# A STUDY OF REPRODUCTIVE PHYSIOLOGY IN THE MALE OCEAN POUT MACROZOARCES AMERICANUS



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# A STUDY OF REPRODUCTIVE PHYSIOLOGY IN THE MALE OCEAN POUT *MACROZOARCES AMERICANUS*

by

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## ABSTRACT

To provide more information on the reproductive biology of an internally fertilizing marine species, a two-year study of the reproductive physiology of the male ocean pout (*Macrozoarces americanus*) was undertaken. The physiological characteristics of the spawning season and sperm physiology, including the changes in the gonadosomatic index (GSI), plasma androgenic steroids, the timing of spermiation, sperm motility and sperm concentration, and the variations of pH, osmotic pressure and the biochemical composition of seminal plasma during the spawning season were investigated. The spermiation response began in June, and by July all mature males spermiated. Spermiation ended in late September or early October. Sperm motility rose and fell in a similar manner from June to September. Compared with the milt of most investigated male teleosts, ocean pout milt had a low sperm concentration (0.91 - 6.81 x $10^8$ spz/ml), and was correlated with spermatocrit values during most of the spawning season ($r^2$=0.79, $p<0.0001$). The GSI of the males was relatively low, ranging between minimum values in September and October (0.26 - 0.31%) and a maximum in July (1.9%). Plasma testosterone levels rose rapidly seven weeks in advance of spermiation and peaked one week before the onset of spermiation, and decreased thereafter. Plasma levels of 11-ketotestosterone paralleled those of testosterone during the spawning season and reached a peak coincident with onset of spermiation.

Biochemical composition of seminal plasma also varied through the spawning

season. The pH of seminal plasma increased from 7.4 to 7.9 during the period of spermiation, and the average pH (7.78±0.03) for the spawning season remained close to an experimentally determined optimum pH range for ocean pout sperm motility (pH 8 - 9). Although the values for seminal plasma osmolality fell from 416 to 339 mmol/kg during the reproductive season, the average osmolality value (356±3 mmol/kg) was within the optimum for ocean pout sperm motility (300 - 400 mmol/kg). In comparing fluctuations in sperm motility with the biochemical composition of ocean pout seminal plasma during the spawning season, this study showed that increased $Mg^{++}$ levels were correlated with the summer period of maximum sperm motility. A seasonal decline in $Na^{+}$ and $Cl^{-}$ ion levels was reflected in lower seminal plasma osmolality values.

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# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 THE REPRODUCTIVE PHYSIOLOGY OF MALE TELEOSTS

Reproduction in male fishes, as in females, is regulated by the brain-pituitary-gonadal axis (Redding and Patiño 1993). Since much of the existing knowledge of fish reproduction is formed on the basis of the study of teleosts, and the ocean pout is classified as teleost, the following literature review will focus on the teleostean species.

As in all vertebrates, external environmental cues influence the teleostean brain-pituitary-gonadal axis by the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus which reaches the pituitary via direct penetration of fibres, or as in other vertebrates may access the pituitary by a specialized portal blood supply (Redding and Patiño 1993). After reaching the pituitary, GnRH binds to gonadotropic cell membranes stimulating the release of gonadotropic hormone (GtH) into the circulation. Via the circulation GtH reaches the testis where it binds to specific receptors in Leydig cells or some other somatic cells of the testis, resulting in the synthesis of steroid hormones. Androgenic steroid hormones from the testis induce development, growth and final maturation of male germ cells and ultimately spermiation. In addition, steroid hormones also regulate GnRH and GtH secretion by either positive or negative feedback action at hypothalamic and pituitary levels to regulate the spermatogenic cycle (Figure 1.1).

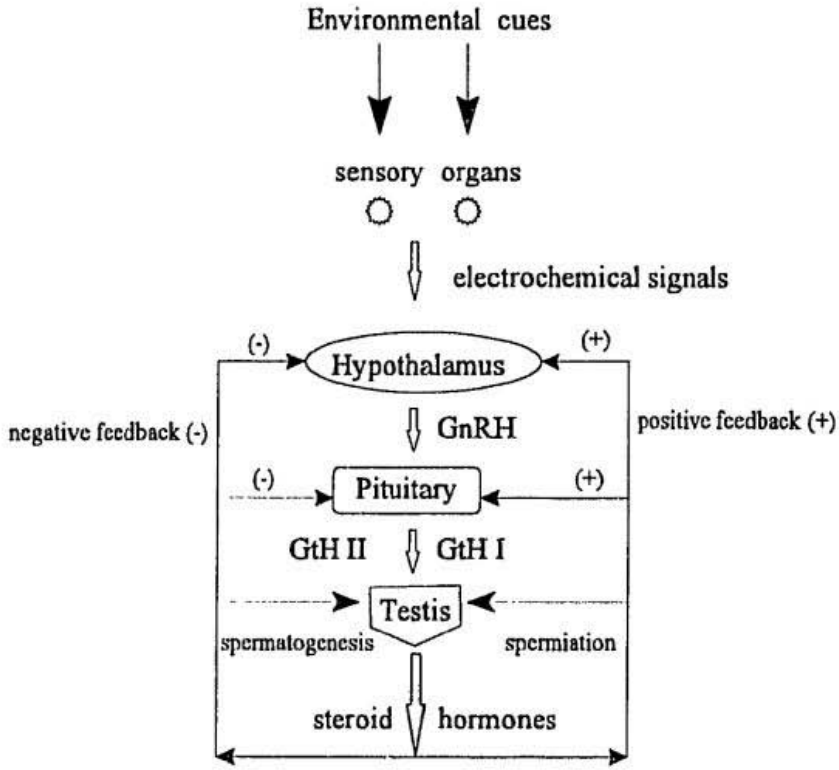


Figure 1.1 Schematic representation of the hypothalamic-pituitary-gonadal axis in teleostean fish (based on Redding and Patiño 1993) .

### 1.1.1 Environmental regulation of the seasonal testicular cycle

The reproductive cycle in teleosts, beginning with the recrudescence of the gonads and eventually final maturation of gametes, usually occurs at a particular time of a year,

particularly for teleosts of the temperate zone (Munro *et al.* 1990). Since the timing of reproduction for each species is related to conditions which optimize survival and growth of the progeny, each species prepares for breeding by undergoing seasonal-specific gonadal development. Certain predictable external environmental factors, such as photoperiod and temperature cues are important in the control of gonadal development, activating specific sensory organs, e. g. photoreceptor (pineal), which produce electrochemical signals that are transformed by the central nervous system into hormonal signals.

The effects of such environmental cues differ from species to species (Billard 1990a; Redding and Patiño 1993), and different stages of gonad development may be affected by different environmental cues. Photoperiod is a major factor influencing spermatogenesis in salmonids. Long photoperiod in the spring results in increases of gonadotropin (GtH) in both pituitary and plasma, followed by the onset of spermatogenesis. The completion of spermatogenesis in rainbow trout (*Oncorhynchus mykiss*) normally requires a decreasing photoperiod regime from 16L:8D to 8L:16D in 6 months. However, photoperiod has no effect on spermiation in rainbow trout when the temperature is between 15°C and 18 °C and in this species low temperatures favour spermiation (Breton and Billard 1977). In goldfish (*Carassius auratus*) where temperature also plays a major role in spermatogenesis interaction with photoperiod is different from that found in the trout, as long days are more favourable than short ones. The

spermatogenesis in goldfish normally occurs between 10 - 24°C from February to May and cannot be initiated if the fish are reared at 30°C. GtH content in pituitary is lower in goldfish held at 30°C and higher while at lower temperatures. Furthermore, goldfish do not spermiate under constant rearing temperatures (10, 17, 24°C), but they do under natural conditions, suggesting that fluctuating temperatures favour spermiation (Gillet *et al.* 1978).

In addition to photoperiod and temperature, social environmental cues are also an important factor, especially for spermiation. It has been demonstrated that female pheromones, e.g. rainbow trout and poeciliids, attract and stimulate the males during spawning season. Milt production, and plasma levels of testosterone (T), 11-ketotestosterone (11-KT), 17α-hydroxy-4-pregnen-3-one, and 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-OHP), increase in male rainbow trout when paired with ovulated females compared with isolated males (Olsén and Liley 1993). In goldfish and carp (*Cyprinus carpio*), GtH levels are increased and spermiation stimulated after the males are placed with females (Billard 1986).

### 1.1.2 Endocrine regulation of spermatogenesis and spermiation

Spermatogenesis and spermiation are regulated by the endocrine (brain-pituitary-gonadal) axis. Experimental disruption of any part of this axis interferes with the process

of spermatogenesis and spermiation.

GnRH is an important neural mediator of spermatogenesis and spermiation in teleosts, being a primary regulator of GtH release. Biochemical and immunocytochemical investigations demonstrate that the hypothalamus of males and females contain as many as three different forms of GnRH (Crim and Bettles 1996). Seasonal changes in the hypothalamic content of GnRH are associated with the release of pituitary gonadotropic hormones (GtHs), testicular recrudescence and maturation (Yu *et al.* 1991; Amano *et al.* 1993). GnRH treatment of male teleosts increases circulatory levels of GtH and sex steroid hormone (e.g. testosterone and 11-ketotestosterone) stimulating spermiation (Donaldson and Hunter 1983; Ngamvongchon *et al.* 1987; Harmin and Crim 1993; Harmin *et al.* 1995).

Gonadotropins regulate spermatogenesis and spermiation via steroid hormones synthesized in the testis. Many studies associate increased pituitary and blood concentrations of GtH with the onset of spermatogenesis and spermiation in male teleosts (Crim *et al.* 1975; Escaffre and Billard 1976; Breton *et al.* 1980; Billard 1986). Experimental treatment of male teleosts with exogenous GtH preparations or with GnRH which induces GtH secretion, increases sex steroid levels from testis and stimulates spermiation (Donaldson and Hunter 1983; Ueda *et al.* 1985; Ngamvongchon *et al.* 1987; Harmin and Crim 1993; Harmin *et al.* 1995). Studies of hypophysectomized male teleosts and *in vitro* studies of testicular steroid production in response to GtH have shown that

the degree to which spermatogenesis and spermiation depend on the pituitary varies according to the developmental stage of testis and species. In general, removing the pituitary after full maturation of the testis causes rapid testicular degeneration and inhibition of testicular steroidogenesis (Billard 1986; Billard 1990a). Such treatment restricts spermiation in some species such as goldfish and winter flounder, but has no effect on other species such as salmonids and plaice, *Pleuronectes platessa* (Billard 1990a). The relative sensitivity of testicular steroid production in response to GtH was found to be minimum at the onset of spermatogenesis and maximal during the spermiation period (Le Gac and Loir 1988; Sakai *et al.* 1989). Spermatogenesis and spermiation are restored after replacement hormone therapy with homologous GtH or pituitary extracts.

Recent studies indicate that the male teleostean pituitary contains two types of GtH cells. Two distinct glycoproteins, GtH-I and GtH-II, have been isolated and characterized from the pituitaries of chum (*Oncorhynchus keta*), coho (*Oncorhynchus kisutch*), and masu salmon (*Oncorhynchus masou*), rainbow trout, carp, and African catfish (*Clarias gariepinus*) (Kawauchi *et al.* 1989; Swanson *et al.* 1991; Van der Kraak *et al.* 1992; Amano *et al.* 1993; Zandbergen *et al.* 1993). Both types of GtH are steroidogenic differing only in their relative potencies (Suzuki *et al.* 1988). GtH-I is found to predominate in pituitary and blood during spermatogenesis, declining during the time of final testicular maturation and spermiation. In contrast, the levels of GtH-II which are low during spermatogenesis, increase dramatically to become dominant during the time

of final testicular maturation and spermiation. On this basis, it is suggested that GtH-I is primarily involved in spermatogenesis while GtH-II actions are reserved for the final aspects of testicular maturation and spermiation.

Several steroids from the testis, including testosterone (T), 11-ketotestosterone (11-KT), 17α-hydroxy-4-pregnen-3-one, and 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-OHP), are induced by GtH and are important in the development and maturation of the teleostean testis. A number of studies of the steroid profiles during the reproductive cycle demonstrated that plasma levels of T and 11-KT are high during the later stages of spermatogenesis and decline after the beginning of spermiation (Fostier *et al.* 1983; Ueda *et al.* 1983a; Scott *et al.* 1980; Hunt *et al.* 1982; Kime and Manning 1982; Baynes and Scott 1985). Based on *in vitro* and *in vivo* studies, it has been suggested that T stimulates and maintains spermatogenesis while both T and 11-KT induce spermiation (Fostier *et al.* 1983) although 11-KT may also play a role in the regulation of the late stages of spermatogenesis as well as initiation of milt production (Fostier *et al.* 1987). The testis of some species also produces 17α, 20β-OHP and plasma levels of this steroid peak during spermiation following the decline of T and 11-KT (Ueda *et al.* 1983b; Kobayashi *et al.* 1986 ). In the rainbow trout, the changes in plasma concentration of 17α, 20β-OHP were associated with sperm production and the $K^+$: $Na^+$ ratio in the seminal plasma suggested that 17α, 20β-OHP might be involved in the process of spermiation by regulating the ionic composition of the seminal plasma (Baynes and Scott 1985).

In addition to local action on the gonads, steroids also act on the endocrine cells of the hypothalamus and pituitary, regulating the release of GnRH and GtH and ultimately controlling the spermatogenic cycle (Billard 1986; Goos 1987). Sex steroids can exert

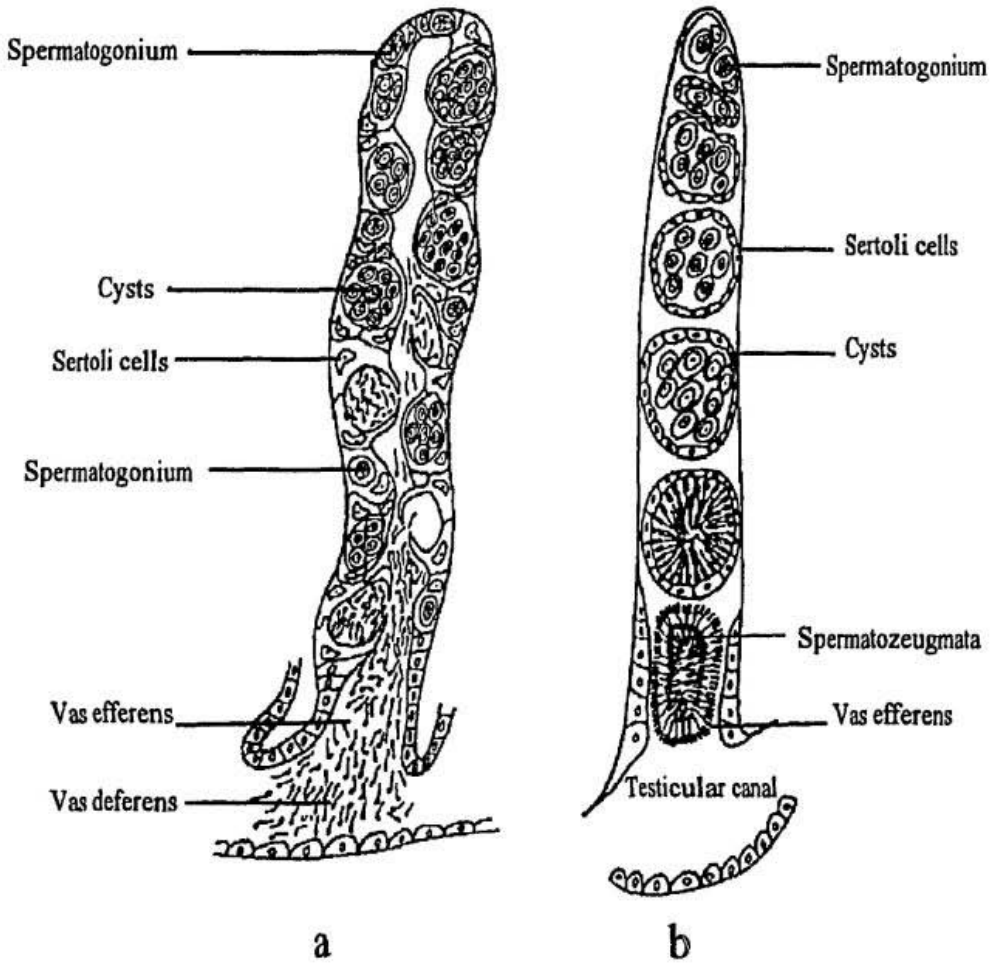


Figure 1.2 Schematic representation of lobular (a) and tubular (b) testis in teleosts (modified from Billard 1990a).

both a negative and positive feedback action on GnRH and GtH secretion, depending on the stage of testicular development in teleosts. In adult teleosts actively undergoing spermatogenesis, testicular steroids exert negative feedback on the hypothalamus-pituitary since castration initiates and steroid replacement inhibits GtH release (Billard 1986; Schulz *et al.* 1993). However, in immature or sexually regressed teleosts, testicular steroids exert positive feedback on the hypothalamus-pituitary stimulating GnRH and GtH production (Crim and Evans 1979; Crim *et al.* 1981; Crim and Evans 1983; Billard 1986; Weil and Marcuzzi 1990).

### 1.1.3 Testicular structure and function

The testis of teleosts, which is usually a paired elongated organ attached to the dorsal body wall, contains both germ and somatic cells (Sertoli cells, boundary cells, Leydig cells; Billard 1990a). Gametes are produced by germ cells, while somatic cells support, nourish and regulate the development of germ cells. Based on the distribution of spermatogonia, the teleost testicular structure can be assigned to either the lobular (unrestricted) or the tubular (restricted) type (Grier *et al.* 1980; Nagahama 1983; see Figure 1.2, page 9). In the former, the mitotically active spermatogonia are found randomly along the entire length of the testicular tubules, usually immediately beneath the tubular basement membrane (Figure 1.2a), as in salmonids and carp (Grier *et al.*

1980). In the latter type, found in the guppy and goodeid teleosts, spermatogonia are completely restricted to the blind end (apex) of the tubules, immediately beneath the testicular capsule (Figure 1.2b).

The teleostean testis consists of lobular (tubular) and interstitial compartments (Figure 1.3; Nagahama 1983; Billard 1990a). The tubular component contains germ and

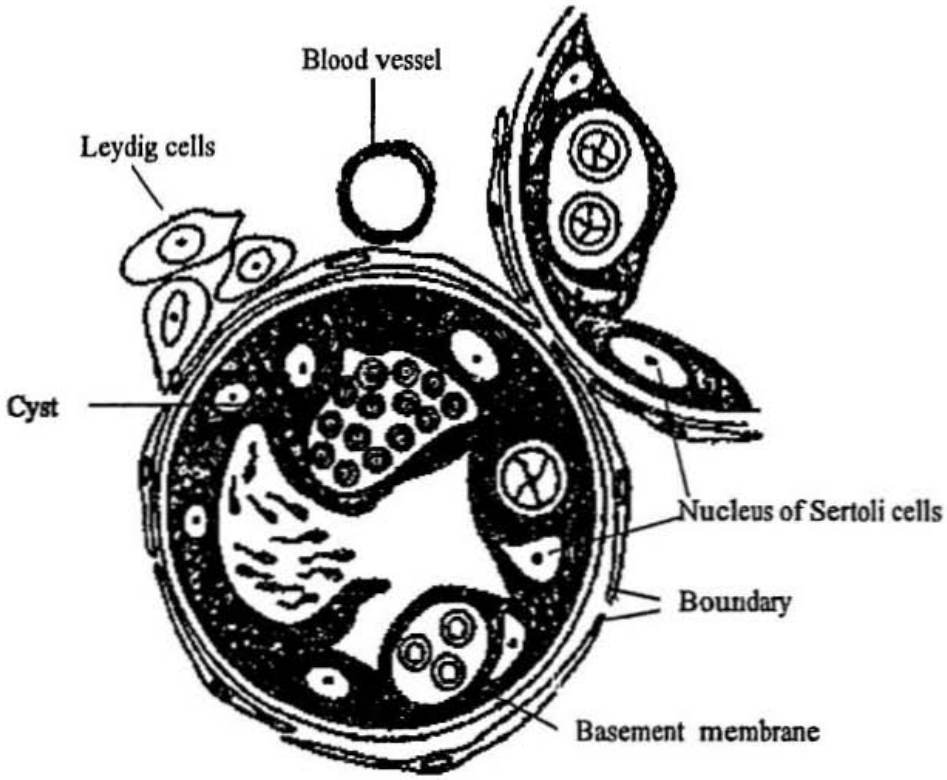


Figure 1.3 Cross-section of a testicular tubule showing its lobular and interstitial compartments (modified from Billard 1990a).

Sertoli cells, which form the germinal cysts. Germ cells develop within cysts with cytoplasmic bridges linking germ cells undergoing synchronous maturation. Evidence suggests that Sertoli cells not only provide the physical support and nurturing of germ cells, but they are also involved in phagocytosis of residual bodies cast off by developing spermatids, as well as the formation of spermatozeugmata (Grier 1981). A basement membrane layer lining the tubular surface and a discontinuous boundary-cell layer separates the germinal cysts from interstitial tissue. The interstitium of the teleost testis contains various interstitial cells, including blood and lymph vessels, and Leydig cells.

Leydig cells are considered the main testicular steroidogenic site (Nagahama 1983; Fostier *et al.* 1983). Sertoli cells, and some epithelial cells of the sperm duct and seminal vesicles of some teleosts are also capable of producing androgenic steroids (Nagahama 1983; Fostier *et al.* 1987; Schoonen *et al.* 1987), the most important being testosterone (T), 11-ketotestosterone (11-KT), and androstenedione (Fostier *et al.* 1983; Loir 1990; Bourne 1991). The testis of some teleosts also produces progesterone, 17α-hydroxy-4-pregnen-3-one, 17α, 20β-dihydroxy-4-pregnen-3-one, 11-deoxycorticosterone, and perhaps small amounts of estrogens (Fostier *et al.* 1987; Barry *et al.* 1993). Taken together, these steroids are involved in a variety of physiological activities including the regulation of spermatogenesis and spermiation, secondary sex characteristics and reproductive behaviours, secretory activity of the hypothalamus and pituitary, and general metabolism (Fostier *et al.* 1983; Billard 1990a).

## 1.2 A COMPARISON OF SPERM PHYSIOLOGY BETWEEN EXTERNALLY AND INTERNALLY FERTILIZING TELEOSTS

Spermatozoa[1] in externally fertilizing teleosts are immotile in the male genital tract or after milt[2] collection until motility, which is normally of brief duration up to several minutes (Billard 1986), is initiated by release into the external aqueous environment. Usually, the concentration of spermatozoa (spz) in milt of externally fertilizing teleosts is high ($10^9$ - $10^{11}$ spz/ml; Doi *et al.* 1982; Kruger *et al.* 1984; Ciereszko and Dabrowski 1994; Harmin and Crim 1993; Suquet *et al.* 1992). This characteristic is also reflected by the high spermatocrit ([the volume of packed spermatozoa/the volume of total milt]x100%), usually ranging from 40% - 80%. By contrast, the spermatozoa of internally fertilizing teleosts are already motile in seminal plasma (Koya *et al.* 1993; Pavlov and Radzikhovskaya 1991) and are transferred directly into the female genital tract. In some cases, the sperm are grouped together into spermatophores or spermatozeugmata (Billard 1978; Grier 1981) which become motile upon transfer into the female genital tract. In these species, the concentration of spermatozoa and spermatocrit

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[1]spermatozoa=sperm

[2]milt=sperm+seminal plasma

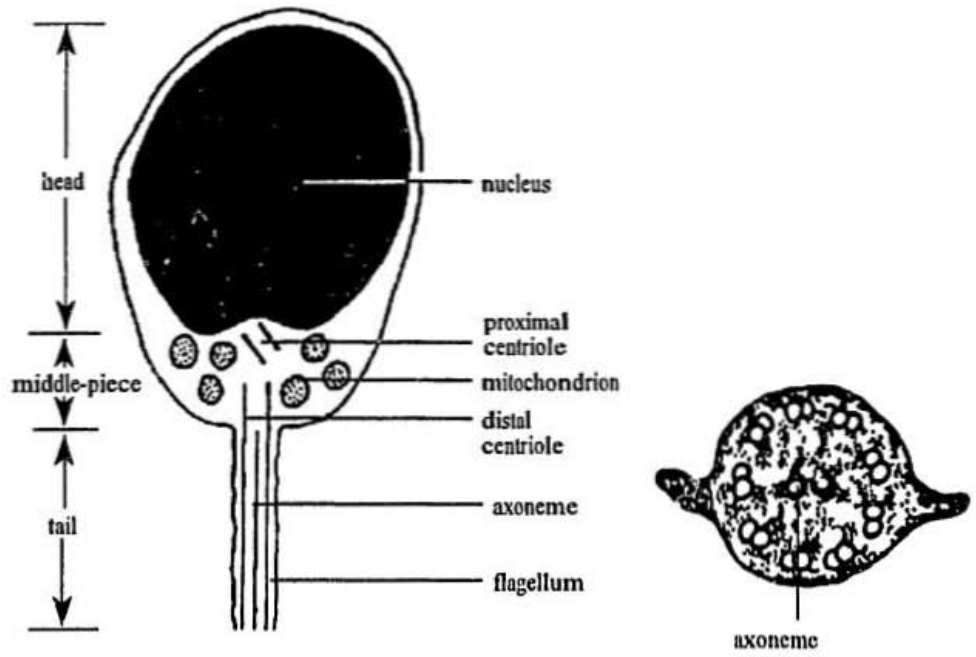


Figure 1.4 Basic sperm morphology in teleosts (after Jamieson and Leung 1991).

are generally lower ($10^6$ - $10^8$ spz/ml and 1 - 10%, respectively) compared with the externally fertilizing teleosts (Pavlov and Radzikhovskaya 1991) and the duration of sperm motility in internally fertilizing teleost is longer, up to several hours or days.

### 1.2.1 Sperm morphology

In a review of teleost sperm morphology Jamieson and Leung (1991) stated that teleost sperm can be structurally divided into a head, the mid-piece, and an elongated tail (Figure 1.4). Typically, fish sperm heads are spherical or oval shapes in the absence of an acrosome characteristic of teleosts which is probably related to the presence of the

micropyle in teleost eggs. The mid-piece consists of a mitochondrial sheath and a central flagellum. In most teleost sperm, the mitochondria are few in number, not modified, and situated in a low collar immediately behind the nucleus. The flagellum of most teleost sperm consists of a typical 9+2 axonemal pattern (nine pairs of peripheral microtubules and one pair of central microtubules). Aflagellated sperm and biflagellated sperm are found in some species.

Generally sperm morphology seems to be related to the biological mode of fertilization. For example, the spermatozoa from external fertilizers exhibit simple structures and do not appear modified (Figure 1.5a; Billard 1990a). The nucleus of sperm generally is round or spherical shape and the chromatin is less condensed, because of limited replacement of histones by protamines. A reduced mid-piece contains few mitochondria, which probably results in short duration motility after sperm activation. These sperm cells, without any modified structure (no sperm bundles), are freely released from cysts, then shed into water (Billard 1983a). In teleosts which have evolved internal fertilization, sperm are complex and greatly modified (Billard 1990a). The sperm nucleus is elongated as a blade by a series of microtubules during spermiogenesis (Figure 1.5b). Meanwhile, the histones of the chromatin are replaced by protamines, resulting in high nucleus condensation. The mid-piece is extremely well developed, and contains numerous large mitochondria, which is probably related to the long duration of sperm motility. Glycogen particles are visible in the mid-piece area in some internally fertilizing species

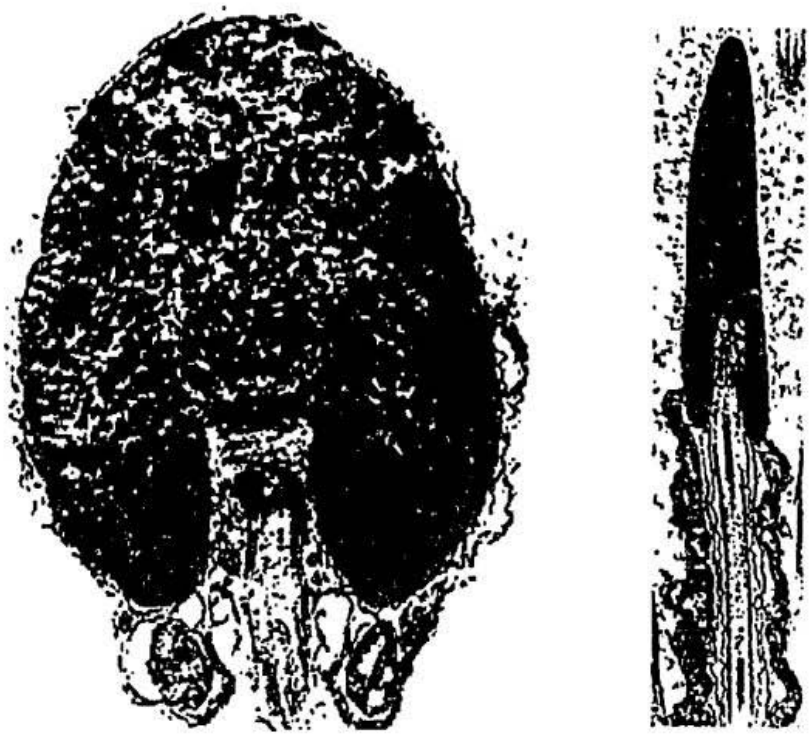


Figure 1.5 The differences of sperm morphology in externally and internally fertilizing teleosts: a) trout spermatozoa. 40,000x; b) guppy spermatozoa. 21,000x (after Billard 1990b).

(guppy, *Poecilia reticulata*). In many internally fertilizing teleosts, sperm are packed together either as unencapsulated (spermatozeugmata; see Figure1.2b) or encapsulated (spermatophores) balls. Sperm transfer in bundles is coupled with the evolution of the appropriate anal fin (gonopodium) and behavioral mechanisms to ensure efficient transfer of male gametes into the female reproductive tract.

In summary, the sperm morphology of externally fertilizing teleosts is primitive with a round nucleus and short mid-piece. In teleosts evolving the internally fertilizing mode, sperm morphology is more complex, with elongated nucleus and well developed mid-piece.

### 1.2.2 Sperm activation and motility

It is suggested that the stimulation of sperm motility could be owing to a change of membrane potential, since sperm motility does not arise in the presence of desmethoxy verapaniel, a special inhibitor of voltage-dependent calcium channels (Billard 1990b). Osmotic pressure, ionic composition and pH may depolarize the sperm cell membrane and are the most important factors determining the activation and motility of the sperm in external fertilizers (Stoss 1983).

Changes in osmotic pressure initiates sperm motility in externally fertilizing teleosts. Hypotonicity generally activates sperm motility in freshwater teleosts, especially in carp (Redondo *et al.* 1991). On the other hand, the motility of spermatozoa in marine teleosts, such as cod (*Gadus morhua*), flounders (*Limanda yokohamae* and *Kareius bicoloratus*) and halibut (*Hippoglossus hippoglossus*), is induced by hypertonic suspension media (Billard *et al.* 1993). Motility occurs in a wider range of osmotic pressures in marine teleosts than in freshwater teleosts.

Besides osmotic pressure, ionic composition is also involved in inhibiting or stimulating the motility of spermatozoa in both freshwater and marine teleosts. Ionic regulation of sperm motility in externally fertilizing teleosts is very complex, and is mainly determined by potassium ($K^+$), calcium ($Ca^{++}$) and magnesium ($Mg^{++}$). Although the mechanism of ionic regulation of sperm motility is still unclear, potassium inhibits, and calcium and magnesium activate sperm in salmonids (Morisawa and Suzuki 1980). In contrast, potassium ion increased the motility of carp sperm (Billard and Cosson 1992). In marine teleosts, potassium ion had no inhibitory effect on sperm motility in the summer whiting, *Sillago ciliata* (Goodall *et al.* 1989), flounder, *Platichthys flesus*, and cod, *Gadus morhua* (Morisawa 1985). The study by Goodall *et al.* (1989) showed that both calcium and magnesium ions increase the duration of sperm motility in the summer whiting. Clearly, ionic regulation of sperm motility varies from species to species.

External pH also affects sperm motility. Generally, alkaline pH favours increased motility and fertility of sperm, while acidic pH inhibits sperm motility in many externally fertilizing teleosts (except in a few teleosts whose sperm is acid-tolerant, e.g. chain pickerel [Duplinsky 1982]). An activating solution below pH 7.8 failed to induce the motility of rainbow trout sperm, and the optimum motility of sea bass sperm was reached in pH 9 seawater (Stoss 1983). The highest motility of halibut sperm occured at pHs ranging between 7.5 to 8.5 (Billard *et al.* 1993).

Knowledge of the regulation of sperm motility in the teleosts with internal

fertilization is limited. Sperm motility is achieved in the guppy, goodeid and *Horaichthidae* teleosts, after the breakdown of the spermatozeugmata or spermatophores in the female genital tract. Billard (1978) demonstrated that motility is initiated without dilution immediately after the sperm bundle of the guppy is dissociated. According to Morisawa and Suzuki (1980), however, breaking up the sperm bundle and initiating sperm motility of guppy and the topminnow (*Gambusia affinis*) occurred in electrolyte solutions such as NaCl or KCl solutions at concentrations between 50 and 300 mmol/kg, especially in KCl solution, but not in a nonelectrolyte solution such as mannitol (100 - 700 mmol/kg). They suggested that ionic concentrations, especially potassium concentrations, might initiate sperm motility in these internally fertilizing teleosts. The spermatozoa of the elkhorn sculpin (internal insemination), *Alcichthys alcicornis* (Koya *et al.* 1993) and wolffish (*Anarhichas lupus*) are already active in freshly collected milt without apparent initiation of activation. Koya *et al.* (1993) showed that the greatest motility of elkhorn sculpin sperm occurred in $Na^+$-supplemented medium rather than other ions and that the optimum ranges for pH and osmotic pressure were pH 7 - 9 and 300 - 400 mmol/kg, respectively. Therefore, osmolality, pH and ionic composition appear to be important factors regulating sperm motility in internally fertilizing teleosts.

### 1.2.3 Sperm metabolism

The duration of sperm motility is very short in externally fertilizing teleosts where osmotic shock may be one factor (Billard 1983b). For example, the spermatozoa of trout (*Salmo trutta*) and carp are quickly damaged after dilution in freshwater. On the other hand, some teleost sperm from external fertilizers are capable of withstanding osmotic shock and still display short-term motility, e.g. tilapia (*Oreochromis mossambicus*). Interestingly, the duration of motility is not extended if sperm are activated in a isosmotic saline medium (from 45 sec. to 90 sec.; Billard 1978). The limited supply of metabolic energy during motility may be an important factor affecting the duration of motility. The energy, mainly provided by ATP (Billard *et al.* 1995a), is obtained from catabolizing both endogenous and exogenous substrates.

For the sperm of externally fertilizing teleosts, the supply of energy from exogenous substrates is very poor, because the spermatozoa are released in an external aqueous environment (Stoss 1983). Therefore, most of the energy for motility is derived from endogenous substrates, especially those substrates localized in the mid-piece (Billard *et al.* 1995a). Furthermore, the small amounts of mitochondria play an important role in producing ATP. In rainbow trout sperm, the intracellular ATP levels are quickly depleted following the initiation of motility (Billard *et al.* 1995a). Demembranated spermatozoa *in vitro* display flagellar waves for extended periods of time (up to 30 min.) at frequencies

depending upon the ATP concentration used in the medium. Since the decrease of flagellar beat frequency during rainbow trout sperm movement reflects a decrease of intracellular ATP concentrations (Christen *et al.* 1987), these results indicate that sperm cell metabolism is not fast enough to cope with the high energy demand during motility and cannot compensate for the rate of ATP hydrolysis by the dynein ATPase. Therefore, the energy available during motility of rainbow trout sperm is mainly produced by the accumulated ATP before motility initiation.

The spermatozoa of internal fertilizers are directly transferred into the genital tract of the female and the metabolic energy during long-term sperm motility may be provided by both endogenous and exogenous substrates (ovarian and seminal plasma). In the presence of a well developed mid-piece, the large amounts of mitochondria enable sperm to quickly synthesize ATP to meet the high energy demand during extended motility. Billard and Jalabert (1973) suggested that glycogen particles stored in the mid-piece of guppy sperm are a major endogenous substrate after the initiation of sperm motility. They also found that the glycogen stores are much higher in spermatozoa found in the testis than in the ovarian cavity of the female, and glycogen stores were depleted when spermatozoa were diluted in a saline solution without glucose. In surfperch spermatozoa, Gardiner (1978) demonstrated the metabolism of glucose and noticed that the addition of glucose to surfperch and the guppy sperm considerably increased the duration of motility. Following the cessation of spermatozoa movement, they were reactivated by a saline

solution enriched with glucose.

In conclusion, the sperm of external fertilizers display a short duration of motility, which is correlated with accumulated ATP before motility initiation. In contrast, sperm from internal fertilizers are motile for long periods of time and display relatively complex metabolism and catabolism processes possibly dependent upon both endogenous and exogenous substrates.

## 1.3 A REVIEW OF OCEAN POUT REPRODUCTION

### 1.3.1 Distribution and systematics

The ocean pout (*Macrozoarces americanus*), distributed on both sides of the North Atlantic Ocean, is a common species in the Northwest Atlantic, where it can be found from Battle Harbor, Labrador, to the Gulf of St. Lawrence and along the coasts of the Maritime provinces (Olsen and Merriman 1946; Scott and Scott 1988). As a ground fish, the ocean pout resides in depths from 10m to over 183m and is more abundant on hard and semi-hard bottom than on muddy substrate. According to conditions at the time of capture, it prefers a temperature and salinity of 6 - 9$^{0}$C and 32 - 34ppt, respectively, although it can be found in temperatures from 0 - 16.7$^{0}$C.

The ocean pout is in the family Zoarcidae, the suborder Zoarcoidei, the order Gadiformes (Nelson 1976). Common names for this fish include eelpout, 'congo eel', muttonfish and lamper eel (Olsen and Merriman 1946; Scott and Scott 1988). This eel-like species is characterized by a broad and heavy head, a terminal mouth with thick fleshy lips and conical teeth in the front of each jaw and one series on the sides. The small and short pelvic fins are located in front of the large pectoral fins. The dorsal and anal fin are continuous to the pointed caudal fin (Figure 1.6). Dorsal colour varies from muddy yellow to reddish brown mottled with grey or olive green, and the colour of the belly is from white to dull yellow. The smooth scaleless skin is covered with mucus (Bigelow and Welsh 1925; Scott and Scott 1988).

### 1.3.2 Biology of reproduction

A detailed study of the biology of ocean pout reproduction was carried out by Olsen and Merriman (1946). They and others (Bigelow and Schroeder 1953; Kohler 1968)

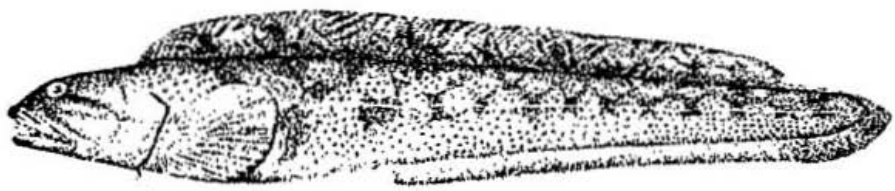

Figure 1.6 Ocean pout, *Macrozoarces americanus* (after Scott and Scott 1988).

reported that ocean pout carry out limited migrations, a reproductive migration to shallow water in spring and a return to deep water in autumn. Evidence for an inshore-offshore migration was also found in the eastern Newfoundland area, which suggested the fish move inshore to spawn in the spring (Keats *et al.* 1985).

Olsen and Merriman (1946) reported that male ocean pout do not mature until reaching a body length of 25 cm, and almost all males of 39 cm in length (4 - 5 year old) are mature. Females mature later where the smallest mature size is approximately 45 cm (5 - 6 year old). Spawning occurs earlier in northern waters and progressively later in southern waters. For example, in the Newfoundland area, spawning occurs in late August (Keats *et al.* 1985), while in southern New England and the Gulf of Maine it takes place in late September and October (Bigelow and Schroeder 1953). Multiple spawnings are unlikely due to the uniform slightly spherical egg masses (Keats *et al.* 1985) which the ocean pout deposits in rocky areas (Olsen and Merriman 1946; Bigelow and Schroeder 1953; Keats *et al.* 1985). After spawning, the egg masses are under parental protection (Bigelow and Schroeder 1953; Olsen and Merriman 1946) which is usually provided by the female during the long period until hatch (Anderson 1985; Keats *et al.* 1985). It takes about 3 months for fertilized eggs to hatch in the laboratory at 4 - 11°C (Methven and Brown 1991).

In recent studies of male and female reproduction conducted at the Ocean Sciences Centre (OSC) of St. John's, Newfoundland, the sperm of ocean pout have been found to

be already motile at the time of collection of milt samples. Upon the addition of seawater to the milt, sperm motility ceases immediately (Crim *et al.* 1995; Yao and Crim 1995a, b; Yao *et al.* 1995). During the spawning season, mature males developed a papilla (protrusive genital pore), and copulation has been observed between the males and females prior to oviposition (Yao and Crim 1995b). Artifical injection of milt into the female ocean pout ovary has yielded fertilized eggs, and the females usually spawned their eggs within 24 hours after insemination (Yao and Crim 1995a). According to these studies, it was suggested that the ocean pout is an internally fertilizing species with biflagellated sperm and the eggs have at least two-micropyles. However, detailed knowledge of the male reproduction and studies of sperm physiology throughout the spawning season were not available.

### 1.3.3 Aquaculture capability

The ocean pout is reported to have many favorable traits for cold water aquaculture. For example, the flesh is delicate with low fat and low cholesterol concentration (Sheeny *et al.* 1977). Market studies have indicated that ocean pout's flesh is acceptable to the American consumer (Sheeny *et al.* 1977; Brown *et al.* 1992). Since the ocean pout contains high concentrations of antifreeze proteins in the plasma and tissues (Fletcher *et al.* 1985; Hew *et al.* 1988), it is able to survive freezing seawater

temperatures (-1.7 °C; Kao *et al.* 1986; King *et al.* 1989). Studies also reported that newly hatched larvae appeared adult-like with functional eyes and mouth, and there was no pelagic stage or apparent metamorphosis. Therefore, the larval survival rates are high (>90%) in the first two months of exogenous feeding and decreased moderately to 75 - 80% in the second year (Methven and Brown 1991; Brown *et al.* 1992).

However, for successful cultivation of the ocean pout, the supply of seed which is limited must be improved through artificial reproduction. Because the ocean pout and wolfish (*Anarhichas lupus*) share similar reproductive biologies (Keats *et al.* 1985; Pavlov and Radzikhovskaya 1991), studies of ocean pout reproduction may also contribute to our understanding of the reproductive physiology of wolfish, another potential new species for cold water aquaculture in eastern Canada.

## 1.4 OBJECTIVES OF THE STUDY

The purpose of this study was to examine the reproductive physiology of the male ocean pout. In particular, the objectives were:

1) identify the reproductive season for male ocean pout;

2) investigate if sperm quality changes during the spawning season;

3) determine the seasonal variations in the testicular index and the plasma

androgenic steroid profiles during the spawning season;

4) study the relationship between the biochemical characteristics of seminal plasma and sperm motility in the ocean pout.

## 1.5 APPROACHES OF THIS STUDY

In accordance with these objectives, two studies were carried out on male ocean pout in 1993 and 1994.

*Study 1.* This study focused on the physiological characteristics of the spawning season in the male ocean pout, including the timing of spermiation, changes in sperm motility, spermatocrit, sperm concentration, the gonadosomatic index (GSI), and plasma androgenic steroids.

*Study 2.* This study focused on the seasonal aspects of sperm physiology and the biochemistry of seminal plasma, including the seasonal variations in pH, osmotic pressure and biochemical composition. In addition, the effects of pH and osmotic pressure on sperm motility were investigated.

# CHAPTER 2

# PHYSIOLOGICAL CHARACTERISTICS OF SPAWNING IN THE MALE OCEAN POUT

## *Macrozoarces americanus*

## 2.1 INTRODUCTION

Ocean pout, *Macrozoarces americanus*, is a benthic marine fish in the Northwest Atlantic Ocean (Scott and Scott 1988), which migrates inshore to spawn in shallow water in the summer. Despite the knowledge that males are summer spawners, the details concerning the timing and duration of spermiation, changes in sperm quality, and the changes in plasma steroids during the spawning season have not been studied.

Since the timing of spermiation and sperm quality are among the major factors influencing the fertilization rate of eggs, a better understanding of male reproductive biology will improve management of brood stock and assist with successful artificial reproduction in captivity.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Experimental animals

Adult male ocean pout (2 - 4 kg) were collected from Newfoundland waters by SCUBA divers during the spawning season, from June to September in 1992 and 1993. They were transported to the Ocean Sciences Centre, and maintained together with

females in flowing ambient seawater (from -1.9 to 18.6$^{0}$C) in indoor round (2 x 2 x 0.4m) fibre-glass tanks. Each tank held 15 fish. The animals were provided with a simulated natural photoperiod and fed chopped capelin twice a week.

### 2.2.2 The gonadosomatic index (GSI)

From September 1993 to August 1994, samples of the testes were obtained from males killed at 1 - 2 month intervals (three males at each time except in September 1993 and January 1994 where only two males were available). Seasonal changes in testicular development were determined according to changes in the gonadosomatic index ([gonad weight / body weight] x 100%).

### 2.2.3 Spermiation

For two years, during the reproductive season from May to October, spermiation was determined at 1 or 2-week intervals by collecting milt from a polyethylene tube inserted through the urinogenital pore into the sperm duct. Ten males in 1993 and 20 males in 1994 were studied and maturation rates were calculated according to the percentage of spermiating fish ([spermiating fish / total fish] x 100%).

### 2.2.4 Sperm motility

Milt samples after collection in borosilicate tubes were briefly kept on crushed ice. Sperm motility was estimated according to the percentage of motile spermatozoa. A 20 µl drop of milt was placed beneath a coverslip on a glass slide at room temperature (~20°C) for light microscopic (400x) observation of the percentage of motile spermatozoa. Three slides were prepared for each milt sample and three fields of view were examined on each slide. After calculation of an average, the relative motility of sperm was ranked according to 5 classes of motility: 0) 0%, 1) 1 - 25%, 2) 26 - 50%, 3) 51 - 75%, and 4) 76 - 100% of motile sperm, respectively.

### 2.2.5 Spermatocrit

In 1993, two small aliquots of freshly collected milt samples from 10 males were transferred into microhematocrit capillary tubes (length=75 mm, ID=1.1 - 1.2 mm). The tubes were sealed with clay, centrifuged for 10 min. at 12,000 x g in a Micro Hematocrit centrifuge (International Equipment Co., A Division of DAMON, made in USA; Model MB), and the spermatocrit was determined (packed spermatozoa volume / total milt volume x 100%).

### 2.2.6 Sperm concentration

The sperm concentration of milt was analyzed in 37 samples collected from 10 males in early July (July 7), middle July (July 19), early August (August 1 and 2), late August (August 31), and early September (September 1) 1993. To inhibit motility and still maintain sperm integrity, milt was diluted in 1:50 DCSB4 buffer (sucrose: 150 mM, $MgSO_4 \cdot 7H_20$: 7 mM, $CaCl_2 \cdot 2H_2O$: 1.7 mM, glycine: 86 mM, and Tris-HC1: 30 mM at pH 8.0 and osmotic pressure 450 mmol/kg [Billard *et al.* 1993]). The spermatozoa were counted using a hemocytometer (American Optical Scientific Instrument Division, 1975) under light microscope at 400x magnification, and the procedure was repeated twice to obtain an average.

### 2.2.7 Androgenic steroids in blood plasma

From May to October 1994, six males were anaesthetized either weekly or biweekly in 100 ppm 2-phenoxyethanol (Sigma Chemical Co.), and a 1.0 ml blood sample was withdrawn from the caudal vein using cool and heparinized 23G needles and syringes. Blood samples were placed in crushed ice after collection before being centrifuged at 4°C for 10 min. at 8,827 x g. Plasma samples were removed and stored frozen at -20°C until androgenic steroid measurement.

The levels of the two major male androgens, testosterone (T) and 11-ketotestosterone (11-KT), were measured in plasma samples by radioimmunoassay (RIA) according to methods described by Harmin and Crim (1993) with some modifications (appendix 1 & 2). Briefly, 100 µl samples of plasma and 10 µl ethanol containing 1,000 CPM of tritiated testosterone (T) were mixed in borosilicate disposable culture tubes. After incubation for 1 - 2 hours at room temperature (20 - 25°C), the mixtures were extracted twice with 2 ml diethyl ether before freezing the aqueous phase over solid $CO_2$ and combining the ether fractions into a clean tube. After evaporating the ether extracts under nitrogen with gentle heating (40°C), the plasma residues were redissolved in 1.0 ml absolute ethanol and stored overnight at 4°C before the RIA was performed.

The plasma extraction efficiency for the androgens in each sample was determined in 100 µl of the ethanol extract by mixing it with 10 ml scintillation cocktail in a scintillation vial. Recovery rates (%) of the tritiated testosterone added to each sample were calculated from the mixtures in a beta counter (Minaxi, β Tris Carb 4000 series). Since recovery rates of 11-ketotestosterone are similar to that of testosterone (± 5% differences; unpublished), recovery rates of testosterone were used for 11-ketotestosterone.

For the measurement of testosterone, 100 µl of the ethanol plasma extracts or standard testosterone in ethanol (1 - 1,000 pg/tube) were pipetted into duplicate 12 x 75 mm borosilicate disposable culture tubes. Following evaporation of the ethanol under nitrogen, 200 µl assay buffer (50 mM $Na_2HPO_4$, 100 mM NaCl, 0.1% $NaN_3$, 0.1%

gelatin, pH 7.4), 100 µl iodinated T (10,000 CPM), and 100 µl diluted T antiserum were added to assay tubes. After a primary incubation period of 1 hr at 22 - 27°C, 1,000 µl of the separating reagent was added to the assay tubes for another 25 min. incubation period prior to centrifugation of the tubes at 1,000 x g for 15 min. The supernatant (containing free testosterone) was then carefully aspirated and the radioactive precipitates were counted in a Packard Auto-Gamma Counter (see appendix 1).

Similar methods were used to determine the 11-KT values in plasma. Again, 100 µl of ethanol extracted plasma was evaporated under nitrogen. A 100 µl aliquot of phosphate buffered saline (PBS: 28 mM $NaH_2PO_4 \cdot H_2O$, 61 mM $Na_2HPO_4$, 154 mM NaCl, 0.1% gelatin, pH 7.0), 100 µl tritiated 11-KT (6,000 - 8,000 CPM), and a 100 µl 11-KT antiserum (1:70,000) were added to the assay tubes. After an overnight incubation at 4°C, 600 µl of a charcoal suspension (1.25 g charcoal, 0.125 g dextran T-70 in 500 ml PBS buffer) was added and the assay tubes incubated for another hour at 4°C. Following centrifugation of the tubes at 1,000 x g for 25 min., the supernatant was decanted into a vial containing 10 ml scintillation cocktail to determine the levels of bound radioactivity in a beta scintillation counter (see appendix 2).

### 2.2.8 Statistical analysis

Regression analysis was applied to 37 milt samples to examine the relationship between sperm concentration and spermatocrit. In addition, this relationship was tested on a subset of 29 milt samples collected from 10 males in middle July, early August, late August, and early September following the disappearance of the relatively large, non-motile spermatids.

Seasonal GSI variation for males was analyzed using ANOVA. A GLM (General Linear Model) procedure (SAS Institute Inc., 1989) was applied, in view of unbalance design data. A Least Square Means test for multiple comparison was utilized to detect the differences between GSI values from September 1993 to August 1994.

## 2.3 RESULTS

### 2.3.1 Seasonal variations in the GSI

From September to January, the GSI of males was low (0.26 - 0.31%; Figure 2.1). By March, however, the GSI began slowly rising indicating that gonadal recrudescence was underway. A major increase in GSI was detected by June reaching a peak in July

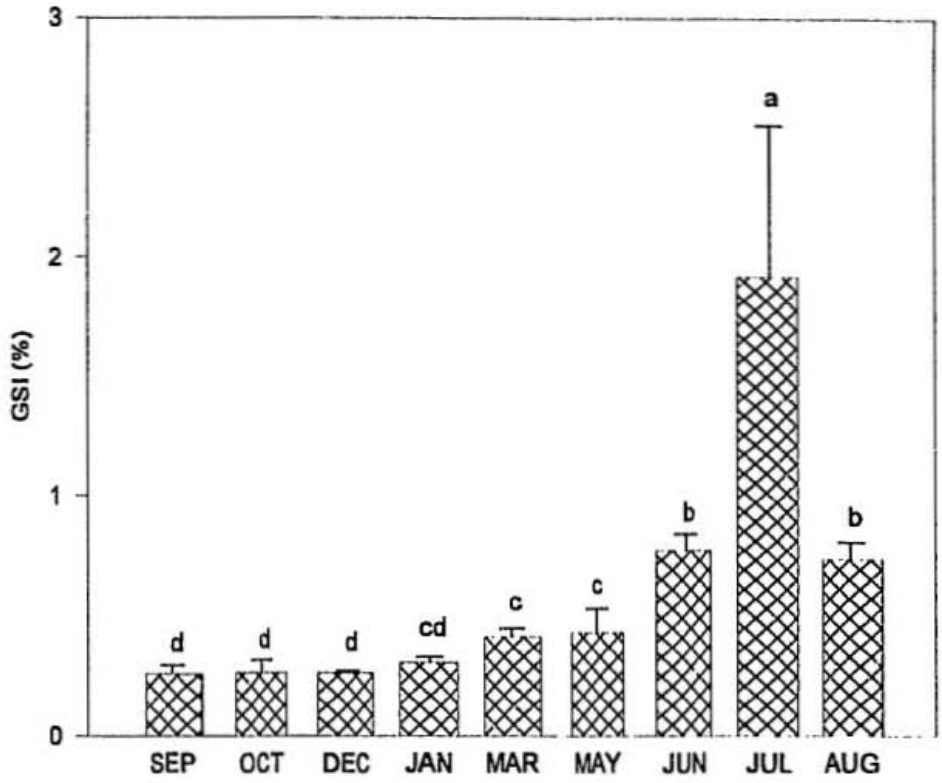


Figure 2.1. Seasonal variations in the gonadosomatic index (GSI) of the male ocean pout (Mean+SE, n=3 except for SEP and JAN where n=2). Months assigned the same letter are not significantly different ($p<0.05$).

(GSI = 1.92%) together with the onset of spermiation. The GSI declined significantly during the spawning season from July to August.

### 2.3.2 The pattern of spermiation

A two-year study of the ocean pout spermiation response yielded a similar pattern

for both years. In 1993 spermiation was detectable in some males (40%) by June 22 while all the males were in spermiating condition by July 18 (Figure 2.2a). Spermiation terminated by September 24, concluding a period of 13 weeks. During the 1994 spawning season, milt was first collected from some males (40%) on June 13 and all males were spermiating by June 30. Spermiation was terminated by October 3 (Figure 2.2a) with the period of spermiation lasting 17 weeks. All males that spermiated in 1993 rematured and produced milt again in 1994.

### 2.3.3 Seasonal variations in sperm motility

Throughout the spawning season, the percentage of motile sperm, rose and fell in a manner similar to the pattern of the spermiation responses (Figure 2.2b). Initially, only half of the spermiating males in 1993 and none of the males in 1994 produced sperm of high motility (percentage of motile cells > 75%). As the season progressed, sperm motility improved, reaching a peak by July and August. By September, however, sperm motility fell again.

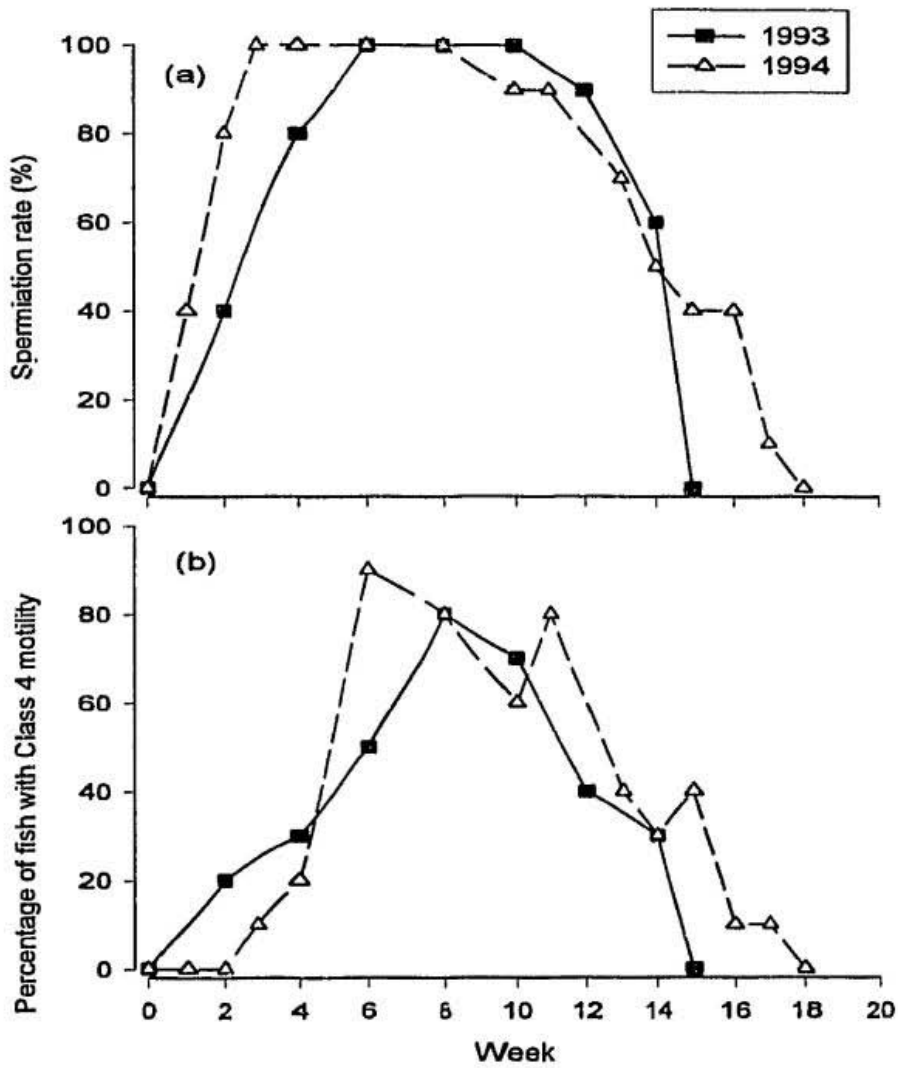


Figure 2.2. Seasonal patterns of spermiation (a) and sperm motility (b) in male ocean pout during 1993 (n=10) and 1994 (n=20). Week 0 represents the time of onset of spermiation (June 22 in 1993 and June 13 in 1994). (a) Changes of the spermiation rate (%) during the spawning season. (b) Percentage of fish with Class 4 motility (76% - 100% motile sperm) during the spawning season.

### 2.3.4 Variations of spermatocrit

In 1993, the spermatocrit declined from >4% to <1% as the season progressed (Figure 2.3). Initially, the spermatocrit was relatively high due to the presence of a high percentage (>50%) of big, non-motile spermatid cells in the early seasonal milt samples (Figure 2.4). As the spermatocrit fell to lower values (1.4 - 1.8%) during the middle of spawning season, the large spermatids had nearly disappeared (Figure 2.5). Finally, the spermatocrit fell to the lowest values by the end of the spawning season.

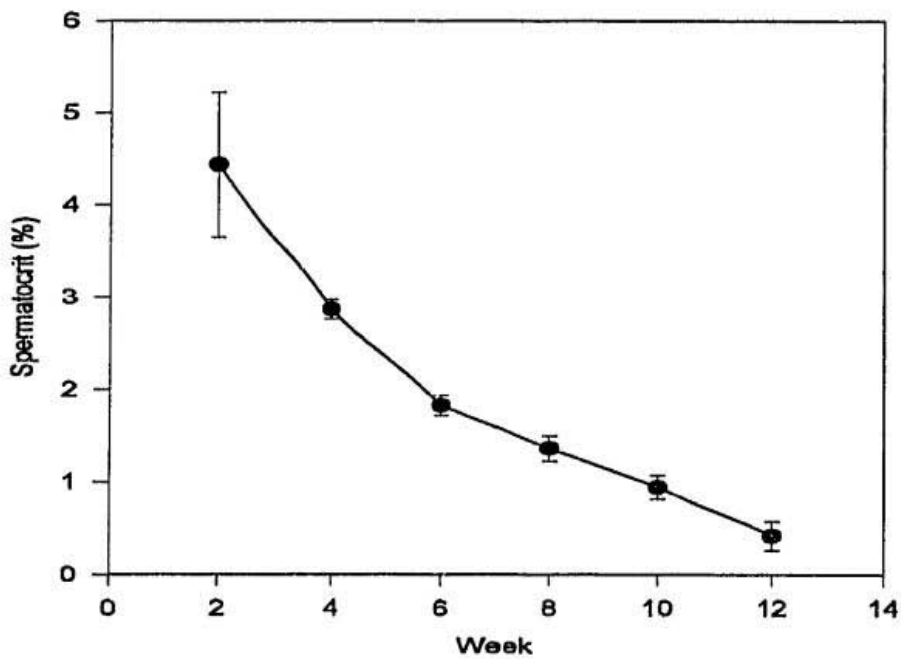


Figure 2.3. The variation of spermatocrit during the 1993 spawning season (Mean±SE, n=10). Week 0 represents the time of onset of spermiation (June 22).

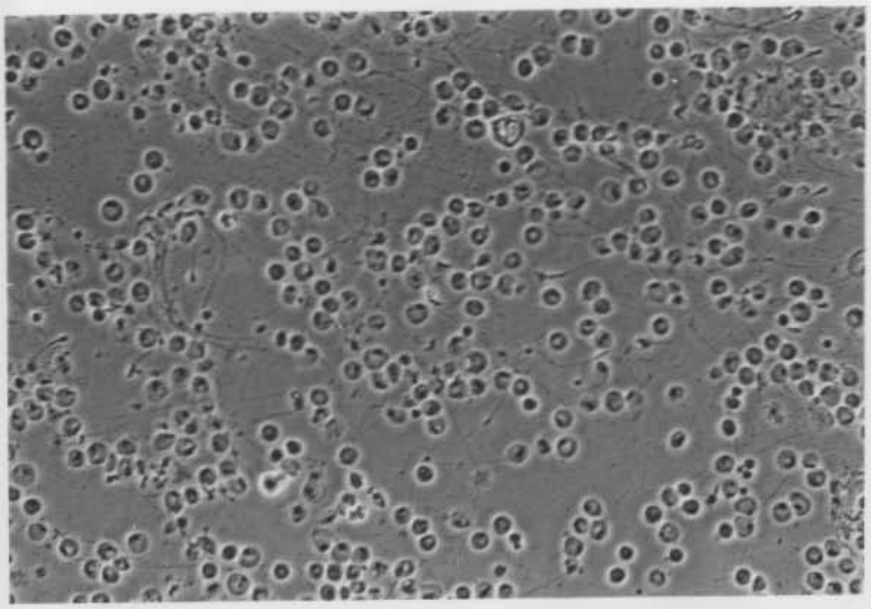

Figure 2.4 A microscopic view (400x) of a milt sample (male E8) collected in June 1993.

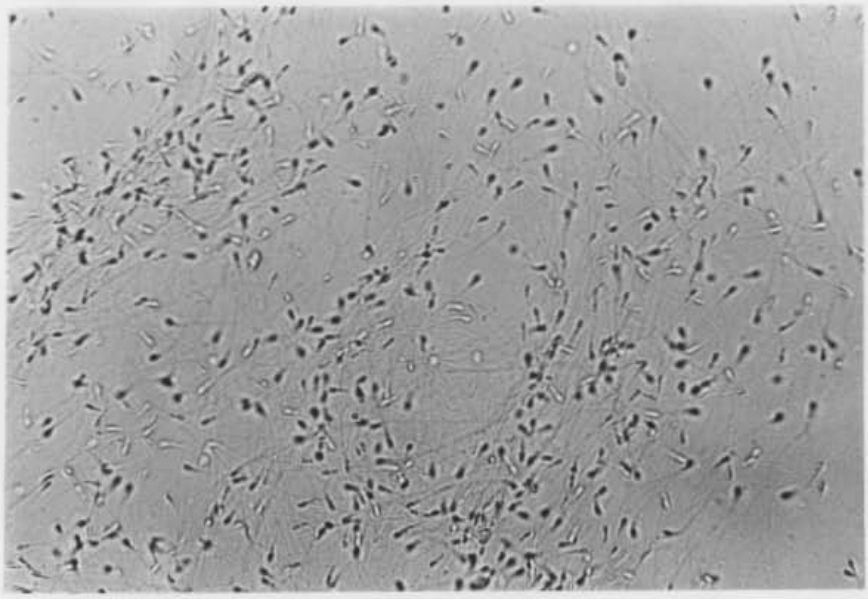

Figure 2.5 A microscopic view (400x) of a milt sample (male E8) collected in August 1993.

### 2.3.5 Sperm concentration

The sperm concentration of ocean pout milt ranged between 0.91 - 6.81 x $10^8$ spz/ml (n=37). Although no linear relationship was found between sperm concentration and spermatocrit from the analysis of all 37 milt samples, linearity was established if samples containing a high percentage (>50%) of spermatids were excluded (Figure 2.6; $r^2$=0.79, P<0.0001, n=29). The linear relationship is described by the equation, Y=1.46+1.48X, where X is spermatocrit and Y is sperm concentration ($10^8$ /ml).

### 2.3.6 Plasma androgenic steroid profiles

A study of the plasma levels of testosterone (T) and 11-ketotestosterone (11-KT) during the spawning season revealed similar patterns for both hormones (Figure 2.7). Plasma T levels increased several weeks prior to the onset of spermiation, briefly reaching a maximum approaching 30 ng/ml just before the onset of milt production. As T rapidly decreased, maximal levels of 11-KT occurred at the time of onset of spermiation. While the period of spermiation continued, T and 11-KT levels began to decline, reaching low levels for the duration of the spawning season (July to September).

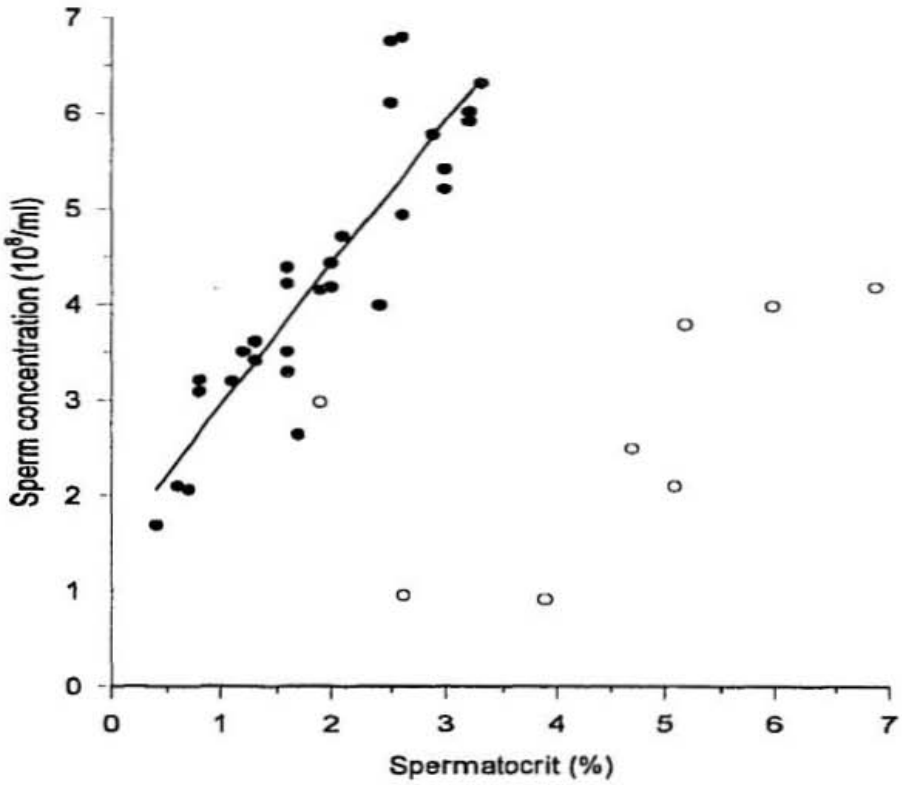


Figure 2.6. The relationship between spermatocrit (%) and sperm concentration ($10^8$/ml) for ocean pout milt. The 8 samples (circles) containing 50% or higher of spermatids were excluded from the regression analysis.

## 2.4 DISCUSSION

This two-year study clearly showed that the reproductive cycle of the male ocean pout is characterized by distinct seasonal variations in the gonadosomatic index, sperm

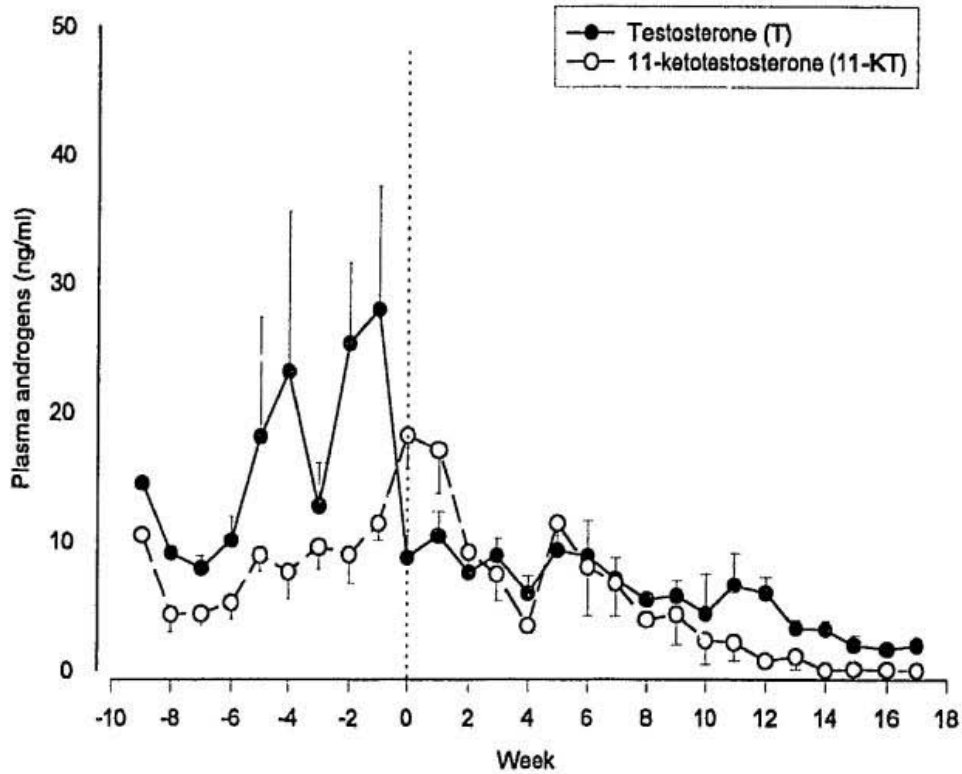


Figure 2.7. Changes in plasma androgens in male ocean pout during the 1994 spawning season (Mean±SE, n=6). Week 0 represents the time of onset of spermiation for each male; negative values indicate weeks before spermiation.

motility, spermatocrit, sperm concentrations of milt, and plasma androgen levels. At the beginning of reproductive development in May, GSI and plasma androgens (T and 11-KT) were low. In June, a significant rise in GSI was accompanied by increases of both T and 11-KT in the plasma. Just before spermiation, T reached its maximum level before rapidly falling. The onset of spermiation coincided with maximal levels of 11-KT and low sperm motility in milt samples characterized by a high spermatocrit and a large

percentage of spermatids. As the season progressed, the GSI values peaked in July and sperm motility improved together with a declining spermatocrit, and lower levels of T and 11-KT. In the middle of the spawning season (July and August), sperm motility reached its maximum along with a range of low spermatocrit and sperm concentration values, and the disappearance of spermatids in milt. Sperm motility, sperm concentrations in the milt, spermatocrit, GSI and plasma androgen values all declined late in the spawning season (September and October).

Büyükhatipoglu and Holtz (1984) noted that sperm motility in rainbow trout improves from the beginning to the middle of spawning season before declining thereafter. A similar pattern was noted for ocean pout in this study, with the best sperm motility occurring in the middle of the spermiation season, in synchrony with the spawning of females. In halibut, however, sperm motility is better at the beginning of spawning before markedly decreasing during the last half of the spawning season, along with spermatocrit values reaching nearly 100% (Methven and Crim 1991). In contrast, the spermatocrit of rainbow trout (Billard *et al.* 1971; Munkittrick and Moccia 1987; Büyükhatipoglu and Holtz 1984), brown trout (Billard 1983c) and ocean pout declines as the spawning season progresses. In the ocean pout, a high spermatocrit value does not indicate a high sperm concentration at the beginning of the spermiation, but rather the presence of a high percentage of immotile spermatid cells in the semen[1].

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[1]Semen=milt

Although the sperm concentration of milt can be accurately determined by hemocytometer methods, this technique is too time consuming for routine measurements. Alternatively, the spermatocrit has been used to measure sperm concentration in fish milt by many authors (Baynes and Scott 1985; Munkittrick and Moccia 1987; Garcia 1991). On the other hand, Suquet *et al.* (1992) used spectrophotometer methods, reporting that the spermatocrit technique is inaccurate for estimates of the sperm concentration in the turbot. After the disappearance of spermatids from ocean pout milt by the middle of July, spermatocrit is an accurate and convenient measure of sperm concentration, which is useful for accurately determining the minimum number of sperm required for artificial insemination techniques.

Both sperm concentration of fish milt and the GSI values in teleosts are related to the mode of male reproduction and the spawning behaviour (Billard 1986; Billard and Cosson 1990). Billard (1986) suggested that high sperm concentrations could compensate for relatively short sperm motility times ranging from 30 sec. to 30 min. and are needed for externally spawning species where fertilization of eggs takes place in an open environment resulting in sperm being quickly diluted. For example, high sperm concentrations ranging from 10 - 55 x $10^9$ spz/ml are found in the milt of salmonids (Ciereszko and Dabrowski 1994), the common carp (Kruger *et al.* 1984), the winter flounder (Harmin and Crim 1993), and the turbot (Suquet *et al.* 1992), and even higher concentrations of sperm, 16.5 x $10^{10}$ spz/ml in semen of the bluefin tuna (Doi *et al.* 1982)

and halibut (Crim *et al.* unpublished). In contrast, the internal fertilization of eggs in the ocean pout is associated with both a prolonged sperm motility time (days) and relatively low sperm concentrations in the milt (1 - 7 x $10^8$ spz/ml), a range also reported for the internally fertilizing wolffish (Pavlov and Radzikhovskaya 1991). Also noted was the high GSI found in males releasing gametes in open or running water without parental care (Billard and Cosson 1990). For example, GSIs varying from 7 - 16% were found in male trout and carp (Billard 1986), the winter flounder (Harmin *et al.* 1995), and Atlantic cod (Trippel and Morgan 1994). However, in some species the males release gametes in protected areas or directly into the female body and in these cases the GSI is low. For instance, the GSI ranges from 0.1 - 2% in male *Tilapia* species (Billard 1986) and the male wolffish (Pavlov and Radzikhovskaya 1991). Likewise, the relatively low GSI in male ocean pout is related to the copulative spawning behaviour (Yao and Crim 1995b).

Previous studies of the relationship between the seasonal plasma androgen profile and reproductive development of male teleosts have usually focused on testosterone and 11-ketotestosterone. Between these two androgens, 11-KT is the predominant androgen in salmonids (Scott *et al.* 1980; Kime and Manning 1982; Fostier *et al.* 1982; ), winter flounder (Harmin *et al.* 1995), and halibut (Methven and Crim 1991), while in this study, T is the predominant androgen in ocean pout. Several studies (Scott *et al.* 1980; Hunt *et al.* 1982; Kime and Manning 1982; Baynes and Scott 1985) have shown that T peaks before 11-KT which corresponds to the spermiation season in salmonids. According to the

ocean pout androgenic hormone profiles, the peak of T is also reached prior to spermiation, followed by a peak of 11-KT in conjunction with the onset of spermiation. While peak levels of the androgens, particularly 11-KT, coincide with the period of spermiation in salmonids (Scott *et al.* 1980; Fostier *et al.* 1982; Kime and Manning 1982), mummichog (Cochran 1987), and winter flounder (Harmin *et al.* 1995), interestingly, there is a brief androgenic peak in the ocean pout with spermiation continuing for several weeks, similar to the halibut (Methven and Crim 1991). Wingfield and Grimm (1977), and Scott *et al.* (1980) suggested that T plays a major role in the late stages of spermatogenesis. A major role of 11-KT could be in controlling spermiation (Scott *et al.* 1980). Fostier *et al.* (1982 and 1984) also demonstrated that the level of 11-KT in plasma was positively correlated with sperm production in rainbow trout during the initiation and arrest of spermiation, which indicates that 11-KT might play a role in the process of spermiation or sperm migration into the vas deferens. On the other hand, there was no relationship between 11-KT levels and sperm production in rainbow trout (Hunt *et al.* 1982; Baynes and Scott 1985). Therefore, Baynes and Scott (1985) considered that a major role of 11-KT could be in controlling the secondary sexual characteristics. The results of the current study, suggest that 11-KT might play a role in initiating spermiation rather than stimulating sperm production due to the rapid decline in plasma 11-KT just after the onset of spermiation, and that T could be involved in the final testis maturation in the male ocean pout.

Some investigations (Scott and Baynes 1982; Ueda *et al.* 1983a, b; Baynes and Scott 1985; Ueda *et al.* 1985) suggest that 17α-hydroxy, 20β-dihydroprogesterone (17α, 20βP) plays a major role in the process of spermiation in fish. However, in the current study, 17α, 20βP was not detectable in ocean pout plasma during the spawning season (data not shown). Since male ocean pout continue to release milt containing high concentrations of motile sperm for several weeks following the androgen decline, further studies are required to determine the presence of other steroids and if they are involved in the control of spermiation and sperm motility in the ocean pout.

# CHAPTER 3

# SEASONAL ASPECTS OF SPERM PHYSIOLOGY AND SEMINAL PLASMA BIOCHEMISTRY IN THE OCEAN POUT *Macrozoarces americanus*

## 3.1 INTRODUCTION

A better knowledge of sperm physiology and seminal plasma biochemistry are essential to improving artificial fertilization procedures for fish. To date, most studies of fish spermiation have focused on the sperm of salmonid, cyprinid and a few marine species which are all external egg fertilizers (Scott and Baynes 1980; Morisawa *et al.* 1983; Stoss 1983; Kruger *et al.* 1984; Billard 1986; Billard *et al.* 1993; Suquet *et al.* 1993; Harmin and Crim 1993; Lahnsteiner *et al.* 1994; Billard *et al.* 1995b). In contrast, studies of sperm physiology and seminal plasma biochemistry in internally fertilizing teleosts have been limited to observations on guppy (Billard and Cosson 1990), and sculpin sperm (Koya *et al.* 1993). Because ocean pout sperm are transferred directly into the female (Crim *et al.* 1995; Yao and Crim 1995a), it is believed that sperm physiology in ocean pout would be different from species releasing sperm into the external environment.

In the past, few studies of fish sperm physiology and biochemistry have focused on seasonal aspects of sperm production, sperm motility, and changes in milt characteristics. Büyükhatipoglu and Holtz (1984) reported that sperm production and motility in rainbow trout increased from the beginning to the middle of spawning season and declined thereafter. There were seasonal fluctuations in sperm concentration, motility, pH, osmolality, and some biochemical constituents of semen in *Cyprinus carpio* and *Oreochromis mossambicus* (Kruger *et al.* 1984). In rainbow trout, Munkittrick and

Moccia (1987) noted that spermatocrit, sperm motility and seminal plasma ion concentrations declined as the season progressed. No seasonal studies of sperm physiology in internal egg fertilizers have been reported. Although some information about male and female ocean pout reproduction has recently become available (Crim *et al.* 1995; Yao and Crim 1995a; Yao *et al.* 1995), detailed studies of the spermiation response and the biochemical and physiological characteristics of ocean pout sperm throughout the spawning season remain to be reported. Furthermore, the extremely long period of motility exhibited by ocean pout sperm provides an excellent opportunity to study various biochemical parameters in relation to fish sperm motility (Yao and Crim 1995a).

In this study, changes in sperm motility were followed in relation to pH, osmotic pressure and the biochemical variations occurring in ocean pout seminal plasma throughout the ocean pout spawning season.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Collection of fish and milt

See Chapter 2 (2.2.1 Experimental animals) for collection and maintenance of fish. Throughout the spawning period, milt samples were collected weekly or biweekly from

mature males by inserting a polyethylene tube into the sperm duct via the urinogenital pore. A total of 98 milt samples were collected from 11 males in 1994.

### 3.2.2 Determination of sperm motility

Sperm motility was estimated from freshly collected samples of milt according to the percentage of motile spermatozoa (Chapter 2). To determine sperm motility for the early, middle and late season, a weighted mean of sperm motility class for each season was used. The weighted mean is the mean sperm motility class weighted by the number of fish in each class. It represents the average of the sperm motility class for all fish in each season.

### 3.2.3 Seminal plasma analysis

After centrifugation of freshly collected milt at 3000 x g for 15 minutes at 4$^0$C, the seminal plasma was removed and divided into aliquots for immediate analysis or for storage at -20$^0$C. The pH and osmotic pressure values of seminal plasma were immediately determined by pH indicator strips between the range of pH 5 and 10 (Em Science, Germany), and by FISKE ONE-TEN osmometer, respectively. Focusing on 6 males, the total protein (TP) concentration, the levels of calcium ($Ca^{++}$), magnesium ($Mg^{++}$), potassium ($K^+$), sodium ($Na^+$), chloride ($Cl^-$), glucose (GLU), and phosphorus (P)

were measured after thawing the seminal plasma. These samples represented three different phases of the spawning season: 1) early season; the first sample of the season collected in June at the initiation of spermiation, 2) middle season; samples collected during the middle of the spawning season in July and August, and 3) late season; the last sample collected near the end of the spawning season in September. The total protein concentration was determined using the Pierce Micro BCA Protein Assay (Pierce 1992). The concentrations of calcium and magnesium were measured colorimetrically using the SYNCHRON CX3 System; seminal plasma potassium, sodium and chloride were measured by indirect potentiometry using ion selective electrodes housed in a flow cell. Glucose was measured in a reaction cup by means of a polarographic electrode and phosphorus was determined using the Phosphorus System Pack for HITACHI 705.

### 3.2.4 Determination of the effects of pH and osmolality on sperm motility

In the middle of the spawning season (August) after pooling milt from 3 males containing highly motile sperm (>75%), a 1 ml sample was centrifuged 1000 x g for 10 min. at 4$^{0}$C (no effect on sperm motility following such treatment). The seminal plasma was removed and the sperm pellet mixture resuspended in 1 ml of a diluent (183 mM NaCl, 10.25 mM $KHCO_3$, 1.45 mM $CaCO_3$, 0.84 mM $MgSO_4 \cdot 7H_2O$, and 0.15 mM glucose) which preserves ocean pout sperm motility (Yao *et al.* unpublished), adjusted with NaOH or HCl at various pHs from 5 - 10 and osmolality values of 320 - 350

mmol/kg, or at different osmolality values (150 - 500 mmol/kg) by changing NaCl content (from 60 - 280 mM) while holding pH constant at 8.0. Both procedures were repeated twice.

### 3.2.5 Statistical analysis

Because samples were taken from the same individuals (repeated measures design), seasonal changes of pH and osmotic pressure of seminal plasma were analysed using a two-way ANOVA model which accounts for the seasonal effect as well as between-fish variability. Duncan's multiple range test was then used to test the seasonal significant differences of pH, osmotic pressure, and biochemical composition of seminal plasma.

## 3.3 RESULTS

### 3.3.1 Seasonal changes in sperm motility

Results of the semen samples collected from a group of 11 males from June to September 1994 showed that sperm motility varies throughout the spawning season. Sperm motility was low at the beginning of the season in June and given a class 1 motility rating (Figure 3.1). However, sperm motility for all males increased greatly

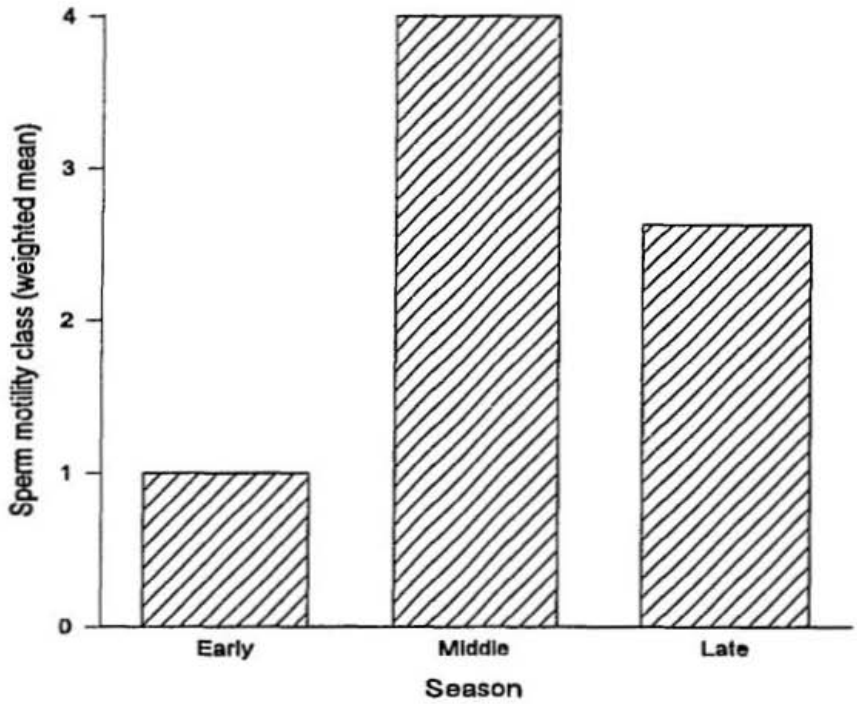


Figure 3.1 Changes in sperm motility (weighted mean) during the spawning season in 1994. The weighted mean is the mean sperm motility class for each season weighted by the number of fish in each motility class.

during July and August as indicated by the class 4 motility rating. After reaching and maintaining maximum motility for about two months during the summer, sperm motility declined again by the end of the spawning season in September.

### 3.3.2 Seasonal changes in seminal plasma pH and osmotic pressure

Data from the pH analysis of 98 seminal plasma samples and 89 osmolality

determinations (the osmolality of 9 out of 98 seminal plasma samples was unreadable presumably due to the presence of antifreeze protein) indicated that the average pH and osmolality values for all seminal plasma samples were 7.78±0.03 (range 7.1 - 8.3) and 356±3 (range 311 - 490) mmol/kg, respectively. At the beginning of the spermiation period in June, the seminal plasma pH value was low (Figure 3.2) compared with pH values obtained in the middle (July and August) and near the end (September) of the

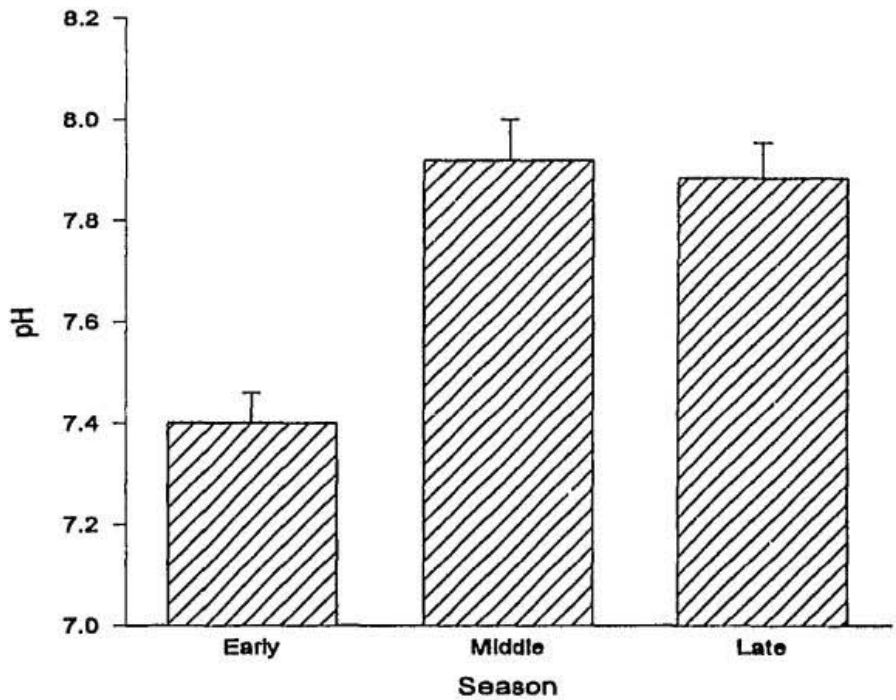


Figure 3.2 pH values (Mean+SE) of seminal plasma at various times of the spawning season. pH of the early season was significantly lower than that of the middle and late seasons ($p=0.0001$, $n=11$).

spawning season (pH=7.4 vs 7.9, P=0.0001, n=11). However, seminal plasma osmotic pressure values (Figure 3.3), which were higher in June, fell by the middle of the spawning season (419 vs 339 mmol/kg, P=0.0005, n=6 [1]) .

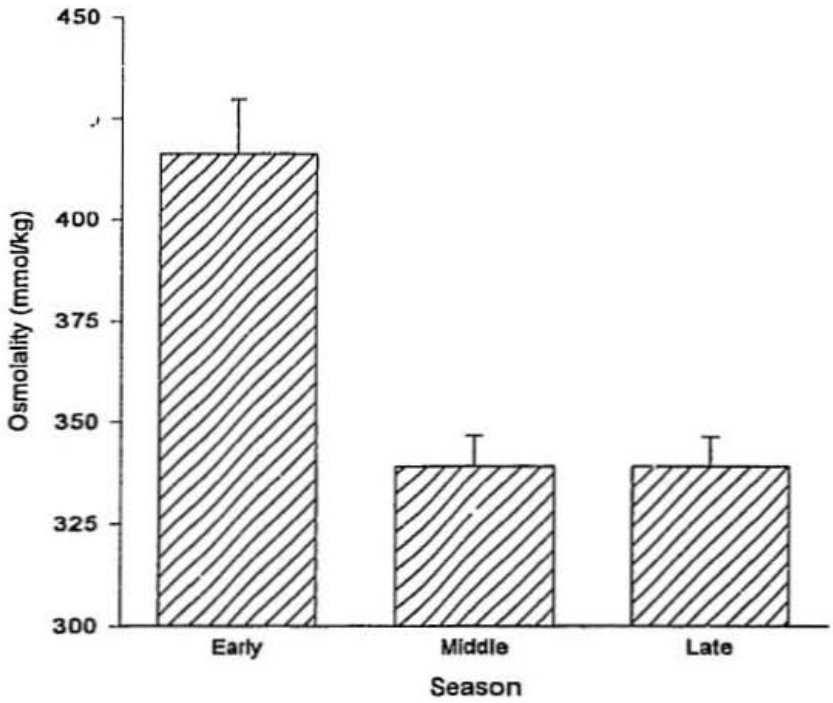


Figure 3.3 A comparison of osmotic pressure (mmol/kg; Mean+SE) of seminal plasma at various times over the spawning season. Osmotic pressure of the early season was significantly higher than that of the middle and late season (p=0.0005, n=6).

[1] Osmolality values from 5 males at the beginning of the spawning were not available.

### 3.3.3 Seasonal changes in biochemical composition of seminal plasma

Although a number of chemical constituents, including ions, glucose and protein, were detectable in ocean pout seminal plasma (Table 3.1), not all were subject to seasonal variation. For example, the concentration of $Mg^{++}$ ion was significantly elevated during the middle of the spawning season ($p<0.05$, $n=6$), coinciding with the period of maximum sperm motility, compared with the beginning or the end of the spawning season (Table 3.1). In contrast, $Na^{+}$ and $Cl^{-}$ levels decreased as the season progressed, reflecting a decline in seminal plasma osmolality (Figure 3.3).

Table 3.1. Biochemical composition of ocean pout seminal plasma throughout the spawning season. Values (mmol/L) are mean ± SEM. Means with the same letter are not significantly different ($p<0.05$, $n=6$).

| Season | $Mg^{++}$ | $Na^{+}$ | $K^{+}$ | $Ca^{++}$ |
|---|---|---|---|---|
| early | 3.825±1.613$^{b}$ | 191.833±2.982$^{a}$ | 8.567±0.741$^{a}$ | 1.673±0.103$^{a}$ |
| middle | 8.633±1.082$^{a}$ | 173.500±1.335$^{b}$ | 8.933±0.858$^{a}$ | 1.518±0.150$^{a}$ |
| late | 4.368±1.365$^{b}$ | 168.833±4.792$^{b}$ | 7.417±1.059$^{a}$ | 1.585±0.156$^{a}$ |
| **Season** | **TP** | **GLU** | **$Cl^{-}$** | **P** |
| early | 0.748±0.224$^{a}$ | 0.100±0.026$^{a}$ | 186.500±1.875$^{a}$ | 0.282±0.244$^{a}$ |
| middle | 0.287±0.087$^{a}$ | 0.017±0.017$^{a}$ | 174.500±1.544$^{ab}$ | 0.150±0.150$^{a}$ |
| late | 0.698±0.411$^{a}$ | 0.150±0.096$^{a}$ | 165.833±6.058$^{b}$ | 0.380±0.380$^{a}$ |

### 3.3.4 pH and osmotic pressure effects on sperm motility

Laboratory tests demonstrated that sperm motility is completely inhibited if pH falls outside of the range from 6.5 - 9.5 (Figure 3.4). Sperm motility increased progressively

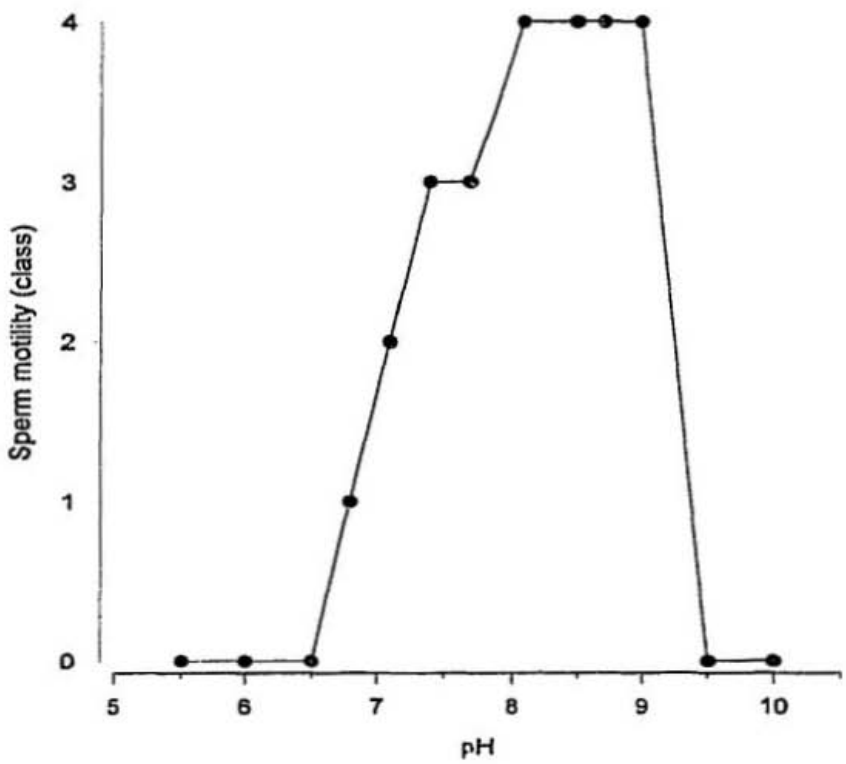


Figure 3.4 The effects of pH on sperm motility (class).
Note: Data were obtained from two repeated tests which showed no difference.

when pH was increased from 6.5 - 8.1. The best sperm motility was observed between pH 8.1 - 9.0. Although some sperm motility was observed outside a range of osmotic pressure from 250 - 400 mmol/kg, sperm motility was optimized (percentage of motile cells > 75%) within a range of osmolality from 300 to 400 mmol/kg. Outside of this osmolality, ocean pout sperm were immobilized in solutions of osmotic pressure 150, 200, 450, and 500 mmol/kg (Figure 3.5).

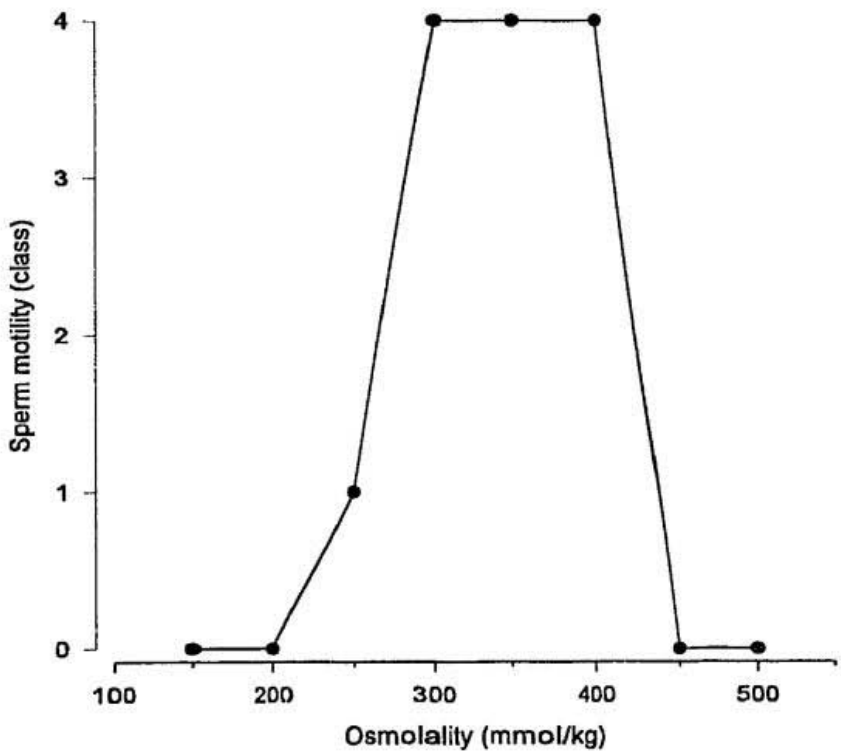


Figure 3.5 The effects of osmotic pressure (mmol/kg) on sperm motility (class).
Note: Data were obtained from two repeated tests which showed no difference.

## 3.4 DISCUSSION

This serial study of seasonal aspects of sperm physiology and biochemistry in the ocean pout clearly showed that concurrent with the increases in sperm motility, fluctuations in some biochemical characteristics of seminal plasma have been discovered with elevated levels of $Mg^{++}$, an increase in pH and a decline in osmotic pressure being associated with the period of maximum sperm motility.

The pH, osmotic pressure, and the ionic composition of seminal plasma are thought to be the most important factors determining the activation and level of motility of fish sperm (Stoss 1983). These factors may be responsible for inhibiting or initiating sperm motility. Although the mechanism for initiation of sperm motility in teleosts is still unclear, it is suggested that the stimulation of sperm motility could be due to a change of membrane potential, depolarizing the sperm membrane (Billard 1990b).

Generally speaking, alkaline pHs appear to offer the best conditions for maintaining fish sperm motility and fertility and ocean pout sperm appear to be no exception since the pH of ocean pout seminal plasma tended to increase during the spawning season, corresponding with improved sperm motility. Throughout the spawning season, the pH of ocean pout seminal plasma averaged 7.78, similar to pH values recorded for seminal plasma (pH=7.83) in the seabream, *Sparus aurata* (Chambeyron and Zohar 1990) but higher than that recorded for the turbot (pH=7.31) by Suquet *et al.* (1993). Even higher seminal plasma

pH values have been reported in the rainbow trout (pH=8.2) and some cyprinid fishes (pH: 7.99 - 8.45) (Lahnsteiner *et al.* 1994). Based upon this laboratory study of the relationship between pH and sperm motility, ocean pout sperm favour an alkaline pH environment ranging from pH 6.5 - 9.0. The motility and fertility of rainbow trout sperm is maximized at pH 9 (Billard 1986). The sperm of halibut and sea bass *(Dicentrarchus labrax)* exhibit the best motility at pHs ranging from 7.5 to 8.5 (Billard *et al.* 1993) and pH 9 (Stoss 1983), respectively. Koya *et al.* (1993) reported that the sperm of elkhorn sculpin (internal insemination), *Alcichthys alcicornis*, showed high motility at pH 7.5, the same pH value found in ovarian fluid. Seminal plasma osmolality is generally found to range from 250 - 450 mmol/kg according to data obtained for various freshwater teleosts e.g., 300 mmol/kg in salmonids (Billard and Cosson 1992), 254.0 - 334.5 mmol/kg in cyprinids (Billard and Cosson 1992; Lahnsteiner *et al.* 1994) and some marine species e.g., 306 mmol/kg in turbot (Suquet *et al.* 1993), the ocean pout (355.6 mmol/kg) and seabream (364.6 mmol/kg) seminal plasma, *Sparus aurata* (Chambeyron and Zohar 1990). Both the sperm of the ocean pout (this study, see Figure 3. 5) and the elkhorn sculpin (Koya *et al.* 1993) showed high motility in solutions of 300 - 400 mmol/kg. During the early stages of the ocean pout spawning season, the osmotic pressure of seminal plasma was relatively high (419 mmol/kg) and sperm motility low. However, by July when sperm motility had improved, the osmotic pressure declined to 339 mmol/kg consistent with the optimal osmolality range for this species. The osmotic pressure requirements for ocean pout sperm motility estimated from this study also reinforces the suggestion that ocean pout are internal egg fertilizers since high

osmolality seawater (~1100 mmol/kg) would quickly immobilize the sperm.

The ionic composition of seminal plasma has an important influence on sperm motility in fish that are external fertilizers. For example, Morisawa and Suzuki (1980) reported that high concentrations of potassium in the seminal plasma favour inhibition of sperm motility in salmonids. In contrast, potassium increased the motility of carp sperm (Billard and Cosson 1992). In marine teleosts, potassium does not have inhibitory effects on sperm motility in the summer whiting, *Sillago ciliata* (Goodall *et al.* 1989), flounder, *Platichthys flesus*, and cod, *Gadus morhua* (Morisawa 1985). Billard and Cosson (1992) showed that calcium, magnesium, and sodium ions can overcome the inhibition by potassium ion and acidic pH, and calcium and magnesium ions can increase the duration of sperm motility in the summer whiting (Goodall *et al.* 1989).

The sperm of internally fertilizing teleosts such as the ocean pout (this study), the marine sculpins (Koya *et al.* 1993) and wolffish (Pavlov and Radzikhovskaya 1991) are unusual in the sense that sperm is already activated directly from the testes and the sperm duct and sperm motility lasts for several days. However, in the guppy, goodeid and Horaichthidae teleosts, sperm gain their motility after breakdown of the spermatozeugmata or spermatophores within the female genital tract.

Hypotonicity generally activates sperm motility in freshwater teleosts, especially in carp (Redondo *et al.* 1991). On the other hand, the motility of spermatozoa in marine teleosts is induced by suspension in hypertonic media (Morisawa and Suzuki 1980) and these

authors suggested that ionic composition, especially potassium concentrations, might initiate sperm motility in the teleosts with internal fertilization. Since the increases in magnesium ion in the seminal plasma parallelled improved sperm motility during the ocean pout spawning season, perhaps magnesium ion plays an important role in the control of ocean pout sperm motility. However, a study by Koya *et al.* (1993) showed that the greatest motility of elkhorn sculpin sperm occurred in $Na^+$-supplemented medium rather than other ions. In this study, a reduction of $Na^+$ and $Cl^-$ levels of ocean pout seminal plasma was correlated with lower osmolality values. The effects of seminal plasma ion composition on sperm activation and maintenance of motility in ocean pout, especially the ions of magnesium, potassium and sodium, and pH and osmolality requires further investigations.

Billard and Cosson (1990) reported that spermatozoa from internal fertilizers, such as guppy and surfperch, could utilize glycogen or glucose (ovarian and seminal plasma) as endogenous or exogenous metabolic energy sources during motility. Although ocean pout spermatozoa are characterized by extremely long periods of motility, this study showed that the protein and glucose content of seminal plasma is not much higher compared with fish possessing sperm of short-term motility (Scott and Baynes 1980; Piironen and HyvArinen 1983; Lahnsteiner *et al.* 1994). Whether ocean pout spermatozoa use endogenous substrates for energy, like the guppy sperm, or use other exogenous substrates in seminal plasma, such as lipid, remains an interesting question for further study.

# CHAPTER 4

# CONCLUSION

## 4.1 SUMMARY OF THE FINDINGS

The major findings from this two-year study of the characteristics of the male reproductive season (Chapter 2) and seasonal aspects of sperm physiology and seminal plasma biochemistry (Chapter 3) in the male ocean pout could be summarized as follows: i) the captive male ocean pout matures and is capable of gamete release from June to September with the best sperm motility shown in July, in synchrony with the female spawning season; ii) plasma androgens (T and 11-KT) rise rapidly seven weeks prior to spermiation with the peak of T appearing one week earlier than that of 11-KT. The peak of 11-KT coincides with the onset of spermiation; iii) based on the changes in GSI throughout a year, the seasonal reproductive cycle of the male ocean pout can be divided into 4 phases, e.g. slow testicular recrudescence beginning in the winter, rapid continued testicular development in early summer, spawning in late summer, and post-spawned testicular regression; iv) the milt contains a low concentration of sperm, which can be determined by spermatocrit during most of the spawning season; v) ocean pout sperm perform best motility in an alkaline environment within an osmolality range between 300 - 400 mmol/kg; vi) increases in seminal plasma $Mg^{++}$ levels parallel improved sperm motility during the spawning season.

## 4.2 CONTRIBUTIONS TO FISH REPRODUCTIVE PHYSIOLOGY AND AQUACULTURE

The findings of this study have both theoretical significance and practical implications. To date, studies of fish reproductive physiology have mainly focused upon externally fertilizing species, teleostean fish in particular. The reproductive physiology of the internally fertilizing teleosts still remains a puzzle, although a few studies have been reported on species such as the Mees, *Lepidogalaxias salamandroides* (Pusey and Stewart 1989), the guppy (Billard and Cosson 1990), the seabream, *Cheimerius nufar* (Garratt 1991), the wolfish (Pavlov and Radzikhovskaya 1991), the sculpin, *Alcichthys alcicornis* (Koya *et al.* 1993), and the ocean pout (Crim *et al.* 1995). This study, with an investigation covering the complete reproductive cycle of the ocean pout, extends our understanding of the reproduction of both a male and female ocean pout, particularly, it provides knowledge of the seasonal reproductive cycle of the male ocean pout, sperm physiology, profiles of plasma androgens during the spermiation period, and possible ionic ($Mg^{++}$) influences on sperm motility.

The ocean pout is a potentially important species for cold-water aquaculture in Eastern Canada (Brown *et al.* 1989), but spontaneous fertilization does not take place under laboratory conditions (Yao and Crim 1995a,b). Being an internal fertilizer, artificial reproduction of the ocean pout is more complicated requiring development of methods for collecting viable sperm and storing sperm of good quality. Likewise efficient use of

sperm for *in vivo* artificial insemination or *in vitro* fertilization of eggs becomes necessary. This study shows that in captivity the male ocean pout produces good quality sperm in July and August (Chapter 2), in synchrony with spawning females, suggesting that high quality sperm can be supplied for artificial insemination yielding techniques for gamete management. The significant linear relationship found between spermatocrit and sperm concentration is useful in determining sperm numbers for artificial insemination. Other useful findings such as optimal pH and osmolality ranges for sperm motility, and the ionic composition of seminal plasma can be applied to improve the storage of sperm. Finally, because wolfish and ocean pout share similar reproductive traits, the knowledge obtained from this study is also valuable to future work on domestication of the wolfish.

## 4.3 IMPROVEMENT AND FURTHER STUDIES

The reproductive cycle of the male ocean pout was divided into 4 seasonal phases based on the changes in GSI throughout the year in this study. However, more precise identification of testicular development in the male ocean pout requires further histological study of spermatogenesis.

In this study, sperm quality was determined by the percentage of motile sperm cells which is influenced by pH and osmotic pressure of the milt. Since sperm motility were only tested in the middle of the spawning season, it would be more convincing if

tests were also conducted both early and late in the spawning season. Sperm flagellar beat frequency has been studied on some species such as trout and carp (Billard and Cosson 1992) and it might be useful to examine the relationship between the flagellar beat frequency and the percentage of motile sperm cells in ocean pout sperm. In addition, the relationship between sperm motility and egg fertilizing capacity of ocean pout sperm should be tested in further studies of sperm quality.

Finally, this study raises some interesting questions concerning the type of steroid involvement (e. g. C-19 and C-21) in the processes of spermiation and sperm motility, since males continue to release high motility sperm for several weeks after the decline in plasma androgens. How do ions and other biochemical components of milt affect sperm activation and the maintenance of motility in ocean pout? What metabolic mechanisms are involved with the extremely long duration of the motility period in ocean pout sperm?

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# APPENDIX

## 1.TESTOSTERONE (T) RADIOIMMUNOASSAY (RIA)

### Assay buffer

50 mM Phosphate, 100 mM Sodium Chloride, 0.1% Azide, 0.1% Gelatin

| | |
|---|---|
| $Na_2HP0_4$ | 6.30 g/l |
| $KH_2P0_4$ | 0.75 g/l |
| NaCl | 5.86 g/l |
| $NaN_3$ | 1.00 g/l |
| $D{\cdot}H_20$ | 1000 ml, pH 7.4 |

After adjusting the pH, add gelatin (1.0 g/l) and heat to dissolve.

### Label

Make iodinated T assay dilution (approximately 1,000 cpm/10µl) using above assay buffer.

**NOTE:** This isotope is stored in the buffer not ethanol and does not need to be dried down.

The recovery estimates use tritiated testosterone with a dilution of 1,000 cpm/10µl.

### Antisera

The working dilution of antisera is made up from the antibody (AB) diluted with the assay buffer (50% binding rate).

**Standard curve**

The standard curve is prepared in ethanol. The final volumes are dried down under nitrogen ($N_2$) in the RIA tube and brought back to 100µl with assay buffer.

Stock solution: 1 mg testosterone / 10 ml ethanol

Working solution: 100x = 100µl stock diluted to 10 ml ethanol

10,000x =(100x) diluted to 10 ml ethanol

The dilutions are made in 10 ml Class A volumetric flasks with a disposable micropipet to measure the 100 µl aliquot. Subsequent 2x serial dilutions of the 10,000x are prepared

**Preparation of samples**

Add 10µl of tritiated isotope into 100 µl blood plasma for the recovery estimate. Vortex 1 min. and incubate 0.5-1.5 hr at room temperature.

Add 2.0 ml of ether, vortex 1 min. Freeze aqueous layer in a dry-ice/ethanol bath. Pour off the aqueous layer (ether extract) into a clean tube, repeat.

Evaporate the ether under $N_2$ and add 1ml of distilled ethanol to the resultant solute. Vortex 1 min. Keep the samples at room temperature for at least 1 hour or overnight at $4^0$ C before doing the recovery estimates. Count 100µl for 20 min.

**Testosterone RIA protocol**

| # | ID | STD# | Vol | buffer | AB | label | SEP/REG* |
|---|---|---|---|---|---|---|---|
| 1,2 | CT | | | - | - | 100µl | - |
| 3,4 | 0 AB | | | 300µl | - | 100µl | 1 ml |
| 5,6 | 0 Hormone | | | 200µl | 100µl | 100µl | 1 ml |
| 7,8 | 0.98 pg | 12 | 100µl | 200µl | 100µl | 100µl | 1 ml |
| 9,10 | 1.95 | 11 | 100µl | 200µl | 100µl | 100µl | 1 ml |
| 11,12 | 3.0 | 10 | 100µl | 200µl | 100µl | 100µl | 1 ml |
| 13,14 | 7.8 | 9 | 100µl | 200µl | 100µl | 100µl | 1 ml |
| 15,16 | 15.6 | 8 | 100µl | 200µl | 100µl | 100µl | 1 ml |
| 17,18 | 31.2 | 7 | 100µl | 200µl | 100µl | 100µl | 1 ml |
| 19,20 | 62.5 | 6 | 100µl | 200µl | 100µl | 100µl | 1 ml |
| 21,22 | 125.0 | 5 | 100µl | 200µl | 100µl | 100µl | 1 ml |
| 23,24 | 250.0 | 4 | 100µl | 200µl | 100µl | 100µl | 1 ml |
| 25,26 | *500.0* | 3 | 100µl | 200µl | 100µl | 100µl | 1 ml |
| 29,28 | 750.0 | 2 | 75 µl | 200µl | 100µl | 100µl | 1 ml |
| 29,30 | 1000 | 1 | 100µl | 200µl | 100µl | 100µl | 1 ml |
| 31,32 | sample | | 100µl | 200µl | 100µl | 100µl | 1 ml |

* SEP/REC: Separating reagent.

NB:all reagents must be at room temperature!!

-Add standards/samples to RIA tubes (in ethanol), evaporate under $N_2$ .

-Add 200µl assay buffer, vortex.

-Add 100µl isotope, 100µl antibody (working dilution), vortex and keep at room temperature

for 1 hr.

-Add 1.0 ml of separating reagent, vortex and keep at room temperature for 25min.

-Centrifuge at 1000 x g for 15 minutes.

-Aspirate supernatant carefully. Count pellet 1.0 min. in gamma counter.

## 2. 11-KETOTESTOSTERONE (11-KT) RADIOIMMUNOASSAY (RIA)

**Assay Buffer**

| | |
|---|---|
| $NaH_2PO_4H_2O$ (28 mM) | 3.87g |
| $Na_2HPO_4$ (61 mM) | 8.66g |
| NaCl (154 mM) | 9.00g |
| $D{\cdot}H_2O$ | 1000ml, pH 7.0 |

After adjusting the pH, add 1.0g Gelatin/l (0.1%) and heat to dissolve.

**Label**

For assay: 6,000-8,000 cpm/100µl

An appropriate volume is dried down under $N_2$ and brought up to volume with assay buffer (check a 10µl aliquot of the stock and calculate how much is needed for the entire assay.

**Standard curve**

(See that for testosterone RIA)

**Separation**

Dextran-Coated Charcoal (Char.)

| | |
|---|---|
| Kodak Charcoal RIA Grade (0.25% w/v) | 1.25g |
| Dextran T-70 (0.025% w/v) | 0. 125g |
| Assay Buffer | 500 ml |

**Sample extraction and Preparation**

(see that for testosterone RIA)

## 11-KETOTESTOSTERONE(RIA) PROTOCOL

| # | ID | STD# | Volume | Buffer | AB | Isotope | Char. |
|---|---|---|---|---|---|---|---|
| 1,2 | CT | | | 800µl | - | 100µl | - |
| 3,4 | 0 AB | | | 200µl | - | 100µl | 600µl |
| 5,6 | 0 Hormone | | | 100µl | 100µl | 100µl | 600µl |
| 7,8 | 0.98 pg | 11 | 100µl | 100µl | 100µl | 100µl | 600µl |
| 9,10 | 1.95 pg | 10 | 100µl | 100µl | 100µl | 100µl | 600µl |
| 11,12 | 3.0 pg | 9 | 100µl | 100µl | 100µl | 100µl | 600µl |
| 13,14 | 7.8 pg | 8 | 100µl | 100µl | 100µl | 100µl | 600µl |
| 15,16 | 15.6 pg | 7 | 100µl | 100µl | 100µl | 100µl | 600µl |
| 17,18 | 31.2 pg | 6 | 100µl | 100µl | 100µl | 100µl | 600µl |
| 19,20 | 62.5 pg | 5 | 100µl | 100µl | 100µl | 100µl | 600µl |
| 21,22 | 125.0 pg | 4 | 100µl | 100µl | 100µl | 100µl | 600µl |
| 23,24 | 250.0 pg | 3 | 100µl | 100µl | 100µl | 100µl | 600µl |

| # | ID | STD# | Volume | Buffer | AB | Isotope | Char. |
|---|---|---|---|---|---|---|---|
| 25,26 | 500.0 pg | 2 | 100µl | 100µl | 100µl | 100µl | 600µl |
| 29,28 | 750.0 pg | 1 | 75µl | 100µl | 100µl | 100µl | 600µl |
| 29,30 | 1000.0 pg | 1 | 100µl | 100µl | 100µl | 100µl | 600µl |
| 31,32 | sample | | 100µl | 100µl | 100µl | 100µl | 600µl |

-Add standards and samples to RIA tubes (in ethanol), evaporate under $N_2$

-Add 100µl assay buffer, vortex. Once assay buffer has been added to tubes, the tubes should be kept in ice/water bath.

-Add 100µl isotope ( 6,000-8,000 cpm/100µl)

-Add 100µl antibody (at appropriate dilution-1:70,000)

-Incubate overnight at $4^0$C

-Add 600µl. charcoal suspension at $4^0$C, vortex and keep at $4^0$C for 1 hour.

NOTE: **Do not add charcoal to tubes #1 & 2!**

-Centrifuge at 1000 x g for 25 min.

-Decant supernatant into scintillation vial, add 10ml scintillation cocktail, cover the vials, shake and count pellet for 2 min.