STUDIES OF SEASONAL GONADAL CYCLES AND HORMONAL CONTROL OF REPRODUCTION IN WINTER FLOUNDER, PSEUDOPLEURONECTES AMERICANUS (WALBAUM)

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By

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ABSTRACT

Studies were conducted using gonadotropic hormone-releasing hormone analog (GnRH-A) in adult winter flounder, Pseudopleuronectes americanus (Walbaum). This species was used as a model fish in an effort to understand seasonal gonadal cycles and the pituitary-gonadal axis regulation of reproduction in a seasonally breeding teleost.

The natural seasonal reproductive cycle was investigated over a two-year period. In females, rapid ovarian recrudescence which begins in August, was largely completed by December. However, ovarian growth continued throughout the winter as shown by further increases in both gonadosomatic index and oocyte diameter. Analysis of oocyte-size frequency distribution indicated that in reproductively active females, previtellogenic and vitellogenic oocytes are present but just one clutch of vitellogenic oocytes matured per reproductive season. Plasma estradiol increased in parallel with gonadosomatic index and oocyte diameter, whereas plasma testosterone remained low during the early stages of ovarian recrudescence. Compared with females, male testicular development was more rapid, with GSI reaching a maximum in October followed by declining values prior to spawning. Plasma testosterone and 11-ketotestosterone levels rose

slowly in association with the progress of testicular development. Plasma sex steroids reached their peak values just prior to the spawning period (May/June) in both sexes, while the minimum seasonal hormone values were observed in fish with regressed gonads.

Seasonal responses of the pituitary, and indirectly the gonads, to GnRH-A were monitored by measuring plasma androgens and estrogen following in vivo hormone treatment. Both males and females were highly responsive to GnRH-A treatment throughout the gonadal recrudescence period and the prespawning phases; little or no response was observed during the regressed period. Plasma levels of estradiol, testosterone and 11-ketotestosterone were elevated following GnRH-A administration and the hormone levels were sustained for prolonged periods of time. The effects of GnRH-A treatment on the reproductive cycle were also observed by noting increases in gonadosomatic index and oocyte diameter in fish during the period of gonadal recrudescence. During the prespawning stage, ovulation and spermiation could be achieved following releasing hormone treatment. In contrast, the low gonadosomatic index remained unchanged by GnRH-A treatment during the regressed period.

The capacity of GnRH-A to accelerate final egg maturation/ovulation and induce spawning was investigated in prespawning females prior to the natural spawning

period. Hormone administration induced ovulation in some females in February, as early as three months prior to the normal spawning season. Egg/larval quality data indicated that GnRH-A can be used to advance spawning of female flounder without having serious detrimental effects on the rates of egg fertilization, hatching and larval survival.

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GENERAL INTRODUCTION

Knowledge of the fundamental aspects of reproduction in teleosts is necessary to be able to control fish reproductive activities and to permit an optimization of these activities with respect to fish culture operations. In particular, with the rapid expansion of aquaculture operations, there is a continuous demand for reliable fry production throughout the year. Such fry production is needed for fish farming purposes since the demand for fish is increasing while the yield from wild fishes is declining (see reviews of Billard, 1989; Yaron and Zohar, 1989). Studies of fish reproductive physiology and the development of fish farming are closely linked. Furthermore, fundamental studies are important towards an understanding of endocrine regulation in this diverse group of vertebrates and are needed to understand basic mechanisms underlying the reproductive processes. According to Zohar (1989), control of reproduction involves two primary objectives: namely to have various species reproduce in captivity and to achieve higher survival of farmed fish.

1.1 Ovarian Development in Teleosts

In most teleosts, the ovaries develop as hollow organs consisting of many connective tissue septa (ovigerous lamellae) projecting into the ovarian lumina (see review of Wallace and Selman, 1981; De Vlaming, 1983). Oogenesis, which is defined as the transformation of secondary oogonia into primary oocytes, occurs within the ovigerous lamellae. It begins with mitosis of primary germ cells or oogonia that later undergo meiosis to form oocytes. Oogonia are released from the germinal epithelium of the lamellae and mature within the folds of follicular epithelium. They appear in section as small, rounded cells with large nuclei each containing a single prominent nucleolus. Usually they are found in small nests.

The oogonia proliferate by mitotic division soon after the ovaries have differentiated, and the oogonia are transformed into primary oocytes. In most teleosts, oogonial proliferation and the formation of new oocytes occurs throughout the reproductive life. In species that have distinct annual breeding cycles, oogonial proliferation has been observed just prior to, during or immediately after the spawning period. On the other hand, in species with prolonged breeding cycles, oogonial proliferation may be continuous.

The transformation of oogonia into primary oocytes represents the first phase of meiosis, the DNA is

replicated and the chromosomes proceed through the leptotene, zygotene and pachytene stages of prophase. From the completion of pachytene till a few days before the eggs are released, the meiotic process is arrested at diplotene. During this period oocytes usually increase in volume several thousandfold.

At the beginning of the primary growth phase oocytes appear very similar to the oogonia (cytoplasm contain central nuclei), but each oocyte becomes invested with a layer of follicle cells. In the course of oocyte growth, the nuclei increase in size and multiple nucleoli appear. The cytoplasm increases in volume. A rather prominent feature of this stage of the oocyte is the formation of the "yolk nucleus" or "Balbiani body", where the function is unclear. The oocyte at this phase is also known as a previtellogenic oocyte (devoid of yolk).

Next is the secondary growth phase (vitellogenic oocyte) which involves the accumulation of yolk precursor by oocytes. Two separate processes occur, endogenous and exogenous vitellogenesis, each of which leads to the formation of a different type of "yolk." In teleosts, endogenous vitellogenesis is observed during the early stages of secondary growth. It is characterized by the appearance of vesicles (vacuoles) in the cytoplasm, that contain a glycoproteinaceous material. It has been established that these organelles later become cortical

alveoli, which at fertilization release their contents into the perivitelline space. The major events, accounting for the enormous growth of oocytes, takes place during the secondary growth phase. It involves the sequestration and packaging of the hepatically derived glycolipoprotein plasma precursor, vitellogenin, into yolk protein. The vitellogenins once taken up by the oocytes are enzymatically cleaved to form lipovitellin and phosvitin.

In fully grown vitellogenic oocytes, the nucleus initially (germinal vesicle) lies in the centre with the chromosomes still remaining at the diplotene or dictyate stage. The resumption of meiosis occurs during oocyte maturation. Oocyte maturation occurs when the germinal vesicle begins migrating towards the periphery of the oocyte and the nuclear membrane disintegrates (germinal vesicle breakdown). The chromosomes condense and proceed to first meiotic metaphase, followed by the expulsion of first polar body, and the remaining chromosomes then proceed to the second meiotic metaphase where meiosis is halted once again. In teleosts, as in other vertebrates, once this second arrest occurs, the oocyte has become "mature" and fertilizable, and can be correctly called an egg. During oocyte maturation, many teleosts take up substantial amount of water (Clements and Grant, 1964; Craik and Harvey, 1984). The process, termed hydration, is very pronounced among marine teleosts that spawn pelagic

eggs; its effect is to reduce the specific gravity of the eggs so that they can float.

Ovulation generally follows oocyte maturation so rapidly that the two events are often considered a single event. However there is a distinct process between the two events. Ovulation is the process whereby mature eggs are released from the follicles into the body cavity (e.g., rainbow trout) or the lumen of the ovary (e.g., turbot and flounder). In most teleosts, the process involves the detachment of follicles from the oocytes and expulsion of the egg by the contraction of smooth muscle fibres which are present in the thecal layers of the follicles. Ovulation is often accompanied by the production of an ovarian fluid. Once the egg has been fertilized, meiosis resumes and one of the two haploid sets of female chromosomes is eliminated (as the second polar body). The remaining set of chromosomes combines with the set contributed by the sperm to reestablish the normal diploid genome.

1.2 Testicular Development in Teleosts

In the males, the testes occupy the same position within the body cavity as the ovaries. They are connected to the exterior by a duct, the vas deferens, which opens at the genital papilla. There are two basic types of testicular structure in teleosts i.e. the tubular and

lobular type (see reviews of Billard et al., 1982; Nagahama, 1983). The tubular type is not so common and found only in the atheriniform fish. The common lobular form is actually composed of tubules but in histological sections it appears lobular because the diameters of the tubules are varied. All the tubules are divided into two compartments i.e. intertubular and intratubular. The intertubular space (interstitium) contains boundary cells, connective tissue, fibroblasts, blood vessels and Leydig (interstitial) cells. The intratubular space contains only germ cells and Sertoli cells.

The proliferation of germ cells takes place within cysts formed by the Sertoli cells. Primary spermatogonia (spermatogonia A) are found singly or in small groups at scattered sites along the tubule walls. In most teleosts these stem cells are found at all seasons. The secondary spermatogonia (spermatogonia B) proliferate from these stem cells by mitotic division. The secondary spermatogonia transform into primary spermatocytes, a process known as spermatogenesis. The first meiotic division of the primary spermatocytes (spermatocyte I) produces secondary spermatocytes (spermatocyte II). The secondary spermatocytes then transform into spermatids through the second meiotic division. The spermatids, although having a haploid set of chromosomes, are still not capable of functioning as male gametes. The spermatids

metamorphose into spermatozoa, a process known as spermiogenesis, and then the cyst ruptures, liberating the spermatozoa into the tubule lumen. During the spawning period, the testes become hydrated, and the sperm are ejected into the sperm duct, a process known as spermiation. In teleosts the mixture of seminal fluid and sperm is commonly referred to as milt.

1.3 Endocrine Regulation of Reproduction in Teleosts

Reproduction in teleost fish, as in many other vertebrates, is characteristically cyclical with precise annual reproductive cycles hormonally regulated by the brain-pituitary-gonadal axis. The release of pituitary gonadotropin is stimulated by luteinizing hormone-releasing hormone (LHRH) also known as gonadotropic hormone-releasing hormone (GnRH), a brain peptide releasing hormone. The primary amino-acid structure of GnRH, pyro-Glu^{1}-His^{2}-Trp^{3}-Ser^{4}-Tyr^{5},Gly^{6},Leu^{7}-Arg^{8}-Pro^{9}-Gly^{10}-NH_2 was first elucidated in the early 1970s in extracts of mammalian hypothalamic tissue (Matsuo et al., 1971; Burgus et al., 1972). Since then various forms of GnRH have been discovered in many vertebrates (see reviews of Sherwood and Lovejoy, 1989; King and Millar, 1990). The primary decapeptide structure of fish GnRH has been identified from material isolated and purified from extracts of the brain of chum salmon. The salmon GnRH sequence Trp^{7}, Leu^{8}-

GnRH differs by two amino acids compared to mammalian GnRH (Sherwood et al., 1983).

The teleost pituitary secretes a variety of hormones including gonadotropin(s) which are involved in regulating reproductive processes. Recent studies have shown that two types of gonadotropin are found in chum salmon; gonadotropin I (GTH I) is more predominant in the plasma during vitellogenesis and early spermatogenesis whereas gonadotropin II (GTH II) levels are higher in mature fish at the time of ovulation and spermiation (Kawauchi et al., 1989). In the past, most GTH radioimmunoassays have been directed towards measuring GTH II (maturational GTH). GTH II levels in a variety of salmonid fishes are known to change during the reproductive cycle from generally low plasma levels at the beginning of spermatogenesis and oogenesis followed by a gradual increase towards spermiation, oocyte maturation and ovulation (Crim et al., 1975; Crim and Evans, 1978).

Sex steroids are synthesized by the gonads and released into the blood as a result of gonadal stimulation by gonadotropin. Teleost oocytes are surrounded by a multilayered follicle composed of at least two cell layers i.e. an inner granulosa layer, which is separated by a basement membrane from an outer thecal layer. These follicular layers are the sites of sex steroid production especially special thecal cells. In females, stimulation

of ovary by gonadotropin results in testosterone and estradiol-17ß production. During vitellogenesis estradiol-17ß stimulates the teleost liver production of vitellogenin (see reviews of Wallace and Selman, 1981; Weigand, 1982) which is incorporated into the growing oocytes. Plasma levels of estradiol-17ß increase in vitellogenic females and the role of estradiol-17ß in vitellogenin synthesis is well established (see review of Fostier et al., 1983). On the other hand, testosterone is also found in the plasma of reproductively active females but the functions of testosterone are less clear. It has been shown that testosterone acts as a precursor for estradiol-17ß production. Nagahama (1983) suggested the presence of a two-cell model such that testosterone, which is produced in the thecal cells of the oocyte, is subsequently converted to estradiol-17ß by aromatase activity in granulosa cells. Testosterone synthesis occurs following gonadotropin stimulation, while the mechanism of aromatase activation is not clearly understood. In vitro studies of isolated follicles showed that testosterone may initiate germinal vesicle migration from the central to the periphery position (So et al., 1985a; Wrigt and Zhao, 1988).

Another steroid, 17α,20ß dihydroxy-4-pregnen-3-one (17α,20ß DHP) is found in the plasma of mature female salmonids and a variety of other female fish at the time

of final oocyte maturation (Goetz, 1983). Although 17α,20ß DHP is thought to be the oocyte maturation hormone in salmonids and cyprinids, in ovulating flatfishes very low amounts of 17α,20ß are detected (see review of Scott and Canario, 1987).

In the males, the major androgens found in teleost plasma at the time of reproductive development are testosterone and 11-ketotestosterone (see reviews Kime 1980, Billard et al., 1982). These gonadal androgens, produced by the Leydig cells under gonadotropin stimulation (Billard et al., 1990) play a role in spermatogenesis. For example, in goldfish, testosterone supports spermatogonial division and meiosis in testicular explants (Remacle, 1976). High levels of testosterone and 11-ketotestosterone in the plasma late in the spawning season suggests that testosterone and 11-ketotestosterone are involved with spermiation (see review of Fostier et al., 1983). Another steroid 17α,20ß DHP has been found in ripe males with running milt or in spawning males of salmonids (Scott and Baynes, 1982). These workers showed that there is a positive correlation between 17α,20ß DHP plasma levels and the volume of milt (sperm count) collected manually (Scott and Baynes, 1982).

1.4 Use of Hormones in Stimulating Gonadal Development and Inducing Spawning

Although the reproductive cycles of fish can be regulated by environmental changes e.g. light and temperature, in fish culture hormonal approaches have been used for stimulating gonadal development and inducing spawning. A variety of hormones of the brain-pituitary-gonadal axis have been used for this purpose (see reviews of Lam, 1982; Donaldson and Hunter, 1983).

In males, induction or acceleration of spermatogenesis has been achieved with hormones such as human chorionic gonadotropin (Yamamoto et al., 1972; Shehadeh et al., 1973a), purified salmon gonadotropin (SG-G100, Funk and Donaldson, 1972; Mackinnon and Donaldson, 1978) and Luteinizing Hormone-Releasing Hormone (LHRH, see review of Lam, 1982). Results of using steroid hormone administration for inducing spermatogenesis in teleosts have been inconsistent (see review of Lam, 1982).

Hormonal stimulation of vitellogenesis in female teleosts appears to be more difficult with some success using pituitary extract in Japanese eel (Sugimoto et al., 1976) and rainbow trout (Upadhyay et al., 1978). Human chorionic gonadotropin treatment has produced inconsistent results; while Khoo (1980) successfully induced vitellogenesis in intact goldfish, administration of human

chorionic gonadotropin to hypophysectomized goldfish did not stimulate vitellogenesis (Yamazaki and Donaldson, 1968). Human chorionic gonadotropin alone is not effective in female eels except when combined with carp pituitary homogenate (Epler and Bieniarz, 1978). Nevertheless, human chorionic gonadotropin is effective during the later stages of gonadal development if given in sufficiently high doses (Shehadeh et al., 1973a).

Crude or partially purified pituitary extract, mammalian gonadotropin such as human chorionic gonadotropin, and purified teleost gonadotropin such as SG-G100 are among the most commonly used gonadotropic agents for inducing spawning of fish. These hormones are applied either alone or in combination e.g. fish pituitary extract in combination with human chorionic gonadotropin, for induction of ovulation. Traditionally, in fish culture, the induction of ovulation and spawning has been done by injection of crude piscine pituitary extract. However, this method has some drawbacks such as the lack of standardization of the potency of pituitary materials. The gonadotropic potency of a pituitary extract depends on the stage of sexual maturity of the donor as well as the methods of pituitary collection and preservation. There may also be differences in hormone potencies due to phylogenetic specificity between the pituitary donor and the recipient fish (Harvey and Hoar,

1979); the pituitary gland may be obtained from the same (homoplastic) or different (heteroplastic) species. Although, the use of these crude pituitary hormone preparations presently is becoming less popular, induction of spawning using crude or partially purified pituitary extracts continues with the recent reports of the use of the technique in grey mullet (Alvarez-Lajunchere et al., 1988; Lee et al., 1988), goldfish (Suzuki et al., 1988) and the South American pacu (Godinho and Godinho, 1986). Despite inconsistent results for inducing ovulation and some other drawbacks, e.g., being too costly, human chorionic gonadotropin is available commercially and continues to be used to spawn fish as reported in the grey mullet (Lee et al., 1988), sabalo (Fortuny et al., 1988), gudgeon (Kestemont, 1988), white sturgeon (Lutes et al., 1987), milkfish (Marte et al., 1988a), walleye (Pankhurst et al., 1986a), sole (Ramos, 1986a), striped bass (Henderson-Arzapalo and Colura, 1987), murray cod (Rowland, 1988), weakfish (Szedlmayer, 1987), catfish (Legendre, 1986), and mudskipper (Zhang et al., 1989).

Recently, the use of GnRH and its analogs for inducing maturation, ovulation and ultimately spawning in fish has increased because of the commercial availability and reduced costs of this hormone (see reviews of Crim et al., 1987; Peter et al., 1988). In some teleosts, regulation of gonadotropin secretion from the pituitary is thought to

include both the action of a gonadotropin releasing hormone and a gonadotropin release inhibitory factor of unspecified structure (see review of Peter et al., 1986). Pimozide, a dopamine antagonist, potentiates the effects of Gonadotropic Hormone-Releasing Hormone Analog (GnRH-A) on gonadotropin release and ovulation in cyprinids (Chang and Peter, 1983; Chang et al., 1984). A combination of pimozide and GnRH-A is highly effective in inducing ovulation of goldfish (Sokolowska et al., 1984), common carp (Lin et al., 1988), African catfish (De Leeuw et al., 1985), and loach (Lin et al., 1985a), gudgeon (Kestemont, 1988), and Chinese carp (Peter et al., 1988). Similarly, reserpine, a drug which causes general depletion of catecholamines, combined with GnRH-A has proven to be effective in inducing spawning in rainbow and redtail black sharks (Shireman and Gildea, 1989).

1.5 Experimental Fish

The winter flounder, Pseudopleuronectes americanus belongs to the family Pleuronectidae (flatfishes) and is found inshore along the Atlantic Coast of North America from Georgia to Newfoundland and Labrador (Liem and Scott, 1966). Winter flounder inhabit inshore, shallow-water soft muddy to moderately hard bottoms, normally at a depth between 1.8 to 36.6 m; however, McCracken (1954) reported that flounder have been found to a depth of 143 m.

Winter flounder have separate sexes with little sexual dimorphism. Scales found on the blind side of males are ctenoid instead of cycloid giving a rough feeling particularly during the period of reproductive maturity. Although this is generally true it should be noted that large females also have rough scales on the blind side (see review of Klein-MacPhee, 1978).

The flounder ovary is described as being an adaptation of the vertebrate gonad, differing slightly from the ovary of most teleosts in having a relatively short hilus and mesovarium (Dunn and Tyler, 1969). The ovaries are connected anteriorly to the peritoneum by a short mesovarium with arteries and veins passing through the hilus. Much less work has been done on the morphology of the testes of the male winter flounder. The bilobed testes are attached by mesenteries. Each testicular lobe contains a regular system of lobules or seminiferous tubules (Harder, 1975; Billard et al., 1982).

Kennedy and Steel (1971) found that males mature earlier at age VI for flounder from Long Pond, Conception Bay, Newfoundland whereas females do not mature until age VII. Approximately 50% of the females and males are matured at 250 and 210 mm, respectively. In Long Pond, Conception Bay, Newfoundland, male gonads began to enlarge earlier than females and males also reached the spawning stage before the females. Previous descriptions of

reproductive cycle in the winter flounder (Burton and Idler, 1984) indicate that seasonal gonadal growth begins in August together with an active summer feeding period. The gonads continue growing more slowly during the winter (January-March) and spawning begins in late Spring (May/June).

During the summer, adult flounder leave inshore areas if the water temperatures rise above 15°C and the fish return to inshore areas in the fall when seawater temperature return to less than 15°C. Both immature and mature fish are found along the shore areas together with spawning fish, and spawning occurs in shallow water over sand or mud bottom in late Winter or early Spring (McCracken, 1963; Scott and Scott, 1987). Spawning occurs once a year and spawning times vary with latitude, being earliest in the southern range of the fish and occurring progressively later as one moves northwards. In Conception Bay Newfoundland, spawning usually takes place in May/June; however, Kennedy and Steele (1971) reported spawning from March to early June. It has been observed that spawning occurs at night between 2200 and 0330 in captive flounder under artificial lights (Breder, 1922). Prior to spawning both males and especially females exhibit an aggressive swimming activity.

The eggs expelled by the circling spawners settle to the bottom either singly or in clumps. Following

winter flounder is examined using various techniques for administering gonadotropic hormone-releasing hormone analog (GnRH-A, Chapter 4) and determining the reproductive stages which are responsive to GnRH-A treatment (Chapter 5). Thirdly, induction of spawning using GnRH-A is investigated in prespawning female winter flounder and the quality of freshly ovulated/spawned eggs following hormone treatment is assessed (Chapter 6).

fertilization the demersal eggs are adhesive and range from 0.71-0.96 mm in diameter, with an average 0.8 mm (Fahay, 1983). The value of demersal eggs is that they would remain inshore where conditions are more favourable for development. Topp (1968) reported egg fecundity of winter flounder from Narragansett Bay, Massachusetts ranges from 435,00 (age III) to 3,329,000 eggs (age V) females. Hatching occurs in about 15-18 days at 3°C. The larvae undergo a period of larval drift on surface waters, and they metamorphose into typical flatfish form in approximately 2.5 to 3.5 months.

1.6 Objectives of Research

The winter flounder has been chosen as an experimental fish in this research to gain a better understanding of the reproductive physiology of flatfish. Because the flounder is readily available, is abundant, and is easily maintained in the laboratory, it is a good experimental animal (Klein-McPhee, 1978).

This thesis is directed towards understanding the basic reproductive mechanisms occurring in fish and to apply this knowledge for the purpose of improved control of fish reproductive cycles in captivity. These objectives can be achieved firstly by developing an understanding of the natural reproductive cycle in winter flounder (Chapter 3). Secondly, manipulation of gonadal development in

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Experimental Fish and Their Maintenance

Adult winter flounder 300-1000 g in body weight, were collected from the inshore waters of Conception Bay, Newfoundland by SCUBA divers and transported by truck to the Marine Sciences Research Laboratory. The fish were held in 250 litre tanks (n=10-15 fish) supplied with flow-through ambient temperature seawater under a simulated natural photoperiod (St. John's, Newfoundland, Canada). Fish were fed (2-5% body weight) with chopped caplin every other day between 1000 and 1200 hr from May through October since winter flounder cease feeding between October and April. Excess food remaining in the tanks was removed the following day. After acclimation to laboratory conditions for 3-7 days the fish were individually weighed and tagged with an identification tag. Winter flounder were always easily handled without the need of anaesthetics.

2.2 Environmental Conditions

Seasonal seawater temperatures in the laboratory were recorded throughout the year and ranged from 0°C (Winter) to 18°C (Summer). During induced ovulation/spawning

experiments conducted during the winter, seawater temperature was elevated to 5°C to mimic the spring temperatures normally occurring during the spawning period (Bigelow and Shroeder, 1953). Data on daylength for the St. John's area was obtained from Bishop (1987, 1988).

2.3 Ovarian Analyses

2.3.1 Determination of Gonadosomatic and Hepatosomatic Index

At the time of autopsy the fish were quickly decapitated; body, gonad and liver weights were determined for calculation of gonadosomatic index = [gonad weight/body weight] x 100, and hepatosomatic index = [liver weight/body weight] x 100.

2.3.2 Staging of Oocytes

Prior to hormone injection or implantation a few milligrams of ovary were collected by a biopsy procedure by aspirating oocytes into a 10 ml syringe using a 14 gauge needle inserted through the dorsal body wall directly into the ovary. A preliminary study confirmed that this oocyte biopsy procedure resulted in minimal bleeding and had no apparent detrimental effect on ovarian development. About 30-40 oocytes from each female were cleared of yolk to determine germinal vesicle position by exposing ovarian fragments to clearing solution

(ethanol:formalin: glacial acetic acid; 6:3:1 v/v) for about 1 min according to the method of Ng and Idler (1978). The position of the germinal vesicle (GV) could be determined sequentially under the microscope and was staged as follows:

(1) - GV in the central position
(2) - GV slightly offcentre
(3) - GV migrated midway to animal pole
(4) - GV located peripherally against the oocyte membrane
(5) - germinal vesicle breakdown (GVBD)
(6) - ovulation.

Mean GV position was calculated from 3-7 females per treatment group.

2.3.3 Oocyte Size-Frequency Distribution

Small amounts (1-10 mg) of ventral ovary were obtained either by biopsy from live fish or from ovarian tissue collected at autopsy. These small pieces obtained from the middle portion of ovary were fixed in 0.6% NaCl containing 1% formalin (Shehadeh et al., 1973b). Preliminary comparison showed that no significant changes in the oocyte diameters occurred up to 5 days after fixation. Individual follicles (hereafter refer to as oocyte) from fixed tissues were separated under the microscope using fine forceps; previtellogenic and vitellogenic (opaque)

oocytes could be distinguished by their size and appearance and 100-250 follicles from each female and about 5-7 females per sampling period were measured by optical micrometer to the nearest 30 μm at 32X magnification. The size-class frequency distributions were constructed and mean oocyte diameter was determined from these measurements.

2.3.4 Oocyte Dry Matter

Dry matter = dry weight/fresh weight x 100 was determined from a known weight of fresh ovary after drying the tissue at 100°C in an oven for 24 hr.

2.3.5 Histological Procedure

Oocytes from the dorsal part of the ovary were fixed in Bouin's fluid for 24-48 hours and moved through two changes of 50% ethanol before storage in 70% ethanol (Humason, 1979). In preparation for sectioning, the tissues were washed twice in 70% ethanol to which a few drops of ammonium hydroxide were added to assist removal of the picric acid in Bouin's fluid. The tissues were dehydrated and cleared with 70% ethanol, 95% ethanol, 100% ethanol and xylene:toluene (50:50, v/v). After three 45 min changes in 100% toluene, the tissues were infiltrated with liquid paraplast (57°C). Tissue sections were cut at 4-10 μm and stained with haematoxylin and eosin (Humason,

1979). In males, the central portion of the dorsal testis was fixed in Bouin's fluid and tissue sections were prepared for histological observation as described above.

2.3.6 Classification of the Stages of Gonadal Development

The stages of previtellogenic and vitellogenic oocyte development were adapted from Ng et al. (1980a) as follows:

- previtellogenic 1 (PREVIT 1) - cytoplasm devoid of yolk or vacuoles
- previtellogenic 2 (PREVIT 2) - cytoplasm contains no yolk but vacuoles (cortical alveoli) line the periphery
- vitellogenic 1 (VIT 1) - cytoplasm contains eosinophilic yolk granules around the periphery
- vitellogenic 2 (VIT 2) - yolk granules occupy the outer half of the cytoplasm
- vitellogenic 3 (VIT 3) - yolk granules occupy most of the cytoplasm, some reaching the nucleus
- vitellogenic 4 (VIT 4) - cytoplasm completely full of yolk

For the testes, histological sections were examined to determine the most numerous and most advanced sperm cell type present as follows (Crim et al., 1983a):

- spermatogonia A & B - These cells contained a pale cytoplasm with a central nucleus. The

nucleolus was distinct due to a meshwork of chromatin fibers which radiate out towards the nuclear membrane

- spermatocytes I & II -identified by the presence of a central nucleus with patches of densely staining chromatin. Groups of spermatocytes II could be distinguished from spermatocytes I by their smaller size and homogenously-staining nuclei
- spermatids and spermatozoa - both spermatids and spermatozoa share an intensely-staining nuclei occupying most of the cell, spermatozoa could be easily recognized by the presence of their distinct tails

2.3.7 Artificial Fertilization and Egg Quality

Ovulation in female flounder was checked daily by applying slight pressure to the abdomen in the direction of the egg pore. The date of ovulation was taken as the time when the first free flow of eggs could be obtained. Freshly ovulated eggs were stripped into an ice-cold 250 ml beaker.

Artificial fertilization was carried out using the "dry" method. Aliquots of 200-300 eggs were pipetted into petri dishes (3-6 replicates per female) and mixed with 30-50 μl of undiluted milt pooled from two or three fully

ripe males. Ripe males were first available in March and continuing through the spawning season (May/June). Prior to fertilizing eggs, sperm motility was checked under the microscope by activating the sperm with 1 μm filtered ultraviolet sterilized chilled seawater (5°C) containing streptomycin (0.1 g/l) and penicillin (0.06 g/l) (Campbell and Jalabert, 1979). Egg fertilization was initiated by addition of 0.5 ml of clean seawater and the eggs and milt were gently mixed. After 1 min another 1-2 ml of seawater was added and the mixture was thoroughly swirled. During this procedure the eggs began to stick to the bottom of the petri dishes. After 2 min the fertilized eggs were rinsed 2-3 times with sterilized seawater to remove the excess milt. Eggs were then incubated at 5°C under static conditions in the dark.

Egg quality was estimated from the fertilization rate after 24 hr of incubation by counting the number of embryo's which were in the blastula stage. Dead eggs and larvae were removed and the seawater replaced daily. Daily observations were made to determine the time of onset of hatching and duration of hatching interval. Based on larval morphological features, normal and abnormal larvae (curvature of spine, abnormal yolk sacs, or enlarged fin folds) were distinguished and counted just after hatching (Rogers, 1976). Prematurely hatched larvae were also considered abnormal since they were short, thickened, and

often curved. Egg quality was calculated as follows:

Fertilization Rate = [Number fertilized eggs/Total number of eggs incubated] x 100%

Total Hatching Rate = [Total larvae hatched/Number of fertilized eggs] x 100%

Normal Larvae = [Number normal larvae/Total larvae hatched] x 100%

2.4 Milt Analyses

2.4.1 Spermatocrit Determination

The genital area of males was blotted and the first watery portion of the ejaculate was discarded before the remaining milt was hand-stripped into clean, dry glass scintillation vials. Spermatocrit was determined on small aliquots of milt in 0.1 ml capillary tubes after centrifugation of samples at 13,000 x g for 15 min. The packed cell level was measured and the spermatocrit value was determined as follows: % spermatocrit = [length of packed cell (mm)/total length of the milt in tube (mm)] x 100 (Baynes and Scott, 1985).

2.4.2 Sperm Density Determination

Total milt volumes were measured in plastic calibrated

syringes. After recording the volume of milt for each fish, the sperm motility was determined under the microscope. A small droplet of milt was placed in a haemocytometer chamber and observed while seawater was slowly added. Sperm concentration for an individual male was determined by diluting the milt 1:1000 with seawater and counting sperm heads in four squares of the haemocytometer chamber, under phase contrast microscope at 400X. The total numbers of spermatozoa were calculated taking into account the total volume of milt and spermatozoan density (number of spermatozoa/ml). The spermatocrit value could be converted to sperm cell concentration using a regression equation which was determined by haemocytometer counts: number of spermatozoa/ml= (0.12 x spermatocrit + 6.40) x 10^9 (Baynes and Scott, 1985).

2.5 Hormone Administration

2.5.1 GnRH-A Injection

[D-Ala^6,Pro^9-NHEt]LHRH analog (GnRH-A) purchased from Syndel Laboratories Ltd., Vancouver B.C., Canada was stored either dry at 4°C or dissolved in distilled water (500 μg/ml) and stored frozen at -20°C. Prior to injection, aliquots of GnRH-A were freshly thawed and further diluted in fish saline composed of 0.113M NaCl, 0.005M KCL, 0.002M $CaCl_2$, 0.001M $MgSO_4$, 0.001M Na_2HPO_4,

0.001M $NaHCO_3$, 0.001M sodium pyruvate, 0.005M glucose, 0.005M Hepes and NaOH (about 0.0025M) for adjusting pH to 7.5 (Wallace and Selman, 1978). GnRH-A was injected intraperitoneally at the base of the right pectoral fin using a disposable syringe and 25G needle. The doses and frequency of GnRH-A injections vary with the experiments and are stated in each chapter.

2.5.2 GnRH-A Pellet implantation

The protocol for making cholesterol pellets was described in Lee et al. (1985). Briefly, GnRH-A was incorporated into slow release cholesterol pellets (40 or 100 μg of hormone) prepared by dissolving 800 μg of GnRH-A in 0.2 ml of 50% ethanol. The solution was then mixed thoroughly with 190 mg of cholesterol until a paste-like consistency was formed. The mixture was dried in an incubator at 37°C for about 1-2 hr. Following evaporation of the ethanol, the dry powder was mixed with 10 mg of cocoa butter which served as a binder. After stirring again thoroughly, about 30 mg was weighed and packed into a plexiglass mold. For making a fast release pellet (50:50), the procedure is the same as above except 95 mg of cholesterol was mixed with 95 mg cellulose. The pellets were stored at 4°C.

In one experiment, GnRH-A was incorporated into slow release cholesterol pellets measuring 2x5 mm (Crim et al.,

1983b) containing 120 μg of peptide hormone. For this experiment each pellet were inserted into a small incision in the epaxial muscles below the dorsal fin. Sutures were not required since the wound healed within 1-2 weeks. In most experiments pellet implantation was carried out using a sharpened trochar which was inserted directly into the muscle.

2.6 Blood Sampling

Blood samples of 0.5-1.0 ml were collected through the caudal vein using a heparinized 1 ml syringe attached to 21-gauge needles. All samples were collected between 1000 hr and 1200 hr to avoid time effects on daily changes in steroid hormone concentrations unless otherwise stated in individual experiment. Blood samples were held over crushed ice until centrifugation at 15,000 x g for 2 min. The plasma was divided into aliquots of 150 μl and stored frozen at -70°C until sex steroid radioimmunoassay analysis.

2.7 Sex Steroid Radioimmunoassays

Radioimmunoassay (RIA) is one of the most important techniques in the clinical and biochemical fields used to determine the concentration of hormones in very small quantities of serum or plasma (nanogram or picogram). It combines the specificity of the immune reaction with the

sensitivity of radioisotope techniques. The assay technique is based on the competition between cold hormone (unlabelled) and a finite amount of the hot hormone (radioactively labelled) for a limited number of antibody binding sites in a fixed amount of antiserum. The basic assumption is that the antibody will bind equally well to either the labelled or unlabelled hormone. A standard curve can be prepared by placing increasing amounts of cold hormone in separate reaction tubes with a constant amount of antibody and constant quantity of labelled hormone. The quantity of labelled hormone bound to the antibody can be determined by precipitating the antibody and counting the radioactivity bound to it. Another method of separating labelled bound hormone from the free is by using charcoal. Unbound labelled hormone is discarded with the supernatant. The radioactivity measured in the precipitated antibody is inversely proportional to the quantity of cold hormone added. The data can then be plotted as ratio of bound to free label to yield a dose-response curve. The concentration of hormone in unknown plasma samples can be assayed in similar way, and the hormone quantity extrapolated from the standard curve using the amount of label bound to the antibody.

2.7.1 Chemicals for Steroid RIA

2.7.1.1 Tris-HCl Buffer

(Tritiated Testosterone RIA)

The Tris-HCl buffer consisted of 0.05M Tris-HCl (pH 8.0), 0.1M NaCl, and 0.1 % w/v gelatin. The Tris-HCl buffer was stored at 4°C.

2.7.1.2 Phosphate Buffered Saline (PBS)

(Tritiated 11-Ketotestosterone RIA)

PBS buffer was made of 0.03M $NaH_2PO_4.H_2O$, 0.06M Na_2HPO_4, 0.15M NaCl, and 0.1 % w/v gelatin. The pH was adjusted to 7.00 and the buffer was stored at 4°C.

2.7.1.3 Phosphate Buffered Saline (PBS)

(Iodinated Testosterone and Estradiol-17β RIA)

The phosphate buffered saline contained 0.04M Na_2HPO_4, 0.006M KH_2PO_4, 0.1M sodium chloride, 0.1 % sodium azide and 0.1% gelatin. The pH of the buffer was adjusted to 7.4 with NaOH and was stored at 4°C.

2.7.1.4 Non-Radioactive Steroids

Testosterone and Estradiol-17β standards were purchased from Steraloids Inc. (Wilton, NH; USA). Steroids stocks (1mg/10ml) including 11-ketotestosterone were dissolved in ethanol and stored

at -20°C.

2.7.1.5 Radioactive Steroids

(i) [1,2,6,7-^{3}H(N)]testosterone (99.1 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). The tritiated testosterone was diluted to 10 ml with ethanol (stock) and stored at -20°C (60,000-100,000 CPM/0.1 ml). The purity of tritiated testosterone and 11-ketotestosterone was checked by paper chromatography every 3 months. Whatman paper #1 was used with the following solvents (v/v) systems: 80% methanol: haptane (50:50) for tritiated testosterone and cyclohexane:toluene:methanol:water (10:7:102.5) for tritiated 11-ketotestosterone.

(ii) [1,2 (n) ^{3}H] 11-ketotestosterone was a gift from Dr. D.R. Idler (Ocean Sciences Centre, St. John's.). Procedure for synthesis of 11-ketotestosterone from tritiated cortisol has been described by Truscott (1981).

(iii) ^{125}I-Testosterone and ^{125}I-Estradiol-17β were purchased from International Diagnostic Services (IDS), Scarborough, ONT., Canada. The iodinated steroids were stored in phosphate buffer at 4°C.

2.7.1.6 Antiserum

(i) Vials of lyophilized antisera for tritiated testosterone and estradiol-17β were purchased from Miles Scientific (Naperville, Ill., USA) and stored at 4°C. Stock antiserum were prepared by adding 5 ml of Tris-HCl buffer. The antisera were diluted 1:6.4 with Tris-HCl assay buffer for radioimmunoassay.

(ii) The 11-ketotestosterone antiserum was kindly provided by Dr. D.R. Idler. Procedure for raising antiserum for 11-ketotestosterone has been described by Idler and Ng (1979).

(iii) Anti-testosterone and anti-estradiol-17β for iodinated radioimunoassay RIA were also acquired from IDS, Scarborough, ONT, Canada. Antibody was diluted in the rabbit gamma globulin solution and was stored at 4°C.

2.7.1.7 Separation of Bound from Free Steroids

(i) Activated charcoal was used to separate bound from free tritiated steroids. Norit A charcoal was purchased from Sigma Chemical Co., (St. Louis, MO., USA) and dextran T-70 from Pharmacia, Inc. (Piscataway, NJ., USA). For the tritiated testosterone radioimmunoassay, dextran coated charcoal was composed of 2.5 g Norit A activated charcoal and 0.25 g Dextran

T-70 in 500 ml Tris-HCl buffer. Dextran coated charcoal for the 11-ketotestosterone radioimmunoassay consisted of 1.25 g charcoal and 0.125 g dextran T-70 in 500 ml phosphate buffer and stored at 4°C (Simpson and Wright, 1977).

(ii) For iodinated testosterone and estradiol-17β radioimmunoassays, bound steroids were separated from free steroids using a glycol precipitating reagent (IDS, Scarborough, ONT, Canada).

2.7.1.8 Liquid Scintillation Fluid

Liquid scintillation cocktail contained 3.0 g of 2-Diphenyloxazole (PPO) and 0.2 g of p-bis-[2-(5-phenyl-oxazolyl)]-benzene (POPOP) in 1 liter of xylene/Triton X-144 (3:1) (BDH Chemical Co., Mississauga, ONT., Canada).

2.7.2 Extraction of Plasma Samples

Mixtures of 0.1 ml plasma and ca. 1000 CPM either tritiated tracer testosterone, 11-ketotestosterone or estradiol-17β contained in 10 μl ethanol were mixed and incubated 1-2 hr at room temperature prior to extraction of samples twice with 2 ml diethyl ether. The aqueous phases were frozen over solid CO_2 and discarded and the combined ether extracts were evaporated under nitrogen

with gentle heating (40°C). The resultant plasma residues were redissolved in 1 ml absolute ethanol and allowed to equilibrate at least overnight at 4°C before aliquoting for radioimmunoassay and an estimate of radioactivity for sex steroid extraction efficiency.

2.7.3 Steroid RIA Protocols

2.7.3.1 Tritiated Testosterone and 11-Ketotestosterone

In preparation for radioimmunoassay, the ethanol reconstituted extracts of plasma or standard steroids in ethanol (1-1,000 pg/tube) were pipetted in quadruplicate into 12 x 75 mm borosilicate disposable culture tubes. After evaporation of the ethanol under nitrogen, testosterone was determined by bringing tubes to 0.7 ml with Tris-HCl buffer containing testosterone antiserum diluted 1:6.4 and approximately 10,000 CPM tritiated testosterone. Following assay incubation for 1 hr at 37°C, the tubes were rapidly brought to 4°C in an ice-bath and 0.2 ml dextran-charcoal suspension was added to separate bound from free steroids. Following centrifugation of the radioimmunoassay tubes at 2,200 x g for 15 min, the supernatant (bound fraction) was decanted into glass scintillation vials containing 10 ml liquid scintillation cocktail for counting (Minaxiβ Tri-carb 4000 Series, Liquid Scintillation Counter, United Technologies

Packard).

For the 11-ketotestosterone radioimmunoassay, the tubes were brought to 0.3 ml with phosphate buffer containing 11-ketotestosterone antiserum at 1:105,000 final dilution and ca. 10,000 CPM tritiated 11-ketotestosterone. Following overnight incubation of the assay at 4°C, 0.6 ml dextran-charcoal suspension was added and the incubation was continued for one additional hr at 4°C. After centrifugation of the tubes at 2,200 x g for 15 min, the supernatant was decanted into a vial containing 10 ml liquid scintillation cocktail for counting (Minaxiβ Tri-carb 4000 Series, Liquid Scintillation Counter, United Technologies Packard)

2.7.3.2 Iodinated Estradiol-17β and Testosterone

100 μl of ethanol extracts of plasma or standard in ethanol (1-1000 pg) were pipetted in quadruplicate into 12 x 75 mm borosilicate glass tubes. After evaporation of ethanol under a stream of nitrogen, estradiol-17β and testosterone were determined by bringing tubes to 0.4 ml with phosphate buffer containing estradiol-17β or testosterone antiserum (100 μl) and ca 10,000 CPM iodinated estradiol-17β or testosterone. The mixture was vortexed briefly and incubated for 1 hr at room temperature. Prior to radioimmunoassay the buffer,

separating reagent and diluent for antibody were brought to room temperature. Following assay incubation, 1 ml of separating reagent was added to separate bound from free labelled steroid. Incubation was continued for another 25 min at room temperature. Following the second incubation, the tubes were centrifuged at 1650 x g for 15 minutes. After centrifugation the supernatant was aspirated and the tubes containing the bound fraction were counted 1 min in a gamma counter (Packard Autogamma 5650 Series, United Technologies Packard).

2.7.3.3 Recovery

During extraction known counts (ca. 1000 CPM) of tritiated testosterone, estradiol-17β or 11-ketotestosterone were added to plasma samples. The extraction efficiency was determined by counting 0.1 ml of the reconstituted extract and the data were adjusted for recovery.

2.7.3.4 Precision

The precision of the assay was evaluated by the intra-assay (within an assay) and inter-assay (between assays) variations. The intra-assay was determined by 5-7 replicate measurements of a plasma pooled sample in a single assay, and the inter-assay was determined using the same pooled sample in different assays. For the tritiated

steroid radioimmunoassays the intra-and inter-assay coefficients of variation for the testosterone assay were 8.2% (n=7) and 12.9% (n=6), respectively. The intra- and inter-assay coefficient of variation for 11-ketotestosterone were 4.9% (n=5) and 24.7 (n=19), respectively. For iodinated steroid radioimmunoassays the intra-and inter-assay coefficients of variation for the testosterone assay were 4.7% (n=4) and 22.0% (n=10), respectively. The intra- and inter-assay coefficients of variation for estradiol-17β were 4.4% (n=5) and 15.7% (n=12), respectively.

2.7.3.5 Specificity

The specificity of steroid radioimmunoassays depends upon the specificity of the antiserum to closely related substance found in the plasma. To test cross-reactions, serial dilutions of the related steroid hormones were prepared and the amount of hormone that yields 50% inhibition of binding was determined. For tritiated steroid radioimmunoassays the testosterone antibody cross-reactivity was 1.3% for 11-ketotestosterone, 1.0% for androstenedione, 0.06% for estradiol-17β and <0.001% for estriol, estrone, and progesterone. The cross-reactivity of the 11-ketotestosterone antibody to testosterone and 11β-hydroxytestosterone was <0.1% (Ng and Idler, 1980b). For iodinated steroid radioimmunoassays the testosterone

antibody cross-reactivity was 0.0009% for estriol, <0.0007% for estrone, 0.0035% for estradiol, 0.96% for androstenedione, 0.19% for 11-ketotestosterone and 0.0034% for progesterone. The cross-reactivity of the estradiol-17β antibody was 0.43% for estriol, 6.37% for estrone, 0.0046% for testosterone, 0.0007% for androstenedione, 0.0035% for 11-ketotestosterone and 0.0004% for progesterone.

2.8 Statistical Analysis

All data analysis were carried out by computer using the Statistical Analysis System package (SAS, 1985). Log or arc-sine transformation were used where necessary to obtain homogenous variances prior to analysis. All data are expressed as means ± SEM. Data on sex steroid hormones, gonadosomatic index, oocyte diameter, hepatosomatic index, milt volume and spermatocrit were subjected to one/two way analysis of variance followed by Duncan's multiple range test for testing the significance of means. The differences between different oocyte size-class frequency distributions was compared using chi-square frequency analysis ($P<0.05$). Pearson correlation coefficients (r) were used to test for the significant relationships between plasma sex steroids, gonadosomatic index, hepatosomatic index, oocyte diameter and dry matter. Data for ovulation were analyzed using Fisher's

exact test. Analysis of body weight was performed on the untransformed percentages since body weights were expressed as both positive and negative values. After performing the one-way analysis of variance, normality of the residuals was tested using either the Shapiro-Wilk statistic ($N<50$) or the Kolmogorov statistic ($N>50$). In all tests, $P<0.05$ was selected as the level of significance unless otherwise stated. Procedures for these statistical analyses is described in Steel and Torrie (1980) and Snedecor and Cochran (1980).

CHAPTER 3

THE ANNUAL REPRODUCTIVE CYCLE OF WINTER FLOUNDER

3.1 INTRODUCTION

A clear correlation between increases in plasma sex steroids and active seasonal reproductive cycles in teleosts has been established by previous investigators, especially for some freshwater fishes including salmonids and the cyprinids (see review of Fostier et al., 1983). By comparison, our knowledge of the reproductive physiology of marine fishes remains greatly limited. In mature winter flounder, Campbell et al. (1976) isolated and identified a variety of steroids in blood plasma. However, these investigators did not identify estradiol-17ß and did not provide a detailed description of the seasonal sex steroid profile due to the fact that they used pooled samples obtained from a limited sampling program.

Some aspects of the reproductive biology of the winter flounder have been previously described. Dunn and Tyler (1969) and Dunn (1970) suggested that oocytes in winter flounder may take 2 or 3 years to reach maturity. Burton and Idler (1984) reported that oogonial proliferation in flounder occurs during spawning and the post-spawned

period and vitellogenesis is initiated in summer.

Despite the fact that seasonal variations in gonadosomatic index are regarded as indications of gonadal activity in teleosts, a clearer understanding of gonadal development in females can only be achieved from direct studies of the dynamics of oocyte development (De Vlaming et al., 1984). Indeed, precise understanding of the progressive stages of gonadal development would increase our opportunities for manipulating fish reproductive cycles such as accelerating gonadal growth (Chan, 1977; Crim et al., 1983a), or inducing spawning (Crim and Glebe, 1984; Weil and Crim, 1983) by the use of hormone therapy or environmental manipulation (see review of Lam, 1982).

The objectives of our current studies were to determine plasma sex steroid hormone profiles during the annual reproductive cycle of male and female winter flounder and to relate these hormonal events to precise changes in gonadal development. In females, the dynamics of oocyte development were monitored while in males the progress of spermatogenesis during the seasonal reproductive cycle was noted. In order to compare plasma sex steroid profiles of wild fish with the hormone profiles for fish retained in captivity, a group of post-spawned female flounder were maintained in the laboratory for more than a year.

3.2 MATERIALS AND METHODS

3.2.1 Fish

Wild adult winter flounder were collected from September 1987 through December 1988. The fish were held 3-4 days in the laboratory without feed. Fish (n=4-6) were obtained twice a month from August to December to closely follow the period of rapid gonadal growth or recrudescence during the periods of active vitellogenesis and spermatogenesis; thereafter sampling was carried out on a monthly basis. One group of females (n=11), after spawning in May 1988, was maintained in the laboratory for more than one year. They were fed (2-5% body weight) with chopped capelin (Mallotus villosus) in the morning every other day from May through October until eating ceases. During the spawning period (May/June) these fish were inspected daily for signs of ovulation, i.e. abdominal softening or the release of free flowing eggs from the egg pore after applying gentle pressure to the abdomen. Eggs stripped from ovulated females were artificially fertilized and the fertilization rate was used as an index of egg viability (see chapter 2, section 2.3.7).

3.2.2 Blood Sampling

For the captive females, serial blood samples (see chapter 2, section 2.6) were collected monthly from

November 1988 through July, 1989. Blood from ovulated wild females was not collected and sampled during this study, thus plasma sex steroid changes were not measured in spontaneously ovulated females.

3.2.3 Determination of Vitellogenic Oocytes Using Polyacrylamide Gels

During the period of early ovarian recrudescence (September), two types of oocytes were present in the flounder ovary, clear previtellogenic oocytes (<150 μm) and other opaque oocytes that were >150 μm. To determine whether the larger opaque oocytes were indeed vitellogenic, fresh ovarian tissues were obtained and approximately 300-400 follicles containing oocytes >150 μm were carefully selected under the microscope and were pooled into an eppendorf tube. Similarly, 400-450 follicles with clear previtellogenic oocytes were also teased apart and pooled into another eppendorf tube. In addition, a crude extract (a mixture of follicles with clear and opaque oocytes) of fresh or fixed (ca. 2 weeks in 0.6% saline containing 1% formalin) ovarian tissues were also used. These follicles and ovarian tissues were homogenized in vitellogenin buffer (So et al., 1985b; 0.05M Tris, 0.5M NaCl, 0.01M EDTA, 3% trasylol, pH 8) and the protein concentrations in these homogenates were estimated by the method of Lowry et al. (1951). Purified

Figure 1. Seasonal variations in mean monthly (A) laboratory ambient seawater temperatures and (B) daylengths (photoperiod) recorded at St. John's, Newfoundland from September 1987 through December, 1988.

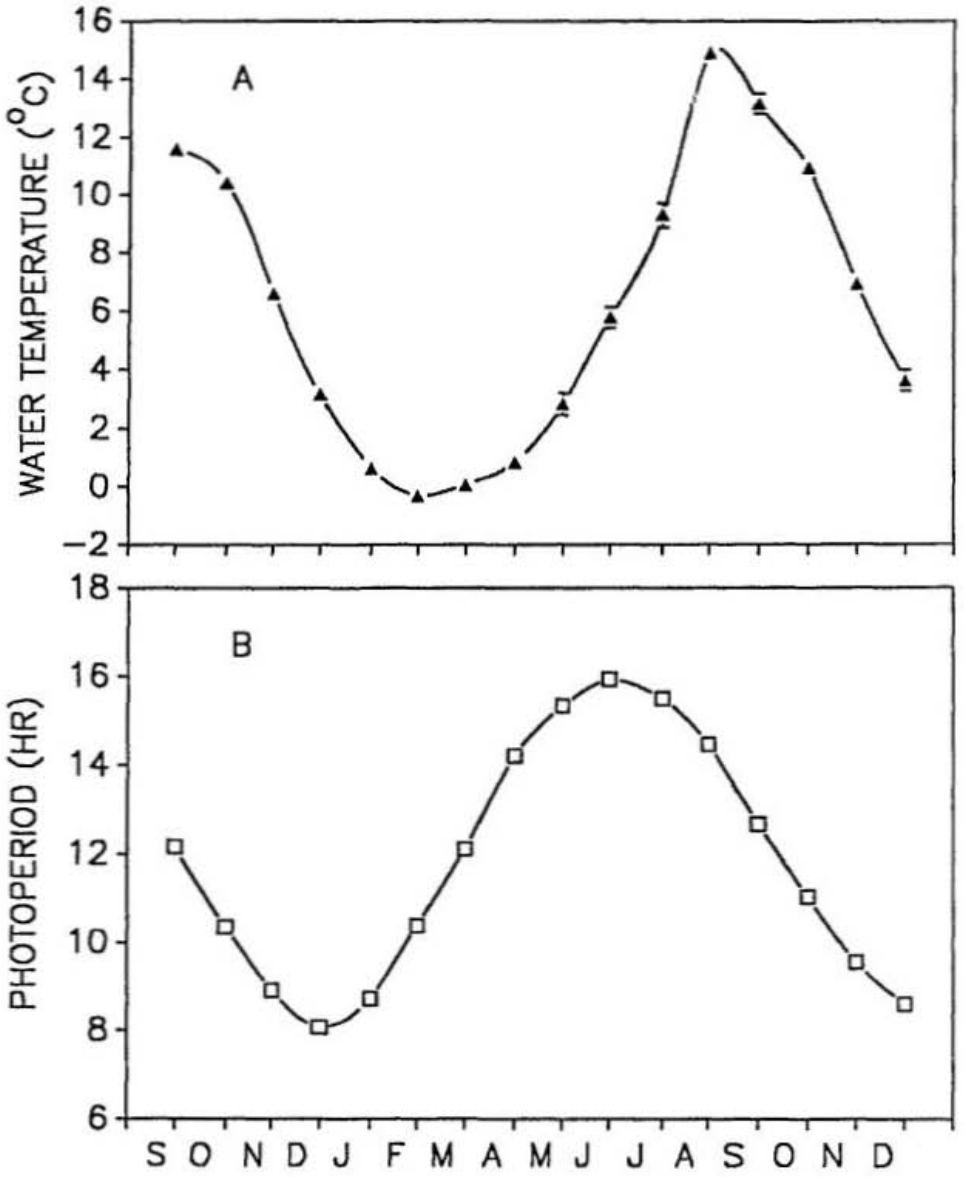
winter flounder vitellogenin was used as standard.

The presence of vitellogenin-like substances in these ovarian tissues was tested by non-denaturing polyacrylamide gel using 5% running slab gel (5% acrylamide, 2.6% cross linkage; pH 8.9) and 4% stacking gel, pH 6.8 at a constant voltage of 300 volts for 2 hr according to So et al. (1985). Proteins were stained with 0.1% Coomassie Blue R-250 (Bio-Rad) and the gels were fixed with 12% TCA for 1 hr before destaining the gel with 7% acetic acid.

3.3 RESULTS

3.3.1 Seasonal Environmental Conditions

The seasonal pattern for environmental fluctuations in laboratory seawater temperature and photoperiod throughout the year are shown in Figure 1 (A,B), respectively. In September, at the beginning of the sampling of wild fish, both seawater temperature and photoperiod were in a declining phase such that, between September and January, seawater temperature and photoperiod fell from 12-0°C and 12-8 hr light/day, respectively. During the winter interval (January-April), seawater temperature remained low while photoperiod began increasing rapidly from the winter minimum. By June, the maximum photoperiod, 16 hr light/day was reached and seawater temperature was rising


A
WATER TEMPERATURE (°C)
16
14
12
10
8
6
4
2
0
−2
B
PHOTOPERIOD (HR)
18
16
14
12
10
8
6
S O N D J F M A M J J A S O N D

towards the peak value, 15°C, observed during the month of August.

3.3.2 Wild Females

Seasonal patterns for gonadosomatic index and oocyte diameter in wild female flounder are presented in Figure 2 (A,B). During the Fall, from August-December (preparatory phase, PREP 1), while the ovaries progressively assumed a yellowish colour, both mean gonadosomatic index and mean oocyte diameters rapidly rose in parallel approaching values >15 and 500 μm, respectively. During the cold winter period from December-February (preparatory phase 2, PREP 2), gonadosomatic index and oocyte diameter both increased more slowly and/or remained plateaued. From March-April (prespawning phase, PRES), the elevated gonadosomatic index and oocyte diameter values reached a peak during the spawning (spawning phase, SPW) period in May/June. After spawning, gonadosomatic index and oocyte diameter rapidly fell to seasonal low values and the flounder ovaries remained in a regressed condition (post spawning phase, POST) from June until August. Both gonadosomatic index and oocyte diameter were significantly correlated in female flounder ($r=0.88$, $P<0.001$, $n=138$) throughout the annual reproductive cycle (Fig. 3).

During the period of most active ovarian recrudescence (PREP 1), the gonadosomatic index of female flounder rose

Figure 2. Seasonal variations of (A) mean gonadosomatic index (GSI) and (B) oocyte diameter in wild female winter flounder from September 1987 through December 1988. Symbols represent means and vertical lines ± SEM obtained from 4-6 fish per sampling period. SEMs too small for scale not shown. Asterisks indicate mean is significantly ($P<0.05$) different from previous sample. The phases of the reproductive cycle in female winter flounder are based on seasonal profiles of the gonadosomatic index, oocyte diameter, plasma estradiol-17β and testosterone. PREP 1, preparatory 1 phase; PREP 2, preparatory 2 phase; PRES, prespawning phase; SPW, spawning phase; POST, regressed period.

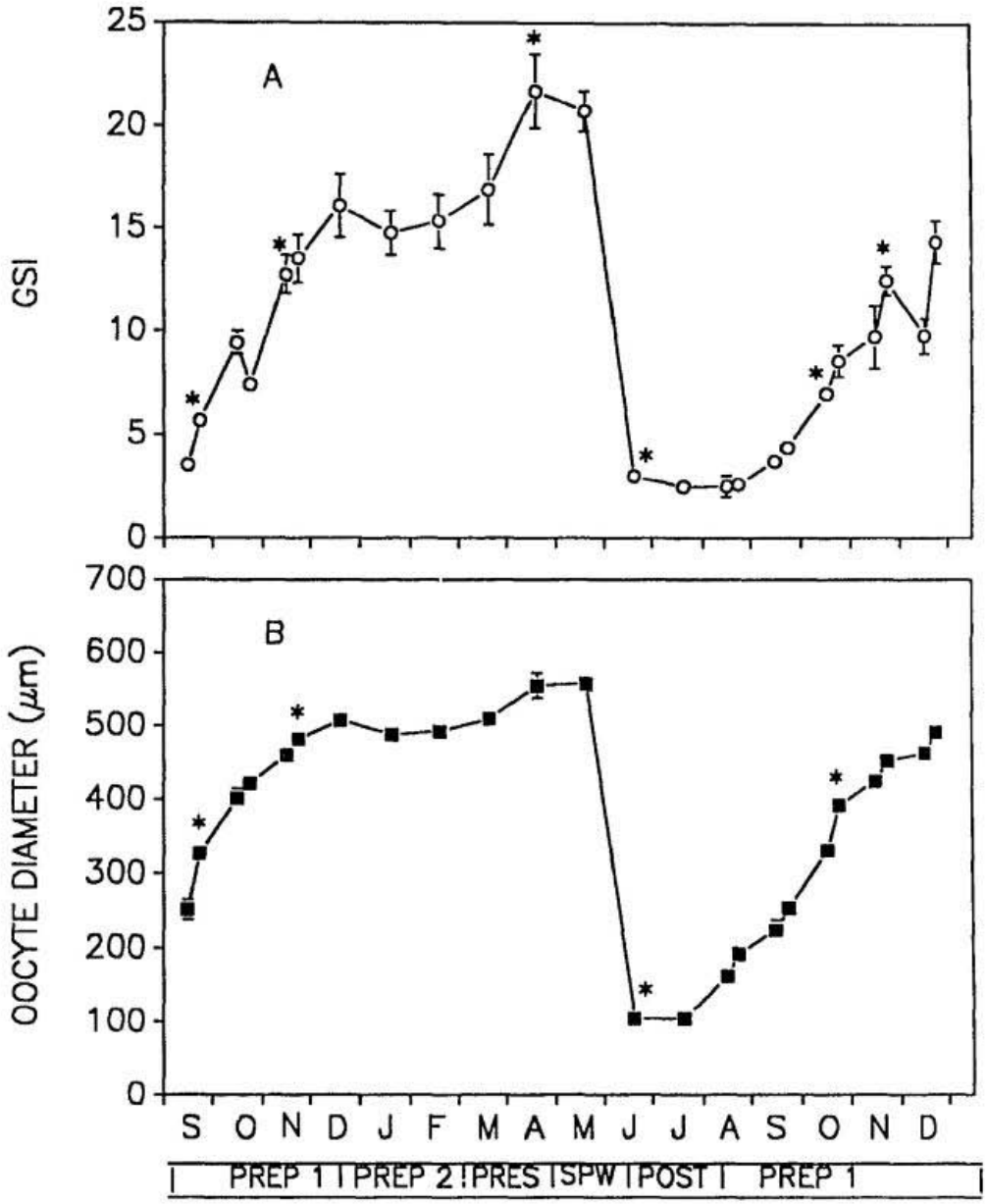
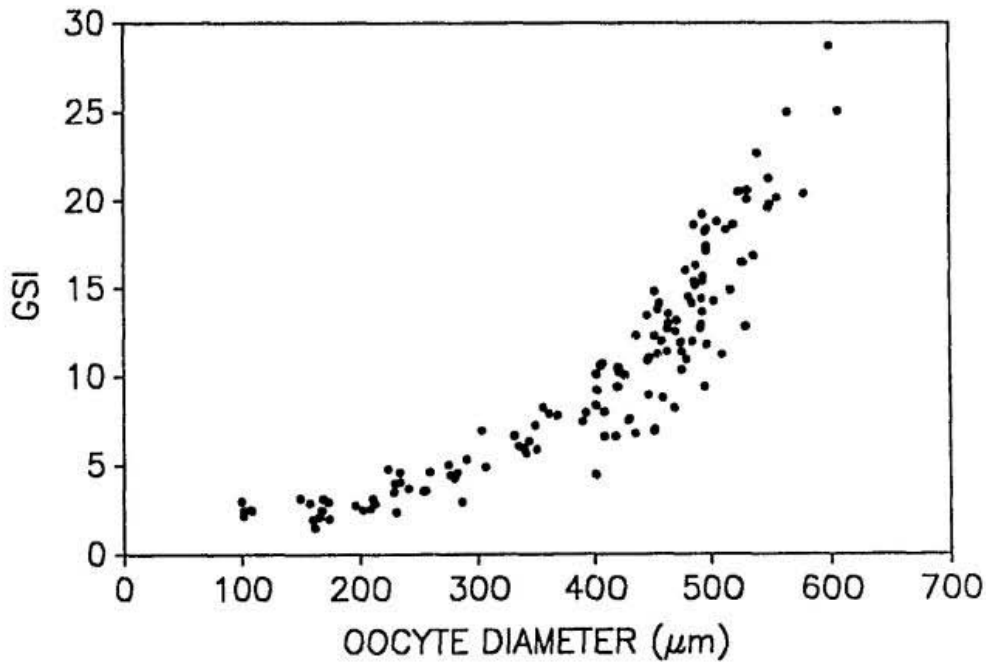

A
B
GSI
OOCYTE DIAMETER (μm)
0
5
10
15
20
25
100
200
300
400
500
600
700
S O N D J F M A M J J A S O N D
PREP 1 | PREP 2 | PRES | SPW | POST | PREP 1

Figure 3. Scatter-plot of gonadosomatic index (GSI) and oocyte diameter of wild female winter flounder from September 1987 through December 1988. Each point represents individual fish.


30
25
20
15
10
5
0
GSI
0
100
200
300
400
500
600
700
OOCYTE DIAMETER (μm)

from about 3 to 15 indicating a very rapid growth of oocytes. The dynamic pattern of oocyte growth was quantitatively analyzed by noting the seasonal changes in oocyte size-class frequency distributions (Fig. 4). The first opaque oocytes (>150 μm) undergoing enlargement were observed in flounder ovaries at the beginning of this PREP 1 phase in August. These populations of opaque oocytes were indeed vitellogenic as shown by the appearance of yolk protein using gel electrophoresis (Fig. 5) and histological sections (Fig. 6). A limited range of different oocyte size classes could be found in the flounder ovary at all times of the year. At the beginning of PREP 1 phase (September) oocytes were still in the early stages of development as reflected by the variable yet small range of oocyte size classes (150-300 μm), with a mode of about 250 μm (Fig. 4., mid September). Oocytes continued increasing in size and by the end of PREP 1 in December the diameter ranged from 400-600 μm (mode 420 μm). Histological sections of early PREP 1 (August-September) ovaries showed the presence of vitellogenic 1 and 2 (VIT 1,2) oocytes indicating yolk uptake had commenced (Fig. 6). However, previtellogenic 1 (PREVIT 1) oocytes were still present in the ovaries of some females. By the end of the PREP 1 (December) phase all ovaries contained vitellogenic 4 (VIT 4) oocytes fully laden with yolk. During the winter (PREP 2, January-February) period,

Figure 4. Size-frequency distribution of opaque vitellogenic oocytes (shaded) of wild female winter flounder from September 1987 through December 1988. Unshaded histogram indicates previtellogenic oocytes from June and July 1988.

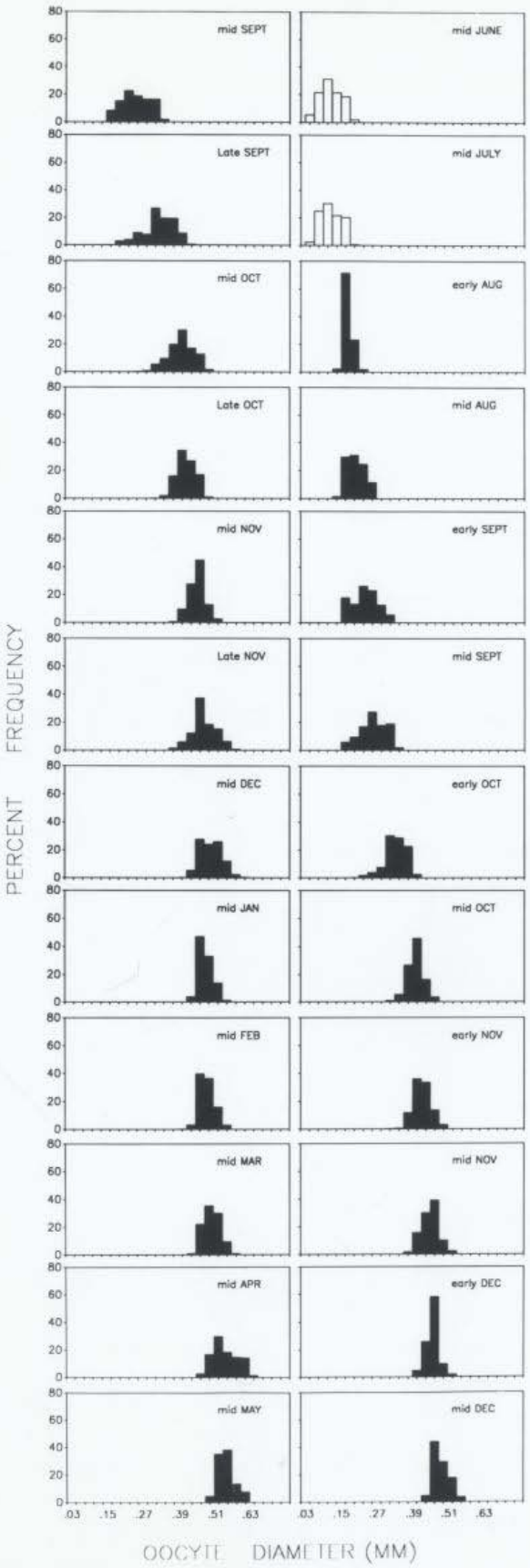

mid SEPT
mid JUNE
Late SEPT
mid JULY
mid OCT
early AUG
Late OCT
mid AUG
mid NOV
early SEPT
Late NOV
mid SEPT
mid DEC
early OCT
mid JAN
mid OCT
mid FEB
early NOV
mid MAR
mid NOV
mid APR
early DEC
mid MAY
mid DEC
0
20
40
60
80
.03
.15
.27
.39
.51
.63
PERCENT FREQUENCY
OOCYTE DIAMETER (MM)

Figure 5. Polyacrylamide gel electrophoresis (5%) of oocyte protein indicating de novo appearance of yolk protein in vitellogenic oocytes of the winter flounder obtained during early ovarian recrudescence. Crude ovarian extracts (lane a), fixed ovarian extract (Lane b), opaque oocytes >150 μm diameter (lane c), previtellogenic oocytes (lane d) and vitellogenin as standard (lane e).

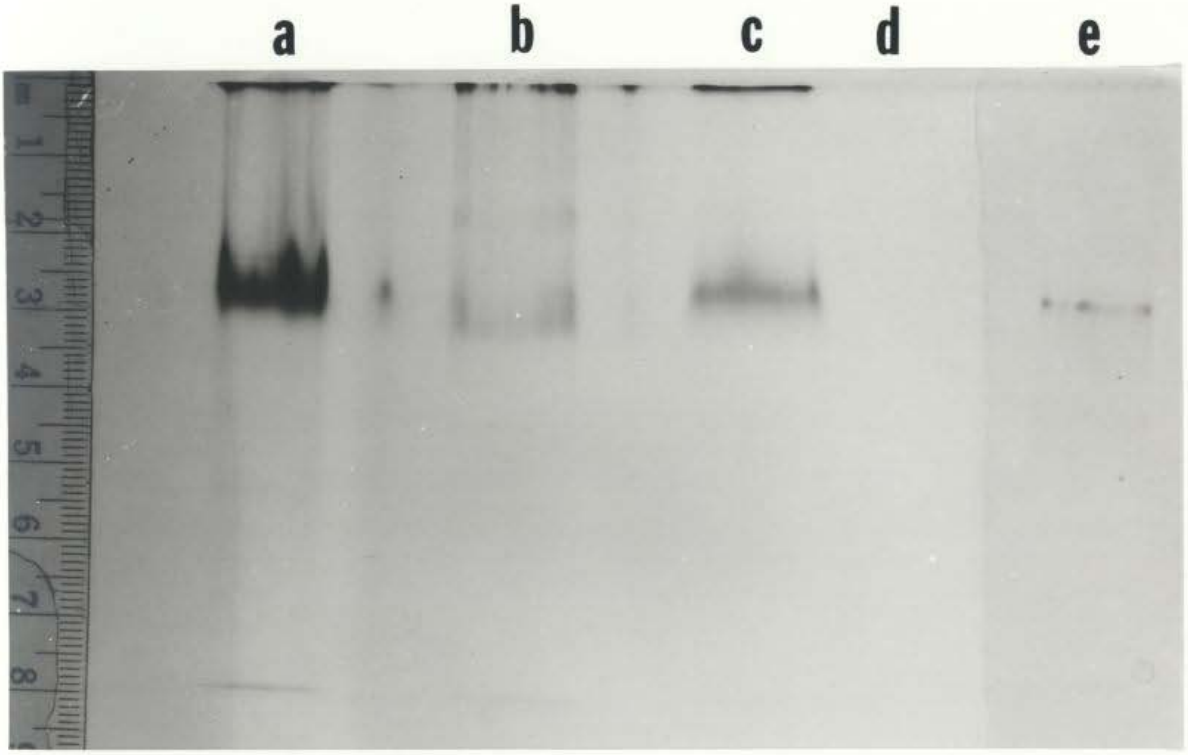
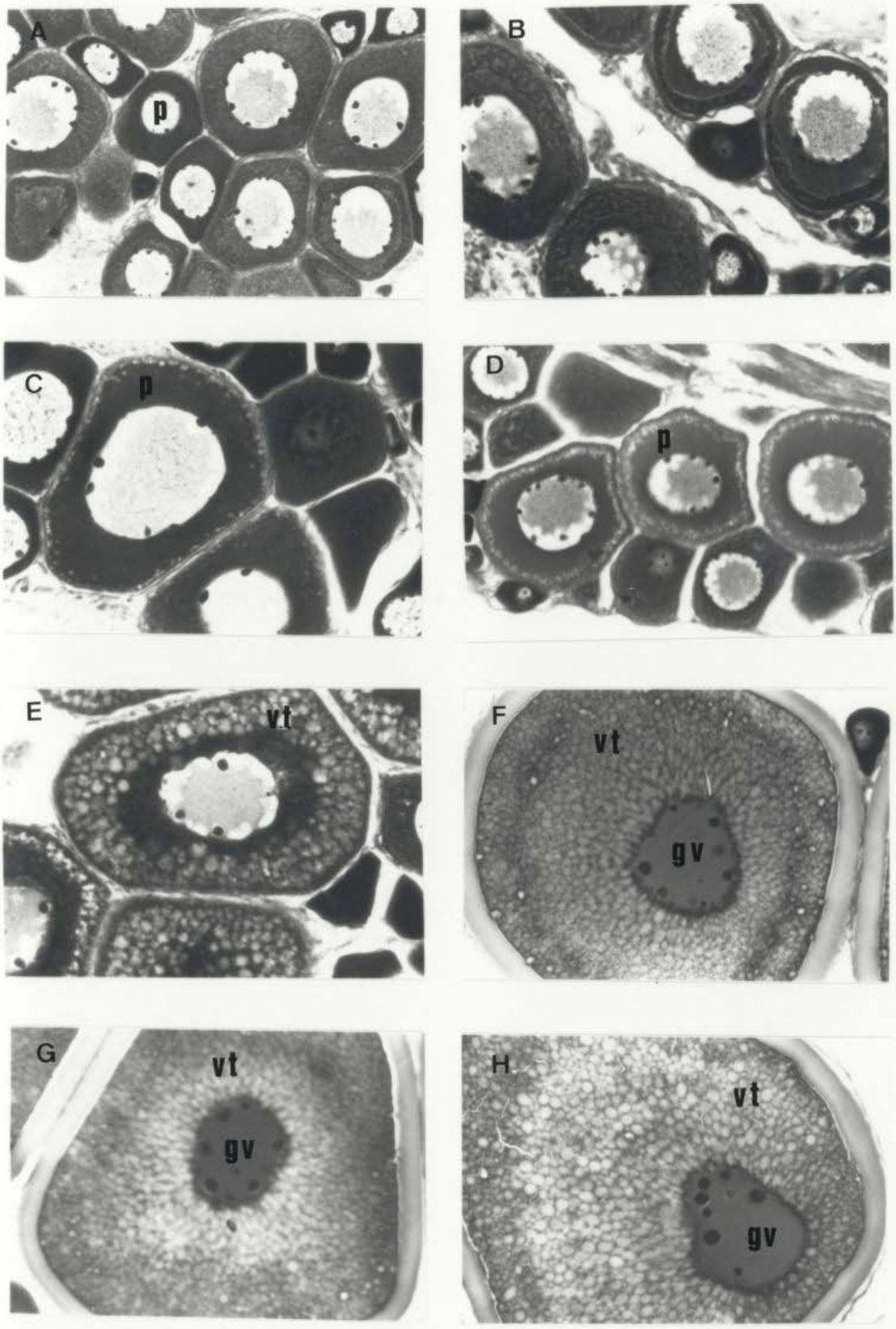

a
b
c
d
e

Figure 6. Histological sections of the winter flounder ovary showing the various types of oocytes.
A. Previtellogenic 1 oocyte (x 25); B. Previtellogenic 1 oocyte (x 40); C. Previtellogenic 2 oocyte (x 40); D. Previtellogenic 2 oocyte (x 25); E. Vitellogenic 2 oocyte (x 40); F,G. Vitellogenic 4 oocyte with centrally located nucleus (x 40); H. Vitellogenic 4 with migrating germinal vesicle (x 40).
p=previtellogenic, vt=vitellogenic, gv=germinal vesicle.


A
p
B
C
p
D
p
E
vt
F
vt
gv
G
vt
gv
H
vt
gv

oocytes continued growing although less rapidly compared to the early stages of ovarian development. Indeed, oocyte size classes became more uniform, an indication of a more synchronous growth of oocytes. Histological sections of these oocytes again showed that an abundance of yolk granules filled the oocyte cytoplasm during this period.

In prespawning females (March-April) the gonadosomatic index (Fig. 2A) ranged from 15-20 and the additional increases in oocyte size (mode 600 μm, Fig. 4) noted were likely associated with oocyte hydration. At the histological level, all oocytes examined were at vitellogenic 4 (VIT 4) stage (Fig. 6). In prespawning females oocytes contained a germinal vesicle in the central position (see chapter 2, section 2.3.2) indicating that the time of spawning remained some distance away; abundant small non-vitellogenic oocytes could also be found within the current clutch of vitellogenic oocytes. Later, during the prespawning phase, the initiation of germinal vesicle migration in vitellogenic oocytes was evident as reflected in germinal vesicle stages 1-3 (see chapter 2, section 2.3.2). As the spawning period approached (May/June), the gonadosomatic index and mean oocyte diameter of female flounder reached 25 and 600 μm, respectively (Figs. 2A, 4), while the germinal vesicle migrated to the periphery of oocytes.

In post-spawned females, the gonadosomatic index fell

to its minimum seasonal value (<3, Fig. 2A) and the ovaries were populated only by clear previtellogenic oocytes when observed macroscopically (mean oocyte diameter 100 μm, Fig. 4). Histological sections of ovaries during this period demonstrated the presence of oocytes containing the Balbiani body and cortical alveoli in the periphery of the cytoplasm of previtellogenic 2 (PREVIT 2) oocytes (Fig. 6).

A rise in the ovarian dry matter, which is a measure of the accumulation of yolk protein elements in oocytes, accounted for the increasing size and consequent increase in mass of the growing oocyte. During PREP 1 (September-December), both dry matter and hepatosomatic index increased very rapidly reaching their peak in January and November, respectively (Fig. 7A,B). In the PREP 2 phase both these parameters showed a declining trend although not significant statistically; finally, hepatosomatic index reached its basal values during the prespawning period (April). Percent dry matter (15%) of freshly ovulated eggs was about half of that post-vitellogenic oocytes. A positive correlation between percent dry matter and gonadosomatic index ($r=0.66$, $P< 0.001$, Fig. 8) and oocyte diameter ($r=0.84$, $P< 0.001$, Fig. 9) was found.

Seasonal patterns for the plasma sex steroids, estradiol-17ß and testosterone, in wild females are presented in Figure 10 (A,B). Initially in the fall and

Figure 7. Seasonal variations of (A) oocyte dry matter (DM) and (B) hepatosomatic index (HSI) in wild female winter flounder from September 1987 through December 1988. Symbols represent means and vertical lines ± SEM obtained from 4-6 fish per sampling period. SEMs too small for scale not shown. Asterisks indicate mean is significantly ($P<0.05$) different from previous sample. Dry matter was not determined in June and July (Fig. 7A). The phases of the reproductive cycle in female winter flounder are based on seasonal profiles of the gonadosomatic index, oocyte diameter, plasma estradiol-17β and testosterone. PREP 1, preparatory 1 phase; PREP 2, preparatory 2 phase; PRES, prespawning phase; SPW, spawning phase; POST, regressed period.

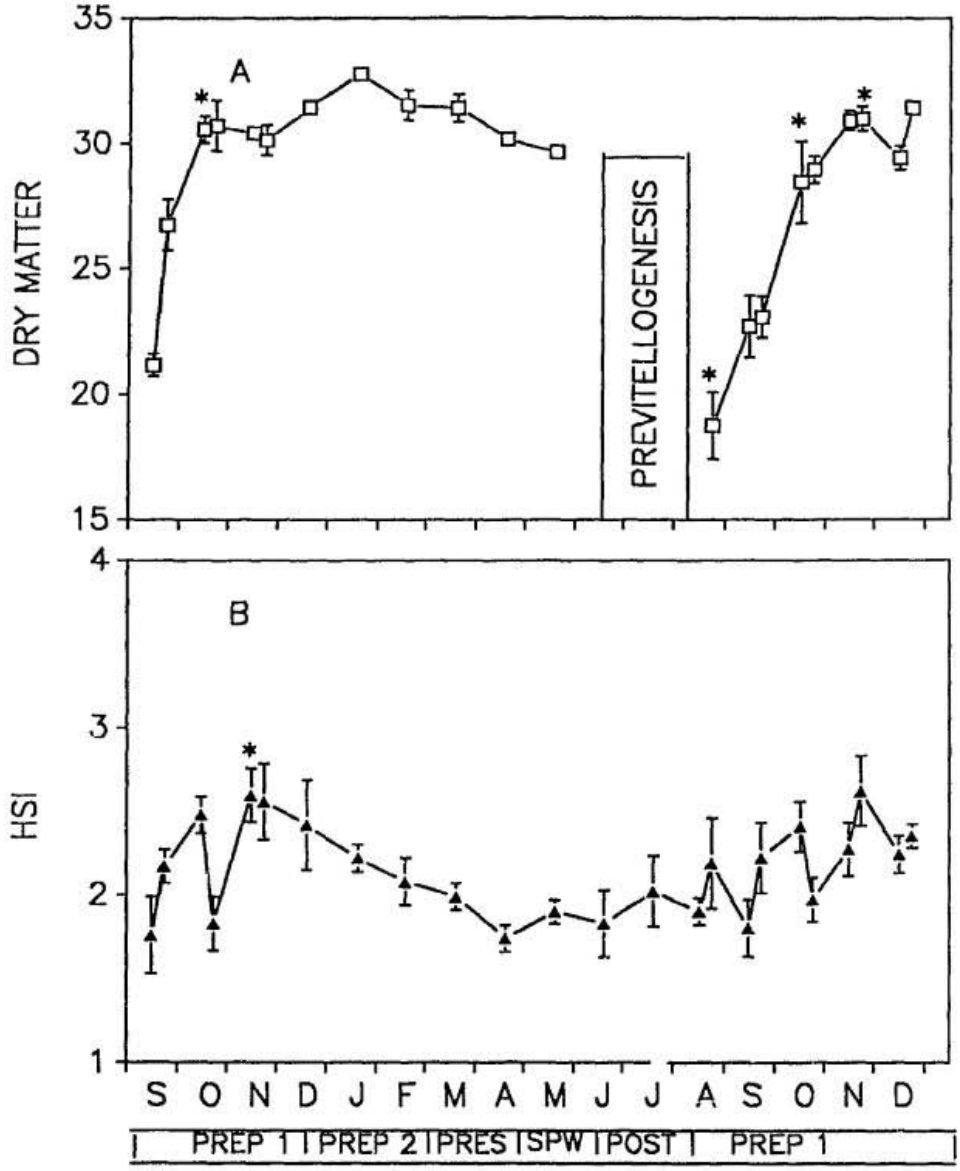

35
30
25
20
15
DRY MATTER
A
PREVITELLOGENESIS
4
3
2
1
HSI
B
S O N D J F M A M J J A S O N D
PREP 1 | PREP 2 | PRES | SPW | POST | PREP 1

Figure 8. Scatter-plot of gonadosomatic index (GSI) and oocyte dry matter of wild female winter flounder collected from Conception Bay from September 1987 through December 1988. Each point represents individual fish.

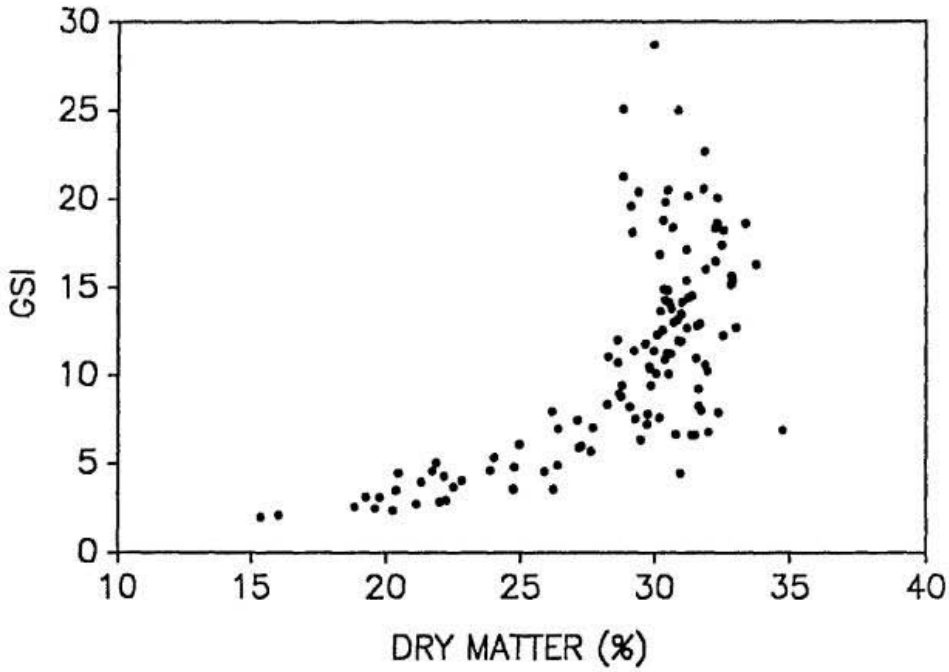

30
25
20
15
10
5
0
GSI
10
15
20
25
30
35
40
DRY MATTER (%)

Figure 9. Scatter-plot of oocyte diameter and oocyte dry matter of wild female winter flounder collected from Conception Bay from September 1987 through December 1988. Each point represents individual fish.

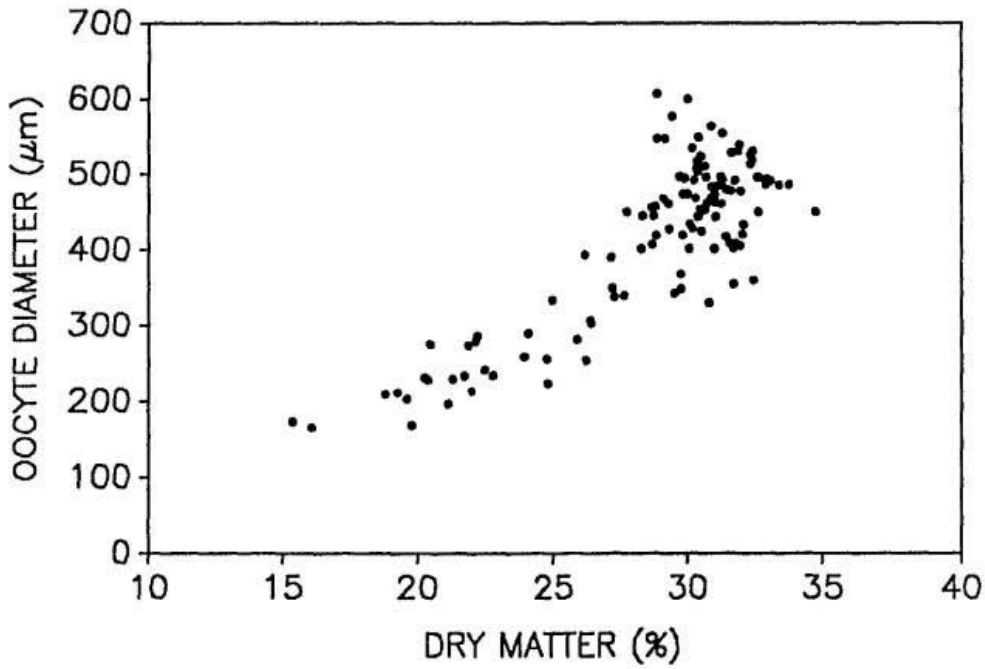
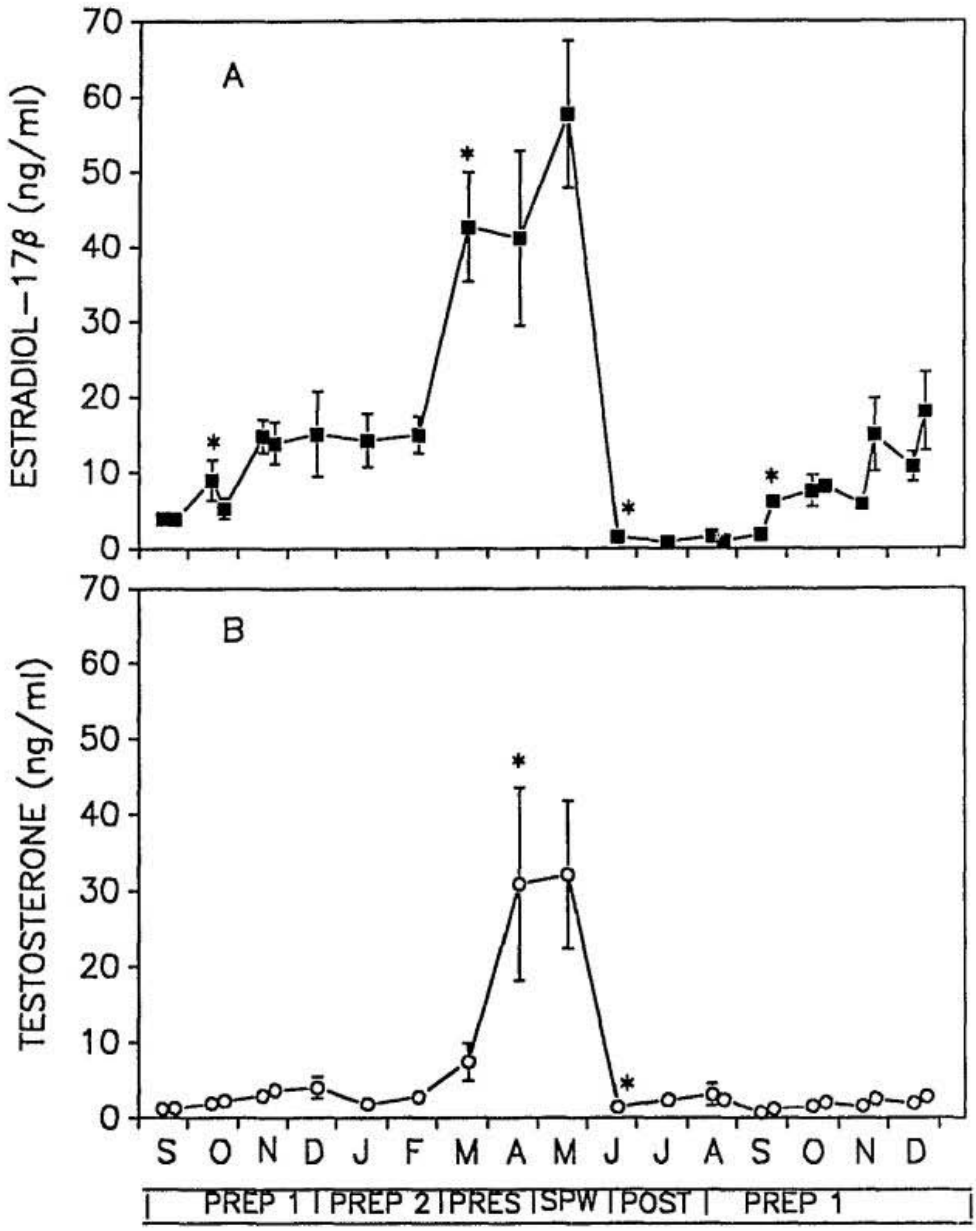

OOCYTE DIAMETER (μm)
700
600
500
400
300
200
100
0
10
15
20
25
30
35
40
DRY MATTER (%)

Figure 10. Seasonal variations in plasma (A) estradiol-17β and (B) testosterone in wild female winter flounder from September 1987 through December 1988. Symbols represent means and vertical lines ± SEM obtained from 4-6 fish per sampling period. SEMs too small for scale not shown. Asterisks indicate mean is significantly ($P<0.05$) different from previous sample. The phases of the reproductive cycle in female winter flounder are based on seasonal profiles of the gonadosomatic index, oocyte diameter, plasma estradiol-17β and testosterone. PREP 1, preparatory 1 phase; PREP 2, preparatory 2 phase; PRES, prespawning phase; SPW, spawning phase; POST, regressed period.


A
B
ESTRADIOL-17β (ng/ml)
TESTOSTERONE (ng/ml)
70
60
50
40
30
20
10
0
S O N D J F M A M J J A S O N D
PREP 1 | PREP 2 | PRES | SPW | POST | PREP 1

winter, while plasma testosterone values remained relatively low and unchanged, an early seasonal increase in plasma estradiol-17ß plateauing at about 15 ng/ml was noted clearly associated with rapid ovarian recrudescence (PREP 1). Further increases in estradiol-17ß combined with a rise in plasma testosterone, achieving peak values of >40 and 30 ng/ml, respectively, were recorded during the prespawning period from March-May (PRES). In contrast to the highest steroid hormone values reached in May, plasma sex steroids fell precipitously during the spawning period in May/June. In females with regressed gonads in June and July, estradiol-17ß and testosterone reached and were maintained at their minimum seasonal values. Plasma estradiol-17ß ($r=0.68$, $P<0.001$), testosterone ($r=0.49$, $P<0.001$) and gonadosomatic index were positively correlated throughout the reproductive cycle (Fig. 11).

3.3.3 Captive females

Only previtellogenic oocytes were observed in the ovaries of post-spawned wild females; however for post-spawned female flounder retained in the laboratory seasonal gonad growth proceeded and eventually ovulation occurred in seven of eight surviving females. Upon autopsy, the gonadosomatic index was observed to be 24 in the only unovulated female and oocytes contained germinal vesicles in the peripheral position. Ovulation was first

Figure 11. Scatter-plot of plasma (A) estradiol-17β, (B) testosterone and gonadosomatic index (GSI) of wild female winter flounder from September 1987 through December 1988. Each point represents an individual fish.

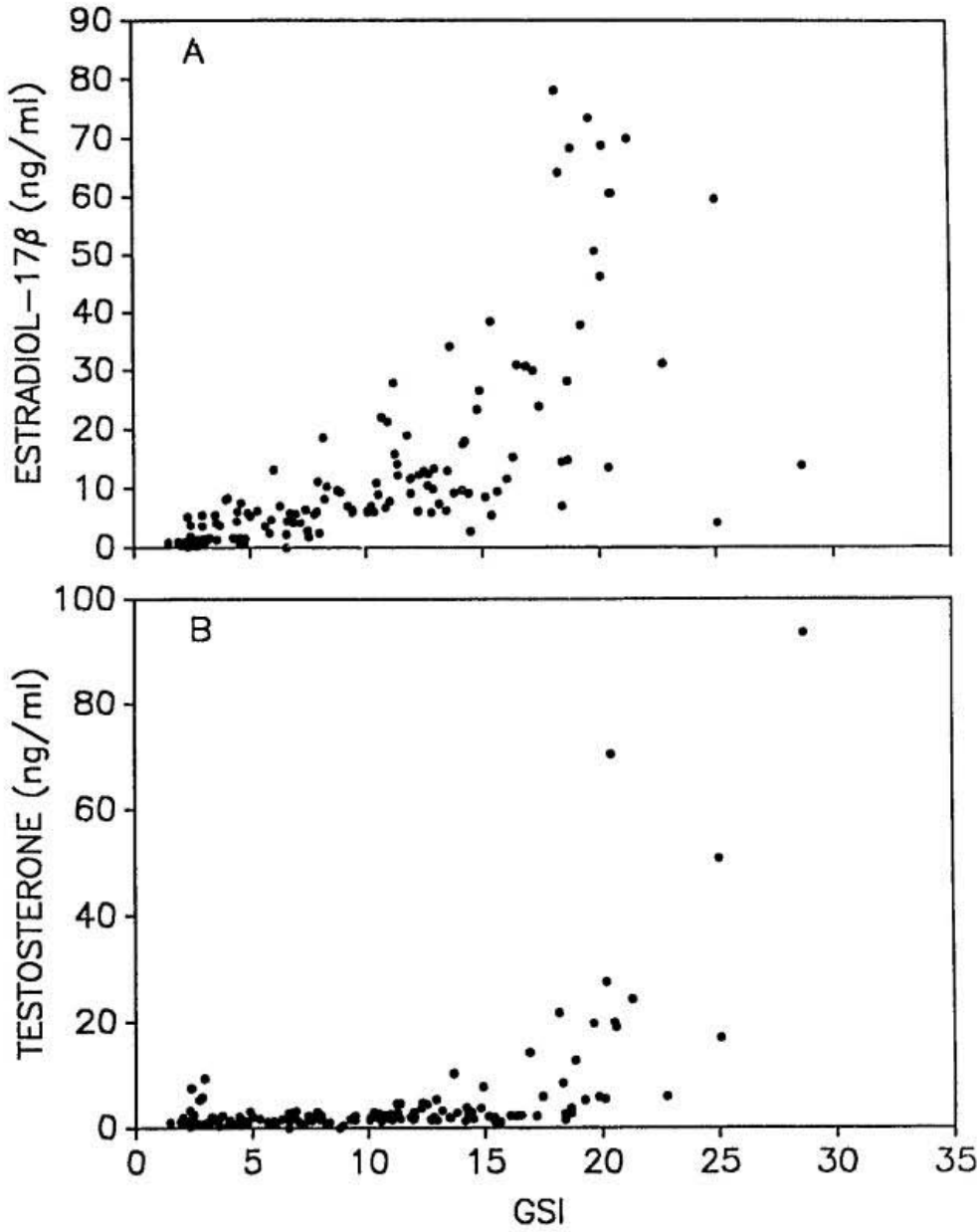

A
ESTRADIOL–17β (ng/ml)
90
80
70
60
50
40
30
20
10
0
B
TESTOSTERONE (ng/ml)
100
80
60
40
20
0
0
5
10
15
20
25
30
35
GSI

Table 1

Date of ovulation and egg fertilization rates of captive female winter flounder maintained in the laboratory

Fish #	Date of Ovulation (1989)	Fertilization Rate (%)
B70	June 16	95.9 ± 0.6
B71	May 31	91.5 ± 0.9
B72	June 28	ND
B73	June 22	91.6 ± 1.0
B74	June 12	ND
B78	June 22	ND
B79	June 22	95.8 ± 0.5

ND: Not determined, values are means ± SEM.

detected in the group of laboratory-reared females on May 31, 1989 (Table 1); all other females ovulated in June. Eggs obtained from captive females appeared normal and high rates of egg fertilization were observed (Table 1).

Serial blood sampling of captive females began in November when plasma estradiol-17ß and testosterone levels were 15 and 10 ng/ml, respectively (Fig. 12A,B). While testosterone levels initially remained steady, plasma estradiol-17ß levels in captive female flounder began to rise; by April, both estradiol-17ß and testosterone attained significantly higher values of 40 and 25 ng/ml, respectively. From May-July, plasma estradiol-17ß and testosterone both began gradually declining (significantly reduced by June). In the post-spawned females in July, plasma levels of estradiol-17ß and testosterone were both very low.

3.3.4 Wild Males

Based on gonadosomatic index (Fig. 13), the seasonal pattern of the reproductive cycle in male flounder mimicked that of females with the exception that males matured earlier as shown by the early peak in the gonadosomatic index. For males the annual reproductive cycle also may be divided into five distinct seasonal phases; first, during an initial preparatory period (PREP 1, August-December), the gonadosomatic index increased

Figure 12. Seasonal variations in plasma (A) estradiol-17β and (B) testosterone in captive female winter flounder. Symbols represent means and vertical lines ± SEM obtained from 4-11 fish per sampling period. SEMs too small for scale not shown. Asterisks indicate mean is significantly ($P<0.05$) different from sample in December.

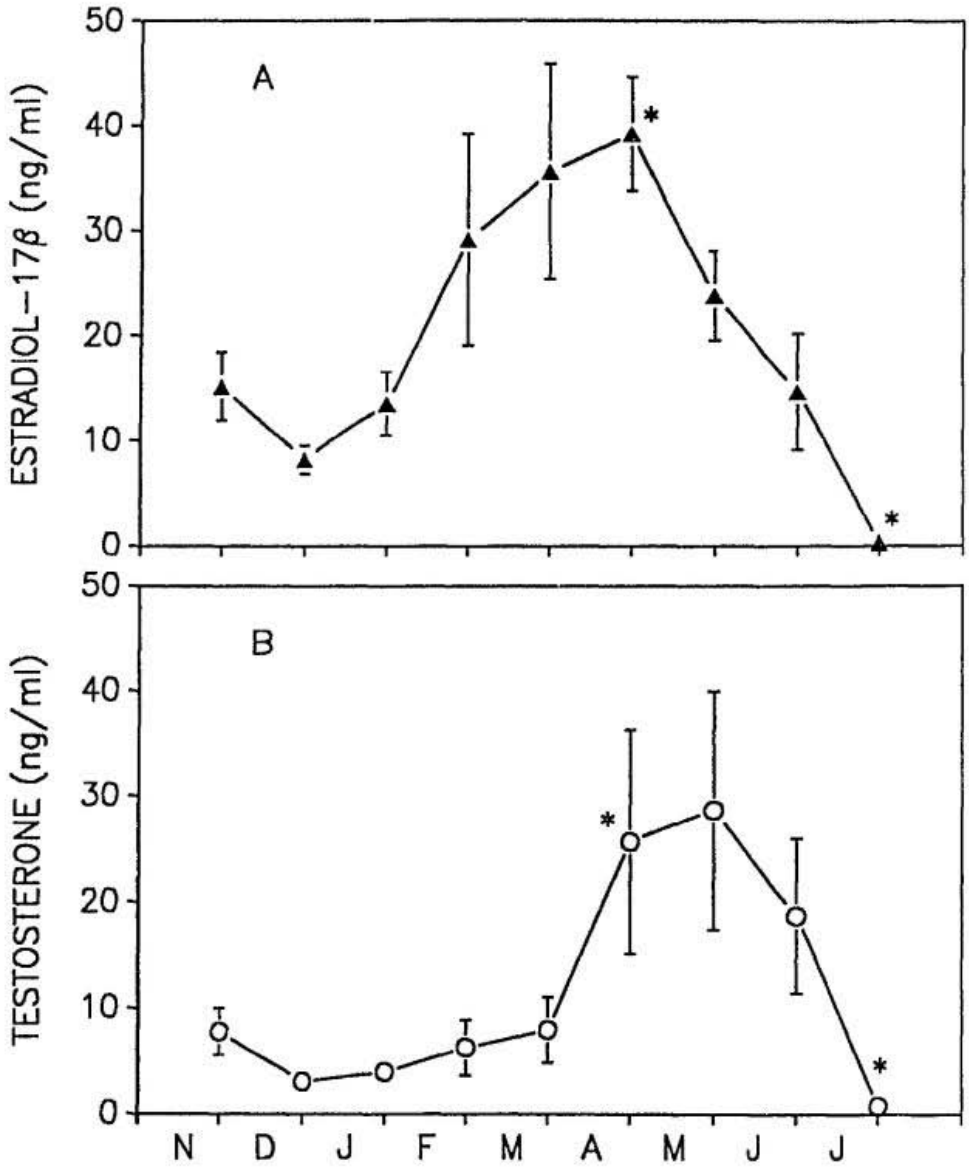
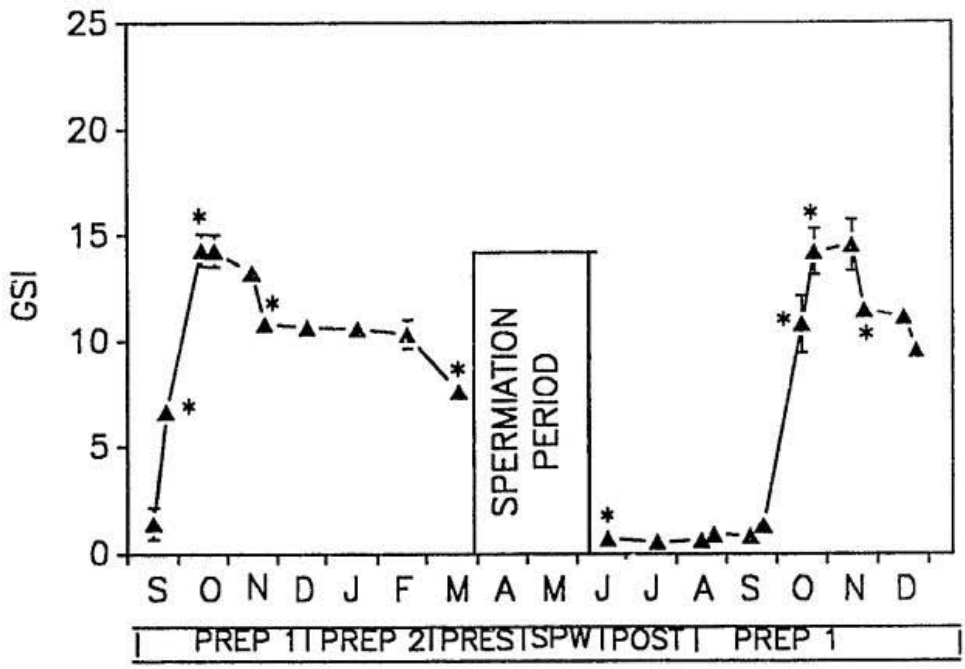

A
B
ESTRADIOL-17β (ng/ml)
TESTOSTERONE (ng/ml)
50
40
30
20
10
0
N
D
J
F
M
A
M
J
J
*

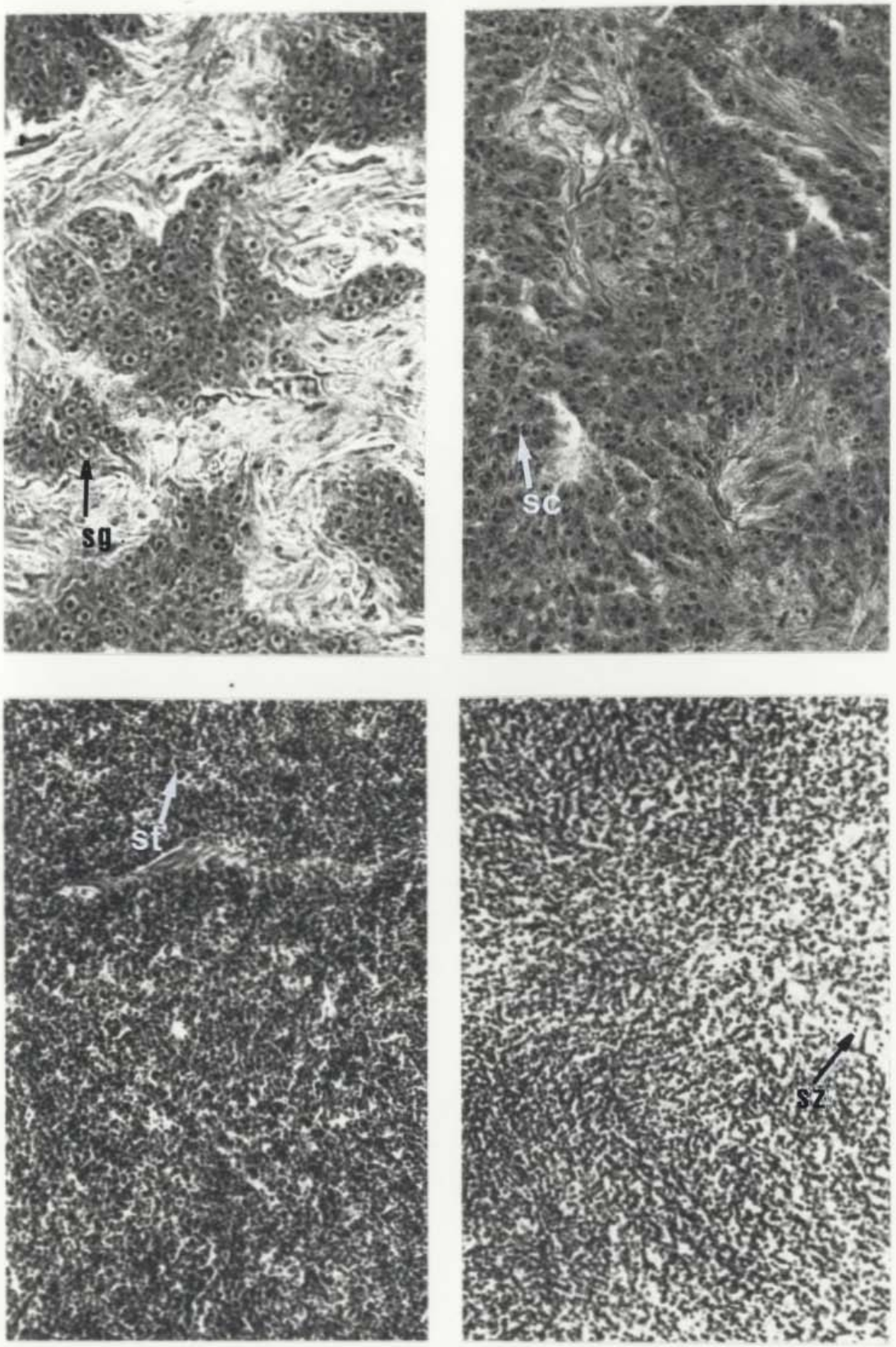
Figure 13. Seasonal variations in gonadosomatic index (GSI) in wild male winter flounder from September 1987 through December 1988. Symbols represent means and vertical lines ± SEM taken from 4-6 fish per sampling period. SEMs too small for scale not shown. Asterisks indicate mean is significantly ($P<0.05$) different from previous sample. Spermiation period indicates that at least some spermiating males are available during the months of March-May. The phases of the reproductive cycle in male winter flounder are based on seasonal profiles of the gonadosomatic index, plasma testosterone and 11-ketotestosterone. PREP 1, preparatory 1 phase; PREP 2, preparatory 2 phase; PRES, prespawning phase; SPW, spawning phase; POST, regressed period.


25
20
15
10
5
0
GSI
SPERMIATION PERIOD
S O N D J F M A M J J A S O N D
PREP 1
PREP 2
PRES
SPW
POST
PREP 1

very rapidly in September from 1 to 15 in October/November before declining to 10 in December. The second preparatory period (PREP 2) represents a period of testicular maintenance in prespermiating males during the winter months (December-February) when few spermiating males were available. The next phase was a prespawning period (PRES, March-April) when the gonadosomatic index values declined and spermiation was detectable in only some individual fish. During May/June, the spawning period (SPW), most males were fully ripe producing relatively large amounts of milt while during June/August, the post-spawned (POST) phase, the testes were regressed (gonadosomatic index <1) and males remained reproductively quiescent.

During active testicular recrudescence (PREP 1) in fall, the gonads grew rapidly to a gonadosomatic index of 15 before declining to 10 in November. At this time the well-developed testes had changed from grey to a uniform creamy white colour retaining this appearance throughout the winter maintenance period. Histological sections in the period of early testicular development showed that the most abundant cell types observed were spermatocytes I and II although some spermatogonia could still be found within the lobules (Fig. 14). By the time the gonadosomatic index in males reached 15, spermatids were the most advanced cell type present, although spermatocytes II were still the dominant cell type. When the gonadosomatic index of

Figure 14. Histological sections of testis from winter flounder showing different stages of reproductive cycle. A. Post-spawned phase (July), testis contain spermatogonia (x 40); B. Preparatory phase 1 (September), testis containing mostly spermatocytes (x 40); C. Preparatory phase 1 (October), testis containing spermatids (x 40); D. Preparatory phase 1 (December) testis containing spermatids and spermatozoa (x 40). sg, spermatogonia; sc, spermatocyte; st, spermatid; sz, spermatozoa.


sg
sc
st
sz

males declined to approximately 10 in the later stages of the PREP 1 phase, the testes contained mostly spermatids and spermatozoa. Spermatozoa were found either in the middle of lobules or close to the ducts. Throughout the winter months (PREP 2) the gonadosomatic index remained elevated (10). Upon approaching the spawning period the gonadosomatic index (8) began to decline significantly ($P<0.05$) and spermiation could be detected in about half of the males.

During prespawning phase (PRES), the white testes contained an abundance of spermatids and spermatozoa. In some males milt could be collected at this time and the spermatocrit (91%) values were significantly ($P<0.05$) higher compared to milt collected from males in the spawning period. Still, milt volume and the total number of collectable spermatozoa were low for prespawning males (Table 2).

During the spawning (SPW) phase all adult males collected from the field were in spermiating condition. While spermatozoa concentrations in the milt were lower in males during spawning phase, milt volume and the total number of spermatozoa collected were significantly ($P<0.05$) increased.

In postspawned male flounder, the grey or black regressed testes were very small (gonadosomatic index <1). Occasionally, the vas deferens contained some residual

Table 2

Monthly values of spermatocrit, milt volume and total sperm collected from spermiating wild male winter flounder

Month	Spermatocrit (%)	Milt vol(ml)	Total Sperm (x 10^9)
March	91.0 ± 2.0[b] (3)	ND	ND
April	84.5 ± 2.0[b] (6)	1.2[a] ± 0.5	21.0 ± 9.8[a]
May	66.5 ± 3.6[a] (6)	3.7[b] ± 0.9	52.2 ± 11.7[b]

Values are mean ± SEM (n), significance ($P<0.05$) indicated by different letters. ND - not determined.

liquid, perhaps milt remaining from the immediately previous spawning period. Spermatogonia A and B were the most prevalent cell types populating the testes of post-spawned males.

Seasonal variations in hepatosomatic index in male flounder are shown in Figure 15. Early in the PREP 1 period, hepatosomatic index (1.8) peaked (August) and then declined significantly. In the winter, mean hepatosomatic index (0.8) remained low; however, a gradual rise occurred during the summer reflecting reconditioning of males during the feeding period.

In post-spawned males (June-early August) plasma testosterone and 11-ketotestosterone were non-detectable. During the late summer (late August) both plasma testosterone and 11-ketotestosterone rose to detectable levels, 2 ng/ml and 7 ng/ml, respectively and by fall these hormones had increased significantly ($P<0.05$) to 5 ng/ml and 20 ng/ml, respectively (Fig. 16). The plasma sex steroids rose in parallel with the increases in gonadosomatic index during the period of spermatogenesis. Compared to 11-ketotestosterone ($r=0.39$, $P<0.001$), plasma testosterone ($r=0.49$, $P<0.001$) appeared more strongly correlated to gonadosomatic index throughout the reproductive cycle (Fig. 17A,B). During the Winter (January-February) the levels of both plasma testosterone and 11-ketotestosterone remained elevated, but further

Figure 15. Seasonal variations in hepatosomatic index (HSI) in wild male winter flounder from September 1987 through December 1988. Symbols represent means and vertical lines ± SEM obtained from 4-6 fish per sampling period. SEMs too small for scale not shown. Asterisks indicate mean is significantly ($P<0.05$) different from previous sample. The phases of the reproductive cycle in male winter flounder are based on seasonal profiles of the gonadosomatic index, plasma testosterone and 11-ketotestosterone. PREP 1, preparatory 1 phase; PREP 2, preparatory 2 phase; PRES, prespawning phase; SPW, spawning phase; POST, regressed period.

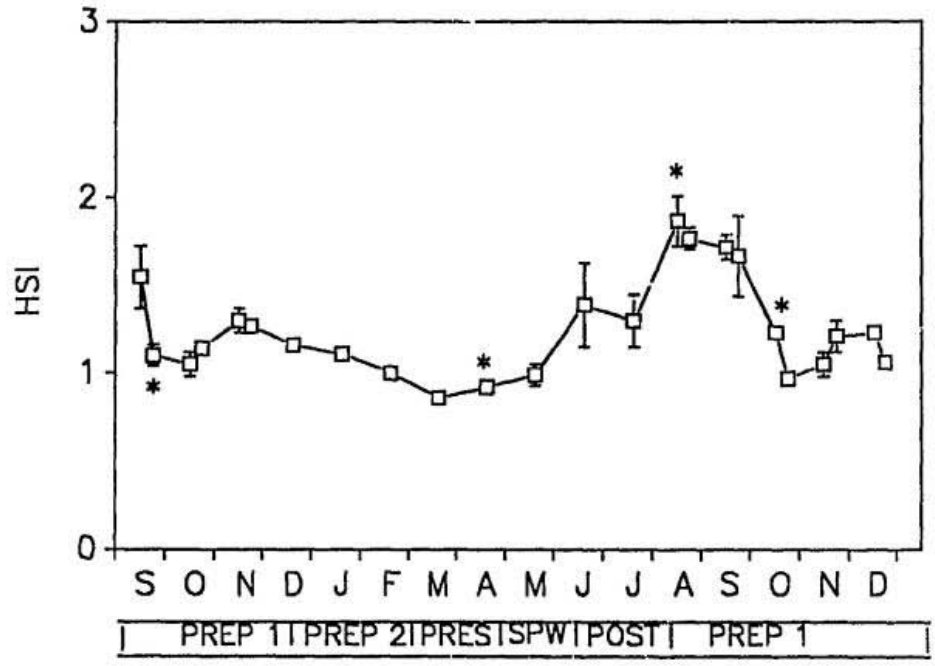

3
2
1
0
HSI
S O N D J F M A M J J A S O N D
PREP 1 I PREP 2 I PRES I SPW I POST I PREP 1

Figure 16. Seasonal variations in plasma (A) testosterone and (B) 11-ketotestosterone in wild male winter flounder from September, 1987 through December, 1988. Symbols represent means and vertical lines ± SEM obtained from 4-6 fish per sampling period. SEMs too small for scale not shown. Asterisks indicate mean is significantly ($P<0.05$) different from previous sample. The phases of the reproductive cycle in male winter flounder are based on seasonal profiles of the gonadosomatic index, plasma testosterone and 11-ketotestosterone. PREP 1, preparatory 1 phase; PREP 2, preparatory 2 phase; PRES, prespawning phase; SPW, spawning phase; POST, regressed period.

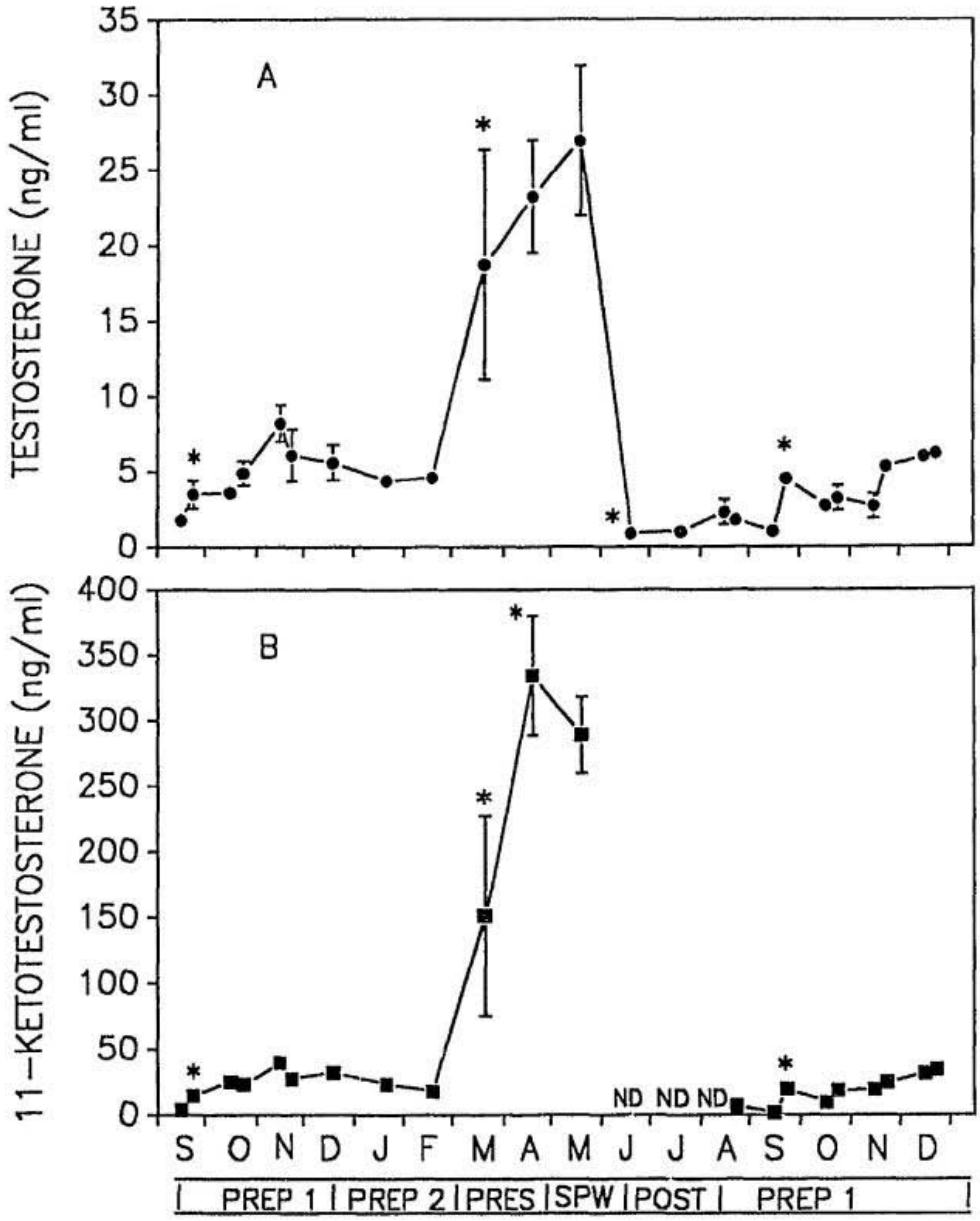
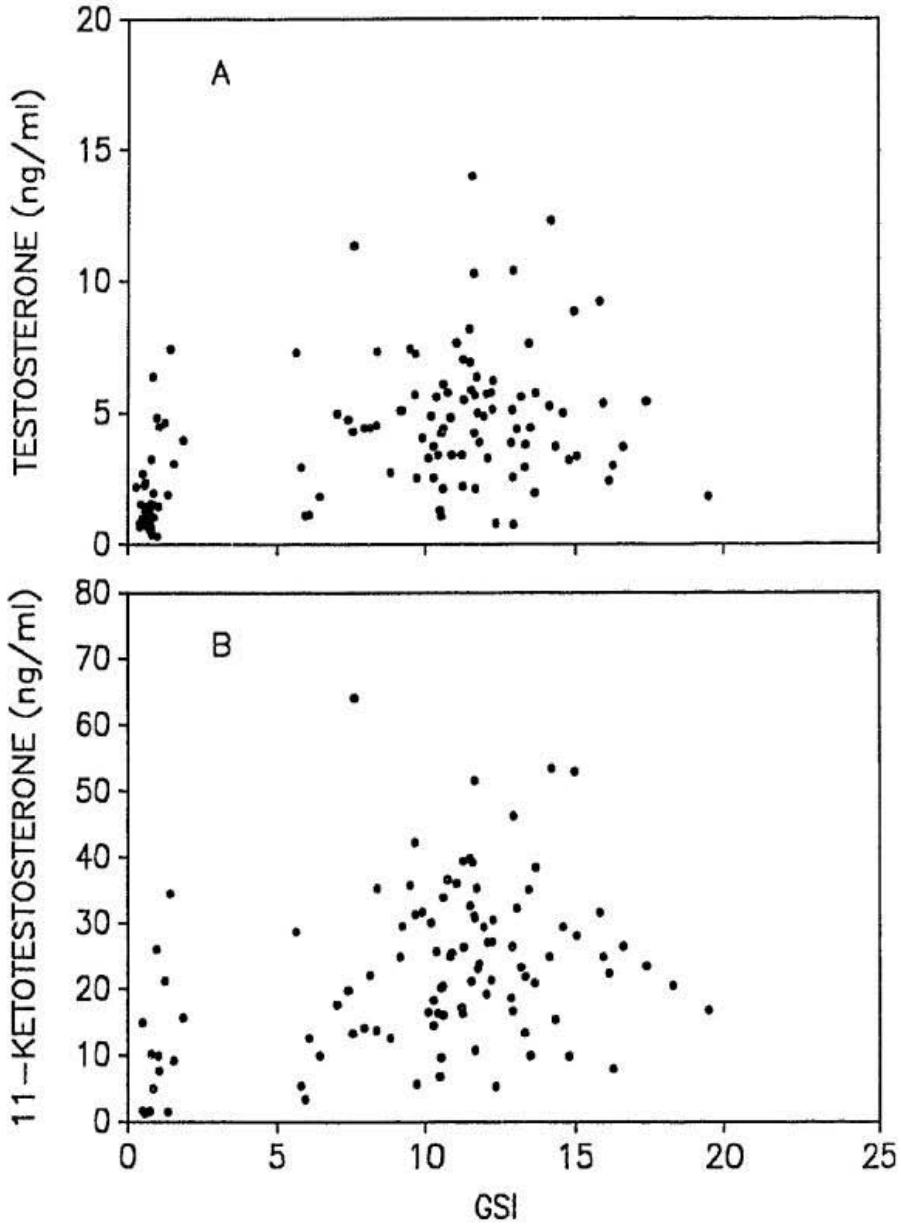
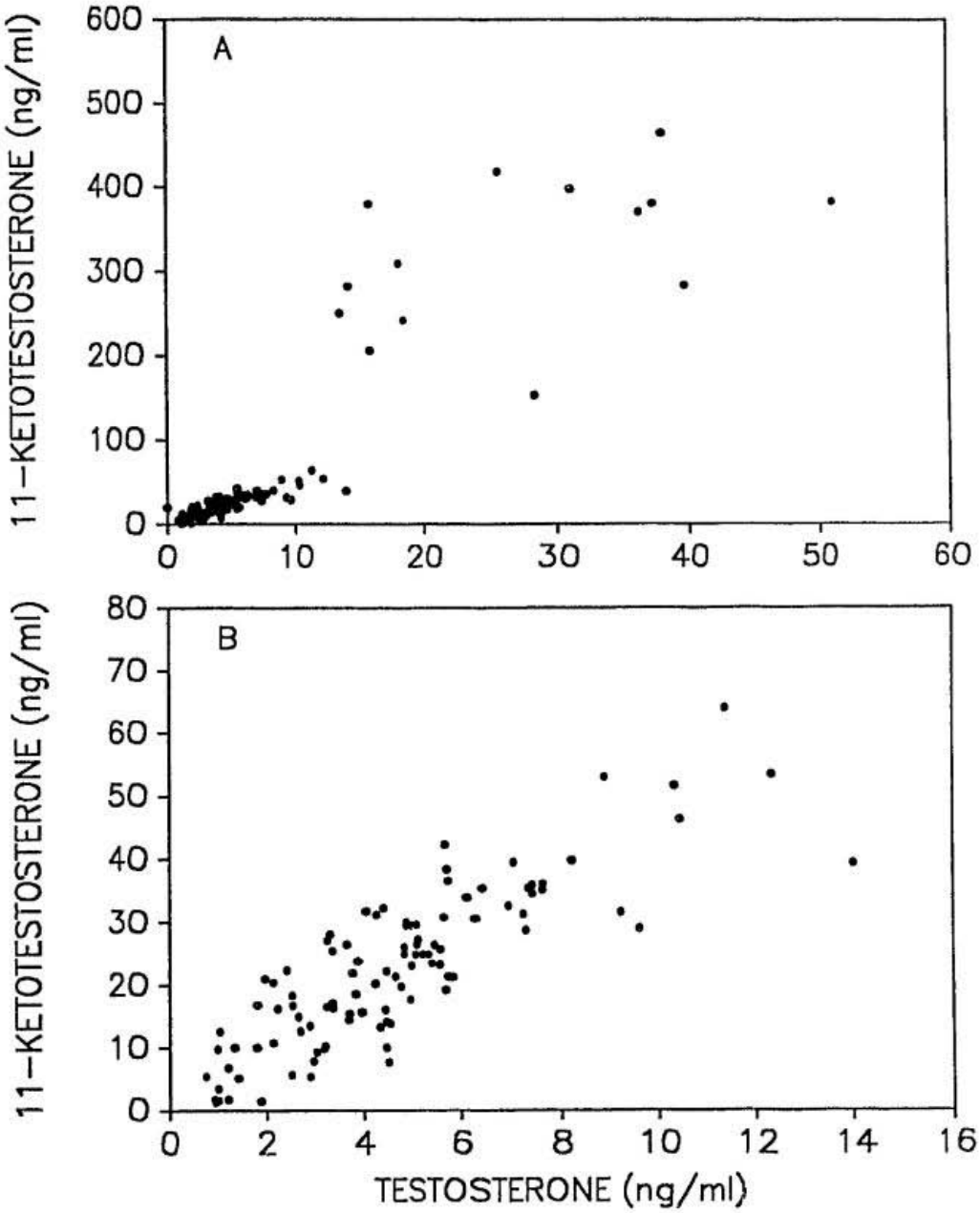

A
B
TESTOSTERONE (ng/ml)
11-KETOTESTOSTERONE (ng/ml)
35
30
25
20
15
10
5
0
400
350
300
250
200
150
100
50
0
ND ND ND
S O N D J F M A M J J A S O N D
PREP 1 | PREP 2 | PRES | SPW | POST | PREP 1

Figure 17. Scatter-plot of plasma (A) testosterone, (B) 11-ketotestosterone and gonadosomatic index (GSI) of wild male winter flounder collected from Conception Bay beginning September 1987 through December 1988. Each point represents an individual fish.


A
TESTOSTERONE (ng/ml)
20
15
10
5
0
B
11-KETOTESTOSTERONE (ng/ml)
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
GSI

increases in both androgens occurred during the prespawning period. In post-spawned males plasma testosterone and 11-ketotestosterone declined to a seasonal low of 0.8 ng/ml and non-detectable values, respectively. Plasma testosterone and 11-ketotestosterone were strongly correlated throughout the annual reproductive cycle ($r=0.89$, $P<0.001$, Fig. 18A) and also during the period of spermatogenesis ($r=0.85$, $P<0.001$, Fig. 18B).

Figure 18. Scatter-plot of plasma 11-ketotestosterone and testosterone of wild male winter flounder from September 1987 through December 1988. Each point represents an individual fish. (A) relationship during entire annual cycle, (B) relationship only during spermatogenesis.


A
11-KETOTESTOSTERONE (ng/ml)
600
500
400
300
200
100
0
0
10
20
30
40
50
60
B
11-KETOTESTOSTERONE (ng/ml)
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
TESTOSTERONE (ng/ml)

3.4 DISCUSSION

The reproductive cycle of male and female winter flounder is characterized by distinct seasonal variations in gonadosomatic index and plasma steroid sex hormones associated with reproductive activity. The seasonal reproductive cycle begins with a period of intense gonadal recrudescence from August-December (PREP 1), followed by more gradual growth and maintenance of the gonads (PREP 2) during the winter from January-March. Subsequently, the prespawning period (PRES) is demarcated (March-May) by a further increase in gonadal weight in females while the relative weight of gonads in males slowly declines. The reproductive phase ends after a short spawning period (SPW) which lasts approximately one month (May/June). Finally, a period of presumed reproductive quiescence (POST) is observed in post-spawned flounder during the summer months of June/July; in this period the gonads remain in sexually regressed condition.

Oocyte Development

The process of ovarian recrudescence in teleosts has been recently reviewed (Wallace and Selman, 1981; Wallace et al., 1987). Generally, teleost oocytes undergo a series of developmental stages beginning with oogonial proliferation, a primary growth phase (previtellogenesis),

the secondary growth phase (vitellogenesis), and final egg maturation and ovulation. In the present study the dynamics of vitellogenic oocyte development was followed through the complete flounder reproductive cycle. Initiation of vitellogenesis occurred in August in winter flounder about the time oocyte size reached 150 μm. This finding was established by various means, namely, by noting the shift towards larger egg sizes in the oocyte size-class frequency distribution (see Fig. 4), demonstrating the onset of yolk-related protein deposition into growing oocytes by gel electrophoresis (see Fig. 5) and finally recording the appearance of yolk globules in histological sections of the ovary (see Fig. 6). The critical oocyte size (150 μm diameter, see Fig. 4) observed in the present study for recruitment into vitellogenesis is in agreement with the histological analyses of oocytes by Dunn (1970) and Burton and Idler (1984). During the active gonadal recrudescent period (PREP 1), the ovaries contain a relatively wide range of oocyte-size classes (see Fig. 4) while later during the slower phase of ovary growth (PREP 2) and prespawning phases, the range variation of oocyte-size classes narrows. Indeed, Tyler et al. (1990) have shown that in the rainbow trout, during early part of vitellogenesis the volume of maturing oocytes within an ovary varied as much as 250 fold; however, at ovulation egg sizes were much

more uniform i.e. less than 2 fold variation in volume. Histological examination of these flounder ovaries shows that they contain oocytes in various stages of development (VIT 1,2,3 or 4) differing in the extent of yolk accumulation and this may contribute to the range in oocyte size classes. When females reach the prespawning stage, gonadosomatic index and oocyte diameter further increase, reaching their maximum during the SPW phase in May/June. In rainbow trout it was established that the increase in ovary weight during vitellogenesis was due to an increase in the size of oocytes rather than the recruitment of more oocytes into development (Tyler et al., 1990).

The annual cyclic pattern of oocyte development and the mode of oocyte recruitment in teleosts can be determined from a histological examination of the ovaries or by plotting successive oocyte size-frequency distributions (see review of De Vlaming, 1983). Analyses of such oocyte size-frequency distributions have revealed three basic patterns of ovary development among teleosts i.e. synchronous, group-synchronous and asynchronous ovaries (see review of Wallace et al., 1987). Previous histological studies of flounder oocytes indicated that at all times of the year at least two populations or year classes of oocytes were present (Dunn and Tyler, 1969; Dunn, 1970; Burton and Idler, 1984). These oocyte classes

consist of a population of small previtellogenic oocytes which develop over a year, and a second population of larger vitellogenic oocytes which are recruited from the previous year's previtellogenic oocyte population. The present oocyte size-frequency distribution analysis in winter flounder indicates that the flounder ovary exhibits just a single mode of progressively developing vitellogenic oocytes. This supports the concept of synchronous oocyte development in winter flounder ovaries. The synchronous type of oocyte development is by far the most common reproductive strategy found among teleosts (see reviews of Wallace et al., 1987; De Vlaming, 1983). Furthermore, the winter flounder can be further designated as a single spawner since all oocytes appear to mature and are spawned synchronously within a short period of time. Some teleosts may contain group-synchronous ovaries and are thought to be fractional spawners i.e. when two or more distinct vitellogenic oocyte classes are observed in ovaries. The latter type of oocyte development has been described in European bass (Mayer et al., 1990), minnows (Heins and Rabito, 1986), Perch (Gale and Deutch, 1985), and mackerel (Asano and Tanaka, 1989). This study shows the presence of previtellogenic oocytes in the flounder ovaries throughout the year (data not shown); these oocytes were the most abundant cell types from June through September. Similar seasonal distributions of

previtellogenic oocytes present throughout the year, with peak abundances in post-ovulatory fish, have been reported in plaice (Barr, 1963), snapper (Crossland, 1977), dab (Htun-han, 1978), goldfish (Khoo, 1979), yellowtail flounder (Howell, 1982), and European bass (Mayer et al., 1990).

Plasma Sex Steroids

It is well established in teleosts that estradiol-17ß stimulates hepatic production of vitellogenin and that estradiol increases in the plasma concomitantly with rising values of gonadosomatic index and oocyte diameter (seasonal gonadal development). In the present flounder study, the highly significant correlation between estradiol-17ß, gonadosomatic index and oocyte diameter provides firm support for the cooperative role of this steroid hormone during vitellogenesis. In flounder, low but detectable estrogen levels (see Fig. 10A). were found in early August in conjunction with the initial signs of vitellogenesis e.g., first appearance of opaque oocytes (Vit 1). Later, when growing oocytes were rapidly enlarging from 250-500 µm (September-December), plasma estradiol-17ß rose to approximately 15 ng/ml levelling off at this intermediate level (see Fig. 10). Testosterone, on the other hand, remained relatively low throughout the winter (see Fig. 10B). During the prespawning period when

elevated gonadosomatic index and oocyte diameter values indicated that the ovaries remain relatively well-developed from March-May, further sharp increases in both estradiol and testosterone were observed, reaching peak seasonal steroid values. These peak sex steroid values, 40-60 ng/ml estradiol-17ß and >30 ng/ml testosterone, were correlated not only with the final rise in the gonadosomatic index but also a lowering of oocyte dry matter reflecting gonadal hydration commonly observed in prespawning female flounder. In the present study, two peaks of plasma estradiol-17ß appeared during the annual reproductive cycle. Although the full physiological significance of plasma estradiol-17ß bimodality in temperate species is unknown, this complex pattern for plasma sex steroid has also been reported in bullhead catfish (Burke et al., 1984). The significance in the present study of the secondary increase in plasma estradiol in March is not so clear. Wallace and Selman (1985) have shown that vitellogenesis continues in Fundulus heteroclitus until the period of final oocyte maturation.

The sudden decline of plasma estradiol-17ß in post-spawned female flounder is consistent with synchronous oocyte development found in other fish such as salmonids (see review of Fostier et al., 1983). In salmonids, plasma estradiol-17ß levels gradually decline concomitantly with

a rise in maturation steroids as spawning approaches. In wild flounder, the expected gradual drop in plasma estradiol-17ß could have been missed due to our sampling regime. On the other hand, studies of captive female flounder indicated the presence of a gradual decline of plasma estradiol-17ß prior to spawning (see Fig. 12A). For individual females where spawning was delayed until the end of June, plasma estradiol-17ß appeared to decline gradually.

A number of investigators have considered the role of testosterone in female teleosts suggesting that it serves as a precursor for estradiol-17ß production (Campbell et al., 1976; Wingfield and Grimm, 1977). Indeed, in vitro estradiol-17ß concentrations in media from vitellogenic follicles incubations were elevated in the presence of testosterone (Kagawa et al., 1984). Since the overall seasonal pattern of testosterone in flounder generally parallels that of estradiol-17ß, it seems reasonable to suggest that testosterone serves an intermediate role for estradiol-17ß production confirming the earlier flounder studies of Campbell et al. (1976).

Because changes in plasma levels of estradiol-17ß and testosterone in captive female flounder appear to mimic the steroid levels in wild fish, it appears that captive flounder are capable of adapting to laboratory conditions such that they continue their normal reproductive cycles.

In contrast, Schoonen and Lambert (1987) reported that catfish which fail to spawn in captivity display steroid patterns that are different from those of wild fish. Although, captive female flounder ovulated relatively late in the normal season, egg viability seemed not adversely affected by the delay in spawning.

Wild Males

In male flounder, the seasonal pattern of the reproductive cycle shares some characteristics of the cycle in females. After the summer feeding period while gonads remain in regressed condition (June-July), testicular development abruptly begins in August, rapidly reaching the peak in gonadosomatic index in October (see Fig. 13). Next, a period of testicular maintenance is observed during the winter months (December-March) but very few males are found in spermiating condition at this time and only a small amount of milt (a mixture of sperm and seminal fluid) is available. Finally, a period of full spermiation is observed from March to May when substantial quantities of milt are readily collectable.

An annual pattern of steroid sex hormone fluctuations occurs in male flounder similar to that found in females. During the regressed period (June-August), when the testes are populated only by spermatogonial cells (see Fig. 14A), plasma testosterone (see Fig. 16A) was at its lowest

seasonal levels while plasma 11-ketotestosterone (see Fig. 16B) was not detectable. Both androgens gradually rose in the plasma in accordance with testicular development which suggests that testosterone and 11-ketotestosterone are involved in spermatogenesis as well as spermiation. Correlation analysis between gonadosomatic index and the androgenic hormones suggests that plasma testosterone has a stronger relationship with spermatogenesis while 11-ketotestosterone is more clearly associated with spermiation. Previous studies have shown that androgens levels are low in spent and regressed winter flounder (Campbell et al., 1976), plaice (Wingfield and Grimm, 1977) and rainbow trout (Scott et al., 1980). The first seasonal increase in plasma androgens coincides with the appearance of spermatocytes in the testes in September. Androgen levels remained relatively steady until onset of the period of spermiation when the maximum concentrations of testosterone and 11-ketotestosterone in male winter flounder were observed suggesting that peak androgen levels are closely associated with spermiation. Peak seasonal levels of 11-ketotestosterone have been found coincident with spermiation in brook trout (Sangalang and Freeman, 1974), brown trout (Kime and Manning, 1982), rainbow trout (Scott et al., 1980; Fostier et al., 1982) and mummichog (Cochran, 1987). Although in flounder both testosterone and 11-ketotestosterone appeared to peak

simultaneously similar to mummichog (Cochran, 1987), in trout the peak of plasma testosterone precedes the maximum levels of 11-ketotestosterone (Scott et al., 1980; Sangalang and Freeman, 1974; Kime and Manning, 1982).

In conclusion, gonad growth is reinitiated in adult male and female winter flounder in August, a time of the year when both water temperature and photoperiod have reached their maximum. In males, the gonads reached maximum gonadosomatic index in October thereafter declining until spawning. The time of spawning correlates with a prolonged period of increasing daylength and slowly rising water temperatures. On the other hand, in the female flounder ovary growth continued more slowly during the cold winter conditions until spawning in May/June. The rise in plasma sex steroids in association with the progress of gonad development reflects the seasonal reproductive activity in adult winter flounder.

CHAPTER 4

METHODS OF GONADOTROPIC HORMONE-RELEASING HORMONE ADMINISTRATION

4.1 INTRODUCTION

Gonadotropic Hormone-Releasing Hormone analog (GnRH-A) has been used to manipulate the reproductive cycles of a variety of teleosts, e.g. to stimulate gonadal development, ovulation or spermiation and induce spawning. In most studies GnRH-A was administered by single or multiple intraperitoneal or intramuscular injections, usually near the completion of vitellogenesis or just prior to the period of spermiation. Recently, (Crim et al., 1983b) showed that in rainbow trout a single application of GnRH-A by implant could accelerate gonadal development and induce spawning.

The mode of administration of hormones to teleosts may be chosen according to specific research objectives. For example, to induce or enhance long term processes such as progressive gonadal development, it is perhaps advantageous to apply a chronic release hormone preparation, especially when fish may be sensitive and stress could lead to ovarian regression (Juario et al., 1984). On the other hand, acute hormone administration may

be applied by injection in support of short-term physiological events such as ovulation or spawning. Plasma GnRH-A profiles differ markedly depending on the mode of administration. For example, Crim et al. (1988) demonstrated in trout that plasma GnRH-A is detectable less than 24 hr following a single injection of the peptide hormone, whereas a single cholesterol pellet implant of GnRH-A resulted in a sustained presence of the peptide hormone in plasma more than 10 days.

The pituitary response to GnRH-A treatment can be traced by gonadotropic hormone release or indirectly by the secretion of steroids from the gonads. However, there have been few studies of sex steroid dynamics and steroid hormone profiles are not well characterized following GnRH-A treatment. Therefore, studies of steroid hormone profiles would yield information on the magnitude of response and the duration of these hormones involved in gonadal development and could prove helpful in optimizing hormone application protocols.

The teleost reproductive processes consist of a cascade of hormonal events operating at the brain-pituitary-gonad axis. In this chapter a series of experiments were conducted to obtain information concerning the gonadal response following GnRH-A treatment. The effects of a single injection or a single pellet implantation of GnRH-A on sex steroid secretion in

male and female winter flounder were compared. In addition, the effect of peptide releasing hormone treatment on sex steroid profiles in intact and hypophysectomized fish was investigated to demonstrate the site of action of GnRH-A.

4.2 MATERIALS AND METHODS

4.2.1 Experimental Fish

Six experiments were conducted with adult male and female winter flounder (Table 3) to study the effects of different methods of GnRH-A administration e.g. injection or implantation on plasma sex steroid profiles.

4.2.2 Hypophysectomy of Fish

On March 29, 1989; 22 prespawning male flounder were divided into the following groups: (1) intact (INTACT, n=7); (2) sham hypophysectomized (SHAM HYPEX, n=7); (3) hypophysectomized (HYPEX) + GnRH-A (HYPEX + GnRH-A, n=4); (4) hypophysectomized + gonadotropin (GTH) (HYPEX + GTH, n=4). The fish were hypophysectomized according to the method of Campbell and Idler (1976). Briefly, the flounder were anaesthetized with MS-222 prior to the operation and a small incision was made (2-3 cm) in the ventral side from the opercular hinge towards the angle of the jaw. The fibres of the mandibulae were retracted and scraped until

Table 3

Summary of experiments on methods of GnRH-A administration

Exp	Date	Temp (°C)	Duration (day)	Sex	Stage	GSI
1	Apr	0.0	2	♂	PRES	8.7
2	Dec	3.0	6	♂	PREP 1	10.0
3	Jan	1.0	6	♂	PREP 2	10.0
4	Mar	0.5	12	♂	PRES	9.3
5	Oct	9.3	11	♀	PREP 1	9.3
6	Nov	7.1	28	♀	PREP 1	10.0

Temperature (Temp) and gonadosomatic index (GSI, initial) are mean values.

a view of the facial nerve emerged. A number eight dental drill was used to produce a hole in the bone anterior to the nerve. Debris was aspirated to keep the cranial cavity free of fluid. A probe was inserted into the cranial opening and the pituitary gland was removed by aspiration.

After removal of the pituitary the cranial opening was covered with aureomycin grease, the muscles returned to their original position, and the skin was sutured. Sham operated fish received the same surgical procedure, however, after exposure of the pituitary, it was left intact. Following surgery the fish were returned to their experimental tank for recovery (5 days) prior to hormone treatment.

Blood samples were collected prior to hormone injection (0) and 24 hr after treatment. However, HYPEX + GTH treated males were sampled at 48 hr post-treatment to examine the effectiveness of GTH in maintaining the 11-ketotestosterone levels. All fish were sacrificed by decapitation and the cranial cavities inspected to determine the completeness of pituitary removal.

4.2.3 GnRH-A Radioimmunoassay

4.2.3.1 Chemicals

Phosphate Buffered Saline (PBS). The phosphate buffered saline for the GnRH-A radioimmunoassay consisted of 0.009M

Na_2HPO_4, 0.001M KH_2PO_4, 0.14M NaCl, 0.0002M thimerosal, 0.1% gelatin and pH was adjusted to 7.6 with NaOH.

Tris-HCl Buffer. The Tris-HCl buffer consisted of 0.025M Tris-HCl, 0.001M Dithiothreitol (DTT), 0.1% BSA and 0.1% NaN_3 and pH was adjusted to 7.5 with NaOH.

4.2.3.2 Extraction of Plasma Samples

Mixtures of 25 µl plasma, 1.0 ml absolute methanol (BDH Chemicals Co., Mississauga, ONT., Canada) and 1000 CPM iodinated GnRH-A in 1.5 ml eppendorf microcentrifuge tubes were vortexed briefly and centrifuged at 15,000 x g for 1 minute. The supernatant was decanted into 12 x 75 mm borosilicate glass disposable tubes and stored at 4°C until analysis for GnRH-A. For recovery estimates, 0.1 ml of the methanol extracts were pipetted into 12 x 75 mm borosilicate disposable glass tubes and counted.

4.2.3.3 GnRH-A Iodination

Labelled [D-Ala^6,Pro^9-NHEt]LHRH (GnRH-A) was prepared using the chloramine T method (Hunter and Greenwood, 1962). Briefly, GnRH-A (2.5 µg), 600 ng Chloramine T, and 0.5mCi $Na^{125}I$ dissolved in 0.05M phosphate buffer, pH 7.5 (60 µl final volume) were mixed together for 4 min at 4°C. After the reaction, mixture was diluted with 150 µl, 0.25 N triethylaminephosphate (TEAP, pH 3.0) buffer A and

applied to a C18 reverse-phase high pressure liquid chromatography radial pack column for purification. A step gradient ranging from 20-100% buffer B (60% acetonitrile in TEAP) in buffer A was performed for 50 min and 1-ml fractions were collected and the radioactivity counted to determine the elution profile. The acetonitrile was evaporated under a stream of nitrogen and the labelled GnRH-A was stored at 4°C diluted with Tris-HCl receptor assay buffer.

4.2.3.4 GnRH-A Radioimmunoassay

In preparation for plasma GnRH-A radioimmunoassay, methanol extracts of plasma or standard GnRH-A (1-1000 pg/tube) in methanol were pipetted in duplicate into 12 x 75 mm borosilicate disposable glass tubes. The methanol was evaporated under a stream of nitrogen. Following removal of the methanol the tubes were brought to 0.5 ml with PBS containing GnRH-A antibody diluted 1:12,000 (courtesy of Dr. Nancy Sherwood, Victoria, B.C.) and approximately 6,000 CPM iodinated GnRH-A. The tubes were briefly vortexed and incubated at 4°C. Following an overnight incubation, the tubes were placed in an ice-water bath (4°C), and incubated for 10 min with 1.0 ml dextran coated-charcoal suspension (1.0 g charcoal, 0.1 g dextran T-70 in 400 ml PBS). Following centrifugation at 2,200 x g for 15 min, the liquid phase (bound fraction)

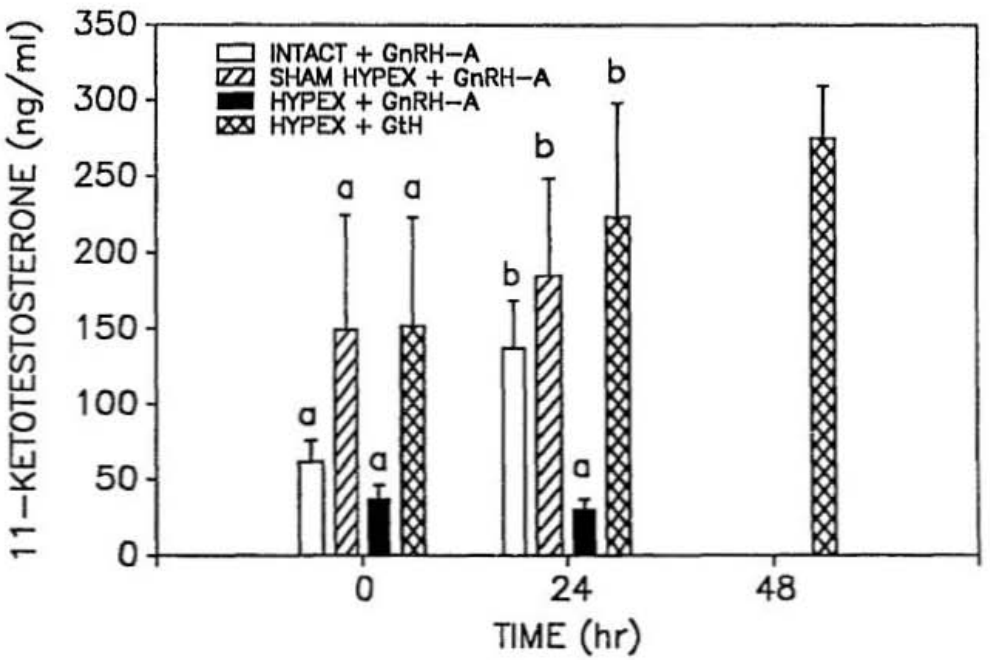
was decanted into clean tubes for gamma counting.

4.3 RESULTS

4.3.1 Plasma 11-ketotestosterone in Hypophysectomized Male Flounder Following Treatment with either GnRH-A or gonadotropin (GTH).

The site of action of GnRH-A was studied in intact and hypophysectomized winter flounder by observing the changes in plasma 11-ketotestosterone levels. On April 2, four groups of male flounder were treated as follows: (1) intact received a single injection of GnRH-A (20 μg/kg body weight); (2) sham hypophysectomized received a single injection of GnRH-A (20 μg/kg body weight); (3) hypophysectomized received a single injection of GnRH-A (20 μg/kg body weight); (4) hypophysectomized received CON AII chum salmon GTH (1.40 mg/kg body weight). At the time of initiation of this experiment, 50% of the males were in spermiating condition (see Table 3, experiment 1); thus, 11-ketotestosterone levels would be expected to be elevated at this stage of reproduction. Prior to hormone injection, plasma 11-ketotestosterone levels were not significantly different between the four treatment groups (time 0, Fig. 19). The levels of 11-ketotestosterone in

Figure 19. Plasma 11-ketotestosterone in male winter flounder following hypophysectomy (hypex) and hormone treatment. Bars and lines represent means ± SEM of 4-7 fish per group. Time=0 indicates blood sampling prior to hormone treatment. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time.

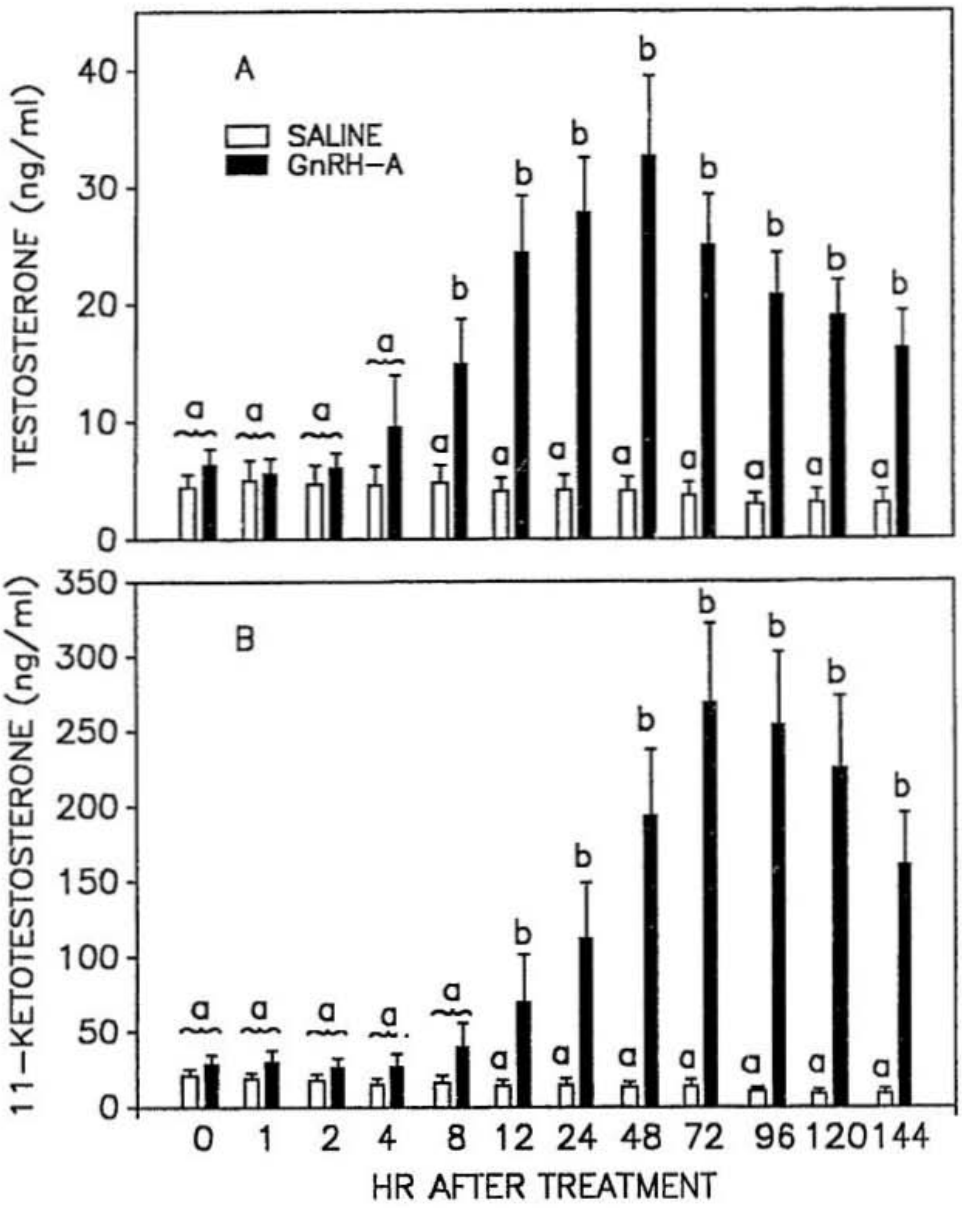

11-KETOTESTOSTERONE (ng/ml)
350
300
250
200
150
100
50
0
INTACT + GnRH-A
SHAM HYPEX + GnRH-A
HYPEX + GnRH-A
HYPEX + GtH
a
a
a
a
b
b
a
b
0
24
48
TIME (hr)

males receiving GTH was maintained at 48 hr. Since the levels of plasma 11-ketotestosterone in hypophysectomized males 24 hr after receiving GnRH-A were significantly ($P<0.05$) lower compared with plasma 11-ketotestosterone in intact males receiving GnRH-A injections or hypophysectomized males after GTH injections, the data support the concept that the pituitary is the site of action of GnRH-A.

4.3.2 Plasma Androgens in Male Flounder Following a Single Injection of GnRH-A.

Two groups of 7 males each were given either a single injection of physiological saline (control) or 20 μg/kg GnRH-A (see Table 3, experiment 2) and serial blood samples (n=12) were collected throughout the 6 day experimental period. Blood samples were collected prior to injection (0 hr) and at 1,2,4,8,12,24,48,72,96,120,144 hr after treatment. For control males (SALINE), both testosterone and 11-ketotestosterone levels remained relatively low and unchanged at approximately 5 and 25 ng/ml, respectively throughout the duration of the study (Fig 20A,B). In contrast, a clear increase in plasma sex steroids was obtained following GnRH-A treatment of prespermiating males. Plasma testosterone and 11-ketotestosterone levels of GnRH-A treated males were

Figure 20. Time-course changes of plasma (A) testosterone and (B) 11-ketotestosterone in male winter flounder following a single IP injection of saline (open bars) or 20 μg/kg GnRH-A (solid bars). Bars and lines represent means ± SEM of 7 fish per group. Time=0 indicates blood sampling prior to treatment. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time (treatment compared to controls).


A
SALINE
GnRH–A
TESTOSTERONE (ng/ml)
0
10
20
30
40
B
11–KETOTESTOSTERONE (ng/ml)
0
50
100
150
200
250
300
350
0 1 2 4 8 12 24 48 72 96 120 144
HR AFTER TREATMENT

significantly ($P<0.05$) increased at 8 and 12 hr, respectively. After reaching their peak levels, 48 hr for testosterone (30 ng/ml) and 72 hr for 11-ketotestosterone (250 ng/ml) both plasma androgens began to decline although the levels remained significantly ($P<0.05$) elevated at the termination of the experiment.

4.3.3 Plasma Androgens in Male Flounder Following a Single Injection of Different Doses of GnRH-A.

Another GnRH-A single injection experiment was conducted with four groups of prespermiating males (see Table 3, experiment 3). The males received either physiological saline control (SALINE) or graded doses of GnRH-A (2 μg/kg, n=6); 20 μg/kg, n=6); 200 μg/kg, n=5; body weight). Blood samples (n=12) were collected prior to injection (0 hr) and at 1,2,4,8,12,24,48,72,96,120,144 hr after treatment.

At this time of the winter both plasma testosterone (4 ng/ml) and 11-ketotestosterone (18 ng/ml) remained detectable; no increases in plasma sex steroid levels were observed in control male flounder, however, by 24 and 72 hr, respectively, plasma testosterone and 11-ketotestosterone levels had declined significantly ($P<0.05$) remaining below pretreatment levels when the 6-day experiment was terminated (Figs. 21,22).

Figure 21. Plasma testosterone in prespermiating male flounder following IP injection of different doses of GnRH-A (2, 20, 200 μg/kg). Bars and lines represent means ± SEM of 5-6 fish per group. Time=0 indicates blood sampling prior to hormone treatment. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time (treatments compared to controls).

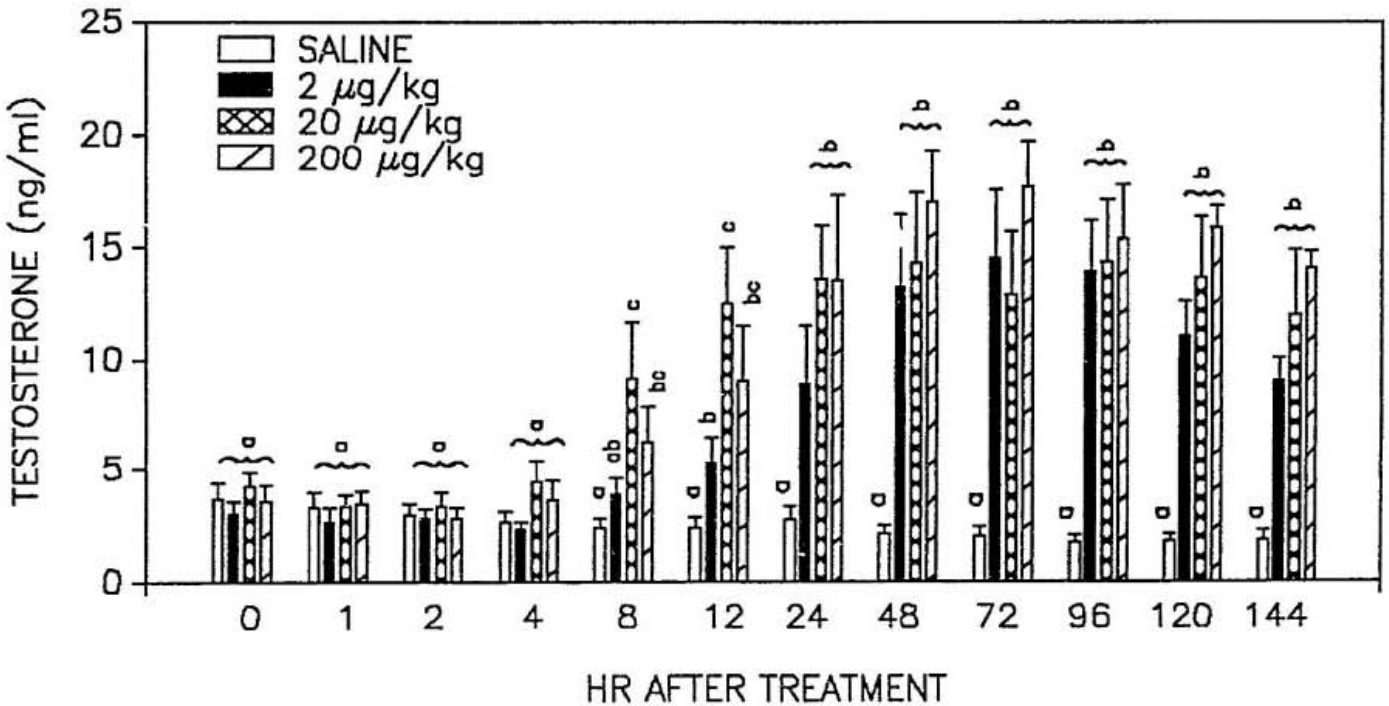
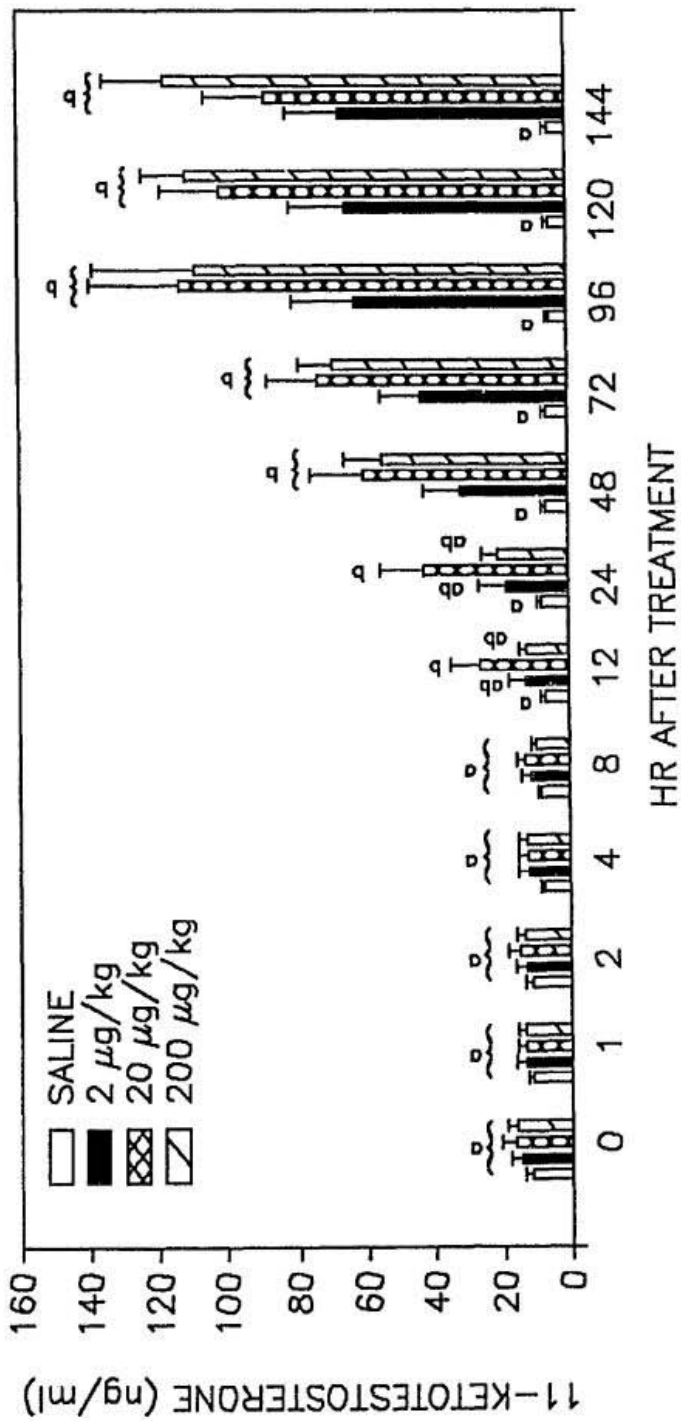

SALINE
2 μg/kg
20 μg/kg
200 μg/kg
TESTOSTERONE (ng/ml)
25
20
15
10
5
0
0
1
2
4
8
12
24
48
72
96
120
144
HR AFTER TREATMENT

Figure 22. Plasma 11-ketotestosterone in prespermiating male winter flounder following IP injection of different doses of GnRH-A (2, 20, 200 μg/kg). Bars and lines represent means ± SEM of 5-6 fish per group. Time=0 indicates blood sampling prior to hormone treatment. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time (treatments compared to controls).


11-KETOTESTOSTERONE (ng/ml)
160
140
120
100
80
60
40
20
0
SALINE
2 µg/kg
20 µg/kg
200 µg/kg
0
1
2
4
8
12
24
48
72
96
120
144
HR AFTER TREATMENT

In contrast, plasma testosterone and 11-ketotestosterone levels were significantly elevated in males receiving peptide releasing hormone treatment. In fish receiving the 2 μg/kg dose of GnRH-A, plasma testosterone was significantly increased ($P<0.05$) 12 hr after treatment and remained elevated throughout the experiment. Plasma 11-ketotestosterone levels also increased in response to the low dose of GnRH-A but compared with testosterone, the initial rise in plasma 11-ketotestosterone levels was delayed until 48 hr. When male flounder were treated with a single injection of 20 μg or 200 μg/kg GnRH-A, plasma levels of testosterone and 11-ketotestosterone were significantly ($P<0.05$) increased by 8 and 12 hr respectively; after the higher doses of GnRH-A treatment, the plasma androgen levels remained significantly ($P<0.05$) increased for the remainder of the experiment.

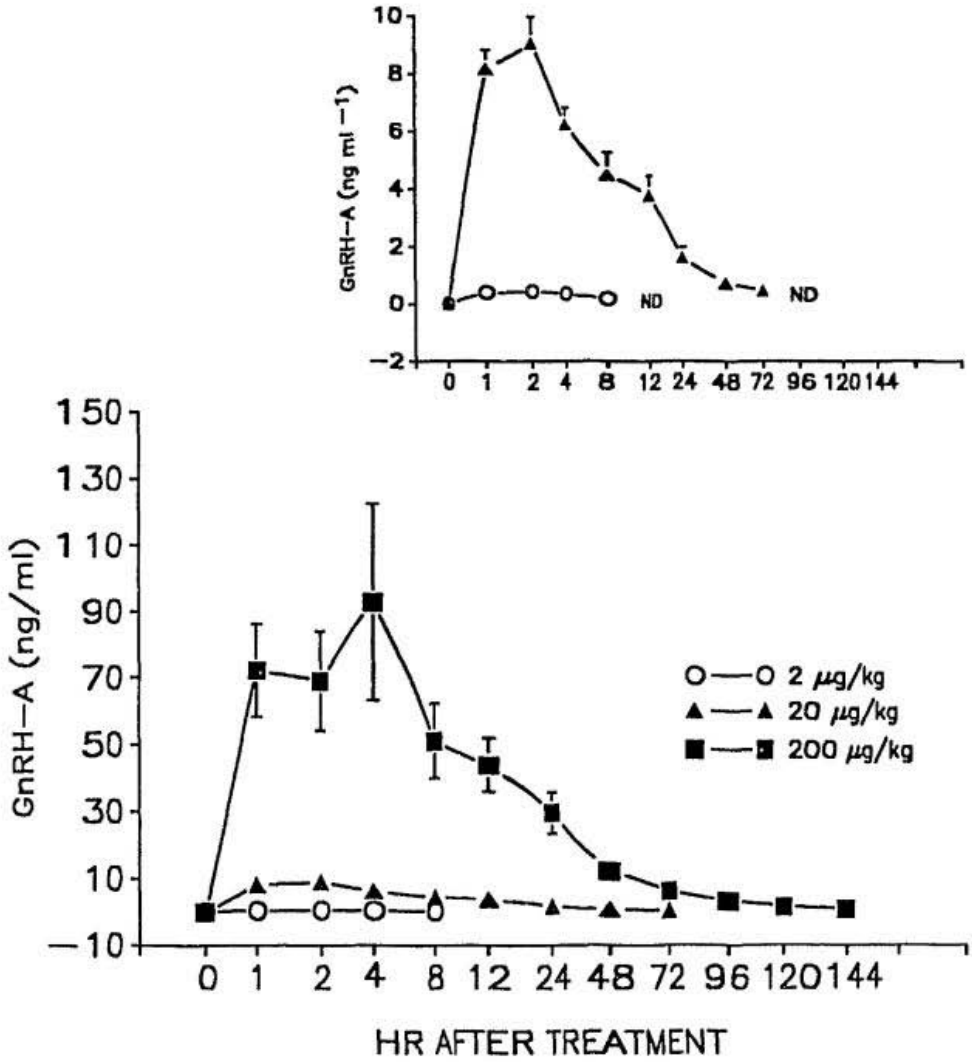
Radioimmunoassay analysis of plasma GnRH-A was conducted to determine plasma GnRH-A levels following exogenous releasing hormone administration. This study indicated that plasma levels of GnRH-A in control fish, not receiving releasing hormone treatment, were always below the radioimmunoassay detection limit (<0.05 ng/ml). However, for all fish receiving GnRH-A treatment, the exogenously administered hormone rapidly appeared in the plasma becoming detectable within 1 hr; plasma GnRH-A

remained elevated for 4 hr, and the peptide plasma hormone levels were significantly declining by 24 hr (Fig. 23). These results also indicate that the length of time before plasma GnRH-A levels declined beneath detection was dose-dependent. When males were administered 2 μg/kg dose, plasma GnRH-A remained elevated for 4 hr, significantly ($P<0.05$) declined by 8 hr and finally disappeared by 12 hr (Fig 23, inset); however, in males receiving the 20 μg/kg dose, plasma GnRH-A levels remained elevated for 4 hr, significantly declined by 24 hr and disappeared from the circulation by 96 hr. High levels of circulating GnRH-A and a long-term detectable GnRH-A profile lasting through 144 hr was observed in the males receiving 200 μg/kg, the highest GnRH-A treatment level.

4.3.4 Plasma Androgens and the Spermiation Response of Male Flounder Following One or Two Injections of GnRH-A.

An experiment was conducted with male flounder when seawater temperatures had fallen to the minimum seasonal level (see Table 3, experiment 4). Three groups (7 fish in each) of males received either 1) saline (SALINE); 2) 20 μg/kg GnRH-A injected once (SINGLE INJ); or 3) 20 μg/kg GnRH-A injected twice (DOUBLE INJ: 48 hr apart) during the study. Blood samples (n=6) were collected on days 0,

Figure 23. Plasma GnRH-A in prespermiating male winter flounder following treatment with different doses (2, 20, 200 µg/kg) of GnRH-A. Bars and lines represent means ± SEM of 5-6 fish per group. Time=0 indicates blood sampling before GnRH-A treatment. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time (treatments compared to controls). Inset shows plasma GnRH-A levels for fish receiving the 2 and 20 µg/kg doses.


GnRH-A (ng ml⁻¹)
10
8
6
4
2
0
-2
ND
ND
0 1 2 4 8 12 24 48 72 96 120144
GnRH-A (ng/ml)
150
130
110
90
70
50
30
10
-10
2 μg/kg
20 μg/kg
200 μg/kg
0 1 2 4 8 12 24 48 72 96 120144
HR AFTER TREATMENT

1,3,6,9, and 12 after hormone injection. Sperm concentrations were determined at the beginning and on the final day of the experiment whereas milt volume was determined only at the end of the study.

This experiment was conducted approximately three months prior to the expected spawning period with prespawning males, some of which were already in spermiating condition. Plasma testosterone (10 ng/ml) and 11-ketotestosterone (50 ng/ml) levels in control fish were elevated at this time of the year. Whereas plasma testosterone and 11-ketotestosterone levels remained high and stable throughout the experimental period in saline control fish, by day 3 and 6, respectively, a significant ($P<0.05$) increase in testosterone and 11-ketotestosterone occurred in the groups of fish treated with GnRH-A (Fig. 24A,B). Although both testosterone and 11-ketotestosterone levels remained elevated through day 9 in males injected once with GnRH-A, by day 12 androgen levels returned to basal values in all the hormone treated groups.

At the beginning of this experiment less than half of the males were in spermiating condition (Table 4). Generally, the milt from ripe males in March was a viscous, creamy white liquid although for the males in group 3 the milt was more dilute (spermatocrit 63.8%). By the final day of this experiment, all surviving males receiving GnRH-A treatment reached the spermiating

Figure 24. Plasma (A) testosterone and (B) 11-ketotestosterone levels in prespawning male winter flounder following a single (20 μg/kg/injection) or double IP injections of GnRH-A (48 hour apart). Bars and lines represent means ± SEM of 7 fish per group. Time=0 indicates blood sampling prior to hormone treatment. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time (treatments compared to controls).

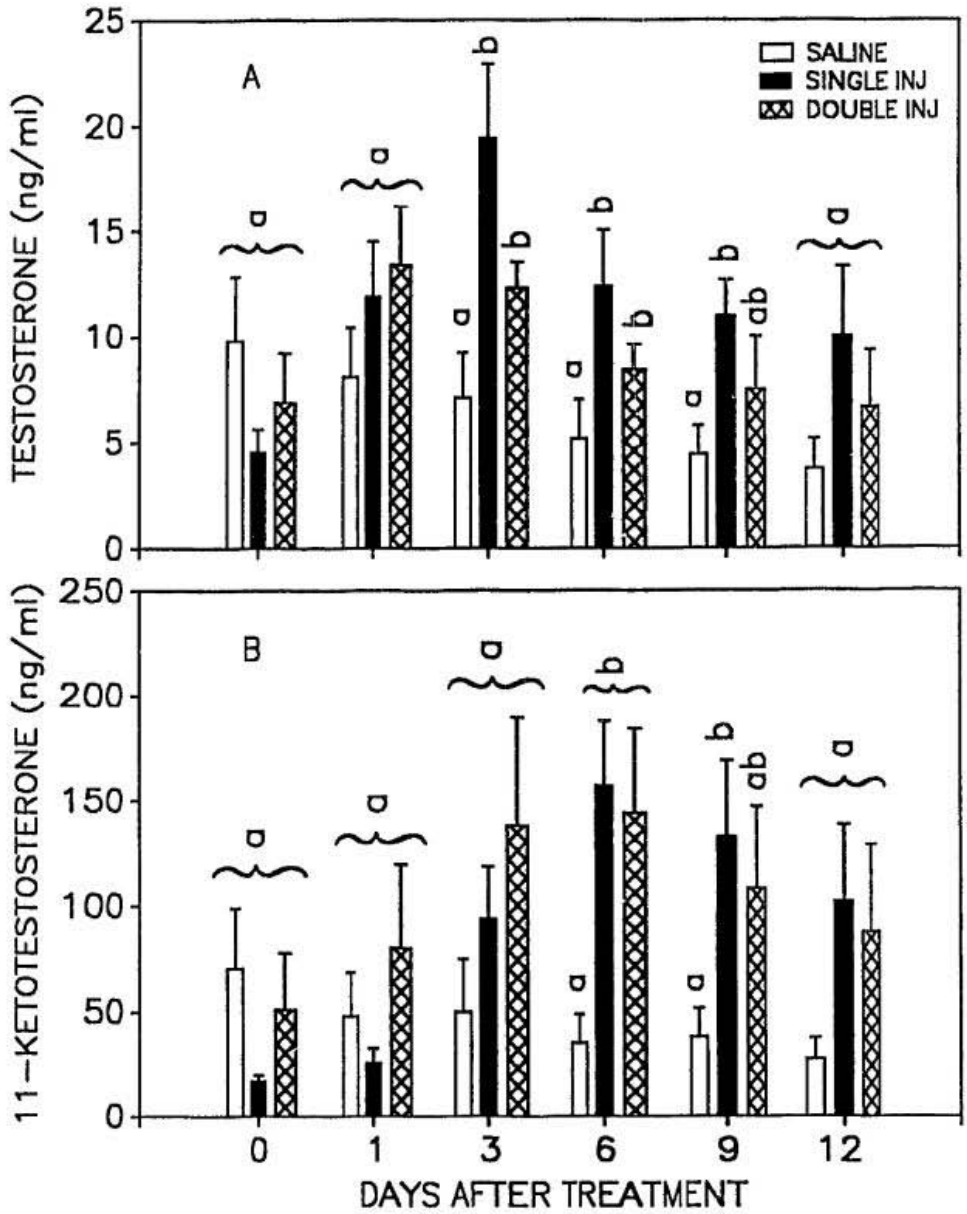

A
B
SALINE
SINGLE INJ
DOUBLE INJ
TESTOSTERONE (ng/ml)
11-KETOTESTOSTERONE (ng/ml)
DAYS AFTER TREATMENT
0
1
3
6
9
12
25
20
15
10
5
0
250
200
150
100
50
0
a
b
ab

Table 4

Effects of GnRH-A treatment on milt volume and total sperm count in male winter flounder

Treatment	No spermiating of total males Initial	Final	Spermatocrit (%) Initial	Final	Milt vol (ml)	Total Sperm ($x10^9$)
SALINE	3/7 (43)	4(67)	83.9 ±7.5	87.3 ±8.1	2.2 ± 0.6[a]	36 ± 9[a]
SINGLE INJ	2/7 (29)	5(100)	88.2	98.1	1.1 ± 0.3[a]	21 ± 7[a]
DOUBLE INJ	3/7 (43)	5(100)	63.8 ±4.1	87.5 ±10.7	7.7 ± 3.9[b]	113 ± 43[b]

Data are means ± SEM; significance ($P<0.05$) indicated by different letters; ( ) indicates percent spermiation. The mortality rate was 1,2 and 2 for SALINE, SINGLE and DOUBLE INJ groups, respectively.

condition, while in control fish, just 4 of 7 males had fully matured and milt could be detected. Milt volume and sperm production was not significantly different between controls and males receiving a single dose of GnRH-A. However, milt volume and sperm production significantly increased in males receiving GnRH-A twice (DOUBLE INJ significantly > SALINE and SINGLE INJ, $P<0.05$).

4.3.5 Plasma estradiol-17ß and testosterone in Female Flounder Following GnRH-A Treatment by a Single Injection or Cholesterol Pellet Implantation.

During the period of gonadal recrudescence (October, see Table 3, experiment 5), an eleven day (264 hr) study was carried out with 28 female flounder which were divided into 4 groups and treated as follows: (1) a single saline injection control (SAL INJ, n=6); (2) a single (20 µg/kg body weight) GnRH-A in saline injection (GnRH-A INJ, n=8); (3) sham hormone cholesterol pellet implant control (SHAM IMP, n=6); (4) 100 µg GnRH-A cholesterol pellet implant (GnRH-A IMP, n=8). A total of 12 blood samples were collected at 0,1,2,4,8,24,48,72,96,120,144, and 264 hr after the treatment.

The steroid data for control (SAL INJ and SHAM IMP) females indicate that plasma estradiol-17ß and testosterone remained unchanged for the initial 24 hr

(Figs. 25,26). Interestingly, from 48-72 the levels of both plasma sex steroids significantly ($P<0.05$) dropped before returning to pretreatment values for the duration of the experiment. In contrast, both modes of GnRH-A application significantly ($P<0.05$) elevated plasma estradiol-17ß and testosterone levels although the steroid profiles differed. Plasma estradiol-17ß and testosterone levels in females receiving GnRH-A by implant were elevated by 24 and 48 hr, respectively, and the elevated levels were sustained to the end of the experiment. On the other hand, plasma estradiol-17ß and testosterone were increased by 24 hr in females injected with GnRH-A. However, by 96 and 72 hr, respectively both plasma estradiol-17ß and testosterone declined and the levels remained undistinguishable from control sex steroid values to the end of the experiment.

By the end of the study, it was apparent that both modes of GnRH-A administration increased gonadosomatic index (Table 5) compared to paired control females reflecting an acceleration of oocyte development.

Figure 25. Time-course changes of plasma estradiol-17β in females following a single IP injection of 20 μg/kg GnRH-A or a single IM cholesterol pellet implant (100 μg GnRH-A). Bars and lines represent means ± SEM of 5-6 fish per group. Time=0 indicates blood sampling prior to hormone treatment. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time (treatments compared to its controls).

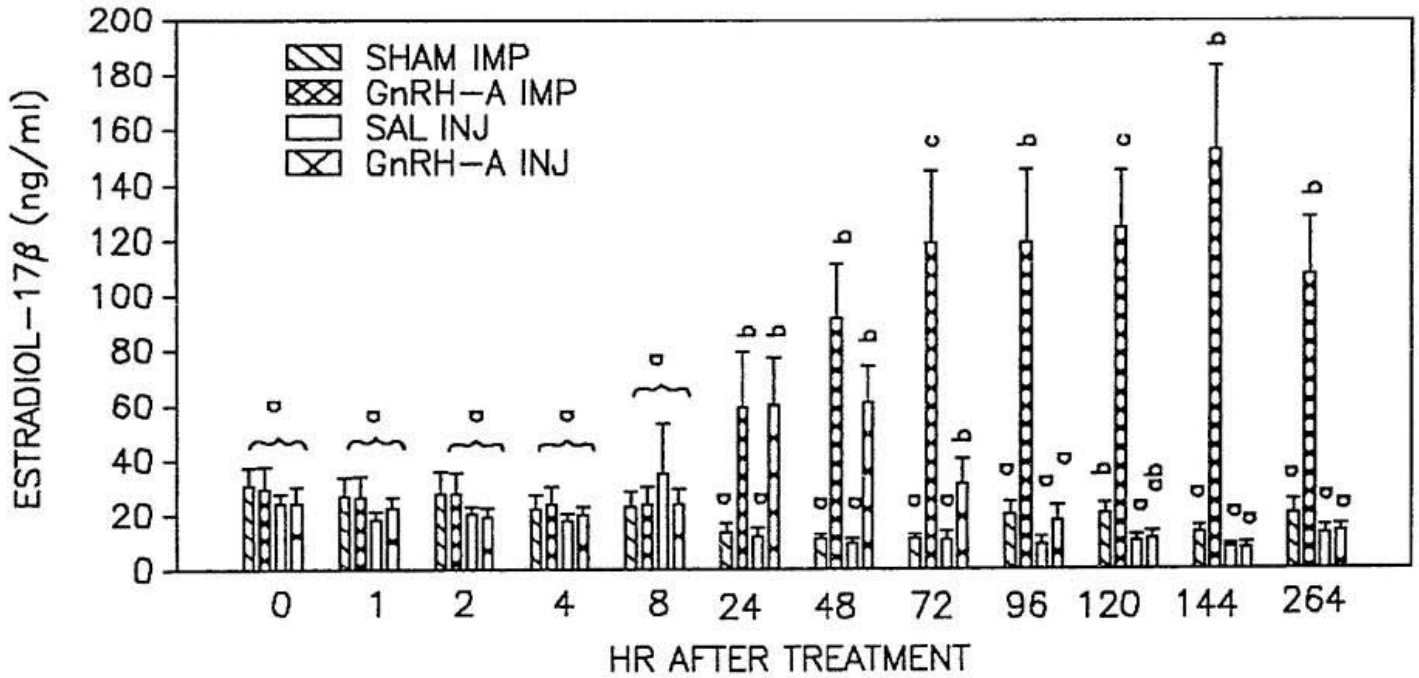

ESTRADIOL–17β (ng/ml)
200
180
160
140
120
100
80
60
40
20
0
SHAM IMP
GnRH–A IMP
SAL INJ
GnRH–A INJ
0
1
2
4
8
24
48
72
96
120
144
264
HR AFTER TREATMENT

Figure 26. Time-course changes of plasma testosterone in females following a single injection of 20 μg/kg GnRH-A or a single cholesterol pellet implant (100 μg GnRH-A). Bars and lines represent means ± SEM of 5-6 fish per group. Time=0 indicates blood sampling prior to hormone treatment. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time (treatments compared to its controls).

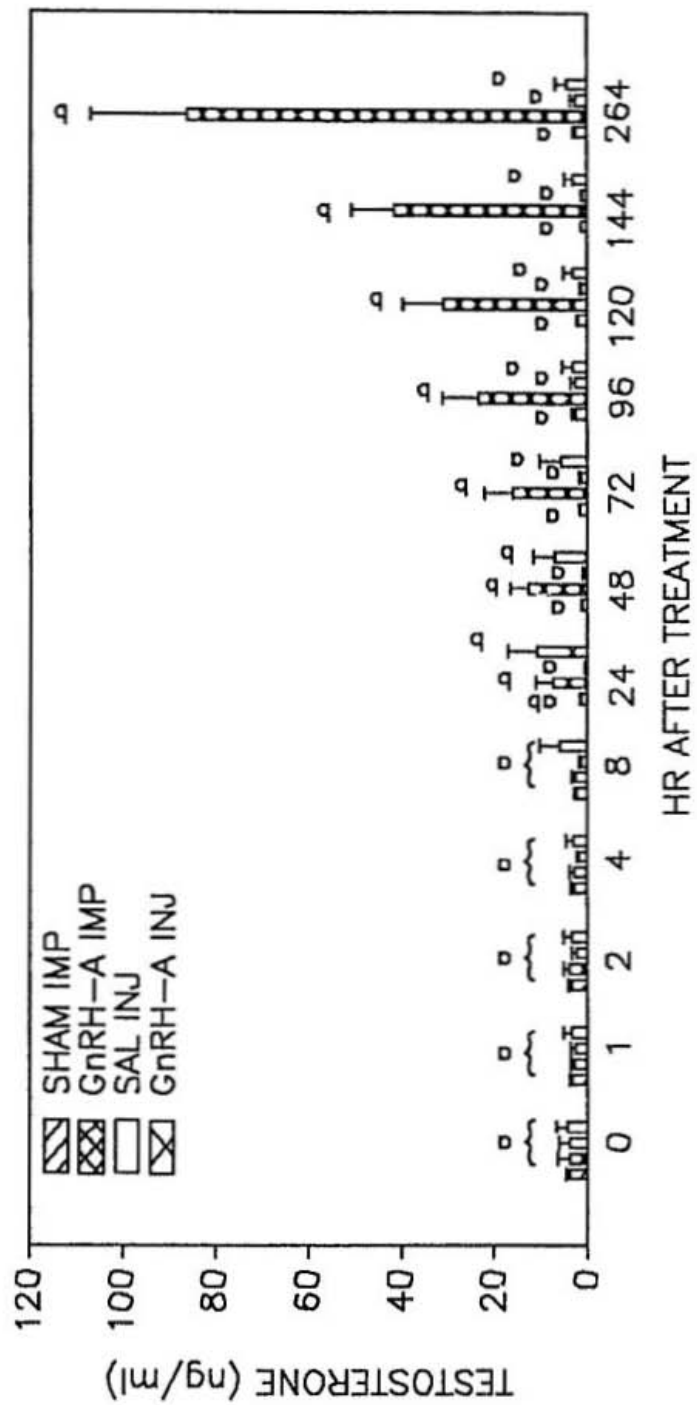

SHAM IMP
GnRH–A IMP
SAL INJ
GnRH–A INJ
TESTOSTERONE (ng/ml)
120
100
80
60
40
20
0
0
1
2
4
8
24
48
72
96
120
144
264
HR AFTER TREATMENT

Table 5

Effects of GnRH-A on gonadosomatic index (GSI) in female winter flounder during the period of gonad recrudescence

Treatment	N	GSI
SHAM IMP	6	8.92 ± 0.25^{ab}
GnRH-A IMP	6	13.27 ± 0.90^{c}
SAL INJ	5	8.49 ± 0.83^{a}
GnRH-A INJ	7	11.07 ± 0.95^{bc}

All values are means ± SEM. Significance ($P < 0.05$) indicated by different letters.

4.3.6 Plasma estradiol-17ß and testosterone in Female Flounder Following GnRH-A Treatment by either Single Weekly Injections or a Single Cholesterol Pellet Implantation.

This experiment was conducted with female flounder which were undergoing gonadal recrudescence in November (see Table 3, experiment 6). Females were divided into 6 groups and treated as follows: (1) A group of females was sacrificed to determine the state of reproduction before initiation of the study (INITIAL, n=6); (2) intact untreated fish (INTACT, n=7); (3) one sham hormone cholesterol pellet implant (SHAM IMP, n=7); (4) one 100 µg GnRH-A cholesterol pellet implant (GnRH-A IMP, n=8); (5) a single saline injection per week (SAL INJ, n=6); (6) a single GnRH-A (20 µg/kg body weight) injection per week (GnRH-A INJ, n=9). For this experiment, blood was collected at 24 hr post-treatment and at weekly intervals just prior to the single weekly injection of GnRH-A giving a total of 5 blood samples throughout the experiment.

Plasma sex steroids in both intact and saline injected control females remained unchanged throughout the 4-wk study period. However, plasma sex steroids declined by the end of the study in control females implanted with a blank cholesterol pellet (SHAM IMP). By 24 hr, although steroids remained unchanged in GnRH-A implanted females, plasma

estradiol-17ß and testosterone significantly increased in females injected with GnRH-A (Fig. 27A,B). At 168 hr, plasma levels of estradiol-17ß and testosterone in GnRH-A implanted females were significantly higher than sham controls and remained elevated until the end of the experiment. On the other hand, from 168 hr until the end of the study, no significant differences were observed in plasma estradiol-17ß and testosterone in females injected with GnRH-A compared to saline controls.

At autopsy, although no significant effect of GnRH-A treatment on gonadosomatic index was observed, in contrast, oocyte diameter was significantly ($P<0.05$) increased by the hormone treatment compared to control females (Table 6). In addition, chi-square analysis of oocyte-frequency distribution showed that oocyte-size class frequencies were significantly ($P<0.05$) advanced in females receiving GnRH-A treatment compared with controls (Fig. 28).

Figure 27. Time-course changes of plasma (A) estradiol-17β and (B) testosterone in females undergoing gonadal recrudescence following single weekly 20 μg/kg GnRH-A IP injections or a single 100 μg GnRH-A pellet implant. Bars and lines represent means ± SEM of 5-6 fish per group. Time=0 indicate blood sampling prior to hormone treatment. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time (treatments compared to its controls).

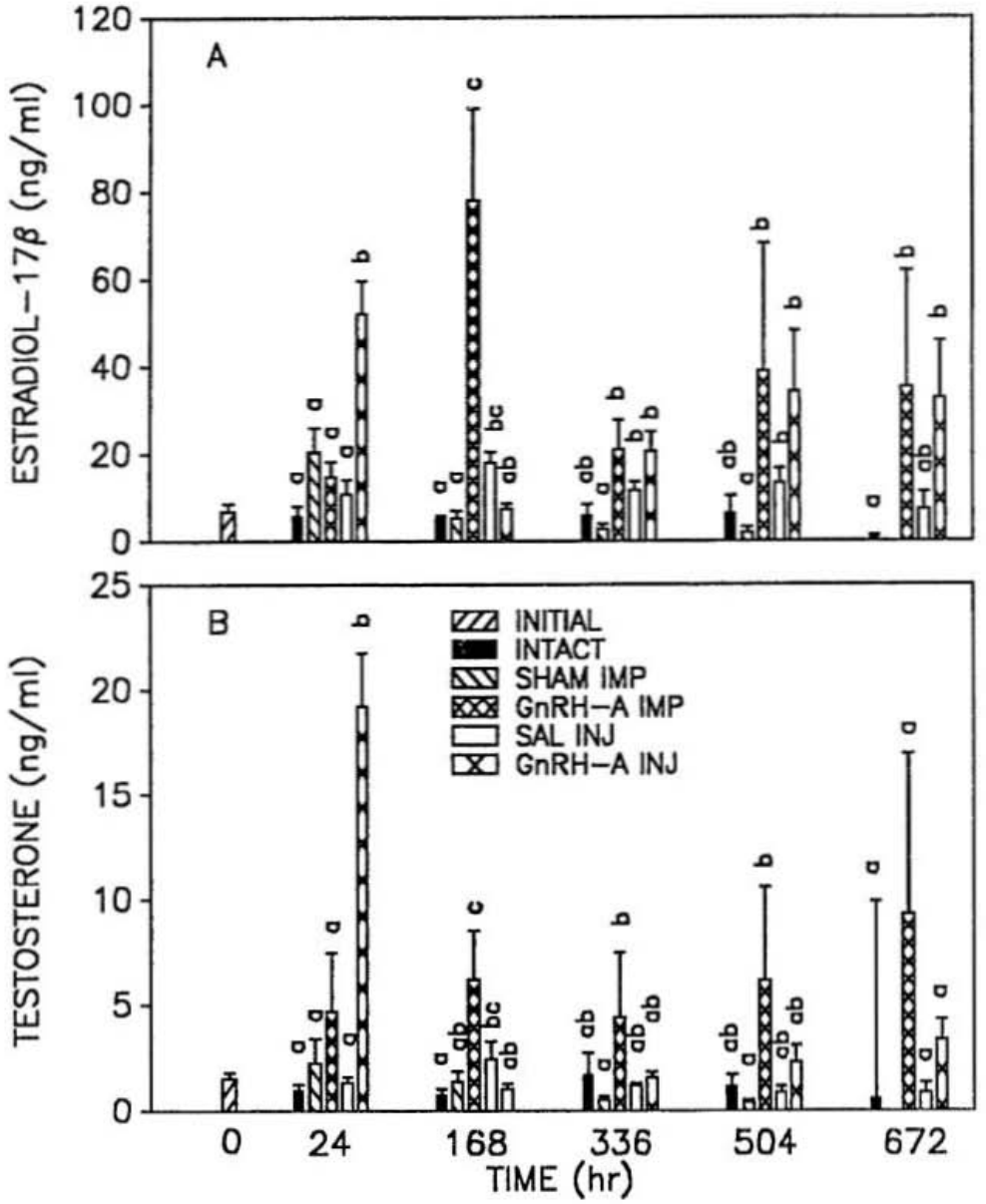

A
B
ESTRADIOL-17β (ng/ml)
TESTOSTERONE (ng/ml)
INITIAL
INTACT
SHAM IMP
GnRH-A IMP
SAL INJ
GnRH-A INJ
TIME (hr)
0
24
168
336
504
672

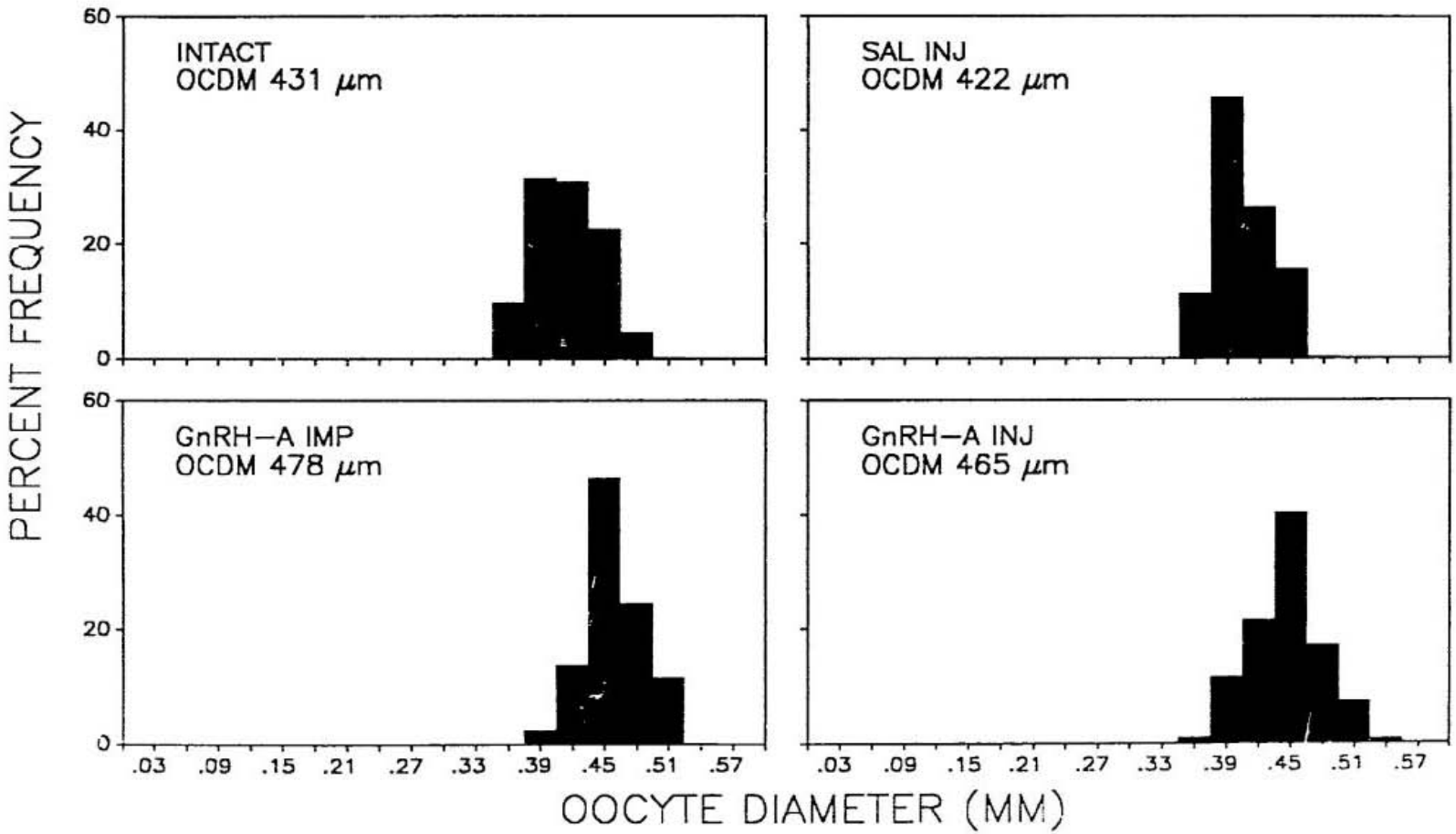
Table 6

Effects of GnRH-A on gonadosomatic index (GSI) and oocyte diameter (OCDM) in female winter flounder during the period of gonadal recrudescence

Treatment	N	GSI	OCDM
INITIAL	6	9.69^{a} ±1.52	423.69^{a} ±8.95
INTACT	4	10.72^{ab} ±0.65	430.69^{a} ±16.95
GnRH-A IMP	3	12.24^{ab} ±1.49	478.11^{b} ±8.95
SAL INJ	4	11.27^{ab} ±1.21	421.73^{a} ±9.70
GnRH-A INJ	7	14.10^{b} ±0.58	464.94^{b} ±8.49

All values are means ± SEM. Significance ($P<0.05$) indicated by different letters. At the end of the study, 100% mortality occurred in SHAM IMP females.

Figure 28. Oocyte-size frequency distributions at the end of a 4-wk experimental period for females injected IP with GnRH-A 20 µg/kg/week or receiving a single 100 µg GnRH-A pellet implant. OCDM, mean oocyte diameter in µm.


INTACT
OCDM 431 μm
SAL INJ
OCDM 422 μm
GnRH–A IMP
OCDM 478 μm
GnRH–A INJ
OCDM 465 μm
PERCENT FREQUENCY
0
20
40
60
.03
.09
.15
.21
.27
.33
.39
.45
.51
.57
OOCYTE DIAMETER (MM)

4.4 DISCUSSION

The present studies clearly demonstrate that both male and female adult winter flounder respond to the administration of GnRH-A resulting in sustained increases of sex steroid levels in the plasma. In female flounder it was also noted that ovary development was accelerated following GnRH-A treatment; in males, GnRH-A injection advanced spermiation and increased milt volume. This provides an indication of the functional nature of the pituitary-gonadal axis during the reproductive period in winter flounder and is consistent with previous studies of teleost fish showing that releasing hormone treatment effectively elevates sex steroid levels in carp (Weil et al., 1980; Takashima et al., 1984; Ngamrongchon et al., 1987), rainbow trout (Weil et al., 1978), walleye (Pankhurst et al., 1986a), goldeye (Pankhurst, 1986b), sea bass (Prat et al., 1987); coho salmon (Sower et al., 1984; Van der Kraak et al., 1984) and steelhead trout (Sower et al., 1984).

GnRH-A profiles in the plasma vary according to the conditions of administration for this peptide hormone. There appears to be a dose-dependent effect of GnRH-A injection with respect to the short or long term profiles of GnRH-A in plasma. Generally, a short term effect is observed following injection (see Fig. 23). For example

GnRH-A disappeared from the circulation after 8 hr when 2 μg/kg was injected. Previously, Crim et al., (1988) demonstrated in trout maintained at 10°C that GnRH-A quickly becomes detectable in the plasma within 5 min of injection and it remains detectable for approximately 5 hr. When male flounder were injected at a higher dose of GnRH-A (200 μg/kg body weight), plasma GnRH-A levels were detectable for 6 days, suggesting the presence of this releasing hormone peptide is prolonged in flounder held under low ambient temperature/seawater conditions (1°C). In addition to dose-dependent effects, the long-lasting action of GnRH in winter flounder could be explained by the protective action of serum GnRH-binding protein as reported in goldfish (Huang and Peter, 1988). Circulating levels of GnRH-A in the plasma depend on its rate of degradation and clearance. Sherwood and Harvey (1986) reported that native GnRH has a very short half-life of approximately 12 min in goldfish. In contrast, GnRH analogs were shown to be relatively resistant to enzymatic degradation compared to native salmon GnRH in vitro in pituitary and kidney (Goren et al., 1987). Thus, the extended presence of GnRH-A in the flounder circulation after a single injection of a relatively large dose of GnRH-A may be an important factor resulting in the prolonged nature of sex steroid elevations lasting several days. The prolonged nature of sex steroid elevations in

flounder could also be due to the nature of pituitary stimulation. The prolonged responsiveness of the pituitary to GnRH-A effects both the synthesis and release of GTH which ultimately acts on the gonads to synthesize sex steroids. Indeed, in vitro GnRH-A stimulates secretion of both GTH I and GTH II, but the pituitary response depends on the stage of reproduction (Swanson et al., 1987). When male flounder were treated with GnRH-A two months prior to their natural spawning period, the onset of spermiation was advanced. The present study showed that both single or double injections of GnRH-A increased the spermiation rate in surviving males (see Table 4). In addition, a greater effect on milt production was achieved in the double injected males. Several studies have shown the effectiveness of GnRH-A in inducing spermiation. A single injection of GnRH-A increased the number of spermiating males in Pacific herring (Kreiberg et al., 1987) and increased the volume of milt (Ngamrongchon et al., 1987). In carp, multiple injections of GnRH-A also increased milt volume and number of spermatozoa collected (Takashima et al., 1984).

The dynamics of GnRH-A after injection was examined only in male flounder but not in the females. However, the results of GnRH-A treatment of females suggested that the mode of releasing hormone application also has an important bearing on the effectiveness of GnRH-A

treatment. For example, when females were tested in October (see Figs. 25,26), injection of GnRH-A induced relatively short term steroid hormone increases; after GnRH-A implantation, however, the elevations of plasma steroids (estradiol-17ß and testosterone) were of much greater duration lasting 11 days suggesting that GnRH-A persists in the plasma particularly after prolonged delivery by pellet implantation. In trout, Crim et al. (1988) demonstrated that a low level of GnRH-A is detectable in plasma for at least 10 days after pellet implantation of gonadotropin releasing hormone. Both short and long-term modes of GnRH-A administration were very effective in increasing gonadosomatic index in females in October during the period of active gonad recrudescence (see Table 5). Although in November, gonadosomatic index was not stimulated by either GnRH-A injection or implantation, oocyte development was clearly accelerated as reflected in the increases of oocyte diameter and also changes in the oocyte size-class frequency distribution. A single implantation of fish with releasing hormone could not only reduce the quantity of hormone required, but this methodology could eliminate the stress of repeated handling that often accompanies protocols requiring serial hormone injections.

The present studies demonstrated that sex steroid profiles would differ depending on the mode of GnRH-A

administration. The fact that winter flounder are relatively large, have a flat shape and remain relatively sedate at cool temperatures, provides convenient access to frequent blood sampling of individual fish. These winter flounder features have been utilized in the series of experiments to precisely characterize the short term gonadal steroid responses of male flounder following GnRH-A treatment as follows: there is an initial increase in plasma testosterone closely followed by the rise in 11-ketotestosterone (see Fig. 20); these elevations in plasma androgens persist in the male flounder for several days. In contrast, the steroid levels in control males remained steady or declined; clearly, GnRH-A is able to prevent the gradual decline of plasma steroids in male flounder held in the laboratory. Ngamrongchon et al. (1987) described the steroid responses in male carp as being composed of rapid increases in testosterone and 11-ketotestosterone within 1 hr of GnRH-A treatment. In the male bullhead catfish, an injection of salmon GnRH resulted in increased testosterone and 11-ketotestosterone levels in the plasma during a period of 4-24 hr (Rosenblum and Callard, 1987). A short term duration of plasma testosterone elevation is observed in the common carp (Takashima et al., 1984) and goldeye (Pankhurst et al., 1986b) following GnRH-A injection. In the present study high levels of androgens were already present in prespermiating male flounder in

March (see Fig. 24). Both types of GnRH-A injection protocols i.e. single or double, appeared to be about equally effective in elevating sex steroids.

The dosage of GnRH-A administered may play an important role in the quantitative aspects of sex steroid release. The GnRH-A dosage study indicated the presence of dose-dependent increases in plasma androgens. While all three doses of GnRH-A were effective in increasing plasma sex steroids, clearly a higher dose of GnRH-A resulted in higher absolute levels of plasma testosterone and 11-ketotestosterone compared to a lower dose of releasing hormone (see Figs. 21,22). A GnRH-A dose-dependent effect on plasma gonadotropin or gonadosomatic index has been previously reported in other teleost including brown trout (Crim and Cluett, 1974; Crim et al., 1981), and in Japanese medaka (Chan, 1977). In contrast, no dose-dependent effects of GnRH-A treatment on plasma gonadotropin or sex steroids were observed in goldfish (Lam et al., 1976; Peter, 1980), or coho salmon (Van der Kraak et al., 1983; 1984). The differences in the response to GnRH may be dependent on several factors, for example mode of GnRH application, type and dose of GnRH, temperature and the reproductive stages of the fish (Pankhurst et al., 1986).

Water temperatures may be an important factor contributing to the nature of the timing and the ultimate

level of circulating testosterone and 11-ketotestosterone in response to GnRH-A. In December when seawater temperatures were approximately 3°C, relatively warm compared to the 0.5°C temperatures recorded in March, the flounder steroid responses to GnRH-A were more rapid and higher absolute levels of plasma testosterone and 11-ketotestosterone were obtained. Still these increases in plasma steroids in flounder were relatively slow when compared with much faster plasma androgen elevations within an hr of GnRH-A treatment recorded for fish held at temperatures above 15°C (Takashima et al., 1984; Ngamrongchon et al., 1987; Pankhurst et al., 1986b). Increased testosterone, 11-ketotestosterone and gonadosomatic index were reported by Kime and Manning (1986) in mature carp following an increase in the temperature of acclimation from 25 to 30°C. Plasma estradiol-17ß was two-fold higher in females implanted with GnRH-A in October compared to females implanted in November for fish held at water temperatures of 9°C and 7°C, respectively. This suggests a greater stimulation of plasma estradiol-17ß at slightly warm or water temperatures.

In summary, both modes of GnRH-A administration were effective in elevating plasma sex steroids in adult winter flounder. In females, GnRH-A given by injection showed a short term effect, whereas hormone incorporated in a

cholesterol pellet produced a prolonged elevation of sex steroids. A low dose of GnRH-A injected into flounder remained detectable in circulation only for brief periods. In contrast, it is speculated that GnRH-A release into the plasma was slow in fish receiving GnRH-A by implant as reflected by the sustained levels of plasma sex steroids over prolonged periods of time. Interestingly, both modes of GnRH-A treatment were effective in accelerating oocyte development in the female flounder at the time of rapid gonadal recrudescence. In males, GnRH-A treatment advanced the onset of spermiation and also produced an increase in milt volume and number of spermatozoa.

CHAPTER 5

MANIPULATION OF THE SEASONAL REPRODUCTIVE CYCLE IN WINTER FLOUNDER USING GONADOTROPIC HORMONE-RELEASING HORMONE

5.1 INTRODUCTION

It has been suggested that the pituitary response to gonadotropin releasing hormone (GnRH) varies throughout the year in accordance with the extent of gonadal development (see review of Crim, 1982). In trout it was demonstrated that GnRH induced gonadotropin (GTH) release from the pituitary changes seasonally and that a greater response occurs in sexually mature individuals (Crim and Cluett, 1974; Weil et al., 1978). Similar seasonal fluctuations in pituitary responsivity to GnRH-A have been observed in goldfish (Lin et al., 1985b; Sokolowska et al., 1985). Still studies of the influence of GnRH or its analogs at the different stages of reproduction are rather limited.

GnRH and its analogs are effective agents for inducing ovulation and spawning in a variety of teleosts (see reviews of Crim et al., 1987; Peter et al., 1987). Most of these studies have been conducted with sexually maturing or mature teleosts when the gonads are nearly fully developed. Few studies have been conducted with teleosts

where hormone therapy is aimed at stimulating the early phases of gonad development including vitellogenesis or spermatogenesis. Control of these early stages of the reproductive cycle could be advantageous especially for teleosts held in captivity. Chan (1977) reported successful induction of vitellogenesis in sexually regressed goldfish following daily treatment of GnRH. Similarly, vitellogenesis and spermatogenesis were stimulated in Japanese ayu (Aida, 1983). In Atlantic salmon Crim and Glebe (1984) observed that GnRH-A implantation stimulates oocyte development during the vitellogenic phase of the reproductive cycle.

In the present studies the effects of GnRH-A treatment on adult winter flounder at different stages of the reproductive cycle were examined in two ways: 1) by examining changes in gonad growth and development and 2) by determining changes in plasma sex steroids which indicates the pituitary/gonadal response.

5.2 MATERIAL AND METHODS

5.2.1 Experimental Design

Six different experiments lasting 1-4 wk were conducted at various stages of the reproductive cycle with both female and male winter flounder (Table 7). The effect of [D-Ala^{6}Pro^{9}NHEt]LHRH (GnRH-A) treatment was

Table 7

Summary of experiments on GnRH-A manipulation of the winter flounder reproductive cycle

Exp	Date	Temp (°C)	Duration (wk)	Stage	Initial GSI	
					Females	Males
1	Sept-Oct	9.5	4	Early PREP 1	3.5	0.6
2	Nov	7.5	1	Mid PREP 1	7.4	14.0
3	Dec	4.0	2 (3)	Late PREP 1	12.6	13.2
4	Jan-Feb	0.5	4	PREP 2	15.0	8.6
5	Mar-Apr	0.5	4	PRES	16.0	8.6
6	Jun-July	10.0	2	POST	2.9	0.7

( ) indicates duration for male flounder. Data shown are mean values.

investigated by testing the seasonal responsiveness of winter flounder at different stages of gonadal development from September-July. The experimental fish were divided into groups of 5-9 fish each and treated as follows: (1) A control group of fish were sacrificed prior to each study to gain baseline information on the initial extent of reproductive development (INITIAL); (2) intact control fish (INTACT), only blood samples were taken from these fish; otherwise, they remained untouched; (3) sham hormone cholesterol pellet implant control (SHAM IMP); (4) 100 μg GnRH-A incorporated into a cholesterol pellet (GnRH-A IMP); (5) three sham control saline injections per week (SAL INJ); (6) three 20 μg/kg GnRH-A in saline injections (e.g. Monday, Wednesday, Friday) per week (GnRH-A INJ).

5.2.2 Blood Sampling and Hormone Analysis

In most experiments, blood samples were collected weekly following the last injection as described in general methods and materials (see chapter 2, section 2.6). Plasma levels of estradiol-17ß and testosterone were determined by iodinated radioimmunoassay and 11-ketotestosterone by tritiated radioimmunoassay (see chapter 2, section 2.7)

5.3 RESULTS

5.3.1 GnRH-A Treatment in September During Early Gonadal Recrudescence (PREP 1).

Females

At the beginning of this experiment in September the females were in the early stages of ovarian recrudescence (see Table 7, experiment 1, initial gonadosomatic index 3.5). By the end of this 4-wk experiment, no significant increases in gonadosomatic index and oocyte diameter values were observed in intact control females compared with initial control females. While no significant hormone treatment effects or seasonal changes were found in hepatosomatic index and dry matter values during this fall experimental period both the gonadosomatic index and oocyte diameter were significantly ($P<0.05$) increased in female flounder implanted with GnRH-A compared with SHAM IMP controls (Table 8). Figure 29 shows the oocyte size-class frequency distribution in controls (INITIAL, INTACT, SHAM IMP, SAL INJ) and GnRH-A treated females. Chi-square analysis revealed that the distribution of various oocyte-size classes was advanced in GnRH-A treated females compared to the control females (SHAM IMP). No significant changes were observed in gonadosomatic index and oocyte diameter in GnRH-A INJ females compared to SAL INJ

Table 8

Effects of GnRH-A treatment beginning September on gonadosomatic index (GSI), hepatosomatic index (HSI), oocyte dry matter (DM) and oocyte diameter (OCDM) in female winter flounder during early gonadal recrudescence (PREP 1)

Treatment	N	GSI	HSI	DM	OCDM (μm)
INITIAL	6	3.50^{a} ±0.43	1.76^{a} ±0.23	21.16^{a} ±0.49	251.79^{a} ±13.17
INTACT	5	4.37^{ab} ±0.74	1.86^{ab} ±0.08	21.57^{a} ±0.71	278.51^{ab} ±24.77
SHAM IMP	5	4.68^{ab} ±0.93	1.90^{ab} ±0.16	23.34^{a} ±1.34	305.16^{b} ±11.56
GnRH-A IMP	5	7.35^{c} ±1.64	2.21^{b} ±0.14	22.61^{a} ±1.60	413.03^{c} ±6.18
SAL INJ	5	5.09^{ab} ±0.65	2.12^{ab} ±0.07	22.71^{a} ±1.10	289.20^{ab} ±19.02
GnRH-A INJ	4	6.16^{b} ±0.49	2.39^{b} ±0.28	21.40^{a} ±1.08	329.19^{b} ±12.39

All values are Mean ± SEM; significance ($P<0.05$) indicated by different letters. OCDM initial controls were taken at the beginning of the experiment, whereas OCDM for other treatments were taken at the end of the experiment.

Figure 29. Oocyte-size class frequencies for female winter flounder during the early gonadal recrudescence (PREP 1) period following GnRH-A injection (INJ) or implantation (IMP). OCDM, mean oocyte diameter in μm.

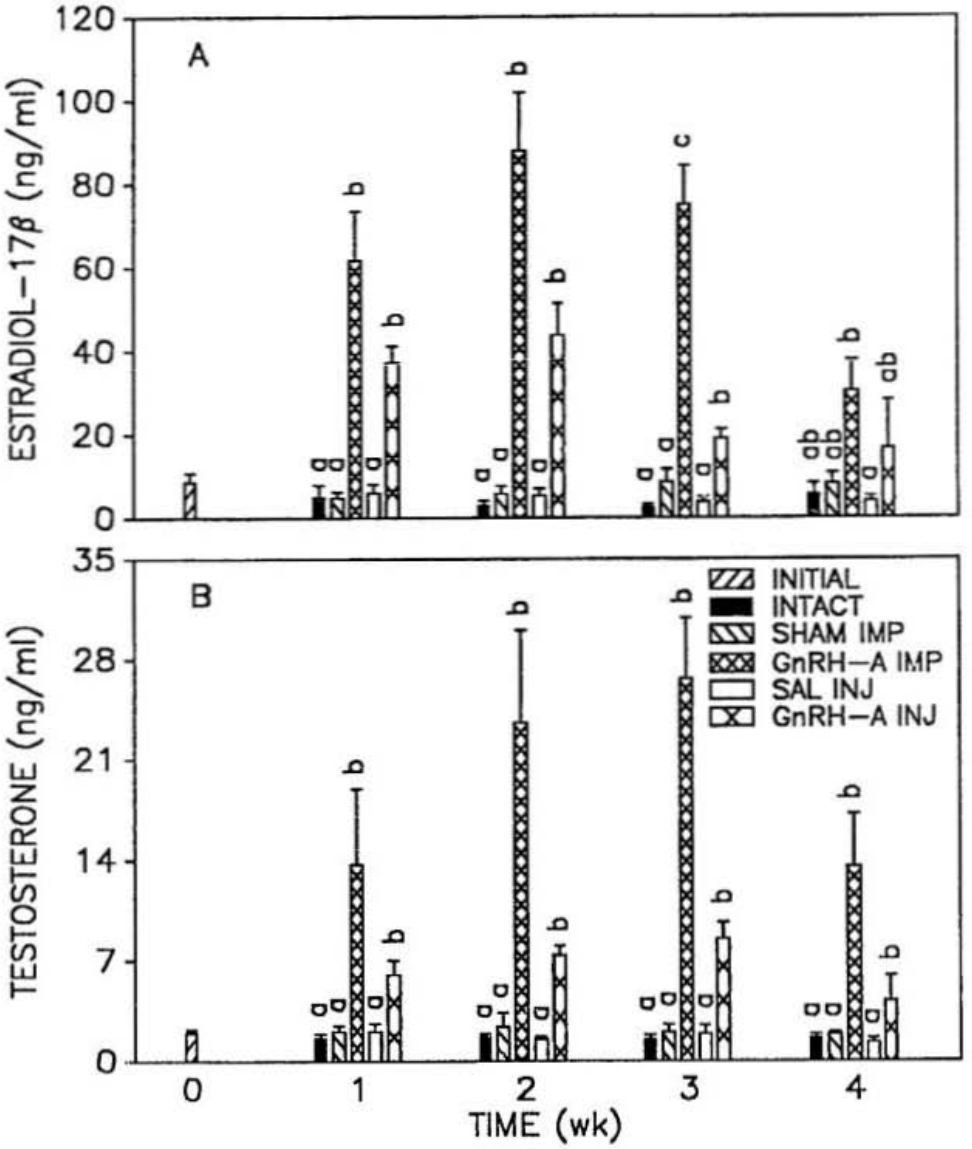
INITIAL
OCDM 251 μm
INTACT
OCDM 278 μm
SHAM IMP
OCDM 305 μm
SAL INJ
OCDM 289 μm
GnRH–A IMP
OCDM 413 μm
GnRH–A INJ
OCDM 329 μm
PERCENT FREQUENCY
OOCYTE DIAMETER (MM)

females. At the beginning of the experiment, oocyte frequency distribution for the initial control females showed that the majority of the oocyte size-class ranged from 210 and 300 μm. At the end of the 4-wk experimental period, although the majority of the oocyte size-classes remained between 210 and 270 μm, larger oocyte size classes ranging between 300 and 410 μm were found indicating progressive seasonal ovarian development.

By the end of the 4-wk study period both modes of GnRH-A administration led to a significant ($P<0.05$) alteration in the oocyte size-class frequency distribution compared to paired controls (SHAM IMP, SAL INJ). In both groups of paired control females the majority of oocyte size-class ranged between 270-300 μm. On the other hand, after GnRH-A treatment the frequency of the larger-sized oocyte classes increased; the majority of oocyte size-class ranged from 300 to 480 μm in both groups receiving GnRH-A (GnRH-A IMP and GnRH-A INJ).

Both estradiol-17ß (8 ng/ml) and testosterone (2 ng/ml) levels were low and remained unchanged for the duration of the experiment in all three groups of control (INTACT, SHAM IMP, SAL INJ) females (Fig. 30A,B). However, both forms of GnRH-A treatment stimulated long term elevations in plasma sex steroid levels; whereas estradiol-17ß declined to control levels by 4 wk, testosterone remained significantly elevated at the end of

Figure 30. Plasma (A) estradiol-17β and (B) testosterone levels at 1,2,3,4 wk following IP injections or IM pellet implantation of GnRH-A during early ovarian recrudescence. Control females were given either saline injections or a blank cholesterol pellet. Bars and lines represent means ± SEM of 4-6 fish per group. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time. Time=0 indicates plasma sex steroid levels in initial controls at the beginning of the experiment.


A
B
ESTRADIOL-17β (ng/ml)
TESTOSTERONE (ng/ml)
TIME (wk)
INITIAL
INTACT
SHAM IMP
GnRH-A IMP
SAL INJ
GnRH-A INJ
0
20
40
60
80
100
120
7
14
21
28
35
1
2
3
4

the 4 wk experimental period. Although plasma steroid levels tended to be higher in females given GnRH-A by implant vs injection, only at week 3 was plasma estradiol-17ß (75 ng/ml) significantly ($P<0.05$) higher between these two groups of GnRH-A treated females.

Males

In males, testicular recrudescence begins in August and progresses very rapidly (see Chapter 3). At the beginning of this experiment in September (see Table 7 experiment 1), the gonadosomatic index was low (Table 9) and the testes of these males were either grey or dark in colour. On the final day of the experiment, the gonadosomatic index of the intact control males were significantly ($P<0.05$) increased compared with initial control males, an indication of seasonal testicular development. However, because the gonadosomatic index of GnRH-A injected males was significantly ($P<0.05$) increased compared with control (SAL INJ) males, the data suggests that testicular development was accelerated by GnRH-A treatment. No spermiation was evident at this stage of the reproductive cycle in control males (INTACT, SAL INJ) or following GnRH-A treatment. In addition, no significant seasonal or hormone treatment effects were observed on hepatosomatic index during this experiment.

Plasma testosterone (1-2 ng/ml) and 11-

Table 9

Effects of GnRH-A treatment beginning September on gonadosomatic index (GSI) and hepatosomatic index (HSI) in male winter flounder during early gonadal recrudescence (PREP 1)

Treatment	N	GSI	HSI
INITIAL	6	0.67^{a} ±0.11	1.55^{a} ±0.18
INTACT	9	4.13^{bc} ±1.47	1.29^{a} ±0.08
SAL INJ	8	2.02^{ab} ±0.69	1.40^{a} ±0.09
GnRH-A INJ	8	5.48^{c} ±1.00	1.33^{a} ±0.12

All values are Means ± SEM; significance ($P \leq 0.05$) indicated by different letters.

ketotestosterone (5-8 ng/ml) both remained low in control males (INTACT and SAL INJ) throughout this experimental period (Fig. 31A,B). In contrast, plasma androgen levels in the hormone injected males were significantly ($P<0.05$) increased at wk 1. By the end of the experiment despite continuing GnRH-A treatment, the levels of both plasma testosterone and 11-ketotestosterone declined, although the hormone levels remained higher compared with control males.

5.3.2 GnRH-A Treatment in November During Mid-gonadal Recrudescence (PREP 1).

Females

This particular experiment was terminated after just 1 week due to a high mortality rate of flounder (see Table 7, experiment 2). In females, in November, the gonadosomatic index (7.4) of initial controls was twice as high compared with females in September. Because of the high number of mortalities the gonadosomatic index and plasma sex steroids from INTACT, SHAM IMP and SAL INJ females were pooled as controls. The ovaries were undergoing rapid development since the gonadosomatic index of pooled controls was significantly ($P<0.05$) higher than the gonadosomatic index of initial control females recorded at the beginning of the experiment (Table 10). No

Figure 31. Plasma (A) testosterone and (B) 11-keto-testosterone levels at 1,2,3,4 wk following IP injections of GnRH-A during early testicular recrudescence. Control males were given saline injections. Bars and lines represent means ± SEM of 6-9 fish per group. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time. Time=0 indicates plasma sex steroid levels in initial controls at the beginning of the experiment.

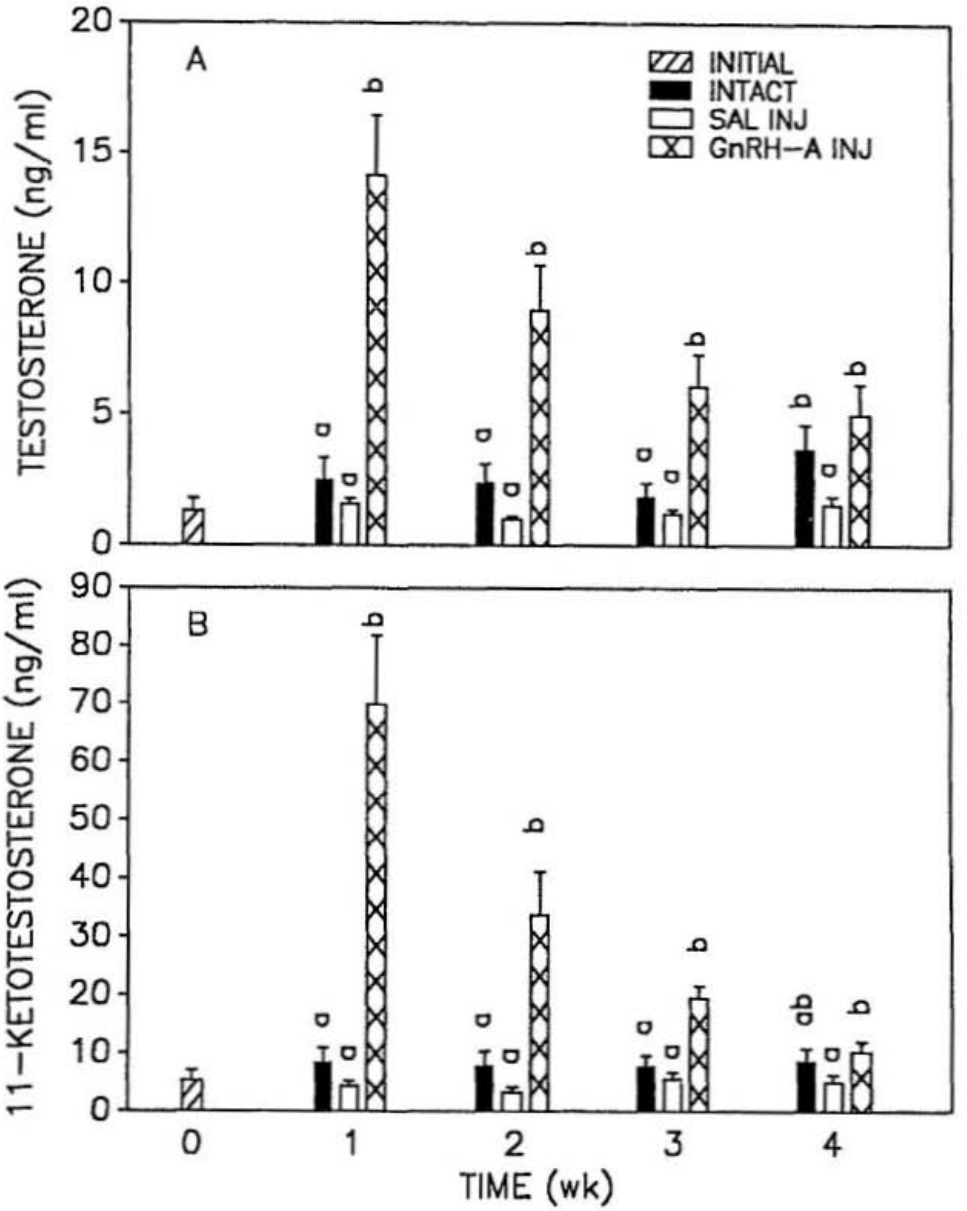

A
B
INITIAL
INTACT
SAL INJ
GnRH–A INJ
TESTOSTERONE (ng/ml)
11–KETOTESTOSTERONE (ng/ml)
TIME (wk)
0
1
2
3
4
a
b
ab

Table 10

Effects of GnRH-A treatment beginning November on gonadosomatic index (GSI) in female winter flounder during mid-gonadal recrudescence (PREP 1)

Treatment	N	GSI
INITIAL	7	7.39[a] ±0.39
CONTROLS	5	11.15[b] ±0.61
GnRH-A IMP	3	11.37[b] ±0.59
GnRH-A INJ	5	12.72[b] ±0.58

All values are Means ± SEM; significance ($P<0.05$) indicated by different letters. CONTROLS consisted of INTACT, SHAM IMP and SAL INJ females.

additional acceleration of gonadal development was observed after just 1 week of GnRH-A treatment.

Both injection and the pellet implant of GnRH-A stimulated a rise in plasma estradiol-17ß compared with control females (Fig. 32A). However, plasma testosterone was significantly elevated only by GnRH-A injection (Fig. 32B).

Males

The testes of males in November were changing to a whitish colour, an indication of the onset of spermatogenesis, compared with the grey, regressed testes found in males in September (see Table 7, experiment 2). Although GnRH-A treatment did not significantly increase the gonadosomatic index of males (Table 11), the hormone treatment advanced the onset of spermiation evidenced by small amounts of milt (<50 µl) expressed from some treated males after gentle stroking of the abdomen.

In control males (INTACT, SHAM INJ and SAL INJ), the steroid hormone levels remained low (testosterone, 5 ng/ml; 11-ketotestosterone, 20 ng/ml) for the duration of this short experimental period (Fig. 33A,B). By contrast, GnRH-A treatment significantly increased plasma levels of both testosterone (15-30 ng/ml) and 11-ketotestosterone (140-160 ng/ml) in hormone treated males with the greatest elevation of plasma testosterone following GnRH-A

Figure 32. Plasma (A) estradiol-17β and (B) testosterone levels at 1 wk following IP injections or IM pellet implantation of GnRH-A during mid-ovarian recrudescence. Controls (pooled from INTACT, SHAM IMP and SAL INJ females) were given either saline injections or a blank cholesterol pellet. Bars and lines represent means ± SEM of 4-7 fish per group. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time. Time=0 indicates plasma sex steroid levels in initial controls at the beginning of the experiment.

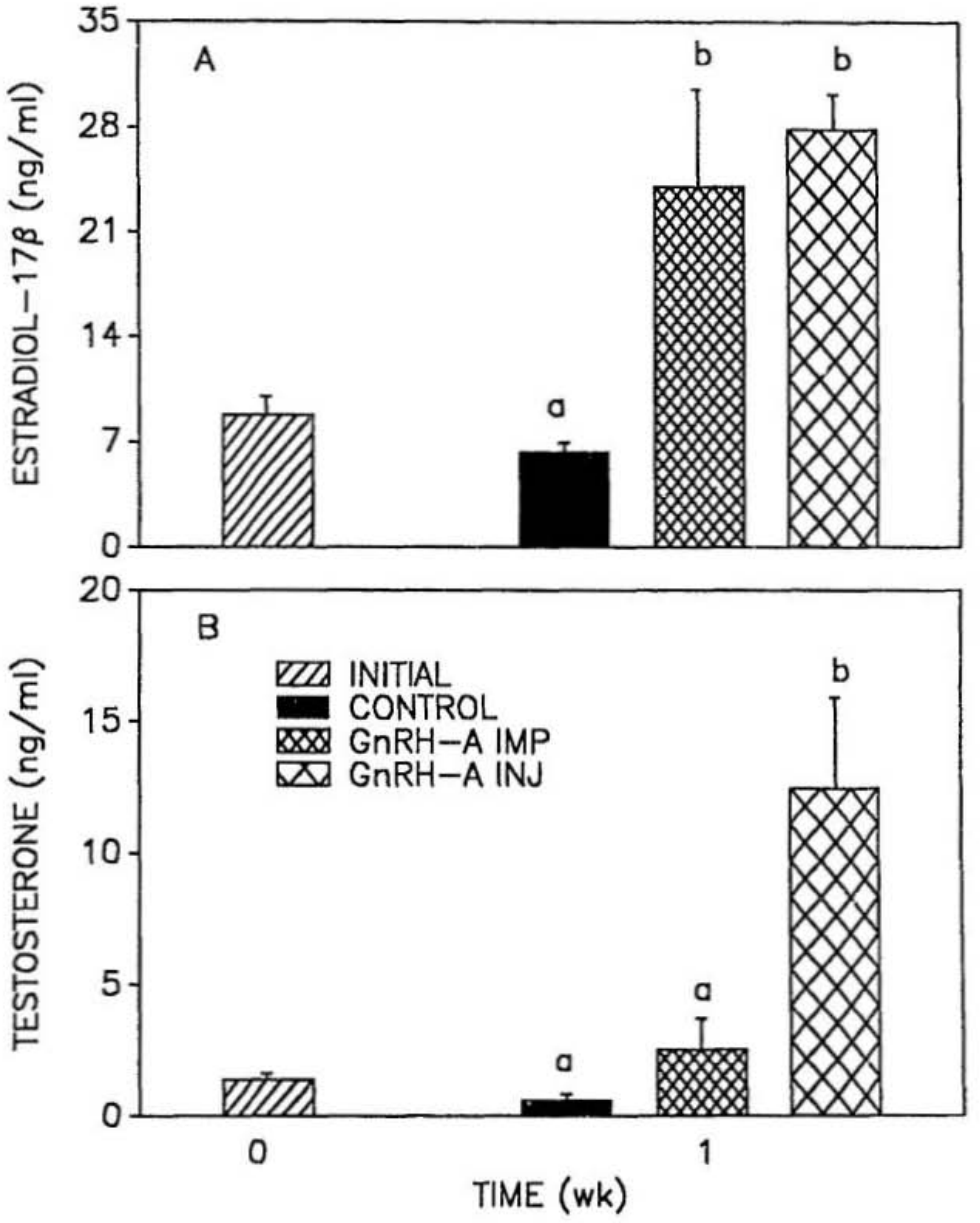

A
B
ESTRADIOL-17β (ng/ml)
TESTOSTERONE (ng/ml)
35
28
21
14
7
0
20
15
10
5
0
a
b
b
a
a
b
INITIAL
CONTROL
GnRH-A IMP
GnRH-A INJ
0
1
TIME (wk)

Figure 33. Plasma (A) testosterone and (B) 11-keto-testosterone levels at 1 wk following IP injection or IM pellet implantation of GnRH-A during mid-testicular recrudescence (PREP 1). Control males were given either saline injections or a blank cholesterol pellet. Bars and lines represent means ± SEM of 4-7 fish per group. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time. Time=0 indicates plasma sex steroid levels in initial controls at the beginning of the experiment.

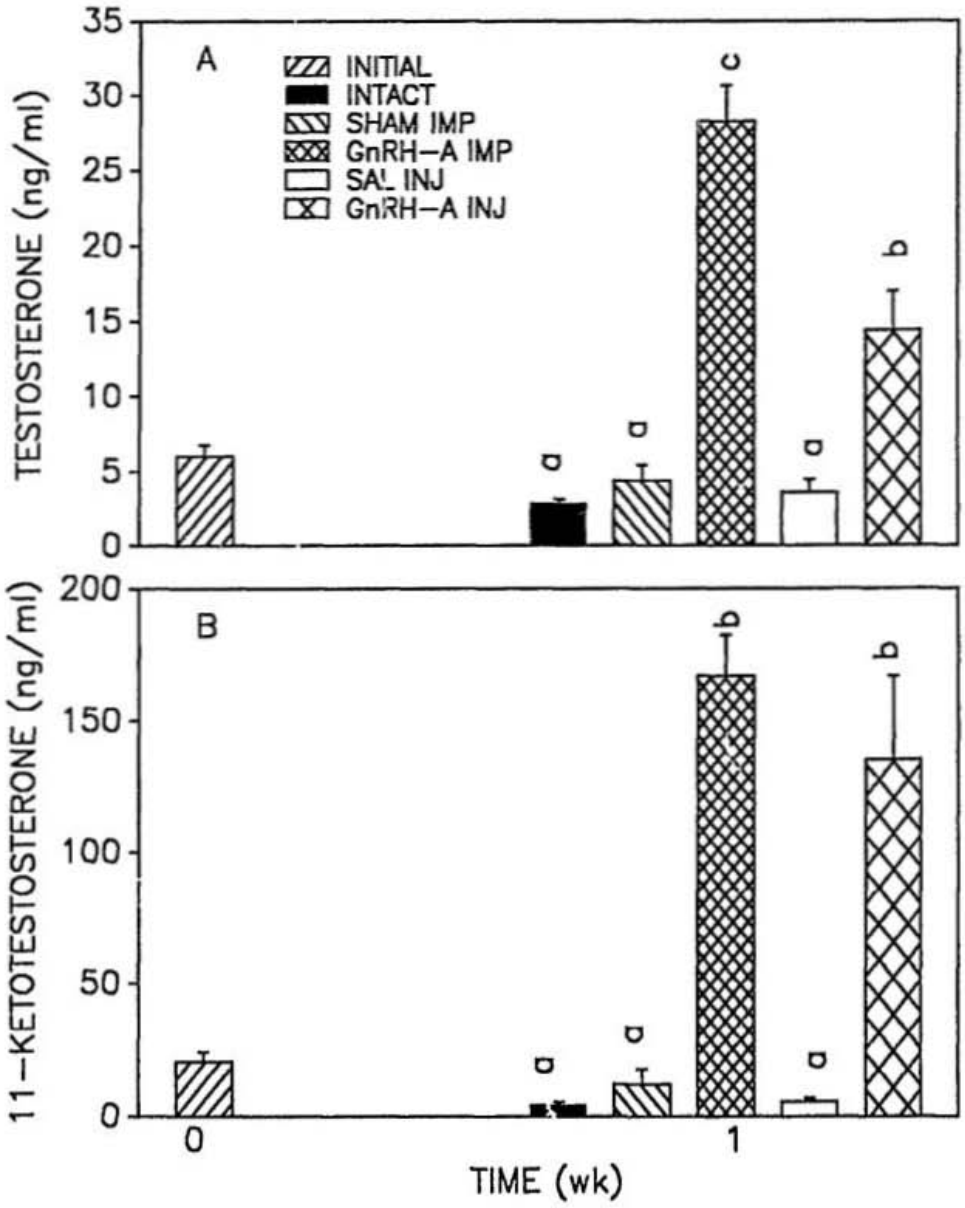

A
INITIAL
INTACT
SHAM IMP
GnRH–A IMP
SAL INJ
GnRH–A INJ
TESTOSTERONE (ng/ml)
35
30
25
20
15
10
5
0
a
a
c
a
b
B
11–KETOTESTOSTERONE (ng/ml)
200
150
100
50
0
a
a
b
a
b
0
1
TIME (wk)

Table 11

Effects of GnRH-A treatment beginning November on gonadosomatic index (GSI) in male winter flounder during mid-recrudescing period (PREP 1)

Treatment	N	GSI	Number of spermiating males
CONTROLS	7	11.71^{b} ±0.48	0 (0)
GnRH-A IMP	5	12.04^{ab} ±0.63	2 (40)
GnRH-A INJ	4	14.05^{ab} ±1.47	2 (50)

All values are Means ± SEM; significance ($P \leq 0.05$) indicated by different letters. () indicates percent spermiation. CONTROLS group consisted of INTACT, SHAM IMP and SAL INJ males.

application by implantation.

5.3.3 GnRH-A Treatment in December During Late Gonadal Recrudescence (PREP 1).

Females

At the end of this two week period (see Table 7, experiment 3), there were no significant differences in gonadosomatic index and hepatosomatic index among the groups of experimental females (Table 12). However, oocyte dry matter was significantly ($P<0.05$) reduced in females injected with GnRH-A compared with saline control females.

Plasma estradiol-17 (6 ng/ml) and testosterone (2 ng/ml) levels remained steady in all control females (INTACT, SHAM IMP, SAL INJ) throughout the study period. In contrast, both modes of application of GnRH-A significantly elevated plasma estradiol-17ß levels (20-30 ng/ml) which were sustained throughout the experiment (Fig. 34A). On the other hand, differences in plasma testosterone in females were observed depending upon the methods of GnRH-A administration. While plasma testosterone levels (18 ng/ml) were elevated in GnRH-A injected females at week 1 and were sustained to the end of the experiment (Fig. 34B), plasma testosterone increases were delayed until week 2 in GnRH-A implanted females (11 ng/ml).

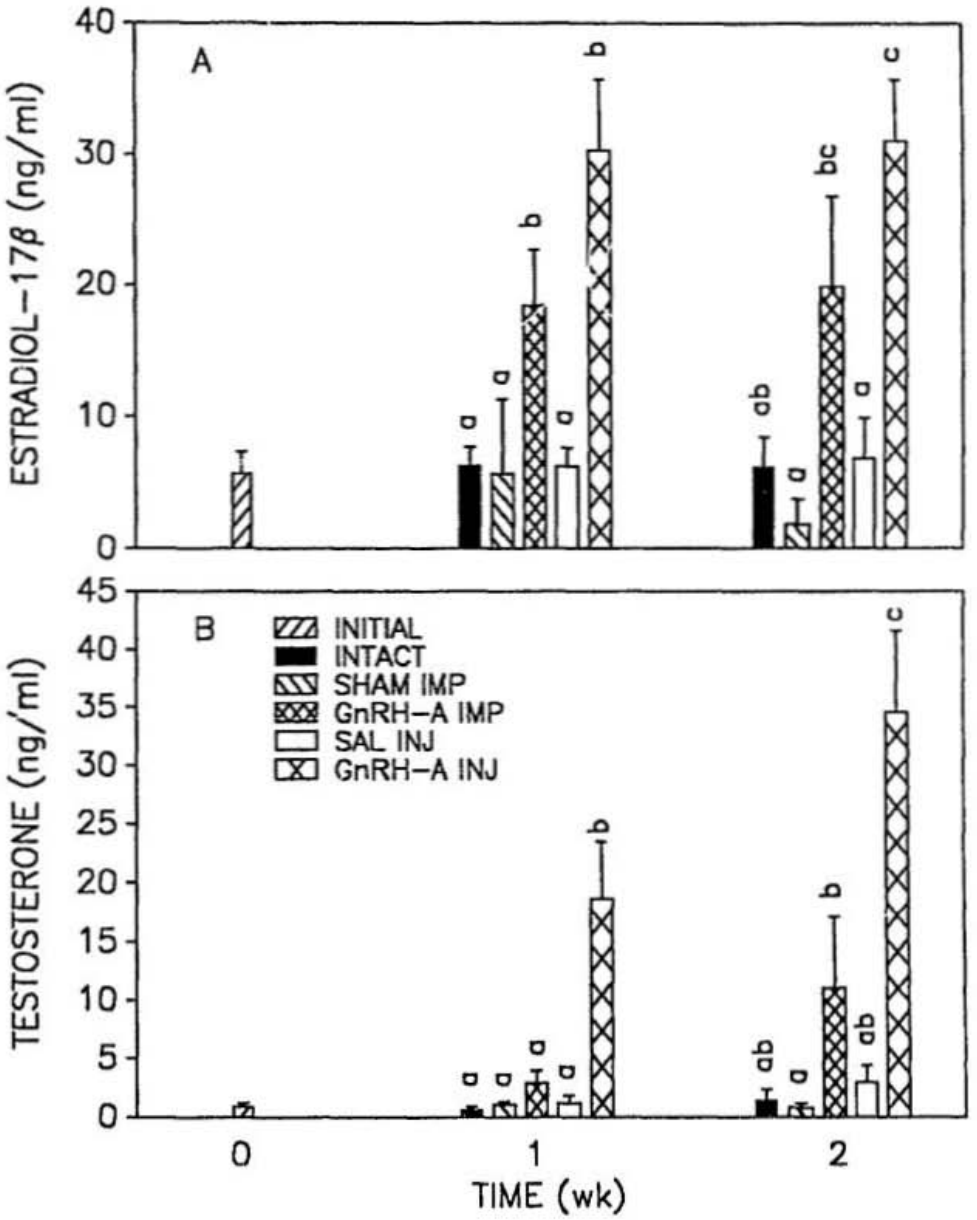
Table 12

Effects of GnRH-A treatment beginning December on gonadosomatic index (GSI), hepatosomatic index (HSI), oocyte dry matter (DM) in female winter flounder during late recrudescing period (PREP 1)

Treatment	N	GSI	HSI	DM
INITIAL	6	12.62^{a} ±1.23	2.47^{a} ±0.08	31.67^{ab} ±0.29
INTACT	4	13.57^{a} ±0.80	2.45^{a} ±0.15	32.06^{abc} ±1.18
SHAM IMP	5	13.71^{a} ±1.44	2.25^{a} ±0.14	34.40^{cd} ±0.56
GnRH-A IMP	5	14.20^{a} ±1.21	2.45^{a} ±0.13	33.23^{bcd} ±1.02
SAL INJ	5	14.45^{a} ±1.64	2.52^{a} ±.08	34.61^{d} ±1.08
GnRH-A INJ	4	13.79^{a} ±0.51	2.54^{a} ±0.10	30.55^{a} ±0.62

All values are Means ± SEM; significance ($P \leq 0.05$) indicated by different letters.

Figure 34. Plasma (A) estradiol-17β and (B) testosterone levels at 1,2 wk following IP injections or pellet implantation of GnRH-A during the period of late-ovarian recrudescence (PREP 1). Control females were given either saline injections or a blank cholesterol pellet. Bars and lines represent means ± SEM of 4-7 fish per group. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time. Time=0 indicates plasma sex steroid levels in initial controls at the beginning of the experiment.


A
B
ESTRADIOL-17β (ng/ml)
TESTOSTERONE (ng/ml)
TIME (wk)
INITIAL
INTACT
SHAM IMP
GnRH-A IMP
SAL INJ
GnRH-A INJ
0
10
20
30
40
0
5
10
15
20
25
30
35
40
45
0
1
2
a
a
b
a
b
ab
a
bc
a
c
a
a
a
b
a
b
ab
a
b
ab
c

Males

The testes of male flounder were white in colour at this stage of development but no spermiating males were observed when the experiment was initiated (see Table 7, experiment 3). On the final day of this study, no significant changes in gonadosomatic index or hepatosomatic index among control males (INTACT, SHAM IMP, SAL INJ) occurred (Table 13). When the experiment was terminated, at least 50% of the males in the control groups had reached the spermiation stage, whereas 100% of the GnRH-A treated males were in spermiating condition. GnRH-A stimulation of spermiation resulted in a more dilute milt and collection of greater numbers of spermatozoa (Table 14).

Plasma testosterone (3 ng/ml) and 11-ketotestosterone (10 ng/ml) in intact and sham control males remained low or declined by the end of the experiment (Fig. 35A,B). Although the steroid profiles differed, both modes of GnRH-A application stimulated significant increases in plasma testosterone and 11-ketotestosterone levels in male flounder. While after 1 wk the highest plasma steroid levels were observed in males receiving GnRH-A by injection, plasma testosterone and 11-ketotestosterone remained elevated to the end of the study period in all males receiving GnRH-A treatment.

Table 13

Effects of GnRH-A treatment beginning December on gonadosomatic index (GSI) and hepatosomatic index (HSI) in male winter flounder during late gonadal recrudescence (PREP 1)

Treatment	N	GSI	HSI	Number of spermiating males
INITIAL	6	13.24^{b} ±0.50	1.32^{a} ±0.07	0 (0)
INTACT	4	11.22^{ab} ±1.28	1.52^{ab} ±0.04	2 (50)
SHAM IMP	4	10.49^{ab} ±1.03	1.56^{ab} ±0.13	3 (75)
GnRH-A IMP	5	ND	1.46^{ab} ±0.08	5 (100)
SAL INJ	5	10.10^{a} ±0.47	1.61^{b} ±0.07	3 (60)
GnRH-A INJ	5	ND	1.48^{ab} ±0.07	5 (100)

Values are Means ± SEM; significance ($P \leq 0.05$) indicated by different letters. () indicates number spermiation. GSI was not determined (ND) in spermiating males.

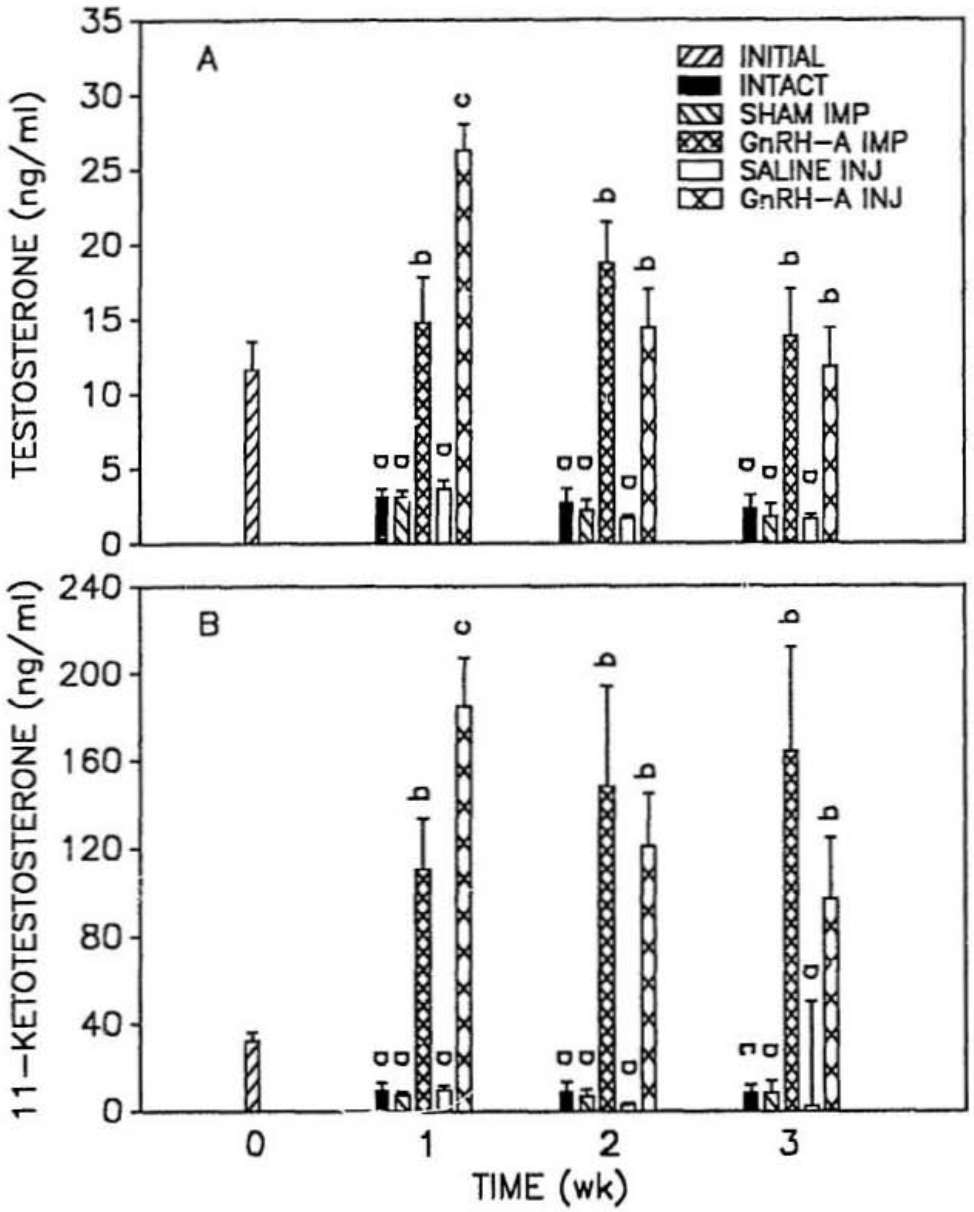
Table 14

Effects of GnRH-A treatment on percent spermatocrit, milt volume and total sperm count on the final day of experiment in male winter flounder

Treatment	N	Spermatocrit (%)	Milt vol(ml)	Total sperm count (x 10^9)
GnRH-A IMP	3	44.1 ± 5.3[a]	2.3[a] ±0.3	27 ± 2[a]
GnRH-A INJ	5	54.9 ± 3.5[a]	4.8[a] ±3.2	61 ± 38[a]

All values are Means ± SEM; significance ($P<0.05$) indicated by different letters. Control males (INTACT, SHAM IMP, SAL INJ) did not spermiate or milt is not enough for determination spermatocrit and sperm count.

Figure 35. Plasma (A) testosterone and (B) 11-keto-testosterone levels at 1,2,3 wk following IP GnRH-A injections or IM cholesterol pellet implantation of GnRH-A during the late stages of testicular recrudescence (PREP 1). Control males were given either saline injections or a blank cholesterol pellet. Bars and lines represent means ± SEM of 4-7 fish per group. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time. Time=0 indicates plasma sex steroid levels in initial controls at the beginning of the experiment.


A
B
INITIAL
INTACT
SHAM IMP
GnRH–A IMP
SALINE INJ
GnRH–A INJ
TESTOSTERONE (ng/ml)
11–KETOTESTOSTERONE (ng/ml)
TIME (wk)
0
1
2
3
35
30
25
20
15
10
5
0
240
200
160
120
80
40
0
a
b
c
d

5.3.4 GnRH-A Treatment in January During the Period of Gonadal Maintenance (PREP 2).

Females

At the termination of this 4-week experiment in winter (see Table 7, experiment 4), although gonadosomatic index and dry matter values remained unchanged in intact control females compared with initial control females, there was evidence of significant stimulation of gonadal development by GnRH-A treatment (Table 15). In females injected with GnRH-A, gonadosomatic index significantly increased and dry matter decreased compared with paired saline control females, but no significant differences were observed in these values after GnRH-A implantation of female flounder. No significant hormone treatment effects were observed on hepatosomatic index. Oocyte diameter increased in GnRH-A implanted females but no change in oocyte diameter occurred in response to GnRH-A injection (Table 15). Oocyte size-class frequency distributions (OSCF) indicated that a narrow range of oocyte sizes occurred in all groups of females at this time of the reproductive cycle (Fig. 36). No significant changes in OSCF were observed in control females when the experiment was terminated. In contrast, GnRH-A treatment significantly ($P<0.05$) shifted the OSCF distribution by the end of the study. Prior to GnRH-A treatment the oocyte size-class ranged between 450-

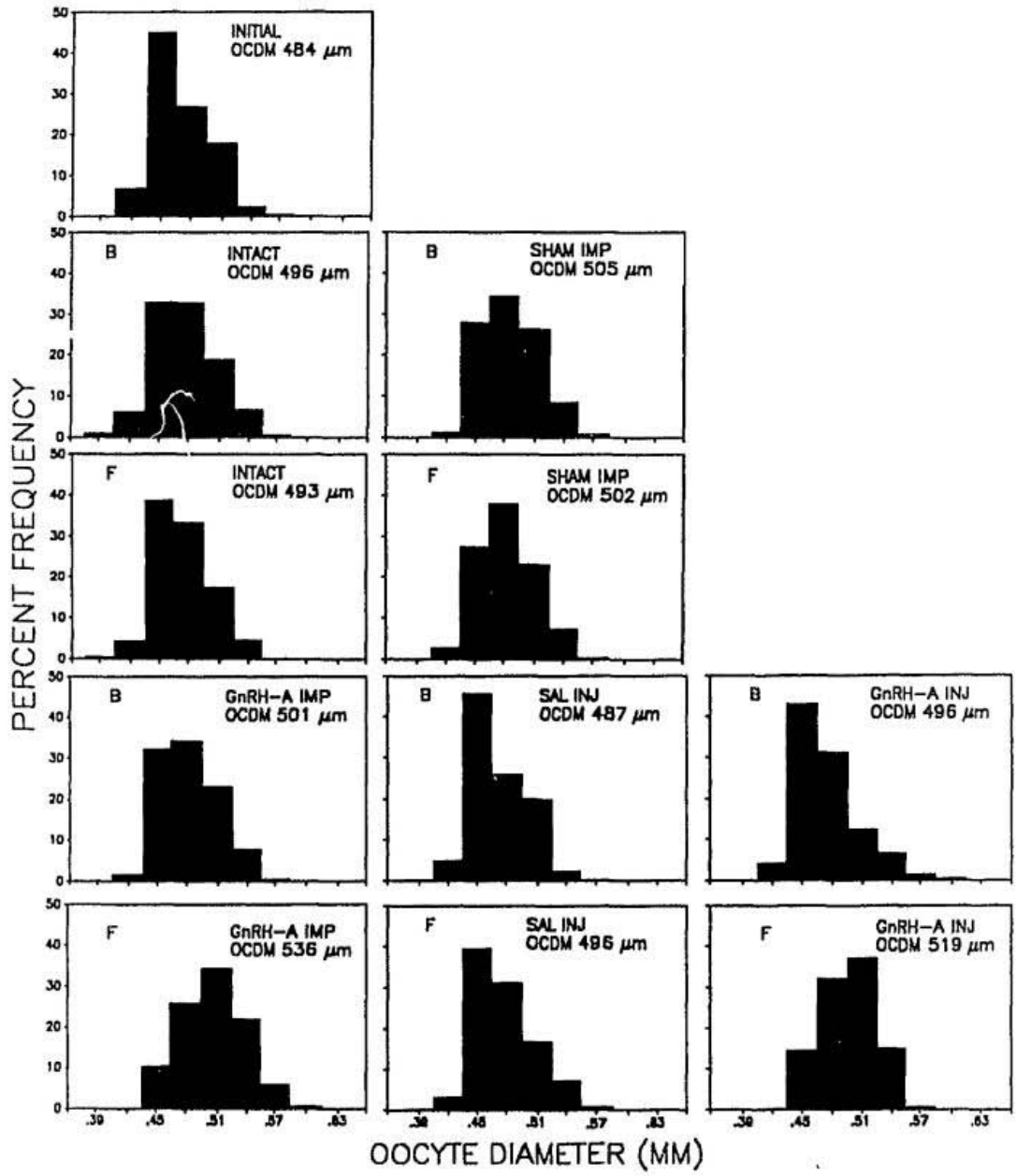
Table 15

Effects of GnRH-A treatment beginning January on gonadosomatic index (GSI), hepatosomatic index (HSI), oocyte dry matter (DM) oocyte diameter (X1 and X2) during the period of ovarian maintenance (PREP 2)

Treatment	N	GSI	HSI	DM	X_1 (μm)	X_2 (μm)
INITIAL	6	14.93^{a} ±1.17	2.16^{a} ±0.07	31.56^{c} ±0.23	484.07 ±5.29	-
INTACT	7	15.17^{a} ±0.75	2.16^{a} ±0.08	31.23^{bc} ±0.25	496.19^{a} ±9.15	493.46^{a} ±7.66
SHAM IMP	7	16.89^{ab} ±1.28	2.16^{a} ±0.10	30.99^{bc} ±0.20	505.07^{a} ±8.07	502.29^{a} ±7.00
GnRH-A IMP	7	19.74^{bc} ±1.47	2.49^{a} ±0.09	28.97^{ab} ±0.52	501.75^{a} ±6.54	536.26^{b} ±9.12
SALINE INJ	6	14.65^{a} ±1.11	2.31^{a} ±0.12	30.75^{bc} ±0.64	487.76^{a} ±7.54	496.64^{a} ±8.22
GnRH-A INJ	6	22.93^{c} ±2.82	2.41^{a} ±0.14	27.48^{a} ±1.58	496.53^{a} ±11.30	519.09^{a} ±7.43

All values are Means ± SEM; significance ($P \leq 0.05$) indicated by different letters. X1 = Oocyte diameter at beginning of experiment, X2 = Oocyte diameter at the end of experiment.

Figure 36. Oocyte-size class frequencies for female winter flounder during the period of continued slow ovarian growth and maintenance (PREP 2) following GnRH-A injection (INJ) or implantation (IMP). (B) and (F) refer to beginning and final day of experiment, respectively. OCDM, mean oocyte diameter in μm.


INITIAL
OCDM 484 µm
B
INTACT
OCDM 496 µm
B
SHAM IMP
OCDM 505 µm
F
INTACT
OCDM 493 µm
F
SHAM IMP
OCDM 502 µm
B
GnRH-A IMP
OCDM 501 µm
B
SAL INJ
OCDM 487 µm
B
GnRH-A INJ
OCDM 496 µm
F
GnRH-A IMP
OCDM 536 µm
F
SAL INJ
OCDM 496 µm
F
GnRH-A INJ
OCDM 519 µm
PERCENT FREQUENCY
OOCYTE DIAMETER (MM)

480 μm for both groups of females receiving hormone application, however; on the final day of the experiment the dominant size class ranged from 510-570 μm.

The oocyte data in Table 16 indicates that a shift in germinal vesicle position (see chapter 2, section 2.3.2) was observed during this experiment particularly in females receiving GnRH-A treatment by injection. At autopsy, partial ovulation was observed in just one female from the GnRH-A injected group achieving gonadosomatic index and dry matter values of at 35 and 20%, respectively.

Plasma estradiol-17ß (10 ng/ml) and testosterone (5 ng/ml) levels in both intact and saline control females remained stable throughout the experiment; interestingly, plasma estradiol-17ß (20 ng/ml) in sham implanted control females (increased significantly at wk 2 compared to values at wk 1 remaining elevated until the end of the experiment (Fig. 37A,B). While both modes of GnRH-A application stimulated rapid ($P<0.05$) increases in plasma estradiol-17ß (40-60 ng/ml) and testosterone (30 ng/ml) within wk 1, plasma estradiol-17ß and testosterone remained elevated to the end of the experiment.

Males

This experiment with prespermiating males was conducted when the ambient seawater temperatures were low,

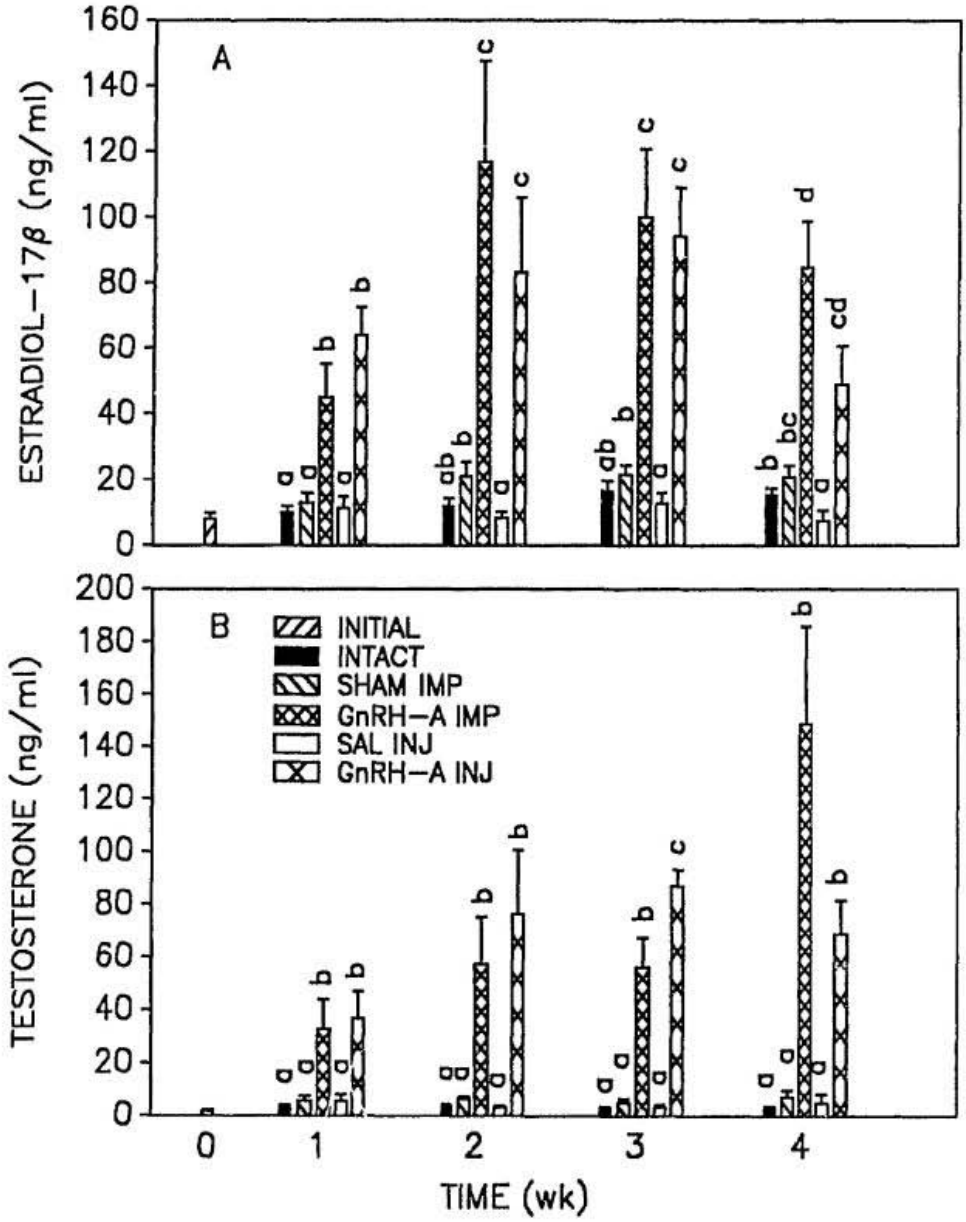
Table 16

Mean germinal vesicle (GV) stage in oocytes from female winter flounder during the period of ovarian maintenance

	GV Stage	
Treatment	T1	T2
INITIAL	1	-
INTACT	1	1.3
SHAM IMP	1	1.6
GnRH-A IMP	1	1.9
SAL INJ	1	1.5
GnRH-A INJ	1	2.5

T1 : Prior to experimentation
T2 : On the final day of the 4 wk experiment

Figure 37. Plasma (A) estradiol-17β and (B) testosterone levels at 1,2,3,4 wk following IP injections or IM pellet implantation of GnRH-A during the period of slow ovarian growth and maintenance (PREP 2). Control males were given either saline injections or a blank cholesterol pellet. Bars and lines represent means ± SEM of 5-7 fish per group. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time. Time=0 indicates plasma sex steroid levels in initial controls at the beginning of the experiment.


A
B
ESTRADIOL-17β (ng/ml)
TESTOSTERONE (ng/ml)
TIME (wk)
INITIAL
INTACT
SHAM IMP
GnRH-A IMP
SAL INJ
GnRH-A INJ
0
20
40
60
80
100
120
140
160
180
200
0
1
2
3
4

approximately 4 months prior to the breeding season in May/June (see Table 7, experiment 4). At the beginning of this experiment, none of the males were in spermiating condition and after 4 wk at the time of autopsy, no significant changes were observed in gonadosomatic index of nonspermiating control (INTACT, SHAM IMP, SAL INJ) males (Table 17). However, some control males were spermiating by the end of the study indicating progressive winter testicular development. Both methods of GnRH-A treatment increased the percentage of males reaching spermiating condition. Both the increase in milt volume and the lowering of the spermatocrit of hormone treated males indicated that GnRH-A treatment induces testes hydration (Table 18).

Both plasma testosterone (2.5-4.0 ng/ml) and 11-ketotestosterone (10-25 ng/ml) in all control groups of males remained steady or declined by the end of the experiment (Fig. 38A,B). In contrast, plasma androgen levels were increased by GnRH-A treatment; whereas the increase in both testosterone and 11-ketotestosterone were sustained until the end of the experiment in males receiving a GnRH-A implant, the elevated steroid hormone values first rose and then began declining towards the end of the experiment in GnRH-A injected males.

Table 17

Effects of GnRH-A treatment beginning January on gonadosomatic index (GSI) and hepatosomatic index (HSI) in male winter flounder during the period gonadal maintenance (PREP 2)

Treatment	N	GSI	HSI	Number of spermiating male
INITIAL	6	8.65^{a} ±0.69	1.10^{a} ±0.06	0 (0)
INTACT	7	10.09^{ab} ±0.15	1.23^{ab} ±0.05	4 (56)
SHAM IMP	7	10.77^{ab} ±0.58	1.33^{bc} ±0.06	3 (43)
GnRH-A IMP	6	ND	1.23^{ab} ±0.09	6 (100)
SAL INJ	7	9.59^{a} ±0.72	1.46^{c} ±0.05	3 (43)
GnRH-A INJ	7	ND	1.48^{c} ±0.0	7 (100)

All values are means ± SEM; significance ($P \leq 0.05$) indicated by different letters. () indicates percent spermiation. GSI was not determined (ND) in spermiating males.

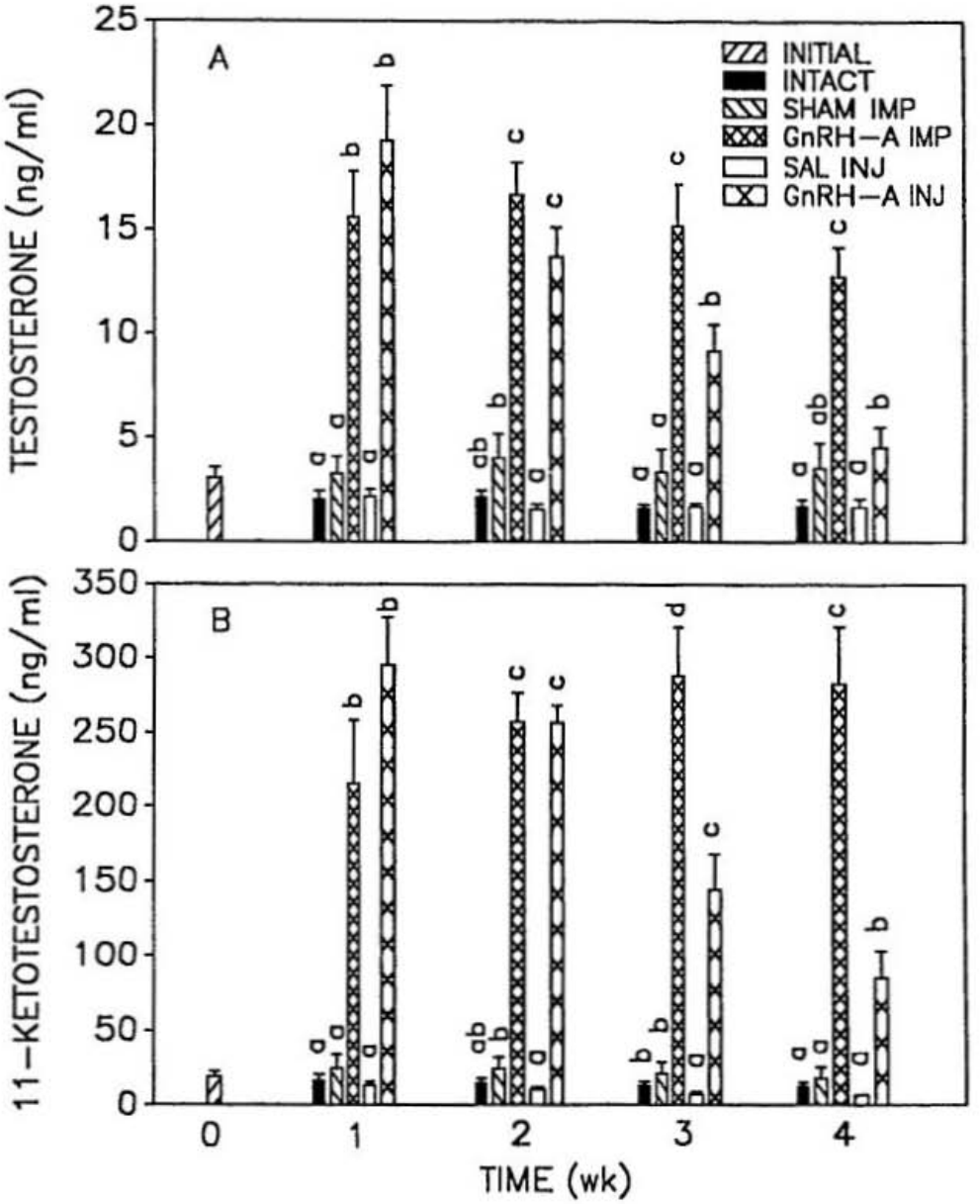
Table 18

Effects of GnRH-A treatment on percent spermatocrit, milt volume and total sperm count on the final day of experiment in male winter flounder

Treatment	N	Spermatocrit (%)	Milt vol(ml)	Total sperm count (x 10^9)
INTACT	1	98.3	0.7	13
GnRH-A IMP	5	44.4 ± 7.0[a]	3.4[a] ±0.8	39 ± 9[a]
GnRH-A INJ	7	48.2 ± 3.8[a]	6.3[a] ±1.1	76 ± 13[a]

All values are means ± SEM; significance ($P<0.05$) indicated by different letters. Control males (SHAM IMP, SAL INJ) did not spermiate or milt is not enough for determination spermatocrit and sperm count.

Figure 38. Plasma (A) testosterone and (B) 11-keto-testosterone levels at 1,2,3,4 wk following IP injections or IM pellet implantation of GnRH-A during the prespermiating stage (PREP 2). Control males were given either saline injections or a blank cholesterol pellet. Bars and lines represent means ± SEM of 5-7 fish per group. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time. Time=0 indicates plasma sex steroid levels in initial controls at the beginning of the experiment.


A
B
INITIAL
INTACT
SHAM IMP
GnRH–A IMP
SAL INJ
GnRH–A INJ
TESTOSTERONE (ng/ml)
11–KETOTESTOSTERONE (ng/ml)
TIME (wk)
0
1
2
3
4
0
5
10
15
20
25
50
100
150
200
250
300
350

5.3.5 GnRH-A Treatment in March During the Prespawning Period (PRES).

Females

This experiment was initiated with prespawning female flounder while the winter water temperature remained low (see Table 7, experiment 5). At autopsy, no significant differences ($P>0.05$) in gonadosomatic index or hepatosomatic index were found between the different treatment groups (Table 19). In contrast, while no change in oocyte dry matter occurred in females injected with GnRH-A, dry matter of oocytes was significantly ($P<0.05$) reduced in females implanted with GnRH-A. Prior to hormone treatment, the germinal vesicle position in all oocytes was slightly offcentre (Table 20, see chapter 2, section 2.3.2). While the germinal vesicle position remained unchanged in oocytes obtained from control females, GnRH-A treatment stimulated germinal vesicle migration since germinal vesicles were found midway or in a peripheral position in some oocytes. At the end of this experiment, the biological action of GnRH-A was evident since, despite holding fish at low water temperatures, partial ovulation occurred in GnRH-A implanted (3 of 6) females. During the course of the experiment 2 females from the injected group ovulated. Mean fertilization rate was 40% for eggs collected from these two GnRH-A injected females. While

Table 19

Effects of GnRH-A treatment beginning March on gonadosomatic index (GSI), hepatosomatic index (HSI), and oocyte dry matter (DM) in winter flounder during the prespawning phase (PRES)

Treatment	N	GSI	HSI	DM
INITIAL	5	15.97[a] ±0.67	2.12[a] ±0.09	31.69[b] ±0.29
INTACT	7	20.13[a] ±2.14	1.79[a] ±0.13	31.03[b] ±0.15
SHAM IMP	7	20.71[a] ±0.80	1.96[a] ±0.07	31.05[b] ±0.23
GnRH-A IMP	6	22.97[a] ±3.92	1.90[a] ±0.27	23.74[a] ±2.58
SALINE INJ	7	17.82[a] ±1.08	2.14[a] ±0.13	30.78[b] ±0.09
GnRH-A INJ	3	20.22[a] ±1.10	2.14[a] ±0.12	29.22[b] ±0.70

All values are Means ± SEM; significance ($P \leq 0.05$) indicated by different letters.

Table 20

Mean germinal vesicle (GV) stage in oocytes from female winter flounder during prespawning (PRES) phase

Treatment	GV stage		
	T1	T2	T3
INITIAL	2	-	-
INTACT	2	2	2
SHAM IMP	2	2	2
GnRH-A	2	2.2	4.0
SAL INJ	2	2	2
GnRH-A INJ	2	2.7	3.3

T1 : Prior to experimentation
T2 : Two weeks after hormone treatment
T3 : On the final day of the 4 wk experiment

hatching rate was low (27%) in eggs from one female, most of the hatched larvae appeared normal (78%). However, 100% egg mortality prior to hatching was observed for the eggs collected from other ovulated female.

At this time of the year the high levels of plasma estradiol-17ß were not further increased by either of the modes of GnRH-A application (Fig. 39A). At the end of the study the levels of estradiol-17ß in the GnRH-A implanted females significantly ($P<0.05$) decreased. By contrast, plasma testosterone significantly ($P<0.05$) increased and these levels were sustained in GnRH-A injected females for 3 weeks. In GnRH-A implanted females the increase in plasma testosterone secretion was delayed until wk 2 (Fig. 39B) and plasma testosterone declined by the end of the study.

Males

Some males were found in spermiating condition at the beginning of this experiment in March (see Table 7, experiment 5). Prior to the study period, at least one male was spermiating in each treatment group (Table 21) and the low spermatocrit values reflected relatively low numbers of spermatozoa. Upon termination of this experiment, all surviving males treated with GnRH-A reached spermiation whereas 85% of the intact control males had advanced to spermiation. Both modes of GnRH-A

Figure 39. Plasma (A) estradiol-17β and (B) testosterone levels at 1,2,3,4 wk following IP injections or IM pellet implantation of GnRH-A during the prespawning stage. Control females were given either saline injections or a blank cholesterol pellet. Bars and lines represent means ± SEM of 4-7 fish per group. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time. Time=0 indicates plasma sex steroid levels in initial controls at the beginning of the experiment.

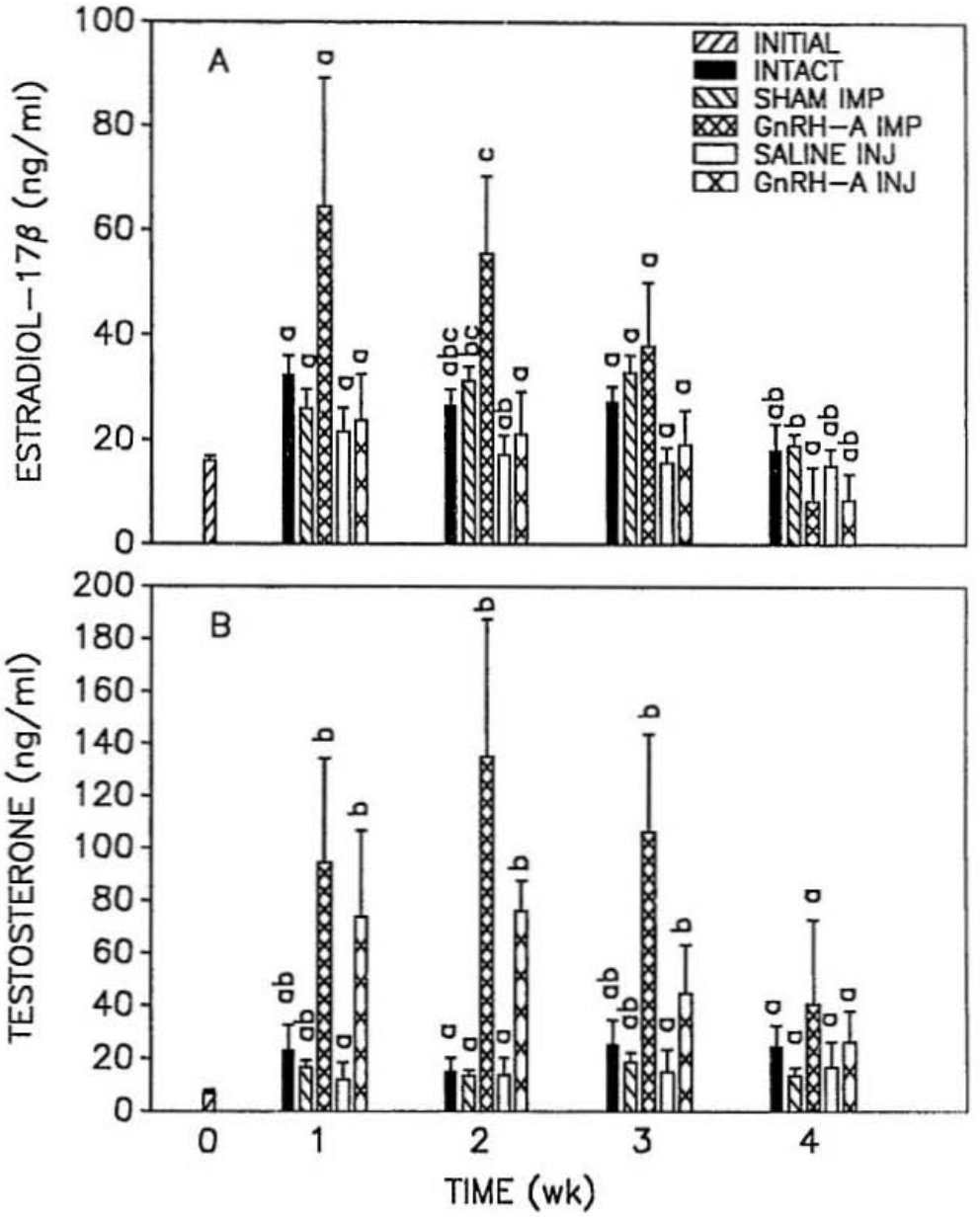

A
B
INITIAL
INTACT
SHAM IMP
GnRH–A IMP
SALINE INJ
GnRH–A INJ
ESTRADIOL–17β (ng/ml)
TESTOSTERONE (ng/ml)
TIME (wk)
0
1
2
3
4

Table 21

Effects of GnRH-A treatment on milt volume and total sperm count in male winter flounder

Treatment	No spermiating of total males Initial	Final	Spermatocrit (%) Initial	Final	Milt vol (ml)	Total Sperm ($x10^9$)
INITIAL	2/6 (33)	ND	56.7	ND	ND	ND
INTACT	2/7 (29)	6(86)	ND	77.4^a ±6.2	0.7 ± 0.1^a	12 ± 3^{ab}
SHAM IMP	1/7 (43)	7(100)	46.3	72.6^a ±7.3	0.6 ± 0.2^a	10 ± 5^a
GnRH-A IMP	1/7 (14)	4(100)	ND	59.1^a ±4.9	6.4 ± 0.9^b	86 ± 11^c
SAL INJ	1/7 (14)	2(100)	43.3	97.2	1.1	20
GnRH-A INJ	1/7 (14)	4(100)	50.1	69.4^a ±5.0	7.3 ± 2.2^b	104 ± 30

All values are means ± SEM; significance ($P<0.05$) indicated by different letters. ( ) indicates percent spermiation. ND - not determined, too little milt.

application significantly ($P<0.05$) increased milt volume and sperm count compared to controls (INTACT, SHAM IMP). No significant differences in hepatosomatic index were observed following GnRH-A treatment of males (Table 22).

Plasma testosterone (12 ng/ml) in intact control males remained stable whereas in sham implant and saline control males the testosterone levels declined by the end of the experiment (Fig. 40). Plasma 11-ketotestosterone (10-30 ng/ml) remained low and unchanged in all control males (INTACT, SHAM IPM, SAL INJ) throughout the duration of the study. Both modes of GnRH-A treatment stimulated increases in plasma T although by wk 4 plasma testosterone returned to levels not significantly different from the controls. At the end of the study, the increased plasma 11-ketotestosterone levels of males implanted with GnRH-A remained elevated, while 11-ketotestosterone levels rose and then declined in males receiving GnRH-A injection.

5.3.6 GnRH-A Treatment of Post-spawned (POST) Flounder in June.

Females

During June/July at relatively warm seawater temperatures (see Table 7, experiment 6) females were found in post-spawned condition (gonads regressed). At this time flounder normally resume feeding in preparation

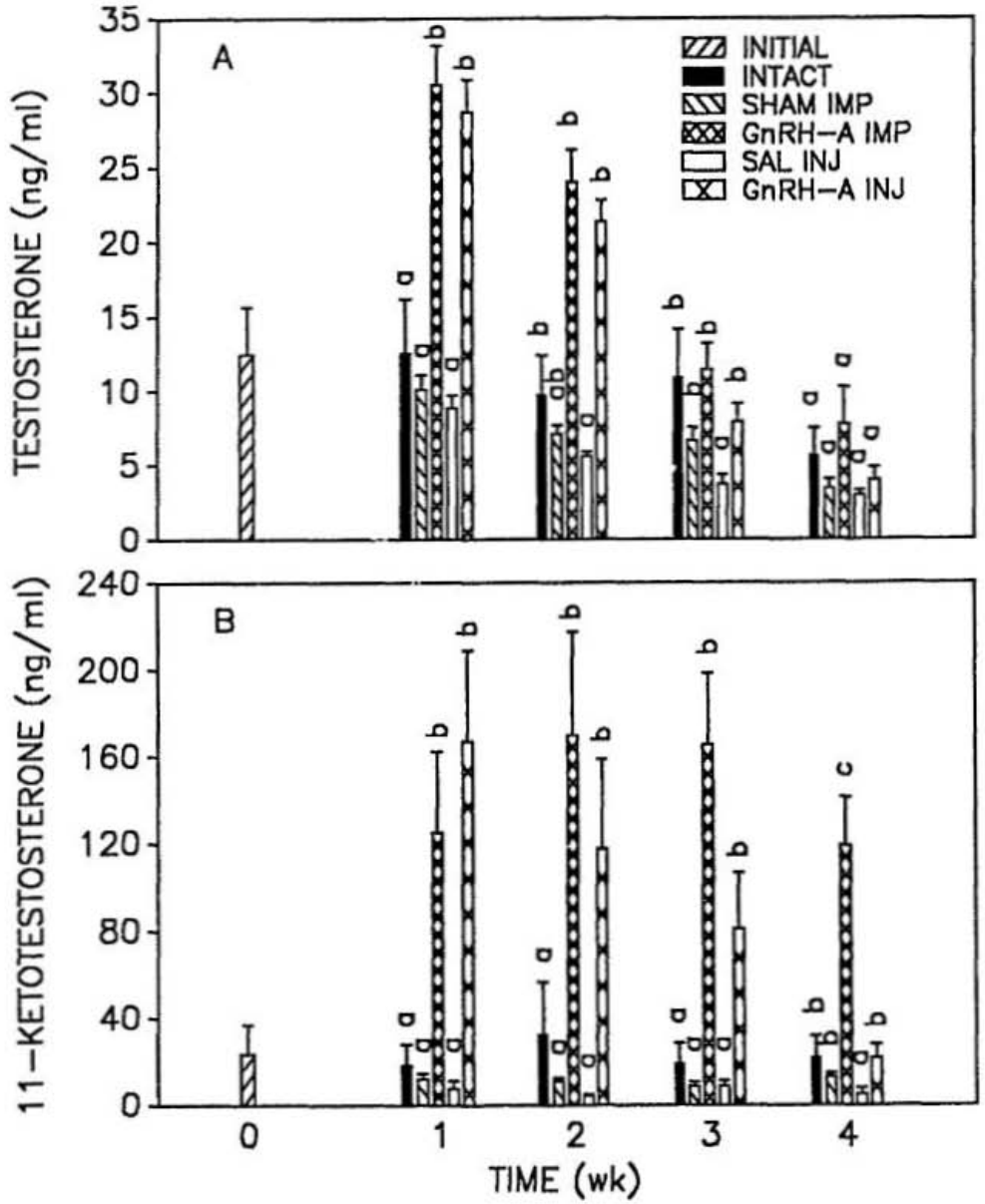
Table 22

Effects of GnRH-A treatment beginning March on gonadosomatic index (GSI) and hepatosomatic index (HSI) in male winter flounder during the prespawning period (PRES)

Treatment	N	GSI	HSI	Number of Spermiating males
INITIAL	6	8.69 ±0.60	0.98[a] ±0.08	2 (33)
INTACT	7	ND	1.21[ab] ±0.04	6 (86)
SHAM IMP	7	ND	1.35[bc] ±0.09	7 (100)
GnRH-A INJ	4	ND	1.20[ab] ±0.12	4 (100)
SAL INJ	2	ND	1.59[c]	2 (100)
GnRH-A INJ	4	ND	1.46[bc] ±0.06	4 (100)

All values are means ± SEM; significance ($P \leq 0.05$) indicated by different letters. () indicates percent spermiation. GSI was not determined (ND) in spermiating males.

Figure 40. Plasma (A) testosterone and (B) 11-keto-testosterone levels at 1,2,3,4 wk following IP injections or IM pellet implantation of GnRH-A during the prespawning stage. Control males were given either saline injections or a blank cholesterol pellet. Bars and lines represent means ± SEM of 4-7 fish per group. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time. Time=0 indicates plasma sex steroid levels initial controls at the beginning of the experiment.


A
B
INITIAL
INTACT
SHAM IMP
GnRH–A IMP
SAL INJ
GnRH–A INJ
TESTOSTERONE (ng/ml)
11–KETOTESTOSTERONE (ng/ml)
TIME (wk)
0
1
2
3
4
0
5
10
15
20
25
30
35
40
80
120
160
200
240

for the next reproductive season. The gonadosomatic index of sexually regressed females was low and no stimulation of gonad weight or hepatosomatic index was found following GnRH-A implantation (Table 23).

Although plasma sex steroids remained detectable in sexually regressed females, the hormone levels were very low (<1.0 ng/ml). These seasonally low plasma estradiol-17ß and testosterone levels were not significantly ($P<0.05$) increased in females following GnRH-A pellet implantation (Figs. 41A,B).

Males

In males, the post-spawned testes also reached the regressed condition at this stage of the reproductive cycle (see Table 7, experiment 6). The gonadosomatic index and hepatosomatic index of sexually regressed males were not significantly ($P>0.05$) changed following GnRH-A implantation (Table 24).

Plasma testosterone (1 ng/ml) in the sham implant males remained detectable but low throughout this experiment. In sexually regressed males GnRH-A treatment produced a low but significant ($P<0.05$) stimulation of plasma testosterone at 2 wk (336 hr, Fig. 42). In contrast, plasma 11-ketotestosterone levels were not detectable in control fish and no increase in 11-ketotestosterone could be detected after releasing hormone

Table 23

Effects of GnRH-A treatment beginning June on gonadosomatic index (GSI) and hepatosomatic index (HSI) in post-spawned female flounder (POST)

Treatment	N	GSI	HSI
INITIAL	6	2.96[a] ±0.39	1.83[a] ±0.20
CONTROL	6	2.61[a] ±0.26	1.94[a] ±0.20
GnRH-A IMP	5	2.84[a] ±0.17	1.97[a] ±0.16

Values are Means ± SEM; significance ($P \leq 0.05$) indicated by different letters. CONTROL consisted of INTACT and SHAM IMP females.

Figure 41. Plasma (A) estradiol-17β and (B) testosterone levels at 24,72,68,336 hr following IM pellet implantation of GnRH-A during the period of gonadal regression. Control females were given a blank cholesterol pellet. Bars and lines represent means ± SEM of 5-6 fish per group. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time. Time=0 indicates plasma sex steroid levels in initial controls at the beginning of the experiment.

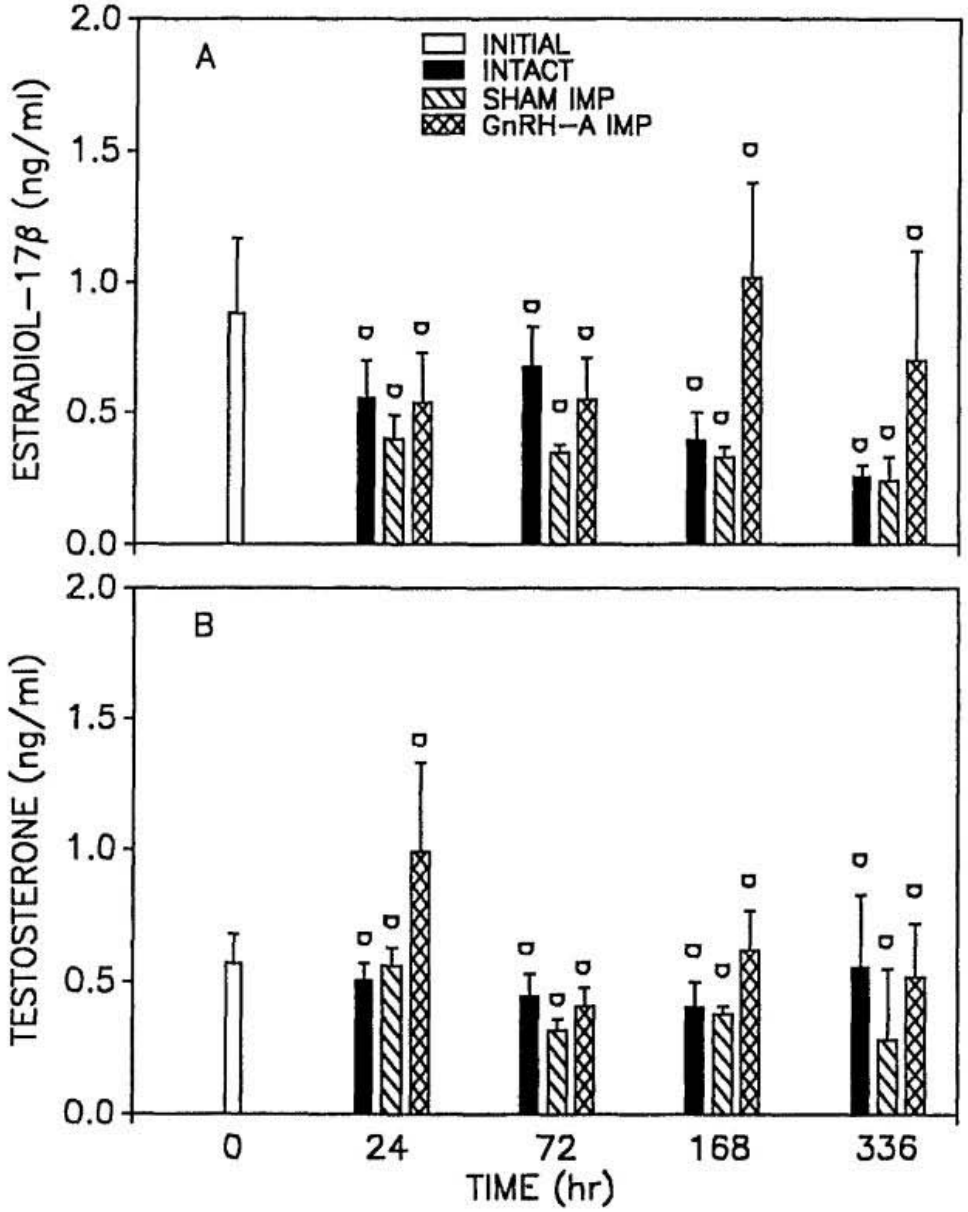

A
B
INITIAL
INTACT
SHAM IMP
GnRH–A IMP
ESTRADIOL–17β (ng/ml)
TESTOSTERONE (ng/ml)
2.0
1.5
1.0
0.5
0.0
0
24
72
168
336
TIME (hr)

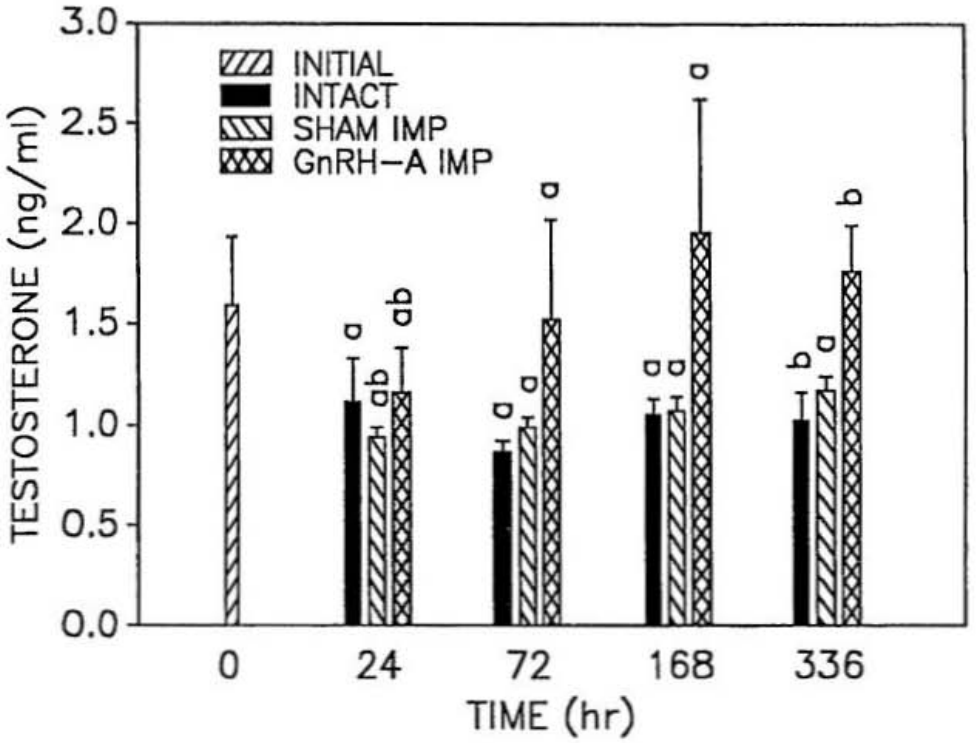
Table 24

Effects of GnRH-A treatment beginning June on gonadosomatic index (GSI) and hepatosomatic index (HSI) in post-spawned male (POST)

Treatment	N	GSI	HSI
INITIAL	6	0.71[a] ±0.04	1.39[a] ±0.24
INTACT	3	0.55[a] ±0.06	1.68[a] ±0.20
SHAM IMP	6	0.70[a] ±0.07	1.63[a] ±0.12
GnRH-A IMP	6	0.55[a] ±0.02	1.64[a] ±0.10

Values are Means ± SEM; significance ($P \leq 0.05$) indicated by different letters.

Figure 42. Plasma (A) testosterone levels at 24,72,168,336 hr following IM cholesterol pellet implantation of GnRH-A during the period of gonadal regression. Control males were given a blank cholesterol pellet. Bars and lines represent means ± SEM of 4-8 fish per group. Values with different letters indicate significantly different values ($P<0.05$) within each sampling time. Time=0 indicates plasma sex steroid levels in initial controls at the beginning of the experiment.


INITIAL
INTACT
SHAM IMP
GnRH–A IMP
TESTOSTERONE (ng/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
ab
a
a
a
a
a
b
a
b
0
24
72
168
336
TIME (hr)

treatment.

5.4 DISCUSSION

To manipulate the various steps of the reproductive cycles of fish with hormone requires a fundamental knowledge of their reproductive cycle particularly when fish will respond to hormone treatment. The present series of experiments demonstrated that the responsiveness of the winter flounder to GnRH-A treatment varies seasonally in association with the different stages of the reproductive cycle. Since no changes in gonadosomatic index and minimal fluctuations in sex steroids occurred in males and females during reproductively the regressed period, it is clear that post-spawned flounder are relatively non-responsive to GnRH-A treatment. On the other hand, the fact that GnRH-A treatment produces changes in plasma sex steroids and stimulates other biological events including gonadosomatic index, oocyte diameter, oocyte size-class frequency distribution, ovulation and spermiation suggests that flounder are very responsive to hormone treatment during most of the remaining periods of the seasonal reproductive cycle. Thus, adult winter flounder clearly responded to GnRH-A treatment both during the period of gonadal recrudescence (PREP 1&2) and also during the prespawning (PRES) phase.

Gonadal Recrudescence (PREP 1&2)

While the effectiveness of GnRH analog for inducing ovulation and spawning of fish has been reported for a variety of teleosts (Crim et al., 1987; Peter et al., 1987), studies of the effects of GnRH-A on stimulating gonadal development in the early phases of fish reproductive cycles are limited. In goldfish, ovary development is accelerated following daily injection of LHRH (Lam et al., 1976) and twelve daily injections of mammalian GnRH induced gonadal development in female medaka (Chan, 1977). In another study in female ayu, which were at the early vitellogenic stage, ovary development was accelerated following a single injection of native GnRH in emulsified solution (Aida, 1983). During the early period of ovarian recrudescence in flounder in September when the GSI was approximately 3, GnRH-A treatment accelerated ovary development as reflected by the increases in gonadosomatic index, oocyte diameter and changing oocyte size-class frequency profile (see Table 8, Fig. 29). In flounder the rate of increases in oocyte diameter are far greater in September compared to the growth of oocytes during the period of ovary maintenance in January (PREP 2). For example in September when oocyte diameter in the female ovary was 250 μm, GnRH-A administration increased oocyte diameter to about 413 μm by the end of the 4-wk experimental period. On the other

hand during the period of ovary maintenance when oocyte diameter was nearly maximum e.g. 500 μm, treatment of GnRH-A also increased oocyte diameter but to a lesser extent. GnRH-A treatment produced shifts in oocyte size-class frequency profiles both in early recrudescence and during the gonadal maintenance periods; however, shifting of the different oocyte size classes particularly from smaller to larger size-classes was limited to September when rapid gonadal growth is normally observed. In milkfish, oocyte size-class distribution plotted after the first ovulation revealed that a new clutch of vitellogenic oocytes appeared 28 days later (Tamaru et al., 1988). In contrast, in female winter flounder oocyte size-class frequency analyses provided no evidence for the appearance of a new clutch of vitellogenic oocytes at the end of the 4-wk study in January. Prior to oocyte maturation, the germinal vesicle moves from a central position in the oocyte to the animal pole where germinal vesicle breakdown occurs. In January, during the period of ovarian maintenance, GnRH-A induced germinal vesicle migration shifting the germinal vesicle away from its central position by the end of the experiment (see Table 16). Indeed, by the end of the experimental period GnRH-A treatment induced partial ovulation in some female flounder. It was determined from these experiments that February is the earliest month for inducing ovulation

following GnRH-A treatment under winter ambient seawater temperature (0.5°C).

The present studies also show that GnRH-A treatment accelerates testicular development in adult male flounder. In September when active testicular recrudescence is underway GnRH-A treatment increased the gonadosomatic index of males but the hormone treatment did not induce spermiation (see Table 9). Spermiation can be induced in male flounder beginning November when a small amount of milt can be collected.

The present steroid data show that female winter flounder are responsive to GnRH-A treatment during both rapid ovary recrudescence (PREP 1, see Fig. 30) and during the phase of slow growth and ovary maintenance (PREP 2, see Fig. 37). Weil et al. (1978) found that sexually mature female rainbow trout are more responsive to GnRH than females at other stages of ovarian development. In the goldfish, Sokolowska et al. (1985) observed that the highest concentrations of serum gonadotropin (GTH) induced by GnRH occurred in females during the late stages of ovarian recrudescence while fish that were sexually regressed were the least responsive.

Judging by testosterone and 11-ketotestosterone stimulation, male winter flounder also respond to GnRH-A treatment during both the period of rapid testicular recrudescence (PREP 1, see Fig. 31) and the testicular

maintenance (PREP 2, see Fig. 38) phases of seasonal reproductive development. Lin et al. (1985b) reported that in the late stages of testicular recrudescence in male goldfish they are more responsive to GnRH-A treatment compared with males at other stages of their sexual development. In male rainbow trout, Weil et al. (1978) demonstrated that the pituitary GTH response to GnRH was greatest in fish when testicular development had at least reached the spermatid stage of development continuing through spermiation. In goldeye, the pituitary GtH responses to GnRH-A were greater in sexually mature males compared to regressed fish or fish during the period of gonad recrudescence (Pankhurst et al., 1986b).

Prespawning Phase (March-April)

During this stage of the flounder reproductive cycle, GnRH-A treatment resulted in positive responses of certain biological events including germinal vesicle migration, ovulation and spermiation. Clearly, during the prespawning phase the seasonal values of gonadosomatic index in females reached 16 and GnRH-A administration was ineffective in further increasing the gonadosomatic index (see Table 19). This suggests that ovaries were nearing maximum development at the prespawning stage. While changes in gonadosomatic index were not observed during this stage of flounder reproductive cycle, changes in

germinal vesicle position clearly followed GnRH-A treatment (see Table 20). Germinal vesicle migration was advanced (mean germinal vesicle 4.0) very rapidly in some individual females during the prespawning phase compared to germinal vesicle position in January (mean germinal vesicle 2.5). This may also indicate that oocytes have almost completed the seasonal vitellogenic process. Indeed, some females ovulated following GnRH-A treatment even though water temperatures (0.5°C) were at a seasonally low point. Eggs from these hormone ovulated females could be artificially fertilized and the embryos developed normally. While ovulation can be achieved from February onwards, in fact the best time to induce ovulation in flounder is between March and June when water temperatures rise to approximately 4-5°C.

In the wild, fully ripe and prespermiating males could be captured during the prespawning phase approximately 2 months before the spawning period. Thus, it is perhaps not surprising that gonadosomatic index was not increased following GnRH-A treatment. Even though milt collections could be made from some untreated males, GnRH-A treatment increased the number of early spermiating males and also increased the abundance of milt (see Table 21). Weil and Crim (1983) showed that giving rainbow trout either multiple injections or a pellet implant of GnRH-A advances spermiation in males; in prespawning landlocked salmon

GnRH-A implant also induced spermiation (Crim et al., 1983a). Pankhurst et al. (1986b) found that milt volumes were increased in goldeye following injection of GnRH-A. Although spermiation in winter flounder can be induced through hormone treatment beginning in November, for practical purposes milt could only be collected in sufficient amounts when male winter flounder were treated with GnRH-A from January onwards.

In prespawning females seasonal plasma estradiol-17ß levels are high and not further elevated following GnRH-A treatment (see Fig. 39). On the other hand, plasma testosterone did increase after GnRH-A treatment and this is consistent with several other studies showing elevations of plasma testosterone levels close to spawning (see review of Fostier et al., 1983). Male winter flounder were also responsive to GnRH-A treatment during the prespawning phase as indicated by increases in plasma androgens (see Fig. 40). Thus, winter flounder like other teleosts are also responsive to GnRH-A during the prespawning phase.

Although GnRH-A administration to the winter flounder during the prespawning phase produced a prolonged period of elevated sex steroids, there was some evidence for the anti-reproductive effects of GnRH-A similar to pituitary self-suppression as described in male goldfish following multiple injections of GnRH or GnRH-A (Peter, 1980). For

example in prespawning males in January (see Fig. 38), multiple injections of GnRH-A initially increased sex steroid levels but eventually plasma testosterone and 11-ketotestosterone decreased by the end of the study. In prespawning males in March, plasma 11-ketotestosterone remained elevated to the end of the study while plasma testosterone showed a declining trend in the males receiving GnRH-A implant (see Fig. 40). Perhaps multiple injections of peptide releasing hormone leads to a depression of the production of gonadal steroids. In goldfish, Peter (1980) showed that multiple injections of GnRH-A depressed plasma GTH levels. Alternatively, stress from repeated handling may affect the levels of plasma sex steroids. Stress has been shown to inhibit reproductive development in fish (Billard et al., 1981) and also depresses sex steroid secretion in rainbow trout (Pickering et al., 1987).

On the other hand, the declining plasma sex steroid values in prespawning females might be viewed as evidence for a shift towards the production of maturational gonadal steroids rather than pituitary self-suppression. While the identity of maturational steroids in winter flounder is presently unknown, studies in a variety of other teleosts species have shown that 17α,20ß dihydroxy progesterone is the maturational steroid (Fostier et al., 1983, Nagahama, 1987) found in increasing amounts in the plasma.

Post-spawned Phase (June/July)

In the summer period, the flounder gonads were found in regressed condition and they remained quiescent while the fish began feeding in preparation for the next reproductive season. Gonadosomatic index of both males and females was not increased following GnRH-A hormone implant (see Tables 23,24). It has been previously reported that the gonadosomatic index of sexually regressed male landlocked salmon is not increased after GnRH-A treatment (Crim et al., 1983a). In contrast, in the sexually regressed goldfish, the gonadosomatic index of both males and females is increased following ten daily 0.01 μg/g or 1.0 μg/g GnRH-A injections (Lin et al., 1985b). While the present study of sexually regressed winter flounder showed that their reproductive system is apparently resistant to GnRH-A treatment, in the medaka (Chan, 1977) or goldfish (Lin et al., 1985b) daily injections of either native GnRH or GnRH-A can stimulate the onset of gonadal development in sexually regressed fish.

Despite GnRH-A implantation, post-spawned female flounder were not responsive to GnRH-A, as indicated by the lack of changes in plasma estradiol-17β and testosterone (see Fig. 41). Post-spawned males were likewise poorly responsive to GnRH-A treatment as reflected by minimal changes in plasma testosterone while 11-ketotestosterone remained undetectable (see Fig. 42).

In sexually regressed male goldeye, plasma testosterone is elevated 6 hr following GnRH-A injection; the steroid levels returned to basal values after 72 hr (Pankhurst et al., 1986b). While plasma sex steroids were not measured in sexually regressed landlocked salmon (Crim et al., 1983a) and goldfish (Lin et al., 1985b), clearly these studies showed that plasma GTH is elevated following GnRH-A treatment.

In summary, adult winter flounder were most responsive to GnRH-A treatment during the periods of gonadal recrudescence (PREP 1&2) and the prespawning phase but poorly or not responsive at all during the post-spawning phase. Stimulation of plasma sex steroids and other biological events occur following GnRH-A treatment but these changes are dependent upon the initial stage of gonadal development when hormone is applied. Some evidence for GnRH-A suppression of elevated sex steroid levels was found in both prespawning males and females.

CHAPTER 6

INDUCED OVULATION/SPAWNING IN FEMALE WINTER FLOUNDER

6.1 INTRODUCTION

Successful induction of spawning in captive winter flounder has been reported using human chorionic gonadotropin (HCG), carp pituitary extract (Smigielski, 1975), and a synthetic GnRH analog (Crim, 1985; Weigand et al., 1987). Although GnRH-A is well known to induce maturation, ovulation and spawning in fish (Crim et al., 1987), few previous studies have also reported on egg quality/larvae development following hormone induction of spawning. Indeed, poor egg quality was observed in various fish following hormone induction of spawning (Smigielski, 1975; Crim and Glebe, 1984; Ramos, 1986a; Lee et al., 1987). In contrast to these reports of poor quality eggs, high hatching rates and a high yield of normal looking fry were observed in catfish (De Leeuw et al., 1985; Manickam and Joy, 1989) and chinese carp (Peter et al., 1988) following GnRH-A treatment.

In the present studies, which were conducted over three consecutive reproductive seasons from 1987-1989, attempts were made to advance ovulation and spawning in

winter flounder using different modes of GnRH-A application and treating fish with hormone at different times prior to the natural spawning period. The influence of HCG, flounder pituitary extract, pimozide (a dopamine antagonist) alone or in combination with GnRH-A on prespawning winter flounder were investigated. In addition, the effects of temperature on the ovulatory responses of winter flounder following GnRH-A treatment were also investigated.

6.2 MATERIALS AND METHODS

6.2.1 Experimental Fish

Sexually maturing prespawning female winter flounder were used in this series of experiments which were conducted from February-May (Table 25). Water temperatures were elevated to 5°C in all experiments.

6.2.2 Collection and Injection of Pituitary Glands

Pituitary glands, removed from mature male and female winter flounder (average body weight 500 g) during the spawning season were removed, immediately frozen on dry ice and stored frozen at -20°C until prepared for injection. In preparation for injection, the pituitaries were homogenized in fish saline, centrifuged at 15,000 x g for 2 min and the supernatant removed for injection. Each

Table 25

Summary of experiments on induced ovulation/spawning

Expt	Date	Duration (wk)	GSI	GV	OCDM (mm)
1	Feb '88	4	17.2	central	ND
2	Mar '87	5	14.0	central	0.47
3	Apr '88	4	17.0	ND	0.53
4	May '88	4	ND	ND	ND
5	Feb '89	7	ND	ND	ND
6	May '89	4	ND	ND	ND

ND - not determined; GSI, gonadosomatic index;
GV, germinal vesicle; OCDM, oocyte diameter.

female was treated with the equivalent of 3 pituitaries in 300 µl.

6.3 RESULTS

6.3.1 Effects of GnRH-A Treatment on Induced Ovulation in February (Period of Gonadal Maintenance).

This experiment was initiated in February about 3 months prior to the expected natural spawning season (Table 25, experiment 1). Thirty female flounder were divided into five treatment groups (6 fish each) as follows: (Group 1) intact, untreated fish; (Group 2) sham control cholesterol pellet IM implant; (Group 3) 100 µg GnRH-A cholesterol pellet IM implant; (Group 4) three control saline IP injections/wk; and (Group 5) three 20 µg/kg GnRH-A IP injections/wk.

Data from oocyte germinal vesicle analysis (Table 26, also see chapter 2, section 2.3.2) indicated that prior to releasing hormone treatment, all flounder oocytes contained a germinal vesicle in central position. After 2 wk, germinal vesicle migration off-centre (stage 2) was evident in oocytes obtained from control females; GnRH-A treatment accelerated germinal vesicle migration to the peripheral position (stage 4) in oocytes of some

Table 26

Mean germinal vesicle stage in oocytes from prespawning female winter flounder in February

Group	Treatment	T1	T2	T3
1	INTACT	1	2	2
2	SHAM IMP	1	2	2
3	GnRH-A IMP	1	2.2	3.4
4	SALINE INJ	1	2	2
5	GnRH-A INJ	1	2.3	4.7

T1 : Prior to experimentation
T2 : Two weeks after hormone treatment
T3 : Final day of the 4-wk experiment

experimental females (mean germinal vesicle stages, Group 3 & 5 = 2.2 & 2.3, respectively). By the end of this experiment, while germinal vesicle remained at stage 2 in oocytes collected from control females, the germinal vesicle position had advanced to stage 4 (peripheral position) in oocytes from some females receiving releasing hormone treatment. Figure 43 shows a sample of the post-vitellogenic oocytes in prespawning females, ovulated eggs and localization of the germinal vesicle position after treating oocytes with clearing solution (see chapter 2, section 2.3.2).

With respect to the ovulatory responses (Table 27), whereas no ovulation occurred in control females held at 5°C in February, GnRH-A treatment induced ovulation in 2 females receiving releasing hormone by implant on the final day of the study. Similarly, 2 females ovulated within 20 days following multiple GnRH-A injections although repeated handling of these females greatly increased the rate of prespawning mortality. Assessment of ovarian development (gonadosomatic index) in females remaining unovulated on the final day of the experiment indicated that GnRH-A treatment increased gonadosomatic index (Gonadosomatic Index in pooled GnRH-A treated females significantly greater, $P<0.05$, compared with controls). Fertilization rate was low (23%) in eggs

Figure 43. A. Post-vitellogenic opaque oocytes (x 32), B. cleared oocytes with GV in central position (x 32), C. GVBD oocytes (x 20), and D. ovulated eggs (x 32) in winter flounder.

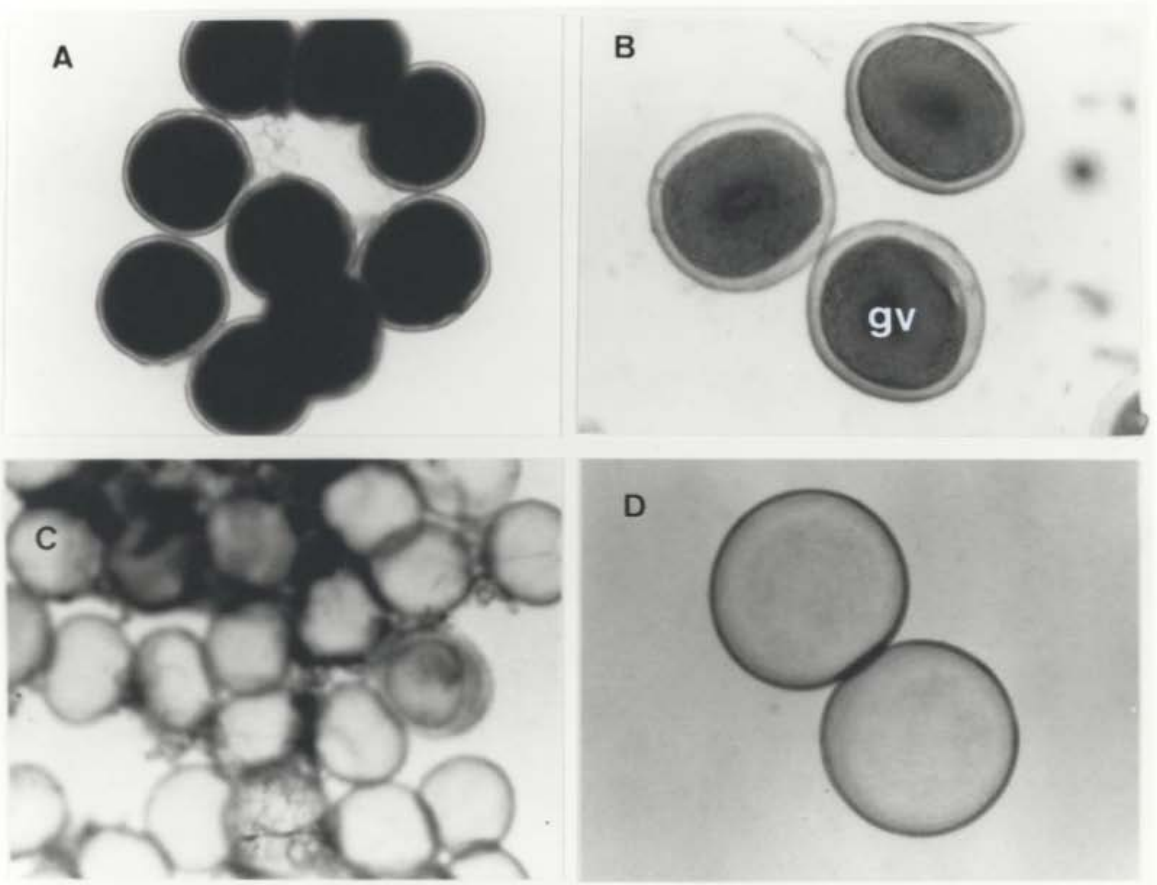

A
B
gv
C
D

Table 27

Influence of GnRH-A treatment on gonadosomatic index (GSI) and ovulation in February (period of gonadal maintenance)

Treatment	Number ovulated	GSI
INITIAL	0	17.2 ± 0.8 (6)
INTACT	0	16.7 ± 0.8 (5)
SHAM IMP	0	19.0 ± 1.9 (6)
GnRH-A IMP	2	21.6 ± 1.2 (4)
SALINE INJ	0	17.3 ± 1.9 (3)
GnRH-A INJ	2	24.8 (1)

At the beginning of the treatment each group consists of 6 fish. ( ) indicate number of unovulated fish surviving on the final day of the experiment.

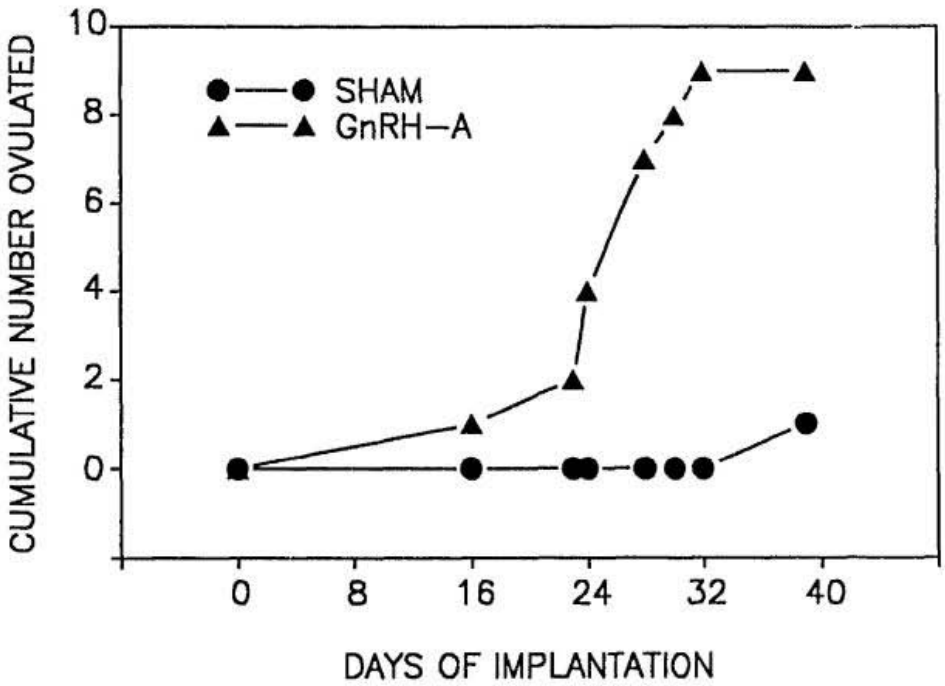
in eggs stripped from just one female flounder induced to ovulate by GnRH-A injection. Eighty-four percent of the embryos hatched between days 11-15 after fertilization and half of hatched larvae appeared to be normal.

6.3.2 Effects of GnRH-A Pellet Implantation on Induced Ovulation (Prespawning Phase).

Another experiment was conducted with 17 prespawning females beginning March approximately 2 months prior to the natural spawning season (see Table 25, experiment 2). Females were divided into 2 groups and implanted IM with either (Group 1, n=8) a blank cholesterol pellet or (Group 2, n=9) a cholesterol pellet containing 120 μg GnRH-A. Autopsies of females (n=4) at the initiation of the experiment in March demonstrated that ovaries were relatively well developed but the fish remained relatively far from spawning e.g. germinal vesicle central (see Table 25).

By the end of this 39 day experimental period, ovulation was obtained in just one sham control female (12% of the group). By contrast (Fig. 44), a significantly greater ovulatory response ($P<0.001$) was obtained in females implanted with GnRH-A; the first ovulation in hormone treated females was recorded 16 days after hormone implantation; most of these females ovulated 3 wk after

Figure 44. The influence of GnRH-A implantation in March upon ovulation responses (cumulative number females ovulated). Day=0 indicates beginning of experiment.


CUMULATIVE NUMBER OVULATED
10
8
6
4
2
0
SHAM
GnRH–A
0
8
16
24
32
40
DAYS OF IMPLANTATION

the GnRH-A treatment and by day 32 following treatment (mean days to ovulation = 25.8), all females (100%) in this group had ovulated.

The spawning response (egg release) of females treated with GnRH-A was quite variable; in 5 females, ovulated eggs were spontaneously released into the tank and thus they were not fertilized. Four females retained their eggs which were removed and fertilized *in vitro*. Egg quality data, in terms of fertilization rate, hatching rate, and larval survival from individual females are shown in Table 28. Hatching began about 12 days post fertilization. Eggs collected from the single ovulating sham control female had a high fertilization rate (92%) but the hatching rate was low and a relatively high proportion of abnormal larvae was observed. By comparison, for females induced to ovulate with GnRH-A, the mean fertilization rate was 58% (range 50-66%). Mean egg survival to hatch was 68% and larval quality (normal development) was very good.

A summary of the gross morphology of embryonic development in winter flounder at 5°C is presented in Table 29 and Figure 45. Mature, unfertilized eggs were almost transparent and had a smooth chorion and a very narrow perivitelline space. The eggs were spherical, demersal and adhesive. Egg diameter varied from 0.62 to 0.83 mm (average 0.70 ± 0.01 mm) and the eggs contained no oil droplet or globules. Due to their adhesive nature,

Table 28

Influence of induced spawning by GnRH-A pellet implant on fertilization rate, hatching rate and presence of normal larvae

Fish #	Fertilization[a] rate (%)	Hatching rate (%)	Normal embryo (%)
Or105	92.2 ±1.9	14.7 ±4.3	64.5 ±8.8
B20	66.4 ±8.9	86.7 ±4.3	98.2 ±0.1
B122	55.7 ±1.8	54.8 ±6.9	75.2 ±2.5
B123	49.8 ±10.8	65.9 ±16.1	97.4 ±1.2
B124	63.2 ±7.2	67.0 ±2.1	97.2 ±0.2

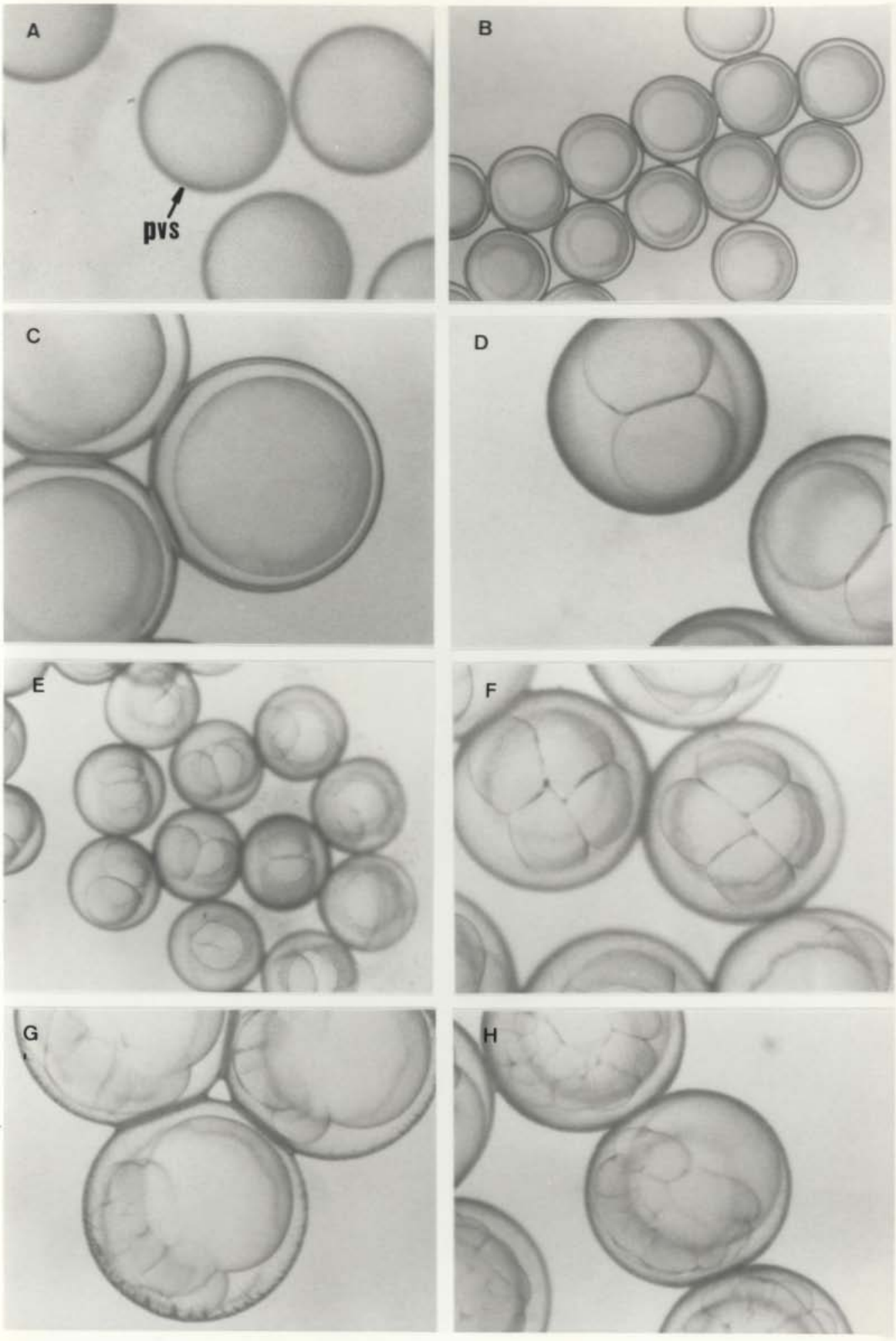
[a] determined from 3 replicate petridishes each containing 200-300 eggs, (OR) sham implant, (B) GnRH-A implant

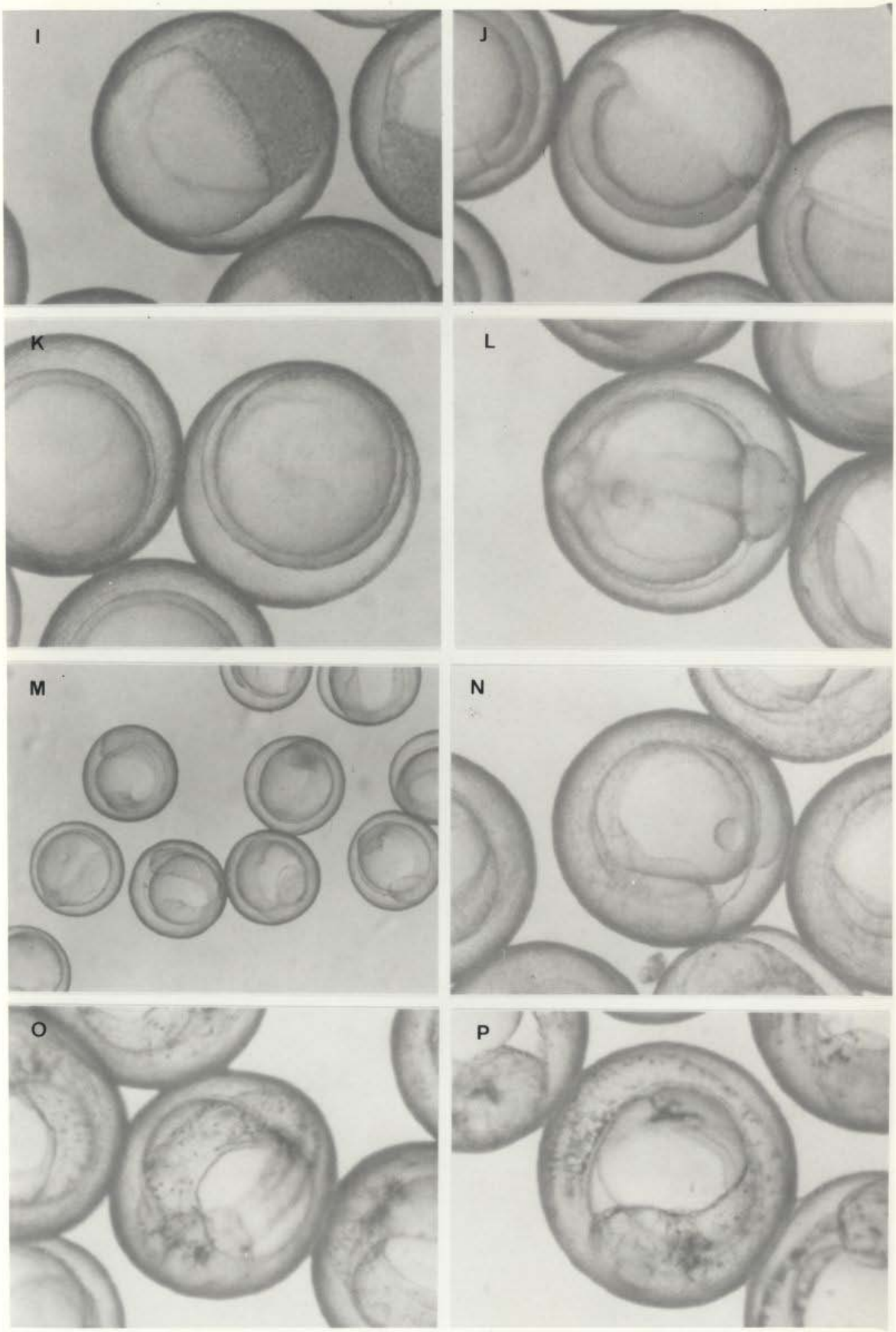
Table 29

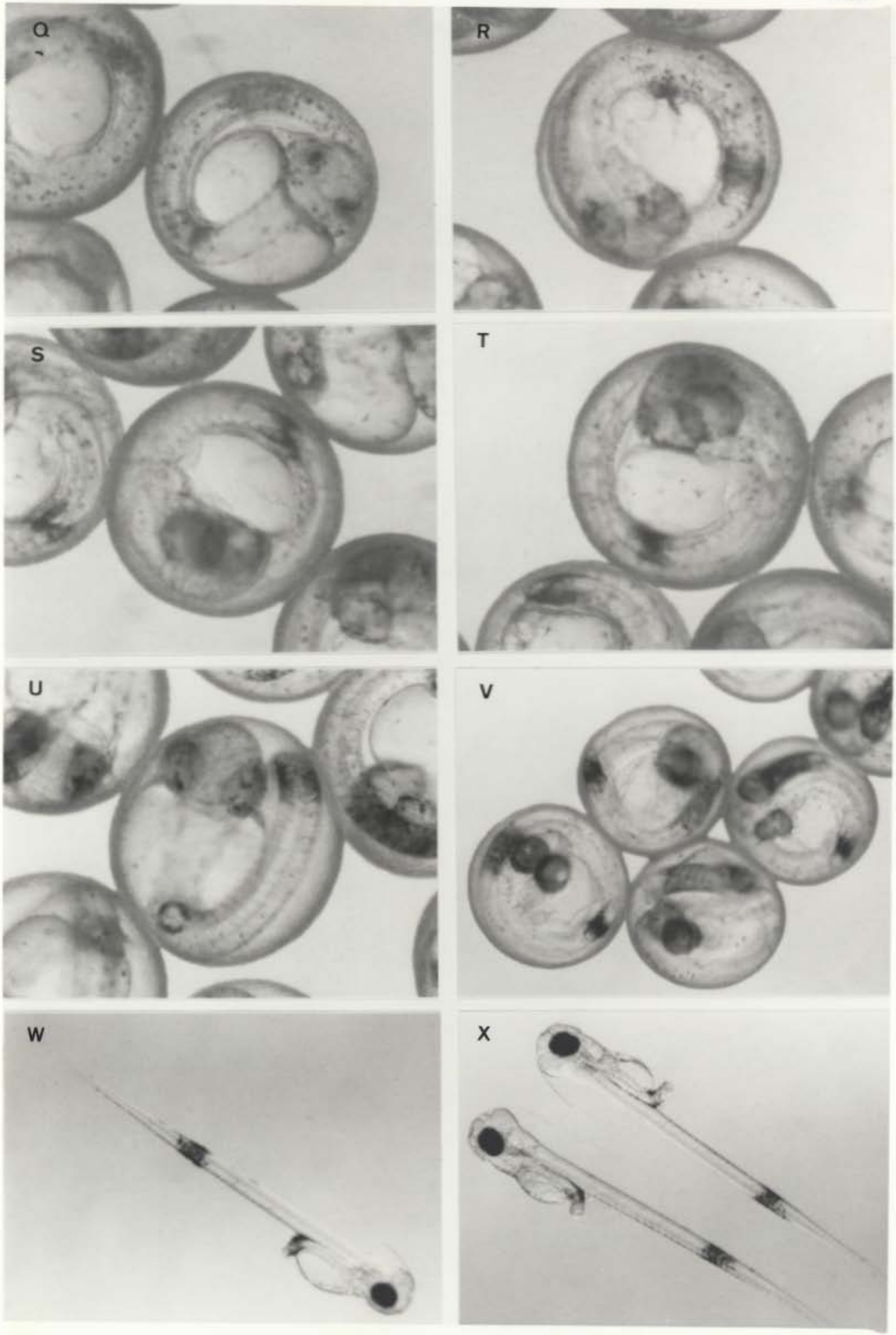
Embryonic development of winter flounder at 5°C

Time after fertilization (hr/day)	Stage	Remarks
0	Unfertilized eggs	narrow perivitelline space.
0.5 hr	Fertilized eggs	0.5 hr after fertilization
4-6.0 hr	2-cells	
7.0 hr	4-cells	
9.0 hr	8-cells	
11 hr	16-cells	
Day 1	Blastula	Blastulation in progress
Day 2	Gastrula	Gastrulation in progress
Day 3	Neurula	Neurulation in progress
Day 4		Embryo 1/2 of egg circumference
Day 5		-
Day 6		Pigments appear on the tail and abdomen
Day 7		Embryo begins occasional body movement
Day 8		-
Day 9		More pigments on the tail and abdomen
Day 10		Eyes well formed
Day 11		-
Day 12	larva	hatching begins

Figure 45. Early developmental stages of winter flounder embryos. A. Unfertilized eggs, perivitelline space (pvs) (x 20), B. Fertilized eggs (x 20), C. Fertilized eggs (x 32), D. 2-cell stage (x 32), E. 2-cell stage (x 20), F. 4-cell stage (x 32), G. 8-cell stage (x 32), H. 16-cell stage (x 32), I. Blastula stage (x 32), J-K. Gastrula stage (x 32), L. Embryo 1/2 of egg circumference (x 32), M. Embryo 1/2 of egg circumference (x 20), N-O. Pigment appear on the caudal and near anal region (x 32), P-Q. Embryo Occasional body movement (x 32), R-T. Well-formed embryo (x 32), U. Prehatching stage (x 32), V. Prehatching stage (x 20), W. Newly-hatched larva (x 20), X. 3-day old larvae (x 20).


A
pvs
B
C
D
E
F
G
H


I
J
K
L
M
N
O
P

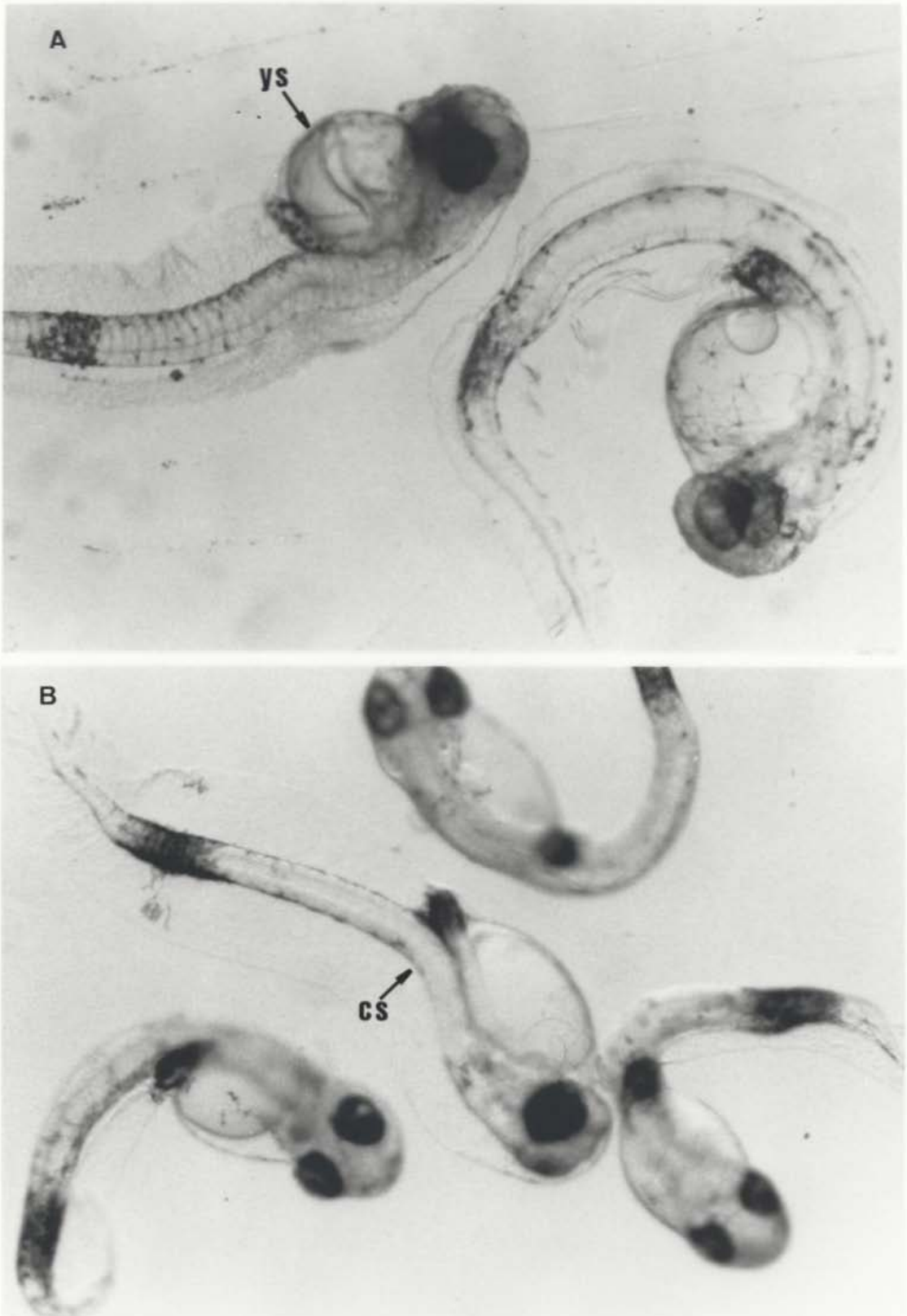

Q
R
S
T
U
V
W
X

the eggs were frequently distorted, in some cases being ovoid, but there was no indication that these eggs produced abnormal larvae. The blastodisk was large and a pale amber colour, while the yolk was colorless. Following fertilization a small perivitelline space developed. The fertilized eggs were spherical, adhesive and transparent. By the third day differentiation occurred and continued until hatching, usually 12-14 days after fertilization at 5°C water temperature. Newly-hatched larvae were inactive and settled to the bottom of the petri dishes. A low percentage of newly hatched-larvae were found to be abnormal (Fig. 46, see chapter 2, section 2.3.7) after hatching.

6.3.3 Effects of Human Chorionic Gonadotropin (HCG), GnRH-A and/or Pimozide on Induced Ovulation in April (Prespawning Phase).

A third experiment was conducted in prespawning females 1 month prior to the natural spawning period (see Table 25, experiment 3). Thirty-six female winter (6 per group) flounder were divided as follows: (Group 1) two control saline injections/wk; (Group 2) two 500 IU/kg HCG injections/wk); (Group 3) two 20 μg/kg GnRH-A injections/wk; (Group 4) 40 μg GnRH-A

Figure 46. Abnormal embryo in winter flounder. A. Larvae with spherical yolk sac (ys) (x 32), B. Curved spine (cs) found in larvae (x 25).


A
ys
B
cs

cholesterol:cellulose (50:50) pellet implant; (Group 5) two 20 µg/kg GnRH-A + Pimozide 10 mg/kg injections/wk; and (Group 6) two 10 mg/kg Pimozide injections/wk.

Prior to the study, an ovarian biopsy of all the experimental females indicated that the germinal vesicle position of oocytes was slightly off-centre (stage 2, Table 30). At the end of the experiment, the oocytes of all the surviving non-ovulated females were cleared to investigate the extent of germinal vesicle migration following hormone treatment. The germinal vesicle of the control fish had migrated further; likewise in females injected with GnRH-A alone, HCG and GnRH-A plus pimozide the germinal vesicle had shifted further into the midway or the peripheral position. However, the germinal vesicle position remained unmoved (slightly off-centre) for groups treated with a GnRH-A implant or pimozide only.

Body weights were monitored for individual females which served as an indicator of ovarian hydration prior to ovulation. Body weights remained stable in females in saline control and pimozide only injection groups of fish for the duration of the study period (Fig 47). In contrast, 10 days after injection of females with HCG, GnRH-A and GnRH-A + pimozide, a 3-5% increase in body weight occurred while a substantial increase in body weight (8%) was observed in females implanted with releasing hormone. By the end of the experiment, body

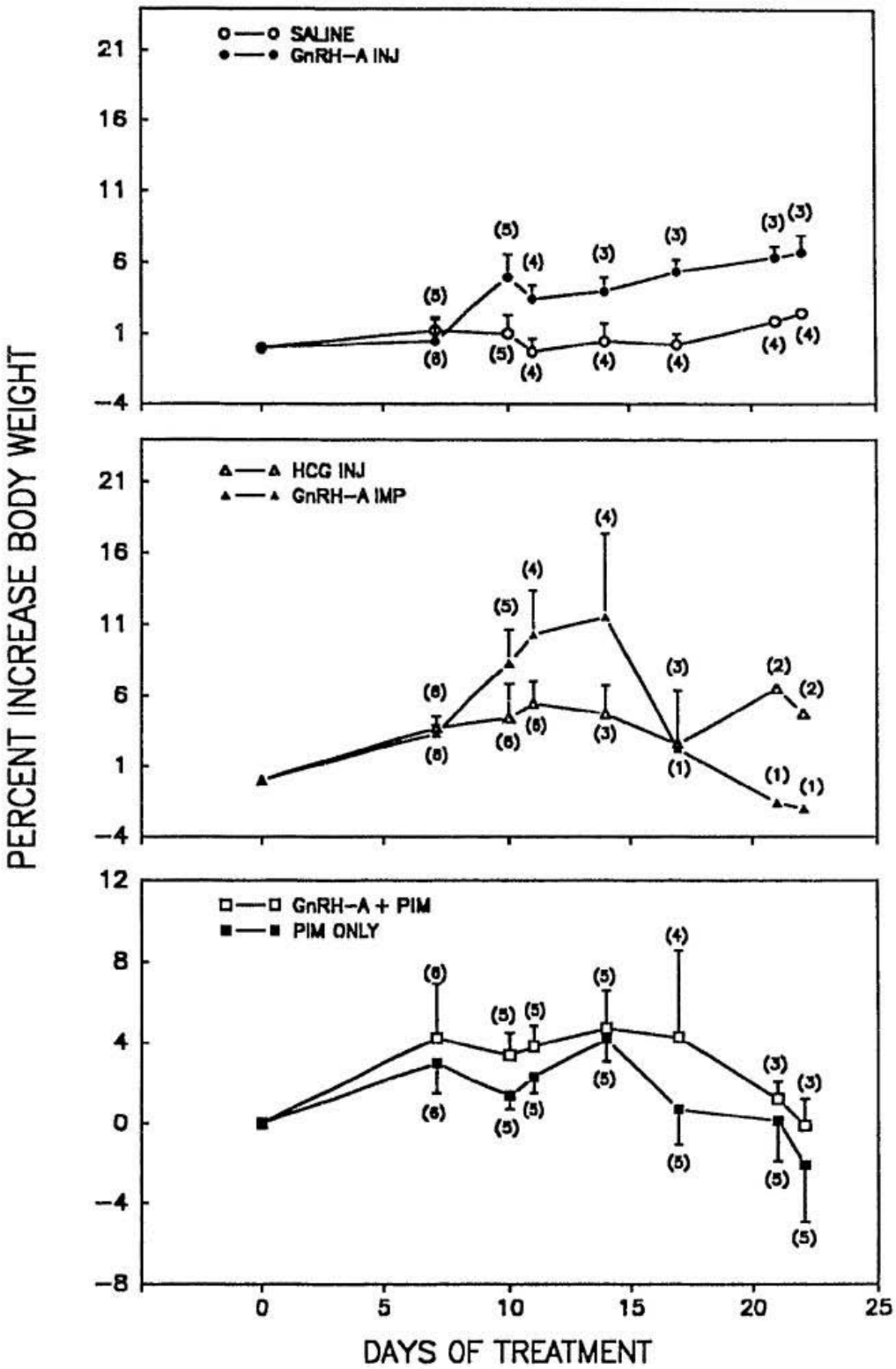
Table 30

Mean germinal vesicles (GV) stage in oocytes from prespawning female winter flounder in April

Group	Treatment	T1	T2
1	SALINE INJ	2	2.7
2	GnRH-A INJ	2	4.0
3	HCG	2	3.0
4	GnRH-A IMP	2	2.0
5	GnRH-A + PIM	2	3.3
6	PIM ONLY	2	2.0

T1 : Prior to experimentation
T2 : On the final day of the 4-wk experiment

Figure 47. The influence of HCG, GnRH-A, Pimozide or combination of Pimozide (PIM) and GnRH-A in April 1988 upon body weights (mean percent increase body weight) of female winter flounder. Control fish were injected with saline vehicle. Data in parenthesis indicate the number of fish. All data are means ± SEM.


SALINE
GnRH-A INJ
HCG INJ
GnRH-A IMP
GnRH-A + PIM
PIM ONLY
PERCENT INCREASE BODY WEIGHT
DAYS OF TREATMENT
21
16
11
6
1
-4
12
8
4
0
-8
0
5
10
15
20
25

weight tended to decline. Gonadosomatic index of all unovulated fish in all the groups was about 20% (Table 31).

A significantly higher rate of ovulation ($P<0.05$) occurred in the female groups treated with GnRH-A (Fig. 48). On the other hand, in females injected with HCG, GnRH-A + pimozide, and pimozide only the ovulation rates were not statistically different compared to the saline controls. The influence of various hormone treatments on the egg fertilization rate is shown on Table 32. Judging by high egg fertilization rates, good quality eggs were obtained from females ovulated with GnRH-A implant and the single female ovulating in the PIM group.

6.3.4 GnRH-A Induced Spawning of Winter Flounder in May and an Evaluation of Egg/Larval Quality.

A fourth study was conducted immediately before the onset of the natural spawning period in May (Table 25, experiment 4). In this experiment males and females (1:1) were placed together in the tank. A total of 19 prespawning females were divided into 2 groups and treated with either; (Group 1,) sham control cholesterol: cellulose pellet hormone implant (n=8) or (Group 2) 40 μg GnRH-A cholesterol: cellulose pellet implant (n=11). Following hormone treatment, body weight changes were

Table 31

Influence of HCG, GnRH-A, pimozide or combination of pimozide plus GnRH-A treatment on gonadosomatic index (GSI) in prespawning female winter flounder in April

Treatment	GSI
INITIAL	17.1 ± 1.3 (5)
SALINE INJ	21.1 ± 0.7 (4)
GnRH-A INJ	26.2 (2)
HCG	21.2 (2)
GnRH-A IMP	21.9 (1)
GnRH-A + PIMOZIDE	21.5 ± 1.2 (3)
PIMOZIDE ONLY	21.4 ± 0.7 (5)

All values are means ± SEM;
( ) indicate number of fish.

Figure 48. The influence of HCG, GnRH-A, Pimozide or a combination of Pimozide (PIM) and GnRH-A upon the ovulatory responses (cumulative number ovulated) of female winter flounder. Day=0 indicates beginning of experiment. Control fish were injected with saline.

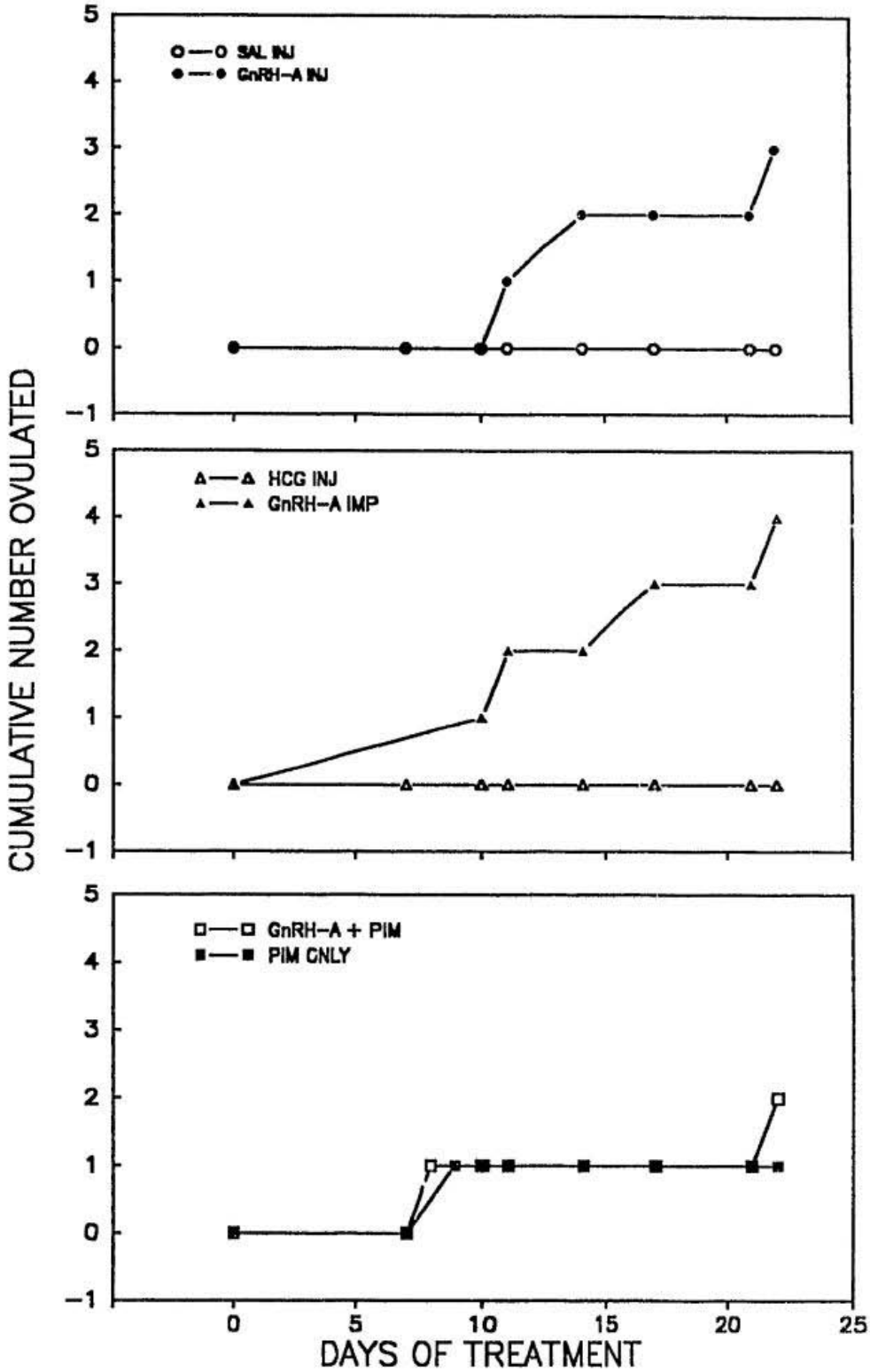

SAL INJ
GnRH-A INJ
HCG INJ
GnRH-A IMP
GnRH-A + PIM
PIM ONLY
CUMULATIVE NUMBER OVULATED
DAYS OF TREATMENT
5
4
3
2
1
0
-1
0
5
10
15
20
25

Table 32

Evaluation of the effects of hormone induced spawning on egg fertilization rate, hatching rate and relative amounts of normal embryos

Treatment/ Fish #	Fertilization[a]	Hatching rate (%)	Normal embryo(%)
GnRH-A INJ			
W192	38.6 ±5.7	91.5 ±1.0	99.6 ±0.3
GnRH-A IMP			
G136	88.9 ±1.7	87.7 ±1.7	97.0 ±2.0
G137	63.9 ±3.4	86.2 ±2.2	95.0 ±1.0
G139	92.7 ±1.1	80.4 ±3.2	99.0 ±0.1
GnRH-A + PIM			
B149	27.7 ±5.3	89.9 ±5.7	97.0 ±2.0
B150	92.4 ±1.3	84.4 ±3.5	96.0 ±2.0
PIM ONLY			
Or193	90.9 ±1.0	90.6 ±1.4	94.0 ±2.0

[a] determined from 5-6 replicate petridishes,each consisting of 200-300 eggs; all values are means ± SEM.

monitored as an indicator of the progress of ovarian hydration and anticipated ovulation (Fig. 49). In sham control females, body weight remained relatively stable throughout the study period, although a 3.5% increase in body weight was observed for the two control females which subsequently ovulated. In contrast, within 5 days of GnRH-A implantation, substantial increases in body weight, reaching a peak of 5% by day 10, occurred in GnRH-A implanted females. A significant ($P<0.05$) increase in body weight was observed in the GnRH-A implanted group (until day 20 after implantation) compared to the sham control females. At autopsy, there was no significant ($P>0.05$) difference in gonadosomatic index between hormone treated fish (24%) and the control fish (20%). By the end of this hormone induced spawning trial, a significantly ($P<0.05$) greater number of hormone treated females had ovulated, 82% (9 of 11), compared with 25% (2 of 8) of the controls (Fig. 50). Mean days to ovulation, was 13 days for GnRH-A implanted females, whereas 17 days were required for ovulation of control females. Most ovulated females spawned within one day after ovulation was detected, however; the time between ovulation and spawning varied among individuals. In some females, eggs were released in a single spawn or in partially ovulated females serial spawning occurred 2 or 3 times after the first spawning. Of the 9 GnRH-A implanted females induced to spawn, eggs

Figure 49. The influence of GnRH-A implantation in May upon body weights (mean percent increase body weight) of female winter flounder. Data in parenthesis indicate the number of fish. All data are means ± SEM.

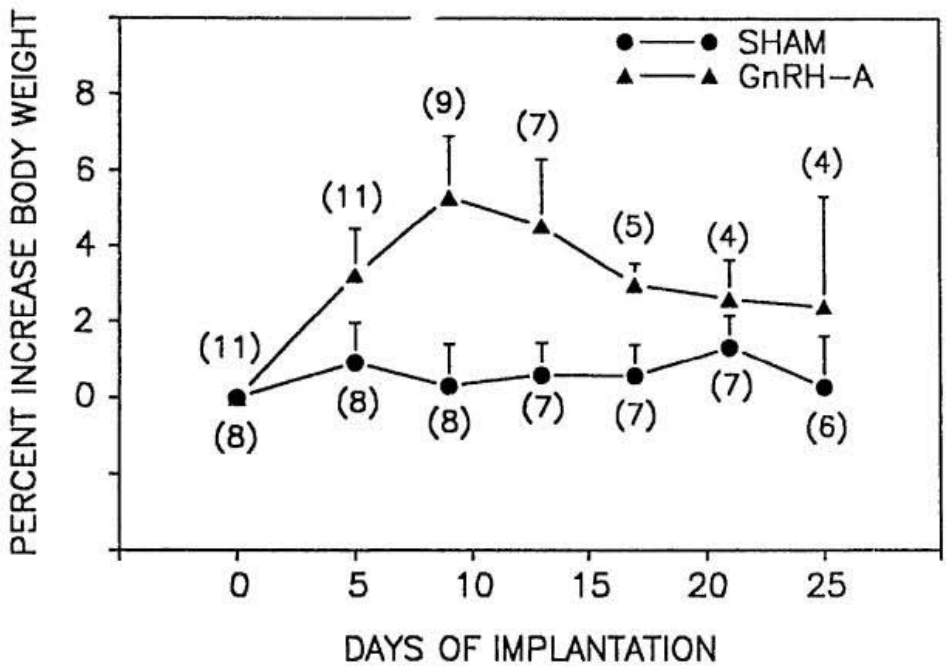
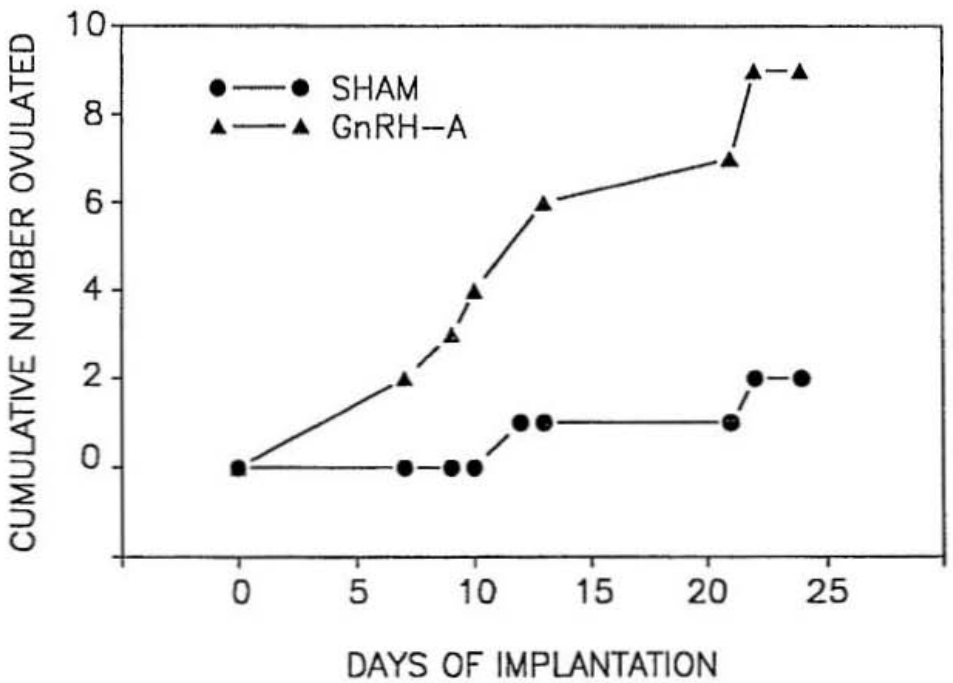

SHAM
GnRH–A
PERCENT INCREASE BODY WEIGHT
8
6
4
2
0
(11)
(9)
(7)
(4)
(11)
(5)
(4)
(11)
(8)
(8)
(8)
(7)
(7)
(7)
(6)
0
5
10
15
20
25
DAYS OF IMPLANTATION

Figure 50. The influence of IM GnRH-A implantation in May 1988 upon the ovulatory response (cumulative number ovulated) of female winter flounder. Day=0 indicates beginning of experiment.


CUMULATIVE NUMBER OVULATED
10
8
6
4
2
0
SHAM
GnRH–A
0
5
10
15
20
25
DAYS OF IMPLANTATION

from 6 females were fertilized in the tank spontaneously; two other females were partially spawned at the end of the experiment and one female had spawned eggs that were of poor quality and were not apparently fertilized. Eggs from only 1 of 2 ovulated control females were fertilized naturally.

Similar to previous experiments, most embryos hatched within two weeks of fertilization at 5°C. Fertilization rates were high, 94%, and 89% respectively, for eggs obtained from the one control fish and from females induced to spawn with GnRH-A (Table 33). Although egg survival to hatch was comparable for eggs obtained from the one control female and females induced to spawn with GnRH-A, good quality larvae were obtained only from females induced to ovulate and spawn with GnRH-A.

6.3.5 Effects of Exposure to Different Water Temperatures on Advanced Ovulatory Responses and Egg Quality in GnRH-A Treated Flounder (Period of Gonadal Maintenance).

The effects of being held at different water temperatures during the winter, upon hormone induced ovulatory responses and resulting egg quality were tested in female winter flounder beginning December 1988 (Table 25, experiment 5). Initially, two groups of females were

Table 33

Evaluation of the effects of induced spawning on egg fertilization rate, hatching rate and relative amounts of normal embryos

Fish #	Ferti-lization[a]	Hatching rate (%)	Normal embryo(%)
Or71	94.0 ±0.5	58.6 ±27.4	3.4 ±1.7
G177	95.7 ±0.6	92.7 ±1.0	98.0 ±1.2
G179	80.7 ±1.6	93.0 ±1.2	98.7 ±0.5
G182	99.3 ±0.1	85.4 ±1.8	97.6 ±0.5
G183	96.8 ±0.6	86.3 ±5.7	94.3 ±1.3
G185	97.5 ±0.2	94.8 ±1.0	97.5 ±0.6
G186	63.4 ±3.8	28.2 ±2.0	68.9 ±2.7

[a] determined from 5-6 replicates of petridishes, each consisting of 200-300 eggs; (OR) sham pellet implant (spontaneously spawn female), (G) GnRH-A implant.

held in the laboratory under either; (Group 1) controlled seawater temperatures maintained relatively warm at approximately 5°C or (Group 2) seasonally declining ambient seawater temperature. Beginning February 2, 1989, when the ambient seawater temperature had reached 0°C, the second group of females was further subdivided into two different temperature groups including; (Group 2A) fish continuing to be exposed to normal seasonally low ambient water temperatures, or (Group 2B) fish immediately returned to 5°C from the low ambient seawater temperatures.

Blank cholesterol pellets (sham control cholesterol pellet implant) or 100 μg GnRH-A pellets were administered to females on February 8, 1989 and body weights and ovulatory responses were followed for a period of 7 wk. The body weight of sham control fish remained unchanged or tended to decrease towards the end of the experiment (Fig. 51). However, following GnRH-A treatment, the body weights of hormone treated females significantly ($P<0.05$) increased (up to 30 days after initiation of the experiment) in fish held at 5°C as well as fish held at low ambient temperature (Groups 1, 2A, and 2B). Autopsies of non-ovulated females at the end of the experiment indicated that (Table 34), GnRH-A treatment resulted in significant increases ($P<0.05$) in gonadosomatic index irrespective of water temperature (pooled GnRH-A compared

Figure 51. The influence of GnRH-A implantation in February 1989 upon body weights (mean percent increase body weight) of female winter flounder (A) females that remained at 5°C never experiencing seasonally low seawater temperature (B) exposed to normal seasonally low ambient water temperatures (C) exposed to the declining low ambient temperatures and then returned to 5°C. Data in parenthesis indicate the number of fish. All data are means ± SEM.

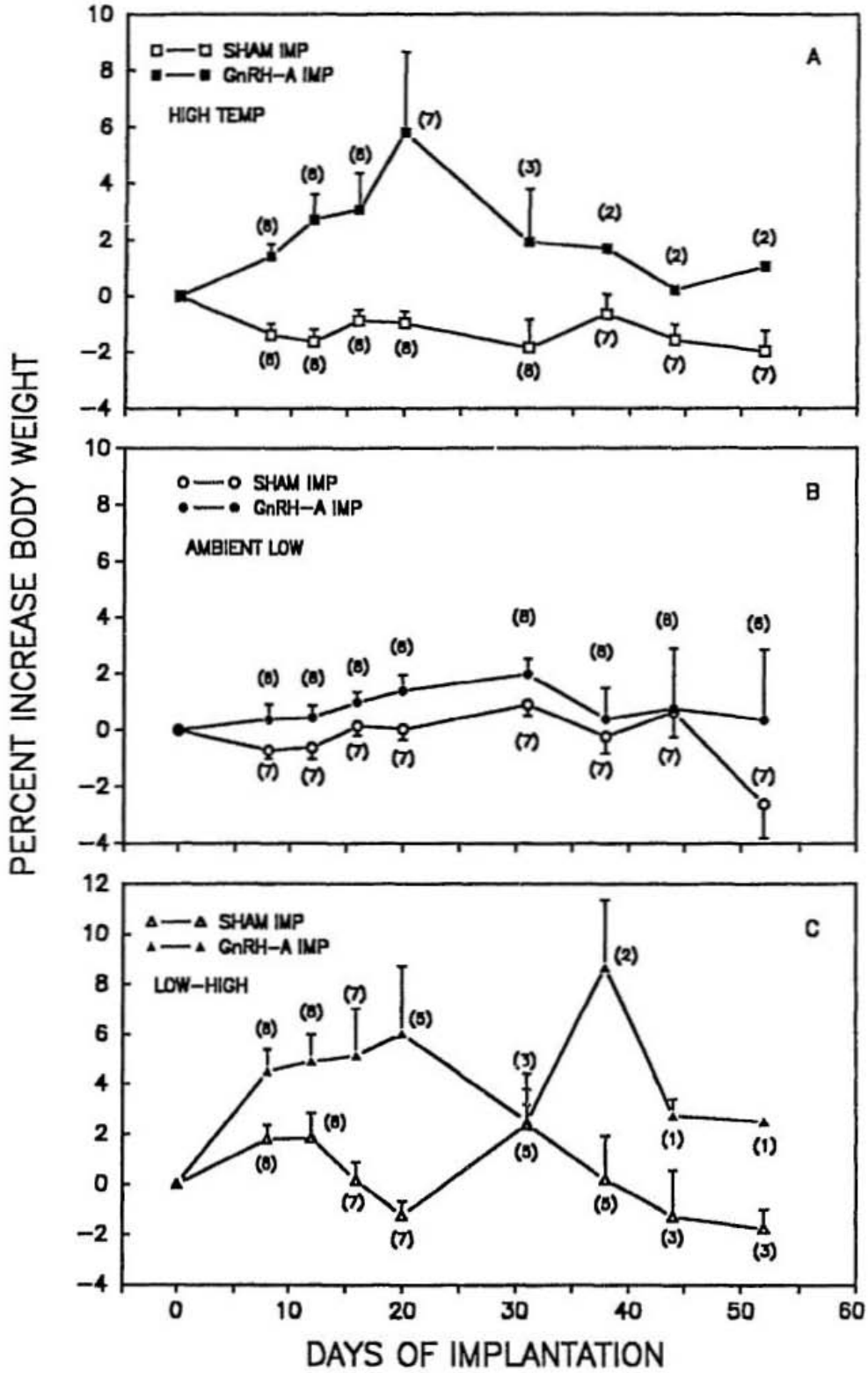

PERCENT INCREASE BODY WEIGHT
SHAM IMP
GnRH-A IMP
HIGH TEMP
A
AMBIENT LOW
B
LOW-HIGH
C
DAYS OF IMPLANTATION

Table 34

Influence of GnRH-A on gonado-somatic index (GSI) in prespawning non-ovulated female winter flounder exposed to different water temperature profiles (February 2 - March 31, 1989)

Treatment	GSI
Controls	15.6[a] ± 0.5 (18)
GnRH-A	21.8[b] ± 0.9 (9)

At the end of the experiment all control and GnRH-A fish, irrespective water temperature, were pooled separately.
( ) number of fish. All values are means ± SEM. significance ($P<0.05$) indicated by different letters.

with pooled control females).

The ovulatory responses of females held under the various experimental temperature regimes are shown in Figure 52. During this particular 51 day winter experiment conducted in February and March, irrespective of water holding temperatures, no control fish ovulated. Similarly, for GnRH-A treated females held at low ambient seawater temperatures, just one of eight females ovulated late in the study period (Group 2A, Fig. 52B). In contrast, the ovulation rate was significantly increased ($P<0.05$) to 83% and 71%, respectively, for GnRH-A treated females held at ambient temperatures and returned to 5°C (Group 2B) or for females held at 5°C and never experiencing seasonally low ambient temperatures (Group 1).

In this experiment egg quality was judged according to egg fertilization rates only (Table 35). Significantly higher ($P<0.05$) mean egg fertilization rates (mean 89%) were recorded for eggs collected from group 2B females which had been exposed to a period of low ambient temperatures and returned to 5°C compared with eggs collected from Group 1 females always held at 5°C (mean 45%).

Figure 52. The influence of GnRH-A implantation in February 1989 upon the ovulatory response (cumulative number ovulated) of female winter flounder (A) females that remained at 5°C never experiencing seasonally low seawater temperature (B) exposed to normal seasonally low ambient water temperatures (C) exposed to the declining low ambient temperatures and then returned to 5°C. Data in parenthesis indicate the number of fish. All data are means ± SEM.

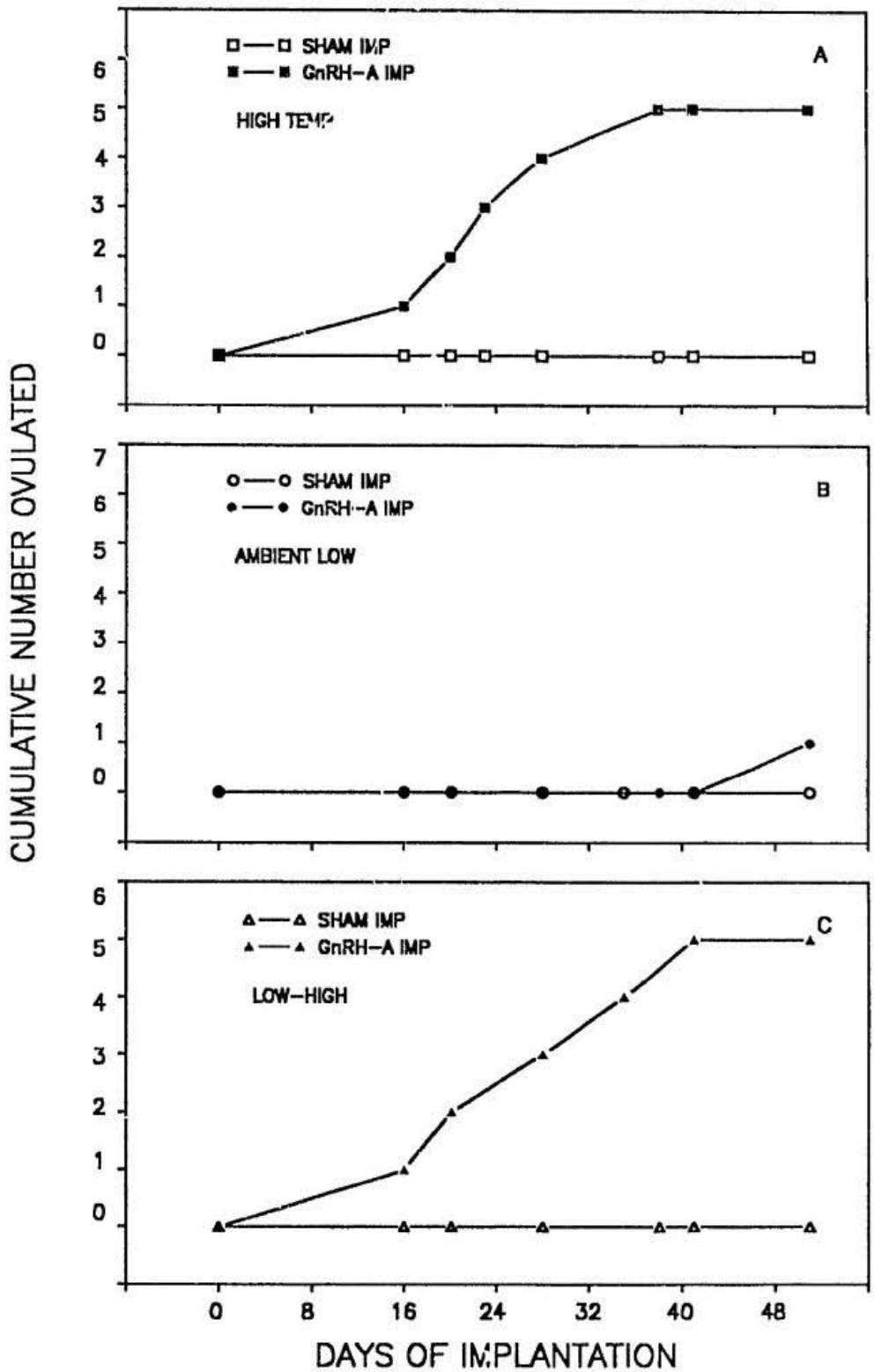

SHAM IMP
GnRH-A IMP
HIGH TEMP
A
SHAM IMP
GnRH-A IMP
AMBIENT LOW
B
SHAM IMP
GnRH-A IMP
LOW-HIGH
C
CUMULATIVE NUMBER OVULATED
0
1
2
3
4
5
6
7
0
8
16
24
32
40
48
DAYS OF IMPLANTATION

Table 35

Influence of induced spawning and temperature on fertilization rate in prespawning female winter flounder (February 2 - March 31, 1989)

Treatment/ fish #	Fertilization (%)
Ambient Temp	
R17	5.5
Low-High Temp	
G174	97.0 ± 0.4
G172	95.9 ± 1.1
G171	71.7 ± 2.1
G169	100 % mort
G175	92.7 ± 0.8
High Temp	
W28	73.0 ± 1.0
W26	73.0 ± 3.7
W25	23.8 ± 2.4
W30	8.7 ± 1.2
W29	ND

Initially each treatment consists of 7 fish; all values are means ± SEM.

6.3.6 Effects of Crude Flounder Pituitary Treatment on Induced Ovulation in May (Spawning Period).

An experiment was conducted just at the onset of the spawning season to determine the ovulatory response of winter flounder treated with crude pituitary extract (Table 25, experiment 6). Beginning May 1, 1989 females were divided into two groups and treated as follows: (Group 1, n=4) controls injected with saline; (Group 2, n=5) females injected (twice/week) with flounder crude pituitary extract (CPE).

Whereas no significant ($P>0.05$) increase in body weight occurred in control females throughout the study period (Fig. 53), the body weight of females injected with crude pituitary extracts increased significantly ($P<0.05$) peaking at 10% by day 15. Thereafter body weight declined.

The ovulatory responses of control females and females treated with pituitary extract is shown in Fig. 54. Only 1 female from the saline injected control group ovulated (May 10, 1989). In contrast, 4 of 5 females (80%) injected with CPE ovulated by the end of this experiment. The first ovulated females in the CPE treatment group also occurred on May 10, 1989 after 4 injections of CPE. Although the number of ovulated females in the CPE group was greater than the controls, the rate of ovulation was

Figure 53. Changes in body weight of prespawning female winter flounder treated with homologous crude pituitary extract (CPE). Control fish were injected with saline. Data in parenthesis indicate the number of fish. All data are means ± SEM.

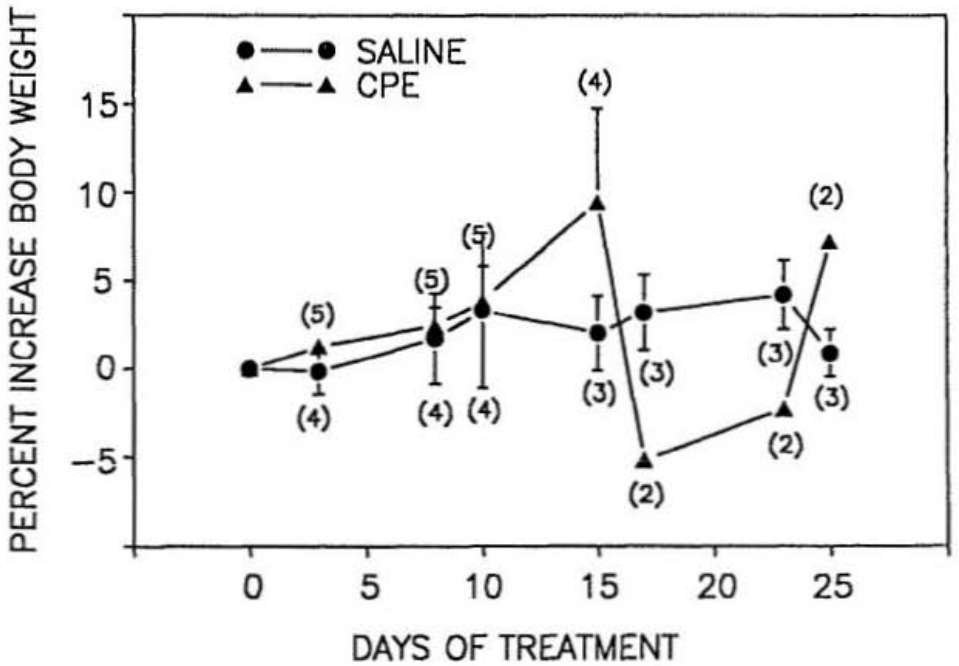
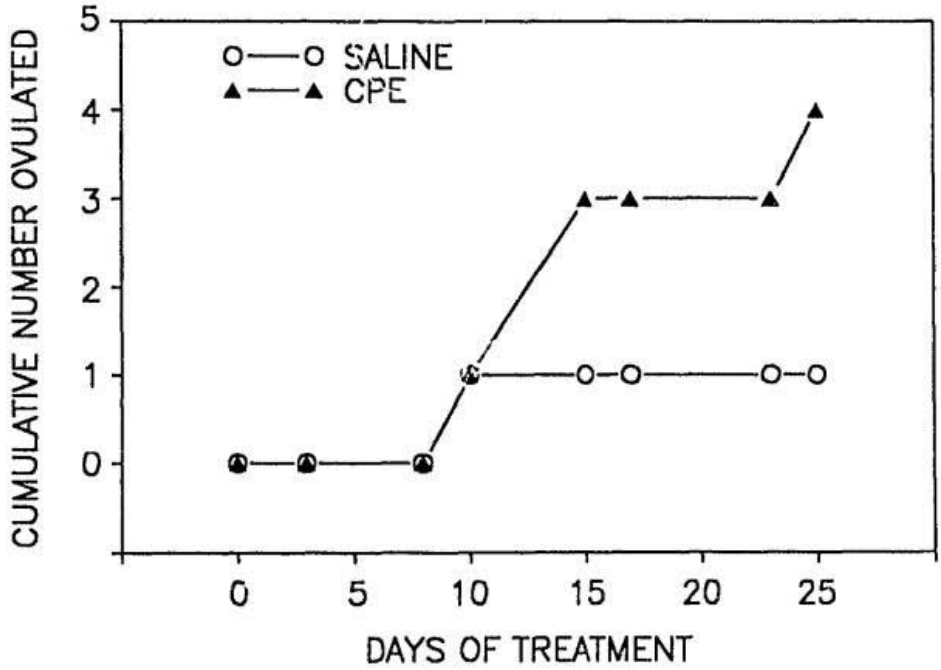

SALINE
CPE
PERCENT INCREASE BODY WEIGHT
15
10
5
0
−5
0
5
10
15
20
25
DAYS OF TREATMENT
(5)
(4)
(5)
(4)
(5)
(4)
(4)
(3)
(3)
(2)
(3)
(2)
(3)
(2)
(3)

Figure 54. Cumulative number females ovulating after treatment with homologous crude pituitary extract (CPE) injection. Control fish were injected with saline.


SALINE
CPE
CUMULATIVE NUMBER OVULATED
0
1
2
3
4
5
0
5
10
15
20
25
DAYS OF TREATMENT

not statistically different between the two groups of females. Although the fertilization rate was not monitored, the eggs had a normal appearance.

6.4 DISCUSSION

The results of these studies clearly show that GnRH-A is a highly effective agent for accelerating final oocyte maturation and inducing ovulation/spawning in prespawning female winter flounder. This is in agreement with results reported for other teleost species, where GnRH-A advances ovulation or spawning in rainbow trout (Crim et al., 1983a), Atlantic salmon (Crim et al., 1983a; Crim and Glebe, 1984), coho salmon (Donaldson et al., 1981; Fitzpatrick et al., 1984; 1987; Sower et al., 1984; Van Der Kraak et al., 1983; 1985), steelhead trout (Sower et al., 1984), and the seabass (Harvey et al., 1985; Almendras et al., 1988; Garcia, 1989; 1990). It is worth noting that captive female flounder held in the laboratory rarely ovulate and spawn spontaneously.

A period of gonadal hydration is commonly observed in teleosts prior to final egg maturation and ovulation (Clements and Grant, 1964). This hydration process is reflected in body weight changes just before ovulation and the female flounder exhibits a markedly distended abdomen at this time (see Figs. 47,49,51). Clearly, untreated control flounder did not experience hydration in the

laboratory in contrast to the hormone-treated females. The present studies are in agreement with the work of Smigielski (1975), who reported that HCG treatment promotes hydration in winter flounder. Increased body weight following hormone treatment has been noted in several other fish species, e.g. striped mullet (Shehadeh and Ellis, 1970), tilapia (Babiker and Ibrahim, 1979), Japanese ayu (Hirose and Ishida, 1974; Hirose et al., 1974), northern anchovy (Leong, 1971), and sablefish (Solar et al., 1987). The practical aspects of this finding are that one may predict the impending ovulatory response in flounder from simply monitoring increases in body weight. Precise knowledge of the timing of ovulation in females increases the opportunity of collection of freshly ovulated, highly viable eggs.

Reliable hormone induction of ovulation and spawning in teleosts is greatly dependent upon the timing of application of the hormone treatment. Although well developed gonads (elevated gonadosomatic index and large oocyte diameters) are found in females as early as December, the earliest ovulations in response to GnRH-A treatment were obtained in February (see Table 27). However, not all of the females responded to hormone treatment in February. It appears that the capacity of GnRH-A to consistently induce ovulation/spawning in winter flounder is probably related to the length of time that

the hormone is administered prior to the natural spawning season. Crim and Glebe (1984) reported in Atlantic salmon, that only 30% of hormone-implanted fish ovulated after treatment with GnRH analog 45 days prior to normal spawning time, whereas 94% of females ovulated when the hormone implantation was delayed until just 28 days prior to spawning time. Similarly, in coho salmon, Fitzpatrick et al. (1987) showed that by delaying GnRH-A treatment until 1 week nearer to the time for spawning, the percentage of responding females increased. The time between the last hormone treatment and the onset of ovulation (latency period) may depend on the stage of development of oocytes (Lam, 1982). While the latent period tended to be longer in February, it was shorter nearer to the spawning season. Water temperatures may have an important influence upon the timing of ovulatory response of female teleosts following GnRH-A hormone treatment. At warmer water temperatures hormone induced ovulatory responses are faster compared to lower water temperatures (Lam, 1982). Perhaps, the relatively long delay between GnRH-A treatment and ovulation/spawning in winter flounder was influenced by the relatively low water temperatures (5°C). Interestingly, the ovulatory responses were no different between female winter flounder held at 5°C and never experiencing seasonal cold water temperatures and those females briefly exposed to cold

ambient seawater temperatures and then returned to 5°C (see Fig. 52). This indicates that there may be other factor(s) serving as environmental cues for fish inducing ovulation/spawning. Substantial delays to ovulation after hormone treatment have been reported in rainbow trout (Crim et al., 1983a), Atlantic salmon (Crim and Glebe, 1984), and sablefish (Solar et al., 1987) where induction of spawning was carried out at relatively low water temperature. The effect of water temperature on ovulation was shown recently in sabalo, a freshwater fish where fish ovulated progressively earlier as the water temperatures were gradually increased (Fortuny et al., 1988). Many reports describe rapid hormonal induction of ovulation or spawning when fish are held at relatively higher temperatures. Normally, ovulation occurs within 24-48 hr after hormone treatment between 17°C and 32°C, e.g. the common sole (Ramos, 1986b), Indian carp (Kaul and Rishi, 1986), milkfish (Marte et al., 1988b), goldfish (Sokolowska et al. 1984) and spotted seatrout (Thomas and Boyd, 1988).

Several types of drugs and hormones have been used to induce ovulation/spawning in fish (Donaldson et al., 1983) and in some species ovulation rate is dependent upon the types of hormone administered. When winter flounder were treated with GnRH-A in April a high percentage of females ovulated. However, human chorionic gonadotropin appeared

to be largely ineffective for inducing ovulation, since only one partially ovulated female was observed at the end of the April study (see Fig. 48). Smigielski (1975), who noted the variable ovulatory response of winter flounder treated with HCG in early autumn, suggested that the failure of winter flounder to respond to HCG probably results from warm water temperatures (>6°C). Still, ovulation is achieved using human chorionic gonadotropin in other flatfish e.g. the sole (Ramos, 1986a). and the Japanese flounder (Hirose et al., 1976; Hirose et al., 1979). The effective dose of human chorionic gonadotropin is variable among different fish species. For example Rowland (1983) showed that 500 IU/kg is the minimal required dose to induce ovulation in golden perch whereas in Asian catfish (Mollah and Tan, 1983) the threshold dose of human chorionic gonadotropin is 2000 IU/kg. Perhaps the dose of 500 IU/kg human chorionic gonadotropin is too low for inducing ovulation in winter flounder. In fact, in the present study the human chorionic gonadotropin dose was higher than reported by Smigielski (1975), and a human chorionic gonadotropin dose-response curve should be conducted to determine the minimum effective dose.

The neuroendocrine regulation of gonadotropin secretion in teleosts involves a dual control, with gonadotropin release stimulated by a gonadotropic hormone-releasing hormone and inhibited by a catecholamine which has been

suggested to be dopamine (Peter et al., 1986). Administration of the dopamine receptor antagonist pimozide greatly potentiates the action of GnRH on gonadotropin release and combined injections of pimozide and GnRH-A are highly effective in inducing ovulation in goldfish (Chang and Peter, 1983; Sokolowska et al., 1984; 1985), common carp (Billard et al., 1984, Lin et al., 1988), African catfish (De Leeuw et al., 1985), loach (Lin et al., 1985b), and in the Asian catfish (Manickam and Joy, 1989). Recent studies have demonstrated that the dopaminergic inhibition of GTH release perhaps is not a universal phenomena in all teleosts. In the present flounder study, pimozide in combination with GnRH-A, did not potentiate the effect of GnRH-A on ovulation, nor was pimozide treatment alone effective. This is in agreement with work of Copeland and Thomas (1989) and Zohar et al. (1987) who found no evidence of dopaminergic inhibition in Atlantic croaker and gilthead seabream, respectively. The use of crude pituitary extracts to induce spawning of fish is a common procedure (Harvey and Hoar, 1979; Lam, 1982; Donaldson and Hunter, 1983). Most of the work using pituitary extract to induce spawning is conducted on mature fish during the normal spawning season where single or double hormone injections are usually sufficient to induce ovulation. In the present studies, however, multiple injections of homologous pituitary hormone

extract were necessary to induce ovulation in winter flounder during the breeding season. Although one might expect induction of the ovulatory response of flounder to be more rapid during spawning season, interestingly, a delay of about 10 days to ovulation was observed following crude pituitary extract injections and a similar latency period is required to achieve ovulation following GnRH-A treatment.

Different modes of hormone administration in teleosts may play an important role in optimizing the protocol for inducing spawning. In these experiments with female flounder which began in February, releasing hormone was administered either by simple rapid acting injections, or long-term hormone release pellet implantation (see Table 27). Early in the reproductive season both modes of hormone application effectively advanced ovulation in a few females; however, GnRH-A pellet implantation proved advantageous because handling stress was minimized and prespawning mortality was reduced. GnRH-A implantation is an effective method for inducing ovulation and spawning in a variety of teleosts including rainbow trout (Crim et al., 1983a), Atlantic salmon (Crim and Glebe, 1984; Crim et al., 1986; Davies et al., 1987), sea bass (Harvey et al., 1985; Garcia, 1989; 1990) and milkfish (Lee et al., 1986; Marte et al., 1987).

The most important concern following hormonal

induction of ovulation is the preservation of egg quality but this issue has rarely been addressed in many previous studies of hormone-induced spawning. Whereas egg fertilization and hatching rates are commonly used to indicate egg quality, it is equally vital to consider even later stages of embryo development and where possible production of normal healthy fry. The best egg quality, i.e., highest hatching rates and greatest proportions of normal larvae, was obtained when female flounder were stripped soon after ovulation and the fresh eggs immediately fertilized (see Tables 28,32,33,35). Still, hormone-induced spawning clearly can produce large numbers of normal larvae when flounder are induced to spawn early, suggesting that GnRH-A treatment does not necessarily produce detrimental effects on larval morphology. Sometimes, low egg fertilization rates following GnRH-A induction of ovulation of female flounder may have been attributed to the advancement of spontaneous spawning which leaves behind very few eggs for testing of <u>in vitro</u> fertilization. Poor egg quality after hormone-induction of spawning could also be due to factors such as egg overripening as reported in ayu (Hirose et al., 1977) and the Japanese flounder (Hirose et al. 1979). The dose of hormone used to induce spawning might influence egg quality e.g. low egg fertilization rates were observed following high doses of HCG in common sole (Ramos, 1986a).

Furthermore, premature seasonal application of hormone treatment may induce precocious egg maturation and ovulation (Fitzpatrick et al. 1984). Early induction of spawning in Atlantic salmon using GnRH-A produces a high number of abnormal eggs (Crim and Glebe, 1984), it lowers egg fertility in coho salmon (Hunter et al., 1981; Fitzpatrick et al., 1984) and it resulted in heavy egg mortalities in rainbow trout (Crim et al., 1983a; Billard et al., 1984). Interestingly, Lee et al. (1987) recorded low egg fertilization rates when grey mullet were induced to spawn with GnRH-A alone, compared to when GnRH-A was injected followed by carp pituitary homogenate. More recently, Garcia et al. (1989) observed that inducing spawning in sea bass with high doses of GnRH-A (350 μg/kg) resulted in a lowering of egg fertilization rates. In contrast, in catfish, normal larvae are produced following releasing hormone treatment (De Leeuw et al., 1985; Manickam and Joy, 1989;). In a previous study on winter flounder, Weigand et al. (1987) showed that GnRH-A treatment may be used to induce ovulation without detectable changes in ovarian chemistry e.g. fatty acids.

In conclusion, GnRH-A treatment is a useful technique to advance ovulation/spawning in prespawning female winter flounder; however, the best egg quality can be achieved when hormone induced ovulation/spawning is performed near the time of the normal breeding season.

CHAPTER 7

GENERAL DISCUSSION

The winter flounder is an excellent model fish for understanding basic endocrine reproductive mechanisms. Such studies have the potential to be used to control the reproductive processes, e.g. accelerating gonadal development and induced ovulation/spawning of fish held under laboratory conditions. Since winter flounder are an inshore species that are readily available all year and are easily maintained in the laboratory, it was possible to undertake these seasonal studies of fish at different stages of the reproductive cycle.

Initially, it is important to describe the physiology of the natural reproductive cycle of winter flounder (CHAPTER 3). It is clear that gonadal recrudescence in both males and females begins in early August immediately following the early summer feeding period. Recruitment of small (<150 μm) previtellogenic oocytes into vitellogenesis begins in August and by tracing the dynamics of vitellogenic oocyte development in female flounder it was found that one clutch of vitellogenic oocytes mature for the current spawning season. This is in agreement with the studies of Burton and Idler (1984) and Dunn and Tyler (1970). The very rapid ovarian growth

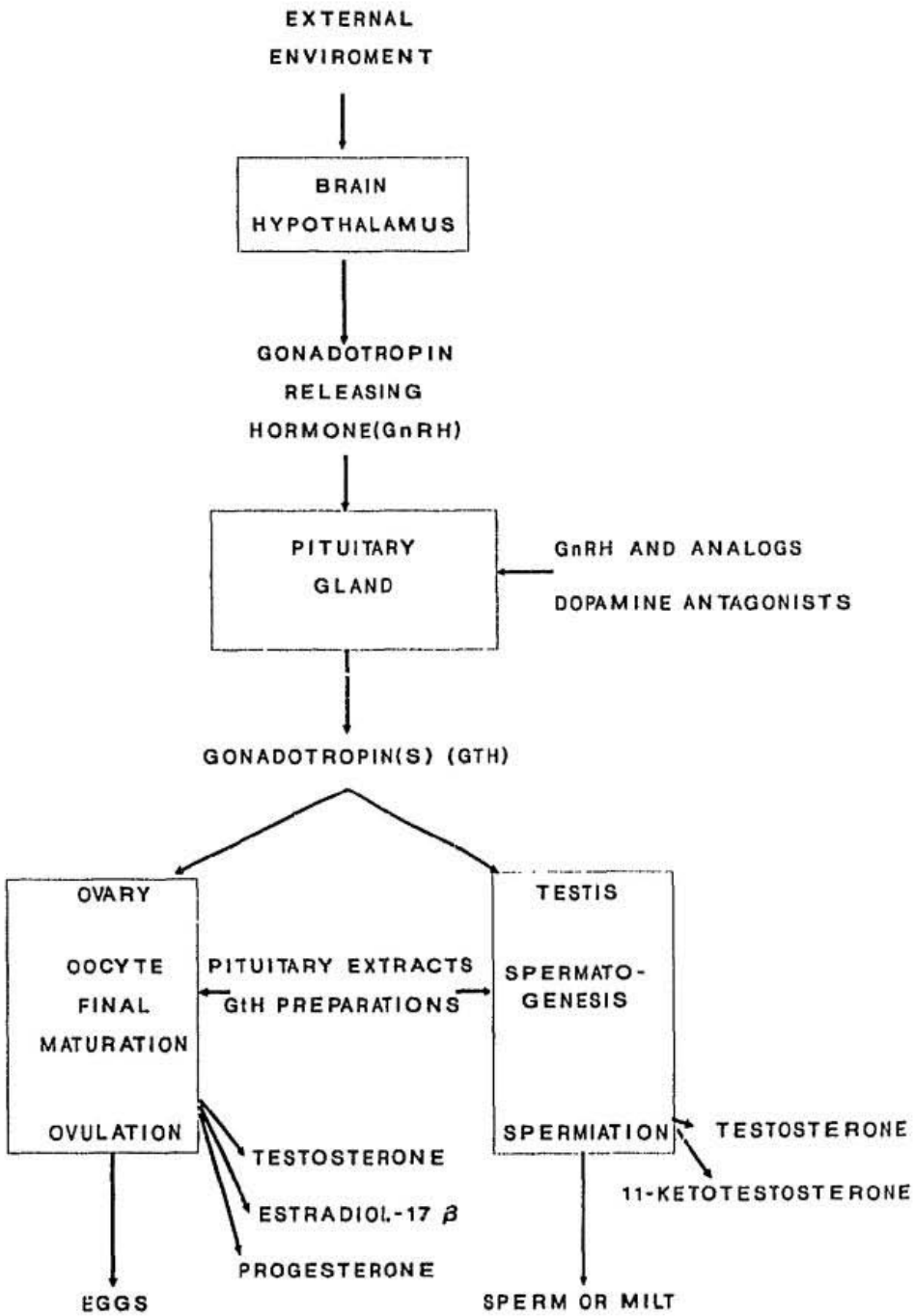
observed from August through November slows by December although oocyte growth continues throughout the winter, as indicated by further increases in both gonadosomatic index and oocyte diameter. During the prespawning phase (March-May), final increases in gonadosomatic index and oocyte diameter associated with gonadal hydration are observed prior to spawning in May/June.

The present study indicates that the sequential development of the gonads is clearly related to increases in the sex steroid hormones (CHAPTER 3). In females, plasma estradiol-17ß provides a good index of early reproductive activity since estradiol-17ß increases in parallel with gonadosomatic index; in contrast, plasma testosterone remains low during the onset of the initial gonadal recrudescent period. Both plasma estradiol-17ß and testosterone reach their highest seasonal levels in prespawning fish; after spawning the steroid hormone levels rapidly decline. It is worth noting that ovarian recrudescence was apparently not impaired in adult female flounder held in the laboratory under seasonally ambient seawater temperature and photoperiod conditions; indeed ovarian growth progressed in parallel with increased plasma estradiol-17ß and testosterone and continued rising until spawning when the hormones declined similar to the pattern found in wild fish. The gonadosomatic index of males reached maximal values in October, indicating that

seasonal testicular recrudescence occurs more rapidly than ovarian development in females. Interestingly, male gonadosomatic index values fell prior to the spawning period in May. Both plasma testosterone and 11-ketotestosterone levels rose slowly in association with progressive testicular development and these hormones reached their peak values during the spawning period before declining precipitously during the post-spawned period. Peak sperm production coincided with the highest plasma androgen levels.

In teleosts, the reproductive process consists of a chain of events involving the hypothalamo-pituitary-gonad axis. The fish endocrine system can be divided into three levels (Fig. 55): first, neuropeptide hormones (gonadotropic hormone-releasing hormone) located in the brain regulate gonadotropin(s) synthesis and secretion; second, pituitary gonadotropin(s) stimulate the production of gonadal hormones or sex steroids i.e. estradiol-17ß, testosterone and progesterone in the females, and testosterone and 11-ketotestosterone in the males, and third, the gonadal steroid hormones directly influence gonadal events such as oogenesis, maturation, ovulation and spawning in the females, and spermatogenesis and spermiation in the males. Many cultured fish species do not spawn in captivity or if spawning occurs they may not spawn synchronously for optimal management. Thus, hormonal

Figure 55. Sites of action in endocrine regulation for the control of reproduction.


EXTERNAL
ENVIROMENT
BRAIN
HYPOTHALAMUS
GONADOTROPIN
RELEASING
HORMONE(GnRH)
PITUITARY
GLAND
GnRH AND ANALOGS
DOPAMINE ANTAGONISTS
GONADOTROPIN(S) (GTH)
OVARY
OOCYTE
FINAL
MATURATION
OVULATION
PITUITARY EXTRACTS
GtH PREPARATIONS
TESTIS
SPERMATO-
GENESIS
SPERMIATION
TESTOSTERONE
11-KETOTESTOSTERONE
TESTOSTERONE
ESTRADIOL-17 β
PROGESTERONE
EGGS
SPERM OR MILT

manipulation of the reproductive cycle becomes of practical value and has become a routine practice in fish farming. Manipulation of reproductive cycles in teleosts is based on intervention in the cascade of endocrine events operating along the hypothalamo-pituitary-gonad axis. Hormones from all three levels have been used for inducing gametogenesis (spermatogenesis and oogenesis), spermiation, ovulation and spawning (Donaldson and Hunter, 1983). [D-Ala^{6},Pro^{9}-NHEt]LHRH (GnRH-A) was used as a chemical messenger in the present studies to intervene at the pituitary level and manipulate different phases of the flounder reproductive cycle. The GnRH-A was selected because it is commercially available and it has proven to be biologically active in a variety of fish species. GnRH-A has other advantages over classical pituitary extracts since application of gonadotropic hormone-releasing hormone stimulates the fish to release its own endogenous gonadotropin.

The present studies showed that winter flounder respond to GnRH-A treatment at different stages of the seasonal reproductive cycle (CHAPTER 5). Adult winter flounder are particularly responsive to GnRH-A treatment in the Fall during the period of gonadal recrudescence and they also spawn early in response to GnRH-A treatment during the prespawning phase. In contrast, they are poorly or not responsive at all during the period when the gonads

are regressed. This is in agreement with findings of Sokolowska et al. (1985) and Lin et al. (1985a) showing that goldfish are more responsive to GnRH-A during the prespawning phase. Similarly, in trout it was demonstrated that GnRH induces greater gonadotropin release in sexually mature individuals as opposed to less mature fish (Weil et al., 1978; Crim and Cluett, 1974). While it is well known that sexually mature prespawning fish are responsive to GnRH-A, these studies on winter flounder clearly showed that they are also very responsive to GnRH-A treatment during the period of early gonadal recrudescence.

During the period of gonadal recrudescence GnRH-A treatment is effective in stimulating ovarian development as reflected by increases in the gonadosomatic index, oocyte diameter and oocyte size-frequency profiles in females. Similarly, gonadosomatic index is increased in males in September and GnRH-A treatment accelerates the onset of spermiation as early as November. In prespawning female winter flounder, GnRH-A accelerates final egg maturation and ovulation early during the winter well in advance of the normal spawning season. GnRH-A administered by either implant or injection in prespawning females induced ovulation three months prior to the normal spawning season. GnRH-A incorporated in a pellet may be the best alternative to multiple hormone injections for acceleration and synchronization of vitellogenesis in

broodstock females as in winter flounder, or promoting repeated ovulations in multiple spawners as in gilthead seabream (Zohar, 1988) or seabass (Almendras, 1988; Garcia, 1990). All these studies make the point that induced spawning by releasing hormone implantation is a good method for minimizing handling stress compared with protocols calling for a series of GnRH-A injections.

Although egg/larval quality data generally indicated that GnRH-A can be used to advance spawning of females without causing serious detrimental effects upon the rates of egg fertilization, hatching and larval survival, the egg quality index appeared to improve when hormone induction of ovulation was performed closest to the natural spawning period (CHAPTER 6). From the points of view of speed/ synchrony of ovulation and of egg quality, it appears that hormone induced ovulation is best achieved during the spawning period.

The work in this thesis has emphasized the importance of understanding the basic endocrine mechanisms regulating the reproductive cycle of winter flounder prior to hormonal manipulation. The present work has demonstrated clearly that GnRH-A manipulation of broodstock reproduction is practical for controlling certain steps of the reproductive processes including gametogenesis, ovulation, and spermiation. During the early phases of the reproductive cycle, GnRH-A treatment can be used to

accelerate gametogenesis. Since this is a long term process, hormone implantation is the recommended method for application compared with administration of a series of hormone injections. In captivity, female flounder seldom ovulate/spawn spontaneously; if it does occur, spawning is often delayed. Clearly, GnRH-A treatment can be used to advance ovulation/spawning to February potentially allowing earlier collection of fry for hatchery operations. In the males, GnRH-A can be used to stimulate the onset of spermiation and increase the amounts of milt collected. Particularly in species where very small amounts of milt are produced, GnRH-A can be applied to increase milt volume especially for artificial fertilization work.

While successful hormonal manipulation of some steps of the winter flounder reproductive cycle have been described in this thesis, clearly there is much more to be accomplished. Investigation of the role of gonadotropin(s) in gametogenesis and steroidogenesis is needed as is knowledge of how gonadotropin(s) levels vary during the entire reproductive cycle. In flatfish, little is known about the process of oocyte final maturation which is under the control of gonadotropin(s) and mediated by unknown steroids secreted by the follicular cells of the oocyte.

REFERENCES

Aida, K. (1983). Effects of LH-Releasing Hormone on gonadal development in a salmonid fish, the ayu. Bull. Jap. Soc. Sci. Fish. 49, 711-718.

Almendras, J.M., Duenas, C., Nacario, J., Sherwood, N.M., and Crim, L.W. (1988). Sustained hormone release. III. Use of gonadotropin releasing hormone analogues to induce multiple spawnings in sea bass, Lates calcarifer. Aquaculture 74, 97-111.

Alvarez-Lajunchere, L., Berdayes-Arritola, J., Laiz-Averhoff, O., and Diaz-Bellido, S. (1988). Positive results of induced spawning and larval rearing experiments with Mugil Liza Val., a grey mullet from Cuban waters. Aquaculture 73, 349-355.

Asano, K., and Tanaka, S. (1989). Ovarian maturation and spawning of the Japanese common mackerel, Scomber japonicus. Nippon Suissan Gakkaishi 55, 1715-1726.

Babiker, M.M., and Ibrahim, H. (1979). Studies on the biology of reproduction in the cichlid Tilapia nilotica (L.): effects of steroid and trophic hormones on ovulation and ovarian hydration. J. Fish Biol. 15, 21-30.

Barr, W.A. (1963). The endocrine control of the sexual cycle in the plaice, Pleuronectes platessa (L). I. Cyclical changes in the normal ovary. Gen. Comp. Endocrinol. 3, 197-204.

Bigelow, H.B., and Schroeder, W.C. (1953). Fishes of the Gulf of Maine. Fish. Bull. U.S. Natl. Mar. Fish. Serv. 53, 1-577.

Billard, R., Bry, C., and Gillet, C. (1981). Stress, environment and reproduction in teleost fish. In. "Stress and Fish" (A.D. Pickering, Ed.), pp. 185-208. Academic Press London.

Billard, R., Fostier, A., Weil, C., and Breton, B. (1982). Endocrine control of spermatogenesis in teleost fish. Can. J. Fish Aquat. Sci. 39, 65-79.

Billard, R., Reinaud, P., Hollebecq, M.G., and Breton, B. (1984). Advancement and synchronisation of spawning in Salmo gairdneri and S trutta following administration of LRH-A Combined or not with Pimozide. Aquaculture 43, 57-66.

Billard, R. (1989). Endocrinology and fish culture. Fish Physiol. Biochem. 7, 49-58.

Billard, R., Le Gac, F, and Loir, M. (1990). Hormonal control of sperm production in teleost fish. In Progress in Comparative Endocrinology: Proceedings of the XIth International Symposium on Comparative Endocrinology, Malaga, Spain 14-20, 1989. (A. Epple, C.G Scanes, and M.H. Stetson, Eds.), p. 329-335. Wiley-Liss, Inc. New York.

Bishop, R.L. (1987). Observer's handbook. The Royal Astronomical Society of Canada, Toronto. Ontario. pp. 210.

Bishop, R.L. (1988). Observer's handbook. The Royal Astronomical Society of Canada, Toronto. Ontario. pp. 212.

Breder, C.M.Jr. (1922). Description of the spawning habits of Pseudopleuronectes americanus in captivity. Copeia 102, 3-4.

Burgus,R., Butcher, M., Amoss, M., Ling, N., Monahan, M., Rivier, J., Fellows, R., Blackwell, R, Vale, W., and Guillemin, R. (1972). Primary structure of the ovine hypothalamic luteinizing hormone-releasing factor (LRF). Proc. Natl. Acad. Sci. USA 69, 278-282.

Burke, M.G., Leatherland, J.F., Sumpter, J.P. (1984). Seasonal changes in serum testosterone, 11-ketotestosterone, and 17β-estradiol levels in the brown bullhead, Ictalurus nebulosus Lesueur. Can. J. Zool. 62, 1191-1199.

Burton, M.P., and Idler, D.R. (1984). The reproductive cycle in winter flounder, Pseudopleuronectes americanus (Walbaum). Can. J. Zool. 62, 1195-1199.

Campbell, C.M., and Idler, D.R. (1976). Hormonal control of vitellogenesis in hypophysectomized winter flounder (Pseudopleuronectes americanus Walbaum). Gen. Comp. Endocrinol. 28, 143-150.

Campbell, C.M., Walsh, J.M., and Idler, D.R. (1976). Steroids in the plasma of winter flounder (Pseudopleuronectes americanus) Walbaum. a seasonal study and investigation of steroid involvement in oocyte maturation. Gen Comp. Endocrinol. 29, 14-20.

Campbell, C.M., and Jalabert, B. (1979). Selective protein incorporation by vitellogenic Salmo gairdneri oocytes

in vitro. Ann. Biol. anim. Bioch. Biophys 19, 429-437.

Chan, K.K. (1977). Effects of synthetic Luteinizing Hormone Releasing Hormone (LH-RH) on ovarian development in Japanese medaka, Oryzias latipes Can. J. Zool. 55, 155-160.

Chang, J.P., and Peter, R.E. (1983). Effects of pimozide and des Gly^{10} [$D\text{-}Ala^{6}$] luteinizing hormone-releasing hormone ethylamide on serum gonadotropin concentrations, germinal vesicle migration and ovulation in female goldfish, Carassius auratus. Gen. Comp. Endocrinol. 52, 30-37.

Chang, J.P., Peter, R.E., Nahorniak, C.S., and Sokolowska, M. (1984). Effects of catecholaminergic agonists and antagonists on serum gonadotropin concentrations and ovulation in goldfish: evidence for specificity of dopamine inhibition of gonadotropin secretion. Gen. Comp. Endocrinol. 55, 351-360.

Clements, H.P., and Grant, F.B. (1964). Gonadal hydration of carp (Cyprinus carpio) and goldfish (Carassius auratus) after injections of pituitary extracts. Zoologica, N.Y. 49, 193-210.

Cochran, R.C. (1987). Serum androgens during the annual reproductive cycle of the male mummichog, Fundulus heteroclitus. Gen. Comp. Endocrinol. 65, 141-148.

Copeland, P.A, and Thomas, P. (1989). Control of gonadotropin release in the Atlantic croaker (Micropogonias undulatus): Evidence for lack of dopaminergic inhibition. Gen. Comp. Endocrinol. 74, 474-483.

Craik, J.C.A, and Harvey, S.M. (1984). Biochemical changes occurring during final maturation of eggs of some marine and freshwater teleosts. J. Fish Biol. 24, 599-610.

Crim, L.W., and Cluett, D.M. (1974). Elevation of plasma gonadotropin concentration in response to mammalian gonadotropin releasing hormone (GRH) treatment of the male brown trout as determined by radioimmunoassay. Endocr. Res. Commun. 1, 101-110.

Crim, L.W., Watts, E.G., and Evans, D.M. (1975). The plasma gonadotropin profile during sexual maturation in a variety of salmonid fishes. Gen. Comp. Endocrinol. 27, 62-70.

Crim, L.W., and Evans, D.M. (1978). Seasonal levels of pituitary and plasma gonadotropin in male and female Atlantic salmon parr. Can. J. Zool. 56, 1550-1555.

Crim, L.W., Evans, D.M., Coy, D.H., and Schally, A.V. (1981). Control of gonadotropic hormone release in trout: Influence of synthetic LH-RH and LH-RH analogues *in vivo* and *in vitro*. Life Sci. 28, 129-135.

Crim, L.W. (1982). Environmental modulation of annual daily rhythyms associated with reproduction in teleost fishes. Can. J. Fish. Aquat Sci. 39, 17-21.

Crim, L.W., Evans, D.M., and Vickery, B.H. (1983a). Manipulation of the seasonal reproductive cycle of the landlocked Atlantic salmon (*Salmo salar*) by LHRH analogues administered at various stages of gonadal development. Can. J. Fish. Aquat. Sci. 40, 61-67.

Crim, L.W., Sutterlin, A.M., Evans, D.M., and Weil, C. (1983b). Accelerated ovulation by pelleted LHRH analogue treatment of spring-spawning rainbow trout (*Salmo gairdnerii*) held at low temperature. Aquaculture 35, 299-307.

Crim, L.W., and Glebe, B.D. (1984). Advancement and synchrony of ovulation in Atlantic salmon with pelleted LHRH analog. Aquaculture 43, 47-56.

Crim, L.W. (1985). Actions of LHRH and its analogs in lower vertebrates. *In* "LHRH and its analogs: Contraceptives and Therapeutic Application, Part I" (B.H. Vickery, J.J.Jr. Nestor, and E.S.E. Hafez, Eds.), p. 377-384. MTP Press Limited, Lancaster, England.

Crim, L.W., Glebe, B.D., and Scott, A.P. (1986). The influence of LHRH analog on oocyte development and spawning in female Atlantic salmon, *Salmo salar*. Aquaculture 56, 139-149.

Crim, L.W., Peter, R.E., and Van der Kraak, G. (1987). The use of LHRH analogs in aquaculture. *In* "LHRH and its analogs: Contraceptive and Therapeutic Application, Part II" (B.H. Vickery, and J.J. Nestor, Jr., Eds.), p. 489-498. MTP Press Limited, Lancaster, England.

Crim, L.W., Sherwood, N.M., and Wilson, C.E. (1988). Sustained Hormone Release. II. Effectiveness of LHRH Analog (LHRHa): administration by either single time injection or cholesterol pellet implantation on plasma gonadotropin levels in a bioassay model fish, the

juvenile rainbow trout. Aquaculture 74, 87-95.

Crossland, J. (1977). Seasonal reproductive cycle of snapper Chrysophrys auratus (Forster) in the Hauraki Gulf. N. Z. Journal of Freshwater Research 11, 37-60.

Davies, B., Glebe, B.D., and Crim, L.W. (1987). Use of pelleted LHRH analogue to induce spawning in Atlantic Salmon. In "Proceedings of the Third International Symposium on the Reproductive Physiology of Fish, St. John's, Newfoundland, Canada, 1987" (D.R. Idler, L.W. Crim and J.M. Walsh, Eds.), p. 25. Marine Sciences Research Laboratory, St. John's, Newfoundland, Canada.

De Leeuw, R., Goos, H.J. Th., Richter, C.J.J., and Eding, E.H. (1985). Pimozide LHRH induced breeding of the African catfish, Clarias gariepinus (Burchell). Aquaculture 44, 295-302.

De Vlaming, V. (1983). Oocyte development patterns and hormonal involvements among teleosts. In: "Control processes in Fish Physiology" (J.C. Rankin, T. Pitcher and R. Duggan, Eds.), pp. 176-199. Croom-Helm, London.

De Vlaming, V., Fitzgerald, R., Delahunty, G., Cech, J.J., Selman, K., and Barkley, M. (1984). Dynamics of oocyte development and related changes in serum estradiol-17β, yolk precursor and lipid levels in the teleostean fish, Leptocottus armatus. Comp. Biochem. Physiol. 77A, 599-610.

Donaldson, E.M., Hunter, G.A., and Dye, H.M. (1981). Induced ovulation in coho salmon (Oncorhynchus kisutch). II. Preliminary study of the use of LH-RH and two high potency LH-RH Analogues. Aquaculture 26, 129-141.

Donaldson, E.M., and Hunter, G.A. (1983). Induced final maturation, ovulation, and spermiation in cultured fish. In "Fish Physiology" (W.S. Hoar, D.J. Randall and E.M. Donaldson, Eds.), Vol. IXB, pp. 351-403. Academic Press, New York.

Dunn, R.S., and Tyler, A.V. (1969). Aspects of the anatomy of the winter flounder ovary with hypotheses on oocyte maturation time. J. Fish. Res. Bd. Can. 26, 1943-1947.

Dunn, K.S. (1970). Further evidence for a three-year oocyte maturation time in the winter flounder (Pseudopleuronectes americanus). J. Fish Res. Bd. Can. 27, 957-960.

Epler, P., and Bieniarz, K. (1978). In vitro ovulation of European eels (Anguilla anguilla L.) oocytes following in vivo stimulation of sexual maturation. Ann. Biol. anim. Biochim. Biphys. 18, 991-995.

Fahay, M.P. (1983). Guide to the early stages of marine fishes occurring in the western North Atlantic Ocean, cape Hatteras to the Southern Scotian Shelf. J. Northw. Atl. Fish Sci. 4, pp. 423.

Fitzpatrick, M.S., Suzumoto, R.K., Schreck, C.B., and Oberbillig, D. (1984). Luteinizing hormone-releasing hormone analogue induces precocious ovulation in adult coho salmon (Oncorhynchus kisutch). Aquaculture 43, 67-73.

Fitzpatrick, S.M., Redding, J.M., Ratti, F.D., and Schreck, C.B. (1987). Plasma testosterone concentration predicts the ovulatory response of coho salmon (Oncorhynchus kisutch) to gonadotropin-releasing hormone analog. Can. J. Fish. Aquat. Sci. 44, 1351-1357.

Fortuny, A., Espinach Ros, A., and Amutio, V.G. (1988). Hormonal induction of final maturation and ovulation in the Sabalo, Prochilodus platensis Holmberg: treatments, latency and incubation times and viability of ovules retained in the ovary after ovulation. Aquaculture 73, 373-381.

Fostier, A., Jalabert, B., Billard, R., Breton, B., and Zohar, Y. (1983). The gonadal steroids. In. "Fish Physiology" (W.S. Hoar, D.J. Randall and E.M. Donaldson, Eds.), Vol. IXB, p. 277-372. Academic Press, New York.

Fostier, A., Billard, R., Breton, B., Legendre, M., and Marlot, S. (1982). Plasma 11-oxotestosterone and gonadotropin during the beginning of spermiation in rainbow trout (Salmo gairdneri R.). Ger. Comp. Endocrinol. 46, 428-434.

Funk, J.D., and Donaldson, E.M. (1972). Induction of precocious sexual maturity in male pink salmon (Onchorhynchus gorbuscha). Can. J. Zool. 50, 1413-1419.

Gale, W.F., and Deutsch, W.G. (1985). Fecundity and spawning frequency of captive tessellated darters-fractional spawners. Trans. Am. Fish. Soc. 114, 220-229.

Garcia, L.Ma.B. (1989). Dose-dependent Spawning Response of mature female sea bass, Lates calcarifer (Bloch), to pelleted luteinizing hormone-releasing hormone analogue (LHRHa). Aquaculture 77, 85-96.

Garcia, L.M.B. (1990). Advancement of sexual maturation and spawning of sea bass, Lates calcarifer (Bloch), using pelleted luteinizing hormone-releasing hormone analogue and 17α-methyltestosterone. Aquaculture 86, 333-345.

Godinho, H.P., and Godinho, A.L. (1986). Induced spawning of the pacu, Colossoma mitrei (Berg 1895), by hypophysation with crude pituitary extract. Aquaculture 55, 69-73.

Goetz, F.W. (1983). Hormonal control of oocyte final maturation in fishes. In. "Fish Physiology" (W.S. Hoar, D.J. Randall and E.M. Donaldson, Eds.), Vol. IXB, p. 117-170. Academic Press, New York.

Goren, A., Zohar, Y., Koch, Y., and Fridkin, M. (1987). Degradation of GnRH and analogs in the gilthead seabream Sparus aurata : An in vitro Study. In "Proceedings of the Third International Symposium on the Reproductive Physiology of Fish" (D.R. Idler, L.W. Crim and J.M. Walsh, Eds.), p. 96. Marine Sciences Research Laboratory, St. John's, Newfoundland, Canada.

Harder, W. (1975). Anatomy of Fishes. E. Schweizerbert'sche Verlagsbuchhandlung (Nägele v. Obermiller), Stuttgart, West Germany. pp. 612.

Harvey, B.J., and Hoar, W.S. (1979). The theory and practice of induced breeding in fish. Ottawa Canada, International Development Research Centre, IDRC-Ts21e: pp. 48.

Harvey, B., Nacario, J., Crim, L.W., Juario, J.V., and Marte, C.L. (1985). Induced spawning of sea bass, Lates calcarifer, and rabbitfish, Siganus guttatus, after implantation of pelleted LHRH analogues. Aquaculture 47, 53-59.

Heins, D.C., and Rabito, F.G. Jr. (1986). Spawning performance in North American minnows: direct evidence of the occurrence of multiple clutches in the genus Notropis. J. Fish. Biol. 28, 343-357.

Henderson-Arzapalo, A., and Colura, R.L. (1987). Laboratory maturation and induced spawning of striped bass. Prog. Fish. Cult. 49, 60-63.

Hirose, K., and Ishida, R. (1974). Effects of cortisol and human chorionic gonadotrophin (HCG) on ovulation in ayu Plecoglossus altivelis (Temminck & Schlegel) with special respect to water and ion balance. J. Fish Biol. 6, 557-564.

Hirose, K., Hirano, T., and Ishida, R. (1974). Effects of salmon gonadotropin on ovulation in the ayu, Plecoglossus altivelis, with special reference to water balance. Comp. Biochem. Physiol. 46A, 283-289.

Hirose, K., Machida, Y., and Donaldson, E.M. (1976). Induction of ovulation in the Japanese flounder (Limanda yokohamae) with human chorionic gonadotropin and salmon gonadotropin. Bull. Jap. Soc. Sci. Fish 42, 13-20.

Hirose, K, Ishida, R., and Sakai, K. (1977). Induced ovulation of ayu using HCG, with special reference to changes in several characteristics of eggs retained in the body after ovulation. Bull. Jap. Soc. Sci. Fish. 43, 409-416.

Hirose, K., Machida, Y., and Donaldson, E.M. (1979). Induced ovulation of Japanese flounder (Limanda yokohamae) with human chorionic gonadotropin and salmon gonadotropin, with special reference to changes in quality of eggs retained in the ovarian cavity after ovulation. Bull. Jap. Soc. Sci. Fish. 45, 31-36.

Howell, W.H. (1982). Seasonal changes in the ovaries of adult yellowtail flounder, Limanda ferruginea. Fish. Bull. U.S. Natl. Mar. Fish. Serv. 81, 341-355.

Htun-Han, M. (1978). The reproductive biology of the dab, Limanda limanda in the North Sea: Seasonal changes in the ovary. J. Fish. Biol. 13, 351-359.

Huang, Y-P., and Peter, R.E. (1988). Evidence for a gonadotropin-releasing hormone binding protein in goldfish (Carassius auratus) serum. Gen. Comp. Endocrinol. 69, 308-316.

Humason, G.L. (1979). Animal tissue technique. W.H. Freeman and Company. San Francisco. pp. 661.

Hunter, G.A., Donaldson, E.M., and Dye, H.M. (1°). Induced ovulation in coho salmon (Oncorhynchus kisutch). I. Further studies on the use of salmon pituitary. Aquaculture 26, 117-127.

Hunter, W.M., and Greenwood, F.C. (1962). Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature (London) 194, 495-496.

Idler, D.R., and Ng, T.B. (1979). Studies on four types of gonadotropins from salmon and carp pituitaries. Gen. Comp. Endocrinol. 38, 421-440.

Juario, J.V., Duray, M.N., Duray, V.M., Nacario, J.F., and Almendras, J.M.E. (1984). Induced breeding and larval rearing experiments with milkfish Chanos chanos (Forskal) in the Philippines. Aquaculture 36, 61-70.

Kagawa, H., Young, G., and Nagahama, Y. (1984). In vitro estradiol-17β and testosterone production by ovarian follicles of the goldfish, Carassius auratus. Gen. Comp. Endocrinol. 54, 139-143.

Kaul, M., and Rishi, K.K. (1986). Induced spawning of the Indian major carp, Cirrhina mrigala (Ham.), with LH-RH analogue or pimozide. Aquaculture 54, 45-48.

Kawauchi, H., Suzuki, K., Itoh, H., Swanson, P., Naito, N., Nagahama, Y., Nozaki, M., Nakai, Y., and Itoh, S. (1989). The duality of teleost gonadotropins. Fish. Physiol. Biochem. 7, 29-38.

Kennedy, V.S, and Steele, D.H. (1971). The winter flounder (Pseudopleuronectes americanus) in Long Pond, Conception Bay, Newfoundland. J. Fish Res. Board Can 28, 1153-1165.

Kestemont, P. (1988). Effects of hormonal treatments on induced ovulation in gudgeon, Gobio gobio L. Aquaculture 68, 373-385.

Khoo, K.H. (1979). The histochemistry and endocrine control of vitellogenesis in goldfish ovaries. Can. J. Zool. 57, 617-626.

Khoo, K.H. (1980). Stimulation of ovarian maturation in fish by sustained hormone preparations. Aquaculture 20, 275-280.

Kime, D.E. (1980). Comparative aspects of testicular androgen biosynthesis in non-mammalian vertebrates. In "Steroids and their mechanism of action in non-mammalian vertebrates." (G. Delrio, and J. Brachet, Eds), p. 17-31. Raven Press, New York.

Kime, D.E., and Manning, M.J. (1982). Seasonal patterns of free and conjugated androgens in the brown trout salmo

trutta Gen. Comp. Endocrinol. 48, 222-231.

Kime, D.E., and Manning, M.J. (1986). Maturational and temperature effects on steroid hormone production by testes of the carp, Cyprinus carpio. Aquaculture 54, 49-55.

King, J.A, and Millar, R.P. (1990). Genealogy of the GnRH family. In Progress in Comparative Endocrinology: Proceedings of the XIth International Symposium on Comparative Endocrinology, Malaga, Spain 14-20, 1989. (A. Epple, C.G. Scanes, and M.H. Stetson, Eds.), p. 54-59. Wiley-Liss, Inc. New York.

Klein-McPhee, G. (1978). Synopsis of biological data for the winter flounder, Pseudopleuronectes americanus (Walbaum). NOAA Tech. Rep. 414. FAO. Fish. Synop. No. 117, 1-43.

Kreiberg, H., Hunter, G.A., Donaldson, E.M., Clarke, W.C., and Baker, I. (1987). Induced ovulation and spermiation in the Pacific herring (Clupea harengus pallasi) using salmon pituitary preparations and a synthetic gonadotropin-releasing hormone analogue. Aquaculture 61, 155-161.

Lam, T.J., Pandey, S., Nagahama, Y., and Hoar, W.S. (1976). Effects of synthetic luteinizing hormone-releasing hormone (LHRH) on ovulation and pituitary cytology of the goldfish, Carassius auratus. Can. J. Zool. 54, 816-824.

Lam, T.J. (1982). Application of Endocrinology to Fish Culture. Can. J. Fish. Aquat. Sci., 39, 111-137.

Lee, C.S., Tamaru, C.S., and Crim, L.W. (1985). Preparation of a luteinizing hormone-releasing hormone cholesterol pellet and its implantation in the milkfish (Chanos chanos Forskal). In "Reproduction and Culture of Milkfish" p. 215-224. Ocean Institute and Tungkang Marine Laboratory, Taiwan.

Lee, C.S., Tamaru, C.S., Bano, J.E., Kelley, C.D., Bocek, A., and Wyban, J.A. (1986). Induced maturation and spawning of milkfish, Chanos chanos Forskal, by hormone implantation. Aquaculture 52, 199-205.

Lee, C.S., Tamaru, C.S., Miyamoto, G.T., and Kelley, C.D. (1987). Induced spawning of grey mullet (Mugil cephalus) by LHRHa. Aquaculture 62, 327-336.

Lee, C.S., Tamaru, C.S., and Kelley, C.D. (1988). The cost and effectiveness of CPH, HCG and LHRH-a on the induced spawning of grey mullet, Mugil cephalus. Aquaculture 73, 341-347.

Legendre, M. (1986). Seasonal changes in sexual maturity and fecundity, and HCG-Induced Breeding of the catfish, Heterobranchus longifilis Val. (Clariidae), reared in Ebrie Lagoon (Ivory Coast). Aquaculture 55, 201-213.

Leong, R. (1971). Induced spawning of the northern anchovy, Engraulis mordax Girard. Bull. U.S. Natl. Mar. Fish. Serv. 69, 357-360.

Liem, A.H., and Scott, W.B. (1966). Fishes of the Atlantic Coast of Canada. Fish. Res. Bd. Can. Bull. 155, pp. 485.

Lin, H.R., Peng, C., Lu, L-Z., Zhou, X-J., Van Der Kraak, G., and Peter, R.E. (1985a). Induction of ovulation in a loach (Paramisgurnus datyamus) using pimozide and D[Ala^{6}, Pro^{9}-N-ethylamide]-LHRH. Aquaculture 46, 333-340.

Lin, H.R., Peter, R.E., Nahorniak, C.S., and Bres, O. (1985b). Actions of the superactive analogue Des-Gly^{10}[D-Ala^{6}]LHRH ethylamide (LHRH) on gonadotropin secretion in goldfish. In. "Current Trends in Comparative Endocrinology" (B. Lofts and W.N. Holmes, Eds.), p. 77-79. Hong Kong. Hong Kong University Press.

Lin, H.R., Van der Kraak, G., Zhou, X-J., Liang, J-Y., and Peter, R.E., Rivier, J.E., and Vale, W.W. (1988). Effects of [D-Ala^{6}, Trp^{7}, Leu^{8}, Pro^{9}NEt]-LHRH (sGnRH-A) and [D-Ala^{6},Pro^{9}NEt]-Luteinizing hormone-releasing hormone (LHRH-A), in combination with pimozide or domperidone, on gonadotropin release and ovulation in the Chinese loach and common carp. Gen. Comp. Endocrinol. 69, 31-40.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

Lutes, P.B., Doroshov, S.I., Chapman, F., Harrah, J., Fitzgerald, R., and Fitzpatrick, M. (1987). Morpho-physiological predictors of ovulatory success in white sturgeon, Acipenser transmontanus Richardson. Aquaculture 66, 43-52.

Mackinnon, C.N., and Donaldson, E.M. (1978). Comparison of the effect of salmon gonadotropin administered by pellet implantation or injection on sexual development of juvenile male pink salmon (Onchorhynchus gorbuscha). Can. J. Zool. 56, 86-89.

Manickam, P., and Joy, K.P. (1989). Induction of maturation and ovulation by pimozide-LHRH analogue treatment and resulting high quality egg production in the Asian catfish, Clarias batrachus (L.). Aquaculture 83, 193-199.

Marte, C.L, Sherwood, N.M., Crim, L.W., and Harvey, B. (1987). Induced spawning of maturing milkfish (Chanos chanos Forskal) with gonadotropin-releasing hormone (GnRH) analogues administered in various ways. Aquaculture 60, 303-310.

Marte, C.L, Sherwood, N.M., Crim, L.W., and Tan, J. (1988a). Induced spawning of maturing milkfish (Chanos chanos) using human chorionic gonadotropin and mammalian and salmon gonadotropin releasing hormone analogues. Aquaculture 73, 333-340.

Marte, C.L, Crim, L.W., and Sherwood, N.M. (1988b). Induced gonadal maturation and rematuration in milkfish: limited success with chronic administration of testosterone and gonadotropin releasing hormone analogue (GnRH-A). Aquaculture 74, 131-145.

Matsuo, H., Baba, Y., Nair, R.M.G., Arimura, A., and Schally, A.V. (1971). Structure of the porcine LH- and FSH-releasing hormone. I. The proposed aminoacid sequence, Biochem. Biophys. Res. Communic. 43, 1334-1339.

Mayer, I., Shackley, S.E., and Witthames, P.R. (1990). Aspects of the reproductive biology of the bass, Dicentrarchus labrax L. II. Fecundity and pattern of oocyte development. J. Fish. Biol. 36, 141-148.

McCracken, F.D. (1954). Seasonal movements of the winter flounder, Pseudopleuronectes americanus (Walbaum) on the Atlantic coast. Fish Res. Board Can. MS. Rep. Biol. Sta. 582, pp. 167.

McCracken, F.D. (1963). Seasonal movements of the winter flounder, Pseudopleuronectes americanus (Walbaum), on the Atlantic coast. J. Fish Res. Board Can. 20, 551-586.

Mollah, M.F.A., and Tan, E.S.P. (1983). Viability of catfish (Clarias macrocephalus, Gunther) eggs fertilized at varying post-ovulation times. J. Fish Biol. 22, 563-566.

Nagahama, Y. (1983). The functional morphology of teleost gonads. In "Fish Physiology" (W.S. Hoar, D.J. Randall and E.M. Donaldson, Eds.), Vol. IXA, pp. 223-275. Academic Press, New York.

Nagahama, Y. (1987). Endocrine control of oocyte maturation. In "Hormones and Reproduction in Fishes, Amphibians and Reptiles" (D.O. Norris and R.E. Jones, Eds.), p. 171-202. Plenum, New York/London.

Ng, T.B., Idler, D.R., and Burton, M.P. (1980a). Effects of teleost gonadotropins and their antibodies on gonadal histology in winter flounder. Gen. Comp. Endocrinol. 42, 355-364.

Ng, T.B., and Idler, D.R. (1980b). Gonadotropic regulation of androgen production in flounder and salmonids. Gen. Comp. Endocrinol. 42, 25-38.

Ngamrongchon, S., Kok, L.Y., and Takashima, F. (1987). Changes in endocrine profiles and spermiation response in carp after LHRH analogues injection. Nippon Suisan Gakkaishi 53, 229-234.

Pankhurst, N.W., Van Der Kraak, G., and Peter, R.E. Peter. (1986a). Effects of human chorionic gonadotropin, Des-Gly^{10}(D-Ala^{6})LHRH-ethylamide and pimozide on final oocyte maturation, ovulation and levels of plasma sex steroids in the walleye Stizostedion vitreum. Fish Physiol. Biochem. 1, 45-54.

Pankhurst, N.W., Van Der Kraak, G., Peter, R.E., and Breton, B. (1986b). Effects of (D-Ala^{6},Pro^{9}-N ethylamide)-LHRH on plasma levels of gonadotropin, 17α,20β-dihydroxy-4-pregnen-3-one and testosterone in male goldeye (Hiodon alosoides Rafineswque). Fish Physiol. Biochem. 1, 163-170.

Peter, R.E. (1980). Serum gonadotropin levels in mature goldfish in response to luteinizing hormone-releasing hormone (LH-RH) and des-Gly^{10} [D-Ala^{6}] LHRH ethylamide. Can. J. Zool. 58, 1100-1104.

Peter, R.E., Chang, J.P., Nahorniak, C.S., Omeljaniuk, R.J., Sokolowska, M., Shih, S.H., and Billard, R. (1986). Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish.

Rec. Prog. Horm. Res. 42, 513-548.

Peter, R.E., Lin, H-R., and Van der Kraak, G. (1987). Drug/hormone induced breeding of Chinese teleosts. In "Proceedings of the Third International Symposium on the Reproductive Physiology of Fish, St. John's, Newfoundland, Canada, 1987" (D.R. Idler, L.W. Crim and J.M. Walsh, Eds.), p. 120-123. Marine Sciences Research Laboratory, St. John's, Newfoundland, Canada.

Peter, R.E, Lin, H.R., and Van Der Kraak, G. (1988). Induced ovulation and spawning of cultured freshwater fish in China: advances in application of GnRH analogues and dopamine antagonists. Aquaculture 74, 1-10.

Pickering, A.D., Pottinger, T.G., Carragher, J., and Sumpter, J.P. (1987). The effects of acute and chronic stress on the levels of reproductive hormones in the plasma of mature male brown trout, Salmo trutta L. Gen. Comp. Endocrinol. 68, 249-259.

Prat, F., Zanuy, S., and Carillo, M. (1987). Effects of LH-RH analog alone or combined with pimozide on plasma sex steroids, egg quality, and spawning in sea bass (Dicentrarchus labrax L). Gen. Comp. Endocrinol. 66, 21-22.

Ramos., J. (1986a). Induction of spawning in common sole (Solea solea L.) with human chorionic gonadotropin (HCG). Aquaculture 56, 239-242.

Ramos, J. (1986b). Luteinizing hormone-releasing hormone analogue (LH-RHa) induces precocious ovulation in common sole (Solea solea L.). Aquaculture 54, 185-190.

Remacle, C. (1976). Actions hormonales sur les cellules germinales des males de Carassius auratus L. en culture organotypiques. Gen. Comp. Endocrinol. 28, 480-491.

Rogers, C.A. (1976). Effects of temperature and salinity on the survival of winter flounder embryos. Fish. Bull. U.S. 74, 52-58.

Rosenblum, P.M., Callard, I.P. (1987). Response of male brown bullhead catfish, Ictalurus nebulosus Lesueur, to gonadotropin-releasing hormone and gonadotropin. Jour. Exp. Zool. 243, 189-199.

Rowland, S.J. (1983). The hormone-induced ovulation and spawning of the Australian freshwater fish golden

perch, Macquaria ambigua (Richardson) (Percichthyidae). Aquaculture 35, 221-238.

Rowland, S.J. (1988). Hormone-Induced Spawning of the Australian Freshwater Fish Murray Cod, Maccullochella peeli (Mitchell) (Percichthyidae). Aquaculture 70, 371-389.

Sangalang, G.B., and Freeman, A.C. (1974). Effects of sublethal cadmium on maturation and testosterone production in vivo in brook trout. Biol. Reprod. 1, 429-435.

SAS Institute Inc. SAS User's Guide: Statistics, 1985 Edition. Cary, NC: SAS Institute Inc., 1985.

Schoonen, W.G.E.J., and Lambert, J.G.D. (1987). Steroid metabolism in the seminal vesicles of the African catfish, Clarias gariepinus (Burchell), during the spawning season, under natural conditions and kept in ponds. Gen. Comp. Endocrinol. 61, 355-367.

Scott,W.B, and Scott, M.G. (1988). Atlantic fishes of Canada. Can. Bull. Fish Aquat. Sci. 219, pp. 731.

Scott, A.P., Bye, V.J., Baynes, S.M., and Springate, J.R.C. (1980). Seasonal variations in plasma concentrations of 11-ketotestosterone and testosterone in male rainbow trout Salmo gairdneri Richardson. J. Fish. Biol. 17, 495-505.

Scott, A.P., and Baynes, S.M. (1982). Plasma levels of sex steroids in relation to ovulation and spermiation in rainbow trout Salmo gairdneri. In "Proceedings of the Second International Symposium on Reproductive Physiology of Fish, Wageningen, THe Netherlands." (Richter, C.J.J., and Goos, H.J. Th., Eds.), p. 103-106. Wageningen Pudoc, The Netherlands.

Scott, A.P., and Canario, A.V.M. (1987). status of oocyte maturation-inducing steroids in teleosts. In "Proceedings of the Third International Symposium on the Reproductive Physiology of Fish, St. John's, Newfoundland, Canada, 1987" (D.R. Idler, L.W. Crim, and J.M. Walsh, Eds.), p. 224-234. Marine Sciences Research Laboratory, St. John's, Newfoundland, Canada.

Shehadeh, Z.H., and Ellis, J.N. (1970). Induced spawning of the striped mullet Mugil cephalus L. J. Fish. Biol. 2, 355-360.

Shehadeh, Z.H., Madden, W.D., and Dohl, T.P. (1973a). The effect of exogenous hormone treatment on spermiation and vitellogenesis in the grey mullet, Mugil cephalus L. J. Fish. Biol. 5, 479-487.

Shehadeh, Z.D., Kuo, C.M., and Milisen, K.K. (1973b). Validation of an in vivo method for monitoring ovarian development in the grey mullet (Mugil cephalus L.). J. Fish Biol. 5, 489-496.

Sherwood. N.M., Eiden, L., Brownstein, M., Spiess, J. Rivier, J., and Vale, W. (1983). Characterization of a teleost gonadotropin-releasing hormone. Proc. Natl. Acad. Sci. USA 80, 2794-2798.

Sherwood, N.M., and Harvey, B. (1986). Topical absorption of gonadotropin-releasing hormone (GnRH) in goldfish. Gen. Comp. Endocrinol. 61, 13-19.

Sherwood, N.M, and Lovejoy, D.A (1989). The origin of the mammalian form of GnRH in primitive fishes. Fish Physiol. Biochem. 7, 85-93.

Shireman, J.V., and Gildea, J.A. (1989). Induced spawning of rainbow sharks (Labeo erythrurus) and redtail black sharks (L. bicolor). Prog. Fish Cult. 51, 104-108.

Simpson, T.H., and Wright, R.S. (1977). A radioimmunoassay for 11-oxotestosterone and its application in the measurement of levels in blood serum of rainbow trout (salmo gairdneri). Steroids 29, 383-398.

Smigielski, A.S. (1975). Hormonal-induced ovulation of the winter flounder, Pseudopleuronectes americanus. Fish. Bull. U.S. Natl. Mar. Fish. Serv. 73, 431-438.

Snedecor, G.W., and Cochran, W.G. (1980). Statistical Methods, 7th Edition. The Iowa State University Press, Ames.

So, Y.P., Idler, D.R., Truscott, B., and Walsh, J.M. (1985a). Progestogens, androgens and their glucuronides in the terminal stages of oocyte maturation in landlocked Atlantic salmon. J. Steroid Biochem. 23, 583-591.

So, Y.P., Idler, D.R., and Hwang, S.J. (1985b). Plasma vitellogenin in landlocked Atlantic salmon (Salmo salar Quananiche): Isolation, homologous radioimmunoassay and immunological cross-reactivity with vitellogenin from other teleosts. Comp. Biochem. Physiol. B81, 63-71.

Sokolowska, M., Peter, R.E., Nahorniak, C.S., Chang, P.J.P., Crim, L.W., and Weil, C. (1984). Induction of ovulation in goldfish, Carassius auratus by pimozide and analogues of LHRH. Aquaculture 36, 71-83.

Sokolowska, M., Peter, R.E., Nahorniak, C.S., and Chow, J.P. (1985). Seasonal effects of pimozide and DesGly^{10} [D-Ala^{6}] LH-RH ethylamide on gonadotrophin secretion in goldfish. Gen. Comp. Endocrinol. 57, 472-479.

Solar, I.I., Baker, I.J., and Donaldson, E.M. (1987). Effect of salmon gonadotropin and a gonadotropin releasing hormone analogue on ovarian hydration and ovulation in captive sablefish (Anoplopoma fimbria). Aquaculture 62, 319-325.

Sower, S.A., Iwamoto, R.N., Dickhoff, W.W., and Gorbman, A. (1984). Ovulatory and steroidal responses in coho salmon and steelhead trout following administration of salmon gonadotropin and D-Ala^{6}, desGly^{10} gonadotropin releasing hormone ethylamide (GnRH). Aquaculture 43, 35-46.

Steel, R.G.D., and Torrie, J.H. (1980). Principles and Procedures of Statistics. New York: McGraw-Hill Book Company.

Sugimoto, Y., Takeuchi, Y., Yamauchi, K., and Takahasi, H. (1976). Induced maturation of female Japanese eels (Anguilla japonica) by administration of salmon pituitaries, with notes on changes of oil droplets in eggs of matured eels. Bull. Fac. Fish. Univ. Hokkaido 27, 107-120.

Suzuki, Y., Kobayashi, M., Nakamura, O., Aida, K., and Hanyu, I. (1988). Induced ovulation of the goldfish by oral administration of salmon pituitary extract. Aquaculture 74, 379-384.

Swanson, P., Suzuki, K., and Kawauchi, H. (1987). Effects of des-Gly^{10}[D-Ala^{6}] gonadotropin-releasing hormone ethylamide (GnRHa) on secretion of two distinct gonadotropins by salmonid pituitaries in vitro. In Proc. 1st Congr. Asia and Oceania Soc. Comp. Endocrinol. (E. Ohnishi, Y. Nagahama, and H. Ishizaki, Eds.). pp. 93-94.

Szedlmayer, S.T. (1987). Hormone-induced spawning of weakfish. Prog. Fish. Cult. 49, 158-160.

Takashima, F., Weil, C., Billard, R., Crim, L.W., and Fostier, A. (1984). Stimulation of spermiation by LHRH

Analogue in carp. Bull. Jap. Soc. Sci. Fish. 50, 1323-1329

Tamaru, C.S., Lee, C.S., Kelley, C.D., Banno, J.E., Ha, P.Y., Aida, K., and Hanyu, I. (1988). Characterizing the stage of maturity most receptive to an acute LHRH-analogue therapy for inducing milkfish (Chanos chanos) to spawn. Aquaculture 74, 147-163.

Thomas. P., and Boyd, N.M. (1988). Induced spawning of spotted seatrout, red drum and orangemouth corvina (Family: Sciaenidae) with luteinizing hormone-releasing hormone analog injection. Contributions in Marine Science. Supp. to Vol. 30, 43-48.

Topp, R.W. (1968). An estimate of fecundity of the winter flounder, Pseudopleuronectes americanus. J. Fish Res. Board Can. 25, 1299-1302.

Truscott, B. (1981). An alternative method for the synthesis of 11-[H^3]ketotestosterone and 11-[^{3}H]-hydoxytestosterone from [^{3}H]cortisol. Gen. Comp. Endocrinol. 12, 491-497.

Tyler, C.R., Sumpter, J.P., Witthames, P.R. (1990). The dynamics of oocyte growth during vitellogenesis in the rainbow trout (Oncorhynchus mykiss). Biol. of Reprod. 43, 202-209.

Upadhyay, S.N., Breton, B., and Billard, R. (1978). Ultrastructural studies on experimentally induced vitellogenesis in juvenile rainbow trout (Salmo gairdneri). Ann. Biol. Anim. Biochim. Biophys. 18, 1019-1025.

Van Der Kraak, G., Lin, H.R. Lin, Donaldson, E.M., Dye, H.M., and Hunter, G.A. (1983). Effects of LHRH and des-Gly^{10} [D-Ala^6] LH-RH-ethylamide on plasma gonadotropin levels and oocyte maturation in adult female coho salmon (Oncorhynchus kisutch). Gen. Comp. Endocrinol. 49, 470-476.

Van Der Kraak, G., Dye, H.M., and Donaldson, E.M. (1984). Effects of LHRH and des Gly^{10} [D-Ala^6] LHRH ethylamide on plasma sex steroid profiles in adult female coho salmon (Oncorhynchus kisutch). Gen. Comp. Endocrinol. 55, 36-45.

Van Der Kraak, G., Dye, H.M., Donaldson, E.M., and Hunter, G.A. (1985). Plasma gonadotropin, 17β-estradiol, and 17α, 20β-dihydroxy-4-pregnen-3-one levels during luteinizing hormone-releasing hormone analogue and

gonadotropin induced ovulation in coho salmon (Onchorhynchus kisutch). Can. J. Zool. 63, 824-833.

Wallace, R.A., and Selman, K. (1978). Oogenesis in Fundulus heteroclitus. I. Preliminary observation on oocyte maturation in vivo and in vitro. Dev. Biol. 62, 354-369.

Wallace, R., and Selman, K. (1981). Cellular and dyanamic aspects of oocyte growth in teleosts. Am. Zool. 21, 325-343.

Wallace, R.A, and Selman, K. (1985). Major protein changes during vitellogenesis and maturation of Fundulus oocytes. Dev. Biol. 110, 492-498.

Wallace, R.A., Selman, K., Greeley, M.S., Begovac, P.C. Lin, Y-W.P., McPherson, R., and Petrino, T.R. (1987). Current status of oocyte growth. In "Proceedings of the Third International Symposium on the Reproductive Physiology of Fish, St. John's, Newfoundland, Canada, 1987" (D.R. Idler, L.W. Crim and J.M. Walsh, Eds.), p. 167-177. Marine Sciences Research Laboratory, St. John's, Newfoundland, Canada.

Weigand, M.D. (1982). Vitellogenesis in fishes. In: "Proceedings of the Second International Symposium on Reproductive Physiology of Fish, Wageningen, The Netherlands, 1982." (C.J.J. Richter and H.J.Th. Goos, eds.), p. 136-146. Pudoc, Wageningen, The Netherlands.

Weigand, M.D., Crim, L.W., Wilson, C.E., Hewitt, C.M., and Loewen, J.M. (1987). A study of oocyte development and induction of spawning in the winter flounder following LHRH analog implantation. In "Proceedings of the Third International Symposium on the Reproductive Physiology of Fish, St. John's, Newfoundland, Canada, 1987" (D.M. Idler, L.W. Crim and J.M. Walsh, Eds.), p. 104-105. Marine Sciences Research Laboratory, St. John's, Newfoundland, Canada.

Weil, C., Billard, R., Breton, B., and Jalabert, B. (1978). Pituitary response to LH-RH at different stages of gametogenesis in the rainbow trout (Salmo gairdneri) Ann. Biol. Anim. Biochem. Biophys. 18, 863-869.

Weil. C., Fostier, A., Horvath, L., Marlot, S., and Berscenyi, M. (1980). Profiles of plasma gonadotropin and 17β-estradiol in the common carp, Cyprinus carpio, as related to spawning induced by hypophysation or LH-RH treatment. Reprod. Nutr. Develop. 20, 1041-1050.

Weil, C., and Crim, L.W. (1983). Administration of LHRH analogues in various ways. Effects on the advancement of spermiation in prespawning landlocked salmon, Salmo salar Aquaculture 35, 103-116.

Wingfield, J.C., and Grimm, A.S. (1977). Seasonal changes in plasma cortisol, testosterone, and estradiol-17β in the plaice, Pleuronectes platessa L. Gen. Comp. Endocrinol. 31, 1-11.

Wright, R.S., and Zhao, W-X. (1988). Steroid release from separated theca and granulosa layers of Atlantic salmon (Salmo salar) ovarian follicles: the effects of a purified salmon gonadotropin preparation. Fish Physiol. Biochem. 5, 131-139.

Yamamoto, K., Hiroi, O., Hirano, T., and Morioka, T. (1972). Artificial maturation of cultivated male Japanese eels by Syanahorin injection. Bull. Jpn. Soc. Sci. Fish. 38, 1083-1090.

Yamazaki, F., and Donaldson, E.M. (1968). The effects of partially purified salmon pituitary gonadotropin on spermatogenesis, vitellogenesis, and ovulation in hypophysectomized goldfish, Carassius auratus. Gen. Comp. Endocrinol. 11, 292-299.

Yaron, Z., and Zohar, Y. (1989). Applications of Comparative Endocrinology in Fish Culture. In "Proceedings of the Satellite Symposium on Applications of Comparative Endocrinology to Fish Culture" Granada, Spain. May 22-23, 1989, p. 11-19

Zhang, Q.-Y., Hong, W.-S., Dai, Q.-N., Zhang, J., Cai, Y.-Y., and Huang, J.-L. (1989). Studies on induced ovulation, embryonic development and larval rearing of the mudskipper (Boleophthalmus pectinirostris). Aquaculture 83, 375-385.

Zohar, Y., Pagelson, G., Tosky, M., and Finkelman, Y. (1987). GnRH control of gonadotropin secretion, ovulation and spawning in the gilthead seabream, Sparus aurata. In "Proceedings of the Third International Symposium on the Reproductive Physiology of Fish" (D.R. Idler, L.W. Crim and J.M. Walsh, Eds.), p. 106. Marine Sciences Research Laboratory, St. John's, Newfoundland, Canada.

Zohar, Y. (1988). Gonadotropin releasing hormone in spawning induction in teleosts: Basic and applied considerations. In Reproduction in Fish: Basic and applied aspects in Endocrinology and Genetics. (Y.

Zohar and B. Breton, Eds.), p. 47-62. INRA Press, Paris.

Zohar, Y. (1989). Endocrinology and fish farming: Aspects in reproduction, growth and smoltification. Fish Physiol. Biochem. 7, 395-405.