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Male Internal Fertilization and Introsperm-like Sperm of the Seaweed Pipefish (*Syngnathus schlegelii*)

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ABSTRACT—Male members of the seaweed pipefish, *Syngnathus schlegelii*, incubate eggs in the brood pouch located on the tail. The eggs are directly spawned into the brood pouch by inverted copulation after intensive courtship, and the fertilization takes place in the brood pouch. During gestation, the brood pouch is filled with viscous fluid that seems to be of maternal origin, indicated by the absence of mucous secretion of the brood pouch epithelium. The spermatozoa are considered to swim in viscous ovarian fluid during fertilization. These findings indicate that the environment for fertilization is equivalent to that for internal fertilization. The pipefish spermatozoa had a bullet-shaped nucleus ($3 \times 0.6 \mu\text{m}$), a spiral mitochondrion and an elongate flagellum (ca. $85 \mu\text{m}$) with centrioles embedded in deep basal fossa. Based on the morphological features, the pipefish spermatozoon may be categorized in introsperm (internally fertilizing sperm). The spermatozoa swim straight by beating the entire length of the flagellum ($84.1 \pm 43.8 \mu\text{m}/\text{sec}$, $\pm\text{SD}$, $n=3$). The number of spermatozoa in the testis was extremely small (1–2 nuclei per whole transverse sectional area). The mode of fertilization is considered to enable the reduction of the spermatozoon density without deteriorating the success of fertilization. Apart from the typical spermatozoa, another type of spermatozoa with the head about 3 times as large as that of typical spermatozoa was observed. The atypical spermatozoa swim in circles (45 turn/min). Possible natures of the atypical spermatozoa are discussed.

INTRODUCTION

Reflecting their varied reproductive strategy, fishes have evolved a variety of functional adaptations of gonads and gametes to the mode of fertilization. Fishes, especially teleosts, display enormous diversity in spermatozoon ultrastructure (e.g. Hara and Okiyama, 1998; Mattei, 1991).

The family Syngnathidae, pipefish and seahorse, is well known for exclusive paternal care of eggs. The eggs are either attached to ventral body surface or enveloped inside the brood pouch on the tail depending on the genus and species (Herald, 1959). While the ovarian structure and oocyte development of pipefishes have been relatively well studied (Begovac and Wallace, 1987, 1988, 1989), attempts to investigate the spermatozoon morphology and fertilization mechanism in pipefishes have failed because of extremely small number of germinal cells observable in the testes. It is of particular interest how spermatozoa are adapted to the unique mode of reproduction of pipefishes.

The seaweed pipefish, *Syngnathus schlegelii*, is distributed in seagrass beds along the coast of all over Japan except for Ryukyu archipelago (Senou, 1993). Among 49

syngnathid species reported in Japan (Senou, 1993), *S. schlegelii* is the most widely spread and common species. Male *S. schlegelii* develops the brood pouch on the tail for incubation of the eggs. In the present study, testicular and spermatozoon morphology, and spermatozoon motility of *S. schlegelii* were investigated in relation to the mating behavior and environment for fertilization.

MATERIALS AND METHODS

Fish

The seaweed pipefish (*Syngnathus schlegelii*) were collected from the seagrass (*Zostera marina* and *Z. caulescens*) beds in the coastal water less than 5 m deep in Otsuchi Bay and the adjacent Funakoshi Bay on the Pacific coast of northern Honshu, mainland of Japan ($39^{\circ}20' \text{N}$, $141^{\circ}54' \text{E}$) by a boat seine ($43 \times 3 \text{ m}$, mesh size $1 \times 1 \text{ cm}$) from 1995 to 1998. The males and females were separately placed in aerated 150-L tanks provided with continuously running filtered seawater at Otsuchi Marine Research Center, University of Tokyo. The pipefish were fed laboratory reared *Artemia* nauplii and natural zooplankters (mainly copepods) collected every morning in Otsuchi Bay.

Reproductive Behavior

Mating behavior was observed in an aquarium. Multiple males (non-brooding) and females were placed in a rectangular aquarium ($150 \times 50 \times 75 \text{ cm}$) to mate: 13 females (152–253 mm SL) and 9 males (147–257 mm SL) for the first trial and 13 females (same as the first trial) and 6 males (163–264 mm SL) for the second trial. The mating behavior was recorded with a video camera until the spawning was

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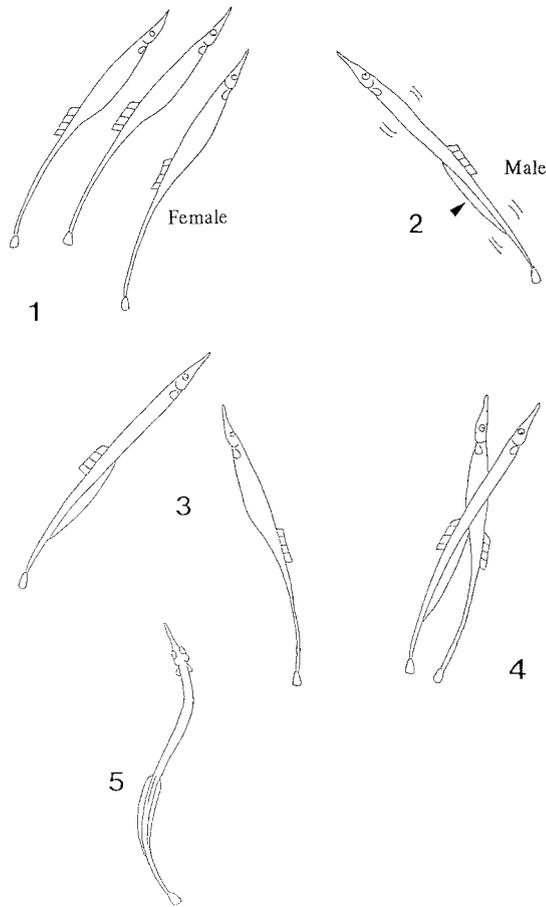


Fig. 1. Five motor patterns seen in the mating behavior of *Syngnathus schlegeli*. Males are shown with the brood pouch (arrow-head). 1) parallel swimming, 2) twitching, 3) rising up, 4) copulation and 5) wiggling. See text for a full description.

completed. Subsequently, behavioral patterns and their sequential order during the courtship and mating were analyzed.

Microscopy

Gross morphology of the brood pouch was observed under a dissecting microscope before the fixation with Bouin's solution. The brood pouch containing prenatal embryos was embedded in paraffin by the conventional method, and the transverse sections (4–7 μm) were stained with PAS (periodic acid Schiff reaction) and alcian blue coupled with Kernechtrot stain solution (Muto Pure Chem. Ltd.) (Okumoto and Inagaki, 1988) in order to detect mucous secretion from the brood pouch epithelium.

Testes were dissected from the body cavity of mature non-brooding males (our preliminary observations of brooding males could identify no spermatozoa) cut transversely into small pieces and fixed in 10% borax-buffered formalin and cold 2% paraformaldehyde –2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for light (n=15) and electron microscopy (n=11), respectively. Most densely packed parts of the testes were selected for electron microscopy.

Testes were embedded in paraffin by the conventional method, and the transverse and longitudinal sections (7 μm) were stained with Haematoxylin and Eosin for light microscopy. Testes for transmission electron microscopy were rinsed overnight in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide in the same buffer for 2 hr and dehydrated through graded ethanol series. Samples were then immersed in QY-1 (Nissin EM Co. Ltd.) and propylene oxide, and embedded in Epon 912 resin (TAAB laboratory equipment Ltd.). Testes were cut into 1 μm transverse sections with a microtome (LKB 2088 Ultratome V), stained with toluidine blue, and observed under a light microscope. Ultrathin sections of the testes were mounted on grids, stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (JEOL JEM-100CX).

For the scanning electron microscopy of spermatozoa, testes in the fixative solution were centrifuged at 2500 rpm for 15 min, and supernatant was discarded. Pellets were then rinsed in 0.1 M cacodylate buffer by centrifugation, mounted on a cover glass coated with 0.1% poly-L-Lysine in distilled water, dehydrated through graded ethanol series, dried with CO_2 in a critical-point drier, sputter coated with platinum palladium, and observed with a scanning electron mi-

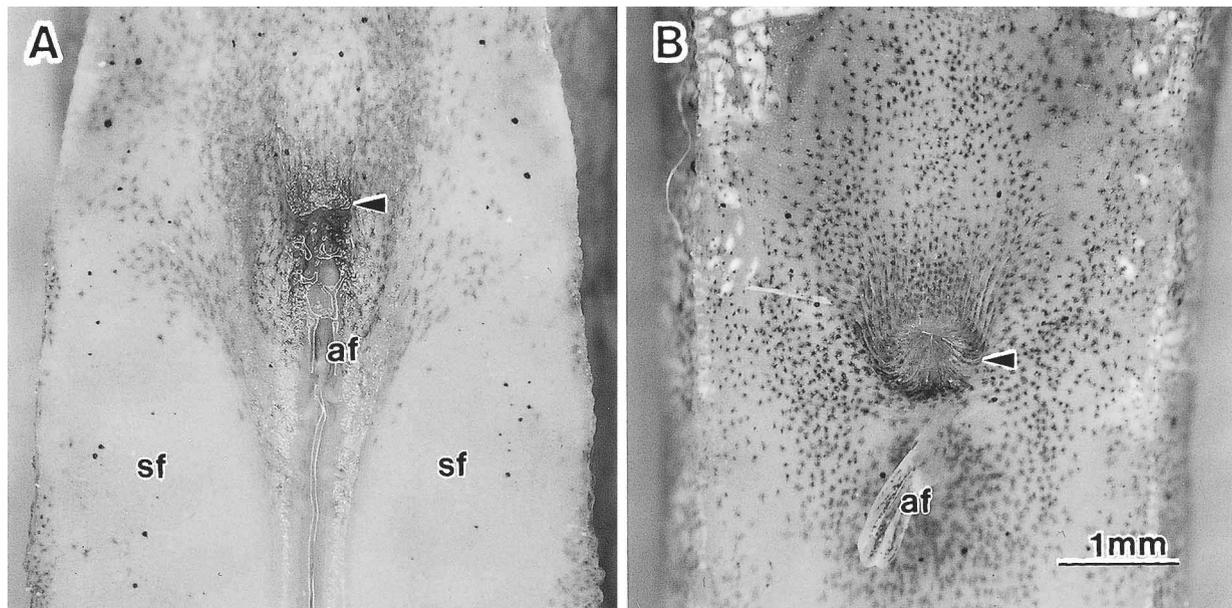


Fig. 2. Genital pore of male (A) and female (B) of *Syngnathus schlegeli*. Male genital pore is located at the anterior end of the brood pouch. Anal fin is partially submerged in the longitudinal fissure of the brood pouch skin folds in male. Female genital papilla is slightly projected. *af*, anal fin; *sf*, brood pouch skin fold; *arrowheads*, genital pore.

croscope (HITACHI S-4500).

Semen samples were observed under a light microscope equipped with a video camera (nac microscopic high speed video) in order to observe the motility of spermatozoa. Testes were cut into small pieces and diluted by environmental seawater on a slide glass. The direction and the speed of spermatozoan movement were analyzed from the video images.

RESULTS

Mating Behavior: Mode of Fertilization

Courtship behavior of *S. schlegeli* consisted of five distinct motor patterns: parallel swimming, twitching, rising up, copulation, and wiggling (Fig. 1). Gronell (1984) originally

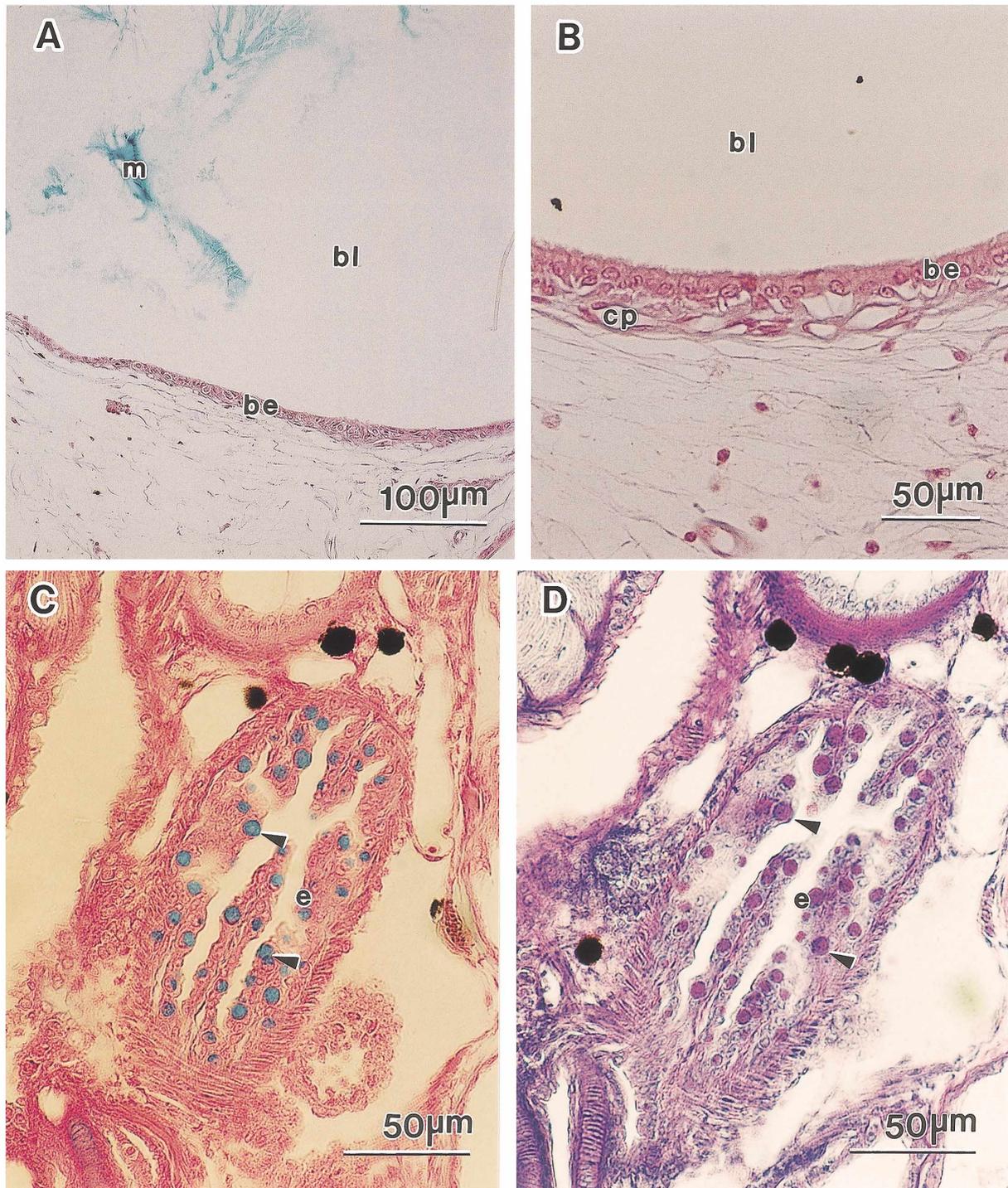


Fig. 3. Mucous detection in the brood pouch and prenatal embryo of *Syngnathus schlegeli*. Remnant mucous material in the brood pouch lumen (A) and mucous cells in embryonic esophagus (C) stained in blue with alcian blue. Embryonic esophagus stained in deep red by PAS (D). Magnified view of brood pouch epithelium (B). Note pouch epithelium is not stained with alcian blue. *be*, brood pouch epithelium; *bl*, brood pouch lumen; *cp*, capillary; *e*, esophagus; *m*, mucous material; *arrowheads*, mucous cells.

described parts of these terms for the courtship in an Indo-West Pacific pipefish species *Corythoichthys intestinalis*. As the definitions by Gronell were not exactly applicable to the mating behavior of *S. schlegeli*, the revised definitions were listed below.

Parallel swimming: Multiple individuals swam around parallel to one another. Two or more females, probably competing against each other, actively performed this behavior. This was also seen between males and females, but did rarely multiple males show this behavior.

Twitching: Females performing parallel swimming and a male approaching to females performing parallel swimming abruptly shook the body.

Rising up: A male started to quickly swim upward and a female deviated from the parallel swimming group to follow it. It was unclear how competition among females ended.

Copulation: A female pressed the genital papilla over the anterior end of the male's brood pouch. The mates were orientated almost vertically and attached obliquely to each other. The female extruded eggs directly into the brood pouch. While the extrusion of the eggs could be identified by quivering of the females, sperm ejaculation was not externally recognizable.

Wiggling: After the copulation, the male slowly twisted the body right and left with a sinuous motion. By this movement, the eggs were transferred to and packed toward the posterior end of the brood pouch. The male performed wiggling even when a single mating filled the brood pouch with eggs.

Male genital pore located adjacent to the anterior end of the brood pouch opens externally, not directly into the brood pouch (Fig. 2A). Female genital papilla is slightly projected

(Fig. 2B). No specializations in copulatory organs for imperviousness to the environmental water are recognized.

Brood Pouch Fluid

Two skin folds extending from the lateral sides of the tail form the ventral brood pouch in *S. schlegeli*. The skin folds merge at the center and are sealed from inside by placenta-like tissue after acceptance of the eggs. The brood pouch was filled with viscous fluid during the incubation.

Although the remnant viscous fluid in the paraffin sections of the brood pouch showed positive reaction to PAS and alcian blue staining (Fig. 3A), the pouch epithelium was not reactive (Fig. 3B). The mucous cells in the esophagus of developing embryos in the brood pouch showed positive reaction to alcian blue and PAS (Fig. 3C and D).

Testis and Spermatozoan Morphology

The testes of *S. schlegeli* are paired slender organs situated dorsad to intestine and are separated along most of their length. The gonadal pore opens at the anterior end of the brood pouch. The longitudinal length of the testes was about 30 to 40% of that of the abdominal cavity. The testis does not form usual cysts. The testis is a semi-transparent hollow tube with cells containing oil-like droplets and holes, and were hardly any germinal cells recognized by light microscopy (Fig. 4). Longitudinal sections of entire testes identified no local specializations in cell compositions. The density of spermatozoa in the testes was extremely low in all specimens.

A small number of spermatozoa were found by electron microscopy (Fig. 5). The number of spermatozoan nucleus observed in a whole transverse sectional area of the testis

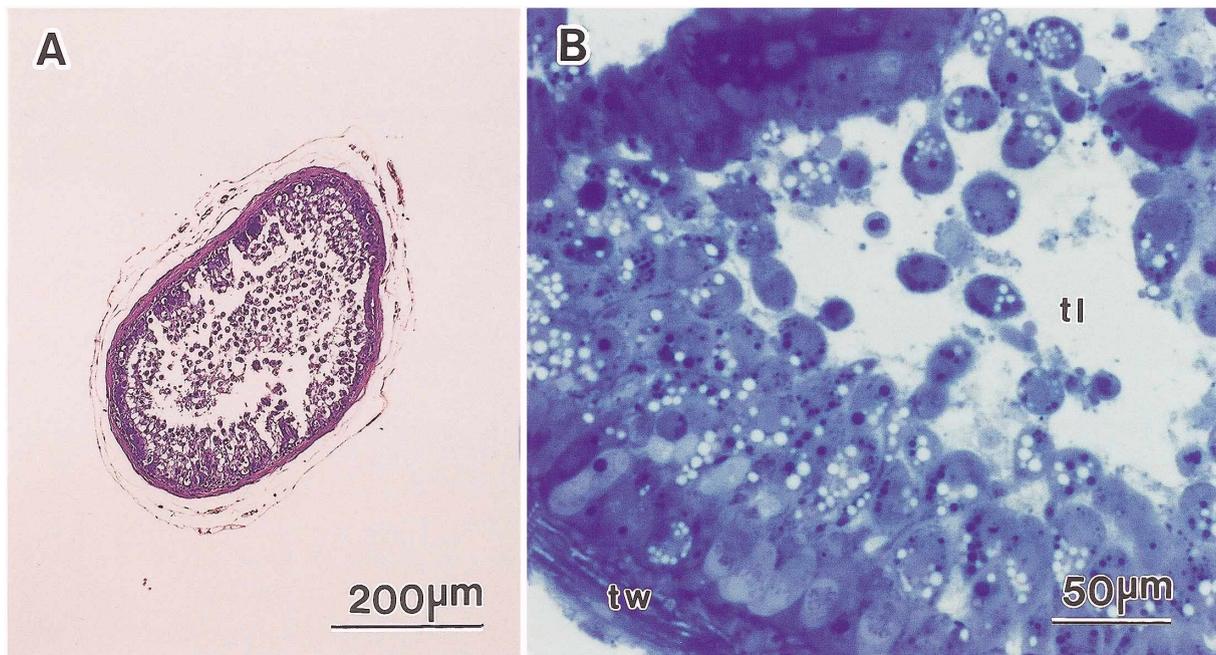


Fig. 4. Light micrographs of testis of *Syngnathus schlegeli*. Transverse section (A, HE stain) and magnified view near the testicular wall (B, toluidine blue stain). The testis being a hollow tube does not form usual cysts. Spermatozoa cannot be identified by light microscopy, and unidentified cells containing oil-like droplets and holes are observed. *tl*, testicular lumen; *tw*, testicular wall.

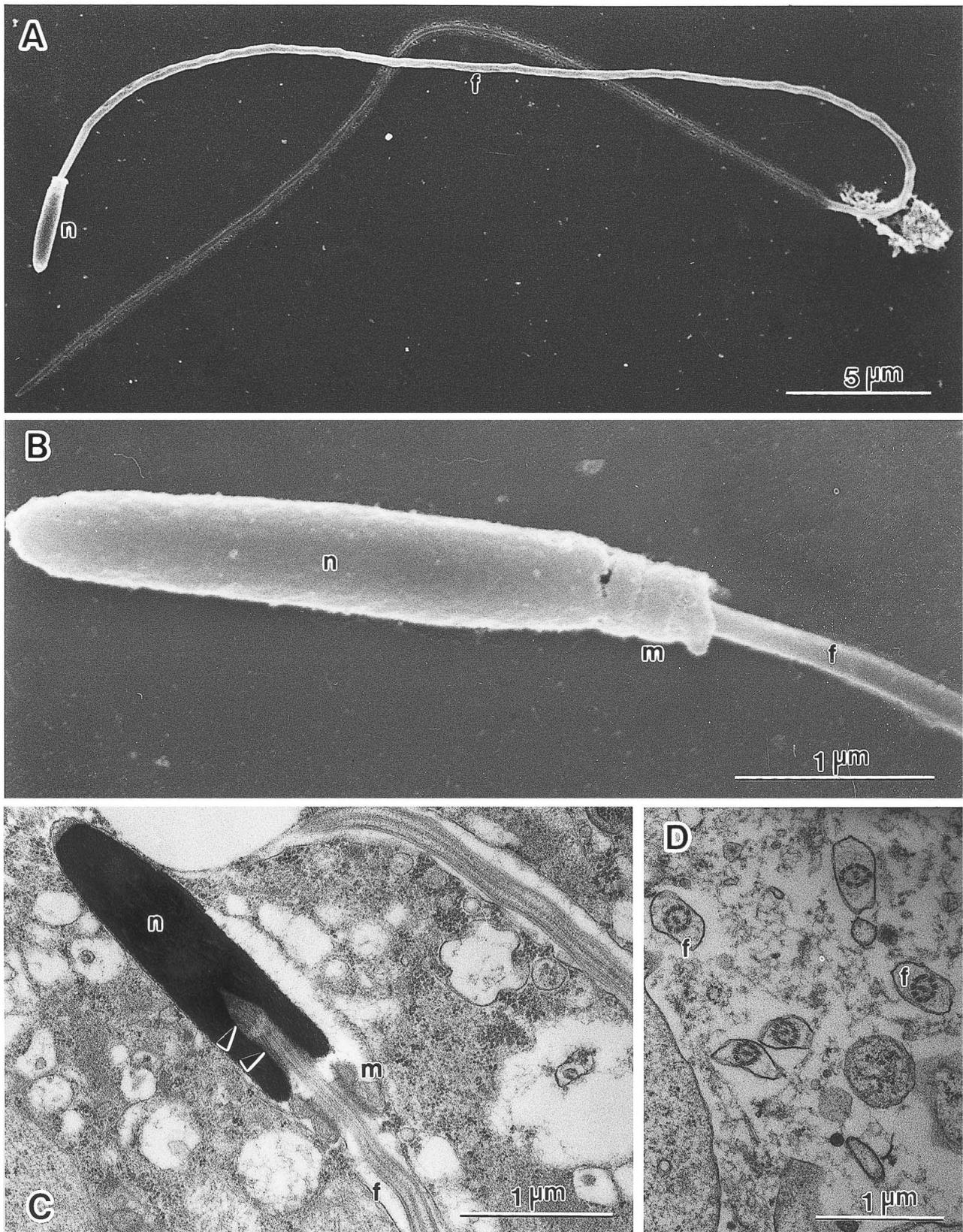


Fig. 5. Electron micrographs of testicular spermatozoa of *Syngnathus schlegeli*. Whole image (A) and magnified view of head region (B). Longitudinal section of head region (C). Transverse section of flagella (D). *f*, flagellum; *n*, nucleus; *m*, mitochondrion; *arrowheads*, centrioles.

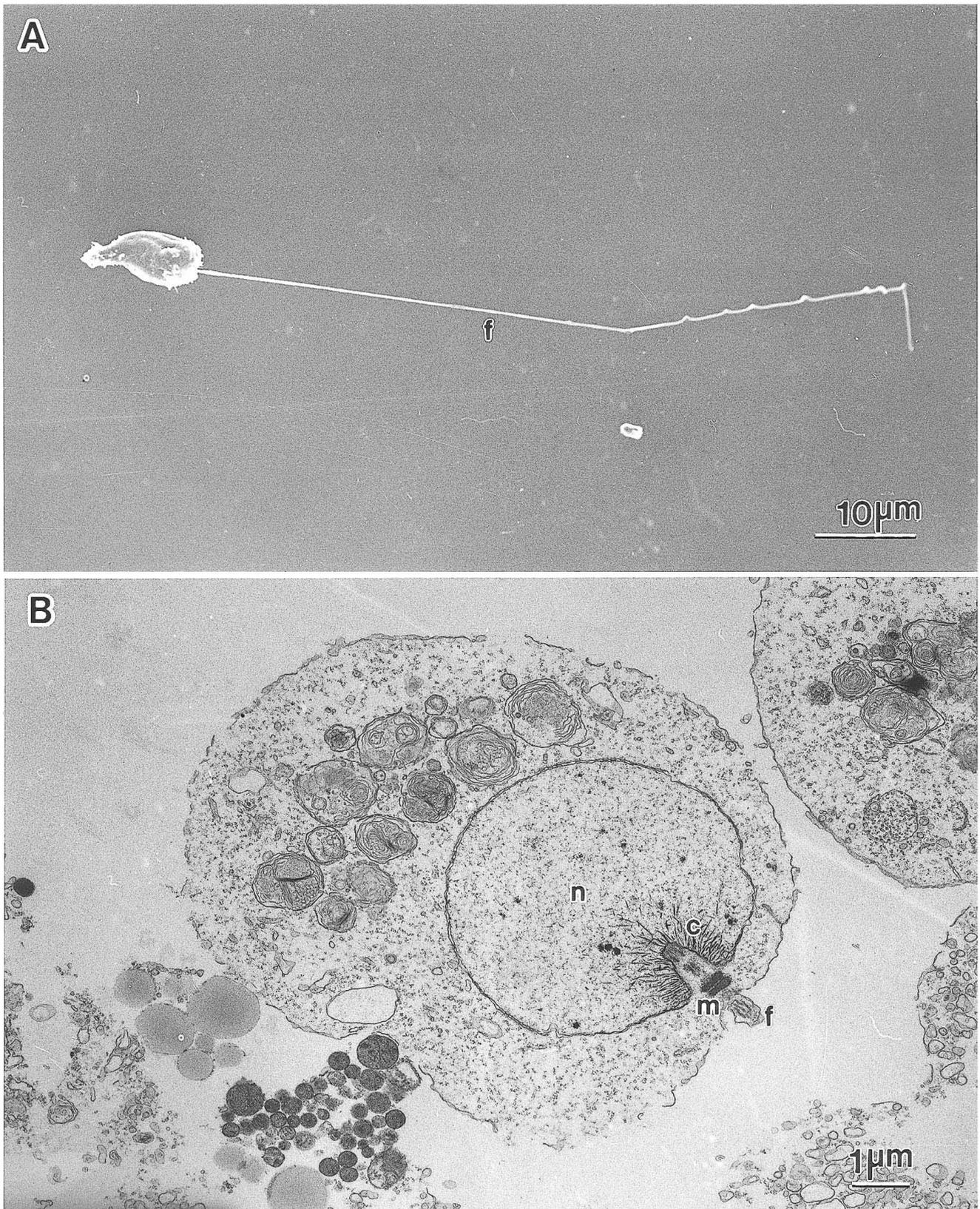


Fig. 6. Electron micrographs of atypical spermatozoa of *Syngnathus schlegeli*. Whole image (A) and longitudinal section of head region (B). The head region contains a nucleus and vesicles among cytoplasm. *c*, chromatin; *f*, flagellum; *n*, nucleus; *m*, mitochondrion.

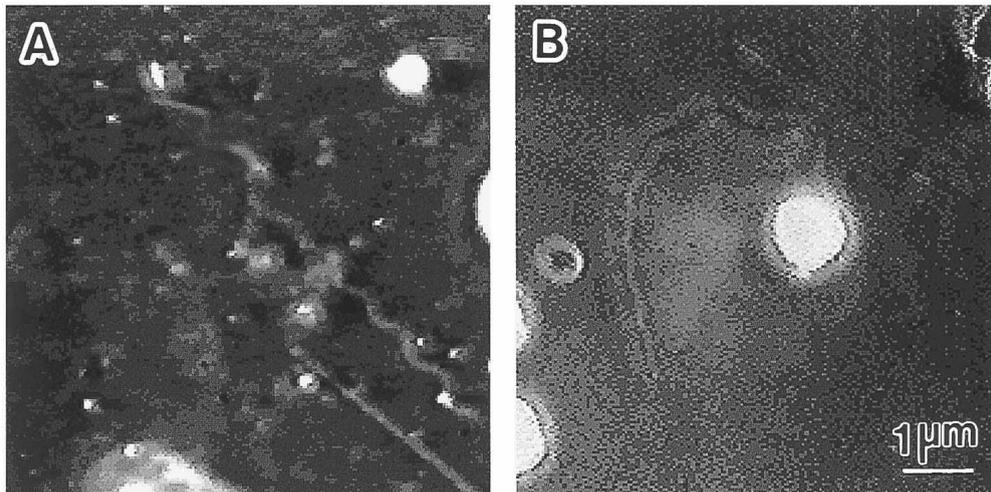


Fig. 7. Video images of motility of two types of testicular spermatozoa of *Syngnathus schlegeli*. Typical spermatozoa (A) swim straight (84.1 ± 43.8 $\mu\text{m}/\text{sec.}$, $\pm\text{SD}$, $n=3$). Atypical spermatozoa (B) swim in circles (45 round/min.).

was no more than 2 in all specimens. The nuclei were seen mostly near the testicular wall, and the direction of the spermatozoon relative to the testis was variable. The spermatozoon consists of a nucleus, a mitochondrion, and a single finned flagellum (Fig. 5A, B and C). The total length, nucleus length and nucleus diameter of the testicular spermatozoa was about $85 \mu\text{m}$, $3 \mu\text{m}$ and $0.6 \mu\text{m}$, respectively. Mitochondrion forming two to three spiral rows around the flagellum is located at the basal part of the elongate bullet-shaped nucleus (Fig. 5B and C). The chromatin is in the form of electron dense masses. Two centrioles mutually forming 45-degree angle were observed in the deep basal fossa at the base of the finned 9+2 flagellum (Fig. 5C and D).

Another type of testicular spermatozoa with the head region about 3 times as large as that of typical spermatozoon was observed by electron microscopy (Fig. 6A). This spermatozoon is hereafter referred to as atypical spermatozoon. The longitudinal sections of the atypical spermatozoon revealed the presence of two centrioles, annular mitochondria, nucleus with chromatin condensed in fibrillate fashion and endoplasmic reticulum-like vesicles among cytoplasm (Fig. 6B).

Spermatozoan Motility

The typical spermatozoa swim straight by beating the entire length of the elongate flagellum (Fig. 7A). Up to 7 waves were recognized in the flagellum. The progressive speed was $84.1 \pm 43.8 \mu\text{m}/\text{sec.}$ ($\pm\text{SD}$, $n=3$). The atypical spermatozoa mostly swim in circles (45 round/min.) also by beating the flagellum (Fig. 7B). The direction is more often clockwise than counter-clockwise.

DISCUSSION

Females were more active than males in the courtship behavior of *S. schlegeli*. Females seemed to compete each other in access to mate through parallel swimming, which is considered to be a kind of lateral display often observed in

fish courtship (e.g. Kuwamura, 1983; Akagawa and Okiyama, 1995). The males appeared to make the final decision to mate. Thus, sex role seemed to be reversed as reported in other species in the genus *Syngnathus* (Berglund *et al.*, 1986a; Berglund and Rosenqvist, 1990, 1993). Not only the sex role but also the mode of fertilization seemed to be reversed in *S. schlegeli*. A pair copulates in order for the female to spawn eggs directly into the male brood pouch, contrary to the case of viviparous fishes in which the sperm are ejaculated into female body during copulation. Although sperm ejaculation was not externally recognizable, given no other chance, fertilization is considered to occur in the brood pouch during copulation and/or wiggling. The mode of fertilization in *S. schlegeli* may be termed "male internal fertilization".

The fertilization environment of *S. schlegeli* seemed to resemble the situation in internal fertilization. While mucous cells in the embryonic esophagus was stained by PAS and alcian blue, the brood pouch epithelium was not stained, indicating the absence of mucous secretion from the epithelium. Electron microscopy has revealed that the brood pouch epithelium consisting of pavement cells and mitochondria-rich cells lacks mucous cells (Watanabe *et al.*, 1999). The absence of the mucous secretion from the brood pouch epithelium indicates that the viscous fluid filling the brood pouch lumen is of exogenous origin, perhaps mainly ovarian fluid and a small amount of seminal plasma. Fish eggs are immersed in ovarian fluid regardless of the species or the type (i.e. cystovarium or gymnovarium) of ovary (Koya *et al.*, 1993). The ovarian fluid and seminal fluid of *S. schlegeli* enter the brood pouch during the copulation and may hardly become dilute, being encased in the brood pouch that is sealed up subsequent to the copulation. Thus, the spermatozoa are considered to swim in viscous ovarian fluid during the fertilization.

Based on the morphological features, the spermatozoon of *S. schlegeli* may be categorized in introsperm (internally fertilizing sperm) (*sensu* Rouse and Jamieson, 1987), which is characterized by an elongate nucleus, midpiece (i.e. the

region bearing mitochondria) and/or flagellum. For instance, the longitudinal section of the spermatozoan nucleus of *S. schlegeli* resembles that of viviparous poeciliid, *Gambusia affinis* (Jamieson, 1991). The spermatozoan flagellum of *S. schlegeli* is more than twice as long as that of the closely related externally fertilizing species, such as *Gasterosteus aculeatus* and *Hypoptychus dybowski* (Hara and Okiyama, 1998). Although adaptive significance of introsperm to internal fertilization has not been understood, the resemblance of the spermatozoon of *S. schlegeli* to introsperm is considered the consequence of convergent evolution for the adaptation to peculiar "male internal fertilization". The bullet-shaped nucleus of the spermatozoa may be advantageous for penetration through viscous ovarian fluid. Furthermore, the spermatozoa were found to swim straight. While many teleost spermatozoa swim more or less in circular motion, straight progression of spermatozoa is typically seen in internally fertilizing elasmobranchs (Ishijima *et al.*, 1998).

Apart from the typical spermatozoa, another type of spermatozoa was observed in the present study. It is not certain from available data alone whether the atypical spermatozoa are immature spermatozoa or functional atypical spermatozoa that have been reported in some organisms, such as moth (Osanai *et al.*, 1987) and sculpin (Hayakawa *et al.*, 1997). The atypical (apyrene) spermatozoa of *Bombyx mori* (moth) swim in circles and are considered to agitate the heterogeneous and highly viscous fluids in the spermatophore, which contain enzymes necessary for the maturation of the typical spermatozoa (Osanai *et al.*, 1987). The atypical spermatozoa of *S. schlegeli*, which were also found to swim in circles, may also stir up and homogenize the seminal fluid, ovarian fluid and seawater in order to assist the motility of the typical spermatozoa. However, further investigations on spermatogenesis and observations of the spermatozoan motility in media other than seawater, such as physiological saline and ovarian fluid, are necessary to elucidate the nature of the two types of spermatozoa in *S. schlegeli*.

While elucidation of the morphology of mature spermatozoa was achieved despite their extremely small number, the amount of data obtained was insufficient to analyze the spermatogenesis of *S. schlegeli*. The effective union of gametes by external fertilization poses a problem for marine fishes. Eggs and spermatozoa become dilute rapidly in seawater; therefore, the number of spermatozoa must be large for successful fertilization. In contrast, small number of spermatozoa of *S. schlegeli* may be sufficient to fertilize the eggs incased in the brood pouch. *S. schlegeli* may produce spermatozoa only enough for fertilization and allocate more energy to physiological maintenance of the embryos in the brood pouch (Quast and Howe 1980; Haresign and Shumway 1981; Watanabe *et al.*, 1999).

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