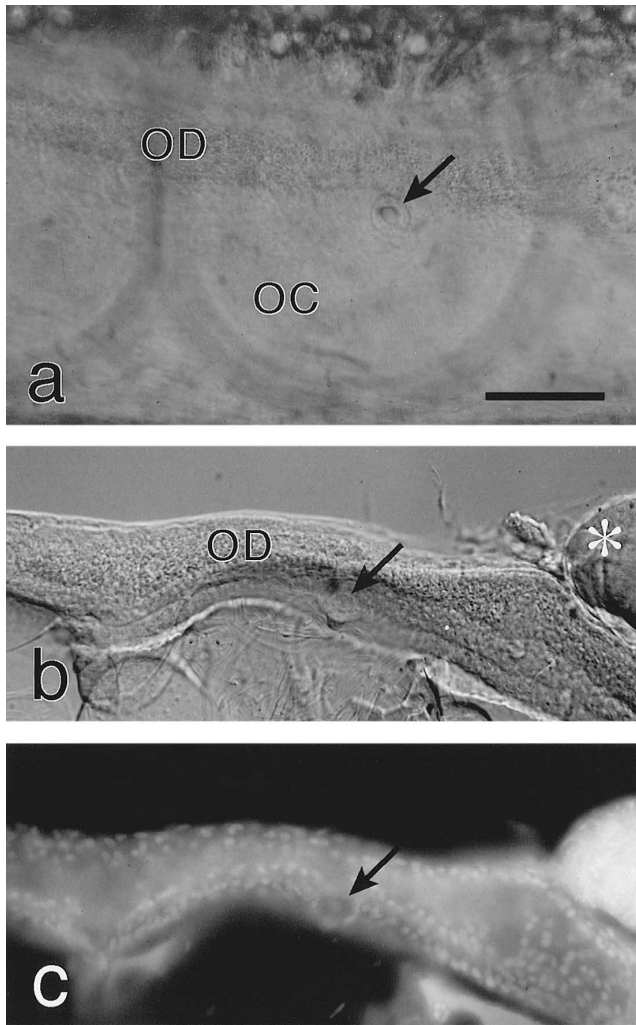


Fig. 2. (a) Fully grown oocyte in a living specimen. A circular structure (arrow) concerning AFCs is located between the oviducal complex (OD) and the oocyte (OC). (b, c) Isolated oviducal complex from a fixed specimen stained with Hoechst dye observed under Nomarski optics (b) and epifluorescence (c). Arrows indicate the circular structure attached on the oviducal complex (OD). Asterisk in b indicates spheroidal "seminal pouch" located at the posterior end of the oviducal complex. Bar = 50 µm.

fluorescence microscope (Fig. 4b). The sperm is filiform (ca. 200 µm) and its thin nucleus is 30 µm long. Therefore, the fertilization occurs after GVBD. The GVBD oocytes containing these thin-thread sperm nuclei are presumably still in ovarian space. Their rare occurrence (Table 1) is accounted for by the fact that eggs remain in the ovarian space for a relatively short time compared to the time they remain in the oviducal complex after ovulation. In most cases GVBD oocytes contain a small bright spot which is "round up" sperm chromatin (Fig. 3f, Table 1). 26% of GVBD oocytes are without sperm nucleus (Table 1), suggesting that 1) these oocytes are unfertilized or 2) sperm chromatin is temporarily dispersed just after fertilization.

The GVBD oocyte at transition into the oviducal complex shows an hourglass shape and contains round-up sperm chro-

matin (Fig. 3e). This fact verifies that the eggs are fertilized before they move into the oviducal complex and are stored until being laid.

DISCUSSION

Participation of AFCs in the fertilization of chaetognaths was documented using transmission electron microscopy by Shinn (1994, 1997). In the present study of chaetognath fertilization various stages of oocytes were examined in isolated oocytes stained with fluorescent dyes for DNA.

Oocytes arise from clusters of germinal cells embedded in the medial side of the oviducal complex. As growing oocytes separate from the germinal clusters, they become attached to pairs of specialized oviducal cells that differentiate into AFCs. Only the largest oocytes are involved in vitellogenesis and will be fertilized and released during the next egg-laying event (Shinn, 1997). AFC2 sinks into the cytoplasm of the fully grown GV oocyte at a point where, egg coat being lacking, a sort of micropyle has formed (Stevens, 1910). AFCs were evident from its circular structure in a whole specimen of *S. cephaloptera* through the body surface (Fig. 2a). The isolated oviducal complex stained with Hoechst dye revealed that the circular structure had several nuclei arranged in a rosette-like shape (Fig. 2c). Considering the observation by Shinn (1994) that supporting oviducal sheath cells retract from the sides of AFC1, the circular structure is composed of centrally located AFC1 and supporting cells surrounding AFC1.

In isolated oocytes of *S. cephaloptera* AFC2 was attached only to the fully grown GV oocytes and was never found in younger oocytes. 65% of fully grown oocytes examined (n=439) in the present study did not show attachment of AFC2 (Table 1), suggesting that the connection of AFC2 is relatively weak even to fully grown GV oocytes.

The sperm nucleus of arrow worms is very thin and about 30 µm in length (Fig. 4b). Similar structure was found only in GVBD oocytes in which AFC2 had disappeared. This most likely corresponds the moment just after fertilization and is evidence that fertilization occurs when eggs are at the first meiotic metaphase. Shinn (1994, 1997) found the existence of numerous sperm passing into the fertilization canal and one or more sperm entering the oocyte cytoplasm. Despite the fact that several sperm have a chance to enter an oocyte at the same time, I also never observed more than a single sperm nucleus in an egg. Thus, the present study provides additional evidence that some mechanism to prevent polyspermy exists in chaetognaths.

In *Sagitta*, zygotes pass individually through the medial wall of the oviducal complex through pores that form when AFCs enter the syncytium (Shinn, 1994, 1997). A different mode of ovulation was reported for *Spadella* by Ghirardelli (1968). He states that zygotes become free in the ovarian space and then enter the oviducal complex posteriorly, through a single pore (Ghirardelli, 1968). In the present study, I observed numerous isolated zygotes from a single ovary of *S. cephaloptera* to have the hourglass shape that is indicative of

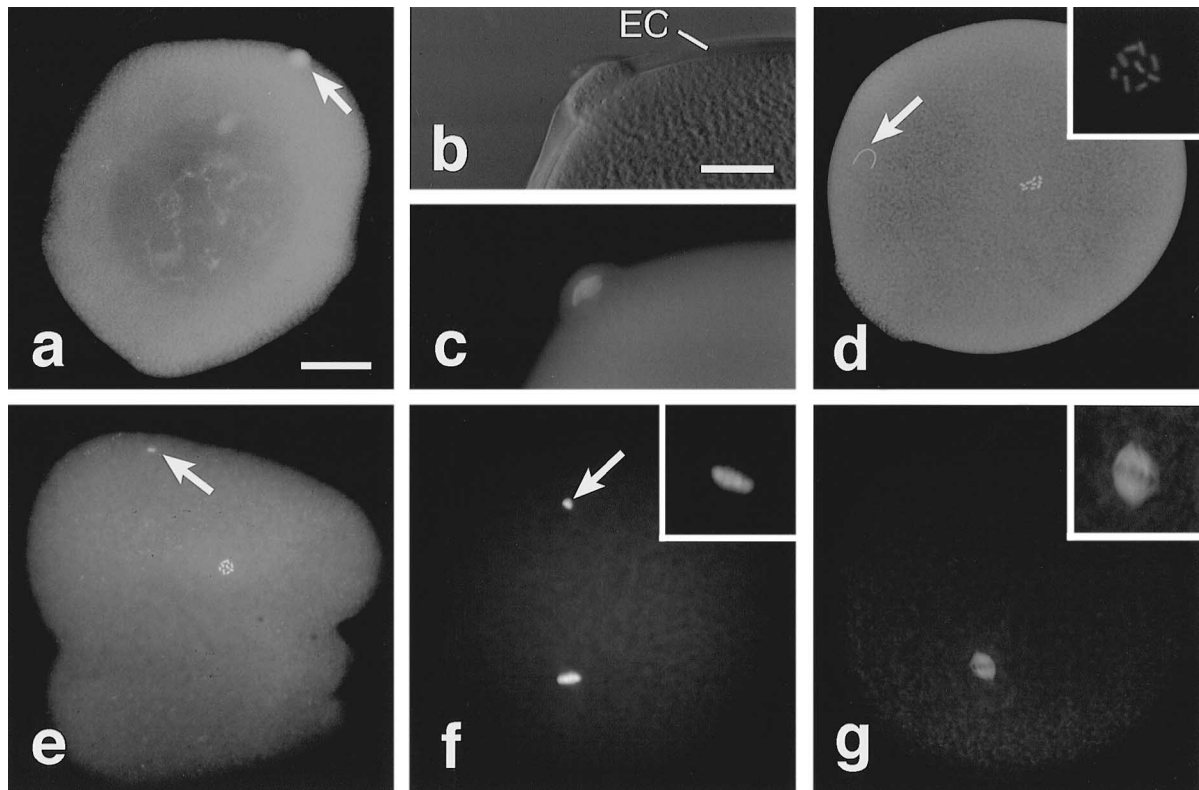


Fig. 3. Wholmount preparations of isolated oocytes from fixed specimens. **(a-e)** Oocytes are stained with Hoechst dye and observed under epifluorescence microscope except for **b**. **(f, g)** An oocyte is stained with DAPI and immunofluorescent staining for tubulin. **(a)** Fully grown GV oocyte. Arrow indicates accessory fertilization cell 2 (AFC2). **(b, c)** Higher view of AFC2 under Nomarski optics **(b)** and epifluorescence **(c)**. EC, egg coat. **(d)** Circular shape GVBD oocyte containing a sperm head (arrow). Inset shows oocyte chromosome bivalents. **(e)** An hourglass shape GVBD oocyte containing "round up" sperm chromatin (arrow). **(f)** Circular shape oocyte containing "round up" sperm chromatin (arrow). Inset is enlargement of the metaphase plate. **(g)** Same oocyte in **(f)** showing spindle. Inset is enlargement of the spindle. Bars = 50 μm in **a, d, e, f, g**; 25 μm in **b, c**. In **d, f**, and **g**, enlargement of insets is double.

Table 1. Presence of AFC2 and sperm nucleus in oocytes.

stages	number of oocytes examined	with AFC2	without AFC2	with sperm nucleus (thin-thread)	with sperm nucleus (round shape)	without sperm nucleus
GV oocytes	439	151	288	0	0	439
GVBD oocytes	373	0	373	22	255	96

the transitive state into the oviducal complex. Thus, in *S. cephaloptera*, eggs enter the oviducal complex individually as previously described for *Sagitta* (see Fig. 91 in Shinn, 1997). The events described by Ghirardelli (1968) presumably correspond to release of eggs from the oviducal complex; ovulation probably occurred several hours before and was not observed or described by him.

Most of the sperm nucleus in GVBD oocyte was round shape. This is different from the male pronucleus which is formed at a late stage of meiotic maturation (Goto, unpublished). The sperm nucleus entering the oocyte may remain condensed as a round shape, although there is a possibility of temporal dispersion of sperm chromatin before condensation.

Fig. 5 is a diagram showing the process of chaetognath

fertilization. The detail of the AFCs is based on the studies of Shinn (1994, 1997). The time course of fertilization and GVBD is not clear in the present study. As reported elsewhere (Stevens, 1910; Shinn, 1994), however, fertilization coincides with GVBD. Shinn (1994) observed the structural change of AFC2 in the specimens preserved a few minutes after GVBD. A substance like a maturation promoting factor (MPF) may influence the structural changes of AFC2. A single mature specimen individually cultured after maturation lays unfertilized eggs, indicating that the fertilization itself does not trigger GVBD of the oocyte. The trigger factor for GVBD is also unknown in chaetognaths, although the existence of follicle cells around oocytes has been found by Shinn (1992).

In *S. cephaloptera*, zygotes arrested at the first meiotic metaphase remain in the oviducal complex for several hours

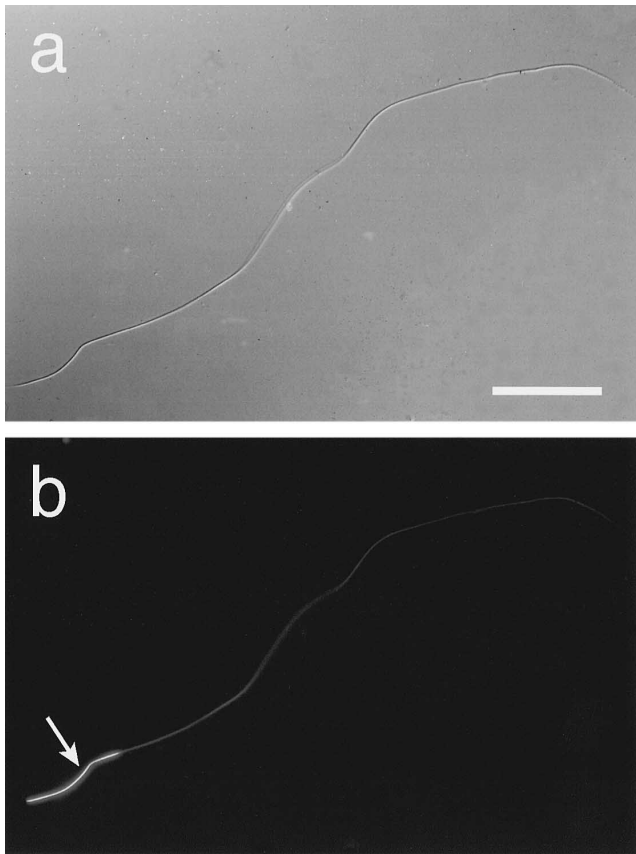


Fig. 4. A single sperm isolated from a seminal vesicle of a living specimen. The same preparation stained with Hoechst dye observed under Nomarski optics (a) and epifluorescence (b). Arrow indicates sperm head. Bar = 30 μ m.

before being laid into sea water. In *Sagitta hispida* egg laying occurs 10 to 15 min after ovulation (Reeve and Lester, 1974; Shinn, per. com.). Ghirardelli (1968) also reported that GVBD normally precedes egg laying by 10 min to 1 hr at the most in *Sagitta* while several hours in *Spadella*. The period that zygotes stay in the oviducal complex seems consistently different between these two genera. It is well known that oocytes arrest at a stage of meiosis and sperm function to initiate the resumption of meiosis (cf, reviewed by Masui, 1985), although temporary delay of the onset of cleavage occurs in diverse organisms having internal fertilization as suggested by Shinn (1994). Trigger factor of the meiotic resumption in metaphase-arrested zygotes has not been examined as yet. To discover the mechanism of fertilization and meiotic regulation including sperm nuclear changes in chaetognaths, we have to use isolated eggs and establish a handling procedure for chaetognath oocytes.

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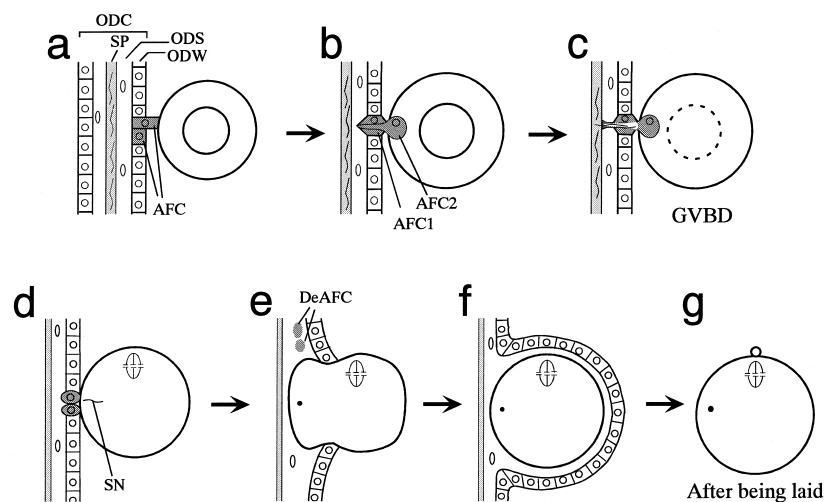


Fig. 5. A schematic diagram showing the process of chaetognath fertilization. Details of the formation and behavior of AFCs are based from the observation by Shinn (1994, 1997). (a) Growing oocyte attaches to pairs of specialized oviducal cells that differentiate into AFCs. (b) AFC2 sinks in the oocyte (refer to Fig. 3a). Cytoplasmic process of AFC2 occludes the fertilization canal formed in AFC1. (c) The cytoplasmic process disappears from the fertilization canal through which sperm enter into egg. (d) A single sperm enters an oocyte after GVBD (refer to Fig. 3d). AFC2 moves outside from the oocyte. (e) Fertilized egg moves into the syncytium oviducal complex through pore that is formed by degeneration of AFCs (refer to Fig. 3e). Sperm chromatin is condensed as a round shape. (f) Fertilized egg is stored in the oviducal complex at the first meiotic metaphase until being laid in sea water (refer to Fig. 3f,g). (g) Resumption of meiosis occurs after being laid in sea water. Sperm chromatin remains a round shape until a late stage of meiosis. AFC, accessory fertilization cell; DeAFC, degenerating AFC; ODC, oviducal complex; ODS, oviducal syncytium; ODW, ovarian wall; SP, sperm.

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