

1999

The role of male brood pouch in the reproduction of the seaweed pipefish,

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ヨウジウオ (*Syngnathus schlegel*) の繁殖における雄の育児嚢の役割

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THE ROLE OF MALE BROOD POUCH IN THE REPRODUCTION OF THE  
SEAWEED PIPEFISH, *SYNGNATHUS SCHLEGELI*

ヨウジウオ(*Syngnathus schlegeli*)の繁殖における雄の育児嚢の役割

by

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March 1999

A Dissertation Presented to  
The Graduate School of Agricultural and Life Science,  
The University of Tokyo

in Partial Fulfillment of the Requirements for the Degree of Doctor of Agriculture

Dissertation directed by  
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## CONTENTS

1 INTRODUCTION-----	1
2 REPRODUCTIVE SEASON, BROODING PERIOD AND NEWBORN----	4
3 REPRODUCTIVE BEHAVIOR AND SPERMATOOZOA-----	19
4 MATE CHOICE, MALE FECUNDITY AND DEVELOPMENTAL STAGE OF NEWBORN-----	33
5 PATERNAL NUTRIENT PROVISION TO EMBRYO-----	51
6 OSMOREGULATION IN THE BROOD POUCH-----	69
7 DISCUSSION AND SUMMARY-----	83
ACKNOWLEDGMENTS-----	89
LITERATURE CITED-----	91
ABSTRACT IN JAPANESE-----	103

## 1 INTRODUCTION

Living organism of any kind makes every effort to increase the survival of its descendants and has developed a variety of tactics to achieve superior reproduction. Various life histories of organisms stem from a large variety of reproductive features. Reproduction is one of the most important components of life history both from parent's and offspring's point of view.

Fishes have evolved reproductive strategies ranging from external fertilization (oviparity) to bearing of embryos (viviparity). In large measure, these differences reflect different approaches to increase fitness. Some oviparous species spawn millions of pelagic eggs in the oceanic waters (e.g. sunfish, Breder and Rosen, 1966; tuna, Schaefer, 1996); some lay smaller number of demersal eggs and protect them until hatching (e.g. filefish, Akagawa and Okiyama, 1995; cardinalfish, Kuwamura, 1983). One of the extreme cases of zygote protection is found in viviparous fishes, such as surferperch (Mizue, 1961; Schultz, 1993), molly (Trexler, 1997), and rockfish (Takemura *et al.*, 1994; MacFarlane and Bowers, 1995).

Another method of parental care is found in the family Syngnathidae, commonly known as pipefishes and seahorses. In the members of syngnathids, males brood eggs by attaching them either to the ventral body surface or to brood organ which may or may not be developed into a pouch (Nelson, 1994). The brood organ, commonly referred to as brood pouch (even if it is not developed into a definite pouch), is located either on the abdomen or tail. Two taxonomic groups were once recognized based on the location of the brood pouch: Gastrophori (abdominal group) and Urophori (tail group) (Duncker, 1915). Among Urophori, Herald (1959) classified the brood pouch into 5 types and advocated a hypothetical evolutionary sequence of the brood pouch development, that is to say, the primitive open-pouch had developed into the complex closed-pouch by the elongation and fusion of the skin folds containing osseous tissue.

Not only are the prenatal embryos in the brood pouch of syngnathids



protected from physical threats, such as predation, they are also considered to be physiologically maintained during incubation. The prenatal embryos in the closed brood pouch are provided with gas exchanges (Nikolsky, 1963). The brood pouch is supposed to provide the embryos with nutrients (Haresign and Shumway 1981; Berglund *et al.*, 1986a; Wetzel and Wourms, 1991). Osmolality in the brood pouch is regulated in order to protect the embryos from hyper- or hypoosmotic environment depending on the environmental salinity (Leiner 1934; Quast and Howe 1980; Watanabe *et al.*, 1999). However, the physiological functions of the syngnathid brood pouch are only superficially known. Azzarello (1991) suggested that the brood pouch does not serve as the primary nutritional source or as an osmotic buffer for the prenatal embryos.

Although quite a large number of ecological study has been conducted on syngnathids (e.g. Howard and Koehn, 1985; Staffe *et al.*, 1989; Lazzari and Able, 1990; Tomasini *et al.*, 1991; Franzoi *et al.*, 1993; Teixeira and Musick, 1995; Vincent *et al.*, 1995), little is known of the Japanese species, perhaps owing to their negligible commercial importance in Japanese market. However, in other parts of eastern Asia, syngnathids, as well as closely related seamoths (Pegasidae), are commonly used in traditional Chinese medicine. Some species are threatened to become extinct because of recent overexploitation.

For the past decade, sex role reversal has been the subject of major interest in reproductive behavioral studies of syngnathids. Because of exclusive paternal care of embryos and consequent depression of male reproductive rate below that of females, sex role is reversed in some pipefish species (Berglund *et al.*, 1986a; Vincent *et al.*, 1992; Berglund and Rosenqvist, 1993). Achievement of reproductive success depends on intrasexual competition for access to mate. Contrary to the usual pattern, females compete more intensely than males for access to mate in some pipefishes. These behavioral studies are mainly restricted to species occurring in the Atlantic. There is only one report on a Japanese species, *Hippichthys penicillus* (Watanabe *et al.*, 1997).

The family Syngnathidae comprises 52 genera with approximately 215

species of pipefishes and seahorses (Nelson, 1994). Most species in the family Syngnathidae inhabit warm temperate to tropical waters. Some species can be found in relatively cool water, from southwestern Alaska to Tierra del Fuego at the southern tip of Argentina (Nelson, 1994). In Japan, 49 species have been reported. The seaweed pipefish, *Syngnathus schlegeli*, probably is the most ubiquitous species among those reported. It lives in seagrass bed in the coastal waters along all over Japan except for Ryukyu archipelago (Seno, 1993). Some reproductive aspects of paternal brooding of *S. schlegeli* have been briefly reported. Male *S. schlegeli* possesses the brood pouch on the tail. The brood pouch consists of two lateral skin folds whose free borders are glued together after deposition of eggs (Noumura, 1957-60). The embryos of *S. schlegeli* hatch in the brood pouch about a week before emergence from the brood pouch as juveniles after exhaustion of yolk (Tamura and Rikimaru, 1957; Takai and Mizokami, 1959).

In this thesis, various aspects of the reproductive biology of *S. schlegeli* were studied. The main scope of the study was to investigate the physiological functions of the brood pouch and their direct and indirect effects on other aspects of reproduction, such as reproductive behavior, mode of fertilization, gonad morphology, and developmental stage of newborn. In chapter 2, general ecology of the pipefish, including reproductive season, sex ratio, habitat and duration of brooding is described. Also, relative growth and feeding and swimming ability of the newborn are briefly described. Chapter 3 deals with courtship behavior, mode of fertilization and spermatozoa. In chapter 4, mate preference, brood size of males and newborn weight, size and developmental stage are described. Chapter 5 probes into the paternal nutritional provision to prenatal embryos through the brood pouch by employing various methods. In chapter 6, osmoregulatory functions of the brood pouch are investigated. A summary and overall discussion are given in chapter 7.

## 2 REPRODUCTIVE SEASON, BROODING PERIOD AND NEWBORN

### Introduction

Pipefishes form an important component of the ichthyofauna of vegetated coastal habitats (Howard and Koehn, 1985). The number of ecological studies of pipefishes have increased in the past decade (Howard and Koehn, 1985; Staffe *et al.*, 1989; Lazzari and Able, 1990; Tomasini *et al.*, 1991; Franzoi *et al.*, 1993; Teixeira and Musick, 1995; Vincent *et al.*, 1995). Wide range of topics are covered in these studies, including habitat selection, diet, reproductive cycle, and population dynamics. However, little is known about the pipefishes in Japan.

The seaweed pipefish, *Syngnathus schlegeli*, is the most common and widespread species in Japan (Seno, 1993). Some aspects of the seaweed pipefish reproduction have been briefly reported by Tamura and Rikimaru (1957) and Takai and Mizokami (1959). However, knowledge on its ecology based on the periodical field data has been extremely limited. In this chapter, the brooding season, sex ratio, development of the brood pouch and the amount of eggs in the brood pouch of *S. schlegeli* in seagrass beds during the period from spring to autumn will be presented. The findings will be discussed in view of the sexual selection theory. Brooding period, swimming and feeding behaviors of newborn were also investigated and will be discussed in relation to the results of relative growth measurements.

### Materials and Methods

#### *Field Collection*

*S. schlegeli* 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°20' N, 141°54' E). A small boat seine (43×3 m, mesh size 1×1 cm) was operated on semi-monthly basis from May to October in 1995, and from April to August in 1996 and 1997. The seagrass beds were distributed in small patches, and an entire patch was encircled with the net for each tow. Time spent on the collection each day was about 3 hr (approx. 10 tows). In 1995, all specimens were retained, but most individuals were released to the collection sites after obtaining data in 1996 and 1997.

Various aspects of the specimens were examined including the quantity, sex, standard length (SL), development of the brood pouch and amount of eggs in the brood pouch. Sex was determined externally by the presence of the brood pouch. Males were recognized by the two lateral skin folds extend from ventral sides of the tail and merge at the center to form the brood pouch. The brood pouch with skin folds not extended enough to merge was considered to be immature. Individuals with a mature or immature brood pouch were categorized as male, and those smaller than them were categorized as juvenile. Bias in sex ratio was statistically analyzed by z-test. The brood pouch fullness was expressed in % of the longitudinal length of the pouch area occupied with eggs in the entire pouch length. Student t-test was conducted to determine statistical differences between SLs of males and females and between SLs of males with full and empty brood pouch for those with mature brood pouch.

Data of the three years were compiled to investigate the monthly trends. The changes in water temperature, one of the most important environmental factors controlling the reproductive cycle of fishes (Bye, 1984), in Otsuchi Bay showed similar trends in the three years (Figure 2.1 after Otobe *et al.*, 1996, 1997, personal communication). Maximum differences in monthly mean temperature from May to October of the three years were within 1.3°C.



### *Brooding Period and Behavior of Newborn*

Brooding period of *S. schlegeli* was determined by rearing 13 males (151 - 254 mm SL) mated in the laboratory. The brooding males were separately stored in 20-L tanks and fed on natural zooplankters and *Artemia* nauplii until parturition. Water temperature of the tanks was measured everyday at 14:00. The water in the tanks was partially changed after measuring the temperature everyday. Neither light nor temperature was controlled. Newborn were fixed in a 10% buffered formalin within 6 hr after parturition for measurements of relative growth.

Newborn were transferred to a 20-L tank for the observation of feeding and swimming behaviors. Rearing method of the newborn followed Hayashi *et al.* (1989). The newborn were fed natural zooplankters everyday during the observations.

### *Relative Growth: Measurements of Body Parts*

Snout, head, caudal fin and total length (TL) of the field-caught and reared *S. schlegeli* were measured. Measurements of juveniles (n=19) and adults (n=17) were done with a digital caliper to the nearest 0.01 mm. Measurements of larvae (n=31) were done using a dissecting microscope with a camera lucida. Length of each part was expressed in % of TL.

## Results

### *Occurrence, Size and Sex Ratio*

*S. schlegeli* was the predominant pipefish resident of the surveyed area. No other syngnathids were collected, except for a small number of *Urocampus nanus*. A total of 447 *S. schlegeli* were collected in the three years. Although the net



collections covered almost entire seagrass beds in the survey area, only 149 *S. schlegeli* were captured a year because the population was relatively small in the area. The total number of male and female collected in the three years was 262 and 168, respectively, and thus the overall sex ratio was biased in favor of males (z-test,  $p < 0.01$ ). Males outnumbered females in June and July. The sex ratio was biased to females in October. (Table 2.1).

The trend in the number of catch per day showed that *S. schlegeli* inhabit the shallow seagrass beds from May to at least October (Figure 2.2). No males were collected in April (Table 2.1). Juveniles first occurred in the seagrass beds in May (Table 2.1). Juvenile occurrence was inconsistent throughout the survey period. Juvenile SL ranged from 74 to 147 mm.

Male and female SL ranged from 133 to 277 and 147 to 277 mm, respectively (Table 2.1). The mean SL of the males ( $212.8 \pm 29.0$  mm,  $\pm$ SD) was significantly larger than that of the females ( $205.9 \pm 29.2$  mm) (two-tailed t-test,  $p = 0.02$ ), indicating the presence of sexual size dimorphism. As for each month, no significant sexual difference in SL was detected in May ( $p = 0.41$ ), June ( $p = 0.75$ ), July ( $p = 0.36$ ) and October ( $p = 0.68$ ), except for August ( $p = 0.004$ ). No trends were recognized in monthly mean SL fluctuation in the males. Female SL decreased through the season.

#### *Male Maturity*

The brood pouch of *S. schlegeli* is a secondary sexual organ developing with sexual maturity of the male. Larger proportion of males had a developing brood pouch in May (23.1%) than in subsequent months (Figure 2.3). The occurrence of immature males with a developing brood pouch decreased towards July (2.0%) and increased again in August (8.6%). In October, only two males were collected, both being mature. Size of the brood pouch development seemed to greatly vary among individuals (Figure 2.4). The largest immature male (215 mm SL) was observed in May, whereas the smallest brooding male was 134 mm SL in June.

### *Reproductive Season*

The reproductive season of *S. schlegeli* was determined by the brooding condition of males. No males appeared in April. The proportions of brooding males out of the total mature males was 33.3% in May, and gradually increased thereafter up to 94.1 - 100% from July to October (Figure 2.5). The end of the reproductive season could not be determined in this study.

### *Amount of Eggs in the Brood Pouch and Male Size*

Although most brooding males had a single clutch of eggs in the brood pouch, 2 to 3 different clutches were identified in some males, evinced by the presence of embryos at different developmental stages. The brood pouches were often found either full of eggs (74.3%,  $n=241$ ) or empty (19.5%). The occurrence of partially filled brood pouch was 6.2%. Full and empty brood pouches were observed in all size ranges from 134 to 277 mm SL. The mean SL of males with a full brood pouch ( $218.6 \pm 26.9$  mm,  $\pm$ SD,  $n=179$ ) was significantly larger than that of empty males ( $205.6 \pm 29.5$  mm,  $n=47$ ) (two-tailed t-test,  $p=0.003$ ). The proportion of male with a partially filled brood pouch was largest in June (8.0%), followed by May (5.1%) and July (4.1%). All males had either full or empty brood pouch in August and October. Most males with a partially filled brood pouch had eggs tightly packed at the posterior (66.6%) or anterior (33.3%) end of the brood pouch. Only one male had eggs scattered in the brood pouch. Partially filled brood pouch was observed in all size ranges from 167 to 245 mm SL, and the proportion of the brood pouch space occupied by eggs ranged from 20% to 90%.

### *Brooding Period*

The brooding period of *S. schlegeli* ranged from 14 to 28 days (mean 22.4 days) under the rearing temperature from 17.2 to 23.3°C. The brooding period had a negative exponential correlation to the mean rearing temperature during

brooding (Figure 2.6).

#### *Swimming and Feeding Behavior of Newborn*

Newborn larvae possessed an inflated swim bladder and were able to swim near the surface of water by beating the tail and caudal fin. Typically, they quickly twisted their body 3 or 4 times, rested for a while, and twisted again. The orientation of the body was mostly parallel to the water surface (Figure 2.7B). As the larvae grew larger, they started to rely more on the dorsal fin and slowly swam with their head up (Figure 2.7C) in the same posture as seen in adults (Figure 2.7A). Horizontally oriented swimming of larger larvae with caudal fin became slower and sinuous. Unlike adult, larvae did not rest on the bottom of the rearing tank but instead stayed drifted in the water.

Newborn larvae were able to feed on small zooplankters. When feeding, larvae quickly flicked their head upward and swam slightly forward, sucking the prey by expanding the snout both horizontally and vertically. Adult also flicked the head for suction feeding, but the snout did not seem to be expandable.

#### *Relative Growth*

The snout length in % of TL increased drastically from about 4% to 7% (i.e. 75% increase from the original proportion) during the early development (Figure 2.8A), while no clear change in the relative head length was observed (Figure 2.8B). Caudal fin length relative to TL abruptly decreased from about 5.5% to 3.5% (36% decrease) (Figure 2.8C) and relative tail length increased from about 45% to 55% (22% increase) also during the early development (Figure 2.8D).

#### Discussion

The reproductive season of *S. schlegeli* in the shallow seagrass beds in Otsuchi

Bay and Funakoshi Bay seemed to begin in May, reach its peak in July and last up to October or later. Few *S. schlegeli* occurred in the survey area in April. Lazzari and Able (1990) reported that *S. fuscus* in northern Mid-Atlantic Bight moves out of estuaries to deeper continental shelf in winter to avoid cooling estuarine waters. *S. schlegeli* may also stay in deeper offshore waters in winter and early spring for a thermal refuge. The concurrence of the appearance in seagrass beds and the initiation of reproductive activity indicates that the pipefish migrate to the coastal seagrass beds in spring in order to breed. Long reproductive season is also reported in other pipefish species. *S. taenionotus* and *S. abaster* in North Adriatic Sea, for instance, reproduce for 5 and 4 months, respectively (Franzoi *et al.*, 1993). The embryos in the brood pouch are considered to be physiologically maintained (Quast and Howe, 1980; Haresign and Shumway, 1981). The extensive paternal care of embryos may enable long-term stable reproduction in syngnathids despite variable environmental conditions in the coastal waters.

A number of species in the genus *Syngnathus* practice polygamous mating system: *S. abaster* (Tomasini *et al.*, 1991), *S. floridae* (Jones and Avise, 1997a), *S. rostellatus*, *S. schmidtii* (Gordina *et al.*, 1991), *S. scovelli* (Jones and Avise, 1997b), and *S. typhle* (Berglund *et al.*, 1988). Occurrence of males with 2 - 3 different clutches of eggs and males with partially filled brood pouch indicates that the mating system of *S. schlegeli* is also polygamous (polygynandrous). As eggs are transferred to the brood pouch through the anterior end by copulation (Chapter 3), further deposition of eggs could be achieved only if embryos are located posteriorly. Males carrying embryos in the anterior part of the brood pouch must wait for the emergence of them before proceeding to the next egg acceptance. Thus, the partial brooding, which was seldom observed in the present study, might not be preferable in terms of superior productivity.

Overall sex ratio of *S. schlegeli* was biased to male. Male-biased sex ratio is also reported in *S. taenionotus* (Franzoi *et al.*, 1993), *Stigmatopora argus* and *S. nigra* (Steffe *et al.*, 1989). Steffe *et al.* (1989) have suggested that one of the



possible explanations to the male-biased sex ratio is females occupying different habitats other than seagrass beds. Gender-based habitat selection has been experimentally demonstrated in *Syngnathus fuscus*, in which the males remain in protected seagrass habitats perhaps to offset the burden of reproduction costs (Roelke and Sogard, 1993). The variability in the sex ratio of *S. schlegeli* observed in the present study implies similar gender-based habitat selection.

Sex role reversal has been the focus of interest in pipefish reproduction for the past decade. The female-biased operational sex ratio (OSR, i.e. the sex ratio between individuals available for reproduction) is considered to be a driving force of sex role reversal (Berglund *et al.*, 1986; Berglund and Rosenqvist, 1990, 1993; Berglund, 1993; Vincent, 1994). According to these studies, OSR in pipefishes is biased to female by greater potential reproductive rate of the females than that of the males caused by the limitation of the males' brooding capacity. The female-biased OSR results in the females competing more intensely for access to mate and the males being fastidious in mate choice.

At the beginning of reproductive season, the sex ratio of *S. schlegeli* was equal, and there was no significant sexual size difference. Takai and Mizokami (1959) have reported a close match of male brood pouch capacity and similarly sized female's batch fecundity in *S. schlegeli*. Thus, assuming that all mature individuals are available for reproduction, the OSR is predicted to be equal. Given that choosiness of the males with respect to mate choice is not a genetically fixed trait (Berglund, 1993 and 1994), sex role may not be reversed at the onset of the breeding season. During the peak of reproductive season, although the sex ratio was biased to male, the OSR is predicted to be female-biased due to that almost all males were brooding and that eggs are continuously produced by the females (Begovac and Wallace, 1987; Chapter 4). Therefore, the sex role is considered to be reversed.

The prolonged brooding period of *S. schlegeli* seems to cause high occurrence rate of brooding males. Increased water temperature within optimal range reduces incubation period of embryos and increases larval growth rate of fishes in



general, and it was not an exception in *S. schlegeli* even though the embryos are incubated inside the brood pouch. Sea surface temperature in Otsuchi Bay reaches about 20°C in the mid summer; therefore, the possible shortest brooding period in the bay is predicted to be around 20 days. Water temperature goes below 10°C in the spring, and brooding period may exceed 30 days.

After approximately one month of brooding, *S. schlegeli* released larvae that were capable of feeding and free swimming immediately after parturition. As the larvae did not rest on the bottom of the rearing tank but instead drifted in the water, they seem to spend planktonic life. The newborn larvae swam by beating the caudal fin. As larvae grew, the dorsal fin was started to be used for vertically orientated slow swimming. Relatively large caudal fin of larvae may support the locomotory capability during the early planktonic life. Although age determination of *S. schlegeli* was not conducted in the present study, abrupt proportional changes in length of body parts during the early development indicate that the body shape of larvae changes into that of adult in a short period of time after parturition. The snout length relative to total length increased by 75% from the initial proportion during the early development. The pipette-like snout of syngnathids is supposed to play an important role in suction feeding by producing a strong inhalant current (Howard and Koehn, 1985; Colson *et al.*, 1998). The elongation of the snout of larvae may improve the feeding capacity. Not only does the paternal brooding protect prenatal embryos from predation, but it also seems to be advantageous to the post-parturition survival of the larvae by reducing the duration of the most vulnerable early life history stage (Houde, 1987; Watanabe *et al.*, 1995; Watanabe and Kuroki, 1997).

Table 2.1 Standard length (SL, mm) and sex ratio of *Syngnathus schlegeli* collected in Otsuchi Bay and Funakoshi Bay from 1995 to 1997.

Month	Male SL (n) (mean $\pm$ SD)	Female SL (n) (mean $\pm$ SD)	Sex ratio M/M+F (%)	Juvenile SL range (n)	Collection (day)
APR	----	216.0 $\pm$ 22.6 (2)	----	----	2
MAY	213.7 $\pm$ 27.2 (39)	219.1 $\pm$ 30.2 (37)	51.3 $\sigma$ = $\varnothing$	113 - 147 (5)	3
JUN	211.0 $\pm$ 31.6 (138)	209.7 $\pm$ 26.2 (62)	69.0 $\sigma$ > $\varnothing$	97 - 116 (3)	5
JUL	211.2 $\pm$ 26.1 (49)	203.8 $\pm$ 19.1 (12)	80.3 $\sigma$ > $\varnothing$	----	2
AUG	*222.3 $\pm$ 30.8 (35)	*199.4 $\pm$ 32.2 (33)	51.5 $\sigma$ = $\varnothing$	55 - 137 (9)	5
OCT	193.0 $\pm$ 48.1 (2)	185.5 $\pm$ 21.1 (23)	8.0 $\sigma$ < $\varnothing$	----	1
TOTAL	*212.8 $\pm$ 29.0 (262)	*205.9 $\pm$ 29.2 (168)	60.9 $\sigma$ > $\varnothing$		18

The bias in sex ratio was determined by z-test ( $p < 0.01$ ). \* Significant sexual size difference was detected by two-tailed t-test ( $p < 0.05$ )

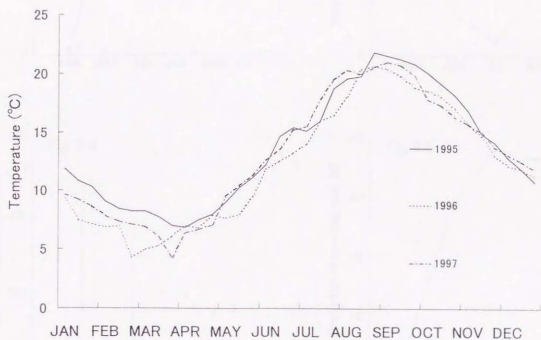


Figure 2.1 Water temperature at 1 m depth in Otsuchi Bay of the three years. (After Otake *et al.*, 1996, 1997 and personal communication) Temperature was measured every 10 min. Tertiary monthly mean temperatures are plotted.

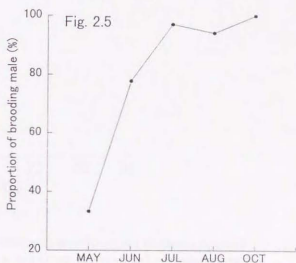
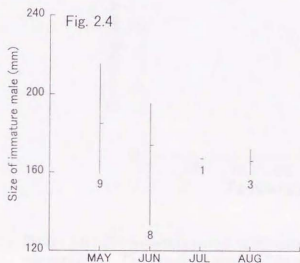
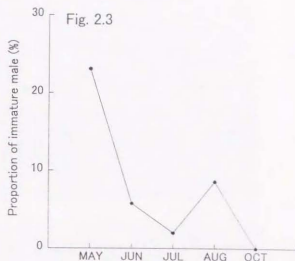
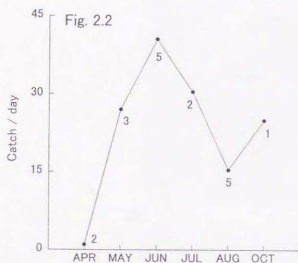


Figure 2.2 Monthly catch per day of *Syngnathus schlegelii* in seagrass beds in Otsuchi Bay and Funakoshi Bay from 1995 to 1997. Numbers indicate the number of days of collection (3 hr/day).

Figure 2.3 Monthly percentage of immature males with a developing brood pouch out of total males of *S. schlegelii* in Otsuchi Bay and Funakoshi Bay from 1995 to 1997.

Figure 2.4 Range and mean standard length of immature males with a developing brood pouch of *S. schlegelii* in Otsuchi Bay and Funakoshi Bay from 1995 to 1997. Numbers indicate the number of males.

Figure 2.5 Monthly percentage of brooding males out of total males of *S. schlegelii* in Otsuchi Bay and Funakoshi Bay from 1995 to 1997.

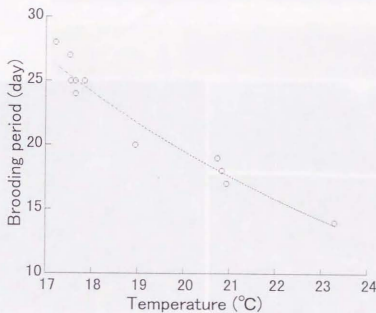


Figure 2.6 The brooding period of *Syngnathus schlegelii* as a function of mean rearing temperature. The brooding period ( $B$  days) had a negative exponential correlation to the mean rearing temperature ( $T$  °C) during brooding;  $B=163.2e^{-0.11T}$ , ( $p<0.001$ ,  $n=13$ ). The temperature was measured at 14:00.



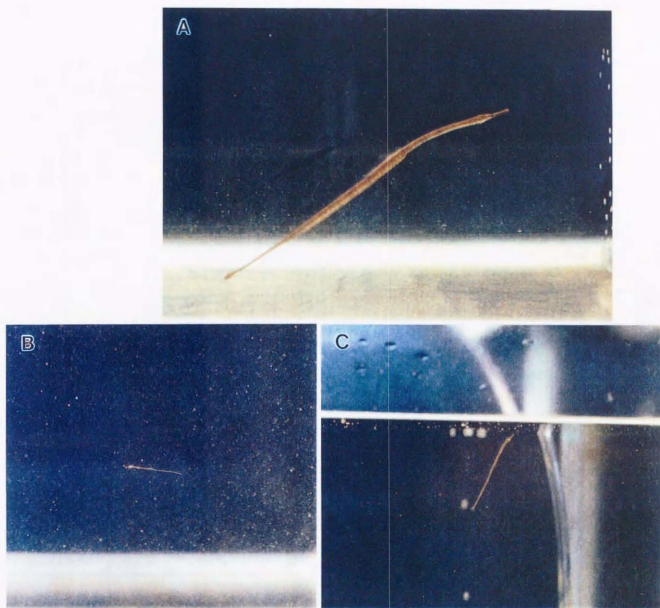


Figure 2.7 Swimming postures of adult male (A), newborn larva (B) and larva (C) of *Syngnathus schlegelii*. Adult typically swims with the head up by the wavy motion of the dorsal fin unless disturbed. Newborn larva swims by beating the caudal fin. As the larva becomes larger, it starts to rely more on the dorsal fin for swimming.

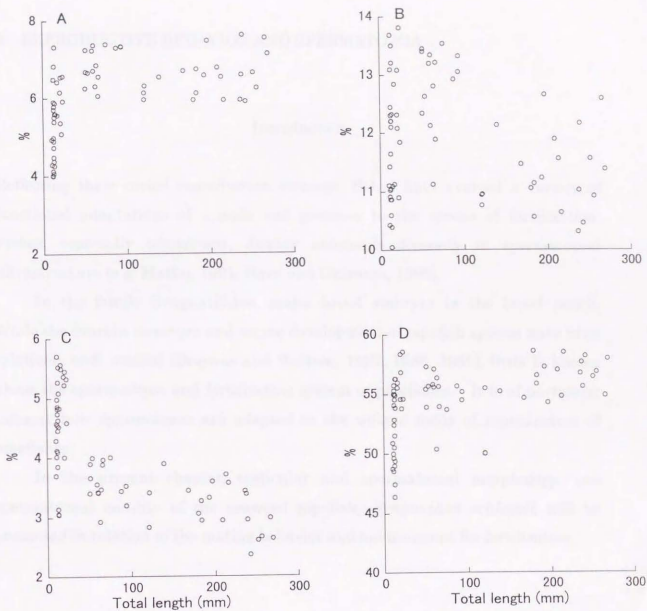


Figure 2.8 Scatter diagram of the relative growth of *Syngnathus schlegelii*. Snout length (A), head length (B), caudal fin length (C) and tail length (D). Each part is expressed in % of total length.

### 3 REPRODUCTIVE BEHAVIOR AND SPERMATOOZOA

#### Introduction

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

In the family Syngnathidae, males brood embryos in the brood pouch. While the ovarian structure and oocyte development of pipefish species have been relatively well studied (Begovac and Wallace, 1987, 1988, 1989), little is known about the spermatozoa and fertilization system of pipefishes. It is of particular interest how spermatozoa are adapted to the unique mode of reproduction of pipefishes.

In the present chapter, testicular and spermatozoal morphology, and spermatozoal motility of the seaweed pipefish, *Syngnathus schlegelii*, will be presented in relation to the mating behavior and environment for fertilization.

#### Materials and Methods

##### *Sampling Methods*

The specimens were collected in Otsuchi Bay and adjacent Funakoshi Bay on the Pacific coast of northern Japan (39°20' N, 141°54' E) by a boat seine (43×3 m, mesh size 1×1 cm) from 1995 to 1997. The males and females were separately placed in aerated 150-L tanks provided with continuously running filtered seawater at Otsuchi Marine Research Center. The pipefish were fed laboratory reared *Artemia* nauplii and natural zooplankters (mainly copepods) collected every

morning in Otsuchi Bay.

#### *Reproductive Behavior Observations*

Mating behavior was observed in an aquarium. Multiple males (non-brooding) and females were placed in a rectangular aquarium (150×50×75 cm) to mate: 13 females (152 - 253mm SL) and 9 males (147 - 257mm SL) for the first trial and 13 females (same as the first trial) and 6 males (163 - 264mm SL) for the second trial. The mating behavior was recorded with a video camera until the spawning was 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 was embedded in paraffin by the conventional method, and the cross sections (4  $\mu$ m) were stained with PAS (periodic acid Schiff reaction) and alcian blue coupled with Kernechtrot stain solution (Muto Pure Chem. Ltd.) in order to detect mucous secretion (Okumoto and Inagaki, 1988) from the brood pouch epithelium.

Testes were dissected out from the body cavity of non-brooding males, cut transversely into small pieces and fixed in cold 2% paraformaldehyde - 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for light and electron microscopy. 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<sub>2</sub> in a critical-point drier, sputter coated with platinum palladium, and observed with a scanning electron microscope (HITACHI S-4500).

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  $\mu$ m 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).

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 seawater on a slide glass. The direction and the speed of spermatozoal movement were analyzed from the video images.

## Results

### *Mating Behavior: Mode of Fertilization*

Courtship in *S. schlegeli* consisted of five distinct motor patterns: parallel swimming, twitching, rising up, copulation, and wiggling (Figure 3.1). Gronell (1984) originally 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*, some new terms were coined, and the revised definitions were listed below.

1. 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.

2. Twitching: Females performing parallel swimming and a male



approaching to females performing parallel swimming abruptly shook the body.

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

4. Copulation: A female pressed the slightly projected genital papilla over the anterior end of the male's brood pouch adjacent to the genital pore. The mates were orientated almost vertically and attached obliquely to each other. The female extruded eggs directly into the brood pouch.

5. 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.

While the extrusion of the eggs could be identified by quivering of the females, sperm ejaculation was not externally recognizable. However, given no other chances for the sperm to enter the brood pouch, fertilization is considered to occur during copulation and/or wiggling.

#### *Brood Pouch Fluid*

Two skin folds extending from the lateral sides of the tail form the ventral brood pouch of *S. schlegeli*. The skin folds merge at the center and are sealed from inside by placenta-like tissue after acceptance of the eggs. The placenta-like tissue, which is initially flat, develops into honeycomb-like structure, to each of whose compartment a single embryo is settled (see Chapter 5 and 6 for detail). The brood pouch was filled with environmental water and viscous fluid before and during the incubation, respectively.

Although the remnant viscous fluid in the paraffin sections of the brood pouch showed positive reaction to PAS and alcian blue staining, the pouch epithelium was not reactive (Figure 3.2A). The esophagus of developing embryos in the brood pouch showed positive reaction to PAS and alcian blue (Figures 3.2B

and 3.2C).

#### *Testis and Spermatozoal 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 were about 30 to 40% of that of the abdominal cavity. The testis is a semi-transparent hollow tube with cells containing oil-like droplets and holes near the wall, and were hardly any germinal cells observed by light microscopy (Figure 3.3). The density of spermatozoa in the testes seemed extremely low.

A small number of spermatozoa were found mostly near the testicular wall by electron microscopy. The number of spermatozoal nucleus observed in a transverse sectional area of the whole testis was usually 1 or 2. The direction of the spermatozoa relative to the testis was variable. The spermatozoon consists of a nucleus, a mitochondrion (Figure 3.4B), and a single finned flagellum (Figure 3.4D). The total length, nucleus length and nucleus diameter of the testicular spermatozoon was about 85  $\mu\text{m}$ , 3  $\mu\text{m}$  and 0.6  $\mu\text{m}$ , respectively. Two to three spiral rows of mitochondrion were situated at the basal part of the elongate bullet-shaped nucleus (Figure 3.4B). The chromatin is in the form of electron dense masses. Two centrioles mutually forming 45-degree angle were observed in deep basal fossa at the base of the finned 9+2 flagellum (Figures 3.4C, D).

Another type of testicular spermatozoa with the head about 3 times as large as that of typical spermatozoon was observed (Figure 3.5A). This spermatozoon is referred to as atypical spermatozoon. The cross section of the atypical spermatozoon revealed the presence of two centrioles, annular mitochondria and nucleus with chromatin condensed in fibrillate fashion among cytoplasm (Figure 3.5B).

### *Spermatozoal Motility*

The typical spermatozoa swim straight by beating the entire length of the elongate flagellum (Figure 3.6A). Up to 7 waves are recognized in the flagellum. The progressive speed was 59.5  $\mu\text{m}/\text{sec}$ . The atypical spermatozoa mostly swim in circles (45 round/min.) also by beating the flagellum (Figure 3.6B). 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). It was the males that 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 to spawn eggs directly into the male brood pouch, contrary to the case of viviparous fishes in which the spermatozoa are ejaculated into female body during copulation. The mode of fertilization in *S. schlegeli* may be termed "male internal fertilization".

The fertilization environment of *S. schlegeli* also seemed to resemble the situation in internal fertilization. The brood pouch epithelium was not stained by PAS or alcian blue. The absence of the mucous secretion from the brood pouch epithelium indicates that the viscous fluid filling the brood pouch lumen is derived from the female, perhaps ovarian fluid. 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 of *S. schlegeli* may

enter the brood pouch during the copulation and not 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 spermatozoal nucleus of *S. schlegeli* resembles that of viviparous poeciliid, *Gambusia affinis* (Jamieson, 1991). The spermatozoal flagellum of *S. schlegeli* is more than twice as long as that of the closely related externally fertilizing species, such as *Gasterosteus aculeatus* and *Hypopttychus dybowskii* (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 to be 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 whether the atypical spermatozoa are immature or aberrant spermatozoa or functional atypical spermatozoa that have been reported in other 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 investigation on spermatogenesis is necessary to elucidate the nature of the atypical spermatozoa.

Because of extremely small number of the spermatozoa, insufficient amount of data was obtained 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).



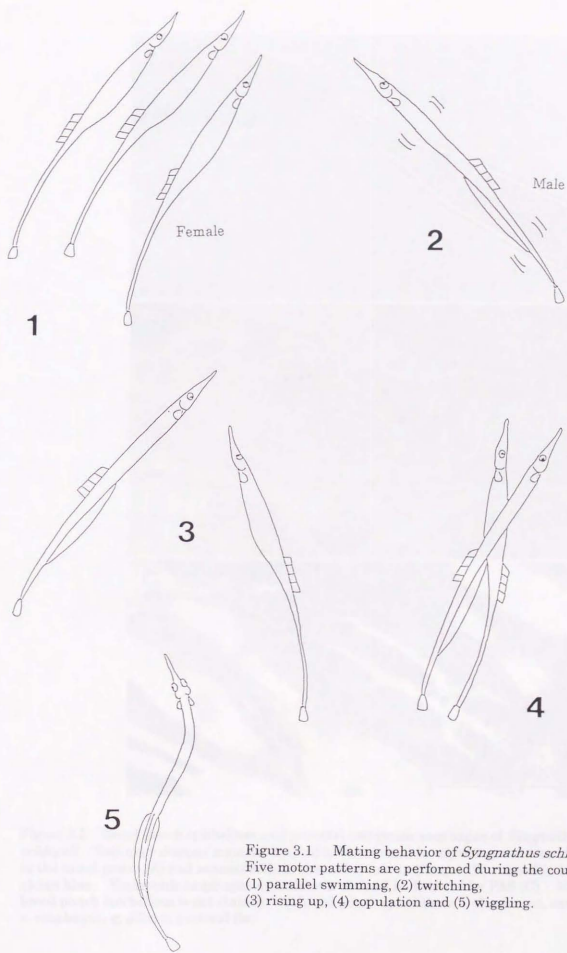


Figure 3.1 Mating behavior of *Syngnathus schlegelii*. Five motor patterns are performed during the courtship: (1) parallel swimming, (2) twitching, (3) rising up, (4) copulation and (5) wiggling.

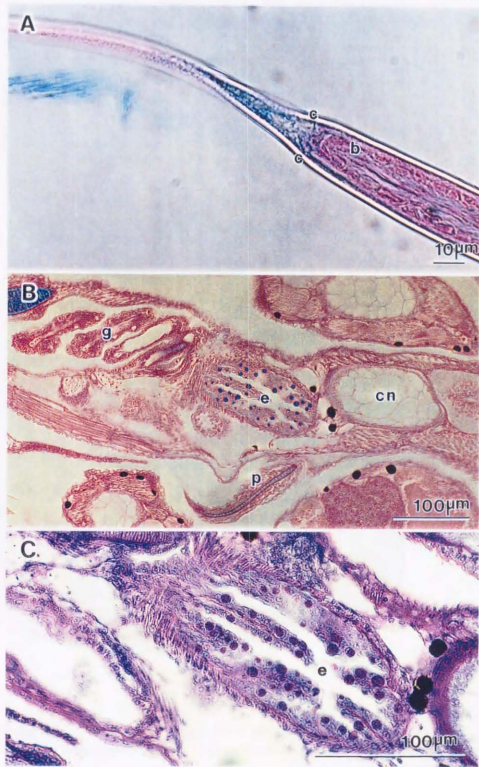


Figure 3.2 Brood pouch epithelium and prenatal embryonic esophagus of *Syngnathus schlegelii*. Remnant mucous material stained in blue bound by adjoining chorions of eggs in the brood pouch (A) and mucous cells in the embryonic esophagus (B) stained in blue by alcian blue. Embryonic esophagus mucous cells stained in deep red by PAS (C). Note brood pouch epithelium is not stained. b, brood pouch epithelium; c, chorion; cn, centrum; e, esophagus; g, gills; p, pectoral fin.

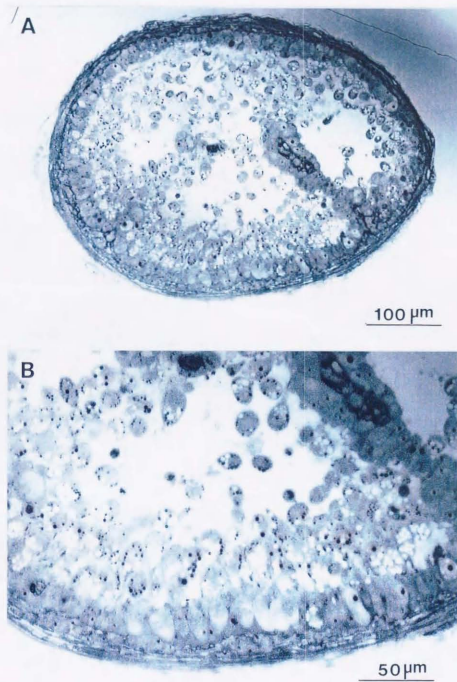


Figure 3.3 Cross section of the testis of *Syngnathus schlegelii*. Whole image (A) and magnified view near the testicular wall (B). 1 µm section stained with toluidine blue.

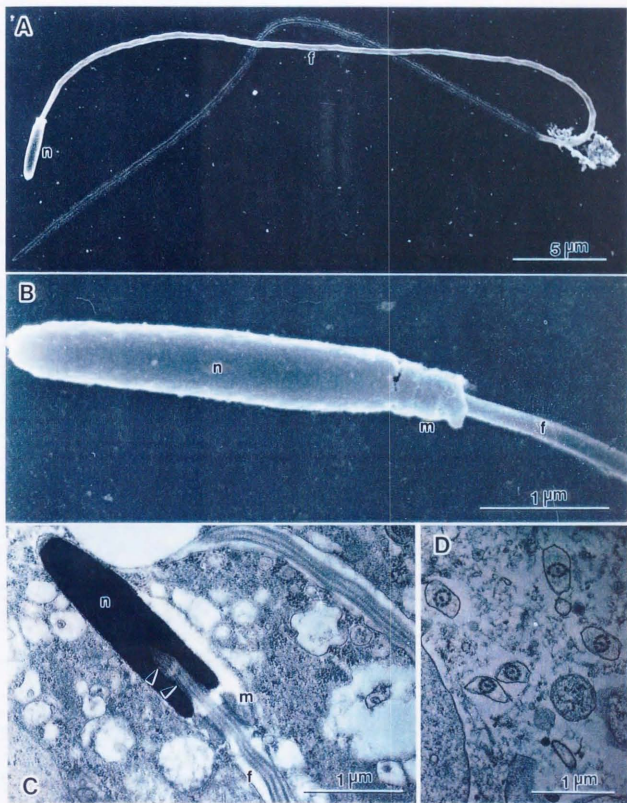


Figure 3.4 Electron micrographs of spermatozoa of *Syngnathus schlegelii*. Whole image (A), magnified view of nucleus (B). Longitudinal section of nucleus (C). Cross section of flagella (D). f, flagellum; n, nucleus; m, mitochondrion; arrowheads, centrioles.



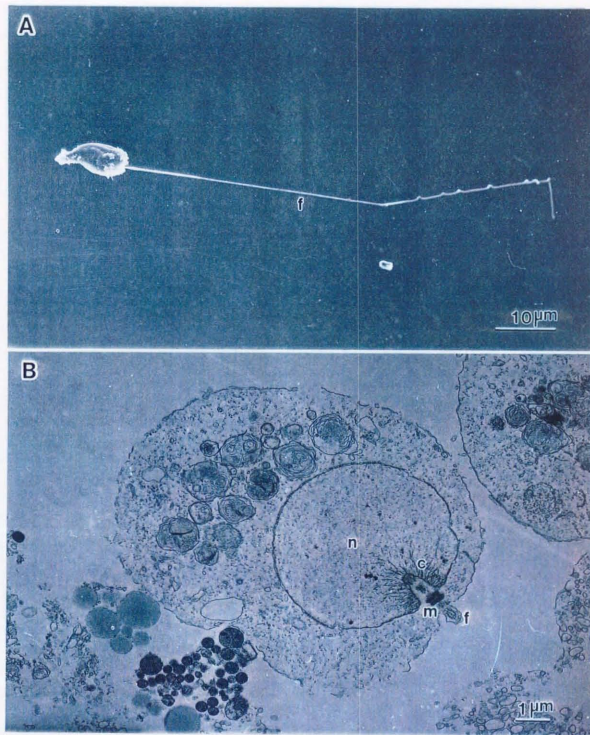


Figure 3.5 Electron micrographs of atypical spermatozoa of *Syngnathus schlegelii*. Whole image (A), longitudinal section of head region (B). The head region contains a nucleus and vesicles among cytoplasm. c, chromatin; f, flagellum; n, nucleus; m, mitochondrion.



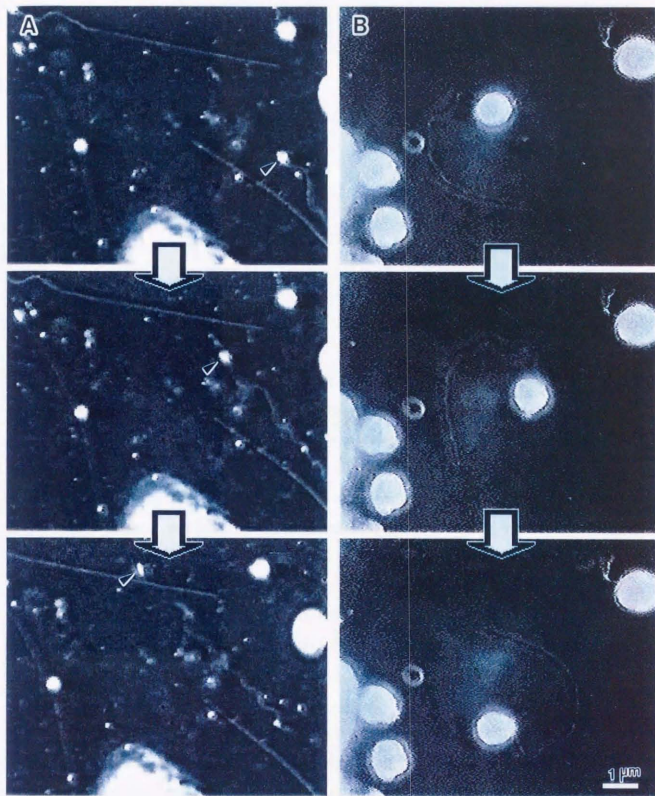


Figure 3.6 Motility of two types of testicular spermatozoa of *Syngnathus schlegelii*. Typical spermatozoon (A) swims straight ( $59.5 \mu\text{m}/\text{sec}$ ). Atypical spermatozoon with a large head (B) swims in circles ( $45 \text{ round}/\text{min}$ ).

#### 4 MATE CHOICE, MALE FECUNDITY AND DEVELOPMENTAL STAGE OF NEWBORN

##### Introduction

Much of the variation among life histories relates to reproduction. Since natural selection favors those individuals with the greatest fitness, it is inevitable that organisms produce robust offspring. The allocation of limited reproductive energy is manifested in the form of trade-off between fecundity and survivorship. Some organisms produce a large number of small vulnerable offspring (i.e. r-selected species); some produce a small number of larger and more developed offspring (K-selected species). In addition to interspecific competition, intraspecific competition is also an important component of reproduction. Members of each sex attempt to maximize their own reproductive success and thereby provoking sexual conflict. Both sexes prefer a mate with superior reproduction ability.

Among teleost fishes with considerable variations in reproductive strategies, syngnathid fishes (family Syngnathidae) display a particularly unique mode of reproduction. Syngnathids serve as a suitable material for the study of both natural and sexual selection.

In the present chapter, preferences in mate size, paternal fecundity, egg and newborn weight and developmental stage of newborn of the seaweed pipefish, *Syngnathus schlegelii*, will be investigated. The results will be discussed in the light of natural and sexual selection. The investigation will also attempt to determine the presence of paternal nourishment during the gestation based on the findings.

## Materials and Methods

### *Sampling Methods*

Specimens used in the present study were collected from seagrass (*Zostera*) beds in Otsuchi Bay and Funakoshi Bay on the Pacific coast of northern Japan (39°20' N, 141°54' E) by a boat seine (43×3 m, mesh size 1×1 cm) from 1995 to 1998. The males and females were separately kept in aerated 150-L circular tanks provided with continuously running natural seawater. They were fed wild zooplankters (mainly copepods) and laboratory reared *Artemia* nauplii during the experiments at Otsuchi Marine Research Center. The females used for egg weight measurements were fixed immediately after collection in a 10% buffered formalin solution.

### *Mate Choice Experiment*

Male's preference for the mate size was investigated in laboratory for the specimens collected in June and July, 1995. Three females of different body size were placed in a 80-L rectangular tank together with one male to examine which female would mate with the male (13 trials). Three standard length (SL) classes were established: small (<180 mm), medium (180 - 220 mm), and large (220 mm<). The females were assigned to one of these three size classes, and three females from the three different size classes were used in the mate choice experiments. The size classes were not necessarily followed, but used as a guide in principle because of the shortage in the specimens. In order to identify the spawned female, a video camera was run continuously to record the mating behavior. In the case where identification from the video images were impossible due to insufficient light, the female whose body depth decreased the most was determined to have spawned. Body depth was measured to the nearest 0.01 mm with a digital caliper before and after the experiments.

#### *Paternal Fecundity and Newborn Weight Measurements*

Only the males with the brood pouch filled with eggs when collected in June and July, 1995 were used in this experiment. The males with the embryos at the final stage of gestation were transferred separately in aerated 20-L tanks, in which they were allowed to give birth to larvae. The timing of parturition was predictable by the eye pigmentation of the prenatal embryos being visible through the brood pouch skin folds. The newborn larvae were fixed in a 10% buffered formalin solution within 6 hr after birth.

The SL and fecundity of the males were measured. The number of dead and unfertilized eggs was counted. The total length (TL) of newborn was measured under a dissecting microscope, and the presence of yolk was examined. Newborn from 20 males were weighed after being rinsed with distilled water and dried for 2 days in a 60°C oven. Because the newborn were small, randomly subsampled 100 siblings were pooled for the measurements of the dry weight. The volume of the brood pouch ( $V \text{ mm}^3$ ) was estimated in the assumption that the pouch is a cylindrical tube:  $V = (D/2)^2 \pi L$ , where  $D$  is the maximum diameter of the brood pouch and  $L$  is the longitudinal length of the brood pouch. The densities of the embryos in the brood pouch of 25 males were obtained as  $N/V$ .

#### *Female Size and Egg Weight Measurements*

Ovaries were dissected out from formalin fixed females collected in May 1998 in order to obtain mature eggs. From each of 15 females, 20 ovulated eggs were randomly collected. The eggs were rinsed with distilled water and dried for 2 days at 60°C for pooled measurements of the dry weight. The relationship between female size and average egg weight was analyzed.

#### *Morphological and Osteological Analysis of Newborn*

Illustration of newborn was prepared using a stereoscopic microscope with a



camera lucida. Osteological analysis of newborn was done by clearing and staining for bone with alizarin red and cartilage with alcian blue following Potthoff (1983). The newborn and adult samples for the osteological analysis were either fixed in 10% buffered formalin or stored at  $-30^{\circ}\text{C}$ . The frozen samples were thawed and fixed in 5% formalin for 1 hr before staining. Terminology of bones followed Gregory (1933), Azzarello (1989) and Colson *et al.* (1998).

Histological sections of eyes of newborn and adults were prepared by conventional paraffin method. Sections ( $4\text{ }\mu\text{m}$ ) cut with a microtome were stained with Mayer's haematoxylin and eosin. Nasal and epidermal sensory organs of newborn were observed by scanning electron microscopy. Newborn were fixed in cold 2% paraformaldehyde - 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 week at longest. The samples were rinsed overnight with 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide in the same buffer for 2 hr, dehydrated through graded ethanol series, dried with  $\text{CO}_2$  in a critical-point drier, sputter coated with platinum palladium, and observed with a scanning electron microscope (HITACHI S-4500).

## Results

### *Mate Choice*

In the 13 experimental replicates, mating took place in 6 instances. During the experiments, the males died one after another, but the females rarely died. Matings usually occurred between dawn to dusk within several days after the initiation of the experiment. In 8 out of the 12 experimental repetitions in which mating took place, the female whose body size was the closest to the male among the three was successful in mating (Table 4.1). In one case, the male mated with two females, in which the female mated first was closer to the male in standard



length. One mating filled at least 1/5 of the brood pouch with eggs in longitudinal length (Table 4.1).

### *Parturition*

The brood pouch expanded near the end of the brooding due to the prenatal embryonic development in the brood pouch. In most cases (48 out of 50), all embryos simultaneously emerged the brood pouch between late evening and early morning except for the two cases in which parturition lasted for two inconsecutive days. In these two cases, dead eggs occupied a great proportion, which were eventually discarded.

The males started slowly rolling and twisting the body several hours before the parturition. In some cases, newborn began to leave the brood pouch by these intermittent movements. The parturition was finished by rapid vigorous swimming and twisting of the body, in which all the embryos, together with dead and unfertilized eggs and parts of the placenta-like tissue of the brood pouch, were shaken out of the brood pouch through the entire length of the longitudinal fissure of the skin folds. The occurrence of dead and unfertilized eggs out of total brood was  $1.36 \pm 0.03\%$  (mean  $\pm$  SD, 27 males).

### *Paternal Size, Fecundity and Newborn Size*

The relationship between the volume of the brood pouch ( $V$ ) and SL of males ( $L$ ) was regressed to an exponential curve:  $V = 77.53e^{0.015L}$  (Figure 4.1;  $r^2=0.91$ ,  $p<0.001$ ,  $n=35$ ). The male fecundity ranged from 690 to 1482 and had a positive linear correlation with  $L$  (Figure 4.2;  $r^2=0.71$ ,  $p<0.001$ ,  $n=30$ ). These resulted in a negative correlation between the embryonic density in the brood pouch and  $L$  (Figure 4.3;  $r^2=0.68$ ,  $p<0.001$ ,  $n=25$ ). No correlation was detected between mean newborn TL and  $L$  (Figure 4.4;  $r^2=0.01$ ,  $p>0.1$ ,  $n=25$ ). The embryos were released at a TL of  $12.9 \pm 0.5$  mm (mean  $\pm$  SD) regardless of the paternal size and embryonic density in the brood pouch. No correlation was detected between

mean newborn dry weight and total length pooled for each parent ( $r^2=0.09$ ,  $p>0.1$ ,  $n=25$ ).

#### *Egg and Newborn Weight*

Mean dry weight of newborn ranged from 0.098 to 0.143 mg, and it decreased as the embryonic density in the brood pouch increased (paternal SL: 188.0 - 255.0 mm) (Figure 4.5;  $r^2=0.28$ ,  $p<0.05$ ,  $n=20$ ). The average newborn weight was  $0.121 \pm 0.010$  mg ( $\pm$ SD, CV=8.3%).

The average dry weight of ovulated eggs was  $0.172 \pm 0.027$  mg ( $\pm$ SD, CV=15.7%), ranging from 0.110 to 0.230 mg. There was a positive linear relationship between female SL (157.5 - 249.0 mm) and egg dry weight (Figure 4.6;  $r^2=0.66$ ,  $p<0.001$ ,  $n=15$ ). The average egg dry weight was significantly heavier than larval dry weight (Mann-Whitney  $U$ -test:  $U=18$ ,  $n_1=15$ ,  $n_2=20$ ,  $z=4.4$ ,  $p<0.001$ ), and the substantial embryonic weight decreased by 29% during the gestation, or the efficiency coefficient of development (Gray, 1927) was 0.71.

#### *Developmental Stage of Newborn*

Almost all newborn ( $95.2 \pm 5.6\%$ , mean  $\pm$ SD, 30 males) had exhausted yolk and were at postlarval stage with the fixed numbers of dorsal (34 - 39) and caudal (10) fin rays (Figure 4.7). The postlarvae seemed to have exhaust yolk shortly before parturition, indicated by the occurrence of yolk-sac larvae almost as large in TL as postlarvae. Fin rays were not present in the pectoral fins and the anal fin of the postlarvae just after the parturition. Finfold remained on dorsal and abdominal sides of the tail, with dorsal one being much smaller. In some postlarvae, the abdominal finfold was connected to the primordial anal fin. In some others, finfold, especially the dorsal one, already disappeared. The postlarvae were semi-transparent and melanophores were observed densely in the abdomen and tail regions.

Bones of the postlarvae were not ossified except for some bones in the head

region, such as premaxilla and maxilla and part of quadrate, symplectic, metapterygoid, ceratohyal and urohyal (Figure 4.8). The axial skeleton and pterygiophores of the postlarvae were cartilaginous. Adult body was enclosed in a series of bony rings, whereas the body surface of the postlarvae was not ossified. Coronet of the adult (Figure 4.8) is not firmly attached to the first bony ring, and this seemed to make the upward flicking of head possible for suction feeding (Chapter 2).

The postlarvae had relatively well developed eyes. Hyaloid body occupied smaller proportion of the eye of the postlarvae (Figure 4.9A) compared to that of adults (Figure 4.9B), and retina with low cell densities almost reached at lens in postlarvae. The retina of the postlarvae possessed all the layers seen in the adult (Figure 4.9C) except for the nerve fiber layer.

Free neuromasts were seen in head and along the body, which seemed to develop into lateral lines in postlarvae (Figure 4.10A). The neuromast was a doughnut-like protuberance with 2 - 4 longer motile cilia and numerous shorter stereocilia (microvilli) at the hollow center (Figure 4.10B). One motile cilium and numerous (approx. 15) microvilli seemed to arise from one sensory cell. No pit organ or opening of lateral lines was observed.

In the postlarva, nostrils were not yet formed, and olfactory epithelium was exposed externally (Figure 4.11A). Ciliated cells and microvillous cells were densely observed on the olfactory epithelium (Figure 4.11B).

## Discussion

The parturition of *S. schlegeli* took place in the nighttime. This may be advantageous in reducing a predation risk of the newborn. *S. schmidtii* in the Black Sea is also reported to give birth to larvae in the evening (Gordina *et al.*, 1991). The embryos are embedded in the placenta-like tissue (see Chapters 5 and 6 for detail). The intermittent movement of delivering males is supposed to

work to loosen up the placenta-like tissue so that it can be detached from the brood pouch inner wall and the embryos are freed.

One copulation filled at least 20% of the brood pouch with eggs, which agrees with the smallest amount of eggs present in the brood pouch of wild males (Chapter 2). Therefore, matings may be repeated up to 5 times to fill the pouch. Berglund *et al.* (1988) suggested that such partial spawning is a consequence of bet-hedging strategy (Stearns, 1976). About 95% of the newborn at the same developmental stage simultaneously emerged from the brood pouch, indicating that the matings are repeated at short time intervals in the wild.

The paternal fecundity of *S. schlegeli* ranged from 690 to 1482, and it is more than tenfold smaller compared with fecundity of concurring species, such as *Engraulis japonicus* (Tsuruta, 1992) and *Hypomesus pretiosus japonicus* (Yanagawa, 1981). *S. schlegeli* is considered to be a relatively K-selected species. Paternal brooding may contribute to the reduction of the newborn number by achieving very low mortality late.

The relationship between male body size and brood size was linear in *S. schlegeli*, as reported in *S. typhle* (Berglund *et al.*, 1988). The linear relationship and the exponential increase of the brood pouch volume with the male size resulted in reduction in embryonic density in the brood pouch with the increase in the male size. Embryos brooded at lower densities were heavier in dry weight. Hence, compared to smaller males, larger males brood embryos at a relatively lower density and give birth to heavier newborn. Similar case has been reported in *S. typhle*, in which heavier males carry heavier embryos than smaller males (Berglund *et al.*, 1986b). Furthermore, males having fewer prenatal embryos are known to give birth to heavier newborn (Ahnesjö, 1992a) whose survival rate is higher than that of those brooded at higher densities (Ahnesjö, 1992b). These authors implied that heavier embryonic weight of larger males was resulted partially from more allocation of paternal nourishment per embryo.

However, there is alternative explanation for the findings in the present study. Larger mates are preferred in both sexes in *S. typhle* (Berglund *et al.*,



1986b). If individuals of both sexes must compromise on the mate size during mating competition, this tendency may result in the close match of the mate size, which has been observed in *S. schlegeli* in the present study. The egg weight of *S. schlegeli* had a positive correlation with the females size. It is, therefore, possible that larger males of *S. schlegeli* give birth to heavier newborn developed from heavier eggs produced by larger females. Thus, the presence of neither the paternal nutritional provision nor the density effect on larval weight may be determined solely by these results.

One of the simplest ways to determine the presence of exogenous nutrient incorporation of embryos during the gestation is to compare the difference in egg weight and newborn weight. The average egg dry weight was significantly heavier than larval dry weight in *S. schlegeli*, and the substantial embryonic weight decreased by 29% during the gestation. Decrease in embryonic weight does not necessarily indicate the absence of paternal nutrient provision. In general, about 1/3 of the energy initially available is expended for metabolism during the incubation. The ratio of larval dry weight to the dry weight of the zygotes termed as the "efficiency coefficient of development" typically has a value of approximately 0.65 in oviparous fishes (Gray, 1927; Scrimshaw, 1945). The value 0.71 in *S. schlegeli* is on the boarder line, indicating that the amount of paternal nourishment contribution is little, if ever present.

The coefficient of variation of egg dry weight (8.3%) was almost twice as large as that of newborn dry weight (15.7%). The parturition took place when the embryos reached a TL of about 13 mm regardless of paternal size or embryonic density in the brood pouch. Eggs with large weight differences seemed to converge to newborn with relatively minor weight differences. In *S. typhle*, metabolic rate of larger embryos are reported to be larger than that of smaller embryos (Berglund *et al.*, 1986b). This may explain the reduction in weight variations of embryos during the gestation.

The newborn were in the postlarval stage with relatively well developed dorsal and caudal fins and sensory organs that seemed functional. They might



have acquired morphological factors advantageous for survival before parturition. The occurrence of dead eggs in the brood pouch was only 1.36%. *S. schlegeli* seems to protect offspring at embryonic and yolk-sac larval stages, which are of highest mortality in many marine fishes producing pelagic eggs. By protecting their offspring rather than providing nourishment, *S. schlegeli* seems to achieve reproductive success.

Feeding function of the brood pouch has been a subject of debate. Haresign and Shumway (1981) reported the permeability of the brood pouch to radio-labeled amino acid in *S. fuscus*, however, they were reluctant to conclude with the embryonic incorporation of the amino acid due to the possibility of contamination with the methods they employed. By comparing the energy contents between eggs and newborn added to the energy converted from respiration rate during gestation, Berglund *et al.* (1986a) claimed that the males provided nourishment to the broods in *S. typhle*. However, a question arises as to the appropriateness of the respiration measurement of embryos removed from the brood pouch with energy consuming osmoregulatory function (Quast and Howe, 1980; Watanabe *et al.*, 1999). On the contrary, Azzarello (1991) reported that embryos removed from the brood pouch completed normal development and concluded that the embryos did not primarily depend on paternal nutrition in *S. scovelli*. For further study of the topics related to paternal brooding of the syngnathids, conclusive direct evidence of the presence of paternal nourishment provision is indispensable.

Table 4.1 Preference in mate body size of *Syngnathus schlegelii*.

Male SL (mm)	Female SL (mm)	Mated female	Remarks
187	165, 218, 241	241 (1/5), 241 (1/3)	Quit when 1/3 filled on the 16th day
223	166, 229, 248	229 (1/2), 229 (3/4), 229 (full)	Mated 3 days in a row
172	174, 221, 243	174 (1/5), 174 (1/3)	No further mating, male died on the 6th day
239	165, 195, 240	195 (1/2)	No further mating
255	182, 219, 250	250 (1/5), 182 (1/3)	No further mating
187	177, 200, 243	177 (1/2), 177 (2/3)	No further mating

One male and three females were placed in the same aquarium to examine the size combination between mating pairs. Numbers in parenthesis indicate the longitudinal length of the brood pouch area occupied with eggs divided by the total brood pouch length.

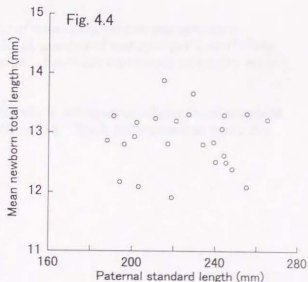
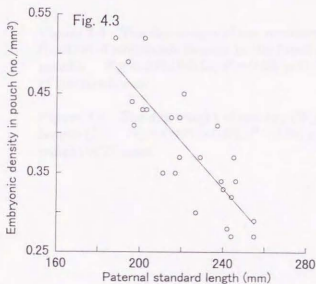
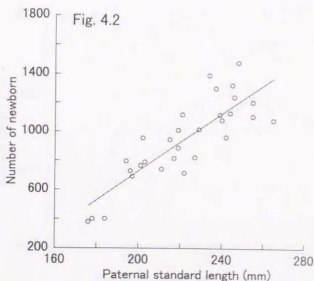
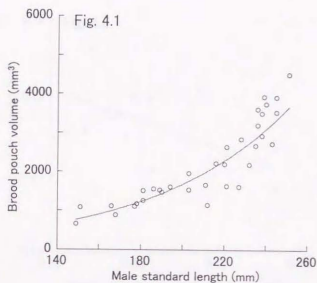


Figure 4.1 The volume of the brood pouch ( $V$ ) of male *Syngnathus schlegelii* as a function of standard length ( $L$ ).  $V = 77.53e^{0.015L}$ ;  $r^2 = 0.91$ ,  $P < 0.001$ ,  $n = 35$ .

Figure 4.2 The number of newborn ( $N$ ) of male *S. schlegelii* as a function of standard length ( $L$ ).  $N = 9.5L - 1261.7$ ;  $r^2 = 0.71$ ,  $p < 0.001$ ,  $n = 30$ .

Figure 4.3 The embryonic density in the brood pouch ( $D$ , number of embryos per  $1 \text{ mm}^3$  of the pouch) of male *S. schlegelii* as a function of standard body length ( $L$ ).  $D = -0.003L + 1.04$ ;  $r^2 = 0.68$ ,  $p < 0.001$ ,  $n = 25$ .

Figure 4.4 The relationship between newborn total length and paternal standard length of *S. schlegelii*.  $r^2 = 0.01$ ,  $p > 0.1$ ,  $n = 25$ . Each dot represents mean total length of 100 newborns.

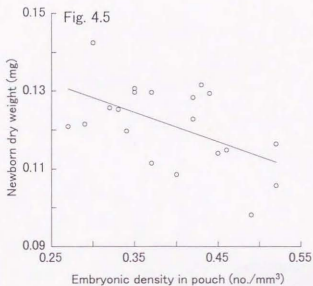


Figure 4.5 The dry weight of one newborn ( $W_n$ ) of male *Syngnathus schlegelii* as a function of embryonic density in the brood pouch ( $D$ , number of embryos per 1 mm<sup>3</sup> of the pouch).  $W_n = -0.075D + 0.15$ ;  $r^2 = 0.28$ ,  $p < 0.05$ ,  $n = 20$ . Each dot represents mean dry weight of 100 newborns.

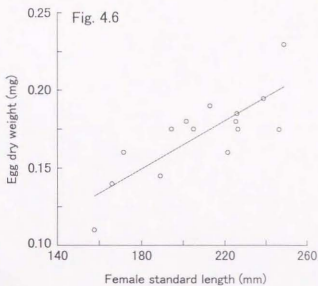


Figure 4.6 The dry weight of one egg ( $W_e$ ) of female *S. schlegelii* as a function of standard length ( $L$ ).  $W_e = 0.0008L + 0.01$ ;  $r^2 = 0.66$ ,  $p < 0.001$ ,  $n = 15$ . Each dot represents mean dry weight of 20 eggs.

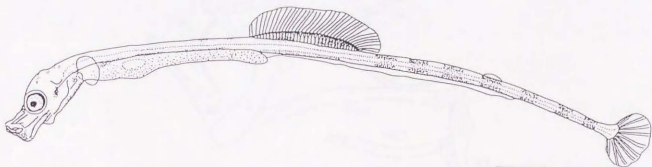


Figure 4.7 Newborn larva of *Syngnathus schlegeli*. Newborn is in postlarval stage with fixed number of dorsal and caudal fin rays. Fin rays are not present in pelvic and anal fins. Finfold remain on dorsal and ventral side of tail. Body is semi-transparent, and melanophores are densely distributed on abdomen and tail, making 7 horizontal bands on tail. The bar is 1 mm.



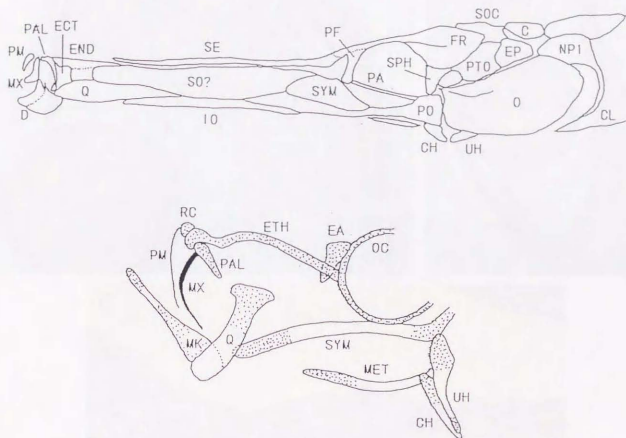


Figure 4.8 Bones of the head region of *Syngnathus schlegelii*. Adult (upper) and newborn postlarva (lower). Bone names and abbreviations are adapted from Gregory (1933), Azzarello (1989) and Colson *et al.* (1998). C = coronet. CH = ceratohyal. CL = cleithrum. D = dentary. EA = ectethmoid arch. ECT = ectopterygoid. END = endopterygoid. EP = epiotic. ETH = ethmoid. FR = frontal. IO = interopercle. MET = metapterygoid. MK = Meckel's cartilage. MX = maxilla. NP1 = first nuchal plate. O = operculum. OC = orbital cartilage. PA = parasphenoid. PAL = palatine. PF = prefrontal. PM = premaxilla. PO = preopercle. PTO = pterotic. Q = quadrate. RC = rostral cartilage. SE = supraethmoid. SO = suborbital. SOC = supraoccipital. SQ = pterotic. SYM = symplectic. UH = urohyal. Cartilage in newborn is stippled.

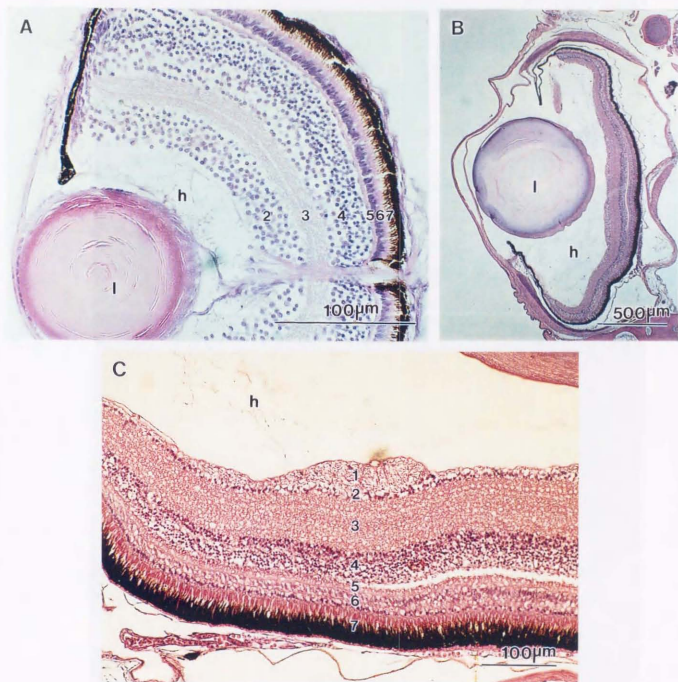


Figure 4.9 Eye of *Syngnathus schlegeli*. Newborn postlarva (A). Adult (B). Magnified view of retina of adult (C). h, hyaloid body; l, lens. Numbers indicate the layers of retina: 1, nerve fiber layer; 2, ganglion cell layer; 3, inner plexiform layer; 4, inner nuclear layer; 5, outer plexiform layer; 6, outer nuclear layer; 7, visual cell layer. The gap between 4 and 5 in the adult retina is an artifact.

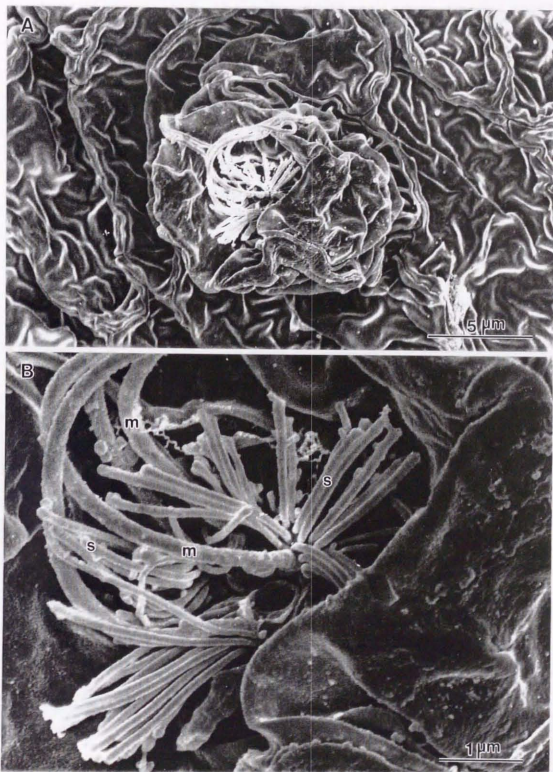


Figure 4.10 Free neuromast of newborn postlarva of *Syngnathus schlegeli* (A) and magnified view (B). Four motile cilia (m) and numerous stereocilia (s) are observed. One sensory cell possesses 1 motile cilium and about 15 stereocilia.



Figure 4.11 Head region of newborn postlarva of *Syngnathus schlegelii* (A) and magnified view of olfactory epithelium (B). Nostrils are not formed. Numerous cilia (c) and microvilli (m) are observed on the olfactory epithelium. e, eye; o, olfactory epithelium; arrowheads, free neuromast.



## 5 PATERNAL NUTRIENT PROVISION TO EMBRYO

### Introduction

In general, two types of viviparity have been recognized in fishes. Ovoviviparous species protect internally fertilized zygotes for a short period of time without providing them with nutrients. True viviparity is defined for conditions with nutrient provision. However, many ovoviviparous poeciliids have been found to provide prenatal embryos with a small amount of nutrients (Scrimshaw, 1945), making the definition of ovoviviparity vague. For this reason, there is a recent tendency to refer to both ovoviviparity and true viviparity simply as viviparity (Wourms, 1981). Nevertheless, it is important to clarify whether the embryos are lecithotrophic (i.e. depend on yolk reserve) or matrotrophic (i.e. rely on maternal nutrients) in order to understand the brooding mechanism and reproductive strategy of the species. Some viviparous species nourish the embryos until sexual maturity (e.g. *Micrometrus minimus*, Schultz, 1993), whereas some others give birth to yolk-sac larvae (e.g. *Sebastes melanops*, Boehlert and Yoklavich, 1984). Naturally, the newborn of these species lead different life history.

The mode of reproduction in Syngnathidae resembles viviparity in respect that the prenatal embryos are enclosed in the male brood pouch and physiologically maintained, (Kronester-Frei, 1975; Quast and Howe, 1980). Paternal energy investment on the embryos in syngnathids has been of much interest in terms of not only physiological but also behavioral ecological point of view since it affects sexual selection and sex role (Svensson, 1988; Berglund *et al.*, 1988; Berglund *et al.*, 1989; Rosenqvist 1990; Berglund and Rosenqvist, 1993). Although these studies are based on the assumption that fathers nourish embryos in the brood pouch (Haresign and Shumway, 1981; Berglund *et al.*, 1986a), actual nutritional relationship between the father and embryos remain unconfirmed.



Some researchers actually doubt the presence of paternal nourishment in syngnathids (Leiner, 1934; Azzarello, 1991). It is important to realize that the study of brood pouch physiological functions, especially paternal-embryonic nutrient transfer, is still in its infancy.

In this chapter, paternal-embryonic nutritional relationships of *Syngnathus schlegeli* will be examined. Tracer experiments have been conducted in an attempt to determine the presence and mechanism of substance transfer. Egg morphology has been observed with regards to the permeability of the chorion to substances. In order to examine the reliance of embryos on paternal nutrition, the effects of paternal starvation on the embryonic nutritional condition has been examined by analyzing newborn RNA/DNA ratio.

## Materials and Methods

### *Sampling Methods*

Adult *S. schlegeli* were collected from seagrass (*Zostera*) beds in Otsuchi Bay and Funakoshi Bay on the Pacific coast of northern Japan (39°20' N, 141°54' E) by a boat seine (43×3 m, mesh size 1×1 cm) from 1996 to 1998. The pipefish were kept in a aerated 150-L circular tank provided with continuously running natural seawater, and fed on laboratory reared *Artemia* nauplii.

### *Microscopy*

Ovaries were dissected out from mature females, cut transversely into small pieces and fixed in 10% buffered formalin and cold 2% paraformaldehyde - 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for light and electron microscopy, respectively. For light microscopy, specimens were embedded in paraffin by the conventional method, and the sections (7 µm) were stained with Mayer's haematoxylin and eosin. For scanning electron microscopy of the

ovulated eggs, specimens were rinsed overnight with 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide in the same buffer for 2 hours, dehydrated through graded ethanol series, dried with  $\text{CO}_2$  in a critical-point drier, sputter coated with platinum palladium, and observed with a scanning electron microscope (HITACHI S-4500). The definition of oocyte developmental stages followed Begovac and Wallace (1988).

### *Tracer Experiments*

Three types of protein tracers were used to determine the substance transfer from paternal abdominal cavity to embryos in the brood pouch. For histochemical detection of a tracer, horseradish peroxidase (HRP, MW 44000, SIGMA Chem. Co.) was used. Biotinylated plasma proteins of tilapia (biotinylated tilapia plasma) and 5(6)-(biotinamidocaproylamido)-pentyl thioureydiylfluorescein (fluorescein biotin, MW 831, SIGMA Chem. Co.) were employed in an attempt to perform the quantitative analysis of substance transfer from father to embryos.

### *(HRP)*

Brooding males were injected with a 0.5% HRP solution in physiological saline in the abdominal cavity (0.1 ml/ind). The fish were kept in tanks with continuously running seawater, and sacrificed after 30 min, 1, 2, 3, 4, 5, and 6 hr after the injection (2 males each).

The procedure for histochemical detection of HRP basically followed Graham and Karnovsky (1966). The brood pouch containing embryos were transversely cut in approximately 1 mm sections and fixed for 4 hr with cold 2% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer (pH 7.4). The specimens were rinsed overnight in the same buffer, and soaked in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB, WAKO Pure Chem. Ind. Ltd.) in 0.05M Tris-HCl buffer (pH 7.6) for 5 min at room temperature before incubating in 0.05% DAB in the same buffer containing 0.01%  $\text{H}_2\text{O}_2$  for 15 min at room

temperature. The specimens were rinsed with 70% ethanol and stored in cold 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide in the same buffer for 2 hr, dehydrated through graded ethanol series, immersed in QY-1 (Nissin EM Co. Ltd.) and propylene oxide, and embedded in Epon 912 resin (TAAB laboratory equipment Ltd.). Ultrathin sections cut with a microtome (LKB 2088 Ultratome V) were observed with a transmission electron microscope (JEOL JEM-100CX) without staining. Two control fish injected with physiological saline were examined after the same procedure.

#### *(Biotinylated Tilapia Plasma)*

Biotinylated tilapia plasma (BTP) was provided by Dr. Takemura, the University of the Ryukyus. Plasma obtained from adult tilapia, *Oreochromis mossambicus*, was dialyzed overnight with cold 0.1 M carbonate buffer. Biotin-N-hydroxysuccinimide (biotin-NHS) dissolved in dimethyl sulfoxide (10 mg/ml) was reacted with the plasma for 4 hr at 4°C (100 µg bitoin-NHS/1 mg proteins in the plasma). Finally the plasma was dialyzed with phosphate buffered physiological saline and stored at -30°C.

BTP was injected to 4 males near the end of brooding in the abdominal cavity once everyday (0.1 ml/injection) until parturition. The males were individually kept in tanks provided with aeration. After injection, the fish were disinfected with Elbaziu (Ueno Finechemicals Ind. Ltd.), and the water in the tanks were replaced. Parturition took place on the 3rd and 4th day (2 males each) after the initiation of the experiment. Newborn were collected, rinsed with 0.1M tris-HCl buffer (pH 7.4), homogenized in a micro test tube (Eppendorf), and centrifuged at 13000 rpm for 3 min. The supernatants were stored at -30°C. Gills were removed from 2 injected males after parturition, and serum was obtained and stored by the same procedure. Newborn from 2 control males without injection were also obtained by the same procedure.

The concentrations of proteins in newborn and serum samples were

measured by BCA (bicinchoninic acid) protein microplate assay (Smith *et al.*, 1985), and the samples were diluted to 3000 µg proteins/ml by distilled water. SDS-PAGE (10% gel) (Laemmli, 1970) was carried out on the newborn and serum samples together with BTP and marker proteins (MW 19400 - 104000). The proteins were transferred to a membrane by Western blotting and stained by HRP method (streptavidin-HRP, SIGMA Chem. Co.). Endogenous peroxidase was blocked by treating the membrane with 0.05% H<sub>2</sub>O<sub>2</sub> in 0.1M tris-HCl buffer (pH 7.4) for 10 min prior to staining.

#### *(Fluorescein Biotin)*

Fluorescein biotin (FB) saturated in physiological saline was injected to 2 brooding males once everyday until parturition (0.2 ml/injection). Both males gave birth to larvae 4 days after the initiation of the experiments. The newborn from the treated males and 1 control male injected with physiological saline were rinsed with 0.1M tris-HCl buffer (pH 7.4), mounted on a glass slide and observed by confocal laser scanning microscopy (Zeiss, LSM310). The 488 nm line of the argon ion laser was used as the excitation wavelength.

Newborn samples were homogenized and centrifuged after the same procedure described above for BTP experiment. The fluorescent intensities of the newborn samples and the FB solution diluted to 1000 times with 0.1M tris-HCl buffer (pH 7.4) were measured from 505 to 550 nm with the excitation wavelength of 494 nm with a fluorescence spectrophotometer (JASCO TFP-700).

#### *RNA/DNA Ratio Measurements*

Brooding males with brood pouch full of eggs were collected in Otsuchi Bay in June 1997. Two males had yellowish embryos, which indicated that they had accepted eggs shortly before the collection. They were kept in a tank at 18.0°C without being fed until parturition (starved group). Parturition took place 17 days and 21 days after the collection by each male. In July 1997, another group



of males was collected in Otsuchi bay and kept in tanks. One male gave birth to larvae on the next day of the collection, and another male on the following day (fed group).

From both starved and fed groups, the newborn were randomly sampled and stored individually in a micro test tube at  $-80^{\circ}\text{C}$  after being rinsed with Tris-HCl buffer (pH 7.2) and measured in TL. The mean temperature at 5 m depth during the last third of July in Otsuchi Bay is reported to be  $18.5^{\circ}\text{C}$  (Otobe *et al.*, 1996). The rearing temperature of the starved group in June was selected so that it would not greatly differ from the environmental temperature in July in order to avoid temperature effects on RNA/DNA ratio (Mathers *et al.*, 1992).

The RNA/DNA ratio in the whole body of each newborn was measured by fluorescent technique using ethidium-bromide, which was originally described by Clemmesen (1993) and modified by Sato *et al.* (1995). The amount of DNA and RNA were quantified with a fluorescence spectrophotometer (HITACHI 850). Salmon sperm DNA (Wako Pure Chem. Co. Ltd.) and yeast RNA (Kanto Chem. Co. Ltd.) were used as the standard. Differences in the RNA/DNA ratio of newborn from starved and fed groups were statistically analyzed by nested ANOVA (Zar, 1996).

## Results

### *Egg Morphology*

A pair of approximately equally sized ovaries coalesced caudally and connected to short oviduct, which lead to the genital pore. The ovary was a cylindrical tube composed of smooth muscle covered by visceral epithelium. The longitudinal length of the ovaries reached about 80% of that of the abdominal cavity. The oocytes at various developmental stages were arranged sequentially in a spiral manner in the ovary (Figure 5.1). Ovulated eggs and germinal ridge (i.e. the



region containing oogonia and chromatin-nucleolus oocytes) were present along the entire length of the ovary.

The ellipsoidal eggs (approx.  $1.0 \times 1.2$  mm) were slightly adhesive and contained a large amount of yolk and numerous oil globules. Openings of pore canals were observed on the entire surface of the egg. The diameter of the slightly raised opening of micropyle was  $9.5 \mu\text{m}$  (Figure 5.2B). The thickness of the chorion of eggs in the brood pouch was  $1.5 - 3.0 \mu\text{m}$ , and 10 strata were recognized (Figure 5.2C). The electron density of the chorion was high, and the pore canals did not penetrate the chorion.

#### *HRP Injection*

The embryos in the brood pouch are embedded in the vascularized placenta-like tissue of the brood pouch. The epithelium of the placenta-like tissue consists of pavement cells (PVC) and mitochondria rich cells (MRC) neighboring capillaries (see Chapter 6 for detail).

Peroxidase activity was observed as small electron-opaque particles in the brood pouch. Small amounts of the particle were detected in all treatment, most densely at the apex of MRCs (Figure 5.3). There were no apparent differences among the treatments sacrificed at different timings. HRP molecules did not seem to be enclosed in a definite membrane. No cytoplasmic budding was observed in MRCs or PVCs. Peroxidase activity was not observed in the embryos and brood pouch epithelium of control fishes.

#### *Biotynilated Tilapia Plasma Injection*

On SDS-PAGE, BTP showed numerous bands in all detectable range from molecular weights of 19400 to over 104000 (Figure 5.4). The serum samples extracted from gills showed bands homologous to BTP, indicating that injected BTP was absorbed from body cavity into the blood circulation. Embryo samples from the injected and uninjected control males showed two bands at about 110

and 80 kDa. The 110 kDa band did not correspond to BTP. The 80 kDa band may correspond to BTP; however, the concomitant appearance in the controls indicates that BTP is not incorporated into embryos.

#### *Fluorescein Biotin Injection*

Fluorescent illumination was observed in the embryos from the injected males, most intensively in the rectum (Figure 5.5), as well as cartilage and pigments. The embryos of the control male did not show fluorescence in the rectum.

The peak of fluorescent reaction of FB was 515 nm (Figure 5.6A). The fluorescent spectrum profiles of the treatment and control did not mutually differ, and fluorescent intensity successively declined from the excitation wave length of 494 nm without showing any increase at 515 nm (Figure 5.6 B-D), indicating the absence of embryonic incorporation of FB.

#### *Paternal Starvation and Newborn RNA/DNA Ratio*

The whole body RNA/DNA ratio of the newborn in fed and starved groups ranged from 1.44 to 2.65 and 1.12 to 2.05, respectively (Table 5.1). The RNA/DNA ratios within the groups were not significantly different ( $p>0.05$ ), and the newborn in the starved group showed significantly lower RNA/DNA ratio ( $1.71 \pm 0.27$ , mean  $\pm$  SD,  $n=24$ ) than those in the fed group ( $2.03 \pm 0.32$ ,  $n=24$ ) ( $p<0.05$ , nested ANOVA). No significant differences were detected in mean paternal SL between fed and starved groups ( $p>0.7$ , two-tailed t-test).

Significant difference was not detected in newborn total length or the amount of DNA between fed and starved groups (Table 5.1.  $p>0.05$ , nested ANOVA). The amount of newborn DNA and total length ( $L_n$ ) of the larger starved male (217mm SL) was significantly smaller than that of the other starved male ( $p<0.05$ , nested ANOVA). However, no morphological defects or locomotory inability was observed in the newborn.

## Discussion

All three tracer experiments performed in the present study to determine the paternal-embryonic substance transfer showed negative results. SDS-PAGE on BTP confirmed that the tracers injected in abdominal cavity are incorporated into the blood circulation of brooding males, supporting the appropriateness of the experimental procedure. HRP molecules seemed to be transported to the brood pouch epithelium through capillaries. While HRP molecules may be released into the brood pouch lumen by MRCs, embryos did not seem to ingest them. This is also evident from the fact that the molecular weight of HRP falls within the limits of detectable range of BTP experiment that showed negative results. Since the measurements of fluorescent intensity did not show any differences between treatment and control, the fluorescence observed in the rectum of the embryos is not FB but perhaps yolk proteins. These results indicate that paternal nutrients of *S. schlegeli* are substances either smaller than 831 Da or larger than 104 kDa if it ever exists.

In viviparous fishes, nutrient provision is achieved largely by 3 mechanisms: oophagy, yolk-sac placenta and placenta analogous (Wourms, 1981). Oophagy or adelphophagy is apparently not applicable to planktivorous *S. schlegeli*. The yolk-sac membrane of embryos in the chorion did not make a direct contact to the brood pouch. One of the possibilities is that the brood pouch excretes nutrients that are small enough to penetrate the electron dense chorion without pores. Takai and Mizokami (1959) reported that the embryos of *S. schlegeli* hatch in the brood pouch about 1 week before parturition. Therefore, alternative interpretation is that, large molecular size nutrients are provided to post-hatching embryos in the brood pouch. However, the periodic acid Schiff negative brood pouch epithelium (Chapter 3) consisting only of PVC and MRC did not possess elaborate structure analogous to trophonemata that excretes histotrophe (uterine milk) as seen in viviparous elasmobranchs (Hamlett *et al.*, 1993). All these results indicate that possible nutrients are small substances transported into the

brood pouch lumen perhaps by simple diffusion from the capillaries.

The results of the RNA/DNA experiment seem to provide a circumstantial evidence of paternal nourishment in *S. schlegeli*. The amount of RNA in the cell is directly proportional to the amount of protein synthesis (Clemmesen, 1996), whereas the amount of DNA is constant in somatic cells (Regnault and Luquet, 1974). The higher RNA/DNA ratio observed for newborn in the fed group compared to the starved group indicates that the paternal nutritional conditions are reflected in embryonic protein synthesis and therefore nutritional conditions. Inasmuch as the experiments were designed so as to eliminate the possible paternal size effects on newborn (see Chapter 4 for detail), the results indicate that the embryos rely, at least partially, on paternal nutritional supply during brooding.

The mean RNA/DNA ratios in the fed *S. schlegeli* ( $2.03 \pm 0.32, \pm \text{SD}$ ) was almost as high as those in feeding larvae of other teleost species, such as *Sardinops melanostictus* (Kimura *et al.*, 1998), *Clupea harengus* (Clemmesen, 1996), *Coregonus* spp. (Steinhart and Eckmann, 1992), *Solea solea* (Richard *et al.*, 1991), and *Theragra chalcogramma* (Canino *et al.*, 1991). Although direct comparison of the RNA/DNA ratio between different species without correcting for the effect of developmental stage, size (Clemmesen, 1994) and temperature (Mothers *et al.*, 1992) may cause a wrong evaluation, the ratio  $2.03 \pm 0.32$  was considered to indicate that nutritional conditions of the newborn are comparable to feeding larvae of other species.

The minimum RNA/DNA ratio required for survival is about 1.0 regardless of fish species (Clemmesen, 1994). The mean RNA/DNA ratio of the newborn in the starved *S. schlegeli* ( $1.71 \pm 0.27, \pm \text{SD}$ ) was well over the fatal value, and the newborn did not show any morphological defects or locomotory inability. Therefore, paternal starvation did not seem to cause fatal deficiency of nourishment to the newborn. The substantial weight of prenatal embryos of *S. schlegeli* decreases by 29% (Chapter 3). While the results of RNA/DNA ratio experiment indicate the presence of paternal nourishment, it does not seem to be

crucial for the development of prenatal embryos. The feeding mechanism of the brood pouch of *S. schlegeli* is considered to be in primitive stage as in viviparous rock fish, *Sebastes flavidus*, in which extra embryonic sources contribute only 3.4% of the energy utilized after fertilization (Hopkins *et al.*, 1995). The embryos of *S. schlegeli* seem to be essentially lecithotrophic depending primarily on yolk reserve.



Table 5.1 RNA/DNA ratio and total length of newborn brooded by starved and fed male *Syngnathus schlegeli* ( $L_m$  = paternal standard length and  $L_n$  = newborn total length).

	$L_m$ (mm)	Starvation (day)	Newborn measured	$L_n$ (mm) Mean $\pm$ SD	DNA ( $\mu$ g) Mean $\pm$ SD (range)	RNA/DNA Mean $\pm$ SD (range)
Starved	217	17	12	10.2 $\pm$ 1.2	3.03 $\pm$ 0.25 (2.53-3.36)	1.74 $\pm$ 0.32 (1.12-2.19)
	214	21	12	13.4 $\pm$ 1.3	3.34 $\pm$ 0.23 (3.03-3.65)	1.67 $\pm$ 0.21 (1.37-2.05)
	Mean			11.8 $\pm$ 2.0	3.19 $\pm$ 0.29	1.71 $\pm$ 0.27
Fed	205	1	12	11.4 $\pm$ 0.5	3.12 $\pm$ 0.14 (2.87-3.33)	2.08 $\pm$ 0.34 (1.44-2.71)
	248	2	12	12.0 $\pm$ 0.5	3.15 $\pm$ 0.33 (2.50-3.95)	1.98 $\pm$ 0.29 (1.55-2.65)
	Mean			11.7 $\pm$ 0.6	3.13 $\pm$ 0.25	2.03 $\pm$ 0.32

DNA: within group,  $p > 0.05$ ; between group  $p > 0.05$ . RNA/DNA: within group  $p > 0.05$ ; between group  $p < 0.05$ . nested-ANOVA

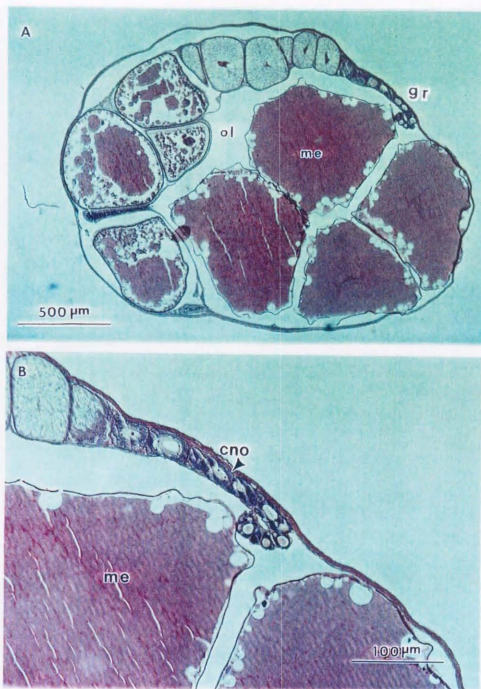


Figure 5.1 Cross section of ovary of *Syngnathus schlegeli*. Whole image (A). Note sequential developmental pattern of follicles. Magnified view of germinal ridge containing oogonia (not seen) and chromatin nucleolus oocytes (B). cno, chromatin nucleolus oocyte; gr, germinal ridge; me, mature egg; ol, ovarian lumen.

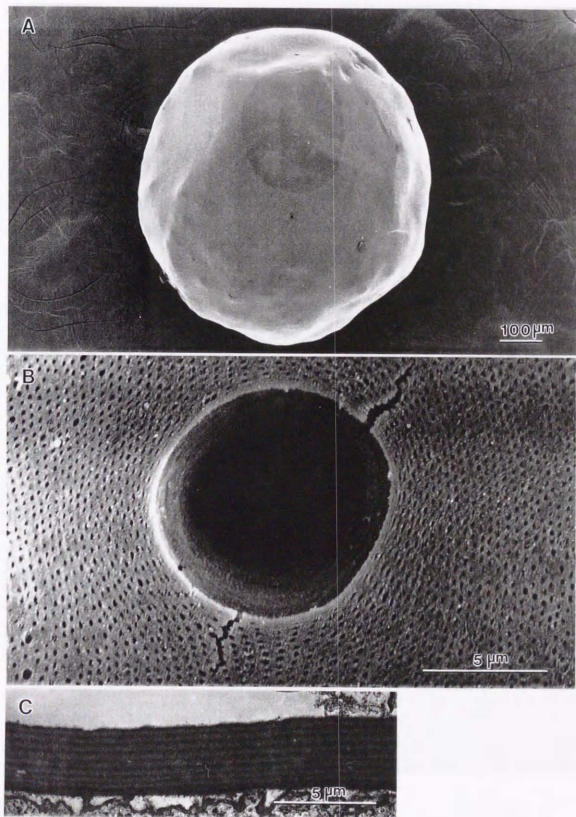


Figure 5.2 Electron micrograph of ovulated egg of *Syngnathus schlegelii*. Scanning electron micrograph of egg (A). Magnified view of the micropyle (B). Note openings of pore canals distributed regularly on the chorion surface. Transmission electron micrograph of chorion in the brood pouch (C). Pore canals do not penetrate the chorion.

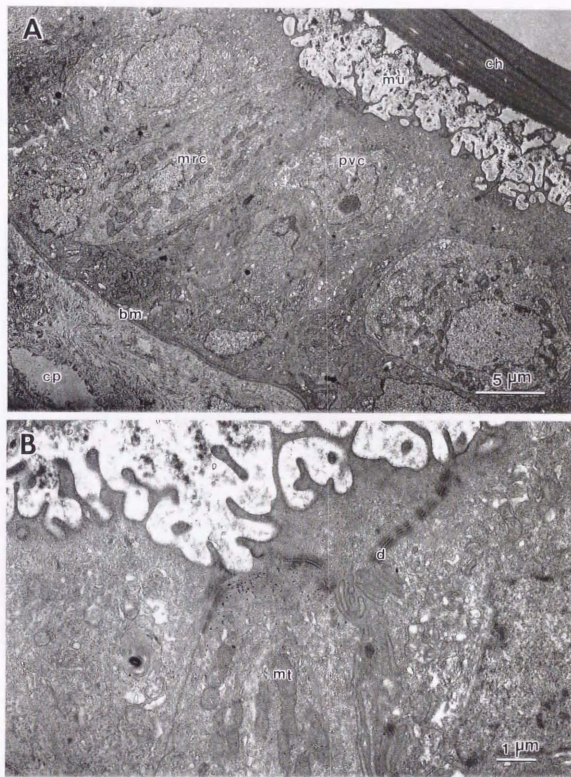


Figure 5.3 Transmission electron micrographs of the brood pouch epithelium of *Syngnathus schlegelii* injected with HRP in the body cavity. The fish was sacrificed 180 min after the injection. Brood pouch epithelium consisting of mitochondria rich cell (MRC) and pavement cell (A). Magnified view of the apex of MRC (B). HRP molecules are observed in the form of opaque particles at the apex of MRC. bm, basal membrane; ch, chorion; cp, capillary; d, desmosome; mrc, mitochondria rich cell; mt, mitochondrion; pvc, pavement cell.



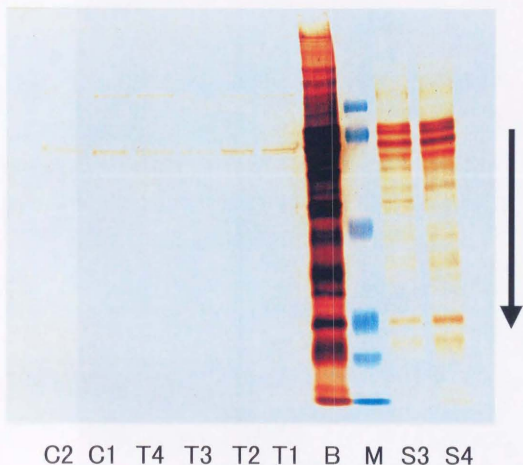


Figure 5.4 SDS-PAGE on newborn of *Syngnathus schlegeli*. Biotinylated tilapia plasma (B) was injected to the abdominal cavity of 4 brooding males, and newborn were obtained (T1 to T4). Control newborn (C1 and C2) were obtained from 2 males. Serum samples (S3 and S4) were obtained from two injected males corresponding to T3 and T4. Marker proteins (M) are, from the top to the bottom, phosphorylase B (104 kDa), bovine serum albumin (82 kDa), ovalbumin (48.3 kDa), carbonic anhydrase (33.4 kDa), soybean trypsin inhibitor (28.3 kDa) and lysozyme (19.4 kDa). Biotinylated tilapia plasma appeared in the serum but not in the newborn. Arrow shows the direction of the current flow.



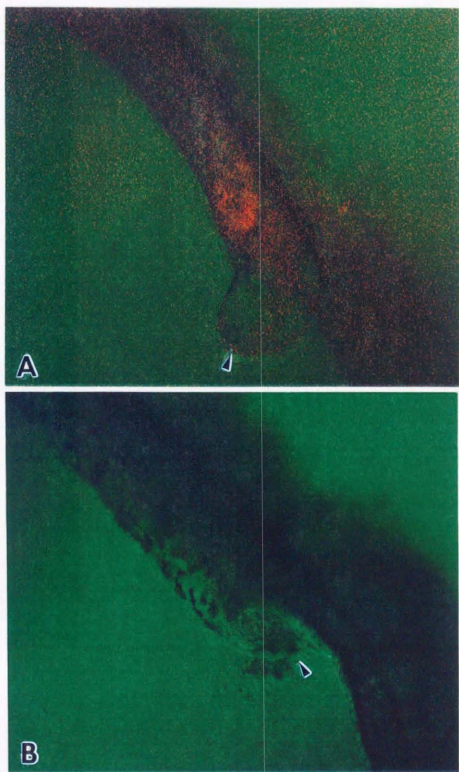


Figure 5.5 Confocal laser scanning micrographs of the rectum of newborn of *Syngnathus schlegelii*. Newborn of a male injected with fluorescein biotin in the abdominal cavity, showing fluorescence (A). Newborn of a control male (B). arrowheads, anus.

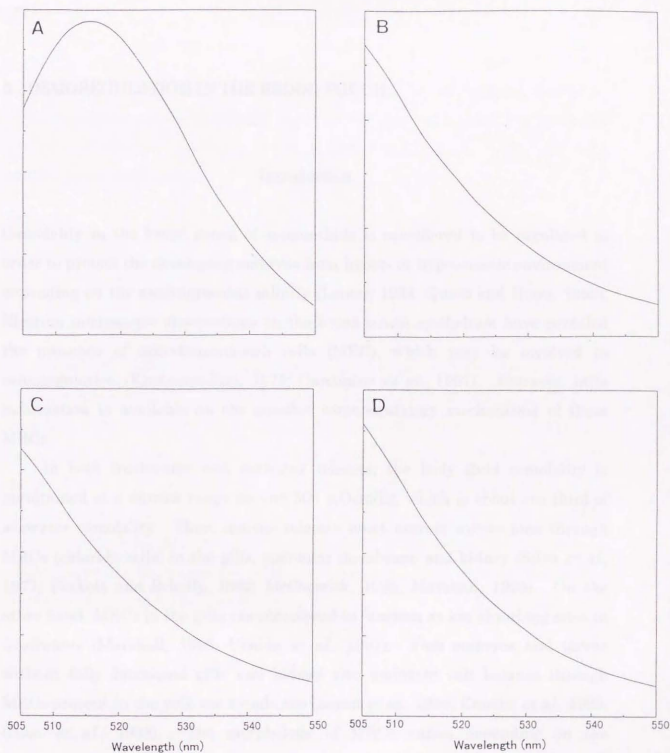


Figure 5.6 Fluorescent spectrum of newborn of *Syngnathus schlegeli*. Fluorescein biotin (FB) injection (A). Newborn from a control male (B). Newborn from males injected with FB (C and D). FB shows the peak at 515 nm. No fluorescence was detected in the treatments and the control. Scales on the vertical axis are arbitrary.

## 6 OSMOREGULATION IN THE BROOD POUCH

### Introduction

Osmolality in the brood pouch of syngnathids is considered to be regulated in order to protect the developing embryos from hyper- or hyposmotic environment depending on the environmental salinity (Leiner, 1934; Quast and Howe, 1980). Electron microscopic observations on the brood pouch epithelium have revealed the presence of mitochondria-rich cells (MRC), which may be involved in osmoregulation (Kronester-Frei, 1975; Carcupino *et al.*, 1997). However, little information is available on the possible osmoregulatory mechanisms of these MRCs.

In both freshwater and seawater teleosts, the body fluid osmolality is maintained at a narrow range around 300 mOsm/kg, which is about one third of seawater osmolality. Thus, marine teleosts must excrete excess ions through MRCs (chloride cells) in the gills, opercular membrane and kidney (Silva *et al.*, 1977; Foskett and Scheffy, 1982; McCormick, 1995; Marshall, 1995). On the other hand, MRCs in the gills are considered to function as ion absorbing sites in freshwater (Marshall, 1995; Uchida *et al.*, 1997). Fish embryos and larvae without fully functional gills and kidney also maintain salt balance through MRCs present in the yolk-sac membrane (Ayson *et al.*, 1994; Kaneko *et al.*, 1995; Sasai *et al.*, 1998). The morphology of MRCs varies depending on the environmental salinity either to excrete or to absorb ions (Uchida *et al.*, 1996; Shiraishi *et al.*, 1997).

The aim of the present chapter is to investigate the involvement of MRCs in the brood pouch epithelium of the seaweed pipefish, *Syngnathus schlegelii*, in the regulation of the brood pouch fluid osmolality. To evaluate the osmoregulatory ability of the brood pouch,  $\text{Na}^+$  concentrations in the brood pouch containing embryos have been measured. The presence of  $\text{Na}^+, \text{K}^+$ -ATPase, a key enzyme of

ion transport, has been investigated in the brood pouch MRCs by immunocytochemistry. Morphology of the brood pouch MRCs has been examined by light and electron microscopy in comparison with the MRCs in the adult gills and the larval epidermis.

## Materials and Methods

### *Sampling Methods*

Adult male *S. schlegeli* were collected from *Zostera* beds in Otsuchi Bay in northern Japan (39°20' N, 141°54' E) by a boat seine (43×3 m, mesh size 1×1 cm) from May 1995 to October 1997. The specimens were maintained in tanks with continuously renewed seawater (salinity 36‰) at Otsuchi Marine Research Center, Ocean Research Institute, the University of Tokyo. The salinity of the collection site was about 33‰. Some brooding males were allowed to give birth to larvae in separate tanks. The brooding males were fed on natural zooplankters (mainly copepods), collected every morning in Otsuchi Bay, and on laboratory reared *Artemia* nauplii.

### *Na<sup>+</sup> Concentrations in the Brood Pouch*

Ten males were anesthetized with 2-phenoxyethanol, rinsed with distilled water and blotted dry. A brood pouch fluid sample was obtained by carefully inserting a glass capillary through a longitudinal incision along the ventral fissure of the brood pouch. The capillary was sealed and centrifuged for 5 min at 10000 rpm in order to separate the brood pouch fluid from possible contaminants, such as yolk and chorion, and were stored at -30°C. The fish was severed posterior to the head, and a blood sample was taken in a glass capillary. The capillary was sealed, and the serum was separated from the blood cells by centrifugation (5 min at 10000 rpm). Analysis of the brood pouch fluid and serum samples, as well as

the environmental water, for  $\text{Na}^+$  concentrations were made on an atomic absorption spectrophotometer (HITACHI 180-50).

#### *Immunocytochemical Detection of Mitochondria-Rich Cell*

The tails of the males with and without embryos in the brood pouch were cut transversely into approximately 5 mm thick portions, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight and stored in 70% ethanol at 4°C. The larvae within 6 hr after birth were likewise fixed and stored.

Histological sections and whole-mount preparations were immunocytochemically stained using an antiserum specific for  $\text{Na}^+, \text{K}^+$ -ATPase. The antiserum was raised against a synthetic peptide corresponding to part of the highly conserved region of the  $\text{Na}^+, \text{K}^+$ -ATPase  $\alpha$ -subunit (Ura *et al.*, 1996). The brood pouch portions were cut into 4  $\mu\text{m}$  sections by conventional paraffin method and stained by the avidin-biotin-peroxidase complex (ABC) method (Hsu *et al.*, 1981), using commercial reagents (Vectastain ABC kit, Vector Laboratories). Newborn larvae and the brood pouch epithelium stripped carefully with forceps was subjected to whole-mount immunochemistry following the method of Ohtani *et al.* (1989). To visualize the immunoreaction, 3,3'-diaminobenzidine and 4-Cl-1-naphthol were used for the sections and whole-mount preparations, respectively. Prepared samples were observed under a light microscope equipped with a Nomarski's differential interference contrast device.

#### *Scanning Electron Microscopy*

Brood pouch portions of about 5 mm in thickness, both with and without embryos, gills removed from the opercular cavity, and newborn larvae were fixed in cold 2% paraformaldehyde - 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for one week at longest, rinsed overnight and postfixes in 1% osmium tetroxide in the same buffer for 2 hr. The samples were then dehydrated through graded ethanol series, dried with  $\text{CO}_2$  in a critical-point drier and sputter coated with platinum



palladium. The surface structure and the size of the apical opening of MRCs were examined with a scanning electron microscope (HITACHI S-4500).

#### *Transmission Electron Microscopy*

For ultrastructural observations, brood pouch portions and gills were fixed, postfixed and dehydrated in the same procedure as described above for scanning electron microscopy. Samples were then immersed in QY-1 (Nissin EM Co. Ltd.) and propylene oxide, and embedded in Epon 912 resin (TAAB laboratory equipment Ltd.). Ultrathin sections cut with a microtome (LKB 2088 Ultratome V) were mounted on grids, stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (JEOL JEM-100CX).

### Results

#### *General Morphology of the Brood Pouch*

The brood pouch of *S. schlegeli* is a ventral organ located on the tail with the anterior end closely associated with the genital pore (Figure 6.1A). Two skin folds with thickened end merge longitudinally relative to the tail axis. The embryos in the brood pouch are attached to highly vascularized placenta-like tissue which seals the skin folds from inside during incubation (Figure 6.1B). The lumen of the brood pouch is filled with viscous fluid. The brood pouch opens loosely and becomes leaky several days before parturition.

#### *Na<sup>+</sup> Concentrations in the Brood Pouch*

The mean Na<sup>+</sup> concentration in the brood pouch fluid with embryos at various developmental stages from blastula to yolk-sac larvae was  $178.8 \pm 14.1$  mM ( $\pm$  SE, n=10). Na<sup>+</sup> concentrations in the serum and the environmental water were 180.8

$\pm 4.7$  mM (mean  $\pm$  SE,  $n=10$ ) and 422 mM, respectively. Thus, the  $\text{Na}^+$  levels in the brood pouch fluid were comparable to those in the serum rather than in the environmental water.

#### *Immunocytochemical Detection of Mitochondria-Rich Cells*

In the brood pouch epithelia of both brooding and non-brooding males, a large number of elongate flask-shaped MRCs were detected by immunocytochemistry with the anti- $\text{Na}^+, \text{K}^+$ -ATPase antibodies (Figure 6.2). The basal part of the MRCs was in contact with capillaries running underneath the epithelium, and the apical end reached the brood pouch lumen (Figures 6.1 and 6.2). Some MRCs of the brooding males possessed an extremely elongate and narrow apical pole (Figure 6.2B). In the brood pouch with embryos, MRCs were larger and lower in density, and the nuclei were more prominent (Figure 6.2B, D) than in those without embryos (Figure 6.2A, C).

In newborn postlarvae, MRCs were observed in the gills, body surface, and fins (Figure 6.2E, F). Although these MRCs are commonly termed chloride cells, they are referred to as MRCs here. Density of MRCs in the body was high around the pelvic fin and along the middle line of the lateral sides. It was frequently observed that some MRCs fuse to form a multicellular complex as indicated by the presence of more than one nucleus (Figure 6.2F). In contrast with MRCs in the brood pouch epithelia, the elongate apical pole was absent in postlarvae. In addition, intensive immunoreaction lay along the gills; however, precise discrimination between MRCs was difficult in the whole-mount preparation because of the high density and three-dimensional distribution of MRCs (Figure 6.2E).

#### *Surface Structures of Brood Pouch Epithelium and Gills*

The surface of the brood pouch epithelium was composed of hexagonal pavement cells (PVCs) with numerous microridges (Figure 6.3). Ovoid pores with

approximate diameter of 0.1 - 0.2  $\mu\text{m}$  were observed between the PVCs in non-brooding males (Figure 6.3B, C). In the brooding males, inner wall of the brood pouch was in the shape of honeycomb (Figure 6.3D), and one embryo was settled in each compartment. Because of the upheaval at the edge of the PVCs and the remnant mucous material, neither the boundaries of the PVCs nor the pores were recognizable in the brooding males by scanning electron microscopy (Figure 6.3E).

In newborn postlarvae, the operculum was not yet fully formed, and rudimentary gills without lamellae were exposed. Apical pits of MRCs were observed at the boundaries of the hexagonal PVCs in the gills and the body surface (Figure 6.4A). Approximate diameter of the pit was 1.5  $\mu\text{m}$  regardless of their location.

In adult fish, 4 pairs of gills and a pair of pseudobranches were completely covered by the opercula. The gill filaments possessed extremely widened and projected lamellae (Figure 6.4B). The pits of MRCs were observed between the PVCs only on the afferent vascular side of the gill filaments and lamellae (Figure 6.4C). Approximate diameter of the pits in the adult gills was 2.5  $\mu\text{m}$ .

#### *Ultrastructure of MRCs in Brood pouch and Gills*

The brood pouch epithelium, which consisted of a single layer of MRCs and PVCs, was in close contact with capillaries (Figures 6.1C and 6.5A, C). Unlike those observed in the larval epidermis, the MRCs in the brood pouch epithelium appeared solitary and did not form multicellular complexes (Figure 6.5A), as was also evident in immunocytochemistry. Elongate MRCs with numerous mitochondria and well-developed tubular systems (Figure 6.5A) were exposed apically to the brood pouch lumen where mucous material was filled (Figure 6.5B). The sectional width of the apical opening was about 0.25  $\mu\text{m}$ , which well matched with the diameter of the pores observed by scanning electron microscopy (Figure 6.3B). The tubular systems were distributed among the entire MRC except for the very end of the apex. The mitochondria in the MRC contained electron dense

particles in the cristae (Figure 6.5D).

The structure of the MRC in the adult gills was typical of chloride cells in marine teleosts (Figure 6.5E). The cytoplasm of the cell is filled with numerous mitochondria packed amongst in well-developed tubular systems. The apical region exposed to seawater was partially covered by overlying PVCs, to which the MRC was tightly linked by desmosomes. An accessory cell interdigitated with the MRC near the apex, forming a loose junction between the two cells.

### Discussion

The  $\text{Na}^+$  concentration in the brood pouch fluid was maintained near that in the serum in *S. schlegeli*. Since  $\text{Na}^+$  and  $\text{Cl}^-$  are the major ion components of fish body fluid osmolality (Bone *et al.*, 1995), the brood pouch fluid is considered to be maintained isosmotic to the serum, as reported in *S. scovelli* (Quast and Howe, 1980). Although changes in the brood pouch fluid osmolality associated with the embryonic development, as such reported in marine seahorse *Hippocampus erectus* by Linton and Soloff (1964), were not examined, the results in the present study indicate that the brood pouch fluid osmolality is reduced from environmental levels to serum levels at the beginning of incubation subsequent to the adhesion of the brood pouch folds. The brood pouch closure becomes loose and leaky toward the end of incubation, and brood pouch fluid osmolality may be equilibrated to the environmental levels in advance of parturition.

Two types of cells were observed in the brood pouch epithelia by transmission electron microscopy, namely MRC and PVC. The observations on the brood pouch epithelium in the present study mostly agree to the observations in *S. abaster* by Carcupino *et al.* (1997) and in *Nerophis lumbliciformis* by Kronester-Frei (1975), except that the size of the MRC opening in the present study was much smaller than that implied by Carcupino *et al.* (1997). Based on the morphological resemblance, the elongate, flask-shaped cells stained with



anti- $\text{Na}^+$ , $\text{K}^+$ -ATPase antibodies are considered to correspond to MRCs identified by transmission electron microscopy. The PVCs, another cell type in the brood pouch epithelium, were not immunoreactive to anti- $\text{Na}^+$ , $\text{K}^+$ -ATPase antibodies. The presence of  $\text{Na}^+$ , $\text{K}^+$ -ATPase in the brood pouch MRCs indicates the possibility that the MRCs are in charge of ion transport (Rossier *et al.*, 1987; Lin and Randall, 1995; McCormick, 1995). The gill filament MRCs in fish are considered to extrude ions in hyperosmotic environments (Foskett and Scheffey, 1982; McCormick, 1995), whereas gill lamellar MRCs, another type of gill MRCs, are considered to absorb ions in hypoosmotic environments (Laurent *et al.*, 1985; Lin and Randall, 1995; Uchida *et al.*, 1996). As the  $\text{Na}^+$  concentration in the brood pouch fluid was lower than that of the environmental seawater, the MRCs may absorb ions, as suggested in gill lamellar MRCs in freshwater fish.

The brood pouch MRC shares the basic structural organization with the gill MRC. Both MRCs contain numerous mitochondria and well-developed tubular systems in the cytoplasm. The tubular system extends almost to the apical opening partially covered by overlying PVCs. The basal parts of MRCs are in close association with the capillaries running underneath the epithelia. In gill MRCs, the tubular system is continuous with the basolateral membrane, resulting in a large surface area for the placement of ion-transporting proteins, such as  $\text{Na}^+$ , $\text{K}^+$ -ATPase (Karnaky *et al.*, 1976; McCormick, 1995). This seems to be also the case with the brood pouch MRCs, as evinced by the extensive staining with anti- $\text{Na}^+$ , $\text{K}^+$ -ATPase antibodies over the entire cell except for the nucleus.

While the brood pouch and gill MRCs share common characteristics, there are important differences in the degree of apical invagination and the occurrence of the accessory cells. The small apical opening of the solitary brood pouch MRCs is invaginated to a lesser extent compared with that of the gill filament MRCs which are often accompanied with accessory cells in seawater. Comparing the ultrastructure of MRCs in the yolk-sac membrane of tilapia, *Oreochromis mossambicus*, larvae adapted to seawater and fresh water, Shiraishi *et al.* (1997) reported that the multicellular complex of MRC with a deep apical pit was almost



exclusively found in seawater-adapted fish. In freshwater-adapted tilapia larvae, however, MRCs in the yolk-sac membrane existed solitarily without forming the multicellular complex. Therefore, the brood pouch MRCs seem to be analogous to MRCs in the gill lamella and the yolk-sac membrane in freshwater, also supporting ion-absorbing functions of the brood pouch MRCs. Furthermore, the brood pouch MRCs show striking morphological similarity with epidermal MRCs of the toad, *Bufo bufo*, which is responsible for active chloride uptake at low salinity (Budtz *et al.*, 1995). While the long apical pole of the toad MRC is considered to protect MRC from physical damages, it is not certain whether this is the case for the brood pouch MRC in *S. schlegeli*.

Carcupino *et al.* (1997) have observed an increase in number of mature MRCs and a concomitant decrease in developing MRCs in the brood pouch of *S. abaster* during incubation, which is in agreement with the present study. In the immunocytochemical detection of the brood pouch MRCs, brooding males possessed well-developed, larger MRCs with distinct nucleus, compared to non-brooding males. Part of placenta-like tissue and MRCs destroyed by parturition seems to be regenerated in advance of egg acceptance. The lower MRC density during incubation probably attributes to the acquisition of larval osmoregulatory ability indicated by the presence of multicellular MRC complexes in the gills and epidermis of the newborn postlarvae.

The present study suggested that, in *S. schlegeli*, the brood pouch MRCs take up ions for the sake of the embryos and larvae until they acquire osmoregulatory ability by developing epidermal and gill MRCs. The ions absorbed by the brood pouch MRCs may enter the blood circulation from capillaries neighboring the MRCs and excreted into the environment perhaps through gills and other osmoregulatory organs. Further studies on the relationship between the number and size of the brood pouch MRCs and the development of embryonic MRCs during incubation may elucidate the integrated ion-transport functions of MRCs in both paternal and larval osmoregulatory surfaces.

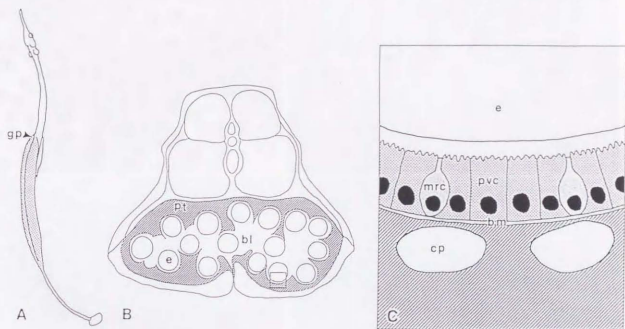


Figure 6.1 Male *Syngnathus schlegeli* with brood pouch (shaded area) (A). Schematic drawing of the transverse section of the tail with the brood pouch (B) and magnified view of pouch epithelium indicated by a box in B (C). bl, brood pouch lumen; bm, basal membrane; cp, capillary; e, embryo; gp, genital pore; mrc, mitochondria-rich cell; pt, placenta-like tissue; pvc, pavement cell.

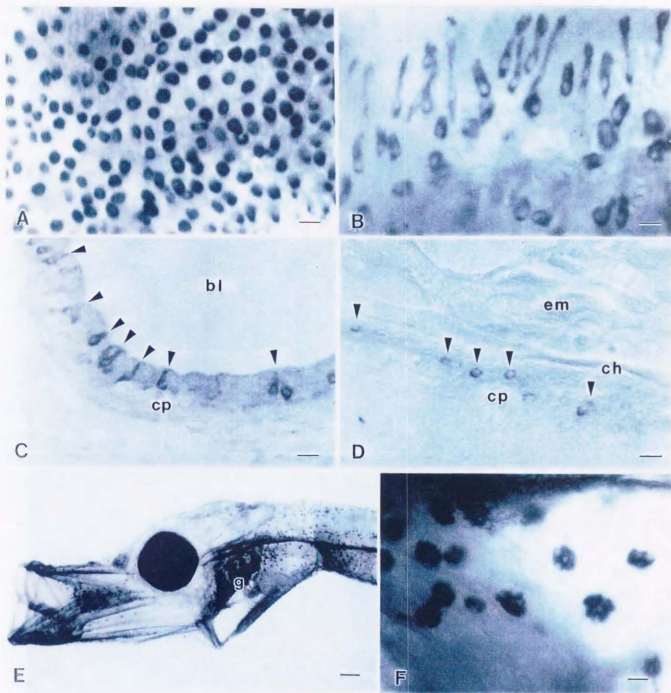


Figure 6.2 Mitochondria-rich cells in the brood pouch epithelium of *Syngnathus schlegelii* detected by whole-mount (A and B) and paraffin section (C and D) immunocytochemistry with anti- $\text{Na}^+$ ,  $\text{K}^+$ -ATPase antibodies. Brood pouch without embryos (A and C). Brood pouch with embryos (B and D). Mitochondria-rich cells in the gills and epithelia covering the head and body of newborn larva detected by whole-mount immunocytochemistry with anti- $\text{Na}^+$ ,  $\text{K}^+$ -ATPase antibodies (E and F). Scattered small spots are mitochondria-rich cells (E). Multicellular complexes of mitochondria-rich cells located anterior to the pelvic fin (F). bl, brood pouch lumen; ch, chorion; cp, capillary; em, embryo; g, gills; arrowheads, mitochondria-rich cells. Bars: 10  $\mu\text{m}$  in A, B, C, D and F; 100  $\mu\text{m}$  in E



Figure 6.3 Scanning electron micrographs of the brood pouch epithelia of *Syngnathus schlegelii* with embryos (A, D and E) and without embryos (B and C). Hexagonal pavement cells with numerous microridges (A). Pores at the boundaries of the pavement cells (arrowheads) (C). Magnified view of the ovoid pore (B). Honeycomb-like structure of the placenta-like tissue (D). One embryo (removed) is settled in each compartment. Remnant mucous material on the pavement cells (E).



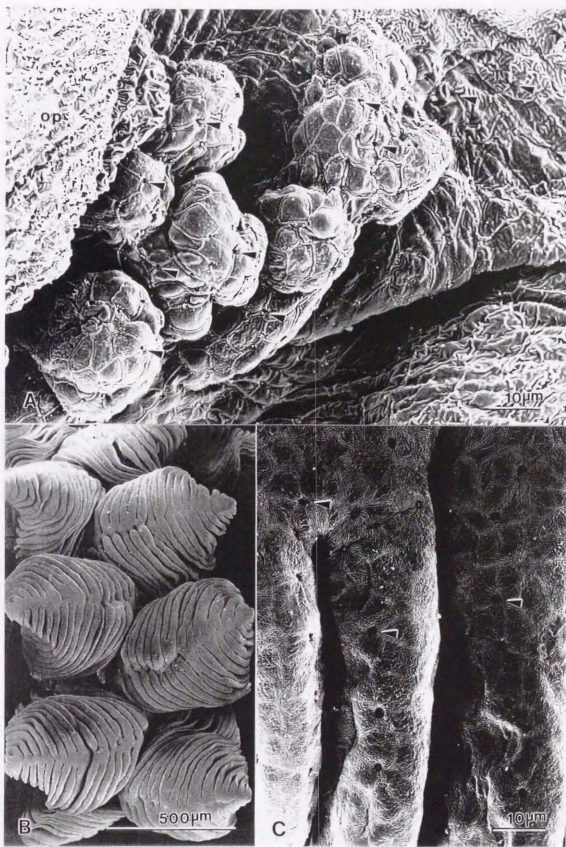


Figure 6.4 Scanning electron micrograph of the gills of newborn *Syngnathus schlegeli* (A). Scanning electron micrographs of the gills of adult (B and C). Gill filaments arranged in alternating positions, forming a row of gill arch (B). Note the extremely widened and projected lamellae alternately inserted to the filaments. Magnified view of the lamellae with the apical openings of mitochondria-rich cells (C). op, operculum; arrowheads, apical opening of mitochondria-rich cells.



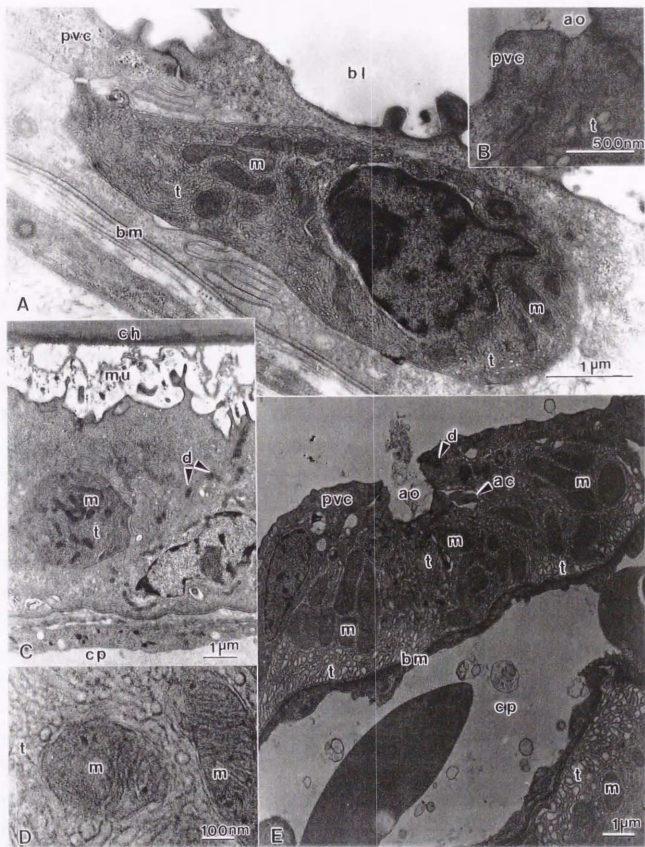


Figure 6.5 Transmission electron micrographs of brood pouch epithelia of brooding *Syngnathus schlegelii*. Mitochondria-rich cell (A). Apical opening of the mitochondria-rich cell between pavement cells (B). Brood pouch epithelium in close contact with chorion and capillary (C). Magnified view of a mitochondrion with electron dense particles in the mitochondria-rich cell (D). Transmission electron micrograph of mitochondria-rich cells in the gill of adult (E). ac, accessory cell; ao, apical opening of the mitochondria-rich cell; bl, brood pouch lumen; bm, basal membrane; ch, chorion; cp, capillary; d, desmosome; m, mitochondrion; mu, mucous material; pvc, pavement cell; t, tubular system.

## 7 DISCUSSION AND SUMMARY

### Physiological Roles of the Brood Pouch

The brood pouch of *Syngnathus schlegeli* did not seem to serve as a primary nutritional source for prenatal embryos, endorsing the standpoint of Azzarello (1991) on *S. scovelli*. While circumstantial evidence of the presence of paternal nutrient provision to prenatal embryos was obtained by the RNA/DNA ratio experiment, long-term starved males did not give birth to larvae with fatally low RNA/DNA ratio (Chapter 5). The three tracer experiments conducted showed negative results (Chapter 5). The efficiency coefficient of development (i.e. newborn dry weight / egg dry weight) was 0.71, which is close to rates typically found for oviparous fishes (Chapter 4). Microscopic analysis of brood pouch epithelium and embryonic epidermis suggested no specialization for nutrient excretion and uptake, respectively (Chapters 5 and 6). On the assumption that nutrient transfer from brood pouch to prenatal embryos exists, these results collectively indicate that only a small amount of nutrients are provided to the embryos perhaps passively rather than actively. *S. schlegeli* seems to be lecithotrophic, depending primarily on yolk reserve.

The osmoregulatory function of the brood pouch of *S. schlegeli* was confirmed. Histochemical analysis of the brood pouch epithelium revealed that MRCs in the epithelium contain  $\text{Na}^+, \text{K}^+$ -ATPase, a key enzyme for active ion transport (Chapter 6). The MRCs are considered to absorb ions in the brood pouch and excrete them into the blood circulation in order to maintain the brood pouch fluid isosmotic to body fluid for the sake of developing embryos. The brood pouch epithelium consisting of MRC and PVC morphologically resembles respiratory epithelia of fishes, indicating that the closed brood pouch of *S. schlegeli* functions in gas exchanges.

The brood pouch of *S. schlegeli* is considered to serve as a protective organ

from physical and physiological disturbances rather than as a trophic organ to nourish embryos.

### Mode of Reproduction

An interesting question arises with respect to the mode of reproduction in *S. schlegelii*: is *S. schlegelii* oviparous or viviparous? Oviparity is a mode of reproduction in which unfertilized eggs are released by the females; fertilization and development of offspring occur outside the maternal body. While the definition of oviparity is rather simple and straightforward, viviparity is harder to define. Wourms (1981) defined viviparity as a process in which eggs are fertilized internally and are retained within the maternal reproductive system for a significant period of time, during which they develop to an advanced state and then are released. While viviparity is practiced by females by definition, it is the syngnathid males, not the females, that brood embryos. Detailed comparisons between the brooding conditions in *S. schlegelii* and viviparous fishes, except for sex difference, are made in an attempt to define the mode of syngnathid reproduction.

The first stage in transition from oviparity to viviparity in fishes involves egg retention without maternal dependency, and further evolutionary changes have involved a loss of yolk concomitant with the specialization of embryonic and maternal tissue as organs of nutrition and internal secretion (Wourms, 1981). In aplacental matrotrophic viviparous teleosts with intraluminal (i.e. within ovarian cavity) gestation, the hindgut or trophotaenia (perianal process derived from embryonic hindgut) provides an effective absorptive surface of nutritive ovarian fluid of serum origin (Schindler and Hamlett, 1993; Hollenberg and Wourms, 1994; Takemura *et al.*, 1995). In viviparous *Sebastes schlegelii* without distinct ovarian structure to supply embryos with nutrients, resorption of unfertilized eggs or dead embryos is considered to enrich the ovarian fluid and supply energy

to surviving embryos (Boehlert *et al.*, 1986). The hindgut of larval teleosts is capable of the uptake of exogenous substances by pinocytosis (Watanabe, 1981, 1982). Regardless of the presence or absence of the maternal nutrition, the mother satisfies respiratory, osmoregulatory and excretory needs of the embryos (Wourms, 1981; Kormanik, 1993; Korsgaard, 1997).

Thus, the conditions in viviparity in teleost fishes are collectively: 1) internal fertilization, 2) retention of embryos within maternal body, satisfaction of embryonic 3) osmoregulatory needs, 4) respiratory needs, and 5) excretory needs. Neither the presence of maternal nor embryonic organs specialized for exogenous nutrition supply is mandatory.

Kuwamura (1987) claimed that the brood pouch is located externally in a strict sense, and he defined syngnathid brooding as external bearing of embryos. Existence of species with the open brood pouch, such as *Corythoichthys* species (e.g. Dawson, 1977), and the fact that the brood pouch of *S. schlegeli* is located on the tail apparently indicate that the brood pouch lumen is not derived from the coelomic cavity. However, the internal ambient conditions of the brood pouch seems to be comparable to that of ovarian cavity; the viscous brood pouch fluid appears to be ovarian fluid (Chapter 3), presumably satisfying the condition 2). In this respect, the mode of fertilization of *S. schlegeli* is analogous to internal fertilization (Chapter 3); therefore, the condition 1) is satisfied. The brood pouch with the epithelium resembling respiratory surface serves as an osmotic buffer (Chapter 6); this seems to satisfy the condition 3) and 4). Although it is not known whether the brood pouch has an absorptive capacity of embryonic nitrogenous wastes (the condition 5), it seems reasonable to conclude that the mode of reproduction of *S. schlegeli* is parallel to viviparity. The reproductive mode of *S. schlegeli* should now be termed "male viviparity or secondary viviparity", one of extreme cases of paternal brood care.



### Evolutionary Sequence of the Brood Pouch

In those teleost fishes which care for the brood, paternal care predominates over maternal care. An imaginary ancestor of male syngnathid probably started to carry embryos by attaching them to body surface, as seen in extant *Doryrhamphus* species. Then some species might have developed the open brood pouch for more elaborate brood care. Herald (1959) proposed a hypothetical evolutionary sequence of the brood pouch development by analyzing the manner in which the brood pouch skin folds close over the embryos. In short, he implied that primitive open brood pouch of *Corythoichthys* species evolved into complex closed brood pouch of *Hippocampus* (seahorse) species, which is closed almost its entire length leaving a postanal pore. The brood pouch of *S. schlegeli* that closes only during gestation is considered to be an intermediate type.

The degree of brood pouch closure in these three genera may be related to the acquisition of physiological functions. The brood pouch of *H. erectus* and *S. schlegeli* functions in osmoregulation and gas exchanges (Linton and Soloff, 1964; Chapter 6). There has been no report on the physiological functions of the open brood pouch of *Corythoichthys* species. Funning of eggs is a common phenomenon in teleost fishes with parental brood care (Krebs and Davies, 1993). While the open brood pouch of *Corythoichthys* species may have a capacity of gas exchanges, it may not function in osmoregulation, as it seems meaningless when the eggs are exposed to seawater. Thus, although it is rather anecdotal, the hypothetical evolutionary line of the physiological functions of the syngnathid brood pouch can be as follows. The open brood pouch first acquired gas exchange capability. Elongation of the skin fold enabled complete encasement of the embryos, and the brood pouch subsequently acquired osmoregulatory function.

Newborn of *C. haematopterus* with the open brood pouch are released at a more immature stage compared to *S. schlegeli*. The newborn larvae lack pectoral and anal fin rays, and large finfolds remain on the lightly pigmented body and tail, with dorsal one extending anterior to the dorsal fin (Watanabe unpublished data).



Embryos of *Hippocampus* species completely concealed within the definite brood pouch develop to a more advanced stage compared to *S. schlegeli*. Newly emerged *H. abdominalis* with the entire body covered uniformly with melanophores possesses all fin rays (Gomon and Neira, 1998). Unlike *Syngnathus* or *Corythoichthys* species, prenatal embryos of *Hippocampus* species develop villous extensions on the anal fin to increase the surface area available for paternal-embryonic exchanges (Wetzel and Wourms, 1991). As it has been repeatedly mentioned in the present thesis, presence of paternal nutrient provision in syngnathid has not been confirmed. However, the morphological features of *Hippocampus* embryos may indicate the presence of paternal nutrient supply during gestation. Application of the tracer experiments conducted in the Chapter 5 of this thesis on *Hippocampus* species may elucidate the feeding function of the brood pouch.

### Summary and Conclusion

Various aspects of the reproductive system of the seaweed pipefish, *S. schlegeli*, have been described with special reference to the male brood pouch in this thesis. The paternal brooding in the brood pouch was found to affect many reproductive features of the pipefish before and after gestation.

Mating behavior and spermatozoal structures of *S. schlegeli* seemed to be indirectly or subsequently affected by the paternal brooding in the brood pouch. About a month of prolonged brooding period (Chapter 2) seemed to reduce the potential reproductive rate of the males, resulting in female-biased operational sex ratio that leads to sex role reversal of the pipefish (Chapter 3). The mode of fertilization was found to be analogous to internal fertilization. The spermatozoa, which seemed to swim in viscous brood pouch fluid during fertilization, had morphological and motional features that resemble introsperm (internally fertilizing spermatozoa) (Chapter 3).

Although the newborn of *S. schlegeli* were still at postlarval stage with immature pigmentation and ossification of bones, they possessed relatively well developed sensory organs and fins (Chapter 4) that made feeding and free swimming possible (Chapter 2). The body shape configuration of larvae seemed to change into that of adult in a short period of time after parturition (Chapter 2). The larvae developed chloride cells in the gills and body surface for osmoregulation before parturition (Chapter 6). Not only does the paternal brooding protect developing embryos during brooding, but it also seems to contribute to post-parturition survival of the larvae.

In conclusion, *S. schlegeli* incubates a relatively small number of embryos during most susceptible egg and larval stages (Houde, 1987; Watanabe and Kuroki, 1997; Watanabe *et al.*, 1995) in the closed brood pouch, which is considered to primarily function as a physical and physiological protective organ. The brood pouch plays an important role in the reproduction of *S. schlegeli* by reducing the mortality of prenatal embryos and post-parturition larvae.

#### ACKNOWLEDGMENTS

I wish to express my sincere gratitude to my supervisor Dr. Y. Watanabe for his encouragement and invaluable instructions throughout this study and my former supervisor Dr. M. Okiyama for assigning this interesting research topic to me. I am grateful to Ms M. Hara, Dr. T. Kaneko and Ms S. Hasegawa, Ocean Research Institute, University of Tokyo, Dr. R. Kimura, National Research Institute of Fisheries Science, Mr. T. Natsusaka, Medical school, University of Tokyo and Mr. J. Hiroi, Kyoto University, for their technical assistance and valuable discussions. I thank Dr. K. Takano and Dr. A. Takemura, University of the Ryukyus, and Dr. Y. Takagi, Otsuchi Marine Research Center, Ocean Research Institute for their useful advice and encouragement. Dr. Takemura also provided me with biotinylated tilapia plasma. I also thank Captain K. Morita, Dr. M. Amano, Mr. K. Hirano, Mr. K. Sado, Mr. M. Kurosawa, and Mr. Y. Iwama, Otsuchi Marine Research Center, Dr. T. Hirose, Japan Sea National Fish Research Institute and Dr. K. Takahashi, Soka University for field assistance. Thanks are also due to Dr. K. Yamauchi, Hokkaido University for supplying me with anti- $\text{Na}^+$ ,  $\text{K}^+$ -ATPase. I am grateful to the colleagues at my laboratory: Dr. T. Saruwatari, Dr. Y. Tsukamoto, Dr. Y. Hayakawa, Mr. M. Yamaguchi, Mr. H. Kato, Mr. T. Takahashi, and Mr. M. Aoyagi, and former colleagues: Dr. T. Otake, Mie University, Dr. I. Akagawa, Miyazaki University, and Dr. M. Kanda, Kochi University for invaluable discussions and field assistance. Gratitude is extended to Dr. K. Kawaguchi, Dr. K. Tsukamoto, Dr. T. Kaneko, Ocean Research Institute and Dr. K. Aida, University of Tokyo for their kind reading and valuable criticism of the manuscripts, and Mr. W. Siu for proofreading of the manuscripts. Finally, I would like to express my gratitude to my parents for the support they have given me to make everything possible.

The contents of the Chapter 6 have been published in Cell and Tissue Research (295 (1999) 1: 141-149), entitled as "Immunocytochemical detection of mitochondria-rich cells in the brood pouch epithelium of the pipefish, *Syngnathus schlegelii*. Structural comparison with mitochondria-rich cells in the gills and larval epidermis" by S. Watanabe, T. Kaneko and Y. Watanabe. The copyright belongs to Springer-Verlag.

## EPILOGUE

Under circumstances which give economical profitability the top priority, curiosity is usually not an important factor. In the academic world, however, curiosity is important because it leads to new discoveries and scientific knowledge. For most of us, it is often difficult to directly utilize scientific knowledge in our everyday life. But we must realize that advancing scientific knowledge is crucial to maintaining and improving our way of life. In my graduate studies, I have worked on the reproductive biology of the seaweed pipefish, one of the fish species with no commercial value in Japan. I hope this thesis will stimulate your curiosity about fish and nature.

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## 論文の内容の要旨

ヨウジウオ科魚類は藻場やサンゴ礁域などに生息する沿岸性の魚類で、世界で215種、日本では49種が報告されている。本科魚類は雄が育児を行うという特異な繁殖生態を持ち、原始的な種では卵を腹部体表に付着させるが、より進化した種では尾部腹面に育児嚢を発達させる。育児嚢は物理的な卵保護のみならずガス交換、浸透圧調節、栄養補給など生理的な胚胎の維持を行うと考えられている。

本科魚類に関する研究は、生態、行動、生理、形態など多岐にわたる分野で行われているが、日本産種についての報告はほとんどなく、本科魚類の繁殖の生理生態を解明する上で鍵となる器官である育児嚢に関しては、世界的にも具体的な知見は乏しい。本研究ではヨウジウオ (*Syngnathus schlegelii*) を対象に野外採集と室内飼育を行い、天然個体群の繁殖生態と育児嚢の生理的機能を明らかにした。

### 繁殖期・育児期間

岩手県大槌湾および船越湾のアマモ場にヨウジウオが出現し始める時期は5月であった。アマモ場への出現と同時にヨウジウオは繁殖を開始し、5月では76.9%の雄が育児嚢を発達させ、そのうちの33.3%が育児中であった。育児嚢を発達させる標準体長は133~215mmと個体差が大きかった。7~10月ではほぼ全ての雄が育児中であり、繁殖期間は6ヶ月以上の長期に及ぶことがわかった。育児嚢内での育児期間は飼育水温と負の相関を示し、17.2~23.3℃では14~28日間であった。水温データからヨウジウオの大槌湾での育児期間は30日以上に及ぶと推定された。アマモ場での性比は繁殖期全体では雄に偏っていた。これは、同属他種で報告があるように育児中の雄が安全な藻場に留まるためであると考えられた。育児中の雄の育児嚢は卵で満たされており、育児嚢内に卵で満たされていない部分が見られた雄は全体の6.2%にとどまった。育児嚢内には発生段階の異なる2つ以上の卵群が認められる場合があり、ヨウジウオの婚姻形態が複婚であることが示唆された。

### 配偶行動・受精環境・配偶子

配偶行動を水槽内で観察したところ、ヨウジウオの求愛行動には Parallel swimming と Twitching の2つのパターンが確認された。配偶者獲得競争であると考えられる Parallel swimming を雌のみが行ったことから、雌が雄よりも活発に求愛行動を行う「性役割の逆転」が確認された。求愛行動の後、雌は生殖孔を雄の生殖孔が位置する育児嚢前端に押しつけ、育児嚢内に直接産卵した。この産卵様式は交尾に相当すると思われる。雄は卵を受け取ると体をくねらせ育児嚢内の卵を整列させた。受精は産卵時或いはこの時点に起こると思われる。

育児嚢は尾部腹側の左右から伸びた皮褶によって形成される。卵を受け取ると育

育児嚢内に胎盤様組織 (placenta-like tissue) が発達して左右の皮褶を接着し、育児嚢は粘液質の液で満たされた。胎盤様組織上皮は PAS およびアルシアン青染色に反応せず、粘液分泌能が確認されなかったことから、育児嚢内の粘液質の液は母体由来の卵巣腔液であると推測された。

精子は細長い核 (約 3  $\mu\text{m}$ ) を持ち、深く陥入する基部には二つの中心子が位置する。遠位中心子からは長い鞭毛 (約 80  $\mu\text{m}$ ) が伸長し、核の基部には鞭毛を螺旋状に取り巻くミトコンドリアが位置する。これらの形態的な特徴は、introsperm と呼ばれる体内受精型精子と一致し、粘性の高い卵巣腔液由来の育児嚢内液中を遊泳する、体内受精に類似した受精様式に適応していると考えられた。精巣内の精子は極端に低密度であったが、精子拡散の少ない育児嚢内では低い精子密度でも十分な受精率を得られると考えられた。

#### 親サイズ・産仔数・仔魚重量・仔魚の発生段階

産仔数は雄の体長と正の相関を示し、690~1482 であった。育児嚢の容積は体長の増加に伴い指数関数的に増加するために、体長の増加に伴って育児嚢の単位容積あたりの胚胎数 (胚密度) は減少した。産出仔魚の乾重量は胚密度と負の相関を示したことから、大きな雄ほど低密度で育児を行いより重い仔魚を産み出すことがわかった。この現象は従来、低密度での育児が胚胎 1 個体当たりの父親からの栄養補給量を増加させるためと解釈されている。しかし、雌の体長の増加に伴って卵の乾重量が増加すること、体長の近い雌雄が配偶ペアを形成したことから、大型の雄は大型の雌から大きい卵を受け取るために産出仔魚重量が増加する可能性が考えられた。産出仔魚の乾重量 ( $0.12 \pm 0.01 \text{ mg}$ ,  $\pm \text{SD}$ ) は、卵の乾重量 ( $0.17 \pm 0.03 \text{ mg}$ ) の 71% にまで減少しており、胚胎への栄養補給が存在するとしても微量であると考えられた。

産出仔魚の全長は親サイズに関わらず  $12.9 \pm 0.5 \text{ mm}$  ( $\pm \text{SD}$ ) であり、 $95.2 \pm 5.6\%$  ( $\text{mean} \pm \text{SD}$ ) が卵黄吸収を終えて間もない後期仔魚であった。後期仔魚は鰭条が発達した背鰭と尾鰭を有し、眼、鼻、遊離感毛などの感覚器は機能していると考えられた。育児嚢内の死卵と未受精卵の合計は全体の  $1.36 \pm 0.03\%$  ( $\text{mean} \pm \text{SD}$ ) であり、ヨウジウオは育児嚢での保護によって胚胎の死亡率を低下させ、運動能力や感覚器を発達させた仔魚を産出することで繁殖を成功させると考えられた。

#### 育児嚢の栄養補給機能

育児嚢内の胚胎は毛細血管に富む胎盤様組織に包まれている。胎盤様組織は繊維質の組織と単層の上皮からなり、上皮には被蓋細胞 (pavement cell) と MRC (mitochondria rich cell) の 2 種の細胞が見られた。雄の腹腔に HRP (horseradish peroxidase, MW 44000) をトレーサーとして投与した結果、胎盤様組織上皮まで

HRP 分子が移動することが確認された。HRP は特に MRC の開口部付近で密に観察されたが、胚胎内への移動は確認されなかった。

ビオチン化したティラピア血清 (様々な分子量の蛋白質を含む) と蛍光標識されたビオチン (MW 831) をトレーサーとして用い、電気泳動法と分光測光法を用いて親腹腔から胚胎への物質の移動を調べた。いずれも胚胎への移動は確認されず、父親から胚胎への分子量 831~104000 までの高分子物質の移動はないと判断された。

成熟卵は楕円形 ( $1.2 \times 1.0$  mm) で、卵門 (直径  $9.5 \mu\text{m}$ ) 付近は緩やかに隆起する。卵膜は 10 層程度からなり、厚みは約  $1.5 - 3.0 \mu\text{m}$  で比較的薄い。卵膜表面には規則正しく管孔が並ぶが卵膜を貫通しない。卵膜の構造が栄養補給など父親との生理的な物質交換に適応しているとは考え難かった。

絶食させた親からの産出仔魚は、摂餌させた親の産出仔魚よりも核酸比 (RNA/DNA) が有意に低く (Nested ANOVA,  $p < 0.05$ )、親の栄養状態が胚胎の栄養状態に反映されると考えられ、栄養補給の存在が示唆された。しかし絶食群仔魚の核酸比は他魚種で報告されている栄養状態の悪化した仔魚の値ほど低くなかったことから、栄養補給があっても、少量であると考えられた。育児嚢に栄養補給能があることは定説となっているがこれまで確定的な証拠は報告されておらず、本種に関しては胚胎の発生や成長を左右する程の栄養補給が存在するとは考え難い。

#### 育児嚢の浸透圧調節能

被蓋細胞と MRC により構成される毛細血管に富む育児嚢胎盤様組織上皮は、魚類の呼吸上皮と同様の構造であった。育児嚢内の胚胎は外部環境から完全に遮断されており、胚胎は胎盤様組織との間でガス交換を行っていると考えられた。育児嚢内液の  $\text{Na}^+$ 濃度は  $178.8 \pm 14.1 \text{ mM}$  ( $\pm \text{SE}$ )、体液と海水ではそれぞれ  $180.8 \pm 4.7 \text{ mM}$ 、 $422 \text{ mM}$  であり、育児嚢内の浸透圧は親の体液と同じ値に保たれていた。

鰓においてイオンの能動輸送に関わる  $\text{Na}^+, \text{K}^+$ -ATPase に対する抗体を用いて育児嚢胎盤様組織に免疫染色を行ったところ、上皮全体に分布する MRC に強い反応が認められた。毛細血管に隣接する育児嚢 MRC の形態はフラスコ状を呈し、細長く伸長する部位の上端が育児嚢内腔に開口すること、細胞全体に管状構造が発達すること、ミトコンドリアを多数持つことが電子顕微鏡下で確認された。成魚の鰓や仔魚の体表に見られる MRC が深く窪んだ開口部を持ち、複数の MRC の複合体を形成する典型的な海水型であるのに対し、育児嚢 MRC では開口部径が 1/10 程度のサイズであること、複合体を形成しないことから、イオンを吸収すると考えられている淡水型 MRC と形態的に類似していた。雄親は MRC によってイオンを吸収して育児嚢内の浸透圧を体液に近い値に保つことで胚胎を保護することが明らかとなった。

以上のように、ヨウジウオでは雄親魚が外部環境から独立した育児嚢内で、ガス

交換および浸透圧調節について胚胎を生理的に保護することが確認された。育児嚢から産出される仔魚は、摂餌及び捕食者から逃避する能力を備えた発生段階に達しており、育児嚢は、生活史のなかで最も死亡率が高い卵仔魚における減耗を著しく小さくすることによって、再生産を成功させる上で重要な機能を持つと結論される。



