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SPERMATOGENESIS INVOLVING PARASPERM PRODUCTION IN THE MARINE COTTOID FISH, *HEMILEPIDOTUS GILBERTI*

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***ABSTRACT.* –** The production of non-fertile parasperm along with fertile eusperm during spermatogenesis occurs widely in animals. The parasperm of some invertebrates are known to promote eusperm fertilization. Parasperm are also produced in some species of cottoid fish. This study showed that the production of parasperm without eusperm and vice versa does not occur during spermatogenesis in Gilbert's Irish lord, *Hemilepidotus gilberti*. Gonadosomatic indices (gonad weight / body weight with gonads × 100), which represent the gonad investment of each male, increased approximately four months prior to the spawning season, corresponding to a spur in the development of eusperm and parasperm. Both sperms may have differentiated from the same spermatocytes and electron microscopy has proven that the spermatids that become eusperm and parasperm were connected by intercellular bridges in a cyst. In the present study, the development of parasperm of *H. gilberti* in relation to its functions on sperm competition is also discussed.

***KEY WORDS.* –** Spermatogenesis, sperm polymorphism, cottoid fish, sperm competition, sculpin, parasperm.

INTRODUCTION

Sperm produced by a species of animal is commonly unified in a single morph but the production of multiple types of sperm within a species has been observed in some species. The phenomenon in which males produce multiple discrete morphological classes of mature sperm within a single ejaculate is called sperm polymorphism (heteromorphism). This is seen in the sea urchin, *Anthocidaris crassispina*, which produces multiple types of fertile sperm (Au et al., 1998). In addition, sperm can be categorized based on their fertilization abilities: fertile sperm (eusperm) and non-fertile sperm. Non-fertile sperm can be further categorized into two categories: a) parasperm which are regularly produced through a constant developmental process along with normal fertile eusperm and b) aberrant deformed sperm which are irregularly crippled by unpredictable errors at several stages during spermatogenesis (Swallow & Wilkinson, 2002; Hayakawa, 2005).

Parasperm are formed through "paraspermatogenesis" and represent the non-gametic products of germ cells during spermatogenesis (Healy & Jamieson, 1981; Swallow & Wilkinson, 2002). In most cases, a single type of parasperm develops, but multiple types of parasperm are produced in some species (Jamieson, 1987; Buckland-Nicks, 1997). Individual variations within a type of parasperm are much less compared to the variations among different parasperm types. Parasperm is different from eusperm, particularly with regards to nuclear characteristics (Meves, 1903; Sivinski, 1984; Jamieson, 1987; Swallow & Wilkinson, 2002). Parasperm can be categorized into three types according to their nuclear state (Meves, 1903): a) apyrene sperm which possess no nucleus; b) oligopyrene sperm which possess less nuclear substance (i.e., chromosome, DNA) compared to eusperm and c) hyperpyrene sperm which possess excess nuclear substance compared to eusperm. All three types of parasperm are incapable of fertilization.

On the other hand, deformed aberrant sperm have to be distinguished from parasperm, mainly because they have no constant developmental process. For example, ejaculates of some primates including humans, contain sperm with deformities such as twin tails, no tails, no acrosome or a combination of these conditions (Gould, 1980). They scarcely contribute to fertilization and such morphological variations are considered abnormal and the result of various errors occurring at various stages of spermatogenesis.

Paraspermiogenesis and euspermiogenesis usually do not occur simultaneously (Jamieson, 1987; Fridländer, 1997; Swallow & Wilkinson, 2002). Euspermiogenesis starts at the early stage of the fifth instar and eusperm appear at the middle of the fifth instar. Paraspermiogenesis occurs at the last phase of the fifth instar and parasperm appear during pupation. The occurrence of parasperm during spermatogenesis can be seen throughout invertebrates and vertebrates (Sivinski, 1984; Jamieson, 1987; Buckland-Nicks, 1997; Hayakawa et al., 2002a; Swallow & Wilkinson, 2002; Hayakawa, 2005).

The occurrence of parasperm is seen widely in various phyla of invertebrates such as Annelida, Rotifera, Pogonophora, Mollusca and Arthropoda (Jamieson, 1987; Swallow & Wilkinson, 2002). Among invertebrates, Mollusca is the major phylum in which parasperm occur. This is especially so in the Class Gastropoda. Parasperm of Gastropoda have been mostly reported from the subclass Prosobranchia (Buckland-Nicks, 1997; Hodgson, 1997). It was reported that parasperm of Gastropoda develop from a distinct lineage of germ cells that can be distinguished from and at a stage equivalent to, the eupyrene spermatogonium (Buckland-Nicks et al., 1982; Winik et al., 2001). However, in most cases, paraspermatogenesis is characterized by atypical cell divisions around meiosis; such as, the primary spermatocyte develops without a maturation division or with asymmetric cell division into apyrene or hyperpyrene (polyploid) sperm (Okura et al., 1988; Buckland-Nicks, 1997; Hodgson, 1997). Such asymmetric cell division is followed by the development of multiple flagella, degeneration of the nucleus as well as the exocytosis of remnant nuclear materials and nutritive granular vesicles.

In contrast, sperm identified as parasperm occur only to a limited extent in vertebrates whereas, it occurs widely in invertebrates as discussed earlier. Cohen (1973) and Harcourt (1991) considered that deformed sperm of mammals might result from unavoidable errors during meiosis since they are functionless. Thus, the deformed sperm of mammals are not equivalent to the parasperm of invertebrates. However, this does not mean that there is an absence of parasperm production in vertebrates. Knowledge of parasperm may be limited in vertebrates due to a shortage of studies. In addition, the absence of clear criteria identifying parasperm has hindered studies in vertebrates. Teleost parasperm are identified by comparing cells in the cyst with those in the semen because irregularly-shaped cells in the seminal duct could either be parasperm or normal spermatids which only complete spermiogenesis in the seminal fluid as free cells (Hayakawa & Munehara, 2004). For example, the large, oval cells seen in the lumen in some teleost fish are spermatids at various levels of development, which are normally released from cysts with breakage of intercellular bridges before completion of spermiogenesis (Lahnsteiner and Patzner, 1990; Lahnsteiner et al., 1990; Koya et al., 1993; Mattei et al., 1993 and references therein; Manni and Rasotto, 1997; Yoneda et al., 1998; Muñoz et al., 2002). Thus, parasperm should be recognized by criteria based on observations of germ cells undergoing spermiogenesis.

On the other hand, cottoid fish parasperm are identified by these well-defined criteria: a) developmental process and morphological uniformity; b) divergence to heteromorphism occurring in meiosis as seen in most invertebrates and c) lack of fertilization capacity owing to the nuclei having aberrant morphology and being larger than the diameter of the micropyle of the egg (Hayakawa et al., 2002a; Hayakawa & Munehara, 2004). Parasperm produced by cottoid fish are usually oval in shape, possess single or multiple nuclei which form masses of highly electron-dense chromatin globules and are rich in cytoplasm (Hann, 1927, 1930; Quinittio & Takahashi, 1992; Hayakawa et al., 2002a). Flagellum formation in parasperm varies among cottoid species (Hayakawa & Munehara, 2004) with Gilbert's Irish lord, *Hemilepidotus gilberti,* produces non-flagellated parasperm (Hayakawa et al., 2002a). Parasperm confirmed by ultrastructural observations are reported from *Blepsias cirrhosus* (Hayakawa & Munehara, 2004), *Cottus hangiongensis* (Quinitio et al., 1989; Quinitio & Tahakashi, 1992), *C. nozawae* (Quinitio, 1989) and *H. gilberti* (Hayakawa et al., 2002a). Although limited to light microscopy observations, the occurrence of parasperm has been suggested in *Calycilepidotus spinosus*, *Cottus asper, Co. bairdii, Co. cognatus, Co. poecilopus, Co. pollux, Co. ricei* and *Trachidermus facsiatus* (Hann, 1930) and *Hemitripterus villosus* (Hayakawa, unpublished data).

In the present study, in order to clarify and compare the development of parasperm and eusperm, annual testicular changes in the marine cottoid fish, *Hemilepidotus gilberti*, were investigated and discussed in relation to their functions on sperm competition.

MATERIALS AND METHODS

***Fish.* –** Eighty seven adult males of *Hemilepidotus gilberti* were collected by angling and gill net sets in the coastal waters off Usujiri, Southern Hokkaido, Japan, from June 1992 to November 1993.

***Light microscopy.* –** Testes of 87 males were collected during the study period for histological observations. Prior to fixation, gonad weights were measured in order to calculate gonadosomatic indices, GSI, (gonad weight / body weight with gonads × 100). The testes were embedded in paraffin after fixation in Bouin's fluid and were serially sectioned at 5 - 7 μm thickness. These serial sections were stained with Delafield's hematoxylin and eosin. Maturity of testes was assessed monthly based on the occurrence of germ cells at

various developmental stages and examinations were conducted twice in January, August, September and October. This is because fish usually migrate from spawning ground to the feeding ground in January in Usujiri (unpublished data) and we wanted to confirm how nutritive conditions (the beginning of feeding activities) affected the gonadal state in that month, thus examinations were carried out twice. Similarly, examinations were carried out twice each from August to October as this period corresponds to the change from feeding period to spawning period where nutritive conditions would also undergo changes (unpublished data). To determine the presence of seminal parasperm, semen collected from the tip of the genital papilla with the use of capillary tubes was smeared on a glass slide and fixed with 10% formaldehyde solution. The nuclei of parasperm and eusperm were stained with Delafield's hematoxylin.

***Electron microscopy.* –** Paraspermatids and euspermatids present in the cyst of testis prior to spermiation (the release of mature sperm from cysts into the lumen) were observed ultrastructurally. The electron microscopy procedure was in accordance with Hayakawa et al. (2002a). Testes of males collected in June 1994 were dissected out and the pieces were fixed immediately in a 4% paraformaldehyde / 2.5% glutaraldehyde mixture in 0.1 M cacodylate buffer (pH 7.4) for about 5 hours at room temperature. The samples were then washed overnight and then post-fixed in 1% osmium tetroxide for 1 hour in the same buffer. After dehydration through a graded ethanol series, the samples were embedded in epoxy resin (Epon 812 TAAB). Sections (silver-gold) were prepared with a Reichert-Jung Ultracut E or Reichert Nissei Ultracut S ultramicrotome and stained with lead citrate and/ or uranyl acetate and examined with an Hitachi H-7000 or JEM 100-CX transmission electron microscope.

RESULTS

***Annual testicular change.* –** An examination of the testis structure of this species showed that the testis contains many lobules which are composed of seminal cysts of varying shapes and sizes, which were distinguishable at different stages of the developing germ cells. As shown in Figure 1, the GSI values among sampled fish began to increase between June and July and remained high from July through November and then decreased in the subsequent months. The seasonal testicular cycle of *H. gilberti* can be divided into six distinct periods as described below, based on histological observations of testis. In addition, changes in GSI values also indicated a division of the seasonal testicular cycle, although the statistical analysis showed no significant difference for GSI values among periods (ANOVA: $F(5, 81) = 1.93$, $p > 0.05$).

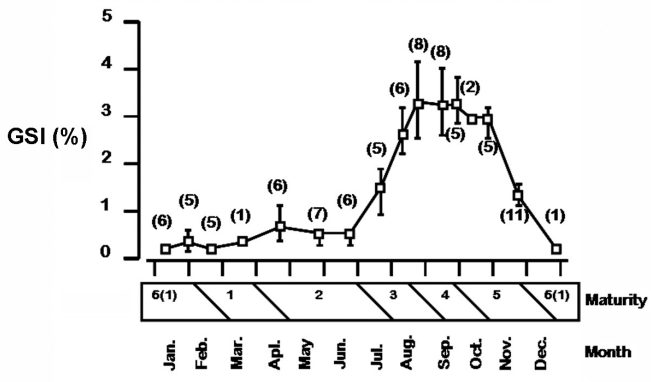

Fig. 1. Mean monthly gonadosomatic indices (GSI) and maturity of testes in *Hemilepidotus gilberti*. Numbers in parentheses in the graph indicate the number of fish sampled. Vertical bars represent standard errors. Numbers in the maturity x-axis represent reproductive periods; 1 = spermatogonial proliferation period; 2 = growth period; 3 = early maturation period; 4 = late maturation period; 5 = functional maturation period; 6 = post spawning period.

***1. Spermatogonial proliferation period (December to March).* –** The testis contained primary and secondary spermatogonia. The primary spermatogonia had 6.0 - 10.0 mm nuclei which were dispersed along the peripheral region within the lobules. The secondary spermatogonia (3.0 - 3.5 μm) were smaller than the primary spermatogonia and make up the seminal cysts. The mean GSI value during this period was low (mean ± S.E. = 0.28 ± 0.18; n = 11).

***2. Growth period (April to June).* –** Spermatogonia grew to form spermatocytes (Fig. 2). The primary spermatocytes are easily distinguished by their larger nuclei (3.0 mm) which stained well with hematoxylin. The secondary spermatocytes were smaller than the primary spermatocyte (2.5 - 3.0 mm) and were stained to a lesser degree by hematoxylin. In some cysts, euspermatid nuclei (< 2.5 μm) that condensed into a mass in the hemisphere were observed. Later in this period, paraspermatids/parasperm could be observed. They were characterized by their large nuclei (5.0 - 7.0 μm) which were strongly stained with hematoxylin. Euspermatids and paraspermatids occurred simultaneously in the same cyst. The mean GSI value in this period was still low (0.45 ± 0.28; n = 19).

***3. Early maturation period (July to early August).* –** Spermatocyte and spermatid cysts increased. Parasperm also increased. The mean GSI value rapidly increased to 2.08 ± 0.93 (n = 11).

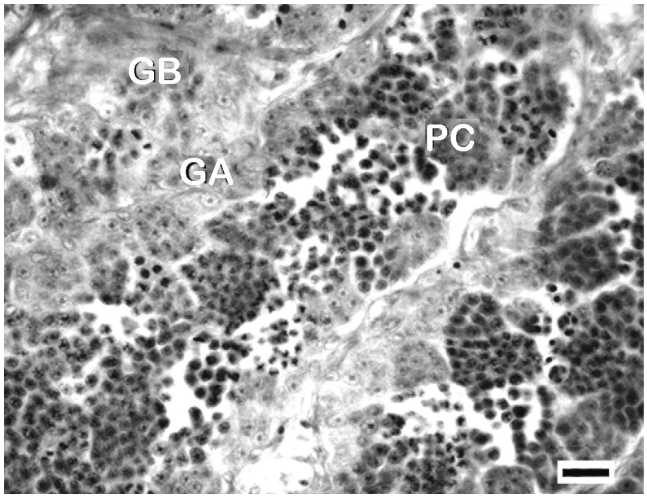

Fig. 2. Cross sections of testes at growth period of the reproductive cycle in *Hemilepidotus gilberti*. Scale bar = 10 μm. GA = primary spermatogonia; GB = secondary spermatogonia; PC = primary spermatocytes.

4. Late maturation period (late August to September). – Secondary spermatocyte and spermatid cysts occupied the testis but sperm appeared in many cysts. Most cysts contain parasperm (Fig. 3). Semen was collected by gently palpating the abdomen of some males. Parasperm were larger than eusperm and could be seen as erythrocyte-shaped cells, strongly-staining with hematoxylin, whereas nucleus of eusperm (< 2.0 μm) is in the shape of a horseshoe (Fig. 4). The mean GSI value in this period reached 3.21 ± 0.71 (n = 21).

5. Functional maturation period (October to November). – Cysts ruptured with sperm and parasperm being released into the lobule. In the lobule, eusperm tended to surround masses of parasperm (Fig. 5). The mean GSI value was 2.48 ± 1.49 (n = 18).

6. Post spawning period (December to February). – Sperm disappeared but parasperm remained in some portions. Spermatogonia were seen along the lobule walls. The mean GSI value was 0.18 ± 0.14 (n = 7).

In the course of spermatogenesis, no feature indicating the initiation of the divergence from normal eupyrene spermatogenesis to parasperm was observed in germ cells before meiosis (Fig. 2). However, paraspermatids, which differ morphologically from normal euspermatids, appeared during/after the second meiotic division in every germinal cyst together with euspermatids (Fig. 3).

Connection between paraspermatids and euspermatids. – As shown in Figure 6, the connection of paraspermatids and euspermatids by intercellular bridges in the cyst was confirmed.

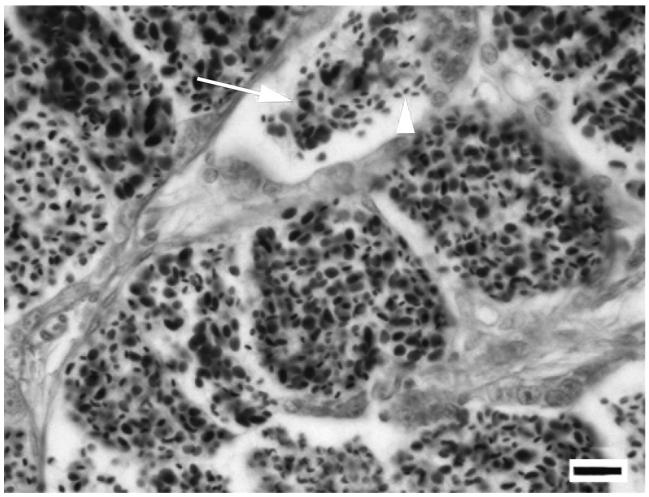

Fig. 3. Cross sections of testes at late maturation periods of the reproductive cycle in *Hemilepidotus gilberti.* Paraspermatids (arrow) can be seen together with euspermatids (arrowhead) in each cyst. Scale bar = 10 μm.

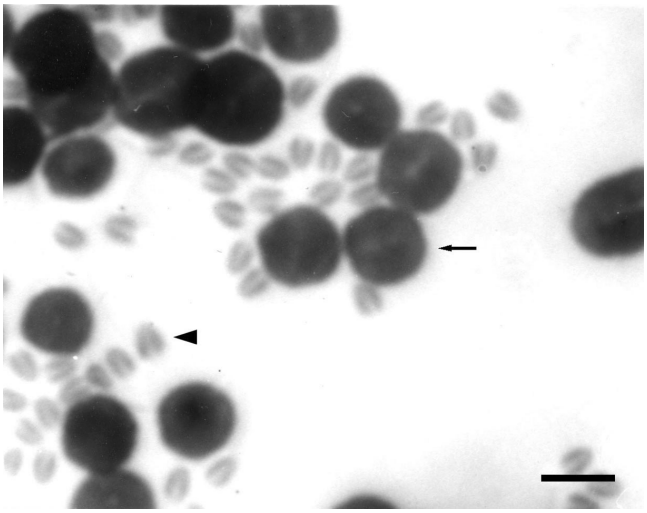

Fig. 4. *Hemilepidotus gilberti* parasperm and eusperm smear on a glass slide stained with hematoxylin. Nuclear of parasperm (arrow) and eusperm (arrowhead). Eusperm flagella are not shown in this micrograph. Scale bar = 5 μm.

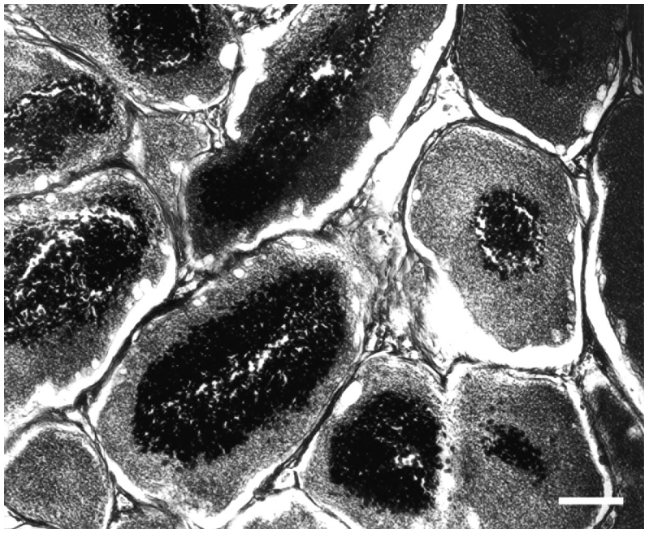

Fig. 5. Cross sections of testes at the functional maturation period of the reproductive cycle in *Hemilepidotus gilberti*. Many eusperm surround the mass of parasperm (more strongly-stained with hematoxylin compared to eusperm) which is located at the central portion in each lobule. Scale bar = 20 μm.

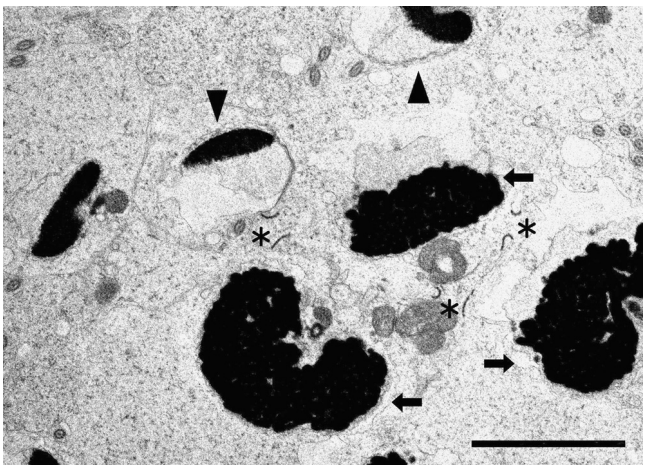

Fig. 6. Electron micrograph of germinal cyst of *Hemilepidotus gilberti*. Arrows and arrowheads represent paraspermatids and euspermatids, respectively. Asterisks indicate the intercellular bridges. Scale bar = 5 μm.

DISCUSSION

Gonadosomatic indices (GSI) values began to increase from June to July, corresponding to the increase of development of eusperm and parasperm in *Hemilepidotus gilberti*. From the spermatogonial proliferation period to the growth period, when spermatogonia and spermatocytes dominated in the testes, there was no indication of the initiation of parasperm development. However, during/after the early maturation period, basophilic germ cells representing paraspermatids appeared along with euspermatids. Paraspermatids and euspermatids occurred simultaneously in the same cyst. Commonly, within the cyst of teleost fishes, the groups of germ cells divide in synchrony and all germ cells derived from a spermatogonium are in the same developmental stage at any given time (Billard et al., 1982; Billard, 1986, 1990; Pudney, 1993). In addition, germ cells undergoing the spermatogenesis are connected to each other by intercellular bridges until spermiation (Faucett et al., 1959). Thus, simultaneous occurrence of paraspermatids and euspermatids in the same cyst indicates that the parasperm and eusperm were derived from the same spermatogonium. This has been verified by electron microscopic observations. Spermatids that become eusperm and parasperm were connected by intercellular bridges within the cyst in both our previous (Hayakawa et al., 2002a) and the present studies.

When parasperm were initially discovered in a cottoid fish, they were regarded as a representation of disease or death of germ cells (Quinitio & Takahashi, 1992). The occurrence of a bridge-partitioning complex (BPC) within the intercellular bridges, which might prevent the spread of disease from one cell to another in the same cyst during meiosis, supported this hypothesis. However, it was reported that the BPC commonly occurs in somatic cell divisions during organogenesis in the embryo of animals in the early stages of development and acts as a regulator of normal cell division (Meithing, 1990). In addition, our recent study on the germ cell culture suggests that spermatocytes may have differentiated autonomously into both types of sperm without contact with Sertoli cells (Hayakawa et al., 2004). Thus, the possibility that differentiation of parasperm is determined before meiosis has to be considered.

Previous investigations on parasperm have shown a number of adaptive roles for these cells. Sahara and Kawamura (2002) examined the roles of parasperm by the "double copulation" experiment using heat-treated males and polyploid males of silkworms. They artificially produced sterile males showing spermatogenesis without apyrene sperm production by heat-treatment of the instars at the spinning stage. The authors also produced polyploid males (triploid and tetraploid) induced by cold-treatment on the eggs at the first cleavage stage. The resultant polyploid males showed spermatogenesis without eusperm production. All the eggs from normal females, fertilized by either the heat-treated or polyploid males were infertile, whereas the eggs from normal females mated with a polyploid male followed by mating with a heat-treated male showed high fertility (more than 70% of the eggs were fertile). Therefore, it was confirmed that parasperm are indispensable for eusperm fertilization in silkworms. The reason why such a complex process is required for fertilization of this species is unclear. However, there may be internal controls to regulate the number of parasperm and eusperm to maintain the males' reproductive (fertilization) success.

On the other hand, it was recently reported that the proportion of parasperm to total sperm produced by males in some Lepidoptera and Prosobranchia is influenced by the change of density or sex-ratio in the population. For example, in the armyworm, *Pseudaletia separata*, males reared in high larval densities increased the production of apyrene sperm but did not alter eusperm production (He & Miyata, 1997). Similar results have been reported from a freshwater snail, *Viviparus ater*. Males in male-biased conditions produced a larger proportion of parasperm than males in female-biased conditions (Opplinger et al., 1998). The role of parasperm in the snail might be to overcome sperm competition (Buckland-Nicks, 1997). Homogenates of the parasperm make eusperm agglutinate, whereas those of eusperm do not have such an effect. Moreover, they often exhibit a sperm plug-like formation in the bursa copulatrix. Thus, the change in the proportion of parasperm to total sperm is thought to be related to sperm competition (He & Miyata, 1997; Opplinger et al., 1998). The reproductive history of each male or change of sex-ratio in the population that they are subjected to for a certain period may predict potential sperm competition and affect sperm production.

In *Hemilepidotus gilberti*, parasperm are released along with eusperm and plays an anti-dispersive role by preventing accidental eusperm loss due to lateral dispersion of semen during ejaculation (Hayakawa et al., 2002c). In addition, parasperm plays a role in assisting kin eusperm to fertilize an egg mass by blocking non-kin eusperm when several males compete during sperm emission (Hayakawa et al., 2002b). The lump formation of parasperm occurs at the boundary of an egg mass where ovarian fluid contacts seawater and it engulfs eusperm from another male that arrives later. This phenomenon suggests that sperm from another male would then be prevented from penetrating through the ovarian fluid surrounding the eggs. Thus, parasperm production likely relates to sperm competition. This ties in with our previous study (Hayakawa et al., 2002a) which suggested that the proportion of parasperm in the semen of *H. gilberti* varies between individuals as the proportion of males in the population becomes larger. What causes such a difference is unclear, but it may relate to the sex-ratio that males are subjected to in the population or the reproductive history of each male. Needless to say, the endocrine mechanism controlling sperm production would differ between invertebrates and fish. However, it has been suggested that sperm characteristics (sperm tail length and swimming speed) of bluegills, *Lepomis macrochirus*, vary among individuals, reflecting the reproductive history or status of each male and its spawning environment (Casselman & Montgomerie, 2004). Since swimming speed is thought to be influenced by ATP stores (Billard et al., 1995), it may represent characteristics of mitochondria (number, size, or metabolic ability) in sperm. Thus, intra-specific factors are a possible influence on the sperm production of fish.

In the present study, it was shown that mean GSI values of *Hemilepidotus gilberti* rapidly increased at the early maturation period, corresponding to an increase in parasperm. Although this species feeds actively for 3 - 4 months prior to the early maturation period, they almost starve from this period to the end of the spawning season (Hayakawa, unpublished data). It is thought that the GSI represent an investment in the gonads and a difference of the GSI values among males indicates a difference in gonadal investment of each male (Marconato & Shapiro, 1996). Thus, the GSI values in male *H. gilberti* are likely to reflect a positive correlation between gonadal investment and feeding, thus affecting sperm (parasperm) production. Taking into account the role that parasperm exhibit in polyandrous mating, it can be predicted that some controls, which respond to the mating environment, are involved in the differentiation of parasperm in *H. gilberti*.

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