Reproductive traits of starspotted smooth-hound *Mustelus manazo* in Tokyo Bay, Japan

(東京湾におけるホシザメ個体群の生殖特性)

Doctoral Thesis

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Contents

I. Preface

Sharks are considered top predators and may have an important role in the regulation of marine ecosystems at lower trophic levels (Cortés, 1999; Stevens et al., 2000; Schindler et al., 2002). In recent years, investigation focusing on elasmobranch (sharks, rays and skates) conservation and management has been increasing. Elasmobranch populations are decreasing worldwide, mainly due to their susceptibility to overfishing and other anthropogenic impacts as well as climate changes (Stevens et al. 2000; Chin and Kyne 2007). Directed commercial fisheries for shark meat and fins, combined with substantial bycatch, are thought to be the main cause of elasmobranch mortality (Bonfil, 1994; McKinnell and Seki, 1998; Megalofonou et al., 2005). Populations with low recruitment (i.e., slow growth, late maturation and low fecundity) are especially vulnerable to overexploitation. Most sharks exhibit slow growth rates, produce relatively few offspring, and have long inter-birth intervals (Manire and Gruber 1990;

Cortés 2000). Consequently, sharks may take a long time to recuperate after a decrease in population size (Stevens et al. 2000).

The starspotted smooth-hound *Mustelus manazo* is distributed from the northwestern Pacific Ocean to western Indian Ocean (Compagno, 1984). *M. manazo* is one of the dominant elasmobranch species in the demersal assemblage in Tokyo Bay, and is caught incidentally during the commercial bottom-trawl fisheries for other target species in the bay (Yamaguchi et al., 1997). The catch-per-unit-effort (CPUE; the number of individuals or the wet body weight of the species caught during a 10-min tow) is commonly used as an indicator of the stock size of a species. Fisheries-independent trawl surveys have revealed that the CPUE of *M. manazo* was low during the 1990s but increased markedly in the early 2000s. CPUE then decreased from 2005 to 2008, but increased again thereafter (Kodama et al., 2010). Furthermore, the number of embryos per litter significantly reduced in comparison with that in 1990s (Yamaguchi et al., 1997). The reason for these fluctuations in stock size remains unknown. To elucidate the actual causal factor(s) for fluctuating *M. manazo* population in the bay, I need to

examine reproductive traits of females and males of *M. manazo*, development and growth of embryos, and several environmental factors, such as prey abundance, temperature and harmful chemical substances, which may have directly or indirectly influenced the reproductive traits that would be recently observed in *M. manazo* population in Tokyo Bay.

II. Chapter 1

Life-history traits of starspotted smooth-hound *Mustelus manazo* in Tokyo Bay: food habit

Introduction

Feeding habits (Yamaguchi et al., 2000) of *M. manazo* in Tokyo Bay have been reported during the 1990s, when the stock size was low (Kodama et al., 2010; Fig. 1-1). However, little is known about the feeding habits of *M. manazo* in Tokyo Bay during the 2000s, when the stock size was greater than that in the 1990s.

The objective of the present study was to clarify the life-history traits of *M. manazo* in Tokyo Bay, detailed about food habit. In this chapter, food habits of this species in Tokyo Bay were investigated. It is indispensable to clarify the life cycle traits needed for stock assessment.

Materials and methods

The samplings were carried out in Tokyo Bay, from May 2007 to

June 2008 and from February 2009 to June 2010 (Fig. 1-2). Samples of *M. manazo* were monthly caught by commercial bottom trawl fisheries (a beam trawl with a mouth 5.5 m and a cod-end mesh size of 3 cm) in the bay. A total of 910 specimens, consisting of 332 males and 578 females, were collected during this study period.

Immediately after collection, the whole stomachs of the specimens were fixed in 10% formalin, recognizable stomach contents were identified to the lowest possible taxon and each prey item was weighed in wet condition to the nearest 0.1 g. To compare the food habits of *M. manazo* quantitatively, in this present study used mean percentage composition of each item by weight (*%W*), divided by the total sum of the individual percentage weight of each item by the number of specimens examined; frequency of occurrence (*%F*), expressed as the percentage of stomachs containing the prey item by the total number of stomachs examined; ranking index (*RI*), calculated by multiplying *%F* by *%W* ($0 \leqq RI \leqq 10000$). The individuals with empty stomachs were excluded from these three methods. The vacuity index was expressed as the percentage of empty stomachs

divided by the total number of stomachs examined. Stomach contents weight was expressed as a percentage of wet body weight. To evaluate dietary variations with predator size, specimens, were sorted into 100 mm total length classes.

Results

Mean stomach contents (weight per body weight, w/bw) of male *M. manazo* were 1.5 % and 0.5% in 2007–2008 and 2009–2010, respectively, and that of females were 1.6 % and 0.5 % in two periods, respectively. The percentage of the stomach contents (w/bw) of male in 2007–2008 was showed highest value of 3.3 % in < 600 mm size class, whereas it was lowest value of 0.4 % in < 700 mm size class. Meanwhile, in 2009–2010, it was showed a value of less than 1% overall (Fig. 1-3).

The dietary composition for *M. manazo* in 2007–2008 and 2009–2010 were indicated in Figure 1-4. Based on three indices (*%W*, *%F*, *RI*), the major dietary component was crustaceans at all locations. There appeared have changes in the frequency of occurrence of several major prey

categories on two periods. The occurrence of crustaceans prey was increased in 2009–2010 than in 2007–2008. Isopods and Hermit crabs appeared a lower frequency in 2007–2008, were not found in prey item in 2009–2010 (Fig. 1-4a). Meanwhile, examined the proportion of the diet categories in the Crustaceans, proportion of crabs were increased, but shrimps decreased (Fig. 1-4b).

Mean percentage weights (*%W*), frequencies of occurrence (*%F*) and ranking indices (*RI*) of each prey items in the diet of *M. manazo* for in 2007–2008 and 2009–2010 are listed in Table 1. Crustaceans constituted the major dietary component for both periods. Further in 2009–2010, crustaceans prey item (*%W*, 95.9; *%F*, 87.1; *FI*, 8359) was occupied the greatest in all prey items.

Diet composition was examined in relation to period and length of *M. manazo* (Fig. 1-5). In 2007–2008, *%W* of important prey items was occurred mantis shrimp, crabs, and shrimps (Fig. 1-5a). Shrimps were the dominant prey item of small length classes, but declining in importance thereafter. In comparison, mantis shrimp was rarely found in the diet of

small length classes, but thereafter increased in importance. In the largest size class, mantis shrimp comprised approximately 30 *%W*. Crabs were found consistently in all length classes. Similarly, this trend was appeared in 2009–2010 (Fig. 1-5b). On the other hand, fishes were declined all length classes in 2009–2010 (Fig. 1-5b).

Discussion

Mean stomach content (w/bw) was dramatically reduced from 2007–2008 to 2009–2010. The differences in mean stomach content weight may reflect differences in the prey environment. It is likely that food limitation is one reason for the differences in the growth rates at each population of *M. manazo*. According to Kodama et al. (2010), stock abundance of mantis shrimp *Oratosquilla oratoria* among major prey item of *M. manazo* in Tokyo Bay was decline in 1990s thereafter.

Figures and Tables

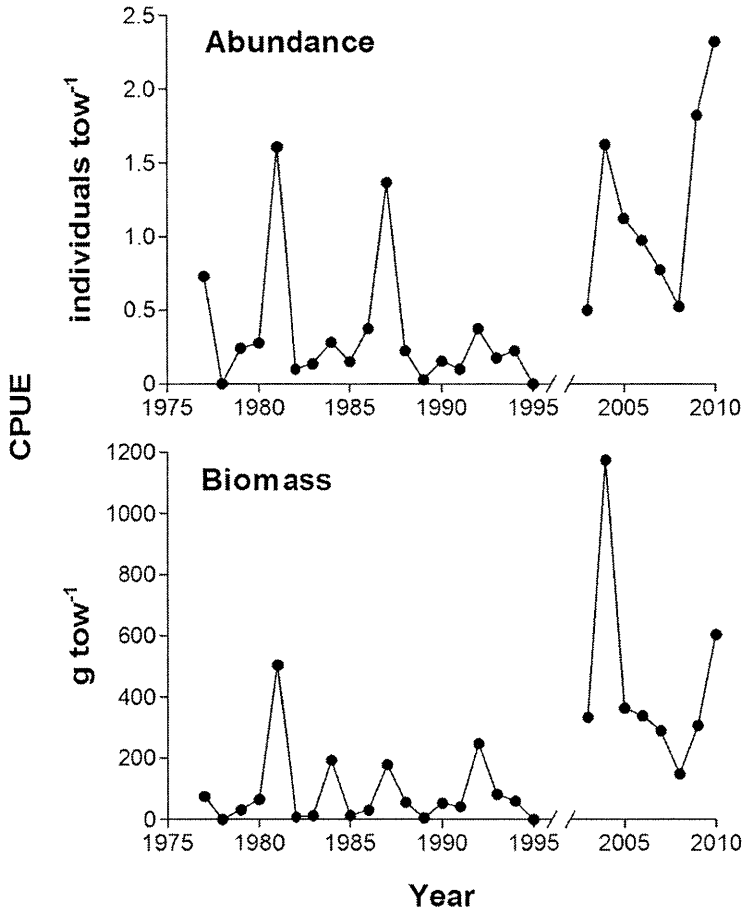

Fig. 1-1. Annual catch-per-unit-effort (CPUE) of *Mustelus manazo* in Tokyo Bay between 1977 and 2010. Data from Kodama et al. (2010)

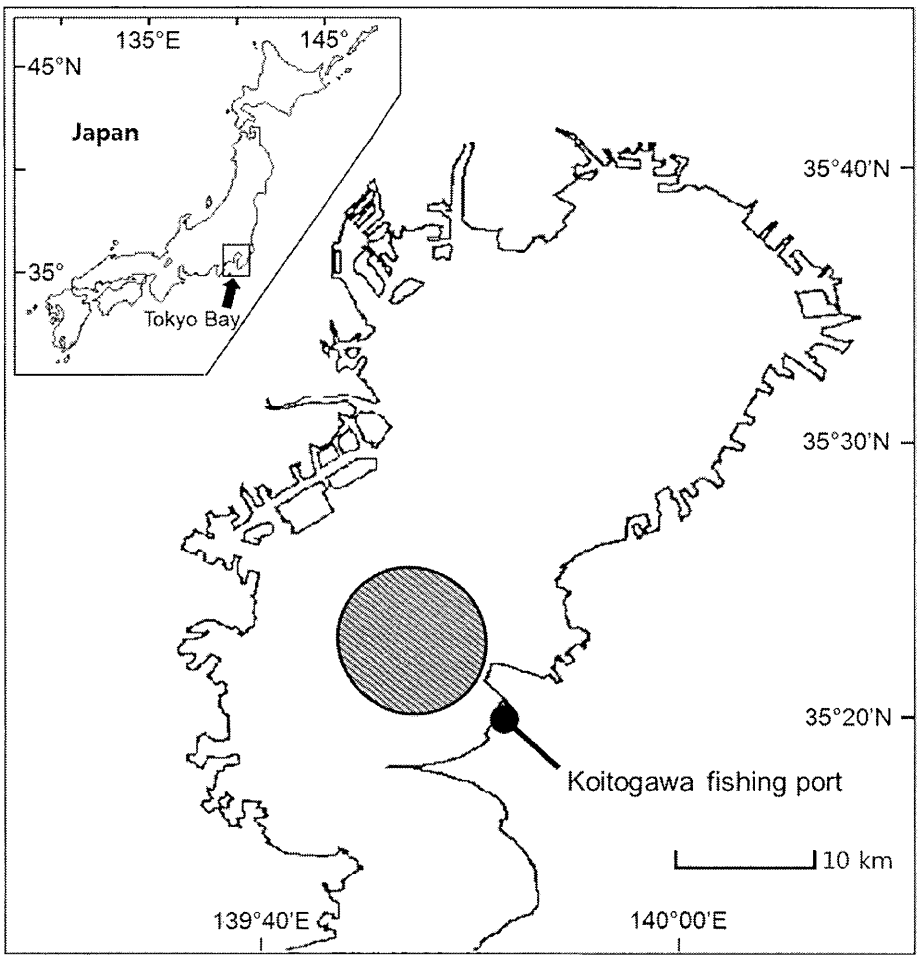

Fig. 1-2. Sampling area of *Mustelus manazo* in Tokyo Bay, Japan.

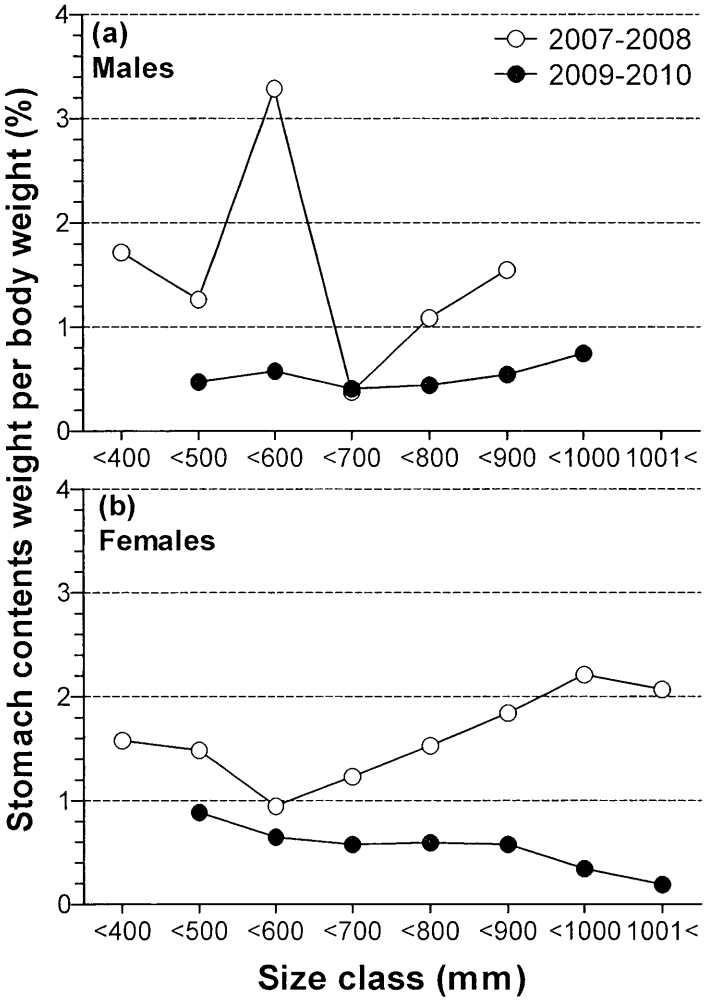

Fig. 1-3. Stomach contents weight per body weight for different size classes of *Mustelus manazo* from in 2007–2008 and 2009–2010 in Tokyo Bay.

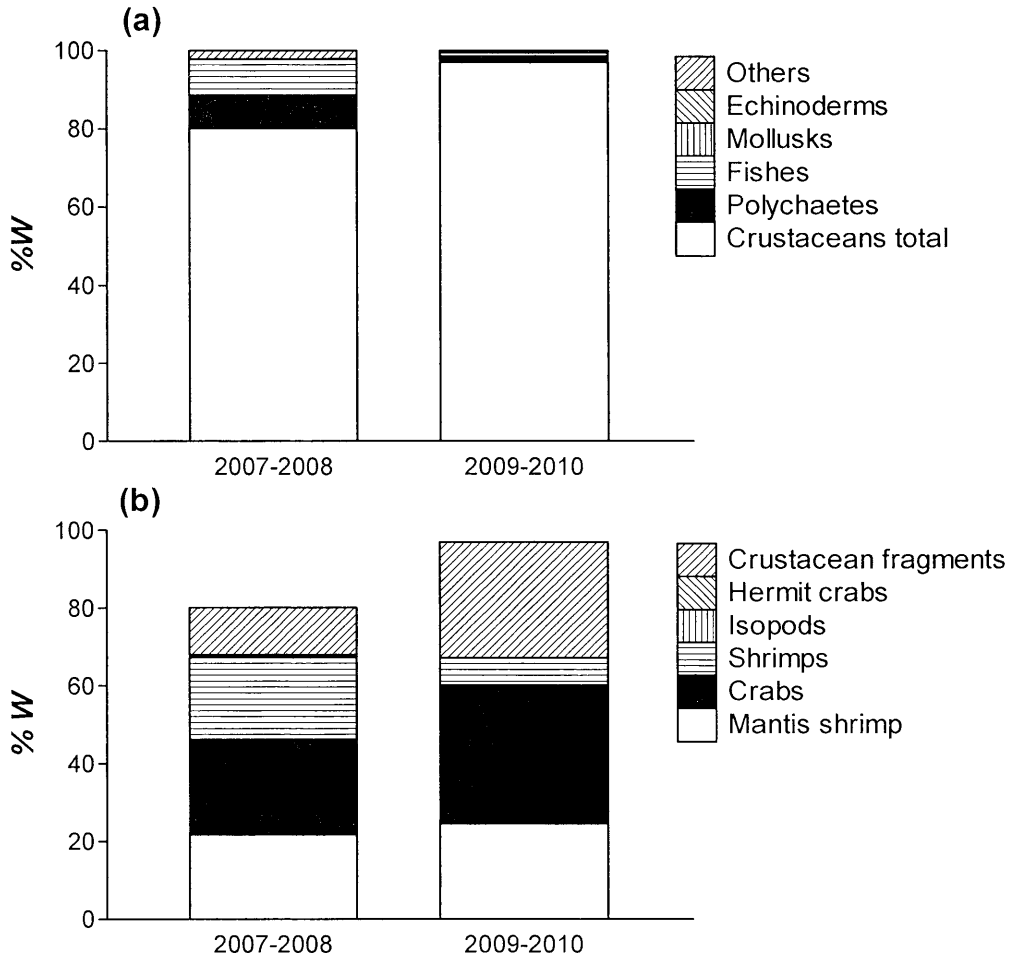

Fig. 1-4. The dietary composition for smooth-hound *Mustelus manazo* from in 2007–2008 and 2009–2010 in Tokyo Bay. (a) Proportion of the diet categories. (b) Proportion of the diet categories in the Crustaceans.

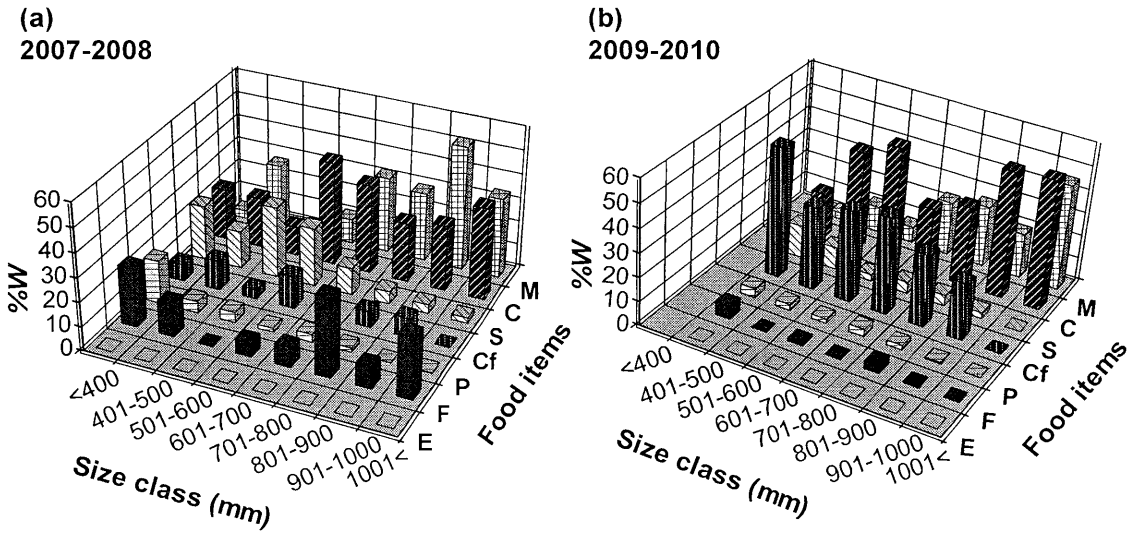

Fig. 1-5. Percentage weight (*%W*) of prey categories for each size class of *Mustelus manazo* from in 2007–2008 and 2009–2010 in Tokyo Bay. *M*, mantis shrimp; *C*, crabs; *S*, shrimps; *Cf*, crustacean fragments; *P*, polychaetes; *F*, fishes; *E*, errant mollusks.

Table 1. Comparison of percentage weight (*%W*), frequency of occurrence (*%F*) and ranking index (*RI*) of prey items in the diet of *Mustelus manazo* from Tokyo Bay.

Prey items			2007-2008			2009-2010		
			%W	*%F*	*RI*	*%W*	*%F*	*RI*
Crustaceans			78.9	96.3	7589.6	95.9	87.1	8358.7
	Mantis shrimp	*Oratosquilla oratoria*	34.3	42.5	1458.5	21.5	27.5	592.6
Crabs			25.4	53.8	1367.6	36.6	2.4	87.6
	Goneplacidae	*Carcinoplax longimana*	0.2	1.3	0.2	–	–	–
		Carcinoplax vestita	21.7	31.3	677.8	26.1	31.4	822.1
		Eucrate crenata	–	–	–	0.5	0.9	0.4
	Portunidae	*Charybdis bimaculata*	0.9	6.3	5.8	1.0	3.0	2.9
	Pinnotheridae	*pinnixa rathbuni Sakai*	0.7	8.8	5.7	2.0	10.5	20.6
		tritodynamia rathbuni	0.1	1.3	0.1	5.1	15.6	79.2
	Cancridae	*Cancer amphioetus*	0.2	2.5	0.4	0.3	2.1	0.7
		Cancer gibbosulus	0.5	2.5	1.2	–	–	–
	Majidae	*Pyromaia tuberculata*	0.2	2.5	0.4	1.1	3.6	3.8
	Unidentified		–	–	–	0.2	–	–
	Fragments		1.1	12.5	14.2	0.3	1.8	0.6
Shrimps			13.3	73.8	981.8	4.5	26.3	118.6
	Crangonidae	*Crangon affinis*	1.8	33.8	60.7	0.8	9.6	7.7
	Alpheidae	*Alpheus distinguendus*	–	–	–	0.5	0.6	0.3
		Alpheus japonicus	9.8	35.0	344.4	1.9	9.0	16.8
	Unidentified		0	1.3	24.2	0.5	3.0	0.9
	Fragments		0.9	26.3	24.2	0.8	6.0	4.8
Hermit crabs						–	–	–
	Galatheidae	*Galathea orientalis*	0.1	2.5	0.1	–	–	–
Isopods			0.3	17.5	4.7	–	–	–
	Fragments		0	2.5	0.1	–	–	–
Crustacean fragments			5.5	35.0	191.1	33.3	68.9	2293.2
Polychaetes			3.3	43.8	144.9	2.1	27.5	58.1
Fishes			17.3	32.5	561.6	1.8	5.4	9.4
	Engraulidae	*Engraulis japonica*	16.3	26.3	428.0	0.3	1.5	0.4
	Sardinella	*Sardinella zunasi*	0.7	1.3	0.9	–	–	–
	Fragments		0.3	6.25	1.7	1.5	3.9	5.7
Errant molluscs			–	–	–	0.1	0.9	0.1
Bivalves			–	–	–	–	–	–
	Semelidae	*Theora lata*	0	2.5	0	–	–	–
	Mytilidae	*Mytilus galloprovincialis*	0	1.3	0	–	–	–
Ophiuroids			0	1.3	0	–	–	–
	Ophiolepididae	*Ophioplocus japonicus*	–	–	–	–	–	–
	Unidentified		0	1.3	0.1	–	–	–
Unidentified			0.5	10.0	5.1	0.1	2.4	0.3
No. fish with food examined					80			328
Vacuity index (%)					3.6			11.6

III. Chapter 2

Reproductive traits of male starspotted smooth-hound *Mustelus manazo* in Tokyo Bay

Introduction

The starspotted smooth-hound *Mustelus manazo* is one of the most dominant elasmobranch species in the demersal assemblage in Tokyo Bay (Yamaguchi et al., 1997). Fisheries-independent trawl survey revealed that catch-per-unit-effort (CPUE: number of individuals or wet body weight of the species caught by 10-min tow) of *M. manazo* was low during 1990s, and markedly increased in the early 2000s (Kodama et al., 2010). Furthermore, CPUE decreased from 2005 to 2008, and increased again from 2009. However the reason of the fluctuations of the stock size remains unknown. In order to explore the cause of the stock fluctuations of *M. manazo*, it is necessary reveal fundamental life history characteristics of this species.

To date, several studies showed that changes in life history traits correspondent with variations in stock-size level have been observed in

other dominant demersal species in Tokyo Bay, for example, Japanese mantis shrimp *Oratosquilla oratoria*; changes in the reproductive patterns concurrent with stock size decline (Kodama et al., 2006), and marbled sole *Pseudopleuronectes yokohamae*; changes in growth between high and low stock-size periods (Lee et al., 2009). Fundamental life history traits (age and growth, Yamaguchi et al., (1996); reproductive traits, Yamaguchi et al., (1997); and feeding habits, Yamaguchi and Taniuchi, (2000) of *M. manazo* in Tokyo Bay have been reported during 1990s, when the stock size was low. However, there has been no scientific report on the life history traits of *M. manazo* in Tokyo Bay during 2000s when the stock size is different from that in 1990s.

In this study, reproductive traits of male *M. manazo* during 2000s were investigated. It is provided the basic reproductive traits of the smooth dogfish in Tokyo Bay.

Materials and methods

Sample

A total of 332 males of *M. manazo* were collected monthly from May 2007 to June 2008 and February 2009 to June 2010 in Tokyo Bay, Japan. Samples of *M. manazo* were monthly caught by commercial bottom trawl fisheries in the bay, and were landed at the Koitogawa fishing port in Chiba Prefecture. The male sex was identified, and measurements of total length (TL, mm), pelvic clasper length (CL, mm) and body weight (BW, g) were carried out. The testes were then dissected out at dockside. After dissection, the weights of the testes and liver (g) were recorded. The testes and epididymis were fixed with 10% neutral buffered formalin for 1 week at room temperature for histological observation.

Development of reproductive organs

Development of reproductive organs relating to sexual maturity of male was classified following the previous study (Yamaguchi et al., 1997). Immature, claspers are not calcified and testes are not well-developed; premature, claspers are more or less calcified and the testes are developed, but no sperm are observed in the seminal vesicles; and mature, claspers are

calcified, and sperm are found in the seminal vesicles.

Size at maturation

To investigate the size at maturity, the TL at which 50% of the individuals achieved maturity (TL_{50}) was estimated by using a logistic equation (Ratkowsky, 1990):

$$P(\mathrm{TL}) = 1/\{1+\exp^{(-a(\mathrm{TL}-b))}\},$$

where P(TL) is the proportion of mature individuals in a given TL class, a is a constant, and b is TL_{50}. The class interval for TL in this analysis was 20 mm. Estimation of the parameters was conducted by means of least-squares fitting using the KyPlot 5.0 software (KyensLab Inc., Tokyo, Japan).

Reproductive cycle

Testis samples after fixation were washed thoroughly in 70% ethanol. The tissues were then dehydrated in a graded ethanol series and infiltrated with paraffin. Sections were cut with a microtome in paraffin at intervals of 6 μm, then stained with Mayer's hematoxylin and eosin.

Thereafter, I confirmed that the development pattern of the germ cells was uniform throughout a testis. On the basis of this screening, a representative cross-section of the testicular tissues was selected from the middle of the right or left lobe for observation under a light microscope. Developmental stages of germ cells in the testes were classified (Fig. 2-4): spermatogonium (stage 1), spermatocyte I (stage 2), spermatocyte II (stage 3), spermatid (stage 4), immature spermatozoon (stage 5), and mature spermatozoon (stage 6). To estimate the progress of maturity in the testes, I used the following equation after Conrath and Musick (2002):

$$P_i = 100 \times L_i / L_t ,$$

where P_i is the proportion of the length of the germ cell cluster at each developmental stage L_i (i = 1~6) as a proportion of the ventrodorsal length of all germ cell clusters combined (L_t; i.e., the total length of a straight line drawn from the central part of the stage 1 germ cell cluster (located in the ventral testis) to the central part of the stage 6 germ cell cluster (located in the dorsal testis) for an image of the preparation of testis used in the calculation). P_i was determined for 1 to 10 mature males per month (Fig. 2-3).

The gonadosomatic index (GSI) of Yamaguchi et al. (1997) was calculated for 1 to 10 mature males per month by using the following equation to investigate the seasonal pattern in the development of the testes:

$$GSI = (\text{testis weight} / TL^3) \times 10^9,$$

Results

Sexual maturation

The relationship between TL and CL for each maturity stage of *M. manazo* was examined (Fig. 2-1). The CL of the immature and mature males gradually increased as TL increased. However, the CL of premature males increased dramatically within TL ranges of 562 to 706 mm in 2007–2008 and 620 to 672 mm in 2009–2010. Males with calcified claspers occurred from a TL of 646 and 648 mm in 2007–2008 and 2009–2010, respectively.

TL_{50} was estimated to be 647 and 650 mm in 2007–2008 and 2009–2010, respectively (Fig. 2-2). The TL_{50} values of between 2007–2008 and 2009–2010 had no difference.

Reproductive cycle

A cross section of the testis is shown in Figure 2-3, and the developmental stages of the spermatocysts are shown in Figure 2-4. In Stage 1, secondary spermatogonia were confined to the spermatocysts, which were delineated by a basement membrane. During this stage the Sertoli cell nuclei began to migrate from their positions lining the spermatocyst lumen and came to lie just internal to the basement membrane of the spermatocyst (Fig. 2-4a). In Stage 2, the primary spermatocyte nuclei contained granular and irregularly aggregated heterochromatin. The Sertoli cell nuclei had completed their migration (Fig. 2-4b). In Stage 3, the presence of secondary spermatocytes was confirmed by their particularly small nuclei (Fig. 2-4c). In Stage 4, spermatids began to transform from round to oval and elongated shapes (Fig. 2-4d), and in Stage 5, the spermatocysts contained mature spermatozoa morphologically, the heads of which were loosely arranged along the basement membrane (Fig. 2-4e). In Stage 6, before spermiation, the spermatozoa were arranged in increasingly dense clumps and had developed further (Fig. 2-4f).

The GSI of mature males peaked in December and then declined until June in 2007–2008, meanwhile it peaked in November and decreased until May in 2009–2010 (Fig. 2-5a). The frequency distribution of the testis maturity stages in mature males also showed seasonal variation in 2009–2010 (Fig. 2-5b). Germ cells at stages 1 to 4 dominated in the testes from June to November. Late developmental stages (5 to 6) increased in proportion from December to March (Fig. 2-5b). The proportion of mature spermatozoa (stage 6) in the testes peaked in April (Fig. 2-5b).

Discussion

Sexual maturation

The minimum size at male *M. manazo* in Tokyo Bay attain sexual maturity was 687 mm TL in 1994–1996 (Yamaguchi et al., 1997), versus 646 and 648 mm TL in 2007–2008 and 2009–2010, respectively. These results suggest that the size at maturity of *M. manazo* in Tokyo Bay was getting smaller in the 2000s than that in the 1990s. Although the reason for this decline remains unknown, it may be related to phenotypic plasticity,

because the size at first maturity of *M. manazo* ranged between 600 and 700 mm TL among different locations in previous research: 600 to 650 mm TL for males and 630 to 700 mm TL for females in the East China Sea (Teshima, 1981) and 621 to 640 mm TL for both sexes near Choshi, Japan (Taniuchi et al., 1983).

Reproductive cycle

Our histological observation of the testes revealed that the frequency of spermatozoa in the testes peaked from April to May, then decreased abruptly in June. On the other hand, the GSI of male *M. manazo* in Tokyo Bay peaked from November to December—almost half a year earlier than the duration of peak frequency of spermatozoa in the testes in both the 1990s (Yamaguchi et al., 1997) and the 2000s (present study). GSI reached its minimum from May to July in both the 1990s (Yamaguchi et al., 1997) and the 2000s (present study). Spermatozoa of *M. manazo* are produced actively in the testes when GSI is declining (Teshima, 1978), and the mature spermatozoa are discharged from the testes through the efferent

duct, then pass through the epididymis and are stored in the seminal vesicles for ejaculation (see; chapter 3). This evidence suggests that ejaculation of *M. manazo* occurred from May to July in Tokyo Bay in both the 1990s and the 2000s.

In conclusion, prominent changes in the reproductive traits (i.e., a decline in size at first maturity) were evident for *M. manazo* in Tokyo Bay during the 2000s. There is a possibility that other life history traits, such as feeding habits may have changed between 1990s and 2000s as observed in the reproductive traits, which may affect stock fluctuation of this species (e.g., decrease in the growth may cause reduced fecundity, or change in feeding habit may affect energy allocation between growth and reproduction). Therefore, further studies are needed to examine if there are any changes in other life history traits of *M. manazo*, which would contribute to elucidate the mechanism of stock fluctuation of *M. manazo* in Tokyo Bay.

Figures and Tables

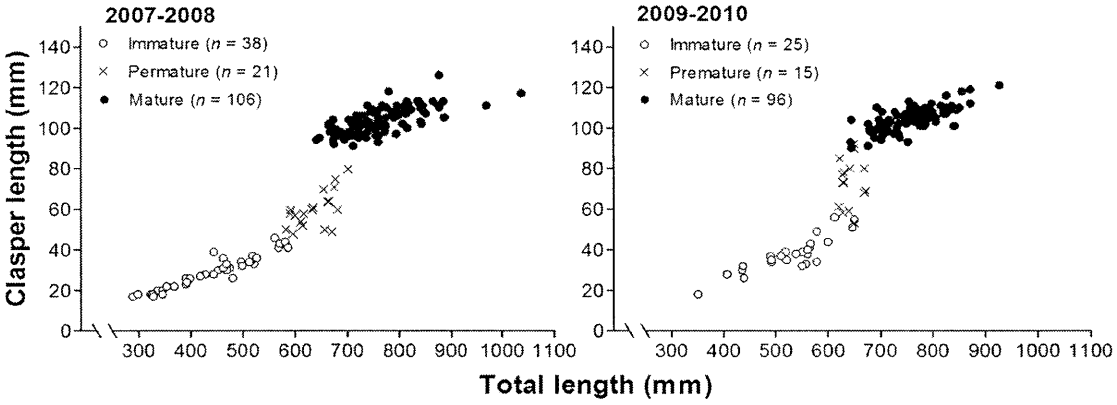

Fig. 2-1. Relationship between clasper length and total length of male *Mustelus manazo* in Tokyo Bay in 2007–2008 and 2009–2010.

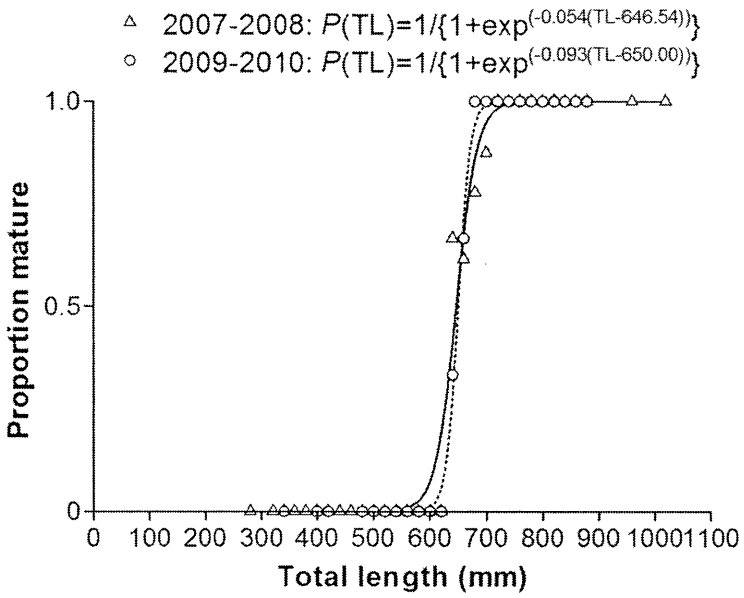

Fig. 2-2. Proportions of mature individual male *Mustelus manazo* in Tokyo Bay as a function of total length (mm) in 2007–2008 (solid lines) and 2009–2010 (dotted lines).

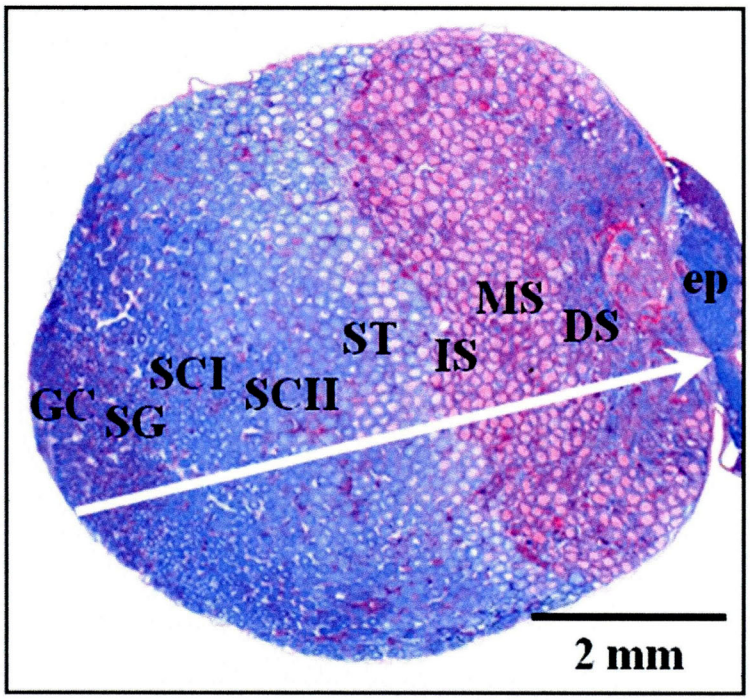

Fig 2-3. Cross section of a *Mustelus manazo* testis, stained with hematoxylin and eosin. *GC* (germinal cell), *SG* (spermatogonia), *SCI* (spermatocyte I), *SCII* (spermatocyte II), *ST* (spermatid), *IS* (immature spermatozoa), *MS* (mature spermatozoa), *DS* (degenerative spermatocyst), *ep* (epigonal organ).

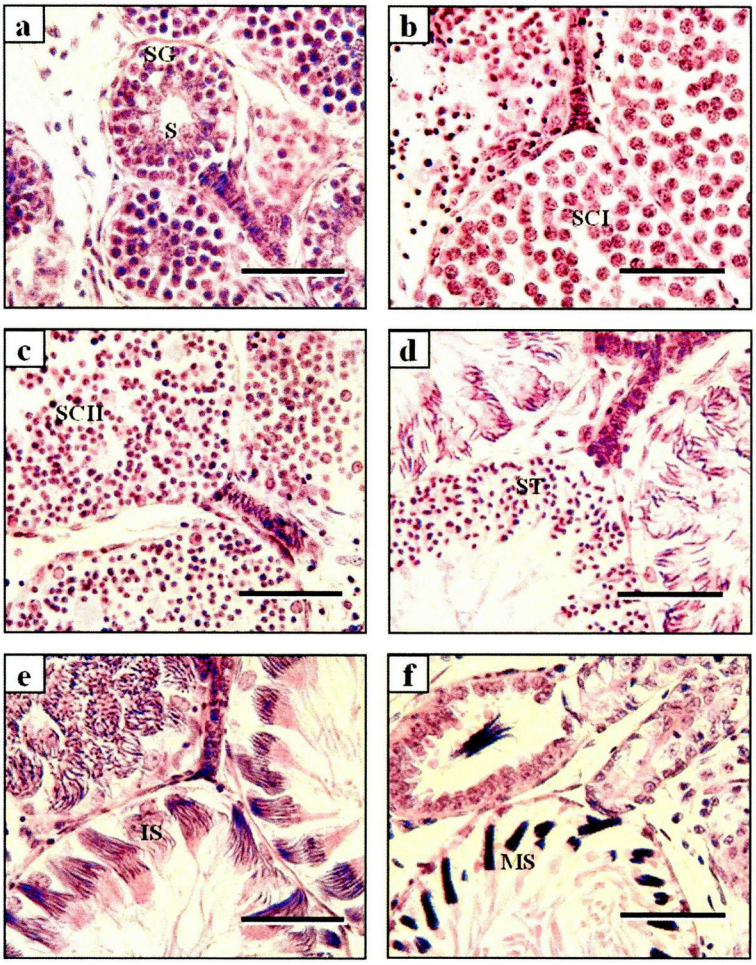

Fig. 2-4. Six stages of spermatogenesis showing spermatocysts in *Mustelus manazo* (stained with hematoxylin and eosin). a: spermatogonia (*SG*) stage (Stage 1), b: spermatocyte I (*SCI*) stage (Stage 2), c: spermatocyte II (*SCII*) stage (Stage 3), d: spermatid (*ST*) stage (Stage 4), e: immature spermatozoa (*IS*) stage (Stage 5), f: mature spermatozoa (*MS*) stage (Stage 6); *S*, Sertoli cell, Bar = 50 μm.

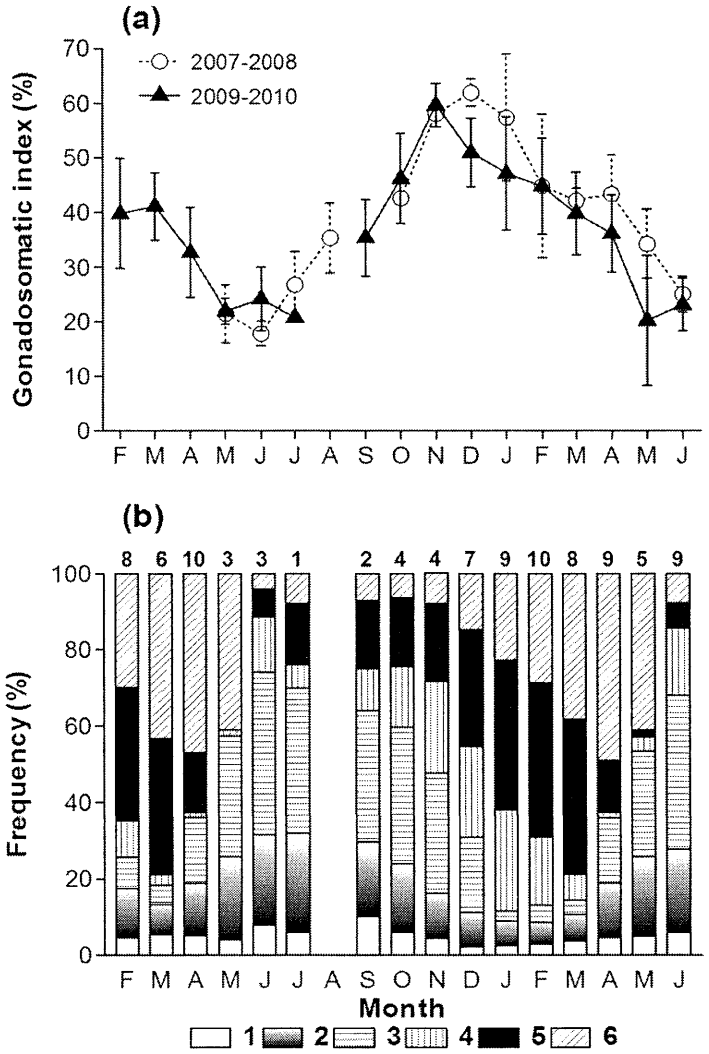

Fig. 2-5. (a) Monthly mean gonadosomatic index in 2007–2008 and 2009–2010. Vertical bars represent SD. (b) mean percentages of germ cell masses of each developmental stage found among mature male *Mustelus manazo* in Tokyo Bay in 2009–2010. Numbers of individuals examined are shown above each column.

IV. Chapter 3

Structure of the intratesticular duct system for sperm emission in the starspotted smooth-hound, *Mustelus manazo*

Introduction

The starspotted smooth-hound *Mustelus manazo* is one of dominant species of the demersal assemblage in Tokyo Bay, Japan. Although *M. manazo* population increased in Tokyo Bay, Japan in early 2000s, it seems to have decreased since the mid-2000s (Kodama et al., 2010). To elucidate the actual causal factor(s) for fluctuating *M. manazo* population in the bay, I need to examine reproductive traits of females and males of *M. manazo*, development and growth of embryos, and several environmental factors, such as prey abundance, temperature and harmful chemical substances, which may have directly or indirectly influenced the reproductive traits that would be recently observed in *M. manazo* population in Tokyo Bay. According to research performed monthly in Tokyo Bay in 2007 and 2008 (see; chapter 4) for the purposes mentioned above, the number of embryos

per litter in this species is now substantially lower than that reported in the 1990s (Yamaguchi et al., 1997). The result invokes a possibility that abnormalities have also occurred in reproductive traits of male *M. manazo* in the bay. As part of a study to elucidate the factors causing the fluctuation in abundance of *M. manazo* population in Tokyo Bay (namely, this decline in embryo abundance, and any abnormalities for male reproductive traits), first of all, I examined the reproductive traits of this species by using female and male specimens collected from the bay by bottom trawlers monthly from February 2009 to June 2010. In the present study, I conducted in depth histological observations on the spermatogenesis and the structure of the intratesticular efferent ducts involved in sperm emission in *M. manazo* to reveal any abnormalities in the male reproductive traits during 2000s.

Another aim of the present study was to reveal the process of sperm emission of *M. manazo*. The testes of elasmobranchs, unlike those of mammals and some teleosts (i.e., blennies, gobies, charids, pimelodids, and catfishes) having testicular glands, testicular blind pouches, and seminal vesicles or sperm duct glands (Chowdhury and Joy, 2007), consist of

seminiferous elements in follicles (spermatocysts) and a unique genital duct system for the emission of sperm into the epididymis. Primary spermatogonia and Sertoli cells lie initially free within the interstitial tissue before becoming sequestered by a basement membrane. The testis is thus composed of a mass of spermatocysts that contain many Sertoli cells, each being associated with a clone of germ cells (Loir et al., 1995). Genesis of spermatocyst proceeds continuously, and the newly formed spermatocysts move steadily away from the germinal sites as they grow and mature, followed closely by successively younger elements (Stanley, 1966). In sharks, such as *Heterodontus portusjacksoni* and *Sphyrna tiburo*, the germinal zone is located at the most ventral part of the testis, and development occurs in a dorsal direction in the order of spermatogonium, spermatocyte, spermatid, and finally spermatozoon in the degenerative zone (Jones and Jones, 1982; Parsons and Grier, 1992).

In the degenerative zone, the mature follicles open onto a collecting ductule system; bundles of spermatozoa are released from the Sertoli cells and flow into the ductules (Stanley, 1966; Rossouw, 1995). Many

investigations of the male reproductive organs of elasmobranchs have focused on the structure of the genital duct system as well as on that of the testis (Stanley, 1966; Dodd, 1983; Maruska et al., 1996). The structure of the rete testis and of the efferent duct connecting with the ductus epididymidis is described in detail in *H. portusjacksoni* (Jones and Lin, 1992; Jones and Lin, 1993). In three sharks, namely *Scyliorhinus caniculus*, *Torpedo marmorata*, and *Squalus acanthias* (Stanley, 1966; Pudney, 1993), and a freshwater stingray, *Himantura signifer* (Chatchavalvanich et al., 2005), brief information has been given on the terminal branching of the duct in the early spermatogonial zone. However, the intratesticular duct system has not been studied in elasmobranchs. Thus, the present study described the detailed intratesticular duct system for sperm emission to the epididymis, which is the first finding in elasmobranchs worldwide (see below).

Materials and methods

Sample collection

A total of 99 sexually mature males of *M. manazo* were collected monthly from February 2009 to June 2010 from Tokyo Bay, Japan. Sharks collected by the bottom-trawl fishery and landed at Koitogawa fishing port in Chiba Prefecture; the sharks were then dissected at the dockside. Maturity in males was defined by calcification of the claspers and the presence of sperm in the seminal vesicles.

Histological procedures

At necropsy, the testes together with the epididymides were removed from at least five male *M. manazo* individuals each month. They were weighed and then fixed in 10% formalin for a week at room temperature. The samples were then washed thoroughly in 70% ethanol. Cross-sections about 5 mm thick were removed at two sites in one testis; the sites were located 1/4 and 3/4 of the way along the length of the testis. The tissues were then dehydrated in a graded series of ethanol and infiltrated with paraffin. Sections were cut in paraffin at 6 μm, stained with Mayer's hematoxylin and eosin and observed under a light microscope. Each stage of

spermatogenesis was classified on the basis of the work of Parsons and Grier (1992), Maruska et al. (1996) and Chatchavalvanich et al. (2005).

In a few of the testes among those treated as above, spermatocysts were broken apart to examine the intratesticular ducts after preservation with 10% formalin. The broken apart spermatocysts were stained with Mayer's hematoxylin and mounted with glycerin to check the connections with the ducts. The remaining ducts were used for examining anatomical network within the testis without the staining.

Transverse sections of the testes were removed from randomly selected three males each in November 2009, March 2010 (non-breeding seasons), and June 2010 (breeding season). Spermatocysts were carefully observed under a light microscope and their number in each stage zone of spermatogenesis was counted. The number of spermatocysts with emission ducts (branch tubules) was also counted in each stage of spermatogenesis. Preparations from the blocks cut 1/4 and 3/4 of the way along the testis, as described above, were used to count the numbers of spermatocysts and the numbers with emission ducts. Number of emission ducts in one

spermatocyst was also carefully checked in serial sections in a part of some testes.

Statistical analysis

Statistical analyses were performed with the Tukey test (KyPlot version 5.0, KyensLab Inc., Tokyo, Japan). Differences with $P < 0.05$ were considered significant.

Results

Zonation of germinal cells and spermatogenesis

The germinal zone was located in the most ventral part of the testis, and development occurred in the order of spermatogonium, spermatocyte, spermatid, and spermatozoon, toward the degenerative zone in the dorsal part of the testis. Newly formed spermatocysts moved steadily away from the germinal sites as they grew and matured (Fig. 3-1a); they were followed closely by successively younger elements. Spermatocysts approaching the middle of the testis contained nearly mature spermatids. The spermatocysts

were bounded by myoid cells.

There were eight stages of spermatogenesis: (1) germinal cell stage, (2) spermatogonium stage, (3) spermatocyte I stage, (4) spermatocyte II stage, (5) spermatid stage, (6) immature spermatozoa stage, (7) mature spermatozoa stage, (8) degenerative spermatocyst stage (Fig. 3-2). In Stage 1, primary spermatogonia surrounded by Sertoli cells were observed within frontal histological sections through the germinal zone (Fig. 3-2a). In Stage 2, secondary spermatogonia were confined to the spermatocysts, which were delineated by a basement membrane. During this stage the Sertoli cell nuclei began to migrate from their positions lining the spermatocyst lumen and came to lie just internal to the basement membrane of the spermatocyst (Fig. 3-2b). In Stage 3, the primary spermatocyte nuclei contained granular and irregularly aggregated heterochromatin. The Sertoli cell nuclei had completed their migration (Fig. 3-2c). In Stage 4, the presence of secondary spermatocytes was confirmed by their particularly small nuclei (Fig. 3-2d). In Stage 5, spermatids began to transform from round to oval and elongated shapes (Fig. 3-2e), and in Stage 6 the spermatocysts contained mature

spermatozoa morphologically, the heads of which were loosely arranged along the basement membrane (Fig. 3-2f). In Stage 7, before spermiation, the spermatozoa were arranged in increasingly dense clumps and had developed further (Fig. 3-2g). In Stage 8, in the degenerative zone, the spermatozoa were evacuated from their spermatocysts into the intratesticular ducts (terminal branch tubules; see below) in the degenerative zone (Figs. 3-2h and 3-3a), and the spermatocysts then underwent degeneration and resorption.

Some spermatocysts were unable to release spermatozoa because of failure of adhesion to the branch tubules. These spermatocysts degenerated *in situ*; spermatozoa spilled into the interstitium, resulting in disorganization of the spermatocysts (Fig. 3-3b). The collapsed spermatocysts were infiltrated by numerous lymphocytes and granular leukocytes containing eosinophilic granules.

The duct system

A complicated branching network of ducts for sperm emission was

evident in the testis (Fig. 3-1c). The intratesticular ducts converged finally into one thick duct (the efferent duct), which was connected to the epididymis (Figs. 3-1c and 3-1d). The intratesticular ducts were composed of terminal branch tubules and stem tubules (Fig. 3-4a) in the spermatogenic zone and collecting tubules (Figs. 3-1b and 3-1c) in the rete testis. The branch tubules attached to the spermatocysts were clearly visible in all zones of spermatogenesis, except the germinal zone where the tubules were close to the differentiating spermatocysts (Figs. 3-2a-g, arrows). One spermatocyst was usually attached to one branch tubule even at the early stage of spermatogenesis. This was verified by serial sections (data not shown). In the germinal and spermatogonial zones, the branch tubules consisted of a single layer of cuboidal cells with or without cilia, covered with a few flattened cells (Fig. 3-4b). The stem tubules were lined mainly by epithelium consisting of columnar or cuboidal cells with cilia, pale round cells (hale cells), and basal cells (Fig. 3-4c). The tubule was surrounded by a single layer of well-developed myoid cells. The epithelium was infiltrated by numerous leukocytes and lymphocytes. The branch tubules grew as the

spermatocysts developed and became similar in structure to the stem tubules near the degenerative zone, where the mature spermatocysts opened onto the branch tubules; bundles of spermatozoa flowed into the stem tubules (Fig. 3-3a).

The collecting tubules were similar in structure to the stem tubules but were thicker in diameter (Fig. 3-4d). The epithelium of the collecting tubules had cell components similar to those of the stem tubules and was surrounded by a few layers of smooth muscle cells. In the dorsal part of the testis, the collecting tubules were connected to the efferent duct, which was lined by epithelium similar to that of the collecting tubules, and had a thick layer of smooth muscle cells (Figs. 3-1b and 3-4d). Large blood vessels observed among the collecting tubules and near the efferent duct contained numerous erythrocytes, as well as a few of lymphocytes and granular leukocytes which were infiltrated into the interstitium of the testis (Figs. 3-1b and 3-3b).

I counted all of the spermatocysts in each zone in transverse sections of the testes randomly selected three males seasonally each. The

number of spermatocysts varied seasonally: in the breeding season (June 2010), the testes tended to have fewer spermatocysts in stages 5 to 7 of spermatogenesis than those in the other seasons (November, 2009 and March, 2010) (Table 1). The proportions of spermatocysts with branch tubules (as counted dimensionally in one transverse section) did not differ significantly among any of the stages at any of the sampling times (Table 2).

Discussion

There were no specific abnormalities in spermatogenesis as well as morphological structure of testes of male *M. manazo* specimens in Tokyo Bay, which may be linked with recent fluctuation in abundance of the population in the bay. Therefore, further studies are needed to elucidate actual causal factor(s) for recent fluctuation in abundance of *M. manazo* population in Tokyo Bay, in terms of sexual maturation of female *M. manazo* specimens, development and growth of embryos, and several environmental factors, such as prey abundance, temperature and harmful chemical substances (Park et al., 2012).

In elasmobranchs, the testis is classified structurally into three types: radial, diametric, and compound (Pratt, 1988). In the testis of *M. manazo*, Teshima (1978) reported that germ cells and Sertoli cells were lined up inside of tubules (seminiferous tubules). However, the present study revealed that the testis contained a lot of cysts with these cells (spermatocysts), but not seminiferous tubules. The germinal zone consists of a strip along the ventral surface of the testis, and spermatocyst development proceeds diametrically, that is, across the width of the testis toward the efferent ducts, which are located dorsally. Thus, as shown by our histological observations, *M. manazo* can be classified as a diametric testis. As reported before (Stanley, 1966; Jones and Jones, 1982; Parsons and Grier, 1992; Rossouw, 1995; Chatchavalvanich et al., 2005; Pratt, 1988; Matthews, 1950; Kassab et al., 2009), I found that in the testes of elasmobranchs the germinal zone was located in the ventral part of the testis, and the spermatocysts moved toward the degenerative zone in the dorsal part; this was accompanied by development of the germ cells. Spermatozoa were conveyed along the ciliated lumina of the intratesticular tubules attached to

the spermatocysts. Spermatocysts that were unable to emit sperm because of failure of adhesion to the branch tubules were disorganized *in situ*, along with their spermatozoa. Since gathering of lymphocytes and granular leukocytes was evident around the spermatocysts undergoing disruption, the residues of spermatocysts seem to be cleared by hemophagocytosis with leukocytes, which may be recruited from the epigonal organ.

The sperm-carrying duct system in the testes of elasmobranch species has been investigated by several authors (Stanley, 1966; Parsons and Grier, 1992; Rossouw, 1995; Chatchavalvanich et al., 2005; Kassab et al., 2009). Most studies have focused on the efferent duct system connected to the epididymis near the degenerative zone where the sperm are released from mature spermatocysts into the attached ducts and subsequently move into the efferent duct in the rete testis. The intratesticular duct system has therefore not been studied beyond a few reports in which the intratesticular ducts were shown to develop in the early stages of spermatogenesis in the germinal and spermatogonial zones in sharks (Stanley, 1966; Pudney, 1993) and a freshwater stingray (Chatchavalvanich et al., 2005). In the present

study, although the testis of *M. manazo* showed seasonal change on the composition of spermatogenic cells in the spermatocyst as has been reported by Teshima (Teshima, 1978), the rate of spematocyst with the branch ducts was approximately the same among each stage of the spermatogenesis. This suggests that development of the ducts is associated with that of the spermatocysts.

The structure of the intratesticular duct system for carrying sperm in *M. manazo* is summarized in Fig. 3-5. The intratesticular ducts were well developed in all zones of spermatogenesis in *M. manazo*. Throughout the testis, most spermatocysts were attached to the termini of branch tubules. The branch tubules were connected via stem tubules to collecting tubules in the rete testis. The collecting tubules converged onto one efferent duct connected to the epididymis. Hence, in the testis, most or all spermatocysts move from the germinal to the maturation zone together with the sperm-carrying ducts until the spermatozoa are expelled from the spermatocysts. Although mechanism involved in the movement of sprematocysts is still unclear, a role of the intratesticular duct system in spermatocyst migration

during spermatogenesis cannot be ruled out. The structure of the epithelium of the intratesticular ducts is similar to that of transitional epithelium, and the myoid cells and smooth muscle surrounding the ducts may contribute to spermatocyst movement and sperm transport. An investigation of the mechanism underlying the formation of new interactions between the spermatocysts and branch tubules in the germinal zone is needed in the near future.

Figures and Tables

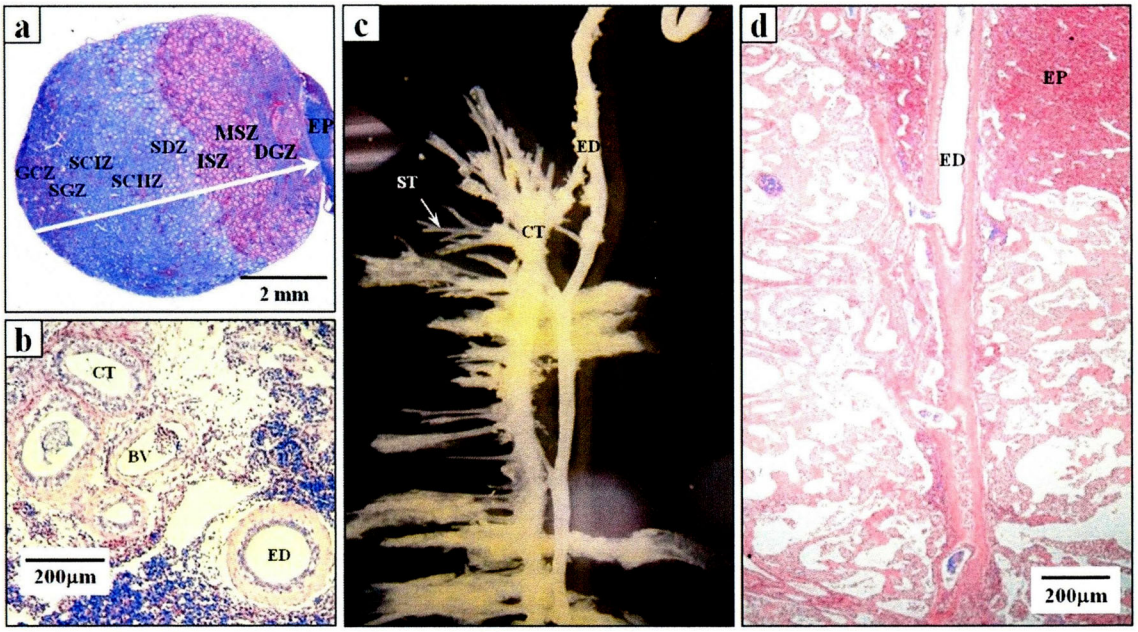

Fig. 3-1. Intratesticular duct system in Mustelus manazo. **a** Growth direction of germ cells in transverse section of the testis (*white arrow*). **b** Collecting tubule and blood vessel in the rete testis and efferent duct in the dorsal part of the testis. **c** Network of the intratesticular duct system, showing the stem tubules, collecting tubules, and efferent duct. **d** Connection between collecting tubules and efferent duct, showing entry of spermatozoa into the efferent duct. a, b, d Stained with H&E. *GCZ* germinal cell zone, *SGZ* spermatogonium zone, *SCIZ* spermatocyte I zone, *SCIIZ* spermatocyte II zone, *SDZ* spermatid zone, *ISZ* immature spermatozoa zone, *MSZ* mature spermatozoa zone, *DGZ* degenerative spermatocyst zone, *EP* epigonal organ, *CT* collecting tubule, *BV* blood vessel, *ED* efferent duct, *ST* stem tubule

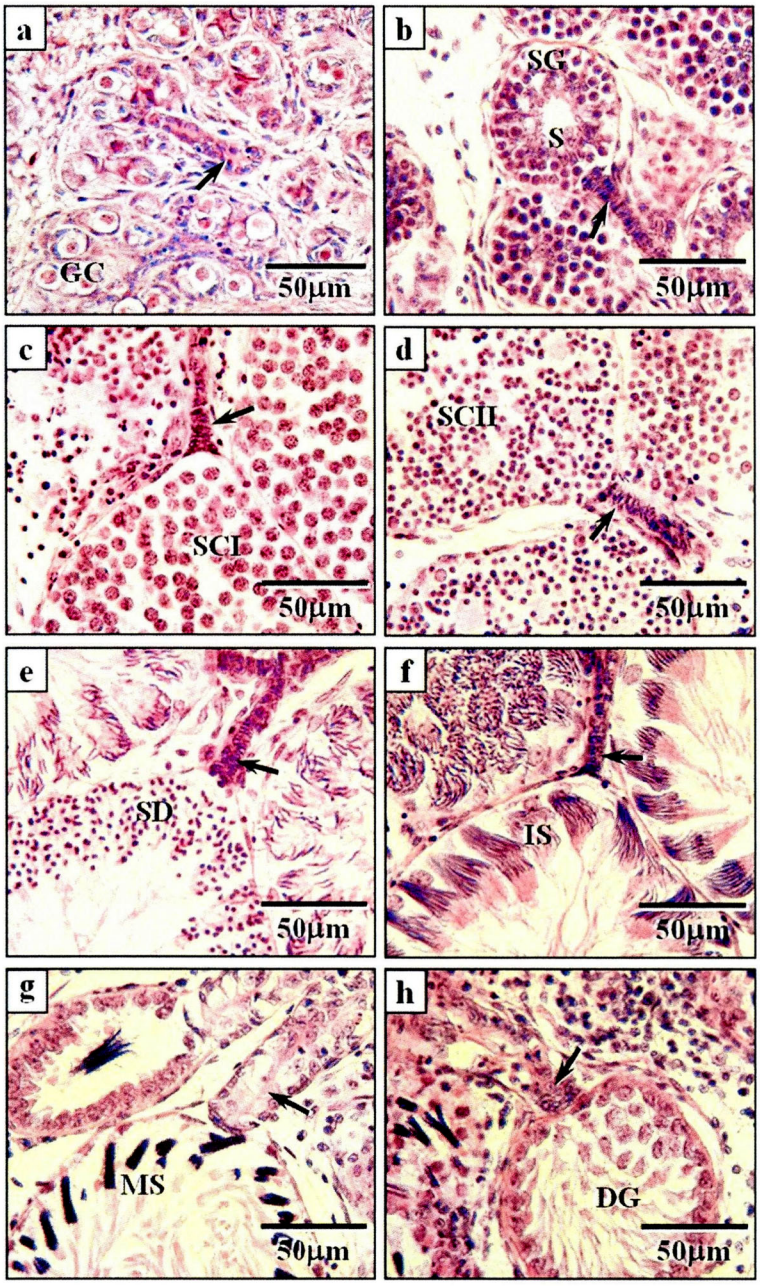

Fig. 3-2. Eight stages of spermatogenesis showing spermatocysts connecting to branch tubules (*arrows*) in *Mustelus manazo* (stained with H&E): **a** germinal cell (*GC*) stage (stage 1), **b** spermatogonia (*SG*) stage (stage 2), **c** spermatocyte I (*SCI*) stage (stage 3), **d** spermatocyte II (*SCII*) stage (stage 4), **e** spermatid (*SD*) stage (stage 5), **f** immature spermatozoa (*IS*) stage (stage 6), **g** mature spermatozoa (*MS*) stage (stage 7), **h** degenerative spermatocyst (*DG*) (stage 8). *S* Sertoli cell

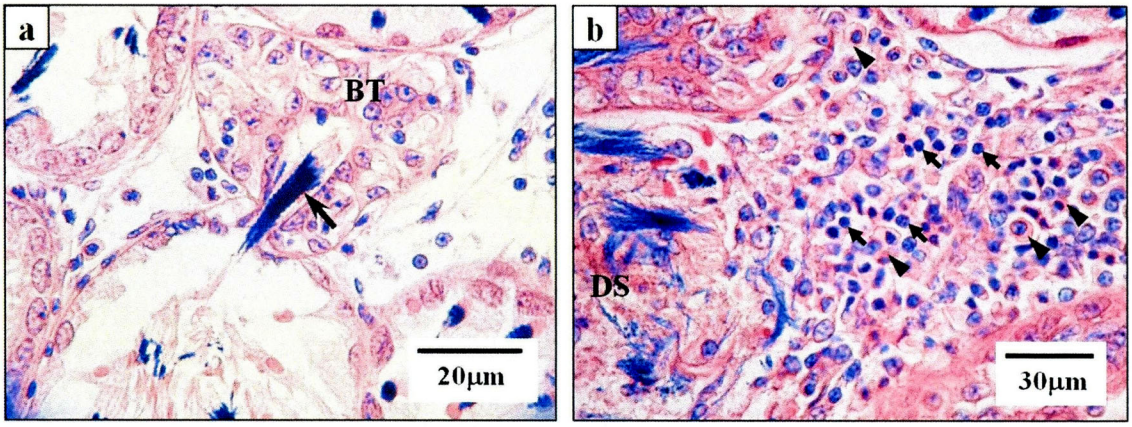

Fig. 3-3. Sperm emission from spermatocysts in the testis of *Mustelus manazo*. **a** Discharge of spermatozoa (*arrow*) into the branch tubule. **b** Disorganized spermatocysts failed to discharge spermatozoa. Infiltration of the interstitium with a lot of lymphocytes (*black arrows*) and granular leukocytes (*arrowheads*). *DS* disorganized spermatocyst, *BT* branch tubule

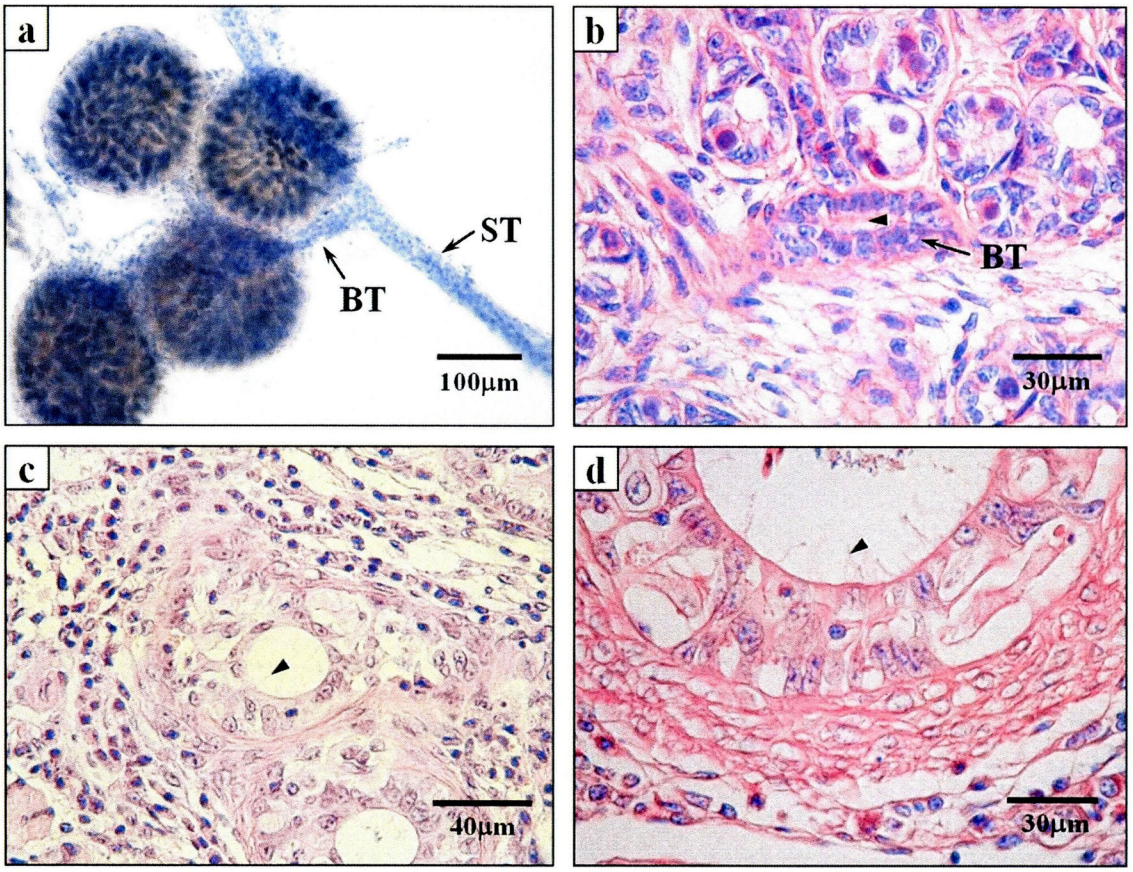

Fig. 3-4. Histological structure of the intratesticular ducts in *Mustelus manazo*: **a** branch and stem tubules connecting to spermatocysts, **b** branch tubule, **c** stem tubule, **d** collecting tubule. *Arrowheads* indicating cilia of the epithelial cells. **a** stained with hematoxylin alone, **b–d** stained with H&E. *BT* branch tubule, *ST* stem tubule

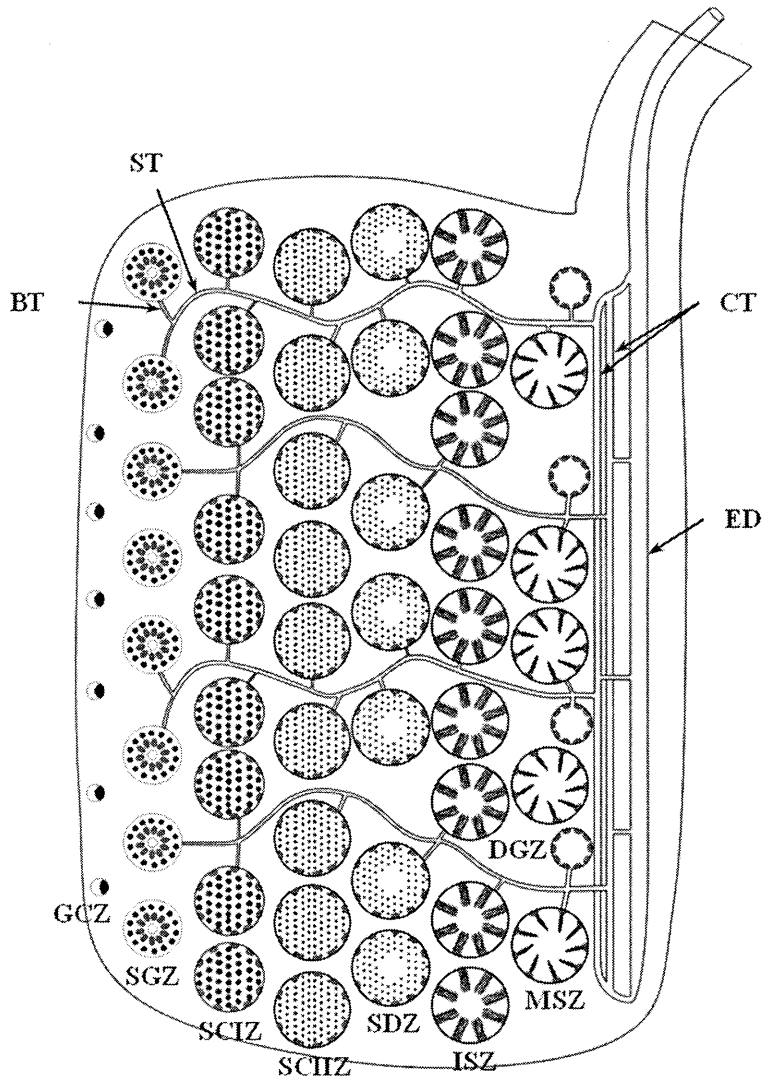

Fig. 3-5. Schematic of the intratesticular duct system in *Mustelus manazo*. *BT* branch tubule, *ST* stem tubule, *CT* collecting tubule, *ED* efferent duct, *GCZ* germinal cell zone, *SGZ* spermatogonia zone, *SCIZ* spermatocyte I zone, *SCIIZ* spermatocyte II zone, *SDZ* spermatid zone, *ISZ* immature spermatozoa zone, *MSZ* mature spermatozoa zone, *DGZ* degenerative spermatocyst zone

Table 1. Numbers of spermatocysts in the testes of *Mustelus manazo* in different reproductive seasons of the year (mean ± SD).

Stage of Spermatogenesis	Nov 2009 (n = 3)	Mar 2010 (n = 3)	Jun 2010 (n = 3)
2	564 ± 125	306 ± 46	772 ± 61
3	499 ± 29	0	522 ± 129
4	544 ± 4	149 ± 119	290 ± 194
5, 6	683 ± 100	702 ± 96	241 ± 6[a]
7	370 ± 66	723 ± 151	219[b]

[a] One of three testes has fewer spermatocysts in stages 5 and 6

[b] Two of three testes have fewer spermatocysts in stage 7

Table 2. Proportion of spermatocysts attaching to branch tubules in the testis of *Mustelus manazo* in different reproductive seasons of the year (%, mean ± SD).

Stage of spermatogenesis	Nov 2009 (n = 3)	Mar 2010 (n = 3)	Jun 2010 (n = 3)
2	14.5 ± 1.3	10.8 ± 2.1	13.1 ± 1.8
3	10.4 ± 1.3	0	14.5 ± 3.2
4	9.7 ± 0.9	7.5 ± 4.4	15.1 ± 8.6
5, 6	8.3 ± 2.0	9.9 ± 1.3	9.9 ± 2.7[a]
7	10.3 ± 2.9	10.3 ± 1.3	10.5[b]

[a] One of three testes has fewer spermatocysts in stages 5 and 6

[b] Two of three testes have fewer spermatocysts in stage 7

V. Chapter 4

Reproductive traits of female starspotted smooth-hound *Mustelus manazo* in Tokyo Bay

Introduction

The starspotted smooth-hound *Mustelus manazo* is distributed from Hokkaido in northern Japan to the northwestern Pacific Ocean. This species is one of the most dominant elasmobranch species in the demersal assemblage in Tokyo Bay (Yamaguchi et al., 1996; 1997; 2000).

According to the recent report, the fisheries-independent trawl survey revealed that catch-per-unit-effort (CPUE: number of individuals or wet body weight of the species caught by 10-min tow) of *M. manazo* was low during 1990s, and markedly increased in the early 2000s (Kodama et al., 2010). Furthermore, CPUE decreased from 2005 to 2008, and increased again from 2009. However the reason of the fluctuations of the stock size remains unknown. In order to explore the cause of the stock fluctuations of *M. manazo*, it is necessary reveal fundamental life history characteristics of

this species.

To date, several studies showed that changes in life history traits correspondent with variations in stock-size level have been observed in other dominant demersal species in Tokyo Bay, for example, Japanese mantis shrimp *Oratosquilla oratoria*; changes in the reproductive patterns concurrent with stock size decline (Kodama et al., 2006), and marbled sole *Pseudopleuronectes yokohamae*; changes in growth between high and low stock-size periods (Lee et al., 2009). Fundamental life history traits (age and growth, Yamaguchi et al., 1996; reproductive traits, Yamaguchi et al., 1997; and feeding habits, Yamaguchi and Taniuchi, 2000) of *M. manazo* in Tokyo Bay have been reported during 1990s, when the stock size was low. However, there has been no scientific report on the life history traits of *M. manazo* in Tokyo Bay during 2000s when the stock size is different from that in 1990s. In the present study, reproductive traits of female *M. manazo* during 2000s in Tokyo Bay were investigated. I examined differences in the reproductive traits between 1990s and 2000s, and then discussed cause of the changes into reproductive traits.

Materials and Methods

Sample collection

A total of 578 females were collected from May 2007 to June 2008 and February 2009 to June 2010 in Tokyo Bay, Japan. Samples of *M. manazo* were caught monthly by bottom-trawl fishery and landed at Koitogawa fishing port in Chiba Prefecture. After collection, sex was identified, and measurements of total length (TL, mm) and body weight (BW, g) were carried out. Then ovary and liver were dissected out at the dockside. After dissection, weight of ovary and liver (g), shell gland width (mm) and maximum ova diameter (mm) were recorded. Ovary, liver and uterus were fixed with 10% neutral buffered formalin for a week at room temperature for later analysis.

Development of reproductive organs

Development of reproductive organs relating to sexual maturity of female was classified following the previous study (Yamaguchi et al., 1997).

Immature, uteri are threadlike and no obviously developed ova are found in the ovary; premature, uteri are thickening but not obviously vascularized, and developed ova are more or less distinct; and mature, embryos are present or uteri are fully flaccid after parturition.

Size at maturation

To investigate the size at maturity, the TL at which 50% of the individuals achieved maturity (TL50) was estimated by using a logistic equation (Ratkowsky, 1990);

$$P(\mathrm{TL}) = 1/\{1+\exp^{(-a(\mathrm{TL}-b))}\},$$

where P(TL) is the proportion of mature individuals in a given TL class, a is a constant, and b is TL_{50}. The class interval for TL in this analysis was 20 mm. Estimation of the parameters was conducted by means of least-squares fitting using the KyPlot 5.0 software (KyensLab Inc., Tokyo, Japan).

Reproductive cycle

The gonadosomatic index (GSI) of Yamaguchi et al., (1997) was

calculated for 1 to 10 mature males per month by using the following equation to investigate the seasonal pattern in the development of the testes:

$$GSI = (\text{ovary weight} / TL^3) \times 10^9,$$

Embryonic growth

To estimate embryonic growth before parturition, TL of all embryos was measured each month. The weight (g) of the external yolk sac of each embryo was also measured.

Fecundity

Fecundity was determined for each mature female by counting the number of embryos per litter. Embryos were counted with or without yolk sac (Figs. 4-1a-1c), and excluded ovulated eggs in the uteri from the counting for estimation of fecundity (Figs. 4-1d and 1e). The relationship between a female's TL and the number of embryos per litter was examined in 2007–2008 and 2009–2010. To examine whether fecundity changed concurrently with variations in stock size, the differences in the slope of the

TL–fecundity relationship were tested between each combination of data from 1994–1996 (Yamaguchi et al., 1997) and data from 2007–2008 and 2009–2010 (present study) by ANCOVA (KyPlot 5.0). The peak of parturition in *M. manazo* in Tokyo Bay occurs in May (Yamaguchi et al., 1997). Our preliminary studies revealed that abnormal parturition occurred in May while gravid females were in the net during the tow of bottom trawl. Abnormal parturition also occurred on the boat before gravid females caught by the bottom trawl were landed at the port. The abnormal parturition may have caused underestimation of fecundity. Therefore, I excluded data of the number of embryo from May in all periods to estimate the TL–fecundity relationship. Data from females that had finished parturition or those that exhibited abnormal parturition had already been excluded in the analysis of TL–fecundity relationship during 1990s (Yamaguchi et al., 1997). I therefore used the original data of Yamaguchi et al., (1997) for comparison of the TL–fecundity relationships in the 1990s and the 2000s.

Results

Sexual maturation

Immature females ranged from 368 to 690 and 436 to 712 mm TL from in 2007–2008 and 2009–2010, respectively. They were characterized by small ovaries, small oocytes, narrow oviduct, thin shell gland, and threadlike uteri (Fig 4-2). These individuals had ovary weights from 0.4 to 6.0 and 1.3 to 6.0 g from in 2007–2008 and 2009–2010, respectively (Figs. 4-2a and 2b), and individuals had shell gland widths from 2.0 to 4.0 and 3.0 to 6.0 mm from 2007–2008 and 2009–2010, respectively (Figs. 4-2c and 2d), respectively. Premature females individuals ranged in size from 620 to 768 and 648 to 752 mm TL from 2007–2008 and 2009–2010, respectively and most had enlarged oviduct, shell glands, uteri, and ovaries. Mature females larger than 654 and 680 mm TL from in 2007–2008 and 2009–2010, respectively, had ovaries with large well-yolked eggs, and fully flaccid uteri.

TL_{50} was estimated to be 684 mm and 683 mm for females in 2007–2008 and 2009–2010, respectively (Fig. 4-3). The TL_{50} values of between 2007–2008 and 2009–2010 had no difference.

Reproductive cycle

The GSI of mature females peaked in May and then declined until August both in 2007–2008 and 2009–2010 (Fig. 4-4). Significant differences were found in the GSI among months for mature females with one-way ANOVA ($P < 0.05$). Tukey's post hoc pairwise comparisons showed that GSI of mature females in May was significantly different from those in August. It was suggested that ovulation occurs during May to June.

The maximum ovum diameter in the ovary of mature *M. manazo* showed obvious seasonal patterns in both 2007–2008 (Fig. 4-5a) and 2009–2010 (Fig. 4-5b). The maximum ovum diameter peaked in June or July (mean ± SD: 16 ± 6 mm in 2007–2008 and 20 ± 6 mm in 2009–2010). The maximum ovum diameter decreased rapidly from July to August, and then increased gradually from August onwards in both 2007–2008 (Fig. 4-5a) and 2009–2010 (Fig. 4-5b). The maximum ovum diameter showed substantial dispersion between 5 and 26 mm during May–July in 2007 and June–July in 2009 (Figs. 4-5a and 4-5b). No eggs were found in the uterus in May 2009. Ovulated eggs and undeveloped eggs were observed from June to August

and from October to May, respectively (Fig. 4-5). The frequency of ovulated eggs in the uterus peaked in July, and then showed a rapid decline starting in August in both 2007–2008 and 2009–2010 (Figs. 4-5c and 5d). Undeveloped eggs were absent or found with low frequency from September to May (0%–20% in 2007–2008 and 0%–29% in 2009–2010; Fig. 4-1e; Figs. 4-5c and 5d).

Embryonic growth

Embryos began to appear from August in 2007 and 2009 (Fig. 4-1a; Fig. 4-6a). The TL of the embryos ranged from 29 mm in August to 285 mm in May in 2007–2008, meanwhile from 26 mm in August to 287 mm in May in 2009–2010 (Fig. 4-6a). Embryos showed rapid growth until January, followed by moderate growth from February to May, in both 2007–2008 and 2009–2010 (Fig. 4-6a). The external yolk sac of the embryos substantially decreased in size after reaching a peak in September (2007–2008) or October (2009–2010; Fig. 4-6b), until embryos grew to more than 200 mm in TL in December (Fig. 4-6a). The yolk sac disappeared from January

onwards (Fig. 4-1c; Fig. 4-6b).

Fecundity

Fecundity of individual females ranged from 1 to 9 embryos per litter in 2007–2008 (Fig. 4-7a). There was a significant positive linear relationship between TL of the female and the number of embryos per litter, and the slope was 0.0091 (Fig. 4-7a). Meanwhile, the fecundity of individual females ranged from 1 to 8 embryos per litter in 2009–2010 (Fig. 4-7b). A significant linear relationship between TL and the number of embryos per litter was also evident in 2009–2010 (Fig. 4-7b). The slope (0.0153) in 2009–2010, however, was significantly higher than that in 2007–2008 (ANCOVA, $P < 0.001$). On the other hand, in 1994–1996, when the stock size was low, the slope was 0.0210, which was significantly higher than those in both 2007–2008 and 2009–2010 (ANCOVA, $P < 0.001$ for both cases; Fig. 4-7c). Slopes of the linear regression in 2007–2008 (0.0091) and 2009–2010 (0.0153) decreased to 43% and 73% of that in the 1990s (0.021), respectively (Fig. 4-7).

Discussion

Fecundity

Comparison of the reproductive traits of *M. manazo* among periods with different stock sizes revealed that fecundity at a given TL in 2007–2008 and 2009–2010 decreased to 43% and 73% of that in the 1990s, respectively. This result implies that a substantial decline in fecundity took place during the 2000s, a period of higher stock size. Although the mechanism responsible for the interannual variation in the fecundity of *M. manazo* in Tokyo Bay remains unknown, it may be related to phenotypic plasticity. Variation in fecundity has been reported for *M. manazo* among five locations during 1994–1996 [Aomori, Maizuru, Shimonoseki, and Tokyo Bay in Japan, and Taiwan; (Yamaguchi et al., 2000)], suggesting that this species exhibits phenotypic plasticity in its fecundity. This plasticity could be driven by changes in population density. For example, the fecundity of Atlantic herring (*Clupea harengus*) in the Western Gulf of Maine was significantly higher in 1982 than in 1969, and a stock size

decline began in the late 1960s, suggesting that increased fecundity may compensate for reduced stock size (Kelly and Stevenson, 1985). Similarly, the high fecundity in 1994–1996 may have contributed to recovery of the stock size of *M. manazo* in Tokyo Bay from the 1990s to the 2000s. Similarly, changes in the fecundity of *M. manazo* in Tokyo Bay in the 1990s may be density dependent with a time lag of several years: the high fecundity in 1994–1996 may be a compensation for the low stock size from the late 1980s to the early 1990s, and it might have contributed to recovery of the stock size from the 1990s to the 2000s. On the other hand, the low fecundity in 2007–2008 and increased fecundity in 2009–2010 might reflect compensation for the high stock size during the mid-2000s and substantial decrease in the stock size until 2008, respectively. However, there is a possibility that the fecundity of *M. manazo* is not only affected by stock size alone, but also affected by a combination of other factors, such as the allocation of resources to reproduction (Ma et al., 1998) and the effects of abiotic factors such as changes in water temperature (Gregersen et al., 2011). Increasing trend of water temperature from 1990s to 2000s has been

reported in Tokyo Bay (Kodama et al., 2010), which is correlated with changes in the demersal assemblage in the bay. However the causal linkage between the changes in water temperature and fecundity of *M. manazo* is still unclear, which deserves further studies.

In the present study, significant changes in the fecundity of *M. manazo* in Tokyo Bay took place within a relatively short duration (4 years) in 2000s. Phenotypic changes in life history traits could occur within short duration in fish species with small body size and short life span (e.g., decrease in growth rate and fecundity of Atlantic silverside *Mendia menidia* within four years (four generations) due to effect of fishing pressure (Conover and Munch, 2002; Walsh et al., 2006)). However, changes in life history traits usually occur in decadal time scale in fish species with large body size and long life span (summarized in (Jørgensen et al., 2007)). I could not reveal the reason of the abrupt change in the fecundity of *M. manazo* which have large body size and long life span (Yamaguchi et al., 1996). Further study is needed to clarify the actual reason(s) and the mechanism.

Sexual maturation

The minimum size at female *M. manazo* in Tokyo Bay attain sexual maturity was 701 mm TL for females in 1994–1996 (Yamaguchi et al., 1997), versus 654 and 680 mm TL in 2007–2008 and 2009–2010, respectively. These results suggest that the size at maturity of *M. manazo* in Tokyo Bay was getting smaller in the 2000s than that in the 1990s. Although the reason for this decline remains unknown, it may be related to phenotypic plasticity, as discussed earlier for changes in fecundity, because the size at first maturity of *M. manazo* ranged between 600 and 700 mm TL among different locations in previous research: 630 to 700 mm TL for females in the East China Sea (Teshima, 1981) and 621 to 640 mm TL for both sexes near Choshi, Japan (Taniuchi et al., 1983).

Reproductive cycle

Gonadosomatic index is the most frequent method used to define mating season for population of shark (Maruska et al., 1996). The present

data show that GSI of mature females peaked in May and then declined until August in both 2007–2008 and 2009–2010. This evidence suggests that mating of *M. manazo* occurred from May to June in Tokyo Bay in both two periods.

The maximum ovum diameter of *M. manazo* in Tokyo Bay during the 2000s exhibited both high and low values from May to July, and the large ova disappeared from the ovaries in August. In addition, the frequency of occurrence of ovulated eggs in the uteri showed high values from June to August. This evidence suggests that ovulation occurred between May and July in the 2000s. Similarly, ovulation of *M. manazo* in Tokyo Bay occurred between May and June during the 1990s (Yamaguchi et al., 1997), suggesting that the timing of ovulation of *M. manazo* in Tokyo Bay did not distinctly vary between the 1990s and the 2000s. Ovulation of *M. manazo* in Tokyo Bay is concurrent with ejaculation, suggesting that mating of this species would take place between May and July. In the 2000s, the frequency of occurrence of ovulated eggs in the uteri of *M. manazo* was high in Tokyo Bay from June to August, followed by the appearance of embryos in the

uteri in August. Similarly, female *M. manazo* with ovulated eggs were found from June to August 1997. Parturition took place between May and June in the 2000s; this was similar to the period in the 1990s (Yamaguchi et al., 1997).

The maximum ovum diameter showed substantial dispersion during the ovulation season (May–July) during 2000s, while that in 1990s exhibited two distinct modes (<5mm and >14mm) (Yamaguchi et al., 1997). The dispersion of the maximum ovum diameter in the 2000s would be derived from asynchronous development of ova in the ovary (i.e., the speed of development may differ among each ovum). In this case, various sizes of immature ova may have remained in the ovary after mature ova were ovulated. On the other hand, the two distinct modes during the ovulation season in 1990s may imply that the development of ova in the ovary would be synchronous (i.e., only ova that are to be ovulated would develop, and the rest of ova remain undeveloped throughout the year). To the best of our knowledge, the change from synchronous to asynchronous development of ova in the ovary has been never reported in elasmobranchs, and the reason

causing this change remains unknown.

Undeveloped eggs (i.e., ova that were ovulated during the peak mating season (May–July) and did not develop into embryos) were present in the uteri at a low frequency of occurrence from October onwards (after the peak season of ovulation) during the 2000s. However, undeveloped eggs were not observed in the uteri from September to May during the 1990s (Yamaguchi et al., 1997). The undeveloped eggs in the uteri found after October may not develop into embryos, as multiple cohorts of embryos were not evident in the uteri. Although the reason why undeveloped eggs occurred in the uteri remains unknown, the occurrence of undeveloped eggs may be related to the decline in fecundity of *M. manazo* in Tokyo Bay during the 2000s.

In conclusion, prominent changes in the reproductive traits (i.e., a decline in fecundity, pattern in the development of ova in the ovary, and presence of undeveloped eggs in the uterus) were evident for *M. manazo* in Tokyo Bay during the 2000s. There is a possibility that other life history traits, such as growth and feeding habits may have changed between 1990s

and 2000s as observed in the reproductive traits, which may affect stock fluctuation of this species (e.g., decrease in the growth may cause reduced fecundity, or change in feeding habit may affect energy allocation between growth and reproduction). Therefore, further studies are needed to examine if there are any changes in other life history traits of *M. manazo*, which would contribute to elucidate the mechanism of stock fluctuation of *M. manazo* in Tokyo Bay.

Figures and Tables

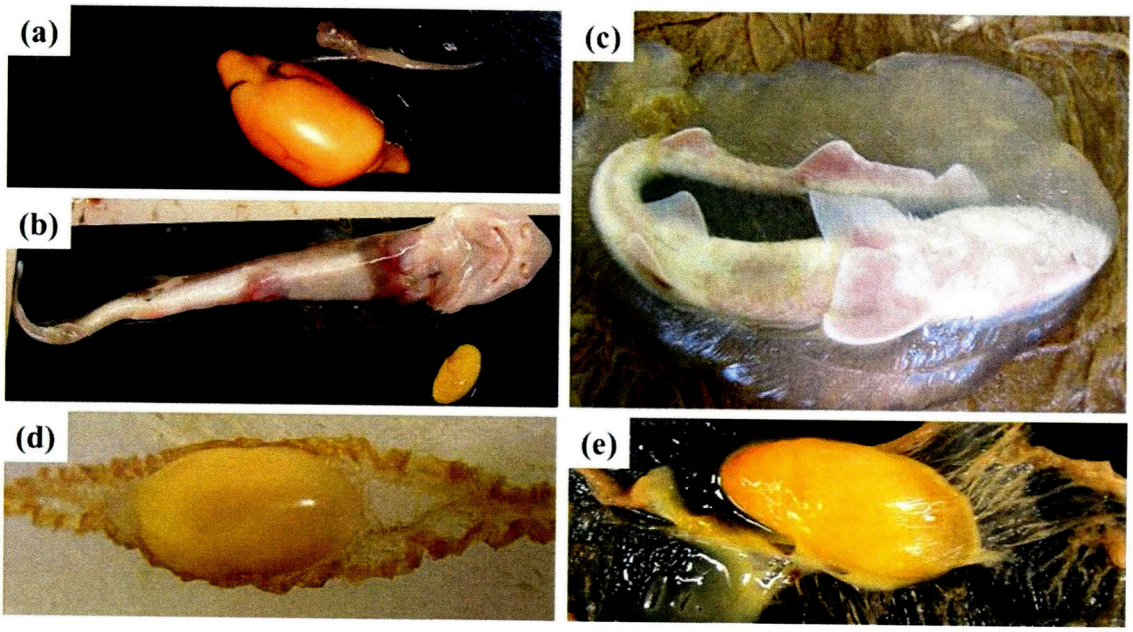

Fig. 4-1. Embryo in the uterus of *Mustelus manazo* with yolk sac collected from Tokyo Bay in (a) August 2008 and (b) November 2006. (c) Yolk sac is absent in embryos collected in March 2009. Ovulated egg found in the uterus in (d) June 2009 and (e) February 2007.

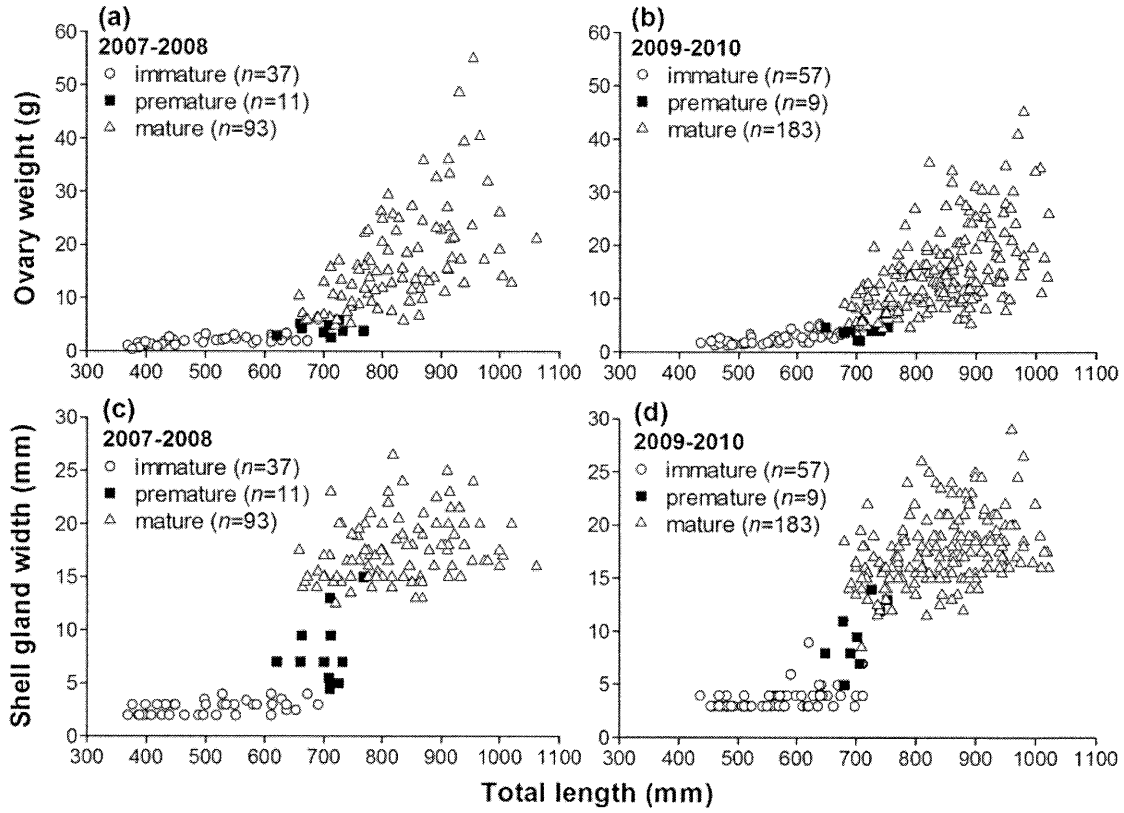

Fig. 4-2. Relationship between total length and (a), (b) ovary weight from in 2007–2008 and 2009–2010, respectively, and relationship between total length and (c), (d) shell gland width from in 2007–2008 and 2009–2010, respectively.

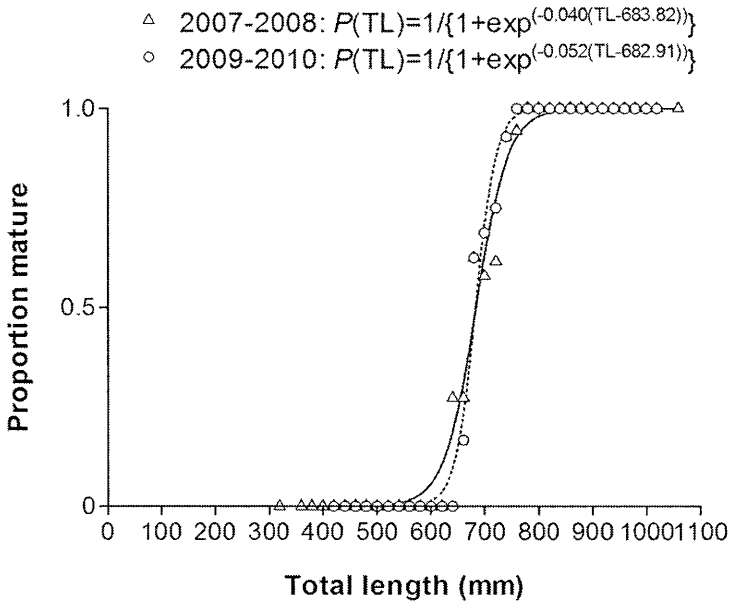

Fig. 4-3. Relationship between proportion of maturity against total length (mm) for *Mustelus manazo* in Tokyo Bay in 2007–2008 (solid lines) and 2009–2010 (dotted lines).

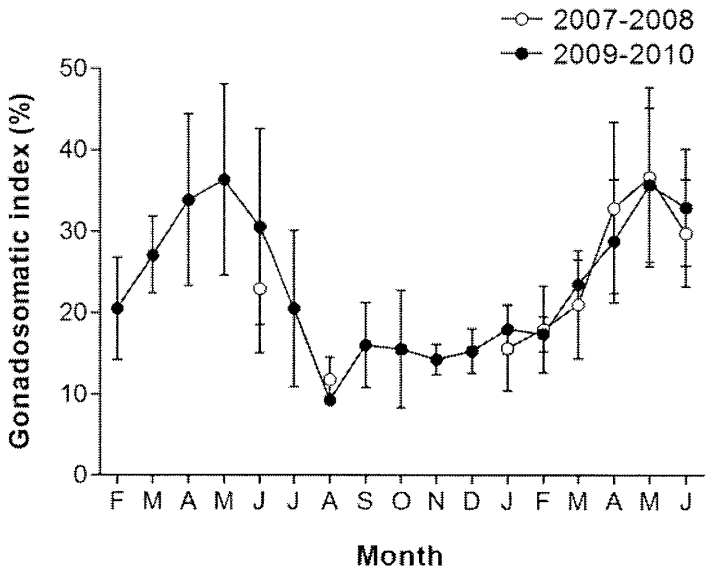

Fig. 4-4. Monthly variations in mean gonadosomatic index among mature female *Mustelus manazo* in Tokyo Bay in 2007–2008 and 2009–2010. Vertical bars represent SD.

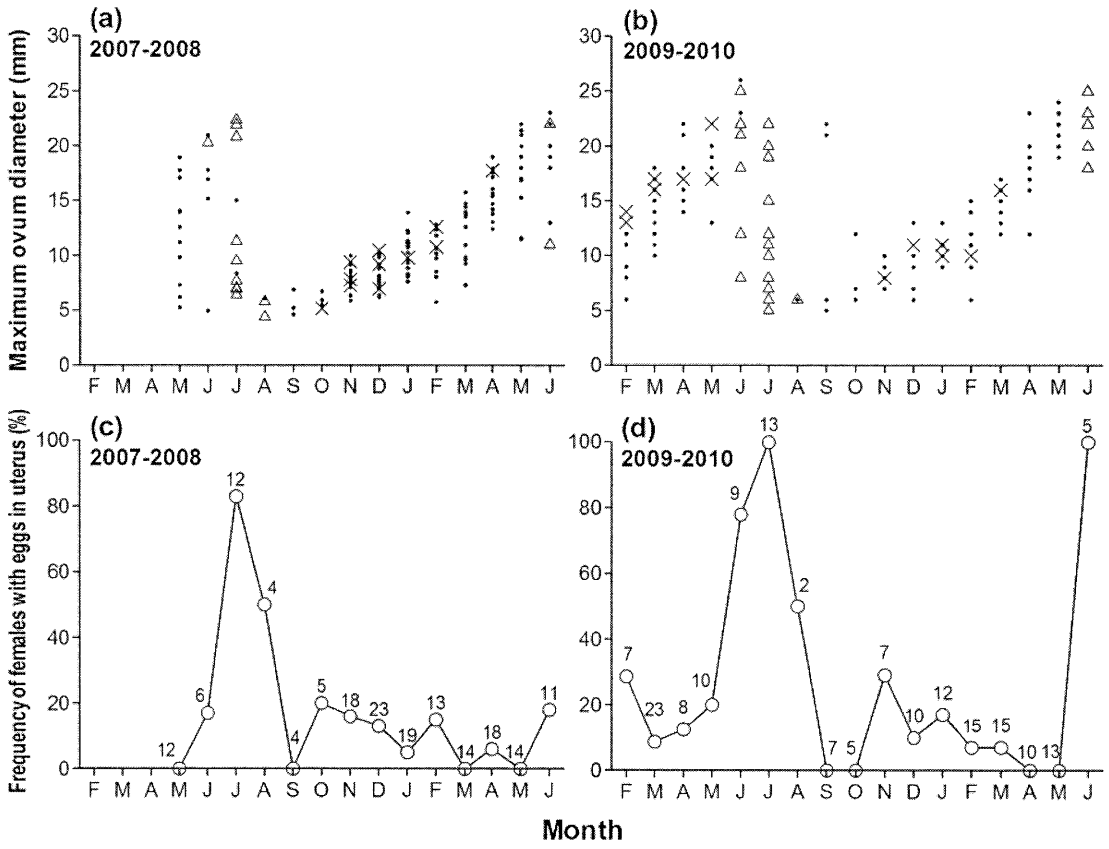

Fig. 4-5. Monthly changes in ovum diameter (maximum size) in the ovaries of mature females *Mustelus manazo* in Tokyo Bay in (a) 2007–2008 and (b) 2009–2010. Open triangles denote individuals with ovulated eggs in the uterus. Crosses indicate individuals with ovulated eggs that did not developed to embryos in the uterus. Frequencies of female with ovulated eggs in their uteri in (c) 2007–2008 and (d) 2009–2010. Number of individuals examined are shown above each symbol.

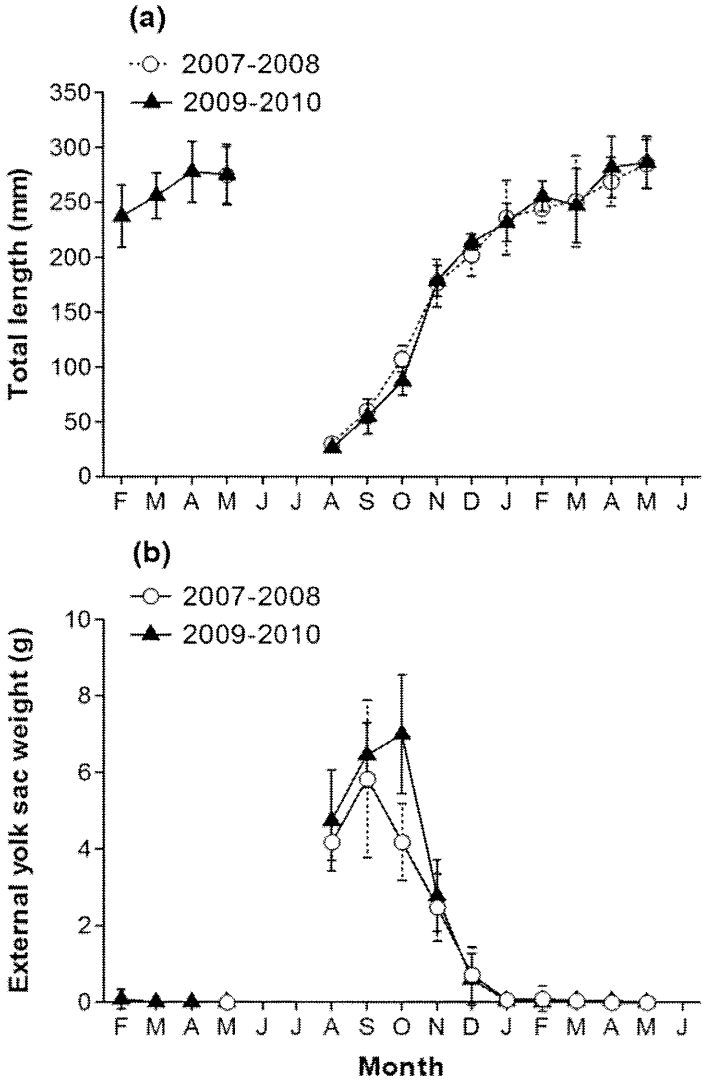

Fig. 4-6. Monthly changes in (a) mean total length and (b) weight of the external yolk sac of embryos in the uteri of *Mustelus manazo* in Tokyo Bay in 2007–2008 and 2009–2010. Values are means ± SD.

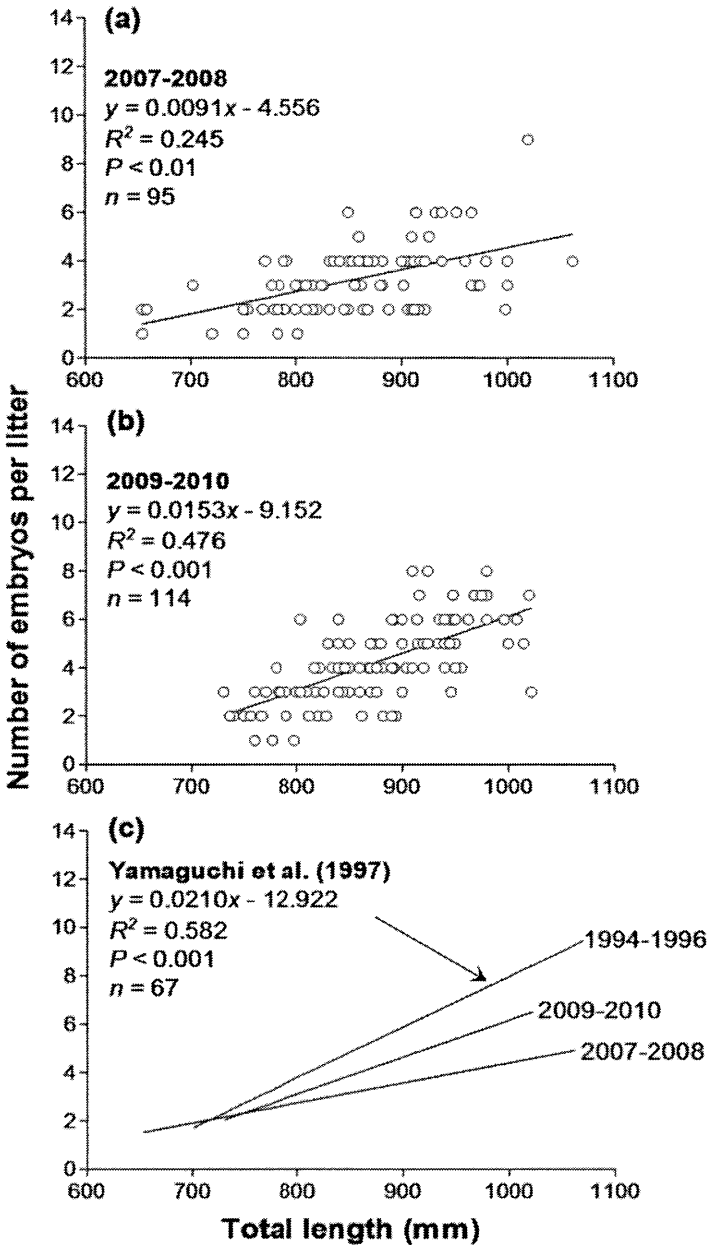

Fig. 4-7. Relationship between total length of female *Mustelus manazo* and number of embryos per litter in (a) 2007–2008 and (b) 2009–2010 in Tokyo Bay. (c) Size–fecundity relationships in 1994–1996 (Yamaguchi et al. 1997) and in 2007–2008 and 2009–2010 (present study).

VI. General discussion

In Tokyo Bay, substantial changes in the stock size of megabenthic species have been observed during the last three decades (Kodama et al., 2010); three characteristics have been found that 1) plural species were decreased drastic synchronously from end of 1980 to early 1990, 2) in the 2000s, their plural species were remained as low as during the 1990 and did not recovered. 3) At the same time, the increase in biomass-based density in the 2000s was attributed to increases in large fish species, such as Japanese sea bass and elasmobranches [starspotted smooth-hound (*Mustelus manazo*), stingray (*Dasyatis akajei*), spiny rasp skate (*Okamejei kenojei*), and Japanse butterfly ray (*Gymnura japonica*)]. The reason for these fluctuations in stock size remains unknown. To elucidate the actual causal factor(s) for fluctuating *M. manazo* population in the bay, I need to examine reproductive traits of females and males of *M. manazo*, development and growth of embryos, and several environmental factors, such as prey abundance, temperature and harmful chemical substances, which may have directly or

indirectly influenced the reproductive traits that would be recently observed in *M. manazo* population in Tokyo Bay.

In present study, changes in the food environment did not have a substantive effect on the growth of embryo, when compared to embryo size of 1994–1996 (Yamaguchi et al., 1997). Meanwhile, size at maturity of *M. manazo* in Tokyo Bay suggests that was getting smaller in the 2000s (present study) than that in the 1990s (Yamaguchi et al., 1997). Although the reason for this decline remains unknown, it may be related to phenotypic plasticity, because the size at first maturity of *M. manazo* ranged between 600 and 700 mm TL among different locations in previous research (Teshima, 1981; Taniuchi et al., 1983).

In the present study, mean stomach content weight per body weight was dramatically reduced from 2007–2008 to 2009–2010. The differences in mean stomach content weight may reflect differences in the prey environment. It is possible to consider that food limitation is one reason for the differences in the growth rate at population of *M. manazo*. According to Kodama et al. (2010), stock abundance of mantis shrimp *Oratosquilla*

oratoria among major prey item of *M. manazo* in Tokyo Bay has declined in 1990s thereafter. Reduction of the prey abundance and decrease the size at maturity both sexes were suggested to be the candidate factors that could have affected the reproduction in *M. manazo* in Tokyo Bay during the 2000s.

In the present study, there were no specific abnormalities in spermatogenesis as well as morphological structure of testes of male *M. manazo* specimens in Tokyo Bay, which may be linked with recent fluctuation in abundance of the population in the bay. The structure of the intratesticular duct system for carrying sperm in *M. manazo* is summarized in Fig. 3-5. The intratesticular ducts were well developed in all zones of spermatogenesis in *M. manazo*. Throughout the testis, most spermatocysts were attached to the termini of branch tubules. The branch tubules were connected via stem tubules to collecting tubules in the rete testis. The collecting tubules converged onto one efferent duct connected to the epididymis. Hence, in the testis, most or all spermatocysts move from the germinal to the maturation zone together with the sperm-carrying ducts until the spermatozoa are expelled from the spermatocysts. Although mechanism

involved in the movement of sprematocysts is still unclear, a role of the intratesticular duct system in spermatocyst migration during spermatogenesis cannot be ruled out. The structure of the epithelium of the intratesticular ducts is similar to that of transitional epithelium, and the myoid cells and smooth muscle surrounding the ducts may contribute to spermatocyst movement and sperm transport. An investigation of the mechanism underlying the formation of new interactions between the spermatocysts and branch tubules in the germinal zone is needed in the near future.

The present study revealed that significant changes in the fecundity of *M. manazo* in Tokyo Bay took place within a relatively short duration (4 years) in 2000s. Phenotypic changes in life history traits could occur within short duration in fish species with small body size and short life span (e.g., decrease in growth rate and fecundity of Atlantic silverside *Mendia menidia* within four years (four generations) due to effect of fishing pressure (Conover and Munch, 2002; Walsh et al., 2006)). However, changes in life history traits usually occur in decadal time scale in fish species with large

body size and long life span (summarized in (Jørgensen et al., 2007)). I could not reveal the reason of the abrupt change in the fecundity of *M. manazo* which have large body size and long life span (Yamaguchi et al., 1996). Further study is needed to clarify the actual reason(s) and the mechanism.

In conclusion, prominent changes in the life cycle traits (i.e., changes in food habit, decrease in the size at first maturity for both sexes, a decline in fecundity, pattern in the development of ova in the ovary, and presence of undeveloped eggs in the uterus) were evident for *M. manazo* in Tokyo Bay during the 2000s. As one of the causal factors of the stock fluctuations of *M. manazo* in Tokyo Bay, the plasticity could be driven by changes in population density, such as the fecundity of Atlantic herring (*Clupea harengus*) in the Western Gulf of Maine (Kelly and Stevenson, 1985). Similarly, the high fecundity in 1994–1996 may have contributed to recovery of the stock size of *M. manazo* in Tokyo Bay from the 1990s to the 2000s. Similarly, changes in the fecundity of *M. manazo* in Tokyo Bay in the 1990s may be density dependent with a time lag of several years. Also in

this study, significant changes in the fecundity of *M. manazo* in Tokyo Bay took place within a relatively short duration (4 years) in 2000s. However, changes in life history traits usually occur in decadal time scale in fish species with large body size and long life span. I could not reveal the reason of the abrupt change in the fecundity of *M. manazo* which have large body size and long life span.

Therefore, further studies are needed to examine if there are any changes in other life history traits of *M. manazo*, which would contribute to elucidate the mechanism of stock fluctuation of *M. manazo* in Tokyo Bay.

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