A SEASONAL STUDY OF MALE REPRODUCTION AND SPERM PHYSIOLOGY OF THREE COLDWATER FLATFISH

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A SEASONAL STUDY OF MALE REPRODUCTION AND SPERM PHYSIOLOGY OF THREE COLDWATER FLATFISH

By

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ABSTRACT

Seasonal patterns of sperm release and changes in sperm quality were studied in winter flounder and Atlantic halibut. While more than half of male winter flounder began producing milt by January, only small amounts of milt continued to be detectable during the prespawning period lasting from January to April. Milt production increased, sperm quality improved and spermatocrit declined during the summer spawning period in May and June, briefly coupled with peak plasma levels of testosterone (T) and 11 ketotestosterone (11-KT). In halibut, the period of milt production in males also began in January and continued into May. Unlike winter flounder, sperm motility in halibut was highest during the earliest stages of the spawning season and a remarkable reduction in sperm motility occurred by the end of the season in association with marked increases in the density of the milt.

Light and electron microscopy observations revealed that primitive features characterize the structure of both the winter flounder and halibut spermatozoa consisting of an unmodified head and a short midpiece enclosing a single unmodified mitochondria ring. The basal body, accommodated in the caudal nuclear fossa, is made up of the distal centriole and a centriolar collar consisting of 9 pericentriolar columns, 9 bridges and an outer ring. Serial microscopic sampling and examination of sperm morphology demonstrated the existence of abnormal spermatozoa in both species throughout the reproductive season. These abnormalities involved the head, tail and cell membrane damage. In both species,

the lowest proportion of abnormal sperm was found during the period that sperm motility was the highest.

The impact of hormonal manipulation of reproduction in mature male winter flounder was examined by means of hypophysectomy (hypex), pituitary replacement therapy (PRT) and the administration of gonadotropin releasing hormone analogue (GnRH-A). During the spawning season, while GnRH-A treatment increased the production of milt and advanced sperm production, milt was diluted and sperm output for the entire reproductive season was not augmented. In hypexed male flounder, by contrast, milt became more concentrated and sperm production declined along with reduced plasma androgen levels. A restoration of milt hydration in hypexed males followed PRT in December and January. Moreover, PRT increased plasma 11-KT levels in intact males at this early seasonal stage of spermiation and appeared to be responsible for advancement of spermiation in a large number of prespawning males. Finally, none of these treatments altering the hormone levels of mature males resulted in detectable changes in either sperm motility or egg fertilization rates.

Studies of the genital tract in male winter flounder indicated that the testicular efferent duct system (TEDS) is composed of a testicular primary duct (PD) and the sperm duct (SD) lined primarily by columnar and cuboidal epithelial cells. Secretion in these cells appeared to be most active during the period of vigorous sperm release in the spawning season and relatively quiescent at other periods, parallelling seasonal changes in milt production and sperm motility. Observations

in vivo indicated that the pH of milt increased and milt density decreased as one proceeded distally along the male genital tract (from PD to SD), since sperm motility was increased perhaps after storage for several weeks in the TEDS in May. This suggests that the testicular efferent duct system plays a role in the mediation and maintenance of sperm function.

Milt properties in the winter flounder were characterized, and showed that seminal fluid osmolality ranged from 356-377 mOsm kg^{-1} with a pH of 8.0. Although protein electrophoretic profiles were very similar between the seminal fluid and blood plasma, the protein, phospholipid (PL), cholesterol (Chl) and some ion (Na^+ and Cl^-) concentrations were lower in the seminal fluid. Of all these substances, only PL and Chl concentrations were found to be negatively correlated with sperm motility.

Like other teleosts, the testicular development in male yellowtail flounder can be classified into 5 progressive stages, i.e. 1) early testis development, 2) rapid testis development, 3) limited spermiation, 4) full spermiation and 5) spent. Despite this apparent progression of development in testes, the seasonal rate of mature and spermiating yellowtail flounder never fell below 40% even during September and December when the lowest gonad-somatic index values were observed. Frequent observations of spermatocytes in the testis of spermiating cultured males and males from the wild suggest that overlapping cycles of spermatogenesis occur in yellowtail flounder, representing a somewhat distinctive mode of male flatfish reproduction in this species.

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LIST OF ABBREVIATIONS

T.....testosterone

11-KT..... 11 ketotestosterone

E2.....17β estradiol

17α, 20β-P.....17α, 20β -dihydroxy-4-pregonen-3-one

GnRH.....gonadotropin releasing hormone

GnRH-A.....gonadotropin releasing hormone analogue

GtH.....gonadotropin

hypex.....hypophysectomy

sham.....sham operation

PRT.....pituitary replacement therapy

TEDS.....testicular efferent duct system

PD.....testicular primary duct

TED.....testicular efferent duct

TMD.....testicular main duct

SD.....sperm duct

GSI.....gonadosomatic index

SEM.....scanning electron microscopy

TEM.....transmission electron microscopy

GA.....primary spermatogonia

GB.....secondary spermatogonia

BW.....body weight

ATP.....adenosine triphosphate

cAMP .....cyclic adenosine monophosphate

CHAPTER 1

General Introduction

1.1. Background for the thesis work

A predicable supply of fish fry represents a major obstacle for rapid development of fish farming since most species reproduce seasonally, spawning once or a few times within a limited time of the year (Zohar, 1989). With an increased knowledge of gonadal development and reproductive regulation, extended gamete production may become possible in some species through manipulation of reproductive cycles (Billard *et al.*, 1993b; Crim, 1991). Still, with reproductive manipulation and adapting fish to captive culture conditions, there is a growing concern about gamete quality. While some research has previously focused on egg quality (Bromage, 1995; Kjorsvik *et al.*, 1990), to date, few investigations have addressed the potential importance of sperm quality (Billard *et al.*, 1995). Although technology for *in vitro* sperm storage has made male gametes available out of season, success in preserving good quality sperm is still limited (Stoss, 1983), depending upon the species, the individual fish and also the timing of sampling (Babiak *et al.*, 1997; Billard *et al.*, 1977; Stoss, 1983). Therefore, in order to improve sperm quality, detailed studies regarding species specific sperm release patterns and sperm

quality changes during the reproductive season or under different conditions are essential. Since fluctuations in gamete quality have been recognized to be a source of recruitment variability in wild fish stocks (Kjorsvik *et al.*, 1990), there is no doubt that research addressing such key questions will not only improve our knowledge of male reproduction and sperm physiology in teleosts, but will also facilitate the development of fish culture and sperm preservation techniques, as well as the conservation of wild stock.

1.2. General review of fish reproduction

1.2.1. The male reproductive system

Development, maturation and storage of sperm in teleosts is undertaken in the male reproductive system, which includes the testes and the genital tract (Billard, 1986) [or the testicular efferent duct system (Lahnsteiner *et al.*, 1993a; Manni and Rasotto, 1997)]. The testes of most teleosts are composed of paired lobes attached to the dorsal body wall by mesentery, encompassing many small tubules where germ cells differentiate and develop into spermatozoa (Nagahama, 1983). Although, there is a wide variation in testicular structure among different species, two basic types (i.e. unrestricted and restricted spermatogonia) are recognized according to the distribution of primary spermatogonia (GA) within the testes (Grier *et al.*, 1980). The unrestricted testis, typically found in most teleosts, can be identified by uniform distribution of GA along the entire length of the testes. In contrast, restricted

distribution of GA occurs in a few species where primary spermatogonia are only found in the distal blind terminus of tubules which are arranged radially from the central testicular cavity (Nagahama, 1983). The development of sperm in the restricted testis starts at the blind end. As they develop sperm migrate to the opposite end of the tubule, and are released into the central testicular cavity when mature. Generally, no central testicular cavity is evident in the unrestricted type of testis which instead contains numerous branched seminiferous tubules. The spermatogonia develop within germinal cysts along the epithelium of the seminiferous tubules and mature sperm are voided into the lumen of the seminiferous tubules as germinal cysts rupture. The restricted testis type is also called tubular testis in some studies, while the unrestricted type corresponds to lobular testis (Billard *et al.*, 1982; Billard, 1986; Nagahama, 1983).

After release, spermatozoa are stored in the testes (the lumen of the seminiferous tubules or the central cavity) or in the genital tract chamber. They are eventually liberated into water or deposited directly into females through the genital pore. The male genital tract, which also varies considerably among species, may be a simple sperm duct (or vas deferens), such as found in the guppy (Billard, 1986). In many species, such as cyprinid fish, however, sperm in the testes first empty into the testicular main duct (TMD) (named the testicular primary duct (PD) in the present study) situated laterally along the testicular lobes before flowing into a second portion of the genital tract, the sperm duct (Lahnsteiner *et al.*, 1994b). An

intratesticular efferent tubule system is present in some species such as European pike, *Esox lucius*, blenniid fish, and grunt, named the testicular efferent duct (TED) (Lahnsteiner *et al.*, 1993a) or "testicular gland"(Lahnsteiner *et al.*, 1990; Seiwald and Patzner, 1987). In these species, mature sperm first flow into the TED and then the TMD before converging in the sperm duct. Extratesticular accessory glands [seminal vesicle (Fishelson, 1991; Fishelson *et al.*, 1994; Har, 1993; Nagahama, 1983) or sperm duct gland (Cinquetti *et al.*, 1990)] are also present in some species which connect with the sperm duct laterally. Although morphological characteristics of the genital tract have been described in a number of teleost species (Lahnsteiner and Patzner, 1990; Lahnsteiner *et al.*, 1990; Lahnsteiner *et al.*, 1993a, b; Lahnsteiner *et al.*,1994b; Manni and Rasotto, 1997; Patzner, 1991; Patzner *et al.*, 1991; Rasotto, 1995; Rasotto and Sadovy, 1995; Rasotto *et al.*, 1992), direct investigations into the role of the genital tract in sperm maturation and sperm maintenance are limited to a few freshwater species (Morisawa and Morisawa, 1986; Miura *et al.*, 1995). According to these studies, the genital tract is the main site of seminal fluid production although other important functions, such as sperm maturation, storage, and nutrition are also suggested (Cinquetti *et al.*, 1990; Miura *et al.*, 1995; Morisawa and Morisawa, 1986; Rasotto and Sadovy, 1995).

1.2.2. Sperm development, maturation and release

For most fish species, development of sperm (spermatogenesis) is completed

within testicular germ cysts, involving serial cell divisions, differentiation, and metamorphosis. Derived from primordial germ cells, primary spermatogonia (GA) from the epithelium of the seminiferous tubules repeatedly divide mitotically, forming secondary spermatogonia (GB) which then develop into primary spermatocytes (I). Spermatocytes I continue to develop into other spermatogenic cells (spermatocytes II and spermatids) within germ cysts surrounded by the Sertoli cells (Nagahama, 1983) through two maturation divisions, and eventually metamorphose into spermatozoa, a process called spermiogenesis involving modification, reorganization and reconstruction of sperm structures or components. For a few species that carry out a process of semicystic spermatogenesis, sperm metamorphosis continues in the genital tract after sperm release from germ cysts (Lahnsteiner *et al.* 1993a; Rasotto and Sadovy, 1995). In addition, in some species, functional maturation of spermatozoa (such as the acquisition of the capacity of movement in sperm) also takes place in the genital tract, although sperm cells are liberated into the testicular lumen after metamorphosis is complete (Morisawa and Morisawa, 1986).

The release of sperm, or spermiation, is somewhat ambiguous terminology for teleosts. Initially, the term spermiation was used in higher vertebrates to describe the detachment of spermatozoa from the Sertoli cells. However in fish with lobular testis it referred to the release of spermatozoa from testicular lobules (Billard and Cosson, 1992). The term spermiation has also been used to indicate

that milt can be collected from the genital pore by manual stripping, correspondent to the release of sperm from the testis into the sperm duct (Baynes and Scott, 1985). In this study, the term spermiation will be used to indicate milt expression by hand-stripping.

1.2.3. Seasonal reproductive cycle

Seasonal reproduction has been described for many fish, particularly for species living in temperate zones and high latitude areas, in response to a changing environment (Bye, 1990). A reproductive cycle may be composed of several consecutive reproductive phases, but at least includes a spawning season and a period of non-spawning. The non-spawning season typically is composed of a regressed phase (sexually inactive or gonadal quiescent period) that typically follows the spawning season, and a period of reproductive preparation which involves an active gamete development phase (gonadal recrudescence). For oviparous species which comprises the majority of teleosts, the spawning season represents the peak of reproductive activity during which mature gametes are vigorously released into water for fertilization and growth of progeny. The reproductive cycle varies considerably from species to species, or even stock to stock. For example, timing, frequency and duration of spawning show large differences depending upon the species, often reflecting synchronization of gametogenesis (Billard, 1986). Seasonal cycles of reproduction in both males and

females are basically similar, except that the development of gametes in males sometimes preceeds maturation of females, resulting in an elongated period of milt production in males, as indicated in carp (Courtois *et al.*, 1986), halibut (Crim et al. unpubl.), and winter flounder (Burton and Idler, 1984; Harmin *et al.*, 1995a).

1.2.4. Reproductive regulation

Although mechanisms underlying the seasonal reproduction cycles still remain incompletely understood, most research to date concludes that the seasonal reproduction of fish results from an interplay between endogenous rhythms and exogenous (environmental) variables that fluctuate seasonally yet remain relatively predicable year after year. Exogenous environmental factors often serve to cue fish to the time of year, and thus induce and regulate gonadal growth and maturation enabling fish to spawn under the most favorable conditions for the young (Munro, 1990). Driven by an innate rhythm, fish perceive and transduce exogenous changes into central nervous system signals, triggering reproductive activities in response to environmental changes. Mediation, coordination, and integration of reproductive activities are accomplished by an endocrine/neuroendocrine unit which includes the hypothalamus-pituitary-gonadal axis. Some components modulate and integrate reproductive events in a paracrine or autocrine fashion, such as various brain neurotransmitters (Breton *et al.*, 1991; Breton *et al.*, 1993; Danger *et al.*, 1991; Kah *et al.*, 1992; Roelants *et al.*, 1990), and bioactive substances in the gonads

(Verhoeven, 1992).

The hypothalamus-pituitary-gonad axis plays a crucial role in the mediation of reproduction in fish. Secretion of pituitary gonadotropic hormones (GtHs) parallels gonadal development in correlation with active gametogenesis (Munro, 1990). A sharp rise of serum GtH occurs at ovulation, spermiation and spawning (Bhattacharya *et al.*, 1994; Lin *et al.*, 1994; Redding and Patino, 1993). Removal of the pituitary (hypophysectomy) from spermatogenic males reduces testicular weight or causes degeneration of developing sperm cells leaving only primary spermatogonia and spermatozoa (Billard, 1974). Restoration of spermatogenesis in hypophysectomized males can be achieved with the administration of pituitary extract or purified gonadotropic hormones, suggesting GtH is necessary to maintain spermatogenesis (Billard et al., 1993b). Spermiogenesis, however, may be independent of GtH's regulation. On the other hand, sperm release (spermiation) is completely inhibited in goldfish (*Carassius auratus*) by deprivation of pituitary hormones or ceases when GtH in serum is low (< 10 ng ml^{-1}) (Billard, 1986). Gametogenesis, and final maturation and spawning may be regulated, respectively, by different forms of GtHs (I and II), as suggested by observations with salmonid fish (Miwa *et al.*, 1994; Planas and Swanson, 1995; Sumpter *et al.*, 1991). Repeated injections of GtH II (maturation hormone) appears to stimulate milt release without inducing spermatogenesis in carp (Billard et at. 1993b).

The secretion of GtH(s) is under dual hormonal control by the brain.

Gonadotropin releasing hormone (GnRH) produced by neurosecretory cells in the hypothalamus and several discrete brain areas (Amano *et al.*, 1991; Kim *et al.*, 1995) stimulates GtH secretion, as indicated by *in vitro* or *in vivo* GnRH treatment (Bhattacharya *et al.*, 1994; Zohar, 1989). Salmon GnRH content in discrete brain areas are high during the spawning season, and significantly increase in the pituitary prior to ovulation (Amano *et al.*, 1992). On the other hand, dopamine (DA) inhibits GtH secretion at both hypothalamic and pituitary levels in fish (Barannikova *et al.*, 1989; Chang *et al.*, 1984a, b). Increasing evidence also indicates that the GtH effects on gonadal development are mediated by gonadal steroid hormones, including androgens [testosterone (T) and 11 ketotestosterone (11-KT)] in males, estrogens (estradiol, estrone) in females, and progestin [17α, 20β-dihydroxy-4-prognen-3-one, (17α, 20β-P)] in both sexes. The major estrogen, 17β estradiol (E2), stimulates the synthesis of vitellogenin in liver and the uptake of the yolk precursor by developing oocytes, while progesterone is considered to be a potent oocyte maturation inducing substance (MIS) in females (Murayama *et al.*, 1994). Information about the steroid involvement in male gametogenesis is more limited (Bhattacharya *et al.*, 1994). Enhancement of T or 11-KT in males, often coinciding with the rise of GtH, is characteristic of serum steroid profiles during gonadal recrudescence (Barnett and Pankhurst, 1994; Hontela and Stacey, 1990; Malison *et al.*, 1994). A higher dose of T is required to maintain meiosis and spermiogenesis in hypophysectomized males (Billard, 1986). Firm evidence is lacking that steroid

hormones are involved in the control of spermiation in teleosts, and the results appear to vary from species to species. In freshwater species, peak levels of T or 11-KT are often found just before the spawning season (Baynes and Scott, 1985; Rosenblum *et al.*, 1994). However, injection or implantation of T or 11-KT fails to stimulate spermiation in several species while only methyltestosterone and progesterone are capable of inducing spermiation in a few of freshwater fish, such as goldfish and trout (Billard, 1986). Peak levels of T and 11-KT were observed to precede the spawning season (Clearwater, 1996; Harmin *et al.*, 1995a; Methven *et al.*, 1992; Ouchi *et al.*, 1988) or persist during it (Carragher and Pankhurst, 1993) in several marine species while the role of progesterone on spermiation remains unclear in most marine teleosts.

1.3. Experimental fish

Winter flounder (*Pleuronectes americanus* Walbaum), yellowtail flounder (*P. ferrugineus* Storer) and the Atlantic halibut (*Hippoglossus hippoglossus* L.) are coldwater flatfish, belonging to the family Pleuronectidae. Previous information indicates that although winter flounder spawn early in the summer, males produce sperm for longer periods lasting several months (Burton and Idler, 1984; Harmin *et al.*, 1995a). Yellowtail flounder are summer spawners as well (Pitt, 1970), with especially prolonged milt production periods observed in captive males (Clearwater, 1996). Although halibut spawn in the winter (Haug and Kjorsvik, 1989; Haug, 1990),

release of milt in male halibut usually lasts for 4-5 months (Crim et al. unpubl.), similar to winter flounder.

Winter flounder were selected for this work because they are a shallow benthic inshore coldwater species, easily collected year-round from North Atlantic waters by SCUBA divers. Besides their tolerance for below-freezing water temperatures, as low as -1.8 °C, winter flounder are hardy and also capable of surviving many laboratory procedures such as reproductive manipulation and hypophysectomy. After ovulation, females spawn sticky-demersal eggs which can be artificially inseminated and conveniently raised in laboratory petri dishes to perform egg fertilization trials.

Winter flounder, yellowtail flounder and halibut are all important cultured species. Since abnormalities in flatfish reproduction have served to evaluate the impact of pollution on wild stocks (Johnnson *et al.*, 1992), it was deemed important to conduct further studies of reproduction in these marine flatfish species in order to facilitate their use in aquaculture and provide better knowledge for effective conservation of these natural resources.

1.4. Objectives and outline of the thesis

This thesis work uses the male winter flounder as a model species, focusing on reproduction and the quality of sperm throughout the season by examining:

1) Seasonal sperm release and changes of sperm quality during the spawning

season (Chapter 3) or throughout the entire spermiation period (Chapter 4 and 5);

2) Role of sex hormones on spermiation, seasonal sperm production and sperm quality (Chapter 3);

3) The seasonal activities of the male genital tract and its role on the development and maintenance of viable sperm (Chapter 6 and 7);

4) Composition of seminal fluid and its relationship with sperm quality (Chapter 8).

5) In addition, seasonal sperm development, sperm release and changes in sperm quality were compared to other flatfish, including halibut (Chapter 4 and 5) and yellowtail flounder (Chapter 9).

CHAPTER 2

General Materials and Methods

2.1. Fish and holding conditions

Three species of fish were used in this study.

2.1.1. Winter flounder

Sexually mature male winter flounder were caught by SCUBA divers inshore in Conception Bay, Newfoundland. The fish were held in a fibreglass tank (either 2 x 2 x 1.2 m or 1.2 x 1.2 x 1 m) together with some females (male: female=5~6:1), supplied with flowing ambient temperature seawater (0-15 °C during a year) and a simulated natural photoperiod for St. John's, Newfoundland, Canada (47°20 N, 52°45' W). Fish cease feeding in winter and spring and resume again during summer. During the feeding season (June to October or November), fish were fed with commercial food pellets (Connors Brothers Co.) at ~1.5% body weight (BW) twice a week.

2.1.2. Halibut

Male Atlantic halibut were selected from a broodstock which was collected by inshore fisherman on the west coast of the Newfoundland or DFO (Department of Fisheries and Oceans, Canada) groundfish survey vessels between 1987 and

1990. Upon arrival at the laboratory, the fish were held in 3 or 5 m (diameter) covered fiberglass tanks at a density ranging from 5-10 kg BW m^{-2}. A relatively stable temperature range was maintained (5 °C in winter and 10 °C in summer, respectively) to reduce the ambient temperature fluctuations. The fish were fed to satiation on a varied small fish diet (herring, mackerel and squid) twice weekly (0.01-1.3% BW day^{-1}) as the rate of food consumption varies seasonally (Keough, 1992).

2.1.3. Yellowtail flounder

Adult male yellowtail flounder were obtained from the inshore waters of Conception Bay by SCUBA divers, or collected by DFO vessels conducting groundfish surveys on the Grand Bank. Fish were held under conditions similar to those applied to the male winter flounder except that the temperature range was maintained between 4-12 °C during the reproductive season by using a mixture of ambient and heated/chilled seawater. Feeding of commercial food pellets (Connors Brothers Co.) was continued throughout the year at a rate of ~1.5% of body weight and given twice a week.

2.2. Milt collection

Presence of sperm in males was checked by gentle compression of the fish abdomen (stripping). Males were considered in nonspermiating condition if no milt

was expressed after 3 consecutive stripping attempts. Once detectable, milt was hand-stripped from the the sperm duct into disposable syringes or glass scintillation, carefully avoiding urine contamination. Milt expressed from the urinogenital pore was discarded if it contained a clear fluid or the pH values fell below 7.5 (milt collected directly from the sperm duct always has a pH > 7.5) , indicating possible urine contamination. After collection, milt samples were stored on crushed ice (0 °C) in an insulated container prior to and during examination.

2.3. Evaluation of milt and sperm production

Milt volume was measured (Chapter 3) using 1, 2, 5 or 10 ml graduated pipettes, depending on the amount of milt collected. Sperm production was estimated according to the following regression equation: number of spermatozoa/ml = (0.1 x spermatocrit - 0.54) x 10^{11} (r^2 =0.71, P < 0.01, N=26). This equation was established by performing sperm counts on milt samples of winter flounder with varying spermatocrit values. A small drop of milt was diluted 5,000 to 10,000 fold and sperm were counted in a haemocytometer. Average cell concentration for each milt sample was obtained from three replicates.

2.4. Measurement of milt spermatocrit and pH

A volume of milt drawn into a 0.1 ml glass capillary tube was centrifuged at 12,000 x g for 25 min in a micro-haematocrit centrifuge (Int. Equipment Co., USA)

to determine spermatocrit according to the formula: spermatocrit (%) = [height of packed cell (mm)/total height of milt (mm)] x 100. Milt pH values was determined by placing 50-100 µl of milt on Colorplast™ pH sticks (EM Science, USA, EM reagent, range 6.5 -10.0, sensitivity 0.2-0.3).

2.5. Evaluation of sperm quality

2.5.1. Sperm motility

Sperm motility was evaluated as soon as possible after milt collection (within 30 min in Chapter 5, or 1-1.5 h in Chapter 3 and Chapter 8), following a double dilution protocol (Billard *et al.*, 1993a): Milt was first diluted 100-300 fold, depending upon sperm concentration, in non-activating $DCSB_4$ sperm buffer (150 mM sucrose, 1 mM $MgSO_4$, 1.7 mM $CaCl_2$, 86 mM glycine, 30 mM trizma base, pH 8.0, Osmotic pressure 290-350 mOsm kg^{-1}, adapted from Chambeyron and Zohar, 1990). The dilution rate was adjusted, up 300-fold, dependent on sperm concentration. In the second step, 2 µl of the diluted sperm suspension was mixed with 50 µl chilled filtered seawater previously loaded on a slide and the simultaneously activated sperm were examined immediately under a darkfield microscope. Sperm motility (the percentage of activated spermatozoa displaying progressively forward movement) and the duration of progressive swimming were recorded. All estimates of sperm motility for each sample was performed 2-3 times.

2.5.2. Sperm fertility trials

The ability of spermatozoa to fertilize eggs was tested using batches of approximately 200 flounder eggs, gently mixed with 40 µl $DCSB_4$ diluted sperm and 2 ml seawater. In two preliminary trials, an appropriate sperm/egg ratio was determined in which detectable differences in embryo development only occurred when this ratio was reduced to 2.48 - 9.46 x 10^3 spermatozoa per egg. After standing approximately two minutes, the eggs were washed 2-3 times with seawater and placed into an incubator (5±2 °C). Water in petri dishes containing developing embryos was changed every 2-3 days. The percentage of eggs containing blastula embryos at 24-48 hr after insemination determined the fertilization rate. The hatched larvae were counted 2 - 4 times from day 12 to 20 following insemination after which all of the eggs were either hatched or died. The number of hatched larvae, divided by the number of fertilized eggs, yielded the hatching rate.

2.6. Histological and EM procedures

Samples (sperm duct and testis) were fixed in Bouin's for 24 -48 h, then embedded into paraffin before cutting into sections of 5-6 um thickness. The sections were stained with haematoxylin-eosin prior to microscopic examination. Sperm samples, fixed in 2.5 % glutaraldehyde, were smeared on a slide and stained with 1.0-2.0% light green. Two hundred spermatozoa were counted randomly from 4-5 microscopic areas (x 1000) for each milt sample to determine

proportions of normal and morphologically abnormal sperm cells (Chapter 5).

For electron microscopy (EM) studies, spermatozoa and sperm duct samples were initially fixed for 2 h in 0.1 M cacodylate buffer (4 °C) containing 2.5% glutaraldehyde. After being fixed again in 1% osmium tetroxide for ~1 h at 4 °C and dehydrated in a series of ethanol solutions, spermatozoa for scanning electron microscopy (SEM) were then subjected to CO_2 critical point drying, coated with silver and observed with a Zeiss 100 scanning EM. Samples (spermatozoa and sperm duct) for transmission electron microscopy (TEM) were embedded in a low viscosity Spurr resin after dehydration. Ultrathin sections were stained with lead citrate and uranyl acetate, then observed under a Zeiss 109 transmission EM.

2.7. Steroid radioimmunoassay (RIA)

The major androgenic hormones, testosterone (T) and 11 ketotestosterone (11-KT), in winter flounder blood plasma (Chapter 3) and seminal fluid samples (Chapter 8) were determined by RIA protocols described previously (Harmin and Crim, 1993; Harmin *et al.*, 1995b) with slight modifications. Steroids were extracted from 0.1 ml plasma or seminal fluid with diethyl ether (2 x 2 ml) after adding 10 µl tritiated T [1000 counts per minute (CPM)] or 11-KT (1000 CPM), then incubated 1-2 h at room temperature (~ 21 °C). After freezing over solid CO_2, the aqueous phases were discarded and the combined ether fractions evaporated in air overnight at room temperature. The resultant plasma residues were reconstituted

in 1 ml absolute ethanol and allowed to equilibrate at least over night at 4 °C before performing RIA and estimating steroid extraction efficiency.

For RIA of T, duplicate samples of 100 µl of the reconstituted plasma extract were evaporated in 12 x 75 mm borosilicate disposable test tubes and mixed with T antiserum (diluted 1:2) and ca. 10,000 CMP of iodinated T [purchased from International Diagnostic Service (IDS), Scarborough, Ontario, Canada] in T assay buffer (50mM phosphate, 100 mM sodium chloride, 0.1 % sodium azide and 0.1% gelatin, pH 7.4). Following incubation for 1 h at room temperature, 1 ml ethylene glycol separating reagent (IDS) was added to the tube, which stood at room temperature for 30 min before centrifugation at 1650 x g for 30 min. The tubes containing the deposited bound steroid fraction were counted for 1 min in a gamma scintillation counter.

In the 11-KT assay, extracted samples were incubated with phosphate buffer containing 10,000 CPM of tritiated 11-KT (prepared by C. Wilson, Newfoundland, Canada, according to Truscott, 1981) and 11-KT antiserum (courtesy D. R. Idler, Newfoundland, Canada; diluted 1:40,000) overnight at 4 °C. After the initial incubation, 0.2 ml dextran-charcoal suspension (2.5 g charcoal, 0.25 g dextran T-70 in 500 ml assay buffer) was added and assay tubes were centrifuged at 2,000 x g for 15 min. The supernatant was decanted into a glass scintillation vial containing 10 ml of scintillation cocktail and antibody bound steroid radio activity was measured in a beta liquid scintillation counter for 10 min.

All steroid data were corrected according to extraction efficiencies estimated simultaneously (97.2% and 95.2% for T and 11-KT, respectively). Plasma samples of each summer experiment in 1994 (Expt. 1 and 2, Chapter 3) were analysed together in the same RIA. For experiments carried out during the winter of 1994-1995 (Expt. 3, Chapter 3), all serial samples collected from individual fish were analysed within a single assay with the number of fish from each treatment group assigned equally across each of the 3 assays to minimize potential effects of interassay variation. T antibody cross-reactivity was 0.19% for 11-KT (Harmin *et al.*, 1995a) and 11-KT antibody cross-reactivity with testosterone was < 0.1% (Ng and Idler, 1980).

2.8. Hormone treatments

2.8.1. GnRH-A pellet preparation

Gonadotropin releasing hormone analogue (GnRH-A) (D-Ala^6, Pro^9-NHEt-LHRH), dissolved in 50% ethanol, was mixed into a cellulose-cholesterol powder (50:50%) and dried briefly at 30 °C. After mixing with a few drops of molten cocoa butter, 27 mg hormone paste was compressed into a cylindrical pellet using a mold. Each pellet contained 110 µg GnRH-A.

2.8.2. Surgical hypophysectomy

The pituitary of males was removed (Chapter 3) according to Campbell and Idler

(1976). Briefly, the pituitary gland was aspirated with a 1 ml glass pipette from a small hole (~2 mm diameter) drilled in the cranium through the lower operculum of fish anaesthetized with 0.1- 0.15% phenoxyethanol (v/v). In sham-control fish, the pituitary was exposed but not removed from the opening in the cranial cavity.

2.9. Analysis of osmolality, ionic and biochemical composition of blood plasma and seminal fluid

The osmolality of the seminal fluid was tested in duplicate samples using a Fiske 110 Osmometer. Measurement of the ionic composition and glucose in the seminal plasma and the blood plasma were performed using a Beckman CX7 system. Sodium, potassium and chloride were determined by indirect potentiometry while calcium and magnesium were measured by means of a colorimetric reaction and glucose by oxido-reductive reaction.

Protein was quantified by the Lowry method (Lowry et al. 1951) using bovine serum albumin (BSA) as the reference. Continuous concentration gradient gel electrophoresis (7.5-20%) was used to study the protein constituents in both body fluids (Hames, 1981). Individual or pooled samples of the seminal fluid were diluted 1:2 with sample buffer (2% SDS, 5% mercaptoethanol, 10% sucrose and 0.002% bromophenol blue, pH 6.8). The dilution for the blood plasma was 1:4. Loading volume was 100 µl for the seminal fluid or 20 µl for the blood plasma, respectively. Samples were denatured by heat treatment at 100 °C for 3 min prior to loading.

Electrophoresis was conducted at 100 v ~1 h for the stacking gel (2.5%) and at 50 v overnight for the resolving gel. Pharmacia protein kits (14.5-94 KD) were used to calibrate the protein molecular weights of the body fluids. The slab gel was stained with 0.1% R250 Coomassie Blue and destained with a solution of 12.5% isopropanol and 10% acetic acid.

Triglycerides (TG), cholesterol (Chl) and phospholipids (Pl) were measured using enzymatic colorimetric test kits from Boehringer Mannheim. After 20 min incubation with reagents at room temperature (~ 21 °C), absorbance of samples was read within 30 min against a reagent blank at 500 nm in a 3550 Microplate Reader (Bio-Rad), calibrated with standard reagents provided by Bochringer Mannheim.

2.10. Statistical analysis

Data was expressed as mean±standard error (s.e.). T-tests, one-way or two-way ANOVA followed by the Duncan's multiple range test were performed by using a SAS program (SAS 6.1) with a P value ≤ 0.05 accepted as the level of statistical significance. Before data was analyzed, they were log, square root or arcsine transformed when necessary to fit the assumptions of ANOVA and t-test.

CHAPTER 3

Sperm Production and Sperm Quality in Winter Flounder, *Pleuronectes americanus*: the Effects of Hypophysectomy, Pituitary Replacement Therapy and GnRH-A Treatment

3.1. Introduction

Previous work studying on the effects of administrating gonadotropin releasing hormone (GnRH) and its analogues in male teleost indicates that the timing of sperm release can be accelerated (Billard *et al.*, 1987; Dabrowski *et al.*, 1994; Garcia, 1993; Harmin *et al.*, 1995b; Kreiberg *et al.*, 1987; Lee *et al.*, 1986; Slater *et al.*, 1995; Sokolowska *et al.*, 1988), presumably acting via pituitary gonadotropin hormones (GtHs) and/or sex steroid hormones (Billard *et al.*, 1987; Crim *et al.*, 1988; Ngamvongchon *et al.*, 1987; Pankhurst, 1994; Takashima *et al.*, 1984). Despite an immediate boost in sperm production following hormonal advancement of sperm release, these short-term studies (Billard and Marcel, 1980; Billard *et al.*, 1987; Courtois *et al.*, 1986; Dabrowski *et al.*, 1994; Garcia, 1991; Kulikova and Burlakov, 1985; Linhart and Billard, 1994; Ngamvongchon *et al.*, 1987; Pankhurst, 1994; Takashima *et al.*, 1984) do not clearly indicate if the annual output of sperm

has been significantly changed. For example, sperm output was augmented by GnRH-A in rabbitfish, *Siganus guttatus*, but only during an initial three weeks (Garcia, 1993). This suggests that hormone-stimulated sperm production may simply represent an advancement of sperm release without stimulation of spermatogenesis.

Apart from influencing the timing of sperm release, hormonal effects on the function of spermatozoa in teleosts remain largely unknown. There is little evidence of an impact of GnRH or GtH administration on sperm quality (Ngamvongchon *et al.*, 1987; Saad and Billard, 1987), although in some species GtH may indirectly regulate sperm activities at the level of sperm duct (Marshall *et al.*, 1989; Marshall *et al.*, 1993). Similarly, a few studies (Defraipont and Sorensen, 1993; Miura *et al.*,1992) reported that 17α 20β dihydroxy-4-pregnen-3-one increased sperm motility. Clearly, hormonal impacts on sperm quality in male teleosts require further investigation.

A previous 4-wk study (Harmin *et al.*, 1995b) showed that GnRH-A treatment enhanced sperm production in prespawning male flounder. The present study was designed to (1) examine the seasonal pattern of sperm production and sperm quality in this species, (2) investigate the effects of pituitary stimulation or deprivation on total sperm output and sperm quality, and (3) determine the effects of pituitary replacement (extract) on spermiation in mature males. In addition, androgen profiles [testosterone (T) and 11 ketotestosterone (11-KT)] associated

with such hormonal manipulations were examined to evaluate the interactions between sex hormone levels and the patterns of sperm release and sperm quality.

3.2. Materials and methods

3.2.1 Design of experiments

Sexually mature male winter flounder were caught in April (for Expt. I and II) or November (for Expt. III) in 1994, then held in a fibre glass tank (2 x 2 x 1.2 m) for at least one week prior to hormonal treatments.

3.2.1.1. Experiment 1

In the first experiment, males were studied throughout their summer spawning season, from April until spermiation ceased in all individuals in August, to investigate seasonal sperm release patterns and the influence of GnRH-A treatment on sperm production and sperm quality. At the beginning of the experiment, six males were killed and the status of testes development determined. On April 20, two groups of males [mean ± s.e. body weight (BW) = 0.455 ± 0.02 kg and 0.392 ± 0.03 kg respectively] were implanted with either a blank cholesterol pellet (control, N=6) or a single hormone pellet (N=5) with each hormone pellet containing 110 µg of GnRH-A (see Chapter 2 for GnRH-A pellet preparation). The fish were weighed, individually identified with a colored floy tag, and placed in a 250-l tank without females.

Presence of milt in control or GnRH-A treated males from each above group was checked weekly for the first 8 wk, then biweekly for the rest of the experiment. Once detectable, as much of the milt as possible was removed from the sperm duct. Upon termination of milt production or soon after death, fish were dissected and body and testis weights were recorded.

Blood samples were obtained using heparinized syringes from all control and GnRH-A treated males before the start of treatment, weekly after the hormone implantation for the first two weeks, then biweekly or monthly for the rest of the experiment. The blood was centrifuged at 9000 x g (5 min, 4 °C) and the plasma withdrawn for storage at -30 °C for steroid analysis.

3.2.1.2. Experiment 2

On May 30, 1994, the pituitary of 9 males (BW=0.450 ±0.06 kg) was surgically removed (see Chapter 2 for hypophysectomy procedure). In sham-control fish (N=8, BW=0.371±0.03 kg), the pituitary was exposed but not removed after opening of the cranial cavity. Milt and blood samples were collected before and after pituitary surgery at weekly sampling intervals for 5 successive weeks. At the end of the experiment, the cranial cavity of fish was checked to confirm the removal of the pituitary from hypophysectomied fish and the body and testis weights were recorded.

3.2.1.3. Experiment 3

In a third experiment beginning November 30, 1994, males were subjected to either pituitary removal (hypophysectomy) or sham surgery. One week following the surgery, 12 hypophysectomized males (BW=0.420 ± 0.03 kg) were injected weekly with pituitary extract (one pituitary per fish, homogenized in 100 µl of 0.65% NaCl) while the two other groups of fish (N=14, BW = 0.421 ± 0.02 kg for hypophysectomy and N=8, BW = 0.427 ± 0.06 kg for sham-control, respectively) received only the 0.65% NaCl vehicle injection (100 µl per fish). Again, the spermiation response was monitored and blood samples were collected weekly before and after these treatments for a 9 week period (week 0: before hypophysectomy, week 1: before the pituitary injection).

3.2.2. Examination of milt and blood samples

Milt production, spermatocrit, and sperm motility were evaluated for all milt samples and the ability of spermatozoa to fertilize eggs was tested five times in the spawning season after GnRH-A treatment in Expt. 1 (May 18, 26, June 1, 8, 30) and three times after the pituitary surgery in Expt. 2 (June 4, 13 and 28) (see Chapter 2). Steroid levels (T and 11-KT) of blood samples were analyzed by radioimmuroassay (RIA) as outlined in the previous chapter (Chapter 2).

3.2.3. Statistical analysis

All data were analyzed by either a one-way or a two-way ANOVA, followed by the Duncan's multiple comparison ($P < 0.05$ or $P < 0.01$) using a SAS program (SAS 6.1). Total seasonal production of milt and sperm number (i.e. accumulative production during the whole period of each experiment) and mean values for other parameters (weekly production, spermatocrit, sperm motility, fertilization and hatching rates) among treatments were compared by t-test (Expt. 1 and 2) or a ANOVA analysis (Expt. 3).

3.3. Results

3.3.1. Male spawning season - Expt. 1

According to autopsy of a small group of males (N=6) on April 20, male flounder were mature (GSI = 10.1± 0.53 %) at the beginning of this experiment when more than 70% males (N=17) were determined to be spermiating. During the following four months, this study demonstrated that the spawning season for normal (control) male winter flounder was characterized by a progressive increase in milt and sperm production in May (a significant rise in milt production occurred in week 4, ml kg^{-1} BW wk^{-1} = 6.1 ± 1.8; t-test; t = 2.74, df = 8, $P < 0.05$) and the spermiation continued in large amount until mid-July in most males, lasting about 2 months (Fig 1A, B). Besides increases in sperm production, the male spawning season was also characterized by an increase in sperm motility values, particularly early in the

season (Fig 1D). Although the onset of the spawning season in May was briefly coupled with high plasma androgen levels, hormone levels quickly declined in June (Fig 2), despite high milt volume, spermatocrit, and thus sperm production values (Fig 1, A - C). By July, a notable drop in sperm motility (ANOVA; $f = 4.94$, $df = 11$, $P < 0.01$) was recorded correlating with the termination of the spawning season (Fig 1D).

3.3.2. GnRH-A treatment - Expt. 1

Advancement of the male spawning season was evident following treatment of males with sustained release GnRH-A pellets. This was indicated by the increased milt volume and sperm production values during the initial 5 weeks of this experiment (Fig 1A, B). Although hormone treatment advanced the seasonal release of sperm (insert in Fig 1B) and boosted total milt production (Table 1), GnRH-A did not significantly augment the total reproductive seasonal output of spermatozoa. In contrast, milt was diluted although no significant difference was found in GSI between GnRH-A and control fish at the end of spermiation.

Although some variations in sperm motility and egg fertility were observed (Figs 1D and 3), significant effects of GnRH-A on sperm quality (sperm motility, egg fertility or larvae hatching rate) were not observed (Tables 2 and 3).

3.3.3. Effects of hypophysectomy - Expt. 2

The effects of hypophysectomy during the male spawning season were examined beginning May 30, 1994. Although milt collection from males was possible for several weeks after hypophysectomy, removal of the pituitary resulted in significantly increased spermatocrit on week 2 and reduced weekly milt volume and sperm production values (Fig 4, Table 4) in association with suppressed steroid levels (Fig 5). Total milt but not sperm production was also decreased (Table 4).

Despite the lower sperm motility (wk 3) and lower larvae hatching rate (wk 2) observed at some sampling dates after pituitary removal (Fig 6), two way ANOVA analysis over a 5 week period indicated that there were no significant changes in sperm quality (Table 5 and 6). This suggests that preservation of sperm quality is neither dependent upon the pituitary nor associated with high levels of plasma androgens in the short term.

3.3.4. Effects of hypophysectomy and pituitary replacement therapy - Expt. 3

Sperm release, sperm quality, and the effect of hypophysectomy on males at an early stage of the reproductive season were studied in December and January. While milt could be expressed from just a small number of normal (sham) males in mid-December (wk 2), the proportion of spermiating males increased to 50% by mid-January (wk 6, Fig 7). Small volumes of milt [0.83±0.29 (sham) and 0.42 ±0.09 (hypex) ml kg^{-1} BW wk^{-1}] and low steroid hormone levels in plasma (Fig 8) characterized both normal males early in the season and the hypophysectomized

males for the duration of this experiment.

While hypophysectomy resulted in significantly reduced plasma T levels (wk 2 and 3, Fig 8A) and an increased spermatocrit (Table 7), by contrast, pituitary hormone replacement therapy (PRT) restored these values to control levels and significantly increased plasma titre of 11-KT (Fig 8A, B). In addition, PRT increased the proportion of spermiating males in January to 100% (Fig 7) and enhanced milt and sperm production without appearing to change sperm quality (sperm motility and swim time) (Table 7 and Fig 9).

3.4. Discussion

3.4.1. Spawning season

According to Harmin et al. (1995a), the annual reproductive cycle of the male winter flounder is composed of several progressive stages, including (1) testes regression following summer spawning, (2) rapid testes recrudescence during the autumn, (3) testes maintenance during the winter, (4) early onset of spermiation in some prespawning males, and finally (5) maximal sperm production during the summer spawning season. These investigations also demonstrated histologically that the testes are filled with spermatids and mature spermatozoa by December. In the present study, it was confirmed that some males are ripe as early as December. Close monitoring indicated that more than 50% of the males started releasing milt by January and continued to produce small volumes of milt until late April.

Thereafter, milt production increased in May when ovulation occurred in wild females from the same site, marking the onset of the spawning season in females. Thus, it can be concluded that male winter flounder mature as early as December and produce milt at a low level for several months. The "true" summer spawning season begins in May, matching the spawning season of female flounder.

3.4.2. Hormonal regulation of spermiation and sperm quality

Onset of the summer spawning season in male winter flounder was found to be correlated to high plasma androgen levels, confirming previous observations (Harmin *et al.*, 1995a). Interestingly, a detailed study of the pattern of sperm release in winter flounder from April through July showed that plasma androgen levels actually declined markedly when milt volume and sperm production were highest well before the termination of the spawning season. Similar changes in the hormonal profiles have been described during spermiation in Atlantic halibut, *Hippoglossus hippoglossus* (Methven *et al.*, 1992) and several other teleosts (Baynes and Scott, 1985; Ouchi *et al.*, 1988; Rosenblum *et al.*, 1987), although there are some species (or strains) where high T and/or 11-KT levels persist during most of the spermiation period (Carragher and Pankhurst, 1993; Fostier *et al.*, 1983). This raises questions about the effect of steroid hormone levels on milt volume, sperm production, and sperm quality throughout the spawning season. In the current study, effects of sex hormones on male sperm release were addressed

by 1) treating male winter flounder with GnRH-A to stimulate the reproductive system, 2) removing the pituitary from mature males to investigate suppressive effects on the male reproductive system, and 3) testing the effects of PRT in hypophysectomized males to determine their potential for recovery of spermiation and sperm production.

It was previously demonstrated in male winter flounder that seasonal sperm release can be accelerated by treatment with hypothalamic hormones analogue (Harmin and Crim, 1993; Harmin *et al.*, 1995b). Despite the greater milt volume in GnRH-A implanted males in this study, the hormone treatment does not significantly augment total seasonal output of sperm cells over the spawning season. Rather, there was increased hydration of the milt, and advancement of sperm release which elevated sperm production briefly, but resulted in earlier termination of the spawning season in most GnRH treated males (4-10 vs 8-16 wks in control group). Hormone-stimulated milt hydration (Clemens and Grant, 1965; Garcia, 1991) and a brief elevation of sperm production (Garcia, 1993) were also observed in some other species. Spermatogenesis is highly synchronous in the winter flounder and completed by December well before onset of the spawning season (Harmin *et al.*, 1995a). Hormonal treatments, therefore, seem unlikely to induce new sperm development during the spawning season, which may explain why total annual spermatozoa production cannot be substantially increased. Thus, it appears that hormone enhanced seasonal milt production in this species largely results from

increased dilution of the milt. The elevated production perhaps is also partially from better recovery of sperm due to hormone induced advancement in the timing of sperm release, avoiding phagocytosis of spermatozoa which was observed within the reproductive tract of this species during sperm storage *in vivo* (Chapter 6).

Hypophysectomy of spermiating males quickly reduced plasma testosterone levels, raised the spermatocrit, and suppressed milt production. Thinning of the milt was restored in hypophysectomized males after injections of pituitary extract which also elevated T and 11-KT and stimulated most males to produce milt in increasing amounts. These results suggest that thinning of the milt and the increased milt production are pituitary dependent, likely mediated by T and/or 11-KT. In contrast to goldfish (Billard, 1986), however, spermiation continued in hypophysectomied male flounder, despite falling plasma androgen levels, suggesting that initial sperm release may be pituitary independent.

The effects of hormone changes on sperm quality in male winter flounder was examined in this study by *in vitro* egg insemination and a serial examination of sperm motility. Despite changes in milt hydration and steroid hormone secretions, no significant differences in either sperm motility or egg fertility were detected until near the end of the season, suggesting that sperm quality was unaffected throughout the spawning season. Similarly, in carp, neither daily nor weekly injections of carp pituitary extract produced a measurable impact on sperm fertility (Saad and Billard, 1987). GtH is thought to influence the motility of trout sperm,

however, by mediating epithelial activities in sperm duct (Marshall *et al.*, 1989; Marshall *et al.*, 1993). Spermatozoa of winter flounder are able to acquire the potential for movement in the testes (unpubl. observation), which is strikingly different from some salmonids where acquisition of sperm motility only occurs during their passage through the sperm duct (Miura *et al.*, 1992). It is likely, therefore, that winter flounder sperm will be mediated in a different way because of differences in the final maturation of sperm.

In summary, it seems that although onset of spermiation may be independent of roles of the hormones examined, GnRH and pituitary hormones appear capable of inducing vigorous sperm release, and milt hydration or secretion of the sperm duct epithelium. The effects of pituitary hormones on sperm release may be mediated by androgens. However, direct effect of androgens on spermiation remains to be further verified, because a large increase in milt volume was not observed immediately following significant increases in PRT-induced 11-KT levels, but occurring 3-4 weeks later (Expt. 3), suggesting that other factors may also be involved in the regulation of the spawning process.

Steroids other than androgens reportedly increase in spermiating fish suggesting their roles in regulating spermiation. For example, 17α, 20β dihydroxy-4-pregnen 3-one (17α, 20β-P) increased during spermiation in snapper, *Pagrus auratus* (Carragher and Pankhurst, 1993) while maximal 17α, 20β-P levels coincided with peak sperm release in rainbow trout, *Salmo gairdneri* (Baynes and

Scott, 1985; Scott and Sumpter, 1989). The role of 17α, 20β-P in mediating sperm release was also demonstrated by *in vivo* treatments (Billard, 1986; Pankhurst, 1994). Moreover, this hormone may also be involved in the regulation of sperm quality, as suggested by fact that the hormone increases sperm motility in salmon (Miura *et al.*, 1992) and goldfish (Defraipont and Sorensen, 1993). This steroid has not been detected in the plasma of spermiating male flounder (Crim unpubl.), or in the plasma of other species, such as blue cod, *Parapercis colias* (Pankhurst and Kime, 1991). The conjugated hormone in blue cod was measurable in its glucuronadated form and increased in spermiating males. Further research is required to ascertain whether this form of hormone is produced and plays a role in regulating sperm release and sperm quality in winter flounder and certain other teleosts.

Table 1 Comparative values for duration of spermiation, terminal GSI, total seasonal milt and sperm production, and spermatocrit in control and GnRH-A treated male winter flounder (Expt. 1).

Treatment	N	Spermiation duration (wk)	Terminal GSI (%)	Total milt volume (ml kg^{-1} BW)	Total sperm production (10^{14} kg^{-1} BW)	Mean spermatocrit (%)
Control	6	11.0±1.3	3.7±1.4	48.6±8.5	3.22±0.64	65.1±3.0
GnRH-A	5	8.4±1.2	2.4±1.1	107.7±21.6 *	4.26±0.81	45.0±2.1 **

Values are mean±s.e.

* $P < 0.05$ (t-test; $t = 2.74$, $df = 9$)

** $P < 0.01$ ($t = 5.2$, $df = 98$)

Table 2 Two way ANOVA analysis for sperm motility, egg fertilization and hatching rates for milt samples of male winter flounder in Experiment 1.

Source of variation	Probability > F (f, df)		
	Motility	Fertilization rate	Hatching rate
Treatment	0.49 (0.33,1)	0.75 (0.1,1)	0.74 (0.11, 1)
Season	0.01(4.58, 12)	0.91(0.25, 4)	0.64 (0.63, 4)
Treat*Season	0.02 (2.46,9)	0.06 (2.44, 4)	0.11 (1.81, 4)

Table 3 Comparative values for sperm motility, and egg fertilization and hatching rates in control and GnRH-A implanted male winter flounder (Expt.1).

Treatment	Motility [a] (%)	Fertilization rate [b] (%)	Hatching rate [b] (%)
Control	38.1±2.9	60.0±4.4	82.5±4.0
GnRH	41.0±2.8	60.0±6.4	82.9±4.7

Values are mean±s.e.

[a] Mean motility values from all samples (N=101) covering whole sampling period

[b] Mean fertilization and hatching rates based on five trials conducted during spawning season

Table 4 Comparative values for mean and total milt volume, mean and total sperm production, and spermatocrit between control (sham) and hypophysectomized (hypex) male winter flounder during the spawning season (Expt. 2).

Treatment	N	Mean milt vol ($ml\ kg^{-1}\ BW\ wk^{-1}$)	Total milt vol ($ml\ kg^{-1}\ BW$)	Mean sperm production ($10^{12}\ kg^{-1}\ BW\ wk^{-1}$)	Total sperm production ($10^{13}\ kg^{-1}\ BW$)	Mean spermatocrit (%)
Sham	8	11.2±1.2	63.2±11.0	6.95±0.78	3.91±0.72	68.4±2.7
Hypex	9	6.8±0.65 **	35.7±4.2 *	4.39±0.45 **	2.30±0.35	76.7±3.2

Values are mean±s. e.

* $P < 0.05$(t-test; $t = 2.42$, $df = 15$)

** $P < 0.01$($t = 3.19$, $df = 90$ for mean milt vol; $t = 2.84$, $df = 90$ for mean sperm production)

Table 5 Two way ANOVA analysis for sperm motility, egg fertilization and hatching rates for milt samples of male winter flounder in Experiment 2.

Source of variation	Probability > F (f, df)		
	Motility	Fertilization rate	Hatching rate
Treatment	0.29 (1.11, 1)	0.11 (2.62, 1)	0.86 (0.03, 1)
Season	0.12 (1.91, 4)	0.06 (2.90, 2)	0.02 (4.08, 2)
Treat*Season	0.13 (1.86, 4)	0.21 (1.65, 2)	0.25 (1.44, 2)

Table 6 Comparative values for sperm motility, egg fertilization, and hatching rates between control (sham) and hypophysectomized (hypex) male winter flounder (Expt. 2).

Treatment	Motility [a] (%)	Fertilization rate [b] (%)	Hatching rate [b] (%)
Sham	33.3±3.2	54.1±5.9	75.9±5.2
Hypex	37.1±3.0	66.8±5.1	76.0±4.9

Values are mean±s.e.

[a] Mean motility from milt samples (N=75) within week 1-5

[b] Mean fertilization and hatching rates based on three trials post-operation

Table 7 Comparative values for weekly milt volume, sperm production, mean spermatocrit, and sperm quality (motility and swim duration) in control (sham) vs hypophysectomized (hypex), and hypophysectomized individuals with pituitary replacement therapy (hypex+PRT) male winter flounder at an early phase of spermiation (Expt. 3).

Treatment	Milt vol (ml kg^{-1} BW wk^{-1})	Sperm production (10^{11} kg^{-1} BW wk^{-1})	Spermatocrit (%)	Motility (%)	Swim duration (sec)
Sham	0.83±0.29^{b}	5.14±0.32^{b}	45.2±5.8^{b}	28.7±4.5	41.1±0.8
Hypex	0.42±0.09^{b}	3.14±0.91^{b}	65.1±7.4^{a}	22.7±4.4	35.8±2.9
Hypex+PRT	4.29±.89^{a}	24.1±5.31^{a}	48.7±3.3^{b}	36.0±4.0	39.2±0.8

Values are mean±s.e. Significance level ($P < 0.05$) within different groups indicated by different letters (ANOVA; $f = 6,45$, $df = 2$, $P < 0.01$ for milt vol; $f = 5.66$, $df = 2$, $P < 0.01$ for sperm production, $f = 3.46$, $df = 2$, $P < 0.05$ for spermatocrit), an values were based on all samples (N=68) during the whole experimental period.

Fig 1 Changes in A) milt volume, B) sperm number, C) spermatocrit, and D) sperm motility during the spawning season in control males (N=6 for weeks 0 - 8, 4 for week 10, 3 for weeks 12 and 14, and 2 for week 16) and males treated with GnRH-A (N=5 for weeks 0 - 4, 4 for weeks 5 - 8 and 3 for week 10) (Expt.1). Significant differences between treatment groups indicated as: ** $P < 0.01$ or * $P < 0.05$ (t-test; milt volume: $t = 3.32$, 4.21, 5.51 and 4.36 for week 2 - 5 respectively, $df = 9$ for week 2 - 4 and 8 for week 5; sperm number: $t = 3.73$, 4.49 and 4.57 for weeks 3 - 5 respectively, $df = 9$ for weeks 3 and 4, 8 for week 5; spermatocrit: $t = 4.43$, 5.55, 4.31 and 6.61 for week 6 - 10 respectively, $df = 9$ for weeks 6 - 8, and 4 for week 10; motility: $t = 1.69$ and 1.30, $df = 9$ and 4 for week 2 and 6 respectively). Differences within the control group compared to the data before the treatment (week 0) are shown by # (t-test; $t = 2.74$, $df = 8$, $P < 0.05$ for milt volume and $t = 4.94$ and 5,12, $df = 8$ for motility). ASP is accumulated sperm production (%) (insert, Fig B). Values are mean±s.e. (vertical bar).

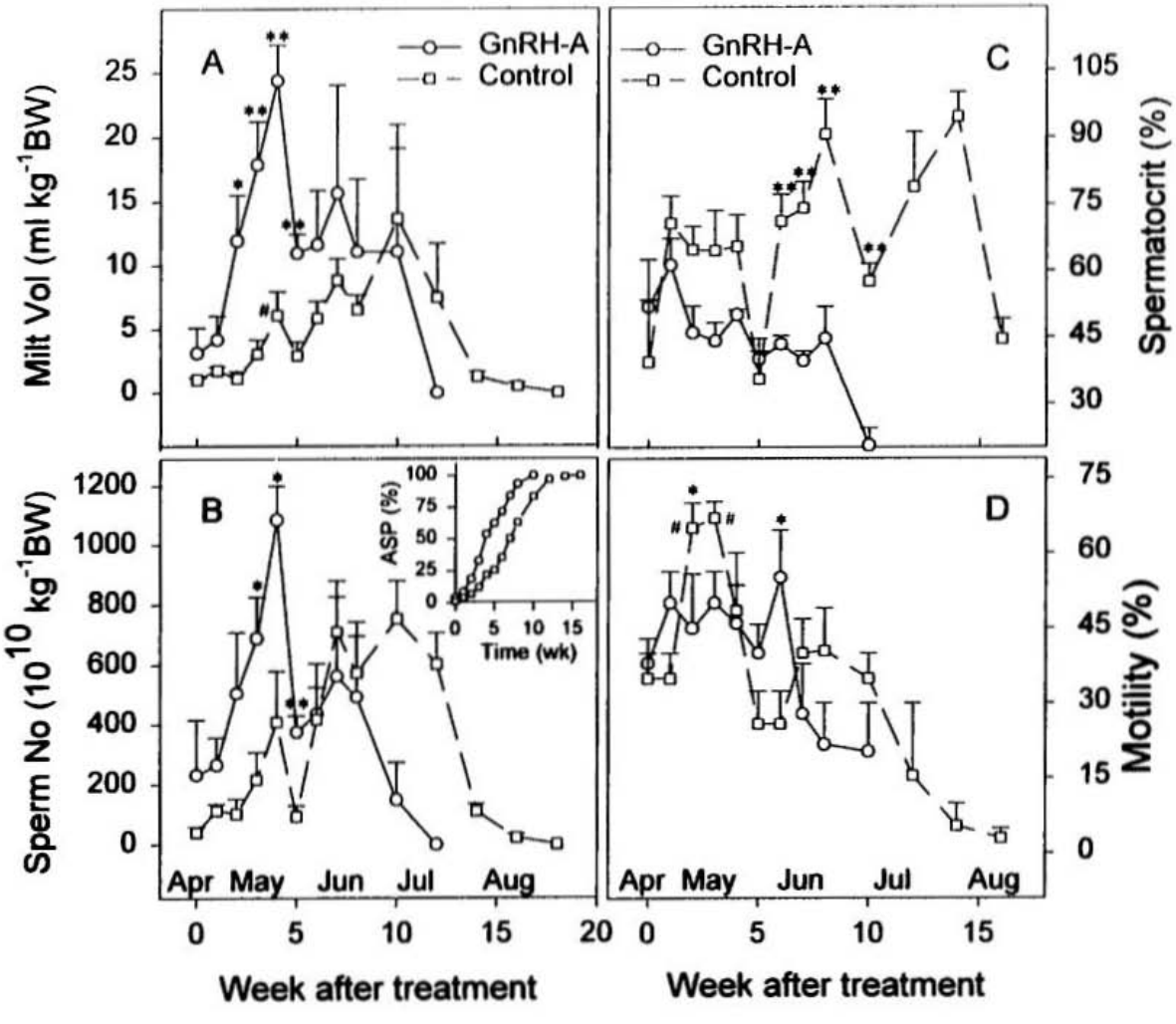

A
B
C
D
GnRH-A
Control
Milt Vol (ml kg-1BW)
Sperm No (1010 kg-1BW)
Spermatocrit (%)
Motility (%)
ASP (%)
Time (wk)
Apr
May
Jun
Jul
Aug
Week after treatment
Week after treatment

Fig 2 Plasma profiles of A) testosterone (T) and B) 11 ketotestosterone (11-KT) in control (N=6 for weeks 1 - 8, N=3 for week 14) and GnRH-A treated male winter flounder (N = 5 for weeks 0 - 4, 4 for week 8) during the spawning season (Expt.1). Differences between two groups at same sampling date: ** $P < 0.01$, * $P < 0.05$ (t-test: $t = 3.96$, $d = 9$ for testosterone and $t = 5.21$, $df = 9$ for 11 ketotestosterone, week 4). Significant differences over the season within each group indicated by different letters (GnRH-A: $f = 4.38$, $df = 4$ for T and $f = 5.89$, $df = 4$ for 11 KT; Control: $f = 10.7$, $df = 5$ for T and $f = 6.18$, $df = 5$ for 11 KT). Values are mean±s.e.(vertical bar).

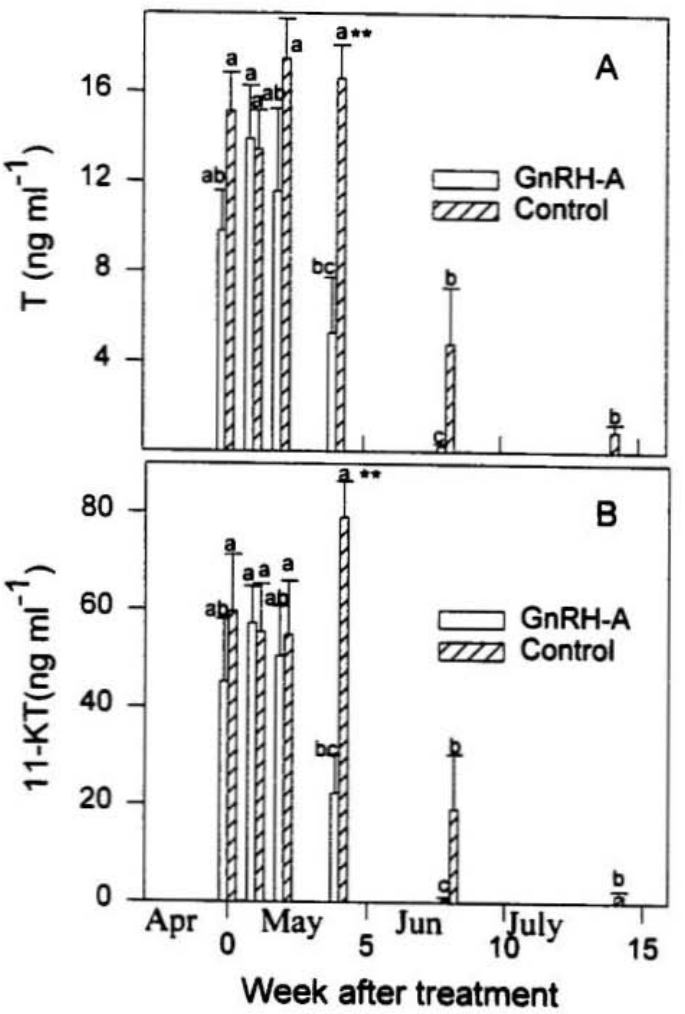

A
B
GnRH-A
Control
T (ng ml-1)
11-KT(ng ml-1)
16
12
8
4
80
60
40
20
0
Apr
May
Jun
July
0
5
10
15
Week after treatment

Fig 3 A) Egg fertilization and B) hatching rates during the spawning season in control males (N=6 for weeks 0 - 8 and 4 for week 10) and males treated with GnRH-A (N=5 for weeks 0 - 4, 4 for weeks 5 - 8 and 3 for week 10) (Expt. 1). Significant differences ($P < 0.05$) between treatment groups indicated as: * (t-test; $t = 3.9$, $df = 8$ for week 5 and $t = 3.47$, $df = 8$ for week 7). Values are mean ± s.e. (vertical bar).

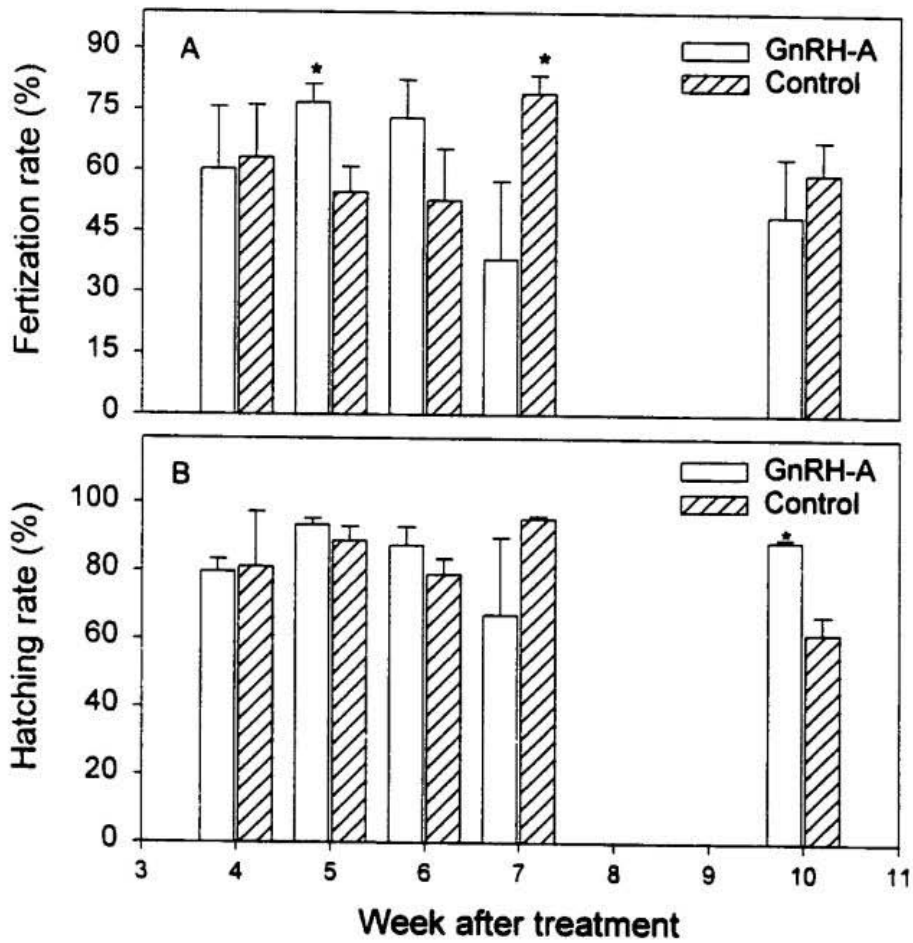

A
B
GnRH-A
Control
Fertization rate (%)
Hatching rate (%)
90
75
60
45
30
15
0
100
80
60
40
20
0
3
4
5
6
7
8
9
10
11
Week after treatment

Fig 4 Changes in **A)** milt and **B)** sperm production, and **C)** spermatocrit values in control (sham) and hypophysectomied (hypex) male winter flounder during the spawning season (Expt.2). Differences between treatment groups of males indicated as: ** $P < 0.01$ or *$P < 0.05$ (t-test; milt volume: $t = 2.34$ and 2.75, $df = 15$ for weeks 2 and 3 and $t = 2.46$, $df = 13$ for week 4; sperm number: $t = 2.62$, $df = 15$ for week 3; spermatocrit: $t = 4.96$, $df = 15$ for week 2). N=9 for weeks 0 - 3, 7 for week 4 and 4 for week 5 in hypex males; N=8 for weeks 0 - 4 and 5 for week 5 in sham males (Note: Number of fish decreased due to mortalities during the experiment).Values are mean±s.e. (vertical bar).

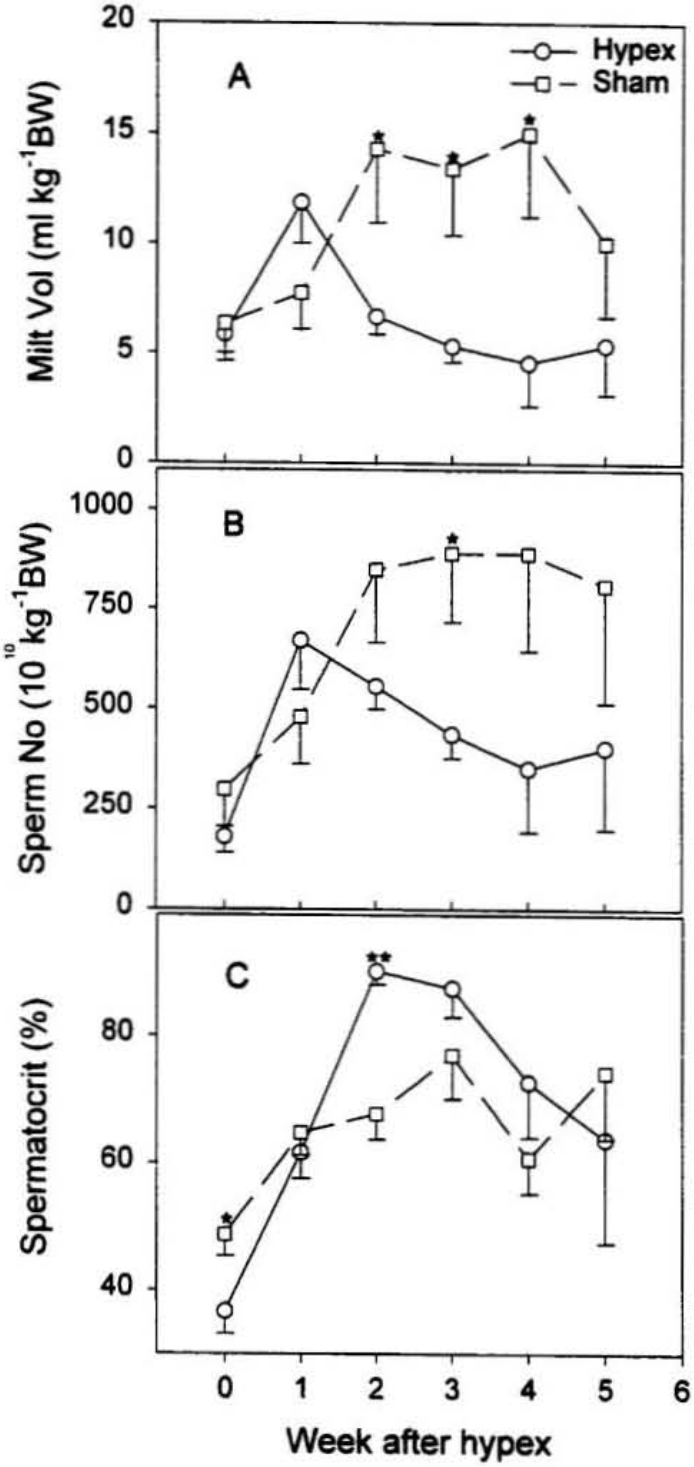

A
B
C
Hypex
Sham
Milt Vol (ml kg-1BW)
Sperm No (10^10 kg-1BW)
Spermatocrit (%)
Week after hypex
0
5
10
15
20
250
500
750
1000
40
60
80
1
2
3
4
6
*
**

Fig 5 Plasma profiles of A) testosterone (T) and B)11 ketotestosterone (11 KT) in control (sham) and hypophysectomized (hypex) male winter flounder during spawning season (Expt.2). Significant differences ($P < 0.05$) between two groups of males indicated by * (t-test; $t = 3.18$, $df = 15$ and $t = 3.05$, $df = 15$ for testosterone and 11 ketotestosterone respectively).N=9 for weeks 0 - 3, 7 for week 4 and 4 for week 5 in hypex males; N=8 for weeks 0 - 4 and 5 for week 5 in sham males (Note: Number of fish decreased due to mortalities during the experiment). Values are mean±s.e. (vertical bar).

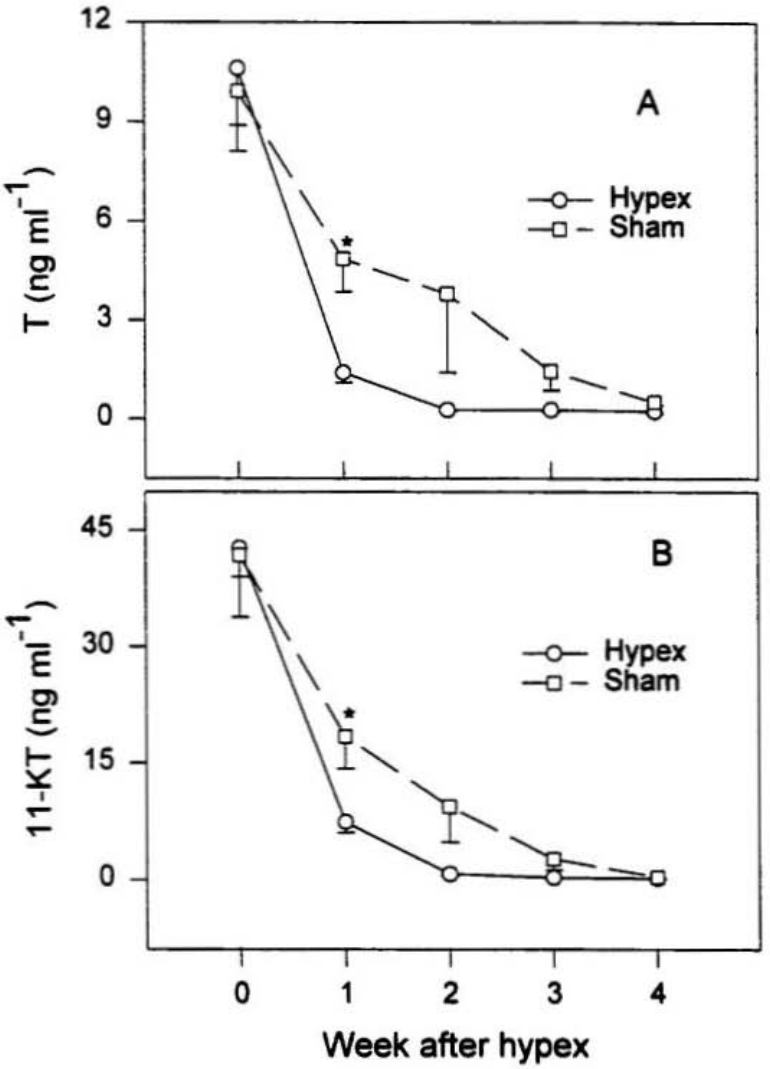

12
9
6
3
0
T (ng ml-1)
A
Hypex
Sham
*
45
30
15
0
11-KT (ng ml-1)
B
Hypex
Sham
*
0
1
2
3
4
Week after hypex

Fig 6 **A)** Sperm motility, **B)** Egg fertilization, and **C)** hatching rates for milt collected during the spawning season from control (sham) males (N=8 for weeks 0 - 4 and 5 for week 5) and hypophysectomized (hypex) males (N=9 for weeks 0 - 3, 7 for week 4 and 4 for week 5) (Exp.2). Significant differences ($P < 0.05$) between treatment groups indicated as: *(t-test; motility: $t = 2.18$, $df = 15$ for week 3; hatching rate: $t = 2.53$, $df = 15$ for week 2 (Note: Number of fish decreased due to mortalities during the experiment). Values are mean±s.e.(vertical bar).

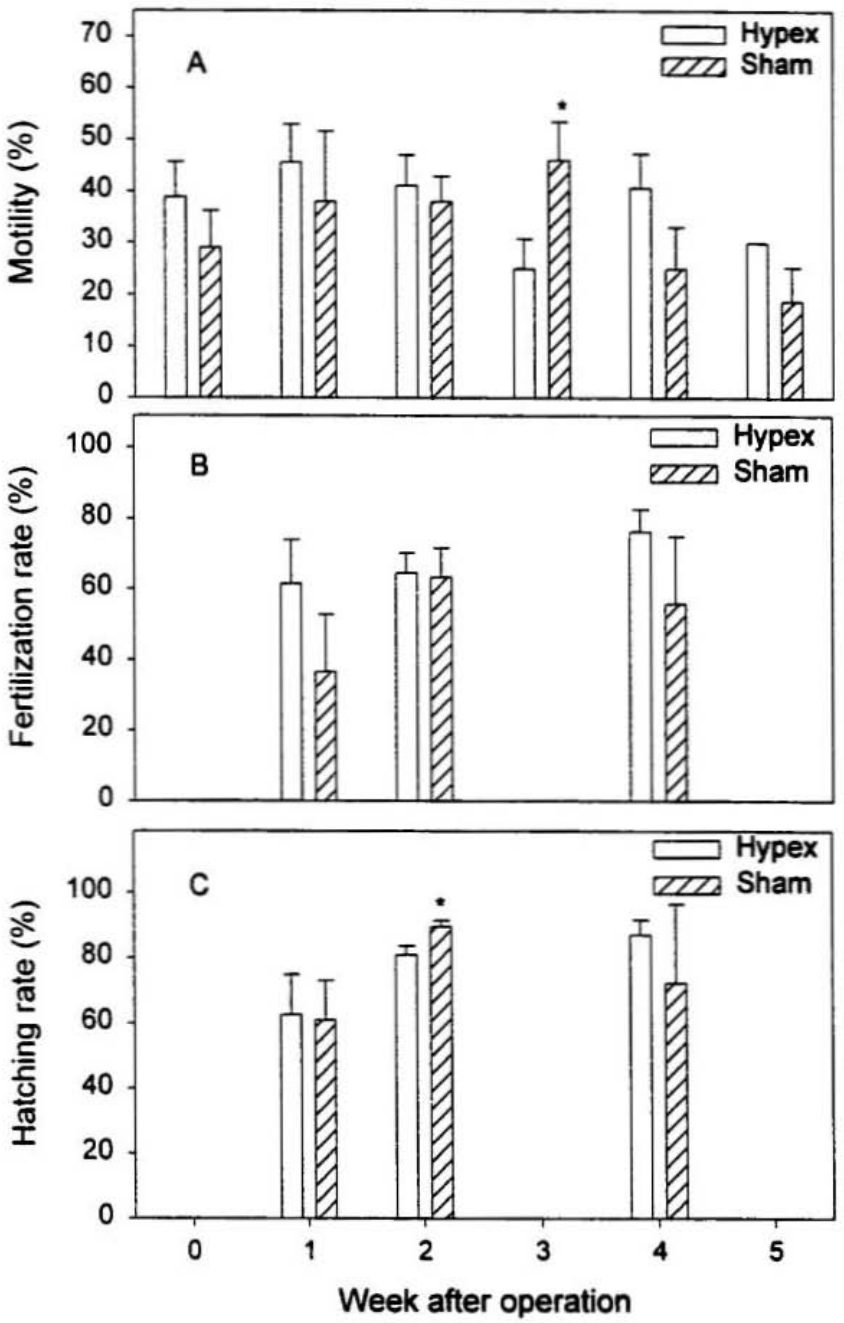

A
Hypex
Sham
Motility (%)
70
60
50
40
30
20
10
0
*
B
Hypex
Sham
Fertilization rate (%)
100
80
60
40
20
0
C
Hypex
Sham
Hatching rate (%)
100
80
60
40
20
0
*
0
1
2
3
4
5
Week after operation

Fig 7 The percentage of spermiating males in prespawning male winter flounder (Expt. 3).. Control (sham): N = 8 for weeks 0 - 3, 6 for weeks 4 - 6, 4 for weeks 7 - 9; hypophysectomized males with pituitary replacement therapy (hypex+PRT): N = 12 for weeks 0 - 2, 11 for week 3, 9 for week 4, 8 for weeks 5 - 6, and 6 for weeks 7 - 9; hypophysectomized males (hypex): N = 14 for weeks 0 - 3, 8 for weeks 4 - 6, and 6 for weeks 7 - 9 (Note: 2 males were sacrificed from each group at week 3 and 6 for histological examination of the sperm duct while some mortality occurred in hypex+PRT and hypex groups during this experiment).

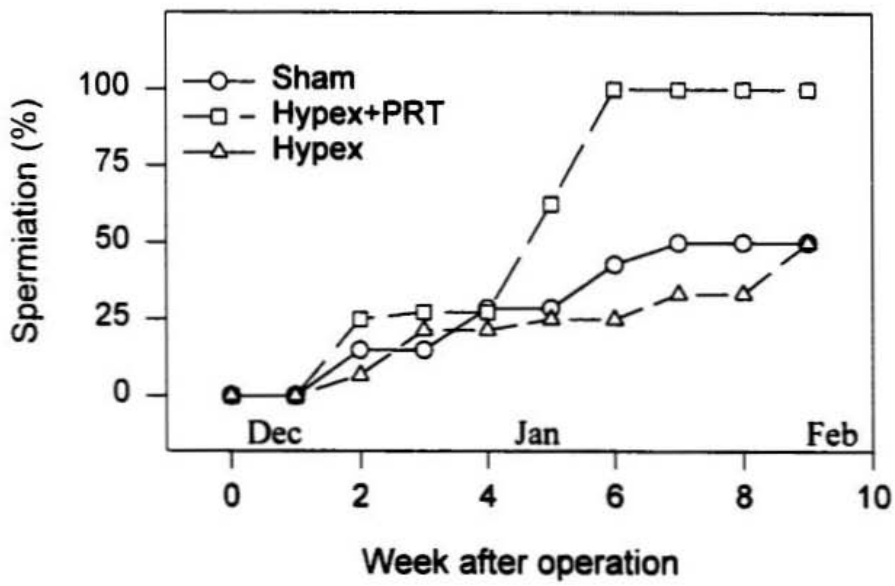

Sham
Hypex+PRT
Hypex
Spermiation (%)
100
75
50
25
0
Dec
Jan
Feb
0
2
4
6
8
10
Week after operation

Fig 8 Plasma profiles of **A)** testosterone (T) and **B)** 11 ketotestosterone (11 KT) in prespawning male winter flounder (Expt. 3). Significant differences ($P< 0.05$) among treatments at the same sampling time are indicated by different letters (ANOVA; testosterone: f = 3.56, 4.14, 4.11, 3.86, 3.52 and 3.37, df = 2 for weeks 1 - 6; 11 ketotestosterone: f = 3.78, 5.38, 6.23, 4.86, 6.61, 8.32, 7.56, 6.03 and 6.07, df = 2 for weeks 0 - 8). Control (sham): N = 8 for weeks 0 - 3, 6 for weeks 4 - 6, 4 for weeks 7 - 9; hypophysectomized males with pituitary replacement therapy (hypex+PRT): N = 12 for weeks 0 - 2, 11 for week 3, 9 for week 4, 8 for weeks 5 and 6, and 6 for weeks 7 - 9; hypophysectomized males (hypex): N = 14 for weeks 0 - 3, 8 for weeks 4 - 6 and 6 for weeks 7 - 9. (Note: 2 males were sacrificed from each group at week 3 and 6 for histological examination of the sperm duct while some mortality occurred in hypex+PRT and hypex groups during this experiment). Values are mean±s.e. (vertical bar).

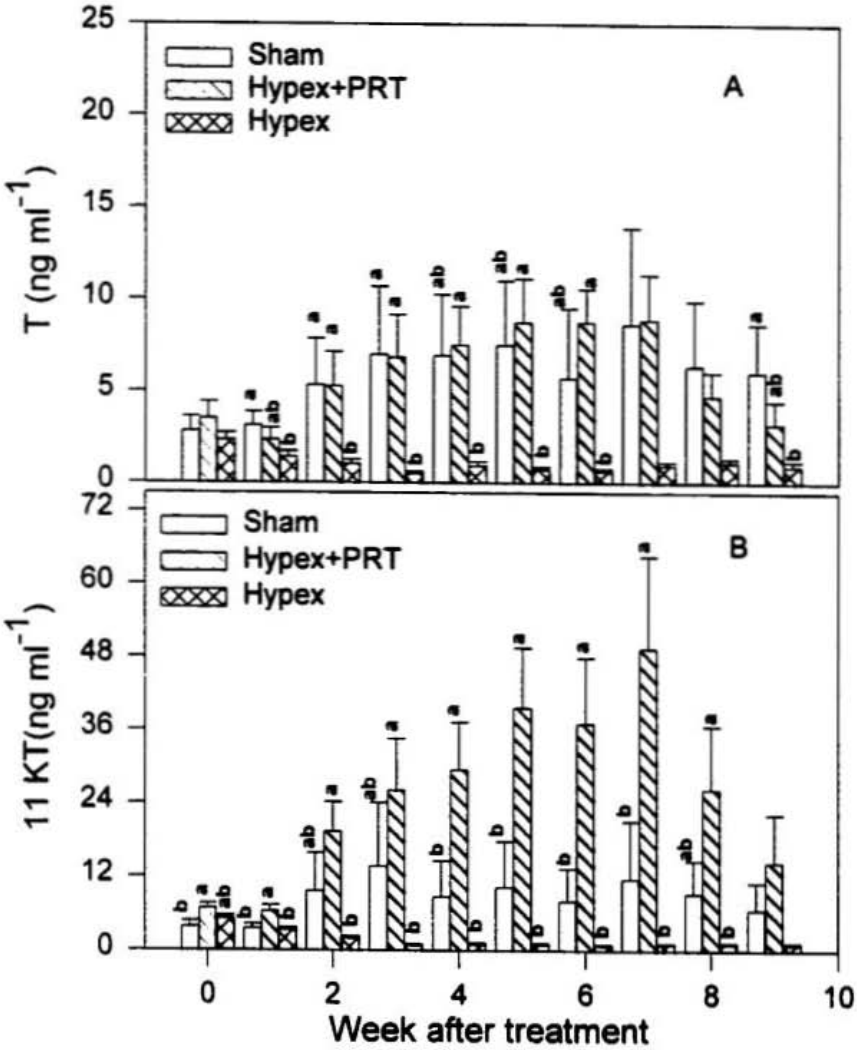
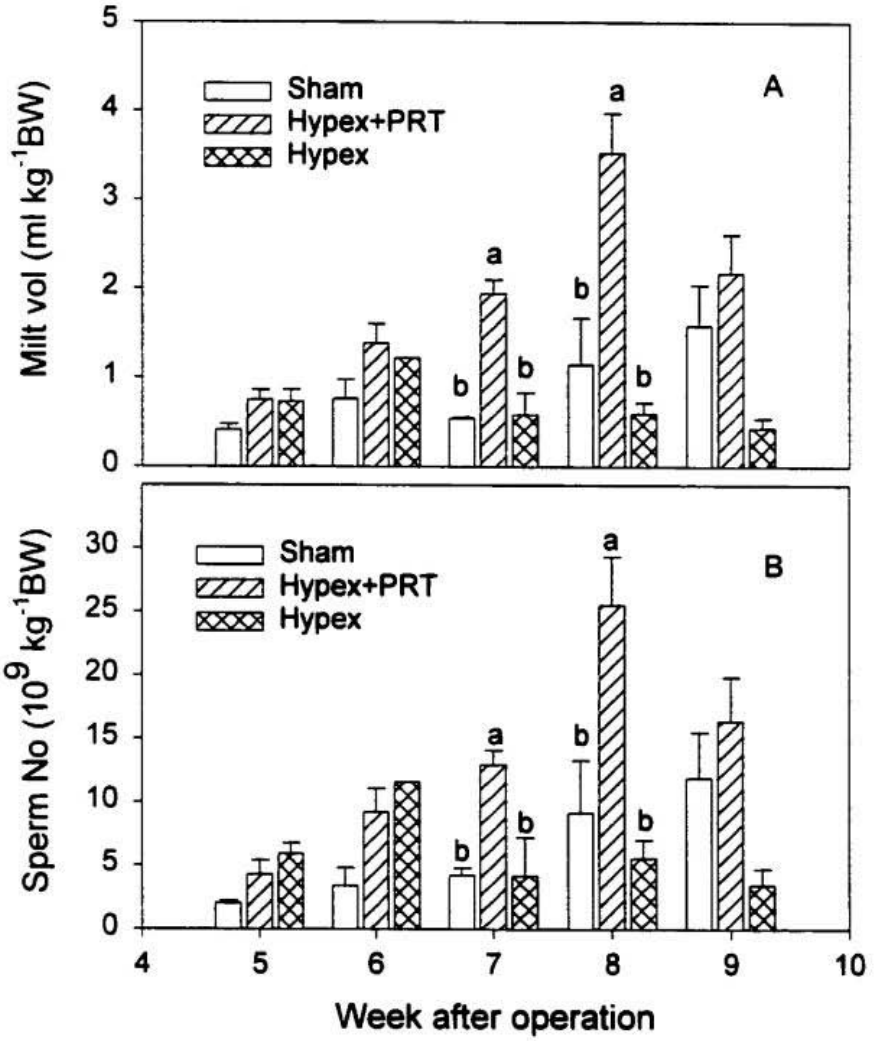

Sham
Hypex+PRT
Hypex
A
T (ng ml-1)
25
20
15
10
5
0
Sham
Hypex+PRT
Hypex
B
11 KT(ng ml-1)
72
60
48
36
24
12
0
0
2
4
6
8
10
Week after treatment

Fig 9 Changes in **A)** milt and **B)** sperm production of male winter flounder during early spermiation (Expt. 3). Significant differences ($P < 0.05$) among treatment groups indicated by different letters (ANOVA; milt volume: $f = 18.2$ and 9.05 for weeks 7 and 8 respectively ($df = 2$); sperm number: $f = 10.8$ and 6.01 for weeks 7 and 8 respectively ($df = 2$). Control (sham): N= 2 for weeks 5, 7, 8 and 9, 3 for week 6; hypophysectomized males with pituitary replacement therapy (hypex+PRT): N = 5 for week 5, 8 for weeks 5 - 6, 6 for weeks 7 - 9; hypophysectomized males (hypex): N =1 for week 6, 2 for weeks 5, 7 and 8, and 3 for week 9. Values are mean±s.e. (vertical bar).


Milt vol (ml kg-1BW)
Sham
Hypex+PRT
Hypex
A
a
b
Sperm No (10^9 kg-1BW)
B
Week after operation

CHAPTER 4

Ultrastructural Features of Spermatozoa in Winter Flounder (*Pleuronectes americanus*) and Atlantic Halibut (*Hippoglossus hippoglossus*)

4.1. Introduction

Although there are certain common features within neopterygii spermatozoa, such as the lack of an acrosome, diversity in this taxa is distinguishable at the level of spermatozoa morphology. While classification of certain fish groups is still considered to be tentative, or even problematic (Lauder and Liem, 1983), information regarding sperm structures is valuable in clarifying phylogenetic relationships among species (Mattei, 1991). However, despite increases in the number of species in which spermatozoa morphology has been studied, a large number of species remains uninvestigated. Besides phylogenetic implications, increased interest is also being devoted to relationships of sperm structures with distinctive functions and metabolic activities, such as species-specific modes of fertilization (Jamieson and Grier, 1993; Stoss, 1983). In this study, the ultrastructure of halibut and winter flounder spermatozoa is described, providing a basis for subsequent studies of variations in sperm quality during the reproductive season while adding new evidence for the evolution of fish.

4.2. Materials and Methods

Winter flounder sperm was sampled from freshly caught males (N=2-3) while halibut sperm was collected from three captive males during the spawning season. Sperm of both species were fixed and prepared for SEM and TEM observations as described in Chapter 2.

4.3. Results

Both winter flounder and halibut spermatozoa are composed of an anacrosomic and unmodified head, a short midpiece, and a long tail (Figs 10A, 11G, H). The length of whole spermatozoa measured about 38 μm for both species.

4.3.1. Head

The head of winter flounder spermatozoa is slightly elongated, only about 2.39 μm in length (including the midpiece) and 1.59 μm in diameter. The head is formed by a domed bullet-shaped nucleus with a deep caudal invagination (the nuclear fossa) for the accommodation of the centriolar apparatus (Fig 10E). Lined with double nuclear envelope, the nuclear fossa is constricted around the central region, dividing the fossa into anterior and posterior chambers, both of them in an orbicular shape (Fig 10E, F). Most of the nucleus appears uniformly electron dense, except for existence of a few electron translucent spots distributed irregularly.

The head of the halibut spermatozoa is almost spherical being about 2.18

μm in length and 2.0 μm in diameter (Fig 11H) and it also contains a caudal nucleus fossa. However, the posterior chamber of the fossa appears wider than in winter flounder on its posterior portion where the centriolar collar is located (Fig 11J).

4.3.2. Midpiece

The midpiece, located at the base of the head, is short and cylindrical, containing a single ring of 8 mitochondria in winter flounder spermatozoa and 9-10 in halibut spermatozoa (Fig 11L) which surrounds the anterior extremity of the axoneme. Mitochondria are spherical in both species with a large compartment containing granular matrix and a few tubular cristae. The cytoplasmic membrane folds into the midpiece at its posterior, forming a short cytoplasmic canal that separates the mitochondrial ring from the axoneme (Fig 10C, 11I).

4.3.3. Centriolar complex

The centriolar complex includes the proximal and distal centrioles, each composed of nine triplets. In winter flounder, the proximal centriole is housed within the smaller anterior fossa chamber, oblique to the longitudinal axis of the flagellum at about 80°. Micro-filaments protrude radially from the outermost microtubule of each triplet, some anchoring the centriole to the outer nuclear membrane lining on the inner surface of the fossa while others connect to an electronically dense ring surmounted on the nucleus-oriented tip of the distal centriole (Fig 10F). Triplets

comprising the distal centriole extend posteriorly, parallel to the longitudinal axis of the axoneme, together with an accessory centriolar collar occupying the larger posterior fossa chamber (Fig 10E, F). Surrounding the distal centriole on the posterior four fifths, the collar appears spherical in outline, constructed by an outer dense ring, 9 dense pericentriolar columns, and 9 bridges between the outer ring and the columns (Fig 10B, F). These bridges are slim at the anterior, progressively becoming wider on the posterior end of the centriole. While each of the inner columns attaches tightly to one of the triplets, the thickened outer ring lies intimately beneath the outer nuclear envelope, connecting with the nuclear envelope by microfilaments (Fig 10F). On the posterior end of the collar, the outer nuclear envelope slightly stretches centrally, holding the collar within the nuclear invagination (Fig 10F). The spherical collar that well fits to the orbicular posterior chamber of the fossa likely functions as a ball-joint in the nucleus-flagellum articulation, establishing a firm but flexible connection between the head and the tail.

The centrolar complex in halibut spermatozoa also consists of the proximal and distal centrioles, as well as a centriolar collar. This collar is similar to that observed in the winter flounder, comprising three elements: pericentriolar columns, bridges and an outer ring, except that the outer ring appears smaller, circumfusing only the posterior half of the distal centriole (Fig 11J, K, M).

4.3.4. Axoneme

The axoneme is derived from the distal centriole, passing through a short cytoplasmic canal that contains the mitochondria and running posteriorly (Fig 10C). Arrangement of microtubules in the axoneme is the typical 9 peripheral doublets plus 2 central tubules in both species (Figs 10D, 11L). Projections that link the peripherical doublets to the central tubules and to the cytoplasmic membrane are evident. The diameter of the tail of winter flounder spermatozoa is slightly reduced in the region immediately behind the head while the remainder appears thicker and extends bilaterally as a barely detectable membranous structure (Fig 10A, C). In contrast, the tail of halibut spermatozoa remains uniform and the membranous ridges are absent (Fig 11H). The end of the tail in both species is slightly pointed (Figs 10A, 11G).

4.4. Discussion

Like the spermatozoa of most neopterygian fishes, no acrosome was found in winter flounder or halibut spermatozoa. In general, these spermatozoa are primitive in form comprising an unmodified, spherical or ovoid head, a non-prominent midpiece containing a single ring of mitochondria and a long tail. In spite of the primitive structure, winter flounder and halibut spermatozoa possess a complex centriolar complex (basal body) within the nuclear fossa which appears unique to the Pleuronectidae species (Jones and Butler, 1988), although various

accessory structures have been found in conjunction with the centrioles (Lahnsteiner *et al.*, 1995; Poirier and Nicholson, 1982). The basal body is made of the distal centriole and an accessory collar consisting of pericentriolar columns, bridges and a outer ring. As seen in the winter flounder, 9 inner columns symmetrically attach to each of the triplets, physically reinforcing the distal centriole. Through these columns and their radially extended bridges, the triplets link to the peripherical outer ring, finally connecting to the outer nuclear envelope. The outer ring has a spherical outline, enabling it to fit intimately within circular posterior chamber of the nuclear fossa. Apparently, it is the basal body that anchors the flagellum in a firm and flexible manner, allowing violent vibration produced by the tail propulsion during sperm movement.

A barely detectable membranous structure was observed extending bilaterally along the length of the axoneme in winter flounder spermatozoa. In traverse section of the flagellum, it could not clearly be identified perhaps due to its extremely fragile nature, easily shrunk during EM preparation. The membranous extensions have not been reported elsewhere in other species of Pleuronectiforms (Jones and Butler, 1988; Mattei, 1991), including yellowtail flounder, *Pleuronectes ferrugineus* (unpubl. observation). On the other hand, similar axonemal extensions are present in other fish spermatozoa (Stoss, 1983), and have been found in exocoetoid and poecillid fish being well developed only in externally fertilizing species (Jamieson and Grier, 1993). The functions of the modification of axoneme

remain unclear although it is believed to improve the efficiency of flagellar propulsion (Stoss, 1983).

A symmetry at the levels of the head and the midpiece seems a common feature in spermatozoa within the order of Pleuronectiforms (Mattei, 1991). Furthermore spermatozoa within the family Pleuronectidae share greater similarities, particularly prominent among small flatfish. For example, both winter flounder and plaice, *Plaichthys flesus,* have a bullet-shaped nucleus while the larger sized halibut displays a spherical head. At the level of the midpiece, the number of mitochondria is 8 for both winter flounder and plaice, but 9-10 for halibut. The spermatozoa of yellowtail flounder, another smaller flounder, also contains a slightly elongated and ovoid nucleus with a slimmer anterior portion of flagellum resembling the tail of winter flounder spermatozoa (unpubl. observations). However, despite large size differences between halibut and other small flatfish, spermatozoa from all these species possess a similar centriolar complex, which all comprises the columns, bridges and a outer ring, including yellowtail flounder (unpubl. observations). Although no structures were observed in winter flounder and halibut spermatozoa that are equivalent to the mushroom-like projection and the central dense granule associated with the proximal centriole in plaice (Jones and Butler, 1988), it is probably due to differences in sectioning planes which were observed. Such great similarities in the spermatozoa morphology is consistent with other morphological, developmental and ecological information that suggests very close

phylogenetic relationships among these species.

Variations in sperm structure have been shown to be associated with sperm function. For example, undeveloped midpiece containing a few unmodified mitochondria is ordinarily associated with species exhibiting external fertilization (Stoss, 1983). This appears the case in winter flounder and halibut (the present study). In addition, mitochondria in winter flounder and halibut spermatozoa contain a few cristae within a large matrix compartment, corresponding to a low level of oxidative phosphorylation (Ross *et al.*, 1995). There is no evidence of intracellular storage of glycogen or lipid in either midpiece or tail regions, although the reserve or resorbtion of glycogen particles has been observed in brown trout, *Salmo trutta* (Baccetti *et al.*, 1975), catfish, *Ictalurus punctatus* (Poirier and Nicholson, 1982) and blenniid fish (Jamieson, 1995). The structural features of flatfish spermatozoa suggest a restricted capacity for intracellular regeneration of ATP, correspondent to brief sperm movement duration following activation.

Fig 10 Electron microscopic (EM) photograph of winter flounder spermatozoa.

A) Scanning EM of flounder spermatozoa showing the head (H) and tail (T) which is slightly slimmer on the region behind the head; laterally extended membranous structures on the tail are barely seen. (x 12,000)

B) Top view of the distal centriole (DC) with the centriolar collar in which dense columns (Cl) are linked to the outer ring (Or) by radially arranged bridges (arrow). N: nucleus, NM: nuclear membrane, M: mitochondria. (x 79,200)

C) The mitochondria (M) and axoneme (A) are separated by a canal (arrow) formed by the invagination of cytoplasm membrane on the central region of the midpiece. N: nucleus, PC and DC: proximal and distal centrioles. (x 32,250)

D) 9+2 microtubular arrangement of axoneme through a cross section on the flagellum. (x 59,400)

E) The head and the midpiece of spermatozoa. The proximal and distal centrioles (PC and DC) located in the nuclear fossa. The spherical centriolar collar (CC) surrounding DC. N: nucleus, M: Mitochondria. (x 41,571)

F) The centriolar complex. Filament-like material on proximal centriole (PC) attaching to surrounding nuclear membrane (NM) or to an electronically dense ring on the top of distal centriole (DC) (small arrow). The bridge (Br) connects the columns (Cl), which attach DC and the outer ring (Or). The outer NM forms loop on the edge of the entrance of the fossa (large arrow). (x 135,700)

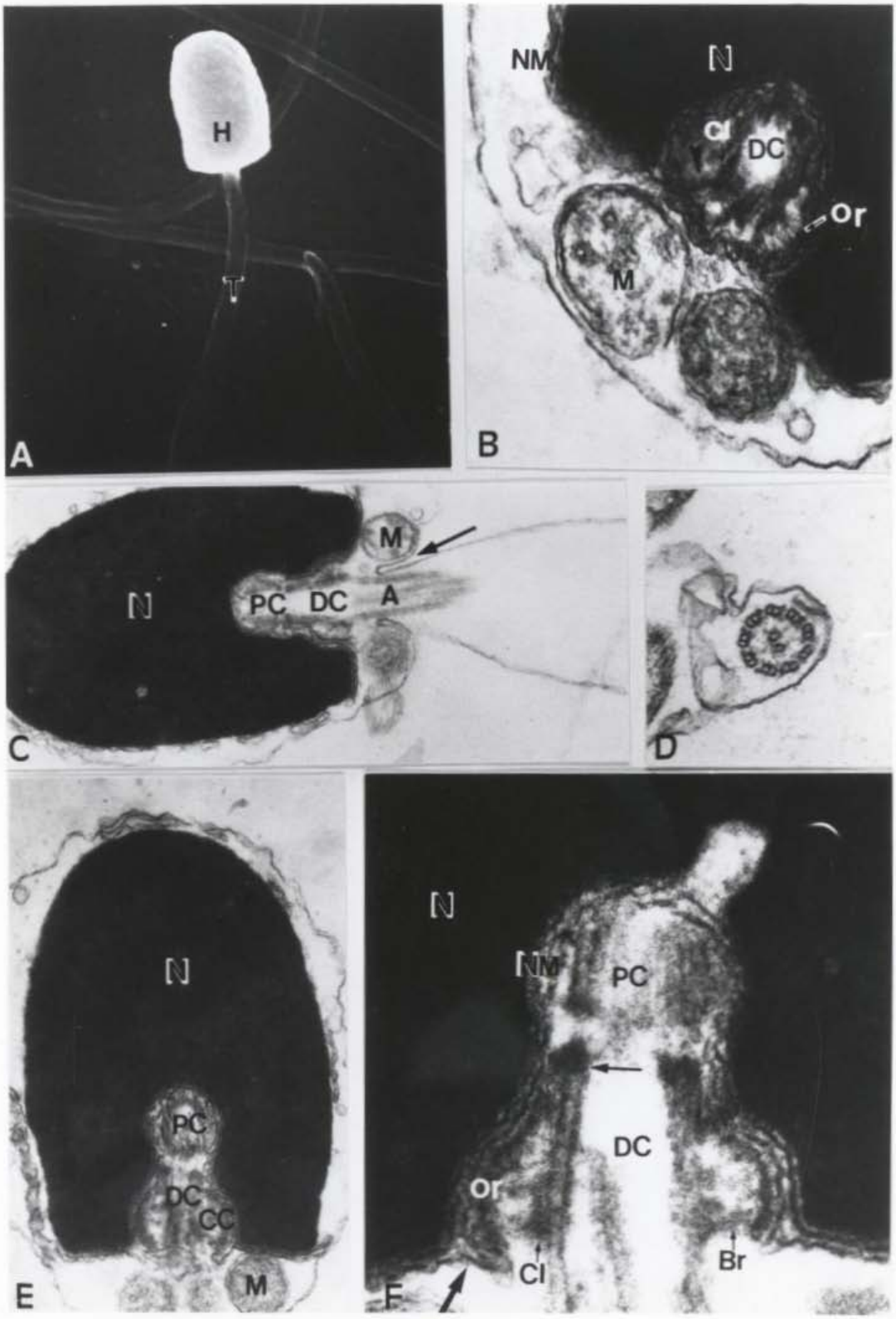

H
T
A
NM
N
Cl
DC
Or
M
B
M
N
PC
DC
A
C
D
N
PC
DC
CC
M
E
N
NM
PC
DC
Or
Cl
Br
F

Fig 11 EM Photograph of halibut spermatozoa.

G) SEM of whole halibut spermatozoa. (x 2,200)

H) Spermatozoa showing the head (H), midpiece (MP) and a part of tail. (x 15,000)

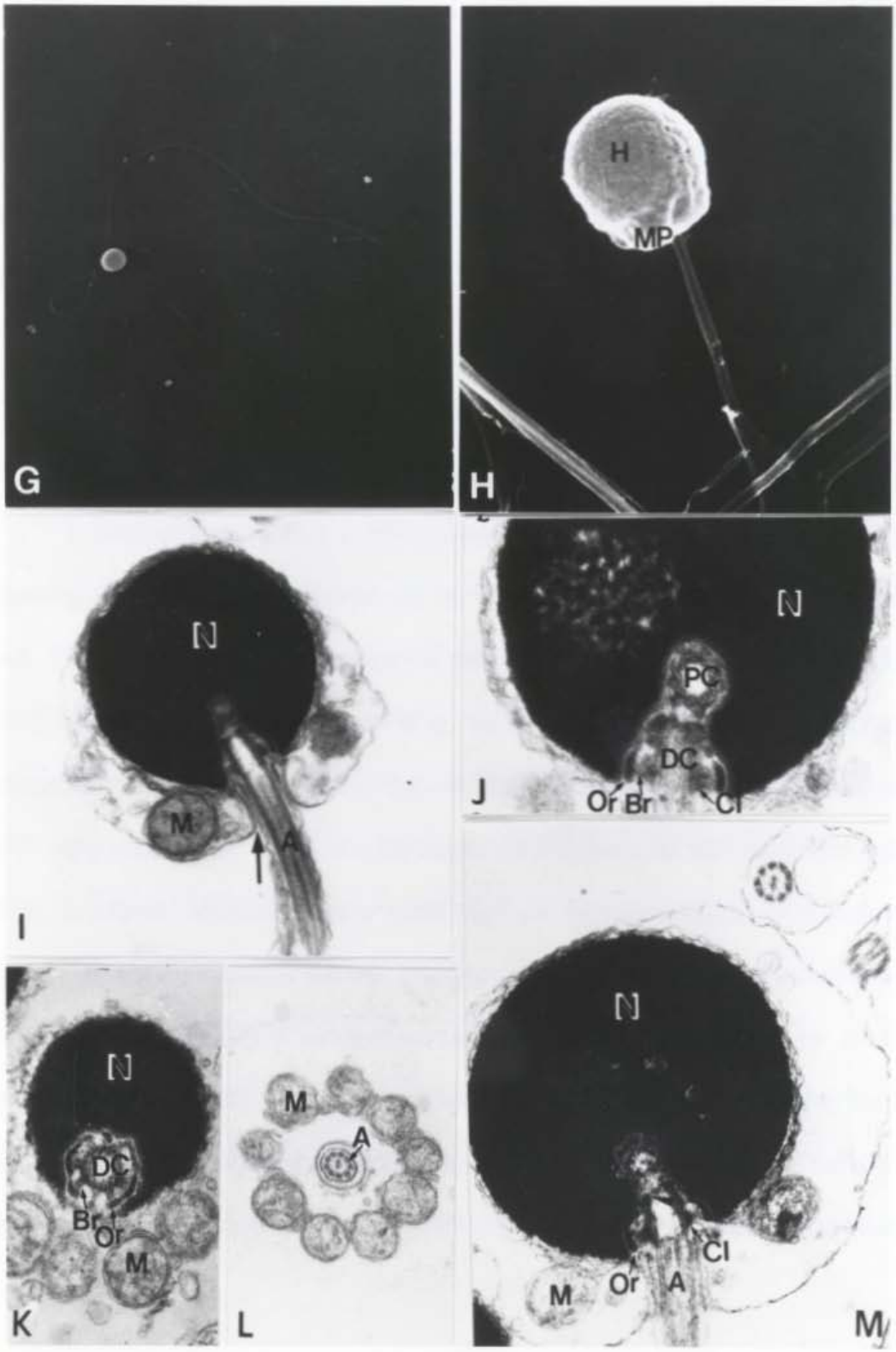
I) Spermatozoa with a spherical head, a midpiece and a part of tail, the axoneme (A) implants into the nuclear fossa through the cytoplasm canal (arrow). N: nucleus, M: Mitochondria. (x 21,500)

J) The proximal and distal centrioles (PC and DC) within the nuclear fossa, slanted to each other at approximately 80°; The DC attached by the centriolar collar which consist of the dense outer ring (Or), the bridges (Br) and dense columns (Cl). N: nucleus. (x 42,300)

K) Top views of the distal centriole (DC) which is surrounded by a centriolar collar in which radially arranged bridges (Br) linking the central triplets to an outer ring (Or) are clearly visible. N: nucleus, M: Mitochondria. (x 28,300)

L) Mitochondrial ring of 9, separated with the centralized axoneme (A) by double cytoplasmic membrane (x 20,000)

M) The axoneme (A) passing through the cytoplasm canal behind the distal centriole; the columns (Cl) of the centriolar collar are clearly visible while the out ring (Or) appears semispherical; a dense ring surmounted on the top of the distal centriole (triangular arrow). N: nucleus, M: Mitochondria. (x 237,600)


G
H
MP
H
I
N
M
A
J
N
PC
DC
Or
Br
Cl
K
N
DC
Br
Or
M
L
M
A
M
N
Cl
M
Or
A

CHAPTER 5

Seasonal Variations in Sperm Quality and Sperm Morphology in Wild and Captive Male Winter Flounder (*Pleuronectes americanus*) and Captive Atlantic Halibut (*Hippoglossus hippoglossus*)

5.1. Introduction

Seasonal fluctuations in milt characteristics or sperm quality during the spawning season have been previously reported in a few teleosts, such as rainbow trout, *Salmo gairdneri* (Buyukhatipoglu and Holtz, 1984; Munkittrick and Moccia, 1987), carp, *Cyprinus carpio* (Kruger *et al.*, 1984; Saad, 1988), tilapia, *Oreochromis mossambicus* (Kruger *et al.*, 1984), the sea bass, *Dicentrarchu labrax* (Billard *et al.*, 1977; Kara and Labed, 1994), and the sea bream, *Sparus aurata* (Kara and Labed, 1994). However, although many male teleosts produce sperm over extended periods, sometimes lasting several months beyond the female spawning season, to date detailed studies of seasonal changes in sperm quality are rare. In winter flounder, *Pleuronectes americanus*, previous observations during the summer spawning season (Chapter 3) demonstrated that sperm quality improves early in the summer but sperm motility abruptly declines towards the end of the reproductive

season. These observations also indicated that some male flounder mature early and produce sperm in December or January, several months prior to spawning in the females. Although it has been noted that motile sperm can be collected in the early phase of spermiation in this species, the question of seasonal changes in sperm quality needs further study. In the Atlantic halibut, *Hippoglossus hippoglossus*, seasonal variations in sperm quality have been demonstrated, showing that sperm quality is higher in winter but declines in late spring during the 4-5 months of milt production (Crim et al, unpubl). However, reasons for the decline in sperm quality remain to be further investigated.

In the present study, milt characteristics and sperm quality of winter flounder were compared throughout a lengthy spermiation period (January to July or August) in collections made from freshly-caught wild males or males that had been held in captivity for one to several months. Changes in sperm structure have been previously reported following various manipulations of sperm including the conditions required for sperm cryopreservation or during sperm activation (Billard, 1983; Gwo and Arnold, 1992; Perchec *et al.*, 1996). In this study, flounder sperm morphology was examined in serially collected milt samples at both the light and electron microscopic levels, in the attempt to investigate reasons for seasonal changes in sperm quality. In addition, seasonal changes of sperm quality in captive halibut was investigated in relation to altered sperm morphology.

5.2. Materials and methods

Male flounder were collected by SCUBA divers monthly, beginning in January (N=13) and continuing through July (N=47, 22, 25, 21, 20 and 24 for February, March, April, May, June and July, respectively) in 1997. For wild males, milt samples were obtained from freshly caught fish within one week of arrival in the laboratory. For captive males, the milt samples for February were collected from the group of males obtained in January; for the remaining months milt samples were collected from a combined group of males caught in January and February. Examination of milt was carried out twice in June (early and late) for both wild and captive males. Because a portion of males brcame unhealthy by June, the number of captive males was reduced in the later portion of this experiment. For male halibut, milt was collected monthly from 3 captive broodstock males during their milt production period (January to May) in 1995.

The pH and spermatocrit of milt and sperm quality (motility) for milt samples of both species were investigated using the procedures described previously (Chapter 2). Four - twelve milt samples from wild flounder and 3 (N=2 in May) from captive halibut collected during the reproductive season were examined by light microscopy for seasonal changes of sperm morphology. Two - three milt samples from the early, middle and late spawning season were also examined under electron microscopy (SEM and TEM, see Chapter 2).

T- test or an ANOVA followed by the Duncan's mutiple range test was used

to test differences between groups or changes over the spermiation period.

5.3. Results

5.3.1. Winter flounder

5.3.1.1. The duration of spermiation

Milt production first become evident in more than half of wild males in January, and continued throughout the prespawning season while the number of spermiating males gradually increased (Fig 12). By May and June, all wild males were spermiating and produced large volumes of milt [1]. The number of spermiating wild males declined abruptly in the middle of July, leaving only a portion (38%) of males producing small amounts of dilute milt. A small number of captive males (~10%) did not spermiate in May; however, spermiation continued in most captive males well into August at which time termination of spermiation had occurred in all wild males.

5.3.1.2. Seasonal changes in milt properties and sperm quality

Sperm motility (ANOVA; $f = 5.25$, $df = 7$, $P < 0.01$) and milt pH ($f = 9.76$, $df = 7$, $P < 0.01$) fluctuated significantly in wild males during the reproductive season, reaching peak values in May and early June (Fig 13A, C). A similar seasonal

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[1] Milt volume is about 0.5 ml or more in each stripping.

pattern of pH change was observed in the captive males. However, while milt spermatocrit values clearly fell, indicating milt hydration, in wild males during the summer spawning season, milt from captive males was significantly more concentrated (Fig 13B) and sperm quality was lower in May compared to wild males [motility=25.5±2.6% cf. 40.5±2.9%, $P< 0.001$ (t-test; $t = 3.79$, $df = 37$); swim duration =19.3±0.5 sec cf. 21.5±0.5 sec, $P< 0.001$ ($t =2.88$, $df = 37$)].

5.3.1.3. Seasonal variation in sperm morphology

Several forms of sperm morphological abnormalities involving the head and the tail were observed in flounder milt samples collected throughout the reproductive season (Fig 14). The malformations included macro-cephalic (2.23±0.41 μm in diameter vs 1.59 μm for the head of normal sperm) or tail-ringed spermatozoa which remain partially or entirely enclosed within a cytoplasmic sac (Fig 15C, G). In addition, spermatozoa with twisted or missing tails (Fig 15B, F) were frequently observed, particularly in the prespawning and postspawning season (Fig 14). Electron microscopy demonstrated that a few of sperm possessed a node head early in the spawning season (Fig 15A), however, the rupture of plasma and nuclear membranes (Fig 15D, E) was frequently observed particularly in the late season (Fig 15F). Serial observations indicated the proportion of abnormal sperm was lowest in May, when sperm motility was observed to be highest (Fig 13A, 14).

5.3.2. Halibut

5.3.2.1. Seasonal changes of milt properties and sperm quality

All male halibut were expressing milt by January (Fig 16), and milt production continued through April and May before terminating in June. Spermatocrit of milt progressively increased throughout the milt production period but a decrease in milt pH value occurred at the end of the spermiation period (Fig 17B, C). In contrast, sperm motility was highest during the earlier phase of the reproductive season (February), decreasing gradually and becoming extremely low at the end of spermiation period (Fig 17A).

5.3.2.2. Seasonal changes of sperm morphology

Spermatozoa with abnormal morphology were present in seasonal collections of halibut milt samples. The abnormalities included macro-cephalic spermatozoa (2.67±0.33 μm in diameter vs 2.0 μm for the head of normal sperm), and spermatozoa with tail ring (Fig 19J, L and insert in Fig 19 I) or tail twist or tail missing (Fig 19I, K). In addition, spermatozoa containing double nuclei were sometimes observed (Fig 19M). Although a small portion of macrocephalic and tail ringed spermatozoa were observed at the beginning of reproductive season (Fig 18A, B), most spermatozoa appeared normal in the early phases of spermiation (Figs 18E, 19H) when sperm were vigorously released. The number of abnormal spermatozoa with tail damage (twist or missing) increased gradually late in the

season (Fig 18C, D) when rupture of cell membranes were frequently encountered (Fig 19K).

5.4. Discussion

5.4.1. Seasonal spermiation in male winter flounder and halibut

The study confirms previous observations of male winter flounder (Chapter 3), showing that about half of the males begin early milt production in January, a prespawning period of initial seasonal milt release that lasts from January to April. By May, however, all of the wild males began spermiating and milt volumes increased. While spermiation in captive males extended into July and August , milt production was completed in mid July for most wild males, despite a few males continuing to express small amounts of clear milt. On the basis of these observations, it is clear that although the spawning season lasts only ~2 months,, most male winter flounder release milt for as long as 6 months of the year. There is a lengthy prespawning phase, followed by the spawning season when the majority of sperm are released, then a brief (wild males) or more extended (captive males) concluding phase of milt release at the end of the season when sperm production is reduced. Male halibut also produced sperm for ~5 months; however, the most active spermiation period for halibut occurs in the winter, followed by a 2-3 month concluding phase when the milt becomes very concentrated.

5.4.2. Seasonal variation of sperm quality

Seasonal variations in sperm quality of winter flounder were observed again in this study, with the proportion of motile sperm increasing significantly during the spawning season. Although some sperm are motile soon after the onset of milt expression, sperm motility remained low in the earlier portion of the spermiation period. Increased sperm motility was also observed in rainbow trout in the early part of the spawning season, followed by a late-season decline (Buyukhatipoglu and Holtz, 1984). These observations agree with a previous study of male halibut: while there is similar length of spermiation period with winter flounder, the quality of halibut sperm appeared higher in winter, during the spawning season, but gradually decreased in late spring (Crim et al. unpubl and the present study). The present study extends previous observations of these two species, linking seasonal variations in sperm quality with changes in sperm morphology and spermatocrit. For example, morphological studies revealed that damage occurs in sperm structures such as the flagellum and membrane systems in both species. In halibut, morphological abnormalities of the spermatozoa increased with the progression of the reproductive season, likely due to sperm aging and degeneration. Similar reasons may account for the increased frequency of flounder sperm with tail abnormalities during the late phase of spermiation. In male winter flounder, the abnormal spermatozoa were also frequently observed during the prespawning season. This may be explained by decomposition of sperm cells during storage,

since intensified phagocytotic activities of the epithelium were observed in the sperm duct during this period of time (Chapter 6). Decomposition of sperm during storage was associated with deteriorated sperm quality found in milt samples stored in the sperm duct for long periods of time, particularly late in the season (Shangguan and Crim, 1995). On the other hand, improved sperm quality during the spawning season may be associated with accelerated release of sperm from the testes thereby reducing time of storage in the sperm duct when milt hydration increases.

Although a higher percentage of normal sperm was found in flounder milt samples collected at the beginning of the spermiation period (January), sperm motility appeared no different from milt samples collected at other times during the prespawning season. In contrast to sperm abnormalities in other phases of the reproductive season, abnormal sperm in the early period of flounder spermiation (January) are composed mainly of tail ringed and macrocephalic spermatozoa. The situation was similar in some male halibut during the early spawning season where sperm motility was low. Although it remains to be clarified whether they represent abnormalities or prematuration of sperm, this may suggest different mechanisms for changes in the sperm quality during the reproductive season.

5.4.3. Impacts of captivity

While broodstock management of aquaculture fish often focuses upon egg

quality, little knowledge of sperm quality is available for the captive male. Reproductive studies have shown that spawning may be inhibited under captive conditions or lead to subtle changes in reproduction such as decreased gamete quality (Crim, 1991). The present study demonstrated that sperm quality was reduced in captive flounder during the spawning season, although it is unclear just what conditions negatively impacted male gametes. Captivity environments may be stressful. Campbell *et al.* (1992) showed that stress reduced sperm counts in male trout and lowered the survival of trout progeny. In the present study, photoperiod and temperature were maintained at levels similar to fish in the wild. However, rapid fluctuations in daily water temperature in tanks may have impaired male spawning activities. Actually, spawning of male flounder appeared, to some extent, suppressed based on the fact that spermiation of some captive males was delayed to June and the duration of spermiation extended at least one month to August. A suppression of spawning may have caused the reduction in sperm quality by aging and phagocytosis, as discussed earlier in this chapter. Effects of temperature on gamete quality also have been documented. Poor egg quality was found in captive broodstock maintained continuously at 14-17 °C, but the quality was significantly improved by a period of low temperature (below 10 °C) in winter, suggesting that some seasonal cycling in temperature is important for maintaining good gamete quality (Bye, 1990). However, the impact of captivity may be temporary if fish adapt to artificial conditions (Pickering and Pottinger, 1985). In captive halibut, stable

sperm release and sperm quality patterns were observed in males over several years of holding in the laboratory (Crim et al. unpubl. and the present study).

Fig 12 Seasonal changes in the percentage of males expressing milt in groups of captive and wild winter flounder. N=13 (Feb), 31 (Mar), 27 (Apr), 22 (May), 9 (June), 7 (July) and 6 (Aug) respectively for captive males, N= 13 (Jan), 47 (Feb), 22 (Mar), 25 (Apr), 21 (May), 20 (Jun) and 24 (Jul) for wild population.

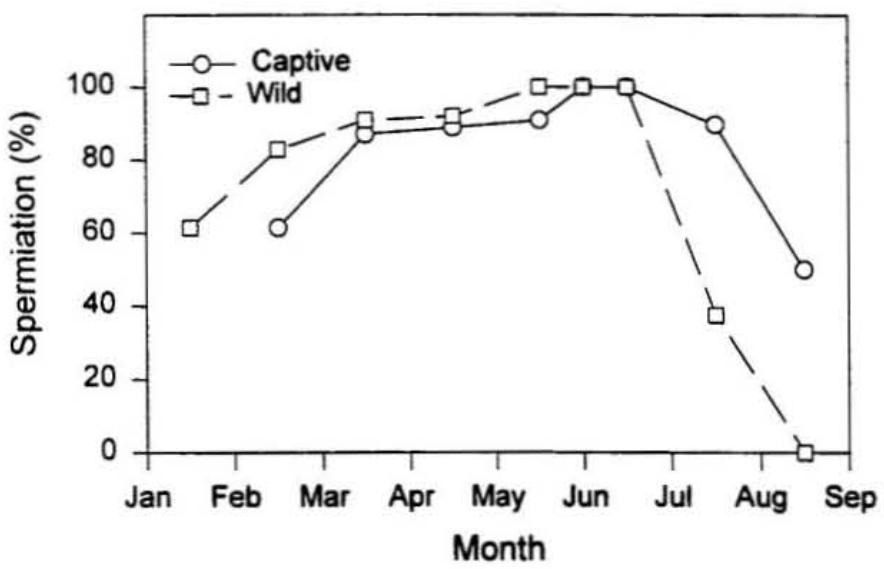

Captive
Wild
Spermiation (%)
100
80
60
40
20
0
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Month

Fig 13 Seasonal changes in A) sperm motility, B) milt spermatocrit, and C) milt pH values in captive and wild male winter flounder throughout the milt expression period. Differences between wild and captive groups are indicated as: *P< 0.01 (t-test; motility: t = 3.79, df = 37; spermatocrit: t = 3.24, df = 36 for May, t = 4.68, df = 21 for early June and t = 4.4, df = 25 for late June) Significant difference (indicated be different letters) along the reproductive season within the group also occurred in wild males (ANOVA; sperm motility: f = 5.25, df = 7, P< 0.01; , spermatocrit: f = 10.78, df = 6,P< 0.001; and pH: f = 9.76, df = 7, P< 0.001) and captive males (spermatocrit: f = 3.23, df = 6, P< 0.01 and pH: f = 3.28, df = 6, P< 0.01).N=8 (Jan), 39 (Feb), 20 (Mar), 23 (Apr), 21 (May), 20 (early Jun), 20 (late Jun), and 9 (Jul) for wild, 8 (Feb), 27 (Mar), 24 (Apr), 20 (May), 9 (early Jun), and 9 (late Jun), and 6 (Jul) for captive males.

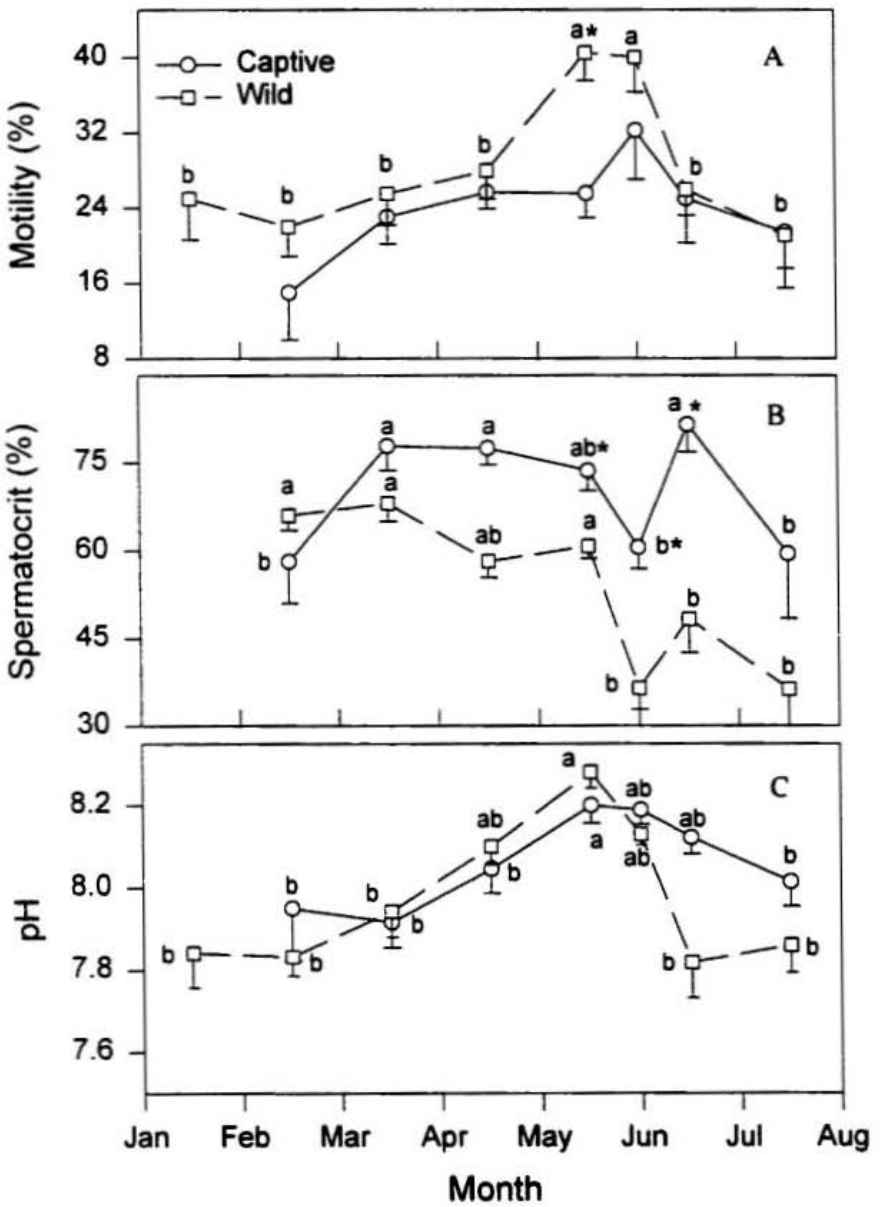

Captive
Wild
A
B
C
Motility (%)
Spermatocrit (%)
pH
40
32
24
16
8
75
60
45
30
8.2
8.0
7.8
7.6
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Month

Fig 14 Seasonal changes in the proportion of normal vs morphologically abnormal spermatozoa for milt collections made from wild male winter flounder. Significant differences over the reproductive season within group indicated by different letters (ANOVA; big head: $f = 3.36$, $df = 4$, $P < 0.05$; tail ring: $f = 6.58$, $df = 4$, $P < 0.01$; tail missing: $f = 5.01$, $df = 4$, $P < 0.01$; tail twist: $f = 3.71$, $df = 4$, $P < 0.05$; normal sperm: $f = 2.71$, $df = 4$, $P < 0.05$). Number of fish in most months: 10-12, except for 6 and 4 in Jan and July respectively.

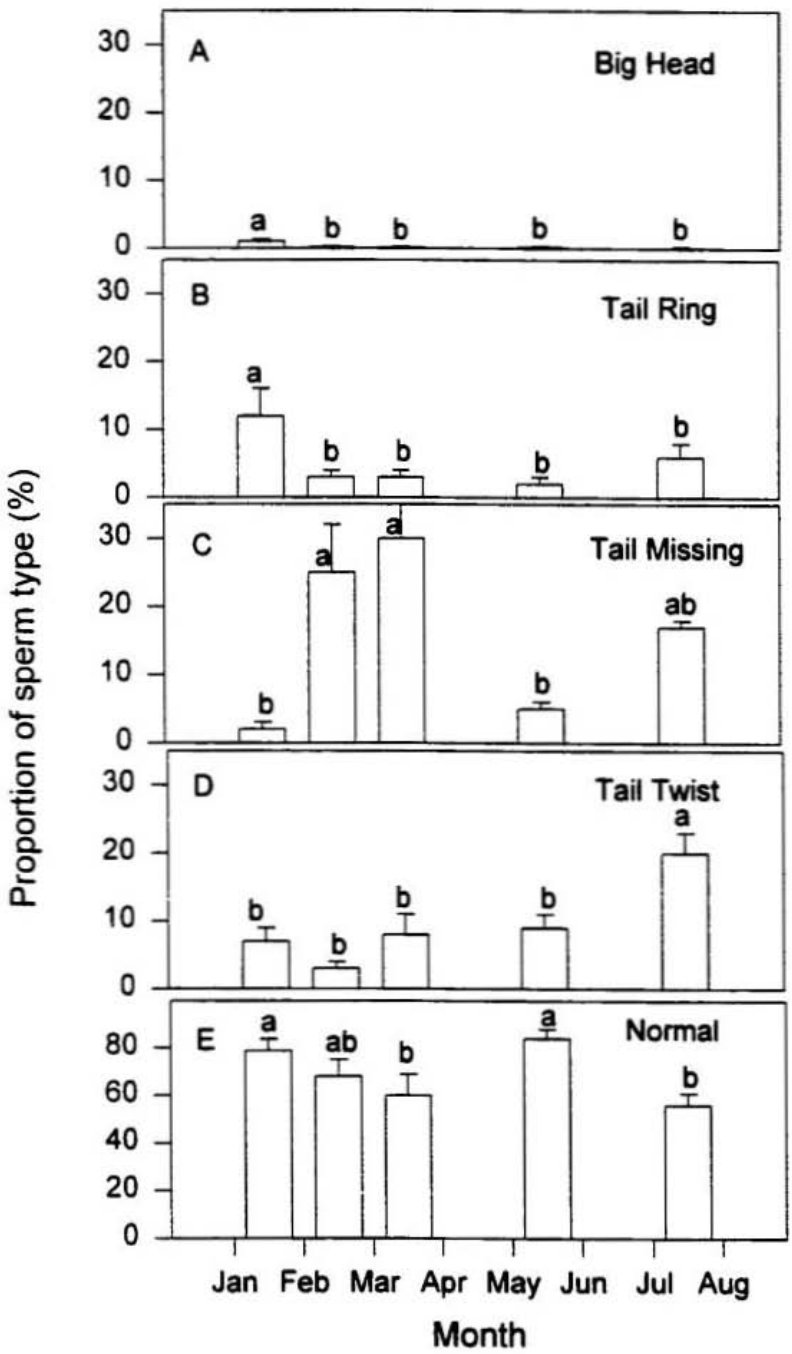

A
Big Head
B
Tail Ring
C
Tail Missing
D
Tail Twist
E
Normal
Proportion of sperm type (%)
Jan Feb Mar Apr May Jun Jul Aug
Month

Fig 15 Seasonal changes of winter flounder spermatozoa morphology [(scanning electron microscopy (SEM) and transmission electron microscopy (TEM)].

A) Flounder spermatozoa (May). Most in normal morphology with few spermatozoa showing broken tail or a node head (*). (x 3,000)

B) Left, tail coil spermatozoa; right, spermatozoa with cytoplasm membrane partially ruptured on the region of the midpiece, resulting in the exposure of mitochondria (arrow). (x 12,000)

C) Spermatozoa with tail ring. (x 6,000)

D) Spermatozoa head (H) showing the rupture of cell membrane. (x 17,000)

E) Spermatozoa showing decomposition of cell membrane and abnormal nucleus (N). M: mitochondria, A:axoneme (x 20,000)

F) Abnormal spermatozoa (July). Most spermatozoa have broken tail with the rupture of cytoplasm membrane. (x 6,000)

G) Spermatozoa with tail ring. Undeveloped axoneme (A) wrapped in a cytoplasm sac (CS) that also embraces a part of nucleus (N). (x 16,000)

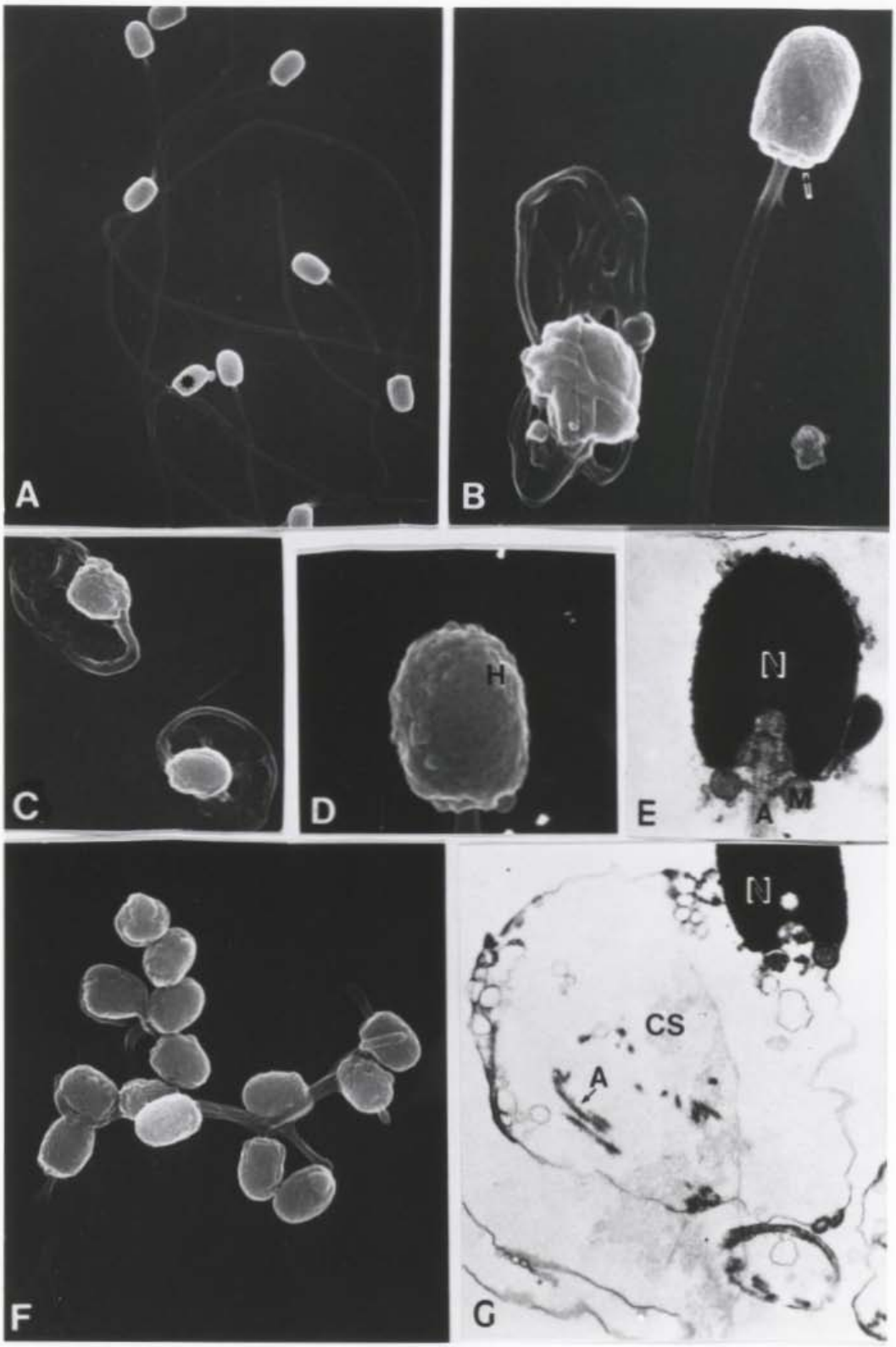

A
B
C
D
H
E
N
A
M
F
G
N
CS
A

Fig 16 Seasonal changes in the percentage of male halibut expressing milt in captivity (N=3).

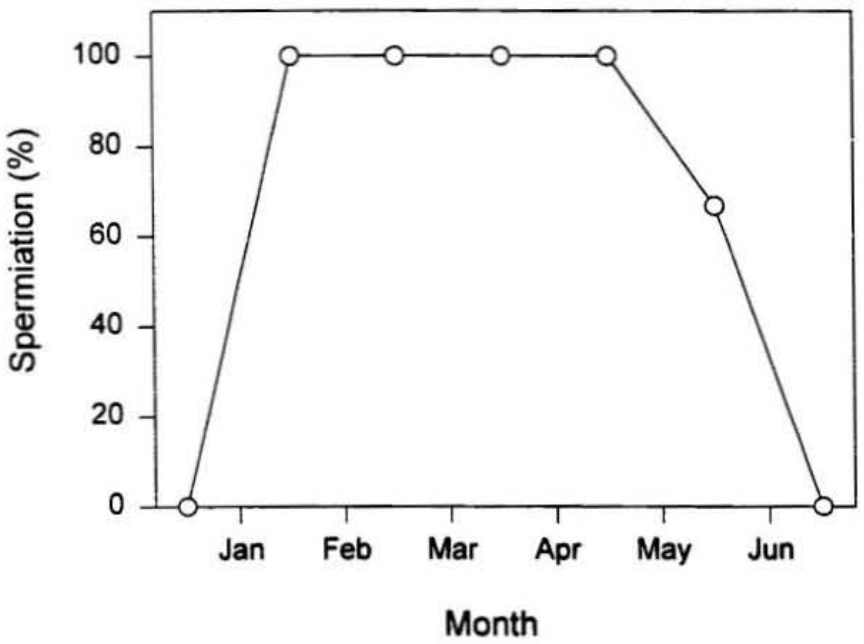

Spermiation (%)
100
80
60
40
20
0
Jan
Feb
Mar
Apr
May
Jun
Month

Fig 17 Seasonal changes in A) sperm motility and B) spermatocrit, and C) milt pH values in captive halibut throughout the milt expression period. Significant differences along the reproductive season are indicted by different letters (ANOVA; motility: $f = 4.23$, df 4, $P < 0.05$; spermatocrit: $f = 19.3$, df = 4, $P < 0.01$; pH: $f = 5.23$, df = 4, $P < 0.05$). N=3 except for 2 in May.

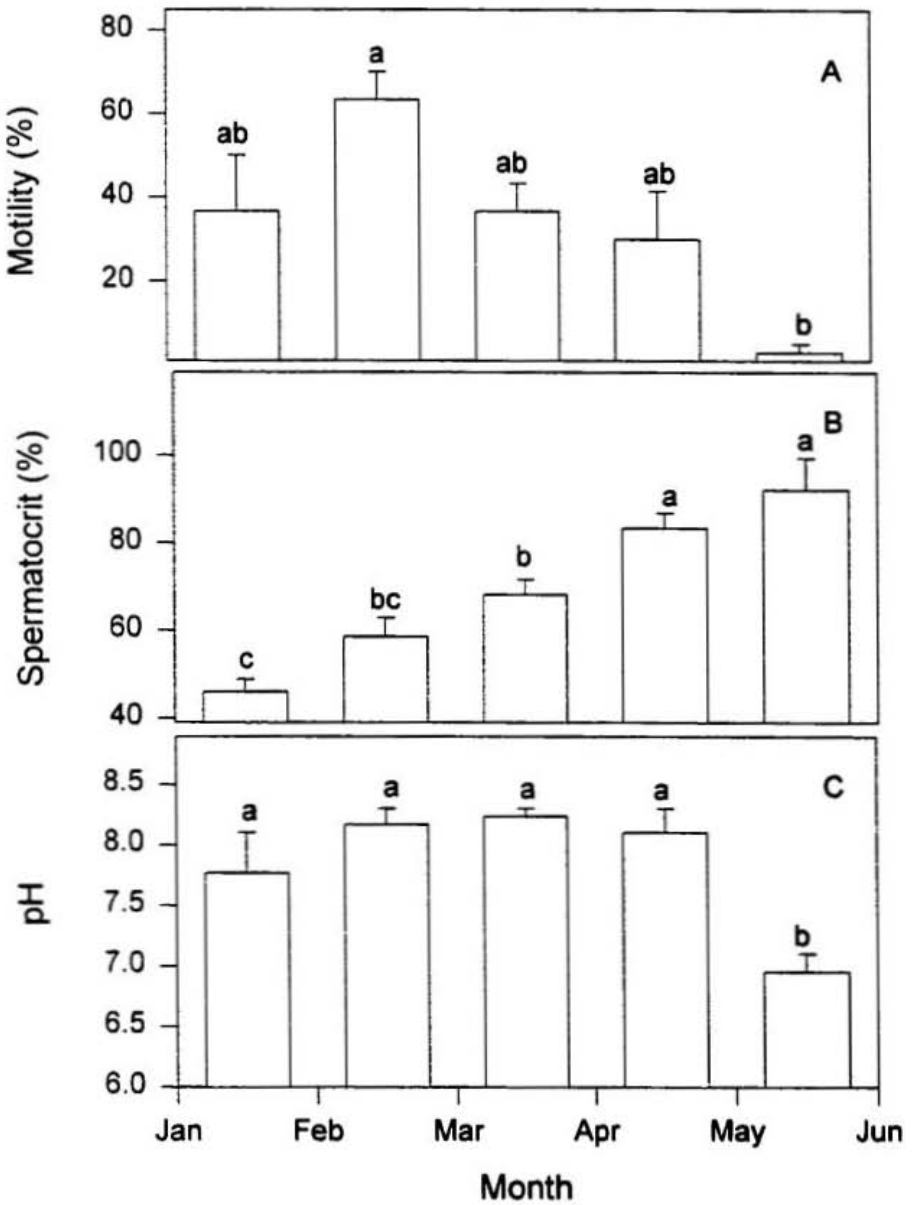

Motility (%)
80
60
40
20
a
ab
ab
ab
b
A
Spermatocrit (%)
100
80
60
40
c
bc
b
a
a
B
pH
8.5
8.0
7.5
7.0
6.5
6.0
a
a
a
a
b
C
Jan
Feb
Mar
Apr
May
Jun
Month

Fig 18 Seasonal changes in the proportion of normal vs morphologically abnormal spermatozoa for milt collections made from 3 captive male halibut. The percentage of normal spermatozoa was square root transformed prior to statistical analysis. Significant differences over the reproductive season within group indicated by different letters (ANOVA; big head: $f = 2.76$, $df = 4$, $P = 0.09$; tail missing: $f = 9.46$, $df = 4$; tail twist: $f = 3.49$, $df = 4$, $P = 0.06$, normal sperm: $f = 9.34$, $df = 4$, $P < 0.01$).

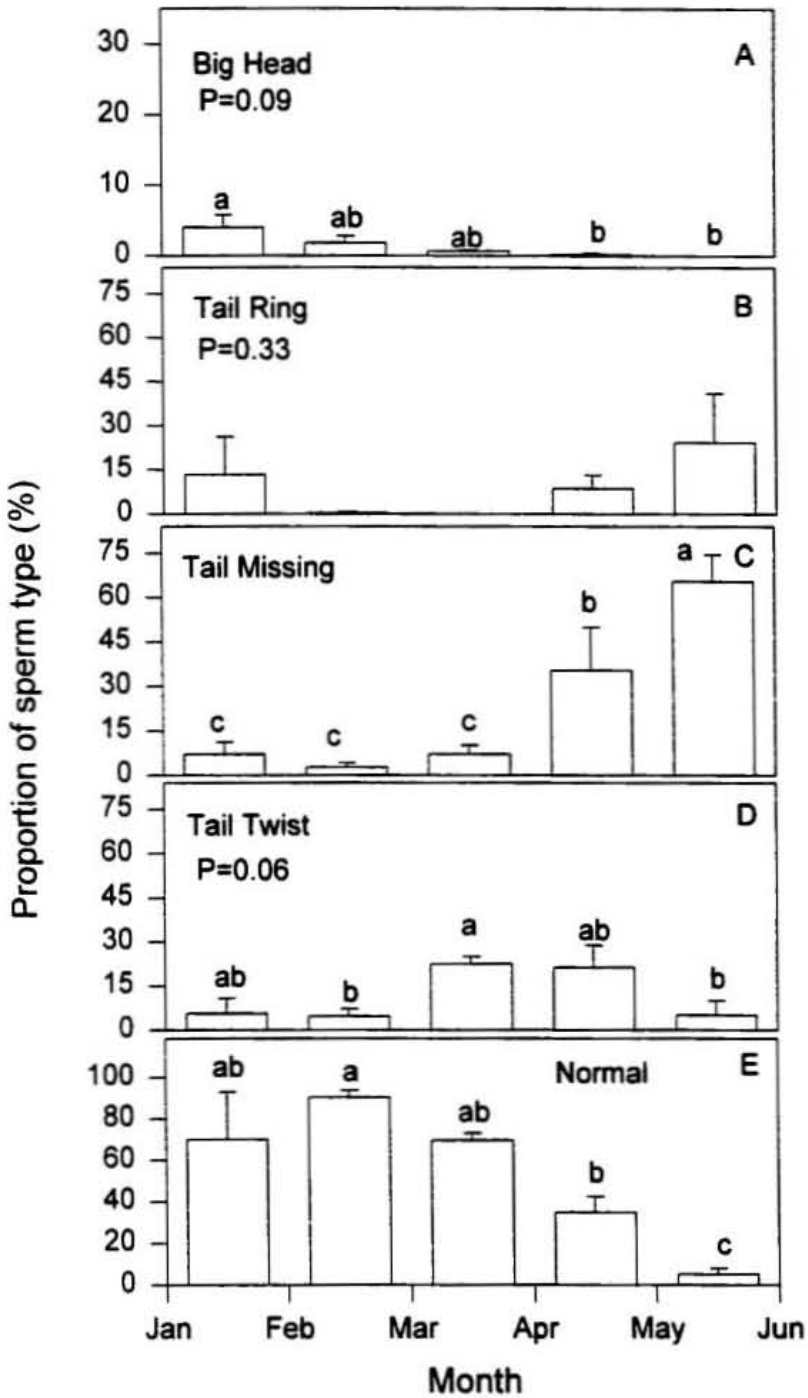

Big Head
P=0.09
A
a
ab
ab
b
b
Tail Ring
B
Tail Missing
C
c
c
c
b
a
Tail Twist
P=0.06
D
ab
b
a
ab
b
Normal
E
ab
a
ab
b
c
Jan
Feb
Mar
Apr
May
Jun
Month
Proportion of sperm type (%)

Fig 19 Seasonal change of halibut spermatozoa morphology (SEM and TEM).

H) Spermatozoa (February). Most show normal morphology. (x 1,200)

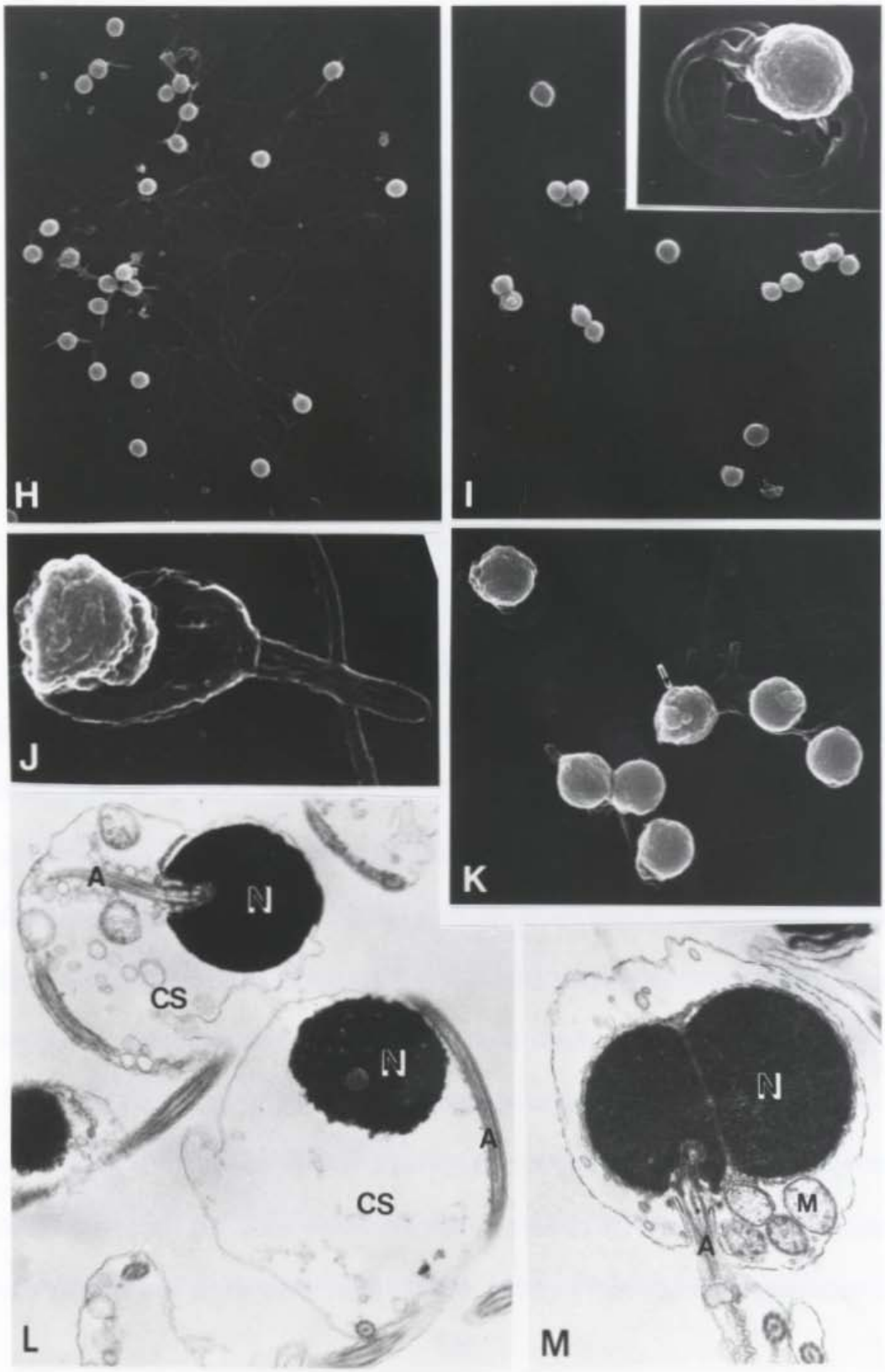
I) Abnormal spermatozoa (April). Most missing tail (x 1,200); Insert: Spermatozoa with tail ring. (x 10,000)

J) Spermatozoa with tail partially wrapped in cytoplasmic sac. (x 12,000)

K) Spermatozoa showing decomposition of cell membrane (arrow) and missing tail. (x 6,000)

L) Spermatozoa with tail ring. Undeveloped axoneme (A) wrapped in a cytoplasm sac (CS) that also embraces the whole or a part of nucleus (N). (x 16,000)

M) Spermatozoa with double nuclei (N). M: mitochondria, A: axoneme (x 16,000)


H
I
J
K
A
N
CS
N
A
CS
L
N
M
A
M

CHAPTER 6

The Testicular Efferent Duct System of Male Winter Flounder (*Pleuronectes americanus*): Studies on the Seasonal Changes of the Functional Morphology

6.1. Introduction

According to previous observations with male winter flounder, sperm are released in a fashion that ensures the best sperm quality occurs within the spawning season, despite a 4-5 month sperm storage in fish (Chapter 5). Although several factors, including sampling regime and captive conditions, have been experimentally demonstrated to affect such a distinct seasonal release (Chapter 5; Shangguan and Crim, 1995), these factors likely influence the release and maintenance of sperm by changing the activities of the male genital tract - the testicular efferent duct system (TEDS) through which sperm are delivered. Several important functions have been ascribed to the TEDS in teleosts, including sperm maturation, nutrition, protection, resorption and storage (Lahnsteiner and Patzner, 1990; Lahnsteiner *et al.*, 1993a, b; Lahnsteiner *et al.*, 1994b; Morisawa and Morisawa, 1988; Morisawa and Morisawa, 1986; Rasotto and Sadovy, 1995). However, in most species examined so far, the final differentiation and maturation of sperm cells are undertaken in the TEDS. Unlike these species, spermatozoa of

winter flounder mature within testicular cysts. Thus, the functions of the flounder TEDS may be distinct from those of other species.

Detailed information on morphological features has been valuable in illustrating the major processes within the TEDS that are responsible for the maintenance and regulation of sperm viability. Production of the seminal fluid, phagocytosis and ingestion of degenerated and damaged sperm have been demonstrated to occur in the duct system of a number of teleosts (Lahnsteiner and Patzner, 1990; Lahnsteiner *et al.*, 1990; Lahnsteiner *et al.*, 1993a, b; Lahnsteiner *et al.*, 1994b; Manni and Rasotto, 1997; Rasotto and Sadovy, 1995; Seiwald and Patzner, 1987), mostly from freshwater-living salmonid, cyprinoid and blenniid fish. From these studies, a close correlation has been defined, linking structures of the duct system to specific functions in species or their distinct reproductive habits (Manni and Rasotto, 1997). In the present study, the morphology and fine structures of the TEDS of winter flounder, a coldwater marine species, were studied with an emphasis on cyclic changes in the epithelial tissue that may contribute to longterm sperm maintenance and the regulation of sperm quality.

6.2. Materials and Methods

Serial samples of the testis and sperm duct of adult males of winter flounder were obtained at monthly intervals during the spawning season (May to July), and at 2-3 month intervals during the rest of the reproductive season in 1995. Males

were anaesthetized with 0.15 - 0.2 % phenoxyethanol (v/v) and the testis and the sperm duct exposed. Tissues were cut from three regions including the central part of the testis with attached testicular primary duct (PD), the proximal sperm duct (PSD) and the distal sperm duct (DSD) (Fig 20). For EM samples, the PD was fixed separately from the testis. Both the testis and the duct samples were prepared for histological and EM examination, as described in Chapter 2.

6.3. Results

6.3.1. General morphology of the testicular efferent duct system

The testicular efferent duct system (TEDS) of male winter flounder is composed of a testicular primary duct (PD) and the sperm duct (SD) (Fig 20). The PD lies along the length of the testicular lobe on the interlateral sides, receiving sperm directly from the testis. Next, the sperm are transported away from the testis into the sperm ducts, terminating at the urinogenital papillae. No accessory glands were found in this species. A number of the tubules within the primary duct in each testicular lobe (Fig 21A) converge into a single large sperm duct which is internally partitioned by branched, longitudinally extended folds (primary folds) into 2 or more channels that remain partially continuous through intercompartment openings (Fig 20). Numerous small folds (secondary folds) are also visible along the traverse sections of the sperm duct (Fig 21B, also see Fig 20 for the organization of TEDS structure).

The architecture of the wall of the TEDS is similar in both PD and SD portions, consisting of three layers: an outmost peritoneum envelope, a thick connective tissue, and an epithelial lining on the inner surface. The connective tissue is abundant with collagen fiber intermingled with some smooth muscle fibers, densely ranged as two (PD) or three (SD) distinctively orientated layers: circular-longitudinal or circular-longitudinal-circular (from the inner surface to periphery).

6.3.2. Types of epithelium and their cytoplasmic features

The TEDS' epithelium, which is adhered to a noncellular basal lamina, is distinctive, alternating between groups of cuboidal, and tall or low columnar cells (Fig 22G). This is in sharp contrast with the epithelium of seminiferous tubules made of mainly squamous cells but becoming mostly cuboidal as the tubules approach the PD. The columnar cells, which predominate in the duct epithelium, are frequently depressed from the surface, forming pits (Fig 21D) which exhibit active secretion during the spawning season (Fig 22F). Besides the columnar and cuboidal cells, a few small round or oval basal cells are also scattered among the columnar cells, resting on the basal lamina. They have a dense nucleus and a scant cytoplasm, often being free of endoplasmic reticulum (ER), Golgi apparatus and mitochondria (Fig 23I).

Microvilli are often observed on the surface of both columnar and cuboidal cells facing into the tubular lumen (Fig 24L). As well as areas of membrane

specialization, the zonula occludens, the zonula adherens and the macula adherens, are situated along the bilateral borders of these cells (Figs 24K, 25M). Tall columnar cells are usually composed of a dense cytoplasmic matrix and an ellipse or rod nucleus with an eccentric nucleolus and an amount of hetero-chromatin peripherally situated (Fig 25O). Rough ER are observed parallel to the long axis of the cells (Fig 23J). Oval or elongated mitochondria appear with lamellar cristae and a dense matrix, usually in a supranuclear location where the Golgi apparatus are distributed (Fig 25M). Vesicles with clear or dense content are present with various diameters from 50-500 nm. Bundles of microfilament are observed around the nucleus while various phagocytic bodies occur in some cells during the reproductive season. Although some of the columnar cells are lower in height and contain an oval or almost spherical nucleus, they are similar to other columnar cells in most ultrastructural features (Fig 26Q).

The fine structure of cuboidal cells includes a less dense cytoplasmic matrix and a round nucleus bearing a centralized nucleolus and lesser amounts of hetero-chromatin. The cuboidal cells in the TEDS (Fig 27R, S) contain abundant rough ER and some mitochondria with tubular cristae. Small vesicles usually are in considerable number while large vacuoles up to 1 μm and microfilament bundles are also frequently encountered. Active secretion is evident on the apical surface of the cells (Fig 27S), which are seldom found in the intratesticular cuboidal cells. Cuboidal cells in testes (Fig 28T, U) differ from their analogues by containing more smooth ER

and Golgi bodies. Mitochondria are not particularly abundant while vacuoles of varying diameter, and large intracellular inclusions showing the features of multivesicle or lamellar body are present.

6.3.3. Alteration of the morphology of testicular duct system during the reproductive season

During early gonadal recrudescence (September), the PD and SD are small in diameter. The epithelium of the TEDS is made up mostly of short columnar cells and cuboidal cells (height x width: 10.0 x 5.2 μm and 9.6 x 8.7 μm, respectively), and few epithelial pits are formed (Fig 21A). The nucleus of the columnar cells appears irregular, containing abundant hetero-chromatin (Fig 24K). A small number of ER cisternae and mitochondria were observed among free ribosomes and, occasionally, a couple of vesicles or vacuoles. When males mature and sperm first becomes expressible (December and January), the epithelium is transformed into mostly tall columnar cells (16.8 x 5.3 um, height x width), frequently forming pits within tubules (Fig 21 B, C). At this point, secretion accumulates in small amounts at the surface of a few epithelial cells, however, most remain inactive (Fig 21D) while phagocytosis of spermatozoa by the epithelium was frequently observed to a variable extent along the duct system.

In May and June, highly active secretion was observed in TEDS, particularly in PD epithelium (Fig 22E, F). Numerous mitochondria with lamellar cristae

characterize most columnar cells, commonly aggregated in the supranuclear region, but also on the bottom the cells (Figs 24L, 25M, O). A great amount of the interdigitation of the cell membrane occurs along the cell border where a considerable number of vesicles with smooth or coated surfaces are produced by active pinocytosis in the vicinity of the basal lamina and neighboring epithelial cells (Fig 25O). Some of these vesicles fuse together or coalesce with existing ER cisternae. Meanwhile, rough ER is increasingly encountered in close proximity to developed Golgi apparatus (Fig 26P). Secretion proceeds in an apocrine mode: The apical portion of the epithelial cell, with dense contents but few intracellular structures, protrudes into the lumen of the ducts, consequently disconnected and shed into the duct chamber (Fig 25N). Vigorous secretory and pinocytic activities are also observed in columnar cells in the pit (Fig 26Q) and in cuboidal cells (Fig 27R, S) found in PD and SD.

Most columnar epithelial cells become reduced in height by the end of the spawning season (July) (Fig 22G), inside which engulfed spermatozoa are frequently visible (Figs 22H, 29W). Secretory activities completely cease in the majority of the epithelial cells within pits and on the surface (Fig 30X, Y), despite apparent activities remaining in some cells (Fig 29W). On the other hand, degeneration of a part of a cell or the whole cell (necrosis) is frequently observed by TEM. Groups of vacuoles aggregate in the supranuclear area of some columnar cells where they fuse together, resulting in the discarding of the apical portion of the

cytoplasm (Fig 31Za). The nucleus in necrotic epithelial cells become highly heterogenous and the nucleolus is dissolved; auto- or hetero-phagosomes, and residual vacuoles are seen in the cytoplasm (Figs 30Y, 31Za). Eventually, the whole cell shrinks and sometimes, the epithelium collapses entirely in a region where the intercellular space of a group of cells enlarges. In this case, these cells become separated from each other or detached from the basal lamina, eventually collapsed or shed from the underlining tissue (Fig 31Zb).

6.4. Discussion

6.4.1. Types of epithelium and their secretory activities in the testicular efferent duct system

At least three basic types of cells have been identified in the epithelial lining of the testicular efferent duct system of winter flounder based on cell shape and location, including columnar, cuboidal, and basal cells. Columnar cells line the inner surface of the sperm duct (or vas deferens) of most teleost species examined so far, including bleniid, *Blennius pavo* and *B. dalmatinus* (Lahnsteiner and Patzner, 1990), grunt (Rasotto and Sadovy, 1995), jawfish, *Opistognathus whitehurstii* (Manni and Rasotto, 1997), European pike, *Esox lucius* (Lahnsteiner *et al.*, 1993a), cyprinid (Lahnsteiner *et al.*, 1994b) and salmonid fish (Lahnsteiner *et al.*, 1993b), while squamous cells reportedly predominated in some blenniid fish (Manni and Rasotto, 1997; Rasotto, 1995). On the other hand, cuboidal cells are only found in the

epithelium of the intratesticular efferent duct (Lahnsteiner *et al.*, 1993a). Although basal cells have not been described in the sperm duct of other teleosts, they do occur in the epithelial tissue of the male reproductive tract in some higher vertebrates, including the ductuli efferentes and the ductus of the epididymis in mammals (diFiore, 1981) and vas deferens in humans (Paniagna *et al.*, 1981). Although the functions of the basal cell remain unclear, they are presumably a stem cell (Ross *et al.*, 1995), perhaps replacing lost epithelium due to necrosis or collapse of cells which was evident in this species, particularly in the postspawning phase of reproduction. Additional evidence for basal cell functioning as sterm cell is that some basal cells increased in size and the amount of cytoplasmic organelles, indicating cell differentiation and functional transformation (Fig 23I, BC1).

Besides the cell types described above, squamous cells were occasionally observed on the top of the epithelium (top cells) without contact with the basal lamina. These top cells sometimes appear in mass, shed into the duct chamber. The morphological equivalent of this type of cell has not yet been reported elsewhere in the reproductive tracts of other male teleost and the origin and functions of the top cells remains obscure. They could originate from the basal cells, or even from the deeper connective tissue, resembling the intraepithelial lymphatic cells within epididymic (Brian *et al.*, 1994) or intestinal epithelium (Ross *et al.*, 1995). In addition, some mitochondria-rich columnar cells were observed, which have been reported in human vas deferens (Paniagna *et al.*, 1981). Furthermore, some squamous cells

with unique fine structural features were occasionally observed within the epithelial pits of the sperm duct of winter flounder (Fig 29V). They are seated on the basal lamina, lacking intercellular junctions and being very active in protein synthesis and secretion (budding form), indicated by developed rough ER, Golgi complexes and electron-dense, secretory granules. The fact that they are completely exposed to the duct lumen and distinct in their cellular appearances is not suggestive of a derivative of the basal cells. Since this type of cell is seldom visualized in the epithelium, further verification is required.

Among these types of cells, the columnar and cuboidal cells make up over 95% of the epithelium. Cuboidal cells are of similar size in all three dimensions, as a general definition (Ross *et al.*, 1995). Cuboidal cells in PD and SD of male winter flounder are considered to be homologous with the square-like Sertoli cells from the testis, possessing similar characteristics of nucleus, cytoplasmic matrix and mitochondria. However they are distinguished by features such as intensive absorption and secretion that are absent in the intratesticular analogues. Active protein synthesis is also suggested by abundant rER cisterns. In contrast, Sertoli cells within the testis are most likely involved in endocytic and lytic processes. On the other hand, cuboidal cells in the duct system are structurally different to columnar cells in their nuclear and cytoplasmic characteristics. Although secretory activity in the cuboidal cells proceeds in a manner similar to the columnar cells, the properties of secretion between these cells may be distinctive according to the

cytoplasmic appearances. The cytoplasmic matrix appears denser in columnar cells and mitochondria in the columnar cells contains lamellar cristae which are substituted by a tubular form in the cuboidal cells, suggesting they may possess different functions.

Despite being different in cell size (short or tall) or location (in pits or on the surface), most columnar cells basically resemble each other in their fine structural appearances of the nucleus, cytoplasm matrix, rough ER and mitochondria. Pinocytosis and secretory activities occur to a great extent during the spawning season, together with abundant amounts of mitochondria found in the cells, suggesting that both surface and pit columnar cells play key roles in the transport of material. However, some pit columnar cells form a huge vacuole containing material with low density which moved to the apical region as the major secretion component. The secretions appear different with most surface columnar which shed a cytoplasm capsule with highly dense content during apocrine activities. This may reflect differences in the properties of the secretory substance.

Overall, the diversity of cell morphology within the duct system may reflect a variety of epithelium functions or seminal fluid component production.

6.4.2. The organization and the functions of the testicular efferent duct system

The simple reproductive tract in male winter flounder consists of only the testicular primary ducts and the sperm ducts without accessory glands. Considering

the formation of spermatozoa in this species, the present results appear to be consistent with Manni and Rasotto (1997), indicating that the organization of the TEDS is closely related to the processes of sperm maturation in teleosts. A well-developed, intratesticular tubular system (the testicular efferent ducts or the testicular gland), which collects sperm from the seminiferous tubules, is present in those species where sperm metamorphosis continues in the testicular efferent duct system (semicystic spermatogenesis). In contrast, cystic spermatogenesis occurs in winter flounder and other species (Lahnsteiner *et al.*, 1993b; Lahnsteiner *et al.*1994b) in the absence or less development of this intratesticular tubular system. This is the type of spermatogenesis characteristic of most teleosts where sperm development is completed within the germ cysts prior to release into the lumen of the seminiferous tubules and the genital tract. The epithelium of the seminiferous tubules in these species may be involved in phagocytosis of the cytoplasmic remnant from sperm differentiation, as observed in the present study.

Despite the absence of the intratesticular ducts and accessory glands in winter flounder, the testicular efferent duct system plays an important role in sperm release and development of sperm function. This is suggested by the active secretory activities of the duct system occurring during the spawning season, coinciding with the seasonal increases in milt production and sperm quality.

The absence of ciliated epithelium in this species, as determined by EM, suggests sperm movement along the genital tract does not depend on cilial

movement. The movement and ejaculation of sperm are more likely facilitated by contraction of the duct wall and a dramatically increased hydration of milt during the spawning season, which greatly reduces the viscosity of milt, presumably increasing the hydrostatic pressure in the duct chamber.

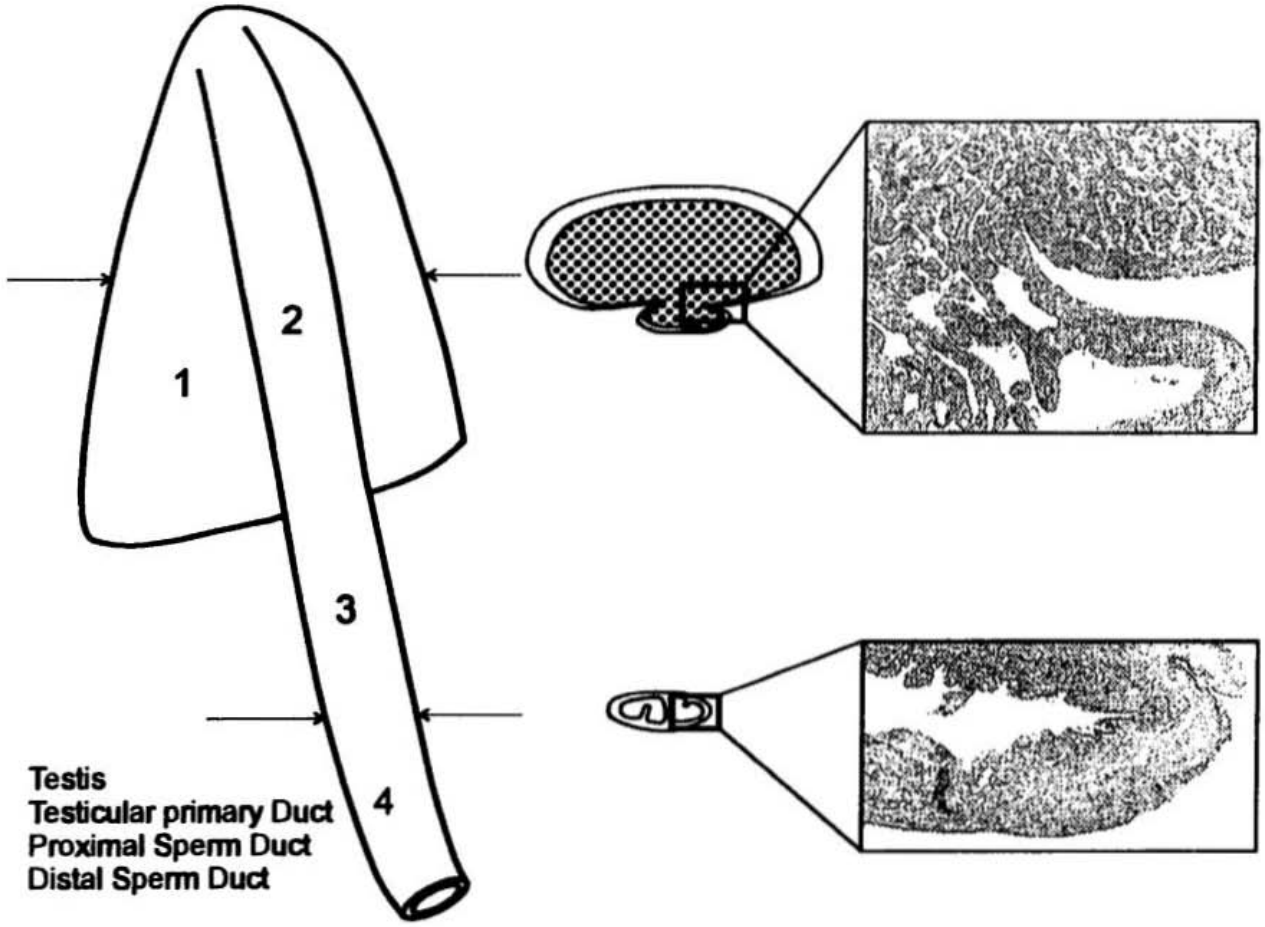
There was no ultrastructural evidence for secretion of glycogen and neutral lipid in the duct epithelium during this study. Again, these phenomena may be associated with the differentiation of winter flounder spermatozoa within the testes before being released into the duct chamber. A similar situation was reported for cyprinid fish (Lahnsteiner *et al.*, 1994b) while the storage and secretion of polysaccharides and/or lipids are prominent in the duct system of jawfish, grunt, blenniid and European pike that all carry out semicystic spermatogenesis (Lahnsteiner and Patzner, 1990; Lahnsteiner *et al.*, 1993a; Manni and Rasotto, 1997; Rasotto and Sadovy, 1995). In rainbow trout, although sperm complete metamorphosis in the testis (van den Hurk *et al.*, 1978a), final sperm maturation takes place in the spermatic duct (Morisawa and Morisawa, 1986; Morisawa and Morisawa, 1988). Likewise, the secretion of lipids by the epithelium of the efferent duct system is evident (Lahnsteiner *et al.*, 1993a). There is no structural evidence suggesting intracellular energy storage of glycogen or lipid droplets within mature flounder spermatozoa. Therefore, the sperm may depend on extracellular energy resources. The utilization of monosaccharides has been demonstrated in sperm of cyprinid fish which are similar to winter flounder in the absence of glycogen and lipid

secretion by TEDS epithelium (Lahnsteiner *et al.*, 1994b). Some lipid components, such as triglycerides, may be the potential energy resources for spermatozoa. It was suggested that spermatozoa of fish employing external fertilization oxidize lipids for energy metabolism (Mounib, 1967). The ability to catabolize these substrates has been confirmed in the spermatozoa of trout and grayling, *Thymallus thymallus* (Lahnsteiner *et al.*, 1991; Stoss, 1983). Based on the ultrastructural demonstration of active cellular synthesis and material transport during the spawning season, the duct system of winter flounder likely contributes to sperm nutrition and maintenance in a manner that needs to be clarified through other approaches, such as histochemical or biochemical analysis.

According to seasonal observations of the milt production, the majority of mature flounder spermatozoa are stored in the testes (or the lumina of the seminiferous tubules) during the prespawing phase, with release into the TEDS only at the onset of the spawning season. While sperm are released in small amounts into the lumina of the duct system during the prespawning phase, there is evidence that they may age and be absorbed by the epithelium (the present study). Phagocytosis is not as prominent in the testis as in the duct during the spermiation period. Therefore, it appears that the testes function as longterm storage sites with spermatozoa stored at a high concentration. The TEDS may accommodate spermatozoa only for a short period of time, in which they are "conditioned" before final sperm ejection.

Parallel seasonal increases in the secretory activity of the TEDS epithelium and sperm quality suggest that the duct system may be involved in the mediation of sperm activities. In salmon, the sperm duct promotes the development of movement capacity to move through mediating pH medium in the seminal fluid (Miura *et al.*, 1992; Morisawa *et al.*, 1993). However, unlike salmon (Morisawa and Morisawa, 1986; Morisawa and Morisawa, 1988), flounder sperm from the testes are motile when diluted by seawater (unpubl. observations). Actual mechanisms by which the TEDS influences the development of flounder sperm function requires further investigation.

Fig 20 Schematic view of the organization of the testis and the testicular efferent duct system of winter flounder.1: Testis, 2: The testicular primary duct, 3: The proximal sperm duct, 4. The distal sperm duct.



1: Testis
2: Testicular primary Duct
3: Proximal Sperm Duct
4: Distal Sperm Duct

Fig 21 Photograph of histological sections of winter flounder testicular efferent duct system.

A) Traverse section through the medial part of testis in the early gonadal recrudescence phase; T: testis, PD: testicular primary ducts.

B) Traverse section through the sperm duct (SD) in the early phase of milt production; many pits (Pt) formed from the surface or within the small (secondary) epithelial folds (F).

C) Traverse section of the testis in the early phase of milt production showing increased pits (Pt) in the primary duct (PD). T: testis; Sp: sperm.

D) Part of primary duct in the early milt production, lots pits (Pt) in inactive secretion; CT: connective tissue; Sp: sperm.

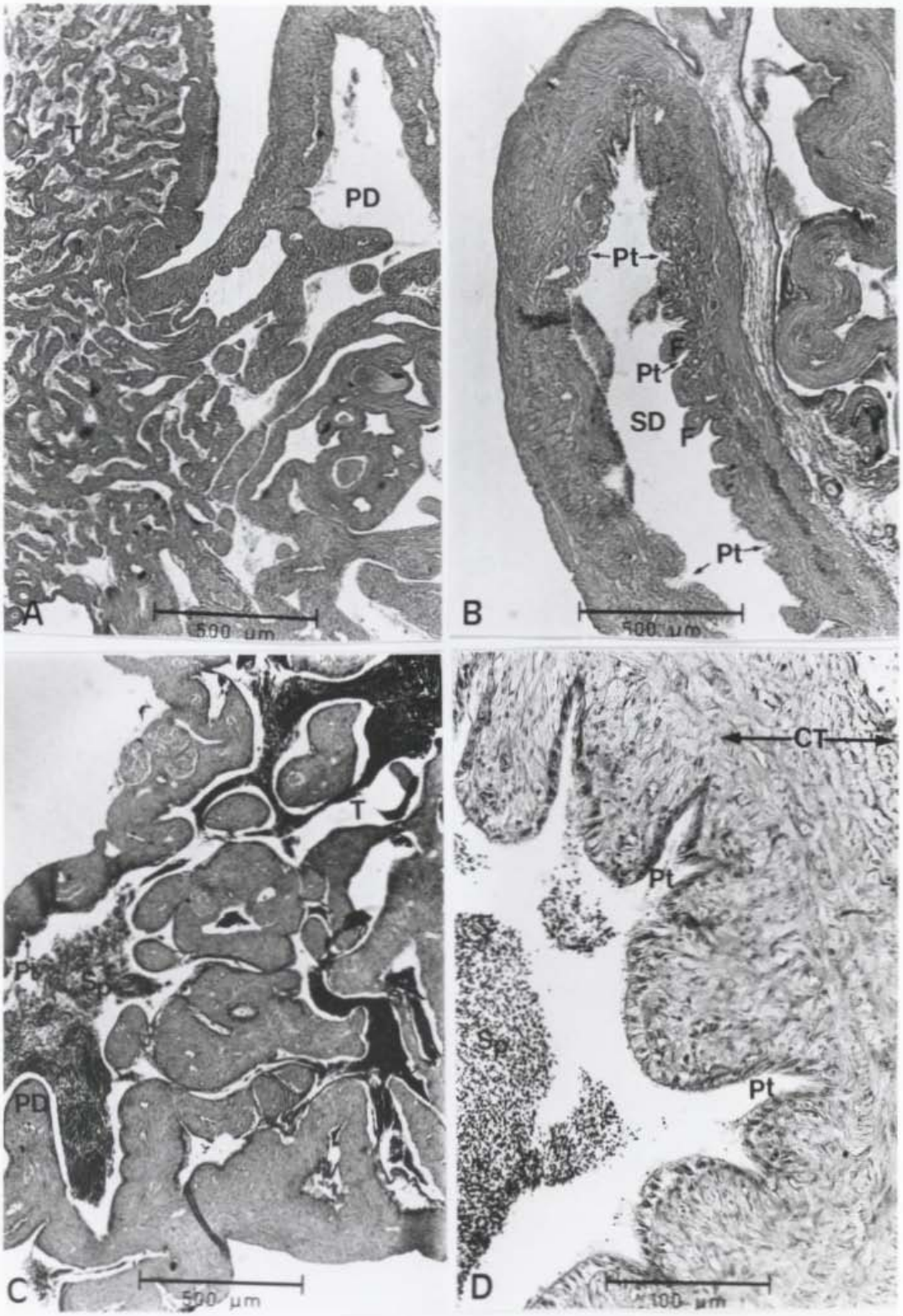

T
PD
A
500 µm
Pt
Pt
SD
Pt
B
500 µm
T
Pt
PD
C
500 µm
CT
Pt
Sp
Pt
D
100 µm

Fig 22 Photograph of histological sections of winter flounder testicular efferent duct system.

E) and **F)** Large amount of secretion (S) on the top of the epithelium (Ep) on the surface (fig E) or within pits (fig F) in the primary duct during the spawning season. Sp: sperm.

G) The epithelium of the sperm duct in the postspawning phase mostly composed of cuboidal (Cb) and low columnar cells (LC) without secretion; No secretion on the tall columnar cells (TC) too.

H) The epithelium (Ep) of the sperm duct showing the vacuolated cytoplasm containing engulfed sperm (arrows).

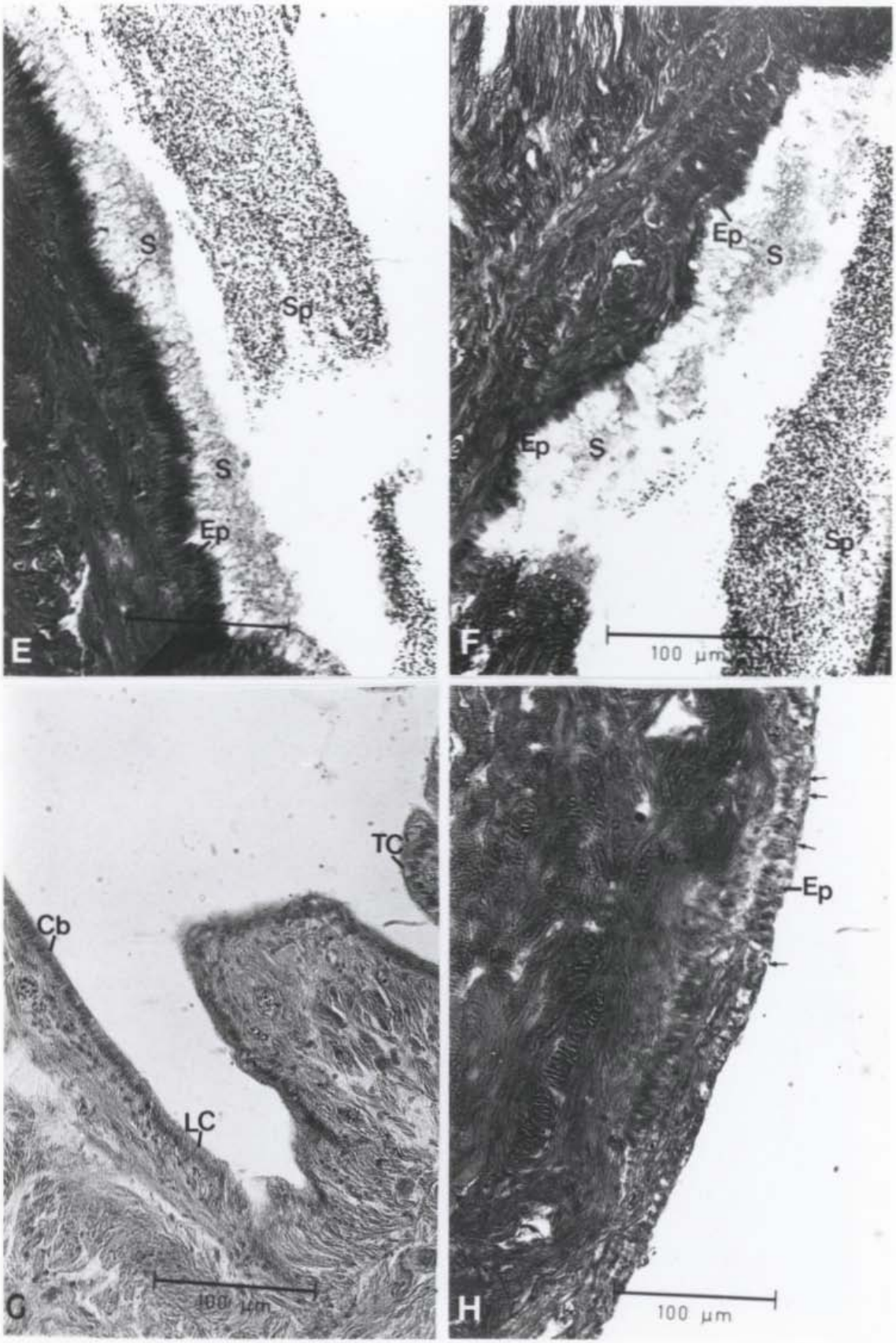
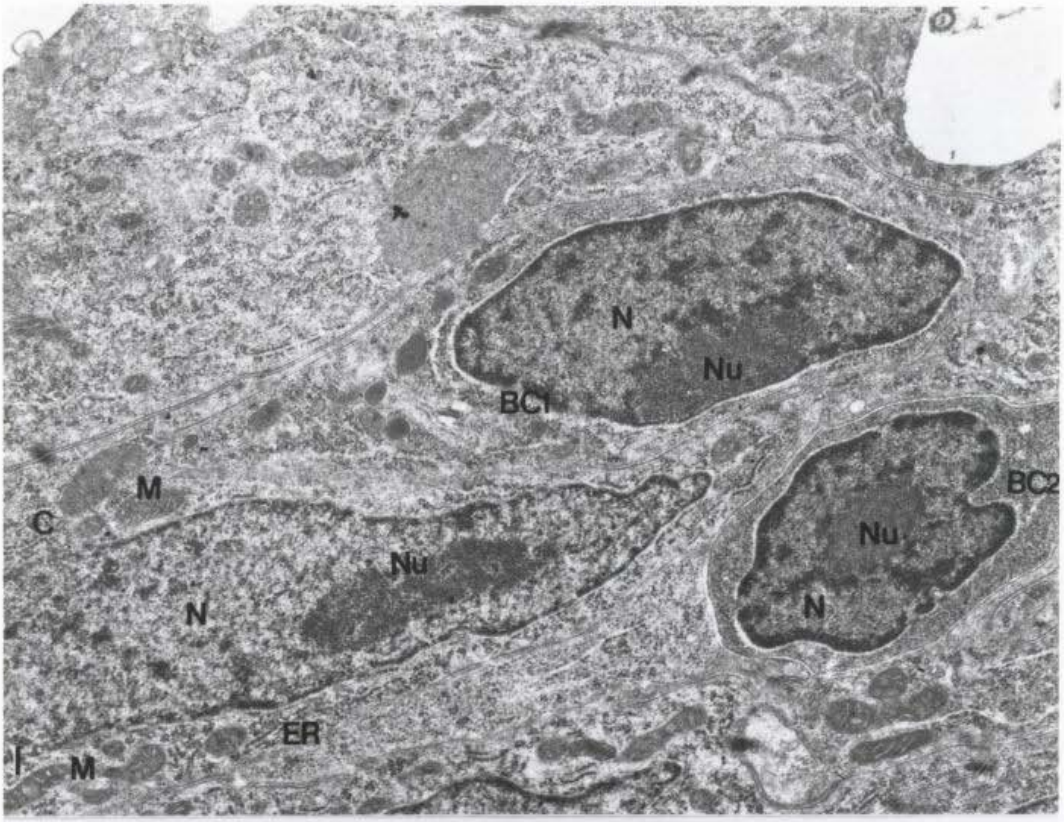

S
Sp
S
Ep
E
Ep
S
Ep
S
Sp
F
100 µm
TC
Cb
LC
G
100 µm
Ep
H
100 µm

Fig 23 TEM photograph of the epithelium of the testicular efferent duct system.

I) Two basal cells (BC1 and 2) located among the columnar cells (C), displaying a large nucleus (N) and small amount cytoplasm with few organelles. Nu: nucleolus, M: mitochondria, ER: endoplasmic reticulum. (x 20,520).

J) Columnar cells, showing mitochondria (M) with lamellar cristae and rough endoplasmic reticulum (rER) parallelly arranged. N: nucleus, BL: basal lamina, M: mitochondria. (x 9,120).


N
Nu
BC1
BC2
M
C
Nu
Nu
N
N
ER
M

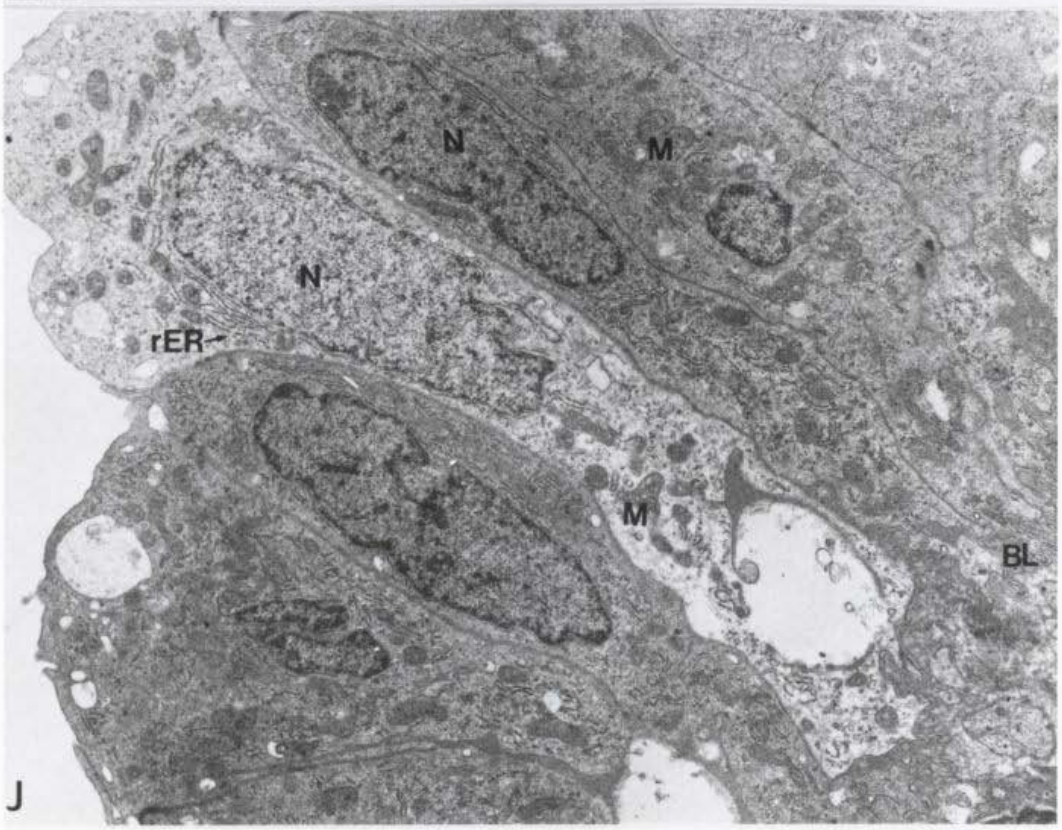

N
M
N
rER
M
BL
J

Fig 24 TEM photograph of the epithelium of the testicular efferent duct system.

K) Columnar cells in the early gondal recrudescence: An irregular nucleus (**N**) with much hetero-chromatin, and a few organelles. **M**: mitochondria, **Vc**: vacuoles, **ZO**: zonula occludens, **ZA**: zonula adherens, **MO**: macula adherens, **Mf**: microfilament. (x 20,520).

L) Columnar cells with numerous mitochondria (**M**) and interdigitation (**ID**) along the cellular border. **N**: nucleus, **Nu**: nucleolus, **Mv**: microvillia, **BL**: basal lamina. (x 6,384).

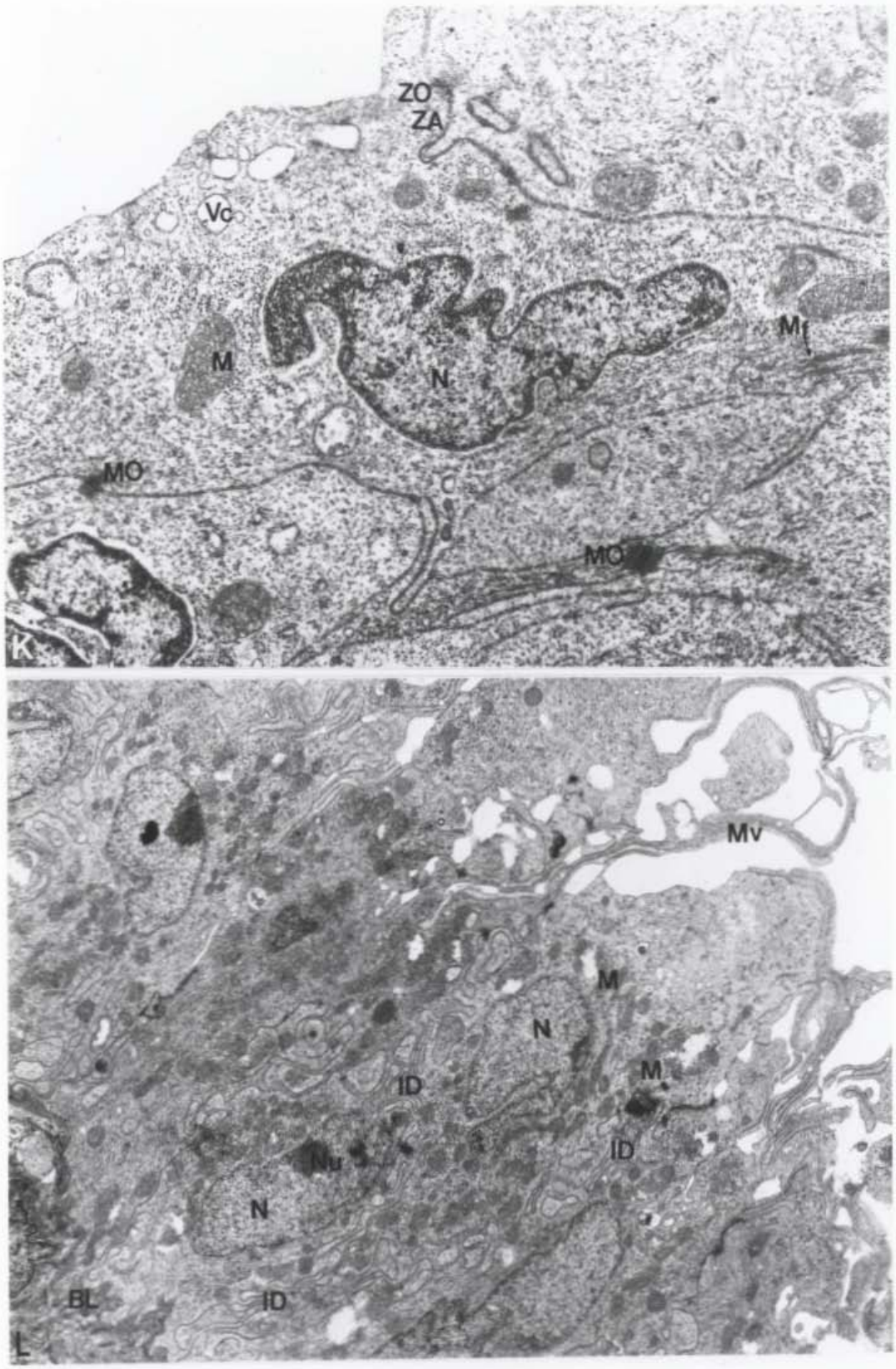

ZO
ZA
Vc
M
N
Mf
MO
MO
K
Mv
M
N
M
ID
ID
Nu
N
BL
ID
L

Fig 25 TEM photograph of the epithelium of the testicular efferent duct system.

M) Large number of mitochondria (M) supranuclearly located in a columnar cell. **N**: nucleus, **Nu**: nucleolus, **rER and sER**: rough ad smooth endoplasmic reticulum, **G**: Golgi apparatus, **ZO**: zonula occludens, **ZA**: zonula adherens, **MO**: macula adherens. (x 16,188)

N) Apocrine secretion(Ap) in a surface columnar cell (C). **M**: mitochondria, **Mv**: microvillia, **Vc**: vacuoles. (x 10,260)

O) Active pinocytosis (small arrows) in a columnar cell; fusion of pinocytic vesicles visible (large arrows). **N**: nucleus, **Nu**: nucleolus, **rER and sER**: rough and smooth endoplasmic reticulum, **Mf**: microfilament, **BL**: basal lamina, **sPv**: smooth pinocytic vesicles, **cPv**: coated pinocytic vesicles. (x 24,282)

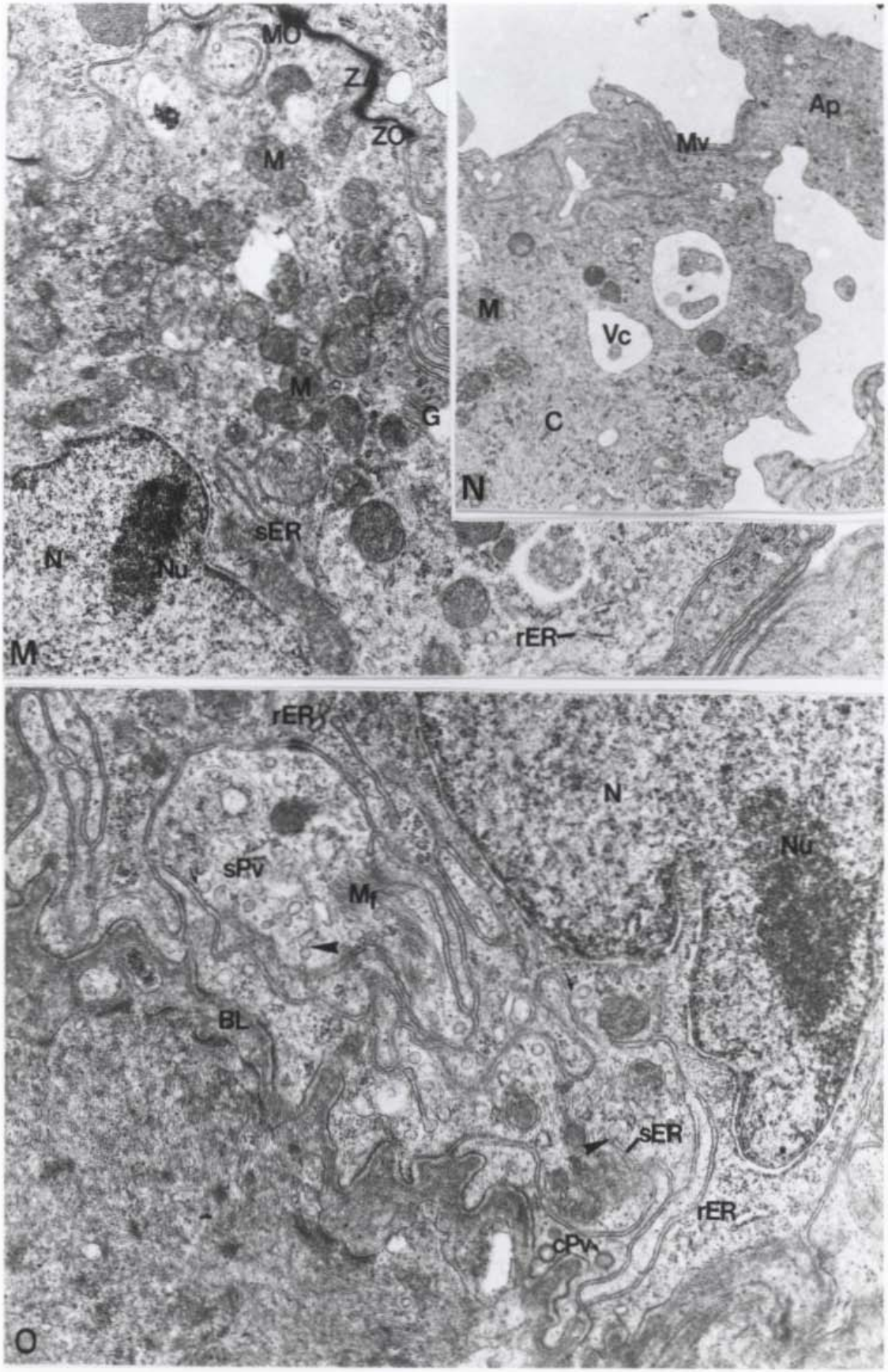

MO
Z
ZO
M
M
G
N
Nu
sER
rER
M
Ap
Mv
M
Vc
C
N
rER
sPv
Mf
BL
N
Nu
sER
rER
cPv
O

Fig 26 TEM photograph of the epithelium of the testicular efferent duct system.

P) Developed Golgi apparatus (G) in a columnar cell closely associated with rough endoplasmic reticulum (rER) and mitochondria (M). N: nucleus, Pv: pinocytic vesicles, GV: Golgi vesicles (lysosome-like). (x 24,282)

Q) Columnar cells within pits showing apocrine secretion (Ap) with low electron-dense contents, and interdigitation (ID) along which numerous pinocytic vesicle are formed. N: nucleus, Nu: nucleolus, M: mitochondria, Vc: vacuoles, BL: basal lamina, Mv: microvilli. (x 12,312)

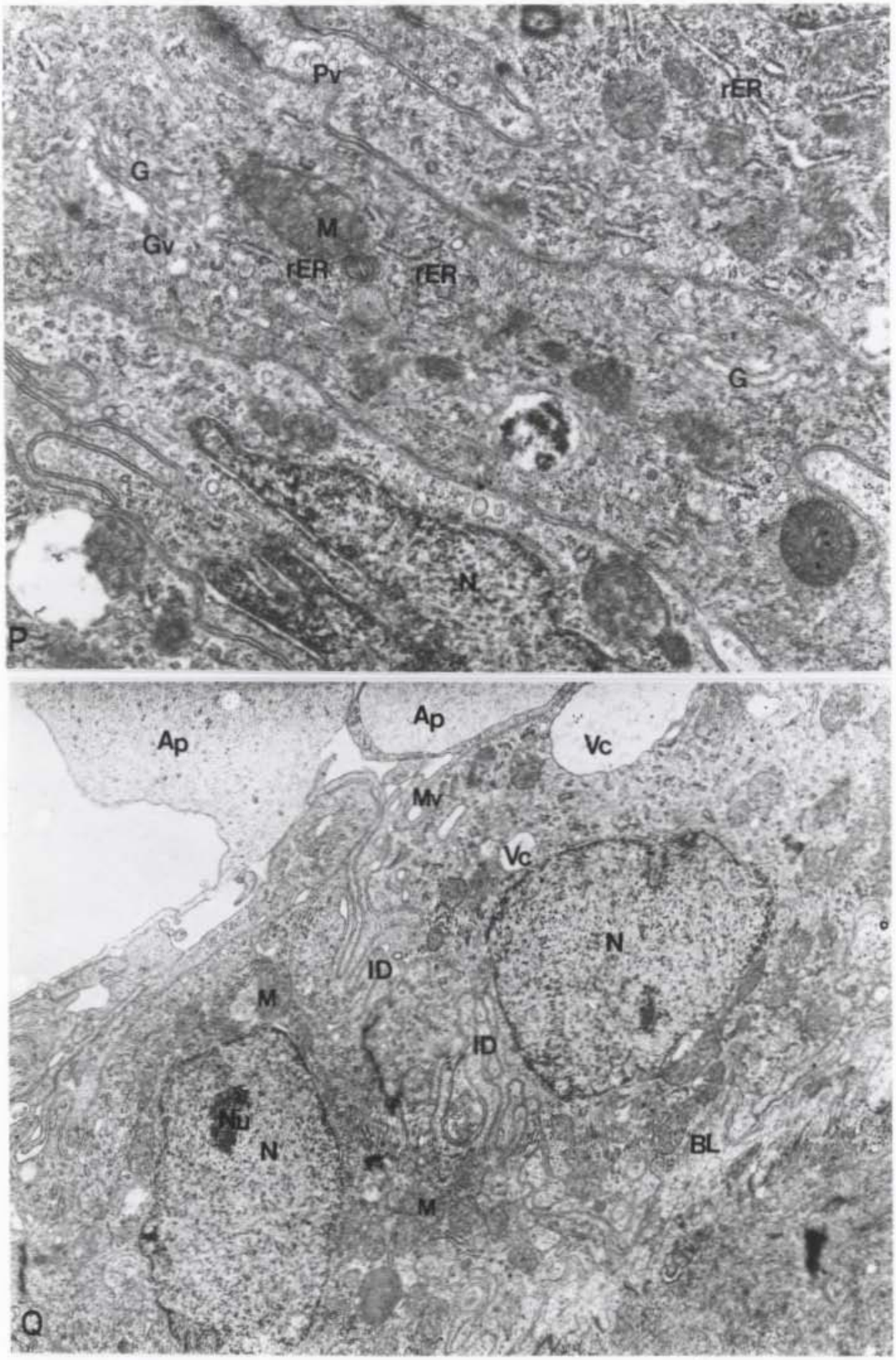

Pv
rER
G
M
Gv
rER
rER
G
N
P
Ap
Ap
Vc
Mv
Vc
N
ID
M
ID
Nu
N
BL
M
Q

Fig 27 TEM photograph of the epithelium of the testicular efferent duct.

R) and **S)** Cuboidal cells in the efferent duct system with developed cytoplasmic organelles, pinocytosis on the cellular border (arrows, fig R), and apocrine secretion (Ap) on the top (fig S). Mv: microvillia; N: nucleus, M: mitochondria, rER and sER: rough and smooth endoplasmic reticulum, Vc: vacuoles, Pv: pinocytic vesicles, ZO: zonula occludens, ZA: zonula adherens, MO: macula adherens, Mf: microfilament. (x 25,082).

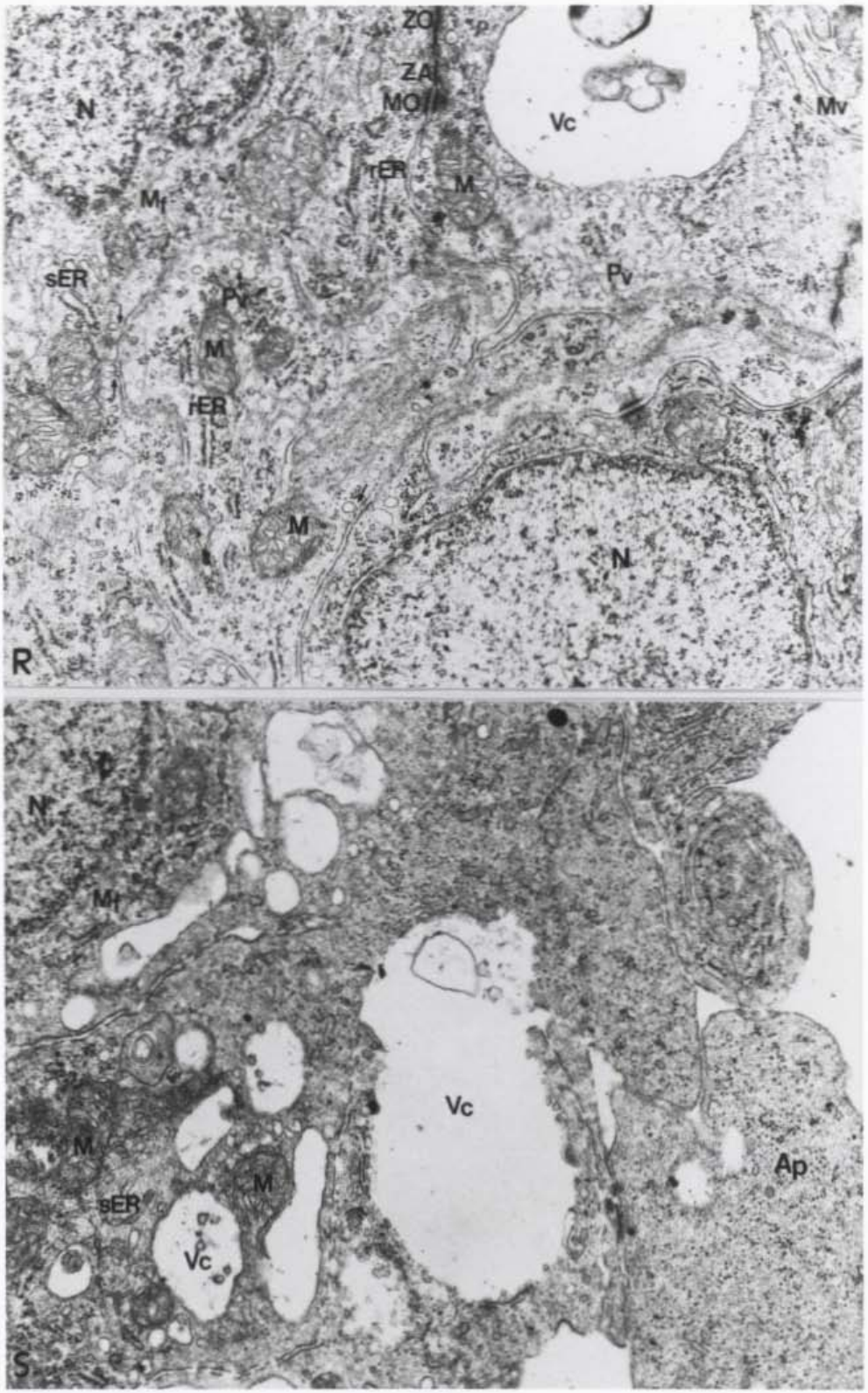

ZO
ZA
MO
N
Vc
Mv
rER
M
Mf
sER
Pv
M
iER
M
N
R
N
Mf
Vc
M
sER
M
Vc
Ap
S

Fig 28 TEM photograph of the epithelium of the seminiferous tubules in testis. **T)** and **U)** Cuboidal cells in the testis. **N**: nucleus, G: Golgi apparatus, **M**: mitochondria, sER & rER: smooth and endoplasmic reticulum, Vc: vacuoles, Vs: vesicles, LB: lamella bodies, RV: residual vacuoles, MVs: multivesicles, Mf: microfilament, Mv: microvillia. (x. 12,141).

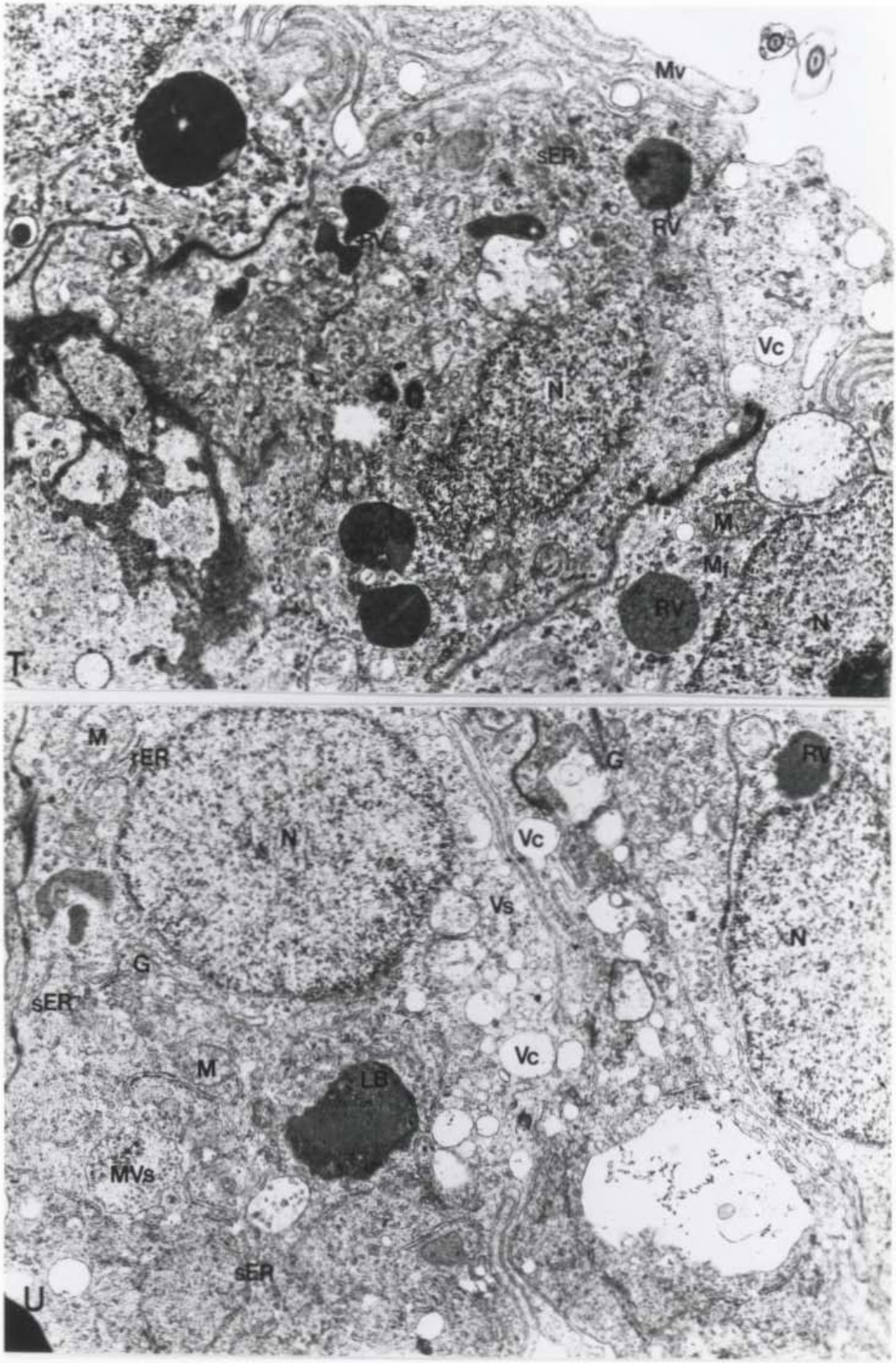

Mv
sER
RV
RV
Vc
N
M
Mf
RV
N
T
M
rER
N
G
sER
M
MVs
sER
U
LB
G
RV
Vc
Vs
N
Vc

Fig 29 TEM photograph of the epithelium of the testicular efferent duct system.

V) Secretory cells in the pits of the epithelium of the sperm duct. **N**: nucleus, **Nu**: nucleolus, **M**: mitochondria, **Sg**: secretory granules, **S**: secretion, **Vc**: Vacuoles, **BL**: basal lamina. (x 11,172)

W) Columnar cells in the postspawning phase; engulfed sperm (Sp) visible within the columnar cells. **N**: nucleus, **M**: mitochondria, **Ap**: apocrine secretion, **S**: secretion, RV: residual vacuoles. (x 6,384)

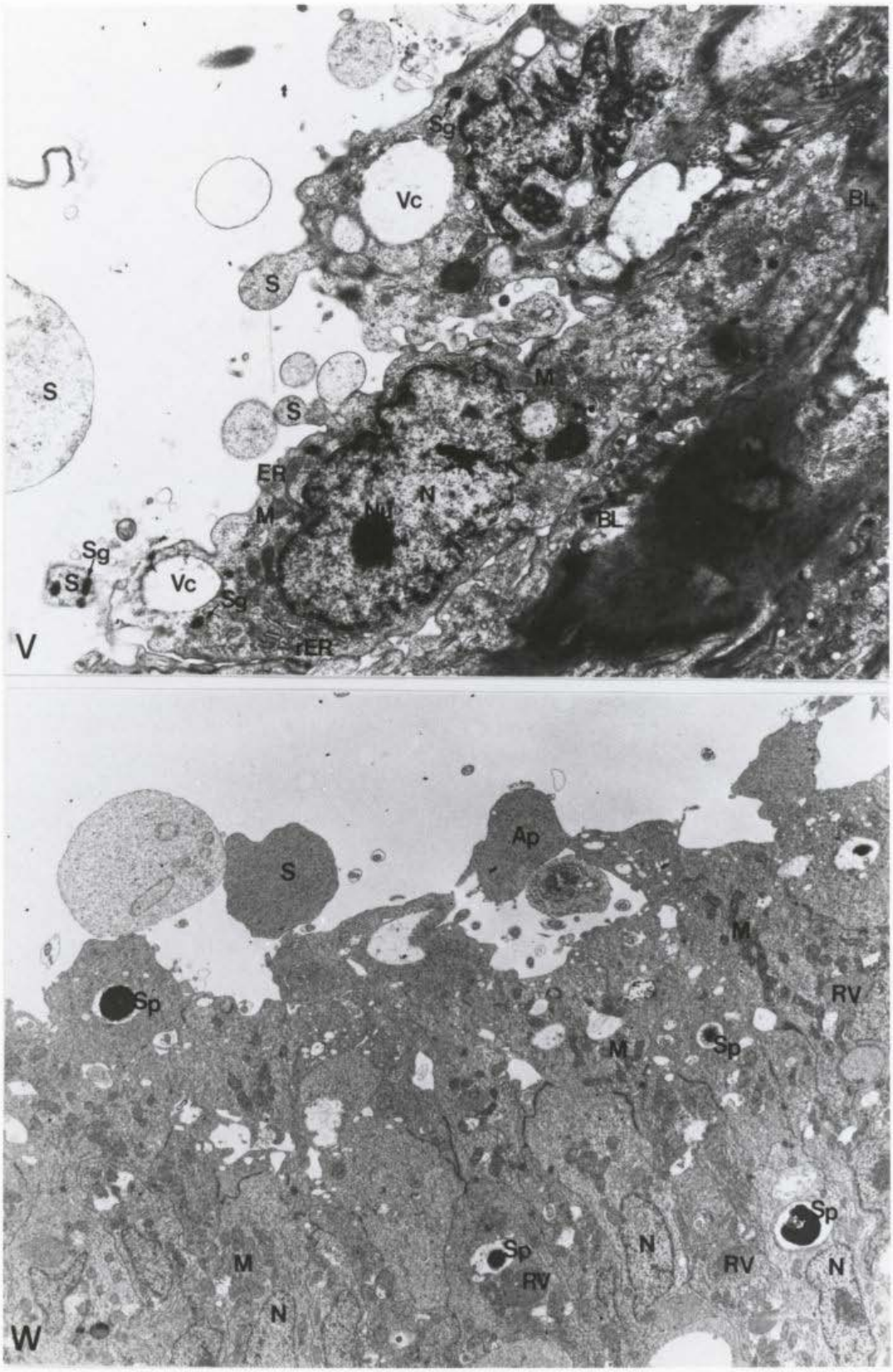

Sg
Vc
BL
S
S
M
S
ER
N
M
Nu
BL
Sg
S
Vc
Sg
rER
V
Ap
S
M
Sp
RV
M
Sp
Sp
Sp
N
M
RV
N
RV
N
W

Fig 30 TEM photograph of the epithelium of the testicular efferent duct system.

X) Columnar cells within pits in the postspawning phase; some of them contain large secretory vacuoles (SV) or residual vacuoles (RV). **N**: nucleus, **M**: mitochondria. (x 5,746)

Y) Necrotic surface columnar cells (**NC**) within which are small, dense nucleus and numerous residual vacuoles (**RV**) and large vacuoles (Vc). **N**: nucleus. (x 7,980)

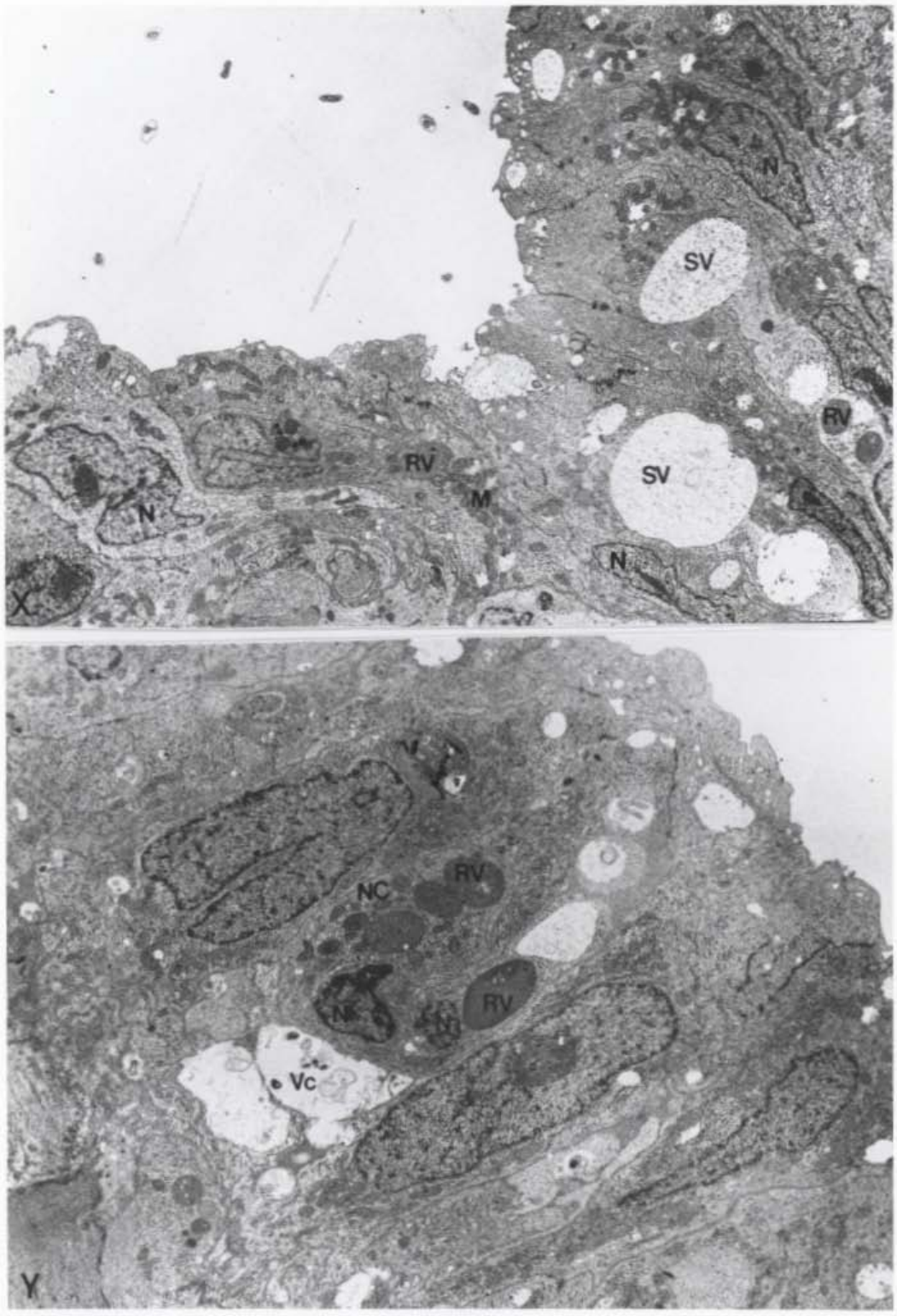
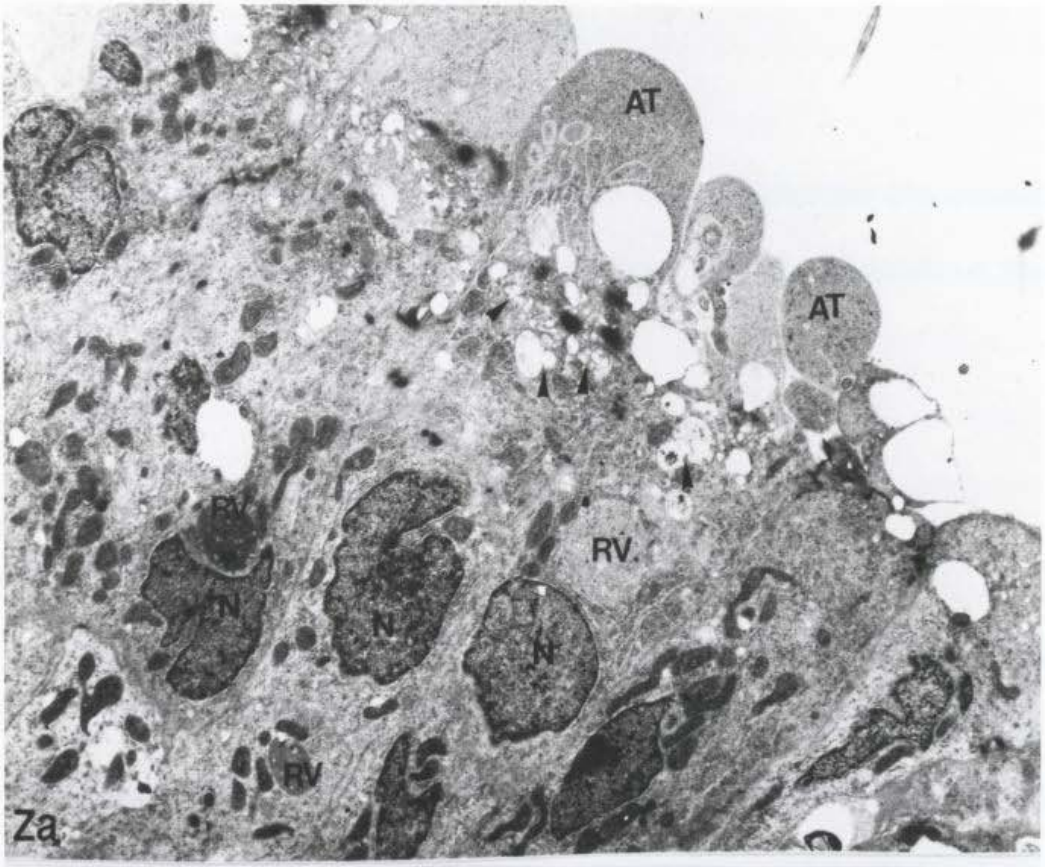
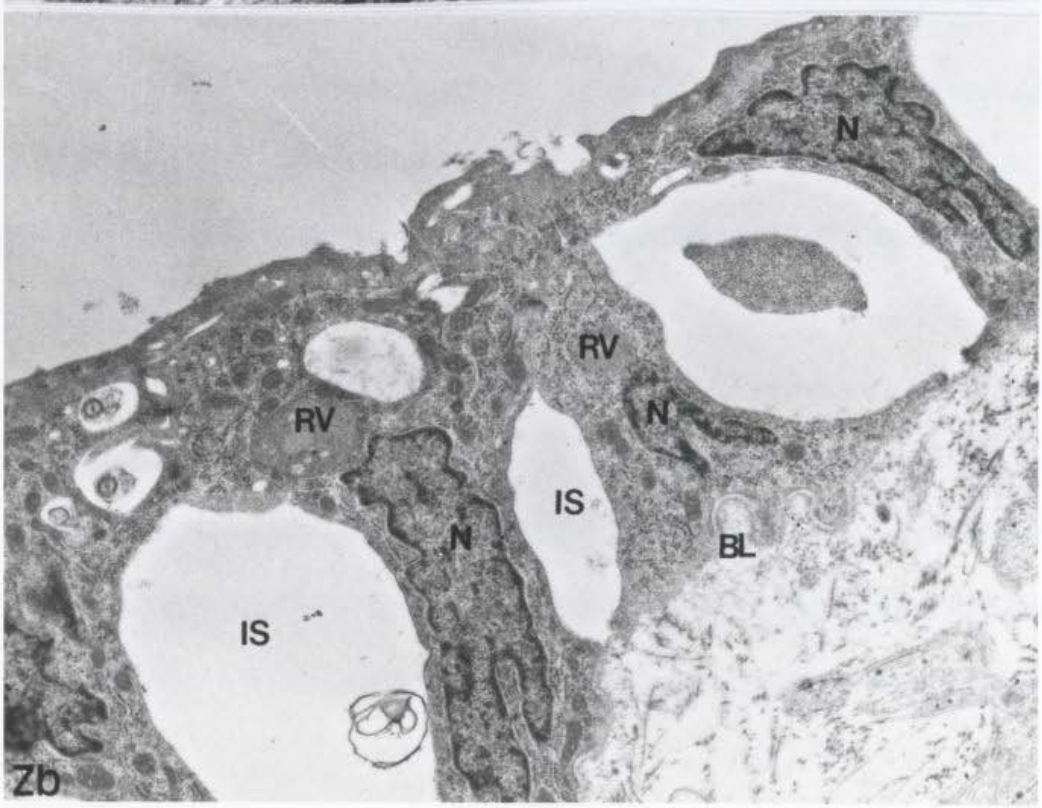

N
SV
RV
SV
RV
M
N
N
X
NC
RV
RV
Vc
Y

Fig 31 TEM photograph of the epithelium of the testicular efferent duct system.

Za) Necrotic epithelium in the postspawning phase. The apical portions (AT) of cell cast off through a fusion of vacuoles (arrows) on the supranuclear region while the rest of the cells also show the sign of the cell degeneration. **N**: nucleus, **BL**: basal lamina, RV: residual vacuoles, (x 5,745)

Zb) The collapse of a group of epithelial cells. **N**: nucleus, **RV**: residual vacuoles, BL: basal lamina, IS: intercellular space. (x 13,680)


AT
AT
RV
RV
N
N
N
RV
Za


N
RV
RV
N
IS
N
BL
IS
Zb

CHAPTER 7

The Testicular Efferent Duct System of Male Winter Flounder *(Pleuronectes americanus)*: Evidence of a Role in Mediation and Maintenance of Sperm Functions

7.1. Introduction

The role of the testicular efferent duct system (TEDS) in sperm development and maturation is critical in teleosts undertaking the "semicystic" type of spermatogenesis, where final differentiation and maturation of spermatozoa occurs in the testicular efferent duct system (Manni and Rasotto, 1997). However, for the majority of teleosts that employ external fertilization, sperm metamorphosis is completed within the testicular germinal cysts prior to release into the testicular efferent duct system. The involvement of the duct system in the development of sperm in these teleosts remains unclear. Studies with salmonid fish and the Japanese eel, *Anguilla japonica,* have demonstrated that the capacity of movement in sperm only occurs after spermatozoa move into the sperm duct (Morisawa and Morisawa, 1986; Morisawa and Morisawa, 1988; Ohta *et al.,* 1997). In one strain of rainbow trout, sperm motility increases in the sperm duct, although motile sperm can be observed in the testis (Koldras *et al.,* 1996). These results suggest that the

duct in teleosts may be responsible for a process similar to the acquisition of the movement potential of spermatozoa in the epididymis of mammals and other higher vertebrates (Orgebin-Crist, 1980). On the other hand, a few studies suggested that the TEDS has an important role in the storage of differentiated spermatozoa (Lahnsteiner and Patzner, 1990; Lahnsteiner *et al.*, 1993b; Lahnsteiner *et al.*, 1994b). Maintenance of the quality of mature sperm appears particularly important, since males often mature considerably earlier than females (such is the case in winter flounder), and spermatozoa may be stored for several months prior to liberation during the spawning season.

The present study investigated the role of the TEDS in winter flounder in the mediation of sperm viability through a comparison of sperm gathered from the testis and different parts of the TEDS. By sampling sperm stored over shorter or longer intervals, changes in milt characteristics and sperm quality within the duct system were examined. In addition, the functional activities of the TEDS was studied during the spawning season.

7.2. Materials and methods

7.2.1. Experiment design

Male winter flounder were obtained from the field about one week before the start of two experiments beginning either in May (groups A1 and A2) or June

(groups B1 and B2), during the middle to late spawning season in 1995 and 1996[2]. Milt from each group of males was collected twice: First, milt was initially handstripped from the genital pore (sample I), following which milt was collected directly from the testis and from different portions of the testicular efferent duct system (sample II) after either a short (9 days, group A1 and B1) or long (17-18 days, group A2 and B2) interval (Table 8). After the first sampling, the males were held without females and checked within 1-2 days for recovery of milt production. For the second sampling, male flounder were anaesthetized in 0.15-0.20 % phenoxyethenol (v/v) and a 1 ml syringe was used to collect milt from the following four portions of the reproductive tract: 1) the testis (through small cuts on the anterior and posterior ends of testis), 2) the testicular primary duct, 3) the proximal sperm duct and 4) the distal sperm duct (Fig 20).

7.2.2. Evaluation of milt properties and sperm quality

Milt spermatocrit, pH values, and sperm quality (motility and swim duration) measurements were determined as previously described (Chapter 2) for all freshly collected milt samples. In addition, sperm motility and swim duration were tested after 24 h storage in $DCSB_4$ buffer (milt : buffer=1:100, 4 °C) for milt obtained in the sampling II from the males in groups A1, B1 and B2. The ability of sperm from

[2] Groups A1, B1 and B2 were examined in 1995 while group A2 was in 1996.

groups B1 and B2 to fertilize eggs was examined by counting gastrula embryos one day after *in vitro* artificial insemination (Chapter 2).

7.3. Results

7.3.1. May experiment

All males had expressible milt within two days after the initial sampling in both groups beginning in May (groups A1 and A2). According to observations of milt obtained in the second collection (sample II), milt characteristics varied depending upon the site of milt collection along the male reproductive tract (Fig 32). Milt was concentrated upon collection directly from the testes as indicated by very high spermatocrit values (> 90%), becoming progressively dilute after collection along the testicular efferent duct system (40~70% spermatocrit). Milt was alkaline in pH throughout the TEDS, although pH was significantly lower when milt was collected directly from the testes.

Sperm from the testes showed the capacity to be activated and swim. However, once into the duct system, sperm motility significantly increased (ANOVA; $f = 9.41$, $df = 3$, $P < 0.001$). While the swim duration was similar for sperm in the testes and in the primary duct, the duration of sperm motility diminished slightly when it was collected at the more distal sites of the sperm duct (sites 3 and 4; Fig 32).

Through a comparison of samples II collected from group A1 and A2,

differences in sperm quality were not pronounced when sperm were stored in the TEDS for varying periods of time (Fig 32). Although, the pH values of milt were lowered by the longer storage time (group A2) in the sperm duct and the testis, the only other changes were a slight reduction in sperm swim time in the distal sperm duct and a lowering of sperm motility in the testis.

Sperm collected in May in the middle of the spawning season retained its quality (motility and swim duration) after dilution with $DCSB_4$ and cold storage for 24 h irrespective of the sites of collection (Fig 33, group A1).

7.3.2. June experiment

Again, in June, males (groups B1 and B2) recovered quickly from milt removal, since milt was collectable within two days of stripping. Based upon the second sampling (II), males studied later in June produced milt of similar characteristics, e.g. spermatocrit and pH values as compared to May in corresponding portions of the genital tract (Fig 34). These milt parameters also changed within the male genital tract in a way which are similar to observations made previously. Generally, sperm motility of sample II in late June was lower compared to earlier in the season (A1 cf. B1, A2 cf. B2) and did not improve after sperm were released into the primary duct. Instead, sperm motility tended to decrease along the TEDS in parallel with gradually decreasing sperm swim duration. Similarly, no significant changes in sperm fertility were demonstrated for

milt collected at the various sites along the reproductive tract at this portion of the season (Fig 35).

After varying periods of storage *in vivo*, reduction in sperm quality in the sperm duct was evident for both groups of males (sample II cf. Sample I), including reduced motility for group B1, and reduced motility and swim time for B2 (Fig 34). Compared with the short sampling regime (group B1), sperm swim time was reduced in all portions of the genital tract of male sampled after the longer interval (B2).

A decline in sperm quality for group B2 became particularly prominent following 24 h storage *in vitro* in extension buffer, while no significant reduction in sperm quality by such storage was observed within most portions of the TEDS in males for milt collected after a short sampling interval (B1, Fig 33).

7.4. Discussion

7.4.1. Effects of the testicular efferent duct system on the mediation and maintenance of sperm quality

Based on these experimental observations, a proportion of testicular winter flounder spermatozoa are mature and have the capacity for movement. This differs from observations made for freshwater species (or strain) showing that testicular sperm are immotile in rainbow trout and chum salmon or nearly immotile in the Japanese eel (Morisawa and Morisawa, 1986; Morisawa and Morisawa, 1988; Ohta

et al., 1997). Evidence for full reproductive capacity of sperm stored in the testes of winter flounder was further demonstrated by showing their ability to fertilize eggs - up to 80% fertilization rate at the sperm : egg ratios examined. However, the motility of winter flounder sperm was significantly increased in the primary duct during the middle of the spawning season, suggesting that the environment of the testicular efferent duct system can improve sperm motility. Improved motility in the sperm duct was also reported for trout sperm (Billard, 1976; Koldras *et al.*, 1996). In the latter study, sperm motility increased from 40 to > 80%, comparing collections from the testis and the sperm duct respectively.

Factors responsible for such increases in sperm motility are not clear. In flounder, the pH is increased in the seminal plasma and the milt has a lower sperm density in the sperm duct as compared with the testes. Increased pH from 7.5 in the testis to 7.9~8.2 in the seminal fluid in the sperm duct of rainbow trout was correlated with the acquisition of motility by sperm (Miura *et al.*, 1992; Morisawa and Morisawa, 1988). The increased pH seems related to sperm motility in flounder, since pH values were found significantly correlated with sperm motility ($r=0.39$, $P < 0.001$, $N=124$ or $r=0.49$, $P < 0.001$, $N=72$ for fresh sperm motility or motility after 24 h storage, respectively). A significant positive relationship between pH and sperm motility was also reported in cyprinid fish, *Alburnus alburnus* (Lahnsteiner *et al.*, 1996). Likewise, a slightly alkaline environment is optimal for halibut sperm motility as demonstrated by Billard, et al (1993a). The increased alkaline pH may

be related to changes in seminal fluid composition and/or sperm metabolic activities. For example, in rainbow trout, bicarbonate concentration in seminal fluid increased in the sperm duct (Morisawa and Morisawa, 1988), possibly the result of increased production of CO_2 associated with elevated cellular respiratory metabolism and energy synthesis. Sperm motility in cyprinid fish is positively linked to the spermatozoa levels of ATPase and key enzymes related to glycolysis, such as lactate dehydrogenase, pyruvate kinase and adenylate kinase, indicating relatively higher metabolic levels in sperm with higher motility (Lahnsteiner *et al.*, 1996). The increase in metabolism may not only be related to maintenance metabolism but also to the storage of energy used during the motile phase. Externally fertilizing fish sperm consuming a large amount of energy during the motile phase require intracellular storage of ATP during the immotile phase (Perchec *et al.*, 1995). Elevated pH promotes the acquisition of sperm motility by increasing the cAMP content in sperm cells (Miura *et al.*, 1992; Morisawa and Morisawa, 1988). However, late in the season, although the pH values and sperm density of milt did not significantly change compared with milt characteristics earlier in the season, improvement of sperm quality was no longer evident. This suggests that other factors are also involved during the development of sperm in the male genital tract (see discussion below).

Besides influencing development or maturation of sperm, the efferent duct system may serve an important role in preserving sperm quality during storage.

Sperm motility remained relatively high for up to 18 days in all three parts of the duct system during the middle of the spawning season. In contrast, for milt stored *in vitro* at cool temperatures without dilution, sperm quality (motility) is usually retained for only 1-2 days based on my observation for this species. The resistance of halibut sperm to cryopreservation declines even more rapidly, within a few hours during storage *in vitro* (Billard *et al.*, 1993a). Therefore, the aging process is apparently slowed by the conditions in the efferent duct system compared with storage of sperm *in vitro*.

In this study, a decline in sperm quality first became detectable in the distal portions of the efferent duct system, as manifested by a reduction in the duration of sperm motility. The reduction in swim time may be related to decreased intracellular energy storage or a less efficient mitochondrial function. In salmonid sperm, the decrease in intracellular ATP levels during the spawning season was associated with shorter duration of swimming in sperm (Benau and Terner, 1980). Stored ATP is thought to be the major energy supply required for sperm movement in carp and trout (Christen *et al.*, 1987; Perchec *et al.*, 1995) since the compensation of intracellular ATP used during the motile phase is limited, likely due to a low mitochondrial oxidative phosphorylation capacity in sperm possessing transient movement (Christen *et al.*, 1987). In carp, ATP synthesis by mitochondria can only maintain about 30% of the initial energy storage measured before sperm activation (Perchec *et al.*, 1995). In halibut, sperm motility was completely blocked

by respiratory inhibitors, suggesting no intracellular ATP storage (Billard *et al.*, 1993a). However this remains to be verified since there are no data concerning halibut sperm ATP levels before and after sperm activation. Although no data exists for changes in ATP levels in winter flounder sperm, structural data do not suggest a high oxidative potential. In addition, although the spermatozoa of flatfish species appear similar in structure, the physiological status of spermatozoa may differ between species. For example, halibut spawn in winter soon after the completion of spermatogenesis, while sperm in winter flounder mature early and may be stored for 5-6 months prior to release during spawning. The long prespawning storage period may also be responsible for a reduced duration of sperm motility in the TEDS of winter flounder in contrast to observations in other teleosts, where sperm swim time is increased along the sperm duct (Koldras *et al.*, 1996).

Results from the present study also indicate that sperm cell aging is a progressive process. Reduction in sperm swim time may be the initial indication of a degradation of cell function. The motile ability of sperm is thereafter lost gradually during aging. However, even when sperm are no longer activated in seawater, some sperm may still remain alive and the capacity for movement may be recovered given certain critical factors. For example, in this study some restoration of sperm motility was observed after 24 h *in vitro* storage in an immobilizing buffer (Fig 33, group B1). Some preliminary observations also indicated that the loss of sperm motility resulting from storage *in vitro* can also be restored by a similar incubation

(Shangguan and Crim, unpubl.). The phenomenon may also account for some deviation between sperm motility and sperm fertility (correlation between sperm motility and fertilization rate in this study: r= 0.73-0.81, P < 0.01) since I also observed *in vitro* stored immotile sperm become activated by egg extract[3].

7.4.2. Seasonal variations in the functions of the testicular efferent duct system

Fluctuations in sperm quality during the spawning season were also evident in this study, similar to previous seasonal observations. These results raise questions such as "Are these seasonal fluctuations in sperm quality related to the functional activities of the testicular efferent duct system ? "

In this study, three types of changes in sperm motility along the efferent duct system were recognized: 1) increasing, 2) increasing followed by a decrease, and 3) decreasing (Fig 36). In males with types I and II, sperm quality increased entering the TEDS; however, the quality did not improve in males of type III Interestingly, the frequency of these changing patterns display a seasonal trend within the period of these observations with mostly type I and II occurring earlier in the season while type III predominates later in the season (Table 9). In addition, results from this study also indicated that good sperm quality lasted up to 18 days

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[3]Freshly collected flounder eggs were homogenized in seawater (~1:10 in volume), and the supernatant was used after centrifuging at 1,500 x g for 10 min.

of storage *in vivo* in the middle of the spawning season (Fig 32). However, late in the season, a similar storage resulted in a decline in sperm quality (Fig 34). Furthermore, comparisons of sperm quality collected from corresponding portions of the TEDS indicated that a decline in sperm quality during the late season only occurred in the TEDS (Fig 37). Taken together, the evidence suggests that the role of the TEDS in the development and maintenance of sperm function is subject to seasonal variations which are likely related to the seasonal changes in sperm quality observed in the present and previous studies.

In rainbow trout, biweekly examination of sperm in the testis and the sperm duct did not show apparent seasonal variation in sperm motility except for a change of testicular sperm motility which peaks in the middle of February just before the largest milt volume can be collected from the sperm duct (Koldras *et al.*, 1996). This may be due to the differences in sperm maturation between species, as well as sampling regime and time. After the first collection, male winter flounder were held without females to suppress spontaneous spawning, which may block sperm release, altering sperm quality. Additionally, this study was carried out during the last portion of spermiation period, and thus different from the study by Koldras *et al.* (1996) which was conducted in the middle of the spermiation period.

Table 8 Milt collection and sampling frequency in male winter flounder during the spawning season.

Group	No of Fish	Sampling time (Date I, II)		Sampling interval (day)
A1	5	May	(May 15, 24)	Short (9)
A2	6	May	(May 20, June 7)	Long (18)
B1	5	June	(June 8, 17)	Short (9)
B2	4	June	(June 5, 22)	Long (17)

Table 9 Seasonal frequency of the sperm motility changing pattern along the genital tract of male winter flounder.

Group	Date of sample II	Type[a]	I	II	III
		No of Fish.	%	%	%
A1	May 24	5	60	40	0
A2	June 7	6	50	50	0
B1	June 17	5	20	20	60
B2	June 22	4	0	50	50
Total		20	35	40	25

[a] Types of motility changing pattern:

Type I: A general increasing pattern although motility of sperm may remain unchanged in the first part or the latter portion(s) of the testicular efferent duct system.

Type II: Sperm motility increases first, followed by a decline in the latter portion(s).

Type III: Decreasing pattern. Usually sperm motility remains unchanged in the primary duct but decreases in more distal parts of the duct system.

Fig 32 Comparison of sperm quality and milt properties for milt collected during the middle spawning season at short (A1) or long (A2) sampling intervals. The left panel shows the data from the first milt sampling (sample I); the right panel represents the data from the second sampling (sample II) where milt was obtained from different locations of the genital tract including 1) the testis, 2) the primary duct, 3) the proximal sperm duct, and 4) the distal sperm duct. Significant differences are indicated by different letters for a comparison among the different sites of the genital tract (ANOVA; $f = 27.33$, $df = 3$, $P < 0.001$ for spematocrit; $f = 7.75$, $df = 3$, $P < 0.001$ for pH; $f = 9.41$, $df = 3$, $P < 0.001$ for motility; $f = 8.54$, $df = 3$, $P < 0.001$ for swim duration) . Difference between two groups in sample II: * $P < 0.05$ or ** $P < 0.01$ (t-test; pH: $t = 6.08$, $df = 8$, $P < 0.001$ for site 1, $t = 4.24$, $df = 9$, $P < 0.01$ for site 3; $t = 4.50$, $df = 9$, $P < 0.01$ for site 4 respectively; motility: $t = 3.63$, $df = 9$, $P < 0.05$ for site 1). Values are mean±s.e..

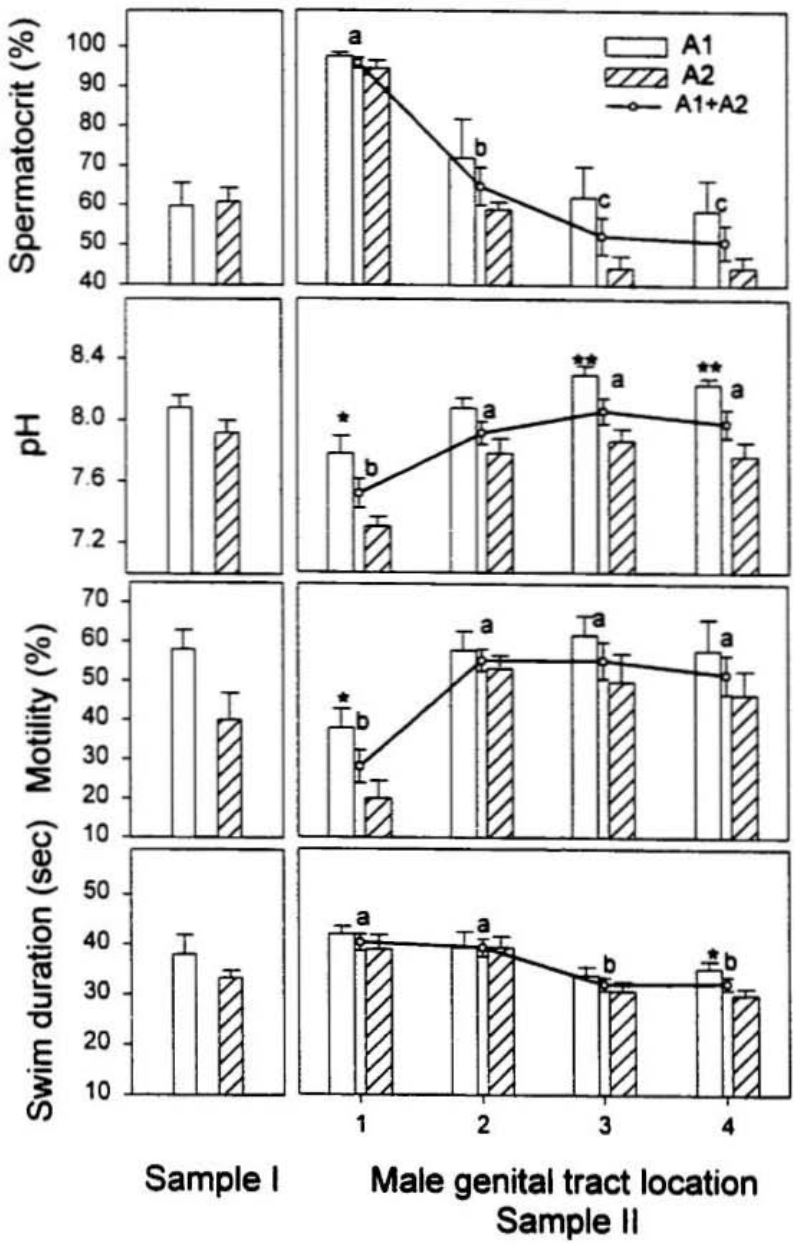

Spermatocrit (%)
100
90
80
70
60
50
40
A1
A2
A1+A2
a
b
c
c
pH
8.4
8.0
7.6
7.2
*
**
**
Motility (%)
70
60
50
40
30
20
10
Swim duration (sec)
50
40
30
20
10
1
2
3
4
Sample I
Male genital tract location
Sample II

Fig 33 Comparison of sperm quality measured freshly or after 24 hr storage in $DCSB_4$ buffer. Milt was collected from different sites of the genital tract including 1) the testis, 2) the primary duct, 3) the proximal sperm duct, and 4) the distal sperm duct from groups A1, B1 and B2. Significant differences between two measurements: * $P < 0.05$, ** $P < 0.01$ [t-test; motility: $t = 2.88$, $df = 8$, $P < 0.05$ (B1, site 3), $t = 6.83$, $df = 6$, $P < 0.001$ (B2, site 2), $t = 4.89$, $df = 6$, $P < 0.05$ (B2, site 3), and $t = 2.95$, $df = 6$, $P < 0.05$ (B2, site 4); swim duration: $t = 4.85$, $df = 6$, $P < 0.01$, (B2, site 2), $t = 2.68$, $df = 6$, $P < 0.05$ (B2, site 3) and $t = 8.04$, $df = 6$, $P < 0.01$ (B2, site 4).Values are mean±s.e..

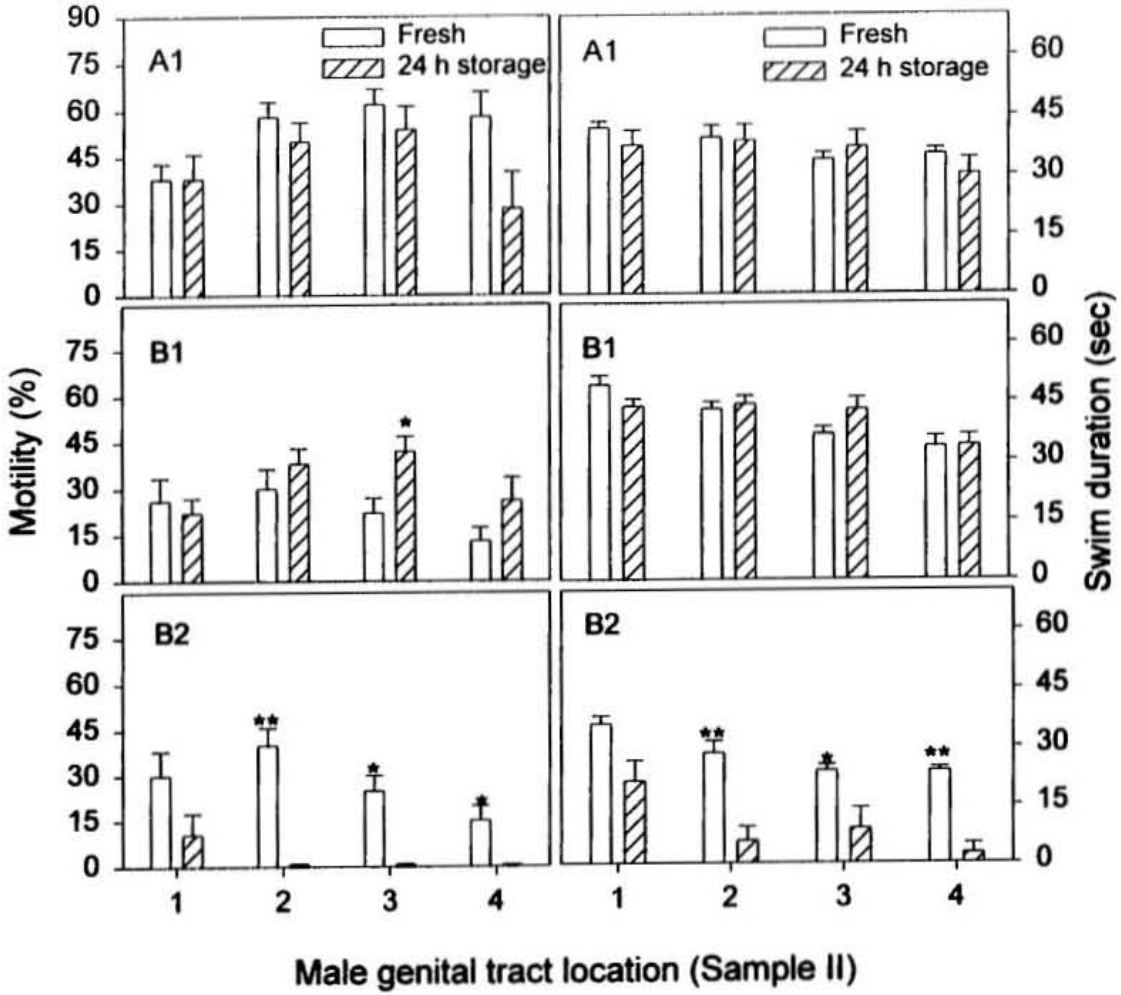

A1
Fresh
24 h storage
B1
B2
Motility (%)
Swim duration (sec)
Male genital tract location (Sample II)
1
2
3
4

Fig 34 Comparison of sperm quality and milt properties for milt collected later in the spawning season at short (B1) and long (B2) sampling intervals. The left panel shows the data from the first milt sampling (sample I); the right panel represents the data from the second sampling (sample II) where milt was obtained from different locations of the genital tract including 1) the testis, 2) the primary duct, 3) the proximal sperm duct, and 4) the distal sperm duct. Significant differences are indicated by different letters for a comparison among different sites of the genital tract (ANOVA; spermatocrit: $f = 7.14$, $df = 3$, $P < 0.01$ for B1, $f = 25.7$, $df = 3$, $P < 0.001$ for B2; pH: $f = 45.44$, $df = 3$, $P < 0.001$ for B1, $f = 50.01$, $df = 3$, $P < 0.001$ for B2; swim duration: $f = 10.11$, $df = 3$, $P < 0.001$ for B1, $f = 8.26$, $df = 3$, $P < 0.01$ for B2). Differences between two groups:* $P < 0.05$ or ** $P < 0.01$ (t-test; swim duration: $t = 3.28$, $df = 7$, $P < 0.05$ for sample I, $t = 4.23$, $df = 7$, $P < 0.01$, $t = 4.13$, $df = 7$, $P < 0.01$, $t = 5.31$, $df = 7$, $P < 0.01$, and $t = 4.8$, $df = 7$, $P < 0.05$ for sample II site 1 - 4 respectively). #: significant differences between sample I and II (site 3 and 4) for milt collected from same group of males [t-test; motility: $t = 4.42$, $df = 8$, $P < 0.01$(B1, site 3), $t = 2.84$, $df = 6$, $P < 0.05$ (B1, site 4), and $t = 2.83$, $df = 6$, $P < 0.05$ (B2, site 4); swim duration: $t = 3.03$, $df = 6$, $P < 0.05$ (B2, site 3) and $t = 2.47$, $df = 6$, $P < 0.05$ (B2, site 4)]. Values are mean±s.e..

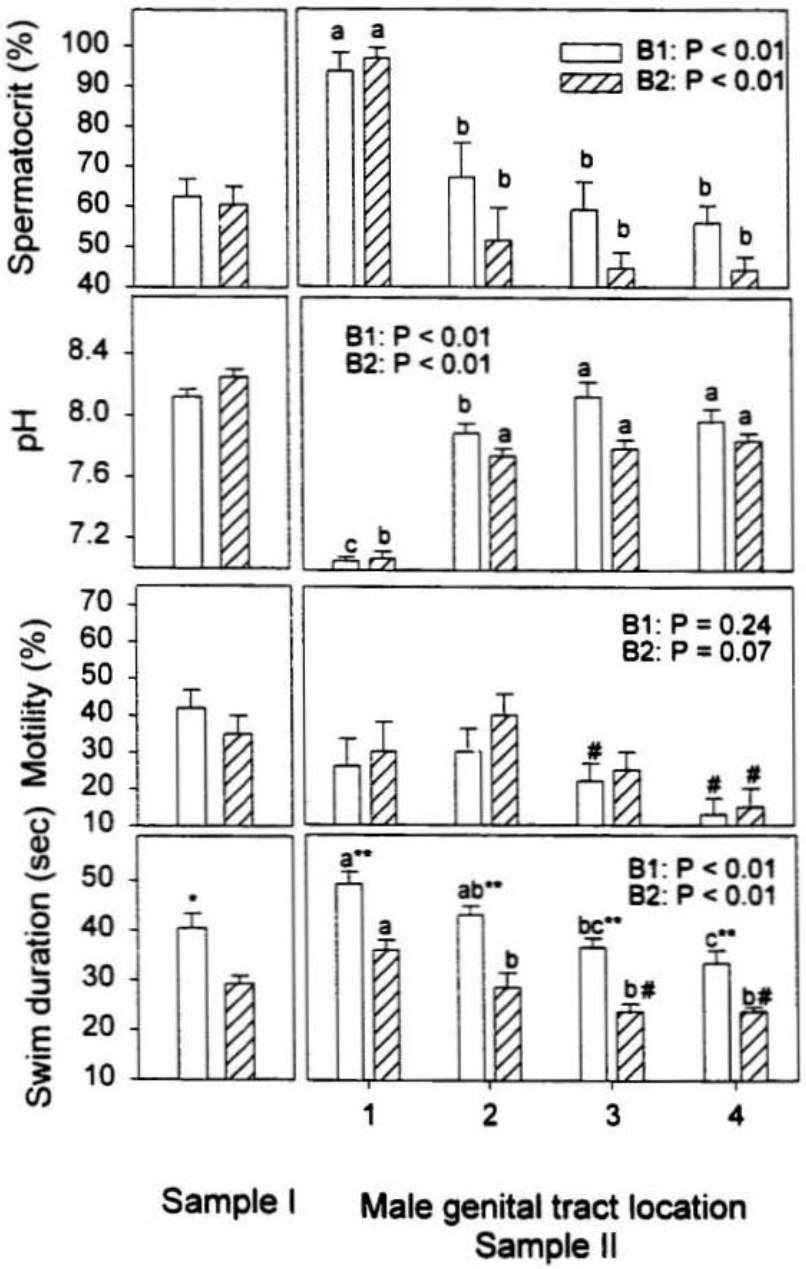

Spermatocrit (%)
100
90
80
70
60
50
40
B1: P < 0.01
B2: P < 0.01
pH
8.4
8.0
7.6
7.2
B1: P < 0.01
B2: P < 0.01
Motility (%)
70
60
50
40
30
20
10
B1: P = 0.24
B2: P = 0.07
Swim duration (sec)
50
40
30
20
10
B1: P < 0.01
B2: P < 0.01
1
2
3
4
Sample I
Male genital tract location
Sample II

Fig 35 Egg fertilization rate for sperm collected in June (groups B1 and B2) from the different sites of male genital tract. 1) the testis, 2) the primary duct, 3) the proximal sperm duct, and 4) the distal sperm duct. Values are mean±s.e..

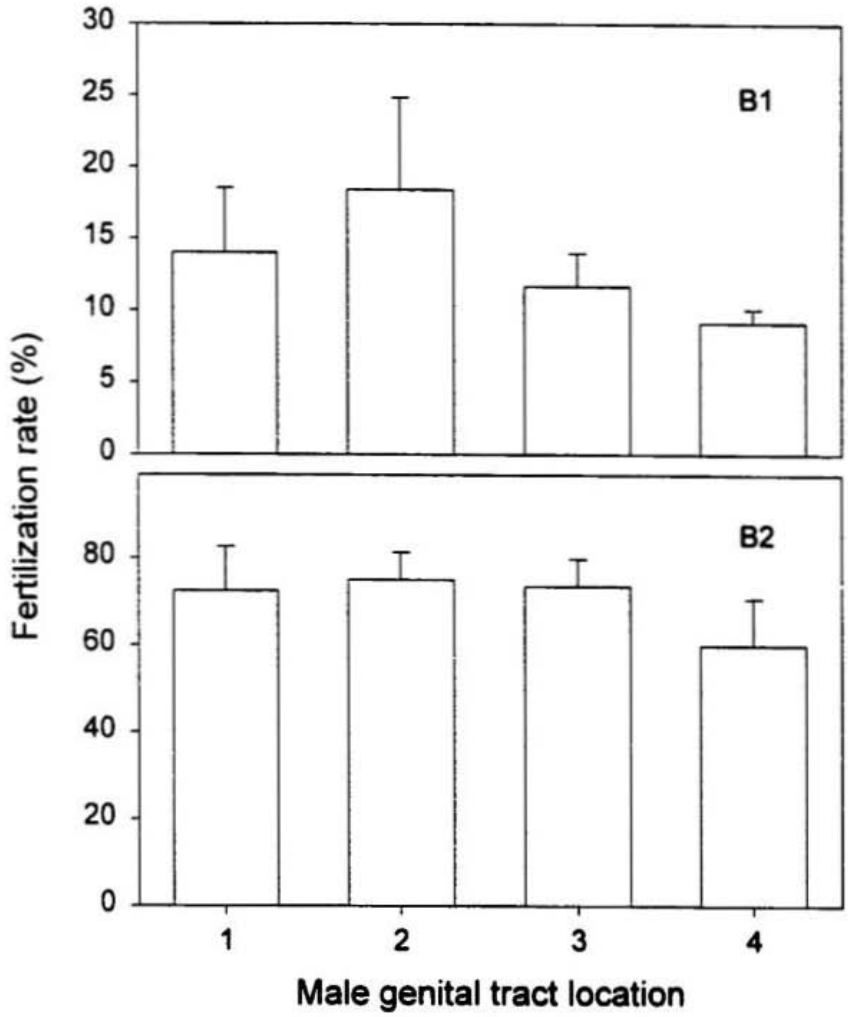

30
25
20
15
10
5
0
B1
80
60
40
20
0
B2
Fertilization rate (%)
1
2
3
4
Male genital tract location

Fig 36 Types of patterns of changing sperm motility along the male genital tract of winter flounder.

Type I: A general increasing pattern although motility of sperm may remain unchanged in the first part or the latter portion(s) of the testicular efferent duct system.

Type II: Sperm motility increases first, followed by a decline in the latter portion(s).

Type III: Decreasing pattern. Usually sperm motility remains unchanged in the primary duct but decreases in more distal parts of the duct system (solid line). An immediate drop in sperm motility in the primary duct has not been observed in the present study (dotted lines).

Large horizontal arrows indicate the release of spermatozoa along male genital tract while lines with small arrows represent changing trends of sperm motility as sperm move towards the genital pore (lines parallel to the X axis: motility does not change; oblique lines: the motility increases or decreases as directed by arrows).

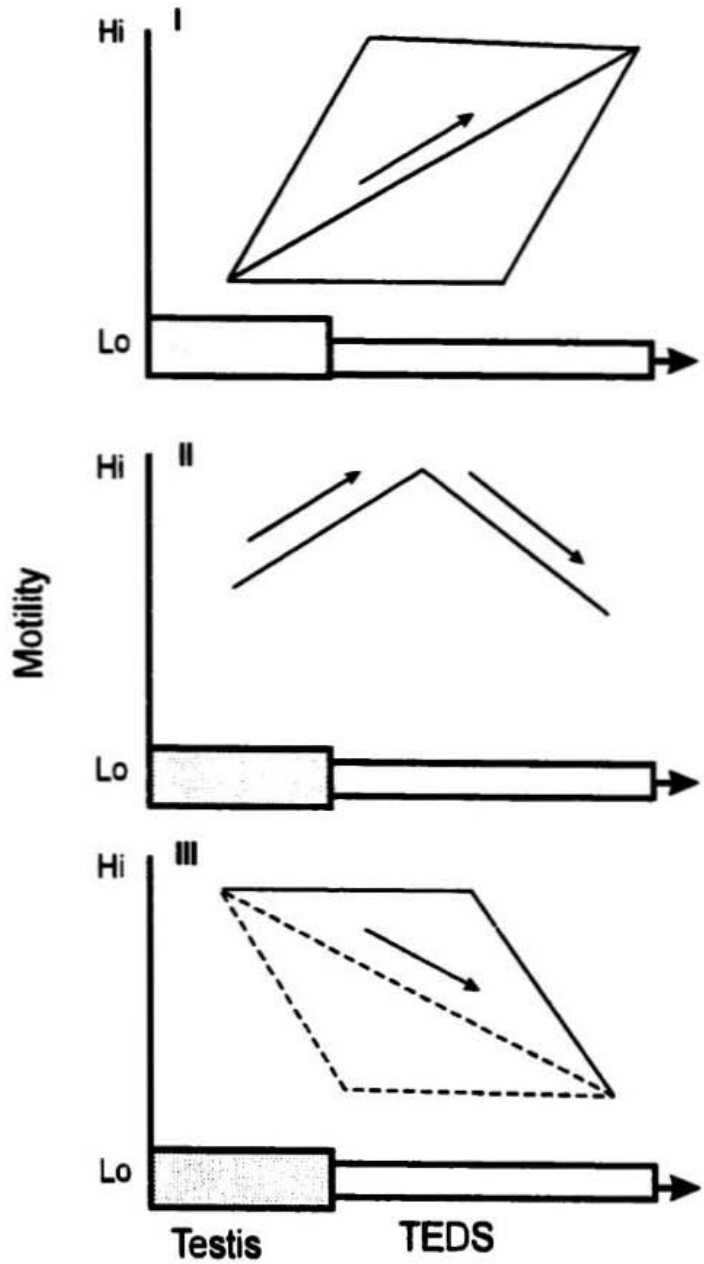
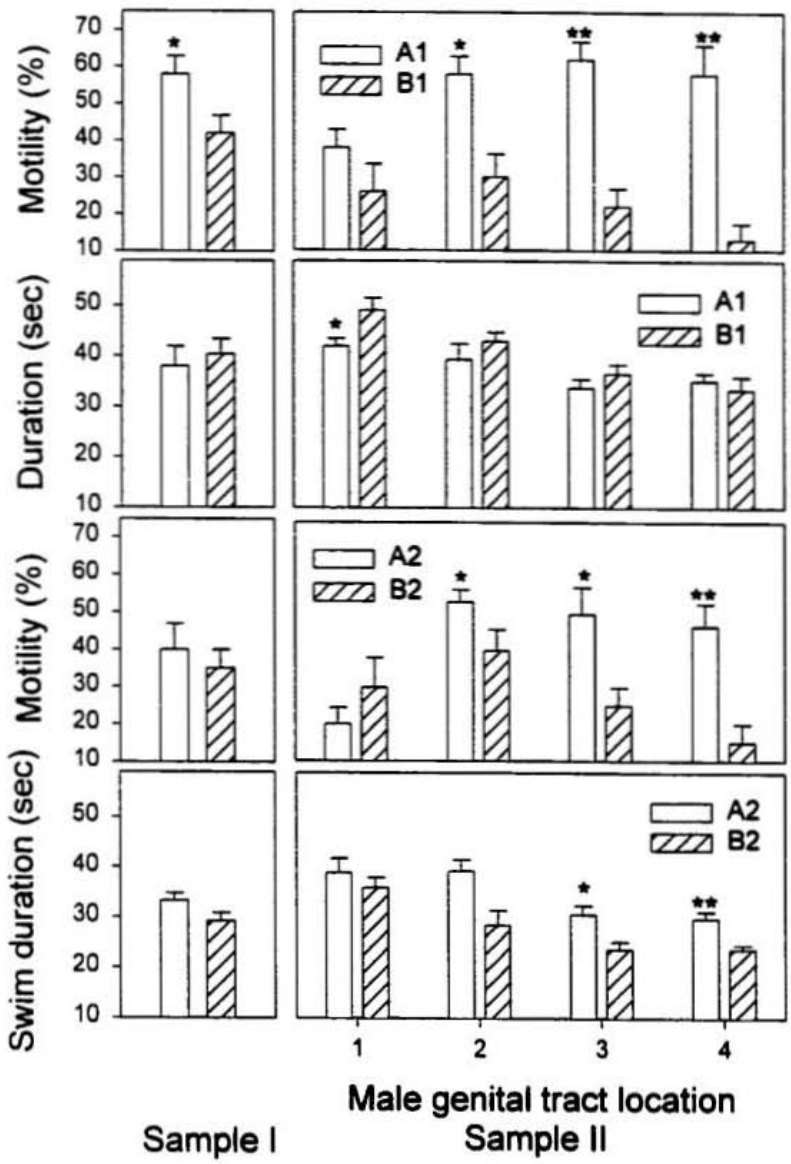

Hi
I
Lo
Hi
II
Motility
Lo
Hi
III
Lo
Testis
TEDS

Fig 37 Seasonal comparison of sperm motility and swim duration along the sperm duct between groups with a short sampling interval (A1 cf. B1) or groups with a long sampling interval (A2 cf. B2). The left panel shows the data from the first milt sampling (sample I); the right panel is the data from the second sampling (sample II) where milt was obtained from different locations of genital tract including 1) the testis, 2) the primary duct, 3) the proximal sperm duct, and 4) the distal sperm duct. Significant differences between two groups: * $P < 0.05$, ** $P < 0.01$ [t-test; motility: $t = 2.31$, $df = 8$, $P < 0.05$ (A1 cf B1, sample I), $t = 3.5$, $df = 8$, $P < 0.01$ (A1 cf B1, sample II, site 2), $t = 5.77$, $df = 8$, $P < 0.001$ (A1 cf B1, sample II, site 3), $t = 4.93$, $df = 8$, $P < 0.01$ (A1 cf B1, sample II, site 4), $t = 2.51$, $df = 8$, $P < 0.05$ (A2 cf B2, sample II, site 3) and $t = 3.99$, $df = 8$, $P < 0.01$ (A2 cf B2, sample II, site 4); swim duration: $t = 2.54$, $df = 8$, $P < 0.05$ (A1 cf B1, sample II, site 1), $t = 2.9$, $df = 8$, $P < 0.05$ (A2 cf B2, sample II, site 2), $t = 2.91$, $df = 8$, $P < 0.05$ (A2 cf B2, sample II, site 3), and $t = 3.62$, $df = 8$, $P < 0.01$ (A2 cf B2, sample II, site 4)]. Values are mean±s.e..


Motility (%)
Duration (sec)
Motility (%)
Swim duration (sec)
A1
B1
A2
B2
Male genital tract location
Sample I
Sample II

CHAPTER 8

Composition of Seminal Fluid in Winter Flounder (*Pleuronectes americanus*): Comparison with Blood Plasma and Association with Sperm Quality

8.1. Introduction

Information regarding the composition of the seminal fluid is essential for an understanding of sperm metabolism, the maintenance of sperm quality, and seasonal changes in sperm quality. Differences in ionic composition between the seminal fluid and the blood plasma have been reported in freshwater and marine fish, leading to the suggestion that potasium (K) plays an important role in suppressing salmonid sperm motility during sperm maturation and maintenance (Morisawa, 1985; Morisawa and Morisawa, 1986). Carbohydrates and lipids are considered to be the major energy sources required for sperm metabolism and function (Lahnsteiner *et al.*, 1991; Stoss, 1983). Changes in seminal plasma protein constituents have been related to the deterioration of sperm structural integrity (Loir *et al.*, 1990), and certain proteinaceous components (such as protease inhibitors) may be involved in the protection of sperm and the regulation of sperm movement (Cosson and Gagnon, 1988; Dabrowski and Ciereszko, 1994, Inaba and Morisawa,

1991). However, despite an increasing knowledge of the properties and composition of the seminal fluid, direct links between the fluid properties or substances and sperm quality still remain unclear. Several components, such as ions, protein, alkaline phosphatase and protease inhibitors, were reported to change along the course of the reproductive season (Dabrowski and Ciereszko, 1994; Kruger *et al.*, 1984; Lou *et al.*, 1990; Pironen, 1985;Wang and Crim, 1997). However, only in rainbow trout (Ciereszko and Dabrowski, 1995) were low levels of seminal plasma ascorbic acid related to reduced sperm motility.

Comparisons of sperm collected from different parts of the reproductive tract demonstrate that the activities of the testicular efferent duct system (TEDS) of the male winter flounder is related to the development and maintenance of sperm function (Chapter 7). Results in chapters 5 and 6 also showed that the secretory activity of epithelium in the sperm duct is highly active during the spawning season, paralleling seasonal variations in sperm production and sperm quality. The studies also indicated significant variations in some milt characteristics, such as milt spermatocrit and pH values during the reproductive season (Chapter 5) or within the TEDS (Chapter 7). This raises questions about the contents of epithelial secretions and whether such secretory substances are linked to changes in the seminal fluid composition, thus resulting in changes in milt properties and sperm quality. The present study characterizes milt properties and seminal fluid composition, including the electrolyte and certain biochemical components, in

comparison with the composition of the blood plasma. Ionic and biochemical constituents of seminal fluid samples from milt displaying a range of sperm quality were also evaluated.

8.2. Material and methods

8.2.1. Collections of seminal fluid and blood plasma

Milt samples were collected during the 1995 spawning season from two groups of male winter flounder (May 15, N=9 and June 5, N=7) after being held in the laboratory for about one week. After the examination of sperm motility, milt spermatocrit and pH values (see Chapter 2 for the description of the procedures), milt samples were centrifuged at 8100 x g for 10 min (4 °C) to separate the seminal fluid and transfered to 0.5 ml plastic vials for storage at -30 °C. Blood plasma samples were collected on June 5, 1994 from six males held in the laboratory for about one month.

8.2.2. Analysis of milt characteristics and biochemical components of seminal fluid and blood plasma.

Osmolality, carbohydrate (glucose), lipids (cholesterol, phospholipids and triglycerides), protein composition and quantity, and some ions (Na, K, Cl, Mg and Ca) in seminal fluid and blood plasma were measured (see Chapter 2). Because the volume of certain blood plasma and milt samples was limited, tests were not

conducted on all samples (see Table 10 for number of sample for each test).

8.3. Results

8.3.1. Sperm quality of milt

Milt samples of differing sperm quality and properties were collected in May and June to evaluate changes in seminal fluid characteristics. For example, sperm quality was greater and spermatocrit lower in May compared with the values recorded in June (Table 11).

8.3.2. Milt characteristics and ionic and organic constituents of seminal fluid and blood plasma

The seminal fluid osmolality ranged from 356-377 mOsm kg^{-1} and was slightly alkaline (Table 12).

According to ionic and organic constituent analysis, both the seminal fluid and the blood plasma contain various ions, lipids and protein (Table 12). Carbohydrate (glucose) was also found in the seminal fluid. However, the data also indicates that lower levels of protein, lipids (PI, ChI), and Na^{+} and Cl^{-} ions exist in the seminal fluid compared with blood plasma.

Electrophoretic profiles demonstrate the existence of major protein bands ranging from 12.5-94 KD in the seminal fluid or 12.5-87 KD in the blood plasma, respectively (Fig 38). Generally, very similar molecular weight proteins were found

in the different individual samples of both fluids except for certain protein bands. For example, a 44 KD protein was absent in some blood plasma samples which instead contain a 12.5 KD protein. The high molecular weight proteins, 74, 80, 87 KD, formed the major blood plasma complement while the 80 KD band was lower in relative amount in the seminal fluid. Additionally, several blood plasma proteins occurred in the 31-35 KD range but a single 33 KD band existed in the seminal fluid.

8.3.3. Seasonal variations in seminal fluid composition

Compared with May, increased levels of protein, lipids (Chl, Pl), ions (Na^+, Cl^-), and androgen (testosterone) concentrations were found in the seminal fluid samples collected in June (Table 13). However, among these particular seminal plasma components, only the concentration of phospholipids ($r=-0.75$, $P<0.05$, $N=9$) and cholesterol ($r=-0.76$, $P<0.05$, $N=9$) were correlated with sperm motility. When the constituents of seminal fluid were analyzed on the bases of sperm motility, lower levels of phospholipids, cholesterol and triglycerides were found in samples with higher sperm motility (Table 14). Although the protein concentration in seminal fluid was not significantly different, variations were observed in the relative quantities of some protein bands (14, 14.5, 20, 26, 37 and 74 KD) in seminal fluid samples pooled according to difference in sperm quality in May and June (Fig 39).

8.4. Discussion

Some aspects of milt properties and the chemical composition of the seminal fluid of winter flounder are comparable with those reported for other marine fish. For example, total protein levels were similar with those reported for turbot, *Scophthalmus maximus* (Suquet *et al.*, 1995), appearing higher than some freshwater species (Lahnsteiner *et al.*, 1994a) but similar to the perch, *Perca fluviatilis* (Lahnsteiner *et al.*, 1995). The seminal plasma is slightly alkaline as with most species (Lahnsteiner *et al.*, 1994a; Lahnsteiner *et al.*, 1995; Pironen, 1985; Suquet *et al.*, 1995). Osmolality is slightly higher among other marine species, including halibut, *Hippoglossus hipoglossus* (Crim et al. unpubl.), turbot (Suquet *et al.*, 1995), gilthead seabream, *Sparus aurata* (Chambeyron and Zohar, 1990), and puffer and black seabream (Morisawa, 1985).

In the present study, changes in ionic levels of the seminal plasma do not correlate with alteration in sperm quality. In salmonid fish, the level of certain ions, such as K^+, in seminal fluid is critical for the maintenance of sperm quality because it suppresses premature sperm motility (Morisawa, 1985). Increased K^+ also improves sperm viability and speed of sperm movement in freshwater cyprinid fish within a concentration range below the level of the seminal fluid (Morisawa *et al.*, 1983). Unlike cyprinid and salmonid fish which migrate between fresh water and sea water, the role of seminal fluid ions in marine fish sperm function is not clear. Ionic ratios between the blood and seminal fluid vary among species. In some

species, the K^+ concentration is lower in the seminal fluid than in the blood plasma while Na^+ remains at the same level (Morisawa, 1985). However, this appears not to be the case for winter flounder, in which the concentration of Na^+ is significantly lower in the seminal fluid while K^+ and Mg^{++} are highly variable among individuals (1.0 ~ 11.8 and 0.69 ~ 15.4 mmol/DL, respectively). Additionally, low Cl^- in winter flounder seminal fluid, as compared to the blood plasma, is contrary to relatively high values observed in the seminal fluid of the black seabream (Morisawa, 1985). In the present study, Na^+ and Cl^- levels were higher in the seminal fluid collected in June compared to earlier samples in mid-May. Na^+ and Cl^- are also variable during the spermiation of halibut (Crim et al. unpubl.). However, although they tend to drop in February, together with peak spawning activities, a clear relationship with seasonal changes in sperm quality has not been identified. Since it has been proposed that low osmolality maintains the immotile status of marine fish spermatozoa during storage *in vivo* (Morisawa, 1985), the capacity to sustain an optimal ionic environment for spermatozoa may be very important for the maintenance of sperm quality. In the winter flounder, the Na^+ level in the seminal fluid is significantly negatively correlated with K^+ concentration ($r= -0.62$, $P < 0.05$, $N=13$) but positively correlated with Cl^- ($r=0.73$, $P < 0.01$, $N=13$). A correlation between ions and seasonal changes in some of the ions in this study may suggest seasonal mediation of the seminal fluid electrolytic balance by the sperm duct, through which an optimal osmotic environment could be established .

Total protein in the seminal plasma of the flounder increased from May to June. However quantitative changes in all protein species did not occur in a parallel manner, as suggested by visual estimation from protein profiles in SDS slab gel. For example, 14, 14.5, 26 and 73 KD proteins were relatively higher in June while the 20 and 74 KD proteins fell in amount. Still, a significant correlation between total protein concentration and sperm quality was not observed in this study, so the functional significance of increased seminal plasma protein levels in June remains to be clarified. Because of similarities between the polyacrylamide electrophoretic patterns of protein in the seminal fluid and blood plasma, it seems impossible to exclude serum-seminal fluid exchanges although a blood-testis barrier has been suggested (Steyn and Van Vuren, 1986). Sertoli cells may produce some serum-like proteins (Mather *et al.*, 1983). Additionally, spermatozoa may also provide some seminal fluid proteins, as suggested for rainbow trout, *Oncorhynchus mykiss* (Loir *et al.*, 1990) in which a number of seminal fluid proteins were shown to react immunologically with an antiserum against spermatozoa. This study also indicated that a 42 KD seminal fluid protein co-migrated with a major sperm membranous protein which was higher at the beginning and particularly at the end of the spermiation period when other sperm membrane proteins were present, suggesting degeneration of spermatozoa particularly at the end of the season. The 42 KD protein was lacking from trout milt containing spermatozoa with a high degree of integrity of the plasma membrane and the best post-thawed fertility (Maisse *et al.*,

1988; Makejac *et al.*, 1990). Besides sperm structural proteins, certain functional proteins, such as enzymes, also vary with the spawning season or among samples differing in sperm quality. Seminal plasma aspartate amino transferase, believed to be released from spermatozoa, increases in activity during storage of rainbow trout semen, paralleling the decline in sperm quality (Ciereszko and Dabrowski, 1994). In contrast, higher levels of protease inhibitor activities were measured in rainbow trout seminal fluid at the beginning of the reproductive season compared to the end of the season (Dabrowski and Ciereszko, 1994). Since morphological damage of winter flounder spermatozoa becomes more evident as the spawning season progresses toward the end of the season, it is not unreasonable to postulate that increased total protein in June and altered amounts of some proteins in the seminal plasma may in part be derived from degenerated sperm.

Seminal fluid phospholipid (PL) and cholesterol (Chl) levels display a significantly negative relationship with sperm quality in this study. Similarly, in *Sparus aurata*, increased seminal fluid TG and Chl concentrations were observed in the beginning and the end of spermiation period (Kara *et al.*, 1996), in association with changed sperm quality (Kara and Labed, 1994). Although some lipid components, such as TG, may be used for energy regeneration, as reported for trout and grayling (*Thymallus thymallus)* spermatozoa (Lahnsteiner *et al.*, 1991; Stoss, 1983), the relationship between sperm quality and PL and Chl concentrations may also be explained as a result of increased collapse of the

cytoplasm membrane of sperm, since lipid secretion was not observed from the epithelium of the sperm duct of winter flounder and both of these lipids are primary components of the sperm cytoplasmic membrane (Lou *et al.*, 1990; Labbe and Loir, 1991).

Finally, the present study confirmed the presence of androgenic hormones [testosterone (T) and 11 ketotestosterone (11-KT)] in the seminal fluid. The presence of steroid hormones (11-KT and 17α, 20β dihydroxy-4-pregnen-3-one) were reported in rainbow trout seminal fluid (Koldras *et al.*, 1996). While they are thought to originate from the testes, other sources of hormone cannot be excluded. There is evidence that the sperm duct possesses the capacity for steroid synthesis since 3α, 3β -HSD and G6PD positive cells were located in the connective tissues underlying the sperm duct epithelium of rainbow trout (van den Hurk *et al.*, 1978a; van den Hurk *et al.*, 1978b). Rainbow trout spermatozoa were also demonstrated to convert 17α, hydroxy-4-pregnen-3-one to 17α, 20β dihydroxy-4-pregnen-3-one *in vitro* but not to synthesize 11-KT (Ueda *et al.*, 1984). 17α, 20β dihydroxy-4-pregnen-3-one has not been detected in winter flounder serum (Crim, unpubl.), while high levels of 11-KT are present in plasma (about 100 µg/ml prior to the spawning season). In addition, the production of androgens by spermatozoa of winter flounder is suggested by findings that androgenic hormones increase after short-term storage of flounder milt samples (2-4 d) at ~ 4 °C (Shangguan and Crim unpubl.). Since the simple cellular structure of winter flounder spermatozoa likely

restricts a capacity for intracellular steroid synthesis, an alternative explanation is that previously stored steroid hormones in spermatozoa are released as spermatozoa die during storage. However, based on present knowledge, a role for androgens in the seminal fluid (or spermatozoa) remains largely unknown, although a positive correlation between seminal fluid 11-KT and Na^{+} levels was demonstrated in the present study ($r=0.72$, $P< 0.05$, $N=8$). Steroid hormones (progesterones) have been suggested to be involved in mediation of pH values in the sperm duct of trout (Miura *et al.*, 1992), and also the regulation of Ca^{++} exchange across cytoplasmic membranes in mammalian spermatozoa resulting in maturation of spermatozoa (Blackmore *et al.*, 1990; Meizel and Turner, 1991).

Table 10 Number of fish used in each biochemical test of winter flounder blood plasma and seminal fluid

Test	Blood plasma	Seminal fluid	
		May	June
Protein	6	7	7
Glucose	0	7	0
Phospholipids	5	5	4
Cholesterol	5	5	4
Triglycerides	5	4	3
Na	5	7	6
K	5	7	6
Cl	5	7	6
Ca	5	7	6
Mg	5	6	5
Osmolality	0	5	0

Table 11 Sperm motility, swim duration, spermatocrit for winter flounder milt sample during the spawning season.

Month	Motility (%)	Duration (sec)	Spermatocrit (%)
May	61.1±4.8	33.3±1.0	53.3±4.7
June	35.7±3.6 **	31.3±1.4	68.6±4.8 *

Values are mean±s.e. (N=9 in May; N=7 in June). Significant difference by t-test: spermatocirt: * $P < 0.05$ ($t = 2.26$, $df = 13.6$); motility: ** $P < 0.01$ ($t = 2.25$, $df = 13.6$).

Table 12 Comparison of ions, and organic components in winter flounder seminal fluid and blood plasma during the spawning season.

Sample	Seminal fluid	Blood plasma
Protein (mg/ml)	4.8± 0.6	23.2± 1.16**
Glucose (mg/ml)	0.44±0.1	N/A
Phospholipids (mg/DL)	118.7±23.2	1084±596*
Cholesterol (mg/DL)	282±56.8	2062±502**
Triglycerides (mg/DL)	11.1±8.3	21.1±4.6
Na (mmol/ DL)	154±1.9	179±4.2**
K (mmol/ DL)	5.7±1.1	3.6±0.6
Cl (mmol/ DL)	141±2.0	161±2.7**
Ca (mmol/ DL)	1.62±0.1	1.93±0.2
Mg (mmol/ DL)	4.18±1.4	1.23±0.6
Osmolality (mOsm/kg)	363±3.7	N/A
pH	8.08±0.1	N/A

Values are mean±s.e.. N/A: no measurement. See table 10 for sample size (values of seminal fluid are means for May and June samples). Significant difference by t-test: * $P < 0.05$ ($t = 3.52$, $df = 4.1$ for PL), ** $P < 0.01$ ($t = 13.83$, $df = 8.1$ for protein, $t = 4.96$, $df = 12$ for Chl, $t = 5.32$, $df = 5.8$ for Na and $t = 5.60$, $df = 8.7$ for Cl).

Table 13 Comparison of winter flounder seminal fluid components during the spawning season.

Month	Prt (mg/ ml)	Chl (mg/ DL)	PL (mg/ DL)	TG (mg/ DL)	Ions (mmol/L)					T (ng/ ml)	KT (ng/ ml)
					Na	K	Cl	Ca	Mg		
May	4.0± 0.5	68.1± 23	162± 47.8	6.1± 3.4	150± 1.8	6.0± 1.5	136± 1.7	1.6± 0.2	5.0± 2.3	1.2± 0.4	3.3± 1.7
Jun	6.4± 0.9 *	159± 26 *	378± 71 *	14.7± 4.7	159± 2.5 *	5.5± 1.9	148± 3.5 **	1.7± 0.1	3.2± 1.4	3.4± 0.3 *	10± 2.6

Values are mean±s.e.. Prt: protein, Chl: Cholesterol; PL: phospholipid, TG: triglycerides, T: testosterone, KT: 11 ketotestosterone. See table 1 for sample size. Significant difference by t-test: * $P < 0.05$ ($t = 2.33$, $df = 9.4$ for prt; $t = 2.55$, $df = 7$ for Chl, $t = 2.5$, $df = 6.6$ for PL and $t = 2.8$, $df = 9.5$ for Na), ** $P < 0.01$ ($t = 5.35$, $df = 10.9$ for Cl)

Table 14 Comparison of winter flounder sperm motility and seminal fluid composition.

Motility (%)	Prt (mg/ ml)	Chl (mg/ DL)	PL (mg/ DL)	TG (mg/ DL)	Ions (mmol/L)					T (ng/ ml)	KT (ng/ ml
					Na	K	Cl	Ca	Mg		
30	6.2± 0.1	173± 28	422± 71	19± 2.5	159± 3.3	5.1± 1.7	146± 3.5	1.5± 0.2	3.3± 1.4	2.6± 0.3	7.0± 2.1
50-70	3.5± 0.6	74.6± 19 *	170± 84 *	5.0± 2.6 *	152± 1.9	6.2± 1.6	138± 2.1	1.7± 0.1	4.9± 2.3	2.5± 0.9	7.9± 3.8

Values are mean±s.e.. Prt: protein, Chl: Cholesterol; PL: phospholipid, TG: triglycerides, T: testosterone, KT: 11 ketotestosterone. Significant difference by t-test: * $P< 0.05$ ($t = 2.85$, $df = 5.5$ for Chl, $t = 3.12$, $df = 4.6$ for Pl and $t = 3.83$, $df = 4.9$ for TG).

Fig 38 Comparison of the protein electrophoretic profiles of winter flounder seminal and blood plasma in June. Column 1-5) individual blood plasma samples, column 6-9) individual seminal plasma samples, column 10) protein markers. Number along column: molecular weight (KD) of protein bands or protein standards.

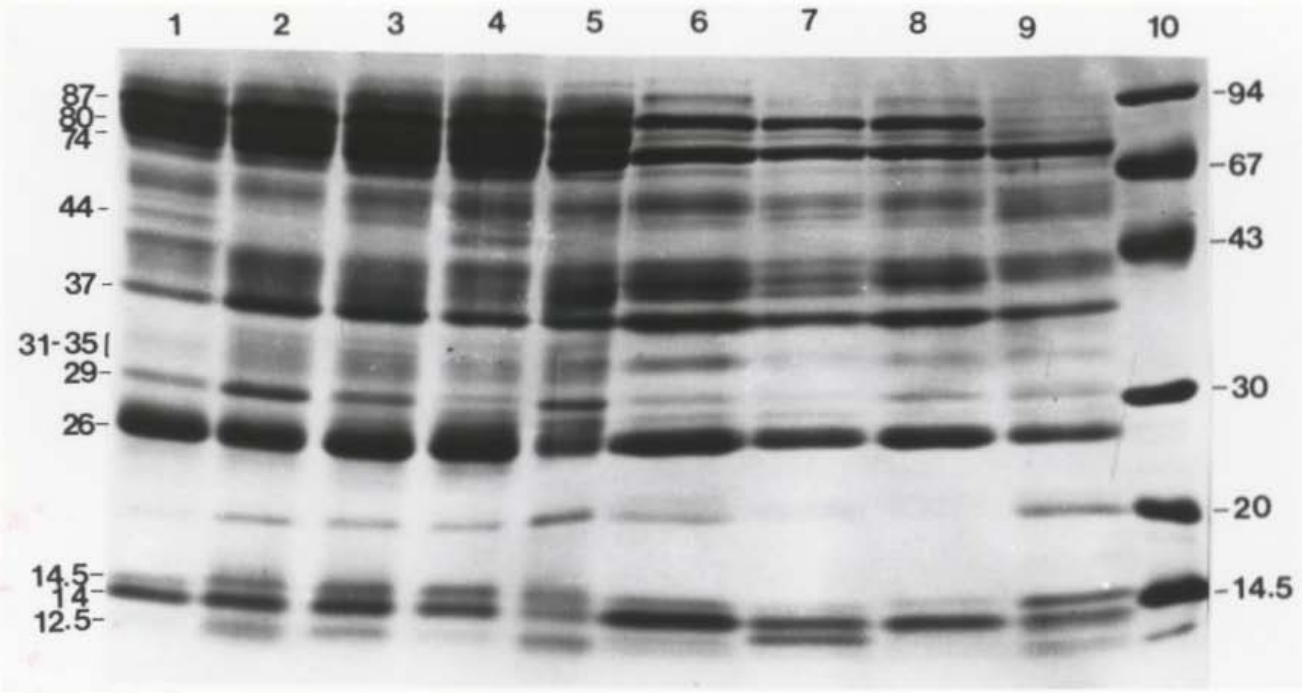
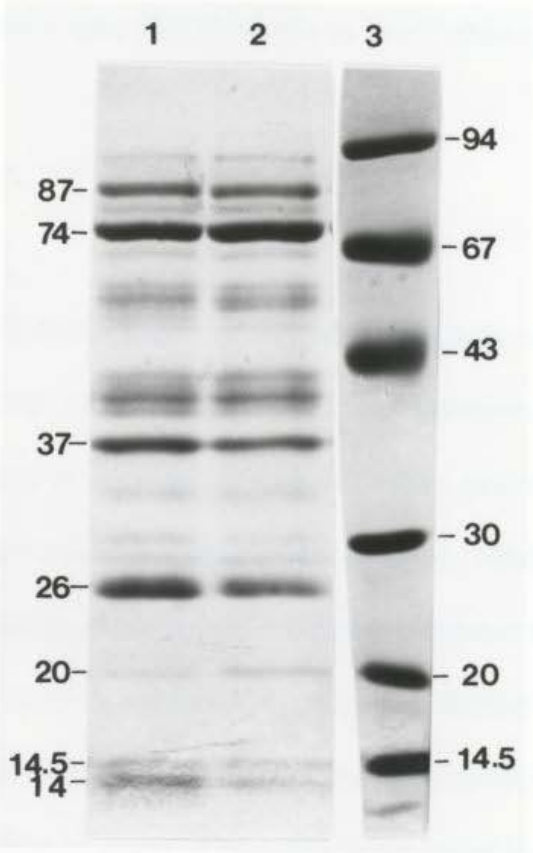

1
2
3
4
5
6
7
8
9
10
87
80
74
44
37
31-35
29
26
14.5
14
12.5
94
67
43
30
20
14.5

Fig 39 A comparison of the protein composition of winter flounder seminal fluid collected in May and June. Column 1) sample pool of 3 in June (sperm motility 30-50%), 2) sample pool of 4 in May (sperm motility 70%), 3) protein markers. Protein loading: column 1:2=1.04:1.00; Number along column: molecular weight (KD) of protein band or protein standards.


1
2
3
94
87
74
67
43
37
30
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20
14.5
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14

CHAPTER 9

Distinctive Male Flatfish Reproductive Strategies: A Study of Spermatogenesis, Testicular Development and Milt Release in the Yellowtail Flounder, *Pleuronectes ferrugineus*

9.1. Introduction

As with most temperate species, reproductive seasonality is remarkable for many cold water teleosts. However, while females tend to spawn over short periods of time in high latitudes (Garcia *et al.*, 1983), mature males generally produce gametes over much longer portions of the year (Harmin *et al.*, 1995a; Crim et al unpubl.) Recently, it was reported that milt is expressible throughout the year from male yellowtail flounder, *Pleuronectes ferrugineus*, a coldwater flatfish (Clearwater, 1996). Because this observation was based on males held in captivity for variable lengths of time, e.g. several months or years, it remains to be clarified whether this elongated spermiation period is normal. Furthermore, if milt production is prolonged in wild populations, this raises interesting questions about whether sperm is stored or produced throughout the year and if there is seasonal variation in sperm viability. Answers to such questions will not only enrich our knowledge of fish reproductive strategies but also may lead to greater success in culturing and farming of fish.

The aim of this study was to investigate seasonal reproduction in male

yellowtail flounder. While milt production was monitored, testis samples were collected throughout the year and examined microscopically to determine the seasonal dynamics of spermatogenesis in wild and captive yellowtail flounder. In addition, results of this yellowtail flounder study were compared with reproduction in other male coldwater flatfish which appear to display more distinct reproductive seasonality compared with yellowtail flounder.

9.2. Materials and methods

Male yellowtail flounder broodstock which had been maintained in the laboratory for at least 6 months preceding this study were monitored for milt production and seasonal testicular development at 2-3 month intervals from July 1996 to May 1997. Three groups of fish freshly caught by SCUBA divers 5-6 days prior to examination were also studied in September (1996), March and May (1997) respectively in an attempt to compare the reproductive performance between wild and captive males. The release of sperm in captive (N=6-13) and wild males (N=3-8) was ranked from 0-4 based upon the amount and properties of milt collected: 0. Absence of milt after three successive strippings; 1. A limited amount of milt (< 0.1 ml) collected after the third stripping; 2. a small volume of thick milt (0.1~0.2 ml) after 1-2 strippings; 3. a moderate volume of thin milt expressible in each of three stripping. 4. large amounts of flowing milt available in all three strippings (Jackson and Sullivan, 1995). Following milt collection, the males were killed and the testes

were removed and weighed. A whole cross section (~3 mm in thickness) was dissected from the central portion of the testis which was prepared for microscopic examination as outlined previously (Chapter 2).

9.3. Results

9.3.1. Gross testis morphology and histology

The male reproductive system of this gonochoristic species is composed of paired testicular lobes located at the posterior edge of the ventral body cavity together with the attached sperm ducts which connect the testes to the urogenital pore between the pelvic fins. At their maximum seasonal development, the testes are enlarged, triangular shaped ivory white organs, shrinking markedly and changing to a light brown colour after most sperm were released.

According to macroscopic and histological observations of testis changes throughout the year, together with some milt properties, testicular development in this species can be classified into five progressive reproductive stages, i.e. I) early testis development - the gonads are small and contain only primary and secondary spermatogonia (GA and GB); II) rapid testis development - the gonads enlarge and contain primarily spermatocytes and spermatids; III) limited spermiation - spermatozoa predominate with a small amount of concentrated milt in the sperm duct; IV) full spermiation - spermatozoa predominate with a large amount of dilute milt in the sperm duct and V) spent - residual sperm present with germinal cysts

filled with GA and GB (Table 15 and Fig 40A-E). However, while only GA or GB cysts were found distributed among a large amount of spermatozoa in the testes of some mature (stage III and IV) males (substaged as III-A and IV-A, Fig 40C), a few spermatogenic cysts containing spermatocytes, often at a status of maturation division, and spermatids are present in testes of other mature flounder (substages: III-B and IV-B, Fig 40D).

9.3.2. Seasonal testicular development and milt production in captive males

In captive male yellowtail flounder, Gonad-somatic index (GSI) values fluctuated seasonally ranging from a high (~2% from March - July) to significantly lower values (<1% from September - December) (Fig 41).

In association with these changes in gonadal development, all male flounder were mature and spermiating (stages III and IV) by March; by July, however, 100% of males were in full spermiation condition (stage IV, Fig 42). During the period from September - December, although a few males possessed small spent testes (stages V), about half the captive males remained mature and at the limited spermiation stage (III) while others (30-40%) were at active spermatogenesis (stage II). Notably, the incidence of mature males in the spermiating condition (III and IV) never fell to less than 40% throughout the year.

According to the study of milt response, the intensity of sperm release and milt hydration significantly changed along the season with a peak spermiation rank

and the lowest spermatocrit value recorded in July (Fig 43A, B).

9.3.3. Seasonal reproduction in wild males

Seasonal changes in GSI values, testis development and milt release in three groups of males, freshly obtained from the field, followed similar patterns to captive males (Fig 41,42, 43A).

9.4. Discussion

9.4.1. Testicular development

A distinct feature of testicular development in male yellowtail flounder observed in this study is the existence of active spermatogenic cysts (meiosis and formation of spermatocytes) in a portion of mature males (stages III-B and IV-B) while the testes of other mature males contain only GB and GA's cysts distributed among spermatozoa (stages III-A and IV-A, Table 16). This observation was based on both wild and captive yellowtail flounder males, suggesting that renewed sperm development can be initiated before the completion of the previous cycle. In teleosts, three types of spermatogenic cycles have been described (Billard, 1986), i.e. continuous, discontinuous with overlapping cycles and well separated cycles, corresponding to asynchronous, group synchronous and synchronous oogenesis observed in females respectively. Spermatogenesis in yellowtail flounder appears to fall into the overlapping spermatogenesis type. Overlapping spermatogenesis in

some temperate male teleosts was indicated by the constant existence of spermatocytes in the testes of some spermiating males during most of the year (Aziz and El Gharabawy, 1990; Bhatti and Al-Daham, 1978; Htun-Han, 1978b). In contrast, well separated cycles of testicular development, when the number of spermatocytes rapidly decreases, and spermatocytes and spermatids completely disappear from the testes during progressive spermiogenesis, occurs in the white perch, *Morone americana* (Jackson and Sullivan, 1995), winter flounder, *Pleuronectes americanus* (Harmin *et al.*, 1995a) and plaice, *Pleuronectes platessa* (Barr,1963). Another common characteristic of the species displaying overlapping cycles of spermatogenesis is that the testes are relatively small even at the time of full gonadal development, GSI=2.1% in yellowtail flounder (the present study) and 3.2% in the dab, *Limanda limanda* (Htun-Han, 1978b), compared with 13% in winter flounder (Harmin *et al.*, 1995a).

In order to be able to indicate the distinctive features that may be compared with other fish (Andrade and Godinho, 1983; Barr, 1963; Htun-Han, 1978b; Jackson and Sullivan, 1995; Scott and Pankhurst, 1992), the testicular development of yellowtail flounder is classified into 5 stages, i.e. I) early testis development, II) rapid testis development, III) limited spermiation, IV) full spermiation, and V) spent, with each of the mature stages further divided into 2 substages (III-A, B and IV-A, B) based on the simultaneous spermatogenesis and spermiation. Testis at stage I (early development) and stage V (spent) are similar to each other in their macro-

and microscopical appearances except for the presence of residual spermatozoa in testes and/or in the sperm duct of spent males. In the present study, in which adult males were selected based on fish size, only one male (39.5 cm in fork length) was found to be in early testis development condition, perhaps caused by slow growth and poor condition. Overlapping cycles of sperm development in males virtually excluded the phase of complete testicular inactivity in this species, a regressed phase which is often evident in other seasonal spawners (Harmin *et al.*, 1995a). Unlike most seasonal spawners, such as winter flounder and the New Zealand snapper (*Pagrus auratus*) (Burton and Idler, 1984; Harmin *et al.*, 1995a; Scott and Pankhurst, 1992), the testes of post-spawned yellowtail flounder (spent stage) do not become bloody or flabby, instead remaining relatively high in weight (GSI 0.2~0.5%) compared to the regressed testis of winter flounder and plaice (~ 0.1 % GSI). The relatively large testes in spent yellowtail flounder are most likely due to a notable proliferation of spermatogonia (GB), some seen undergoing mitosis.

Stage II, or the period of rapid testicular development in yellowtail flounder, is marked by active maturation division and the presence of spermatocytes and spermatids within the testis. A small amount of spermatozoa can usually be observed in the chamber of the testicular efferent duct system or even within the testes of all males at this stage, suggesting they are rematurating. Fish at this stage undergoing their first (pubertal) reproductive development were not observed in this

study.

Since a portion of yellowtail males retained fairly well developed testes containing a large quantity of mature spermatozoa even after the spawning season, the microscopic features of the testis may not clearly distinguish differences in the testicular activities which may be related to seasonal alteration of reproduction in males. Sperm index, spermiation response and sperm production figures have been used to characterize male teleost reproductive activities, while hydration of the milt was associated with active sperm release in several species (Jackson and Sullivan, 1995; Scott and Pankhurst, 1992; Chapter 7). Based on milt volume and milt properties (such as spermatocrit) collected from the sperm duct, testes with similar microscopical features (containing large quantity of mature spermatozoa) can be classified into two development phases (III and IV) which reflect the different intensity of sperm release.

9.4.2. Seasonal reproductive cycle

Overlapping spermatogenesis in the yellowtail flounder results in distinctive cyclic changes of male reproduction. Generally speaking, for most seasonal spawners, rapid testicular development (stage II for this study) represents a period during which active spermatogenesis occurs. Based on seasonal distribution of testis stage, it seems that the rapid development of the testis in yellowtail flounder only occurs in a small portion (30-40%) of males during the period from September

- December. However, since some mature and spermiating males (stages III-B and IV-B) simultaneously undertake their active spermatogenesis even during vigorous spermiation period, together with males at rapid testicular development stage (II), spermatogenesis actually took place in a portion of captive males throughout the year. Consequently, a large portion (> 40%) of the males retained mature testes all-year round (stages III and IV), while only a small number of fish remained relatively reproductively inactive (stage V) in fall and winter seasons. Similar conditions in the testicular development were also observed in wild males with some males containing well developed testes in September after summer spawning (stage III). Consequently, reproductive seasonality becomes less obvious as different phases of development overlap and a postspawning, testicular quiescent phase is eliminated or is extremely brief.

Nevertheless, seasonality of reproduction in male yellowtail flounder is still present, including at least three phases: recrudescence, prespawning, and spawning. Based on the present study, the male spawning season, as defined earlier, mainly occurs in summer at the time when the largest portion of males actively release sperm. This was supported by laboratory observations indicating that sperm production from captive males was maximal in the summer time (July-August in 1993 and June in 1994) (Clearwater, 1996). Based on data from wild fish, recrudescence of the testes, i.e. rapid testis development and sperm formation, likely occurs in the autumn following the summer spawning season, and the

testicular development becomes quiescent by the spring prespawning period. Still, testicular development can occur in early summer in a small portion of wild males, likely continuing throughout the spawning season because the testes of some of the males contain large amounts of mature spermatozoa after the spawning season is over. The timing of the spawning season and gonad recrudescence is also supported by groundfish survey data from the Grand Bank stock, suggesting the spawning season for wild male yellowtail flounder mainly occurs from May to July (peak in June) and recrudescence takes place from July to October (Pitt, 1970).

The distinctive reproductive strategy displayed by male yellowtail flounder appears associated with reproduction in females of this species. Female yellowtail flounder are a group-synchronous egg-batch spawners (Howell, 1983), ovulating eggs at ca. one-two day intervals throughout a period of 1-1.5 months in captivity (Manning, 1996). The spawning season is reportedly widespread in the wild population, occurring over a period from May to September but reaching a peak in May and June (Pitt, 1970; Zamarro, 1988). In the North Sea dab, evidence suggests that spermatozoa are present continuously in testes over the whole year (Htun-Han, 1978b), and group synchronous ovarian development is also evident in females of this species (Htun-Han, 1978a). In contrast, female winter flounder spawn once a year and sperm development in males is highly synchronized, starting in September and being completed by December (Burton and Idler, 1984; Harmin *et al.*, 1995a). The female Atlantic halibut is also an egg batch spawner, ovulating eggs at 2-4 day

intervals in captivity (Norberg *et al.*, 1991). Testicular development in halibut, however, seems similar to winter flounder rather than yellowtail flounder. In halibut, the spermiation period in captivity lasts ca. 5 months during the winter and spring, terminating early in the summer, much like winter flounder. Although one may speculate that spermatogenesis is highly synchronous in male halibut, a histological examination of testicular development is necessary to reveal the duration of spermatogenesis in this batch spawner.

9.4.3. Impacts of captivity

According to this study, although similar patterns of the male reproductive cycle was observed in wild and captive males, influence of captivity on sperm development in yellowtail flounder males is evident. For example, more mature males were observed undergoing active spermatogenesis (the formation of spermatocytes and spermatids) in captivity. Results of the present study also indicate that, under captive conditions, the reproductive cycle of male yellowtail flounder may be altered to some extent. Active spermatogenesis was observed in some captive males from spring to summer while, in contrast, sperm development was found more intensively in testes of wild males in September. These changes are likely associated with warm temperature conditions (4-12 °C) for captive fish during these observations which are different from the temperatures in the wild. Groundfish surveys carried out in April (spring) and September (late summer)

indicated both juveniles and adult yellowtail flounder are distributed mostly within a relatively small temperature range: 0.9 °C in April and 2.2 °C in late summer (Walsh, 1992) and no evidence regarding spawning migration or spawning concentrations was reported (Walsh, 1987). Thus, it is most likely that higher captive holding temperatures during winter and spring seasons stimulated sperm maturation while it in turn suppressed the recrudescence of testes in the fall. Although temperature is considered a less reliable predictive cue for some temperate and subpolar species, a great deal of evidence has indicated that temperature can act as a mediator of gonadal development (Grier *et al.*, 1980). However the scale of temperature change capable of altering gonad development varies, largely dependent on species and cooperative effects of other environmental factors. In this study, slightly increased temperatures appeared to have detectable influences on the testicular development in yellowtail flounder. In contrast, no notable acceleration in testicular development (Chapter 5) occurred in halibut males having been held in higher temperature range, ~5 °C in winter time and ~10 °C in summer, for several years, which may add to evidence for a synchronized spermatogenesis in this species.

Table 15 Stages of testicular development in male yellowtail flounder.

Stage		GSI (%)	Testicular features	
			Macroscopic	Microscopic
I	Early* development	0.2	Light brown in colour and small in volume	Primary and secondary spermatogonia (GA and GB) with abundant connective tissue (Fig 40A)
II	Rapid development	0.5-0.9	Brown colour with firm texture and increased volume; little milt in the center of testis or in sperm duct	Spermatocytes (SC) and spermatids (ST) predominate with a few spermatozoa (Sp) (Fig 40B)
III A, B	Limited spermiation	0.9-3.7	Ivory white or partially brown; small amount of viscous milt in sperm duct; spermatocrit (SCT) >80%	Sp predominate with a few GA/GB cysts (III-A, Fig 40C) or SC/ST cysts (III-B, Fig 40D)
IV A, B	Full spermation	1.2-3.2	Ivory white or partially brown; large amount flowing milt in sperm duct; SCT < 80%	Sp predominate with a few GA/GB cysts (IV-A) or SC/ST cysts (IV-B), similar with III-A or III-B, respectively
V	Spent	0.2-0.5	Brown and firm; little milt in the center of testis or in sperm duct	GB predominate with a few GA and residual Sp (Fig 40E)

* Only observed in one captive male with poor somatic growth (thin body) in May.

GSI: gonad-somatic index.

Table 16 Spermatogenesis in mature testis (stages III and IV) of male yellowtail flounder.

Stage	Source	No of fish	A (%)	B (%)
III	Captive	13	46.2	53.8
	Wild	8	75	25
IV	Captive	13	38.5	61.5
	Wild	4	100	0
Total	Captive	26	42.3	57.7
	Wild	12	83.3	16.7

Note: A: Testis contains mature spermatozoa and GB and GA cysts only; B: Testis contains mature spermatozoa with spermatocytes and spermatids (also see table 15 and Fig 40C, D). The number of fish is the collection of mature males from 5 or 3 sampling dates for captive and wild males respectively.

Fig 40 Photograph of histological preparation of testis in male yellowtail flounder..

A) Testis under early development containing mostly primary and secondary spermatogonia (GA and GB) surrounded by the connective tissue (CT).

B) Testis at the rapidly developing stage filled with spermatocytes (SC), spermatids (ST), as well some residual spermatozoa (Sp); maturation division are frequently observed (arrows).

C) Mature testis predominated by spermatozoa (Sp) with primary and secondary spermatogonia (GA and GB) cysts located along the epithelium of the seminiferous tubules.

D) Mature testis containing mostly spermatozoa and some active spermatogenetic cysts with various developing cells. GA: primary spermatogonia, GB: secondary spermatogonia; SC: spermatocytes; ST: spermatids; Sp: spermatozoa.

E) Testis at the spent stage, secondary spermatogonia (GB) predominate the germ cell population while primary spermatogonia (GA)and some spermatozoa (Sp) are present.

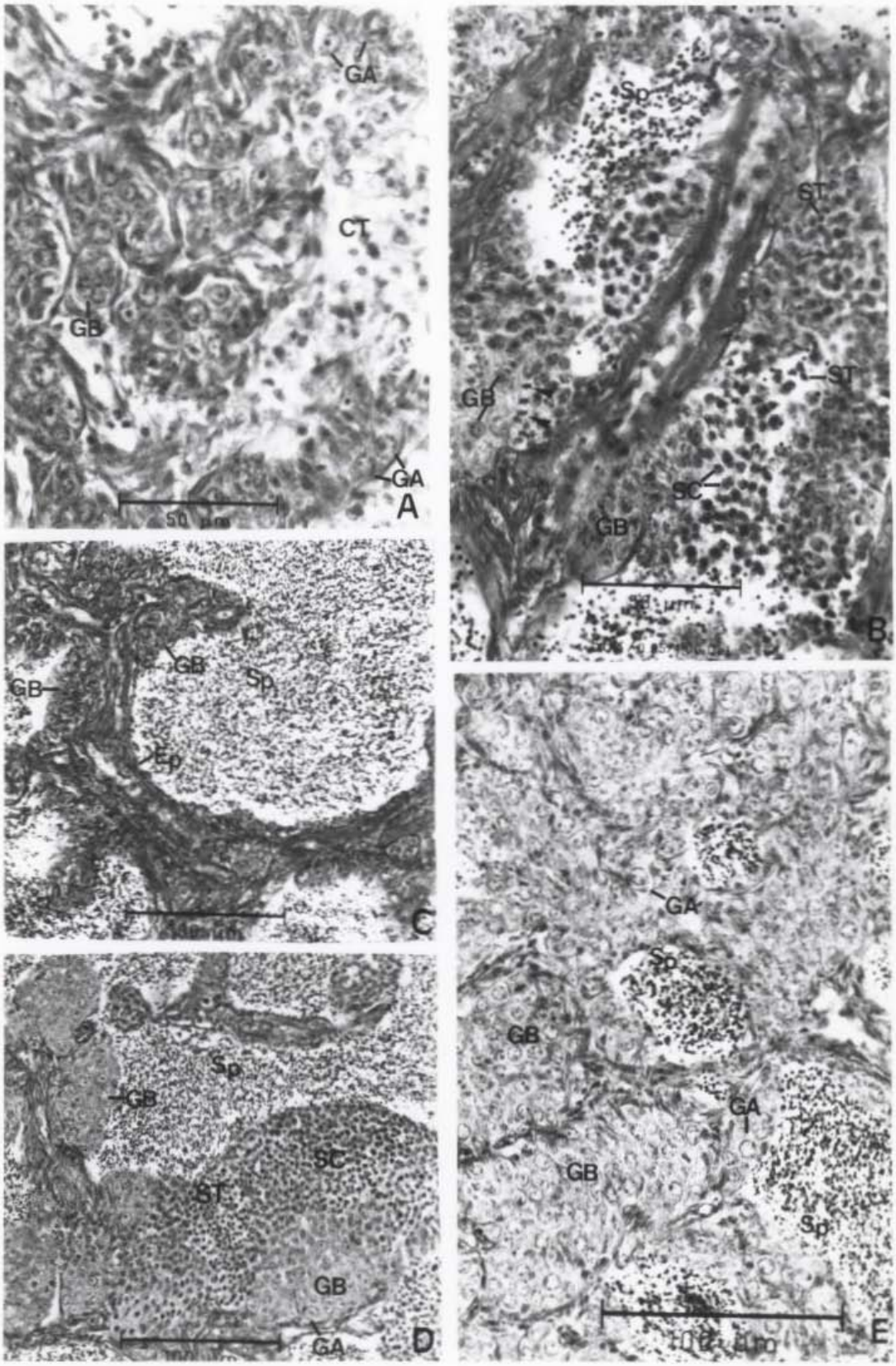

GA
CT
GB
GA
A
Sp
ST
ST
GB
SC
GB
B
GB
GB
Sp
Ep
C
GA
Sp
GB
GA
GB
Sp
E
Sp
GB
SC
ST
GB
GA
D

Fig 41 Seasonal changes in the gonadosomatic index (GSI) in captive and wild male yellowtail flounder. The numbers of captive males: 6 (Mar), 12 (May, exclude one male with poor somatic growth), 7 (July), 6 (Sept) and 6 (Dec); the numbers of wile fish: 3 (Mar), 8 (May) and 3 (Sep). Significant differences ($P < 0.05$) throughout the season indicated by different letters (ANOVA; $f = 4.81$, $df = 4$ for captive males and $f = 6.53$, $df = 4$ for wild fish). Values are means±s.e..

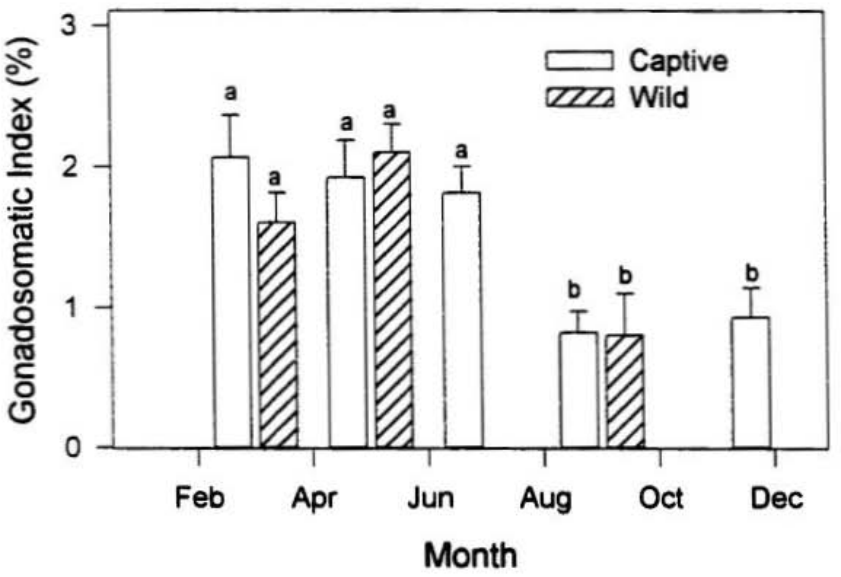

Gonadosomatic Index (%)
3
2
1
0
Captive
Wild
a
a
a
a
a
b
b
b
Feb
Apr
Jun
Aug
Oct
Dec
Month

Fig 42 Seasonal alteration in the stage frequency of testicular development in captive and wild male yellowtail flounder. The numbers of captive males: 6 (Mar), 12 (May, exclude one male with poor somatic growth), 7 (July), 6 (Sept) and 6 (Dec); the numbers of wile fish: 3 (Mar), 8 (May) and 3 (Sep).

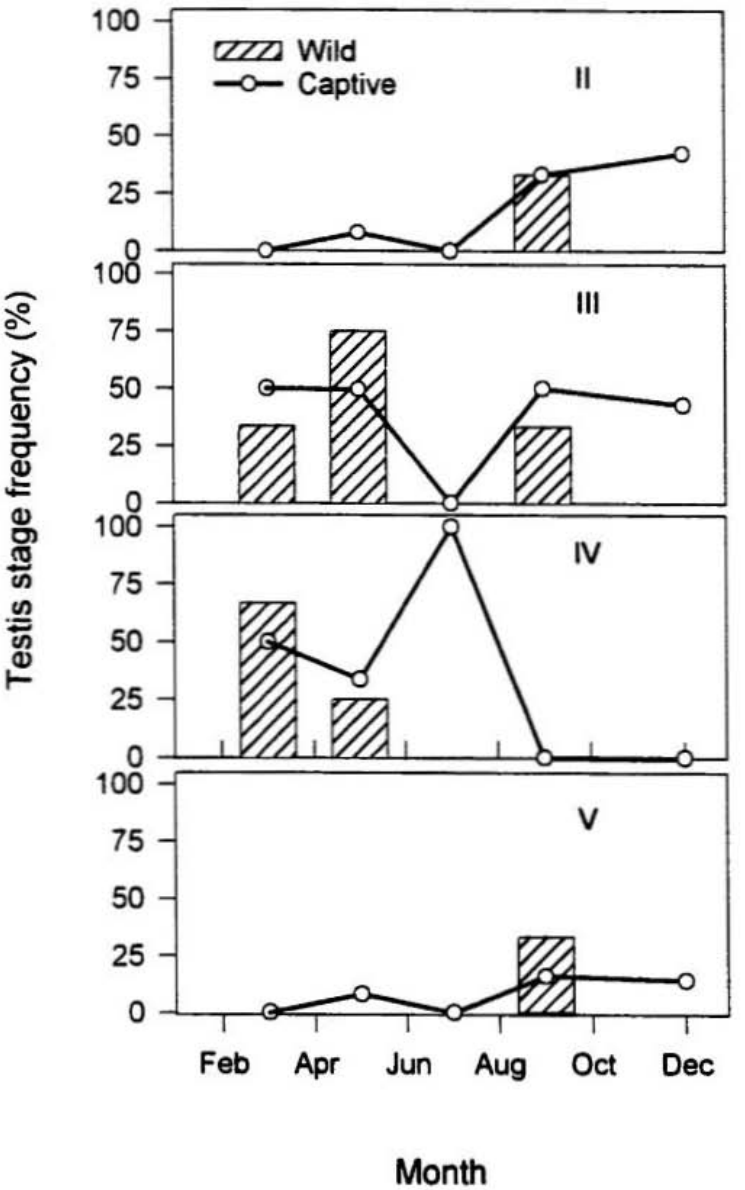

Wild
Captive
II
III
IV
V
100
75
50
25
0
Testis stage frequency (%)
Feb
Apr
Jun
Aug
Oct
Dec
Month

Fig 43 Seasonal patterns of A) spermiation rank and B) spermatocrit in captive and wild male yellowtail flounder. The numbers of captive males: 6 (Mar), 12 (May, exclude one male with poor somatic growth), 7 (July), 6 (Sept) and 6 (Dec); the numbers of wile fish: 3 (Mar), 8 (May) and 3 (Sep). Significant differences along reproductive season indicated by different letters (ANOVA; $f = 8.17$, $df = 4$, $P < 0.01$ for spermiation rank and $f = 3.34$, $df = 4$ for spermatocrit). Values are means±s.e..

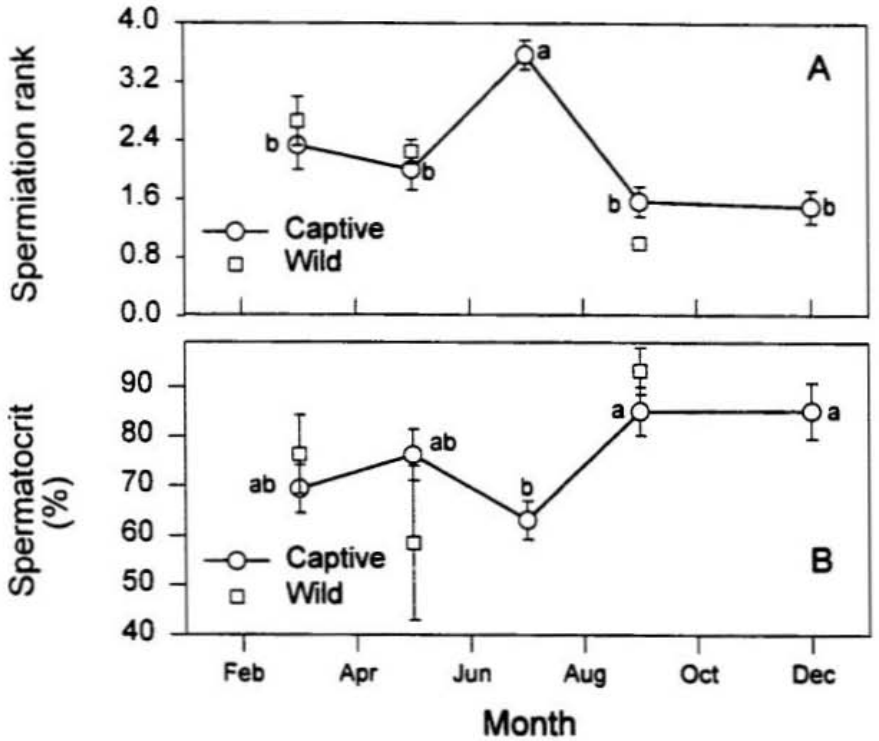
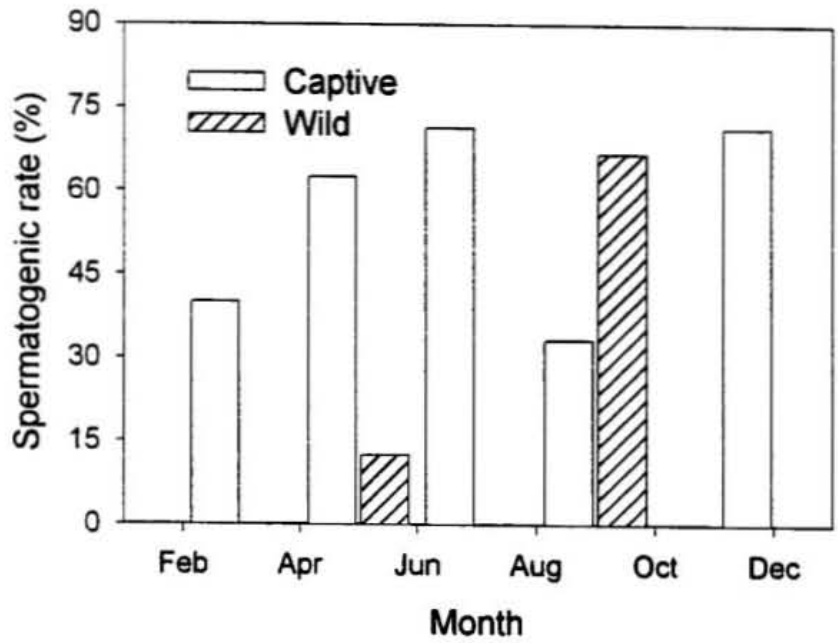

Spermiation rank
4.0
3.2
2.4
1.6
0.8
0.0
A
a
b
b
b
b
Captive
Wild
Spermatocrit (%)
90
80
70
60
50
40
B
a
ab
ab
b
a
Captive
Wild
Feb
Apr
Jun
Aug
Oct
Dec
Month

Fig 44 Seasonal variation in the proportion of captive and wild male yellowtail flounder undergoing spermatogenesis (exclude the male with poor somatic growth in May). Spermatogenic rate: The percentage of males containing testes with spermatocytes and spermatids (active spermatogenesis).


90
75
60
45
30
15
0
Spermatogenic rate (%)
Captive
Wild
Feb
Apr
Jun
Aug
Oct
Dec
Month

CHAPTER 10

General Conclusion

10.1. Seasonal reproduction and its regulation

Knowledge of teleost seasonal reproduction and its regulation is essential for improving broodstock management techniques and manipulating fish reproductive cycles in aquaculture practice. In this study, seasonal reproductive activities were investigated in males of three coldwater flatfish species focusing primarily on the period of release of sperm. In contrast to the rather limited spawning period for females, males are often observed to mature early with the result milt can be expressed for extended period of time. Although males, compared to females, appear to have a "prolonged spawning season", the major period for sperm production does occurs in synchrony with spawning in females. On this bases, therefore, the milt production in males can be divided into three parts including 1) a prespawning phase of initial sperm production in small amounts, 2) the true spawning season when the majority of sperm are produced in large volumes of diluted milt, and 3) the concluding phase at the end of the spawning season when sperm production declines.

According to observations in wild fish, a 4 month prespawning period was

observed in winter flounder males, then sperm were empted from testis within ~ 2 months during the spawning season (May and June), followed by a brief concluding phase when the GSI abruptly dropped (Harmin *et al.*, 1995a, Chapter 3 and 5). By contrast, prespawning release of sperm in halibut males is ralatively shorter while the concluding phase is prolonged. Like winter flounder and halibut, male yellowtail flounder display a specific spawning season in summer, but milt production can be observed throughout the year in a large proportion of the males (Chapter 9). Such a distinctive reproductive cycle in male yellowtail flounder appears to be related to the ability of this species to exhibit periods of overlapping spermatogenesis. In other teleosts, an extended period of milt production has also been reported, such as carp where several months of prespawning milt production precedes the normal spawning season which is about 2-3 months in summer (Courtois et al, 1986).

Since sperm production varies seasonally and both the testis and the sperm duct are involved in sperm release, questions about how hormones control different sperm release phases are of interest. Hypophysectomy data from the present study (Chapter 3) suggested that early release of sperm in small amounts may be independent of regulation of pituitary hormones (GtH). On the other hand, GtH probably is probably responsible for the initiation and maintenance of sperm release in large quantities during the spawning season, since pituitary removal reduced milt production during this period. High levels of serum GtH appear to be required for initiation of the spawning season in teleosts , since a GtH surge has

been reported in some studies preceding the spawning season (see Chapter 1: General discussion). Sperm release in large volumes can even be induced during the prespawning season when GtH levels are increased by injection of pituitaries or GtH hormones (Chapter 3; Courtois, 1986). In goldfish, spermiation is completely blocked by hypophysectomy or it ceases when plasma GtH is low (Billard, 1986), suggesting that the mechanism for spermiation regulation may differ from species to species. The fact that PRT treatment of winter flounder increased plasma 11-KT levels which was followed by significantly increased milt production suggests that this steroid may mediate the action of GtH on spermiation. These results were also observed in the carp (Courtois *et al.* 1986). On the other hand, changes in plasma testosterone concentrations parallel to changes in spermatocrit values (Chapter 3, Exp. 3) may indicate other regulatory mechanisms responsible for milt hydration.

One primary question about hormonal manipulation of the male teleost is whether hormonally induced increases in sperm production resulted from a stimulation of renewed spermatogenesis. Although there is evidence that hormone treatment, such as T or T and LHRH combined, can stimulate spermatogenesis in juveniles (prepubertal) or adult males (see review by Billard et al., 1993), induction of spermatogenesis in mature males remains largely questionable. For example, in this study, administration of a sustained-release GnRH-A pellet into the mature male winter flounder prior to the spawning season did not significantly increase sperm production, suggesting a lack of renewal of spermatogenesis when males

were already mature (Chapter 3). Likewise, repeated injections of carp pituitary extract for seven months in mature male carp during the period of milt production only resulted in continuous milt production with no sign of renewal of spermatogenesis (Saad and Billard, 1987). Such evidence suggests that, at least in some species, either the testes may not be responsive to GnRH and GtH, or spermatogenesis may be inhibited in mature males. It is worth noting that for the many species with well separated spermatogenic cycles, the gonad of mature males contains only spermatozoa and inactive spermatogonia (GA) cysts. Proliferation of spermatogonia normally develops only after spermiation is completed in trout, tench, roach, pike (see review by Billard, 1986) and winter flounder (unpubl. observation). In carp, a few GB containing cysts can be seen in the testes during early spermiation, but they are not observed during the spawning season (Billard *et al.*, 1986). However, in yellowtail flounder, the presence of GB cells in large numbers (some cells in mitotic division) is notable in most mature males throughout the year and formation of spermatocytes and spermatids was frequently observed in mature males. When males were treated with a low dose of GnRH-A (20 µg /pellet) during the spawning season, a significant increase in sperm production occurred in yellowtail flounder over 88 day period (Clearwater, 1996). Perhaps, this hormone treatment stimulated sperm production which could partially be ascribed to accelerated sperm development in this species.

10.2. Seasonal changes in sperm quality and its regulation

This study demonstrated that sperm quality varies on a seasonal basis. The quality of sperm was characterized by examination of sperm motility (percentage of sperm swimming forward) and duration of the swimming. In addition, sperm motility was compared with the ability of sperm to fertilize eggs when eggs were available, which showed they are well correlated. Because changes in temperature from winter to summer may influence the duration of sperm swimming (Billard and Cosson, 1992), swimming duration was not used to monitor seasonal changes of sperm quality over the milt production period.

Based on observations in this study, a period of production of good quality sperm has been identified during the spawning season of winter flounder and halibut, and this is similar to the findings in other seasonally spawning teleosts, including rainbow trout (Buyukhatipoglu and Holtz, 1984) sea bass (*Dicentrarchus labrax*), sea bream, *Sparus aurata* (Kara and Labed, 1994), and the yellowtail flounder (Shangguan and Crim unpubl.).

Studies of sperm morphology of two flatfish (halibut and winter flounder) using electron microscopy (SEM and TEM) revealed that sperm are primitive in these species, having a simple head, an undeveloped midpiece, and a single long flagellum as expected of externally fertilizing species. Furthermore, changes in sperm morphology were related to seasonal variations of sperm quality, indicating that degeneration in sperm structures, such as tail breakage and cell membrane

damage, may be one of main reasons for a deterioration of sperm quality during the season. This is the first evidence helping to identify causes of seasonal changes in sperm quality in teleosts. Similar seasonal variations of sperm morphology have been reported in higher vertebrates, such as lemur, *Eulemur fulvus* (Brun and Rumpler, 1990).

Changes in sperm quality observed in winter flounder males were closely related to seasonal alterations in male genital tract activities. Morphological examination of the winter flounder sperm duct indicated that active epithelial secretion took place in the sperm duct during the spawning season (Chapter 6), correlating with increased seminal fluid production during this period of reproductive season (Chapter 5). However, during the prespawning period and the concluding phase of milt production, epithelial secretions were not so evident while resorption and ingestion of degenerated or aging spermatozoa became the major function of the male genital tract. Direct evidence that the sperm duct is involved in the regulation of seasonal alterations in sperm quality was obtained from observations of sperm collected from different sites within the testicular efferent duct system (Chapter 7). The quality (motility) of sperm was improved while sperm moved from the testes to the sperm duct and well maintained in the sperm duct during the middle of the spawning season but not later in the season.

The role of the sperm duct in mediation and maintenance of sperm quality was further studied through an analysis of the seminal fluid composition (Chapter

8). Seminal fluid likely provides an optimal micro-environment for development and maintenance of sperm function. Differences in ion concentration between the seminal fluid and blood plasma, and a relatively stable osmotic pressure of the seminal plasma (Chapter 8), as well as a general rise of pH values in the TEDS (Chapter 7) suggest that the epithelium of the duct system may regulate ion composition, osmolality and acid-basal environment. In addition, the TEDS may also provide for sperm nutrition during their storage. Glucose, despite the low level, and some lipid components, such as triglycerides (TG), were detected in flounder seminal fluid (Chapter 8) which could be potential energy resources for flounder sperm metabolism, as in other species (Lahnsteiner *et al.*, 1991; Lahnsteiner *et al.*, 1994a; Lahnsteiner *et al.*, 1994b; Stoss, 1983). Higher TG levels in milt samples with low sperm motility mainly later in the season may be partially due to reduction in utilization of energy by reduced numbers of live spermatozoa.

Important seminal fluid components directly related to sperm metabolism and function still remain poorly understood in teleosts. While increases in some organic substances, such as lipids (Chapter 8) and some enzymes (Ciereszko and Dabrowski, 1994), were linked to deteriorating sperm quality, presumably resulting from damage of sperm cells during storage *in vivo* or *in vitro*, other substances may be responsible for the maintenance of sperm viability or mediation of the development of sperm functions. For example, protease inhibitor(s) which have been detected in the seminal plasma of mammals, the sea urchin and fish (Cosson

and Gagnon, 1988; Dabrowski and Ciereszko, 1994; De Lamirande and Gagnon, 1986;) may be important in mediation of metabolic activities for fish sperm related to sperm motility, interaction with proteases present in eggs during insemination, and a protective (anti-protease) role in maintaining viable spermatozoa during storage *in vivo* (De Lamirande and Gagnon, 1986; Dabrowski and Ciereszko, 1994; Inaba and Morisawa, 1991; Cosson and Gagnon, 1988). The anti-protease role may be particularly important for fish species where spermatozoa are stored for long periods of time, such as winter flounder, since seasonal variations of epithelial phagocytotic activities were observed in the sperm duct (Chapter 6).

Based on observations from the present study, the role of the TEDS can be summarized as 1) production of seminal fluid, 2) mediation and maintenance of viable sperm, 3) removal of aging and degenerated sperm, and 4) sperm emission and storage. During the lengthy winter flounder prespawning season, most sperm are stored in testis and rapidly released during the spawning season with improved quality. Thus, it is speculated that sperm metabolic activities may continue at a minimal level or even be inhibited by the testicular environment, possibly due to high sperm concentration, which may facilitate sperm storage within the gonads. As sperm are released into the TEDS during the spawning season, metabolism of sperm cells may be enhanced where sperm density in milt is much reduced by increased seminal fluid production while oxygen and other substrates required for energy synthesis become available. An elevated level of metabolism may correlate

with sperm acquiring the capacity for motility, perhaps through increased utilization of energy resources and oxidative phospharylation. However, the high performance in energy synthesis is restricted due to long-term storage of sperm during the prespawning stage which may cause progressive sperm degeneration. The efficiency of energy synthesis thereby decreases during sperm storage in the TEDS, causing sperm to consume intracellular energy for basic maintenance metabolism as storage time continues. Hence, reduced sperm swim times were observed during the middle of season followed by the gradual loss of the motile ability, particularly later in the season (Chapter 7).

Inconsistent effects of hormone treatment on sperm quality have been reported in some studies. Most evidence suggests that mature spermatozoa are independent of hormonal treatments, including GnRH-A and pituitary hormones (Mylonas *et al.*, 1997; Sorbera *et al.*, 1996; Saad and Billard, 1987; Chapter 3). However, while GnRH-A induced increases in serum 17α, 20β-P levels did not affect sperm quality in sea bass (Mylonas *et al.*, 1997), exposure to a water containing the steroid hormone stimulated male goldfish to produce larger volumes of sperm of better motility (Defraipont and Sorensen, 1993). Similarly enhanced sperm motility following hormone (GnRH-A) treatment was recently reported in yellowtail flounder, together with increased milt production (Clearwater, 1996). Since the volume of milt in this species is very small and during collection urine contamination is common, it remains to be elucidated whether the improvement of

sperm quality was related to increased milt volume by GnRH-A treatment alleviating the urine problem.

The information about seasonal changes in sperm quality and it regulation can be important for many reasons. First, it indicates that males, like females, are an important contributor for successful progeny production through the delivery of good quality sperm during the spawning season. Conservation of sperm quality appears efficient in male teleosts, although mature sperm usually have been stored in males for an elongated period. Since good egg quality usually lasts for only short periods of time, and because eggs quickly over-ripen after ovulation (Kjorsvik *et al.*, 1990), it may be an efficient strategy for sperm to mature early and be ready to fertilize eggs once available. Secondly, the information can also be valuable for sperm preservation, since the seasonal variations in sperm quality have been reported to affect survival of sperm stored *in vitro*. For example, sperm can be stored much longer at 4 °C when milt is collected early in the spawning season (Billard *et al.*, 1977) or the middle of the spermiation period in sea bream and sea bass (Kara and Labed, 1994). Therefore, it is important to collect milt samples at an optimum time of the season to maximize successful sperm preservation.

Seasonal observations of sperm morphology revealed the presence of some abnormal sperm with enlarged head and tail-ring (Chapter 5) which may represent malformation of sperm during spermatogenesis. Some studies suggest that the development of sperm can be distorted if fish are subjected to harmful conditions.

For example, the proportion of morphologically abnormal sperm was increased in male tilapia, *Oreochromis mossambicus* after fish were infected by a bacterium, *Pseudomonas aeruginosa* (Manna and Biswas, 1988). Other changes in the environment may also influence male and female gametes. It was demonstrated that increased concentration of cadmium found in fish gonads as a result of bioaccumulation has a negative impact on sperm motility (Kime *et al.*, 1996). Aberrant environment conditions, such as polluted areas, have became a major challenge for conservation of natural resources recently. Egg and early larval development have been used to evaluate the impact of pollution on wild fish populations in several studies which indicate pollutants (such as petroleum) cause abnormal egg development, or chromosome aberration (Johnnson *et al.*, 1992). Since examination of sperm motility and morphology is very convenient, sperm quality (such as sperm morphology and motility) may become valuable indicators of the environmental impact on fish reproduction.

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