A STUDY OF REPRODUCTIVE BIOLOGY OF THE OCEAN POUT (Macrozoarces americanus L.) IN CAPTIVITY

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A STUDY OF REPRODUCTIVE BIOLOGY OF THE OCEAN POUT (*Macrozoarces americanus* L.) IN CAPTIVITY

BY

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ABSTRACT

The reproductive biology of the ocean pout (*Macrozoarces americanus* L.) was studied. This included (1) the biochemistry of vitellogenin (egg yolk protein precursor), (2) the seasonal reproductive cycle and the associated endocrine changes, (3) the regulation of fish reproduction and (4) the biology of egg fertilization.

Synthesis of vitellogenin in the liver of ocean pout was induced by administration of the ovarian steroid 17ß-estradiol. Vitellogenin was isolated from the plasma by gel filtration and characterized biochemically. It is a glycolipophosphoprotein, 577.8 KD in molecular weight, and contains 17.92% lipid and 3.56% total phosphorus, of which 80.34% (or 2.86% of total vitellogenin) is in the form of lipid-bound phosphorus and the rest (19.1%, equivalent to 0.68% of vitellogenin) is protein bound-phosphorus. The vitellogenin protein contains high levels of the essential amino acids, including arginine, lysine, isoleucine and leucine, valine, threonine and phenylalanine, and some non-essential amino acids, such as aspartic acid, serine, glutamic acid and alanine. With the isolated vitellogenin, antisera were prepared in rabbits and a vitellogenin radioimmunoassay was developed and used for sex determination and for studies of the seasonal changes in plasma vitellogenin.

The seasonal reproductive cycle of female ocean pout was found to be organized into 4 successive phases of reproduction: 1) a post-spawning quiescent phase (August-September to February), in which the small ovaries (GSI 1.21 $\pm$ 0.25) are inactive and predominated by small previtellogenic oocytes (ca. 1.0 mm diameter); a low level of both

sex steroids (testosterone and 17ß-estradiol) and vitellogenin occurs in the plasma; 2) the reproductive preparatory phase (March - May), in which the ovaries develop slowly (GSI 4.61 $\pm$ 2.34) and consist of both previtellogenic and small vitellogenic oocytes (4 - 5 mm diameter); plasma levels of sex steroids and vitellogenin begin rising slowly; 3) the rapid ovarian growth phase (June - August), in which both the diameter of the vitellogenic oocytes (7 - 8 mm); the GSI (16.76 $\pm$ 6.54) and levels of sex steroids in the plasma rapidly increase and 4) the ovulation and spawning phase (August - September), when fish spawn and the plasma sex steroids return to basal levels. The females spawn once per year, producing a single batch of 1200 - 1700 eggs (8-9 mm diameter). The seasonal reproductive cycle in males was found to be composed of two phases, (1) a quiescent phase (October - May) and (2) the reproductive phase (June - September). The spawning season of males (occurrence of spermiation) starts with rapid testicular development, following a dramatic increase in both testosterone and 11-ketotestosterone in the plasma.

Increasing water temperature in the winter stimulated feeding activity but did not alter the timing of the reproductive cycle of the ocean pout. However, lengthening the photoperiod in the winter stimulated steroidogenesis and vitellogenesis and advanced the spawning season. Administration of gonadotropic hormone releasing hormone analog to mature females advanced and synchronized spawning through accelerating the preovulatory decline of plasma testosterone and 17ß-estradiol.

Sperm motility, pH, osmolarity and ionic composition of the ocean pout seminal plasma were studied. Milt collected from the reproductive tract (sperm duct) of mature males contained highly motile spermatozoa (sperm) in a very low concentration. Motility

of the sperm was enhanced in ovarian slime collected from the ovaries of prespawning females but was immobilized instantly upon dilution with seawater, suggesting that the ocean pout is an internal fertilizer. pH and osmolarity of the seminal plasma were in a range of 7.2-7.5 and 365-406 mOsM, respectively. Various ions including Na^{+}, K^{+}, Ca^{++}, Mg^{++} and Cl^{-} as well as glucose were detected and quantified in the seminal plasma. Fertilized eggs were produced by artificial insemination, both *in vivo* (injecting sperm directly into the ovary of mature females) and *in vitro*. The *in vitro* insemination necessitated a contact period of 5 hours between the eggs and sperm for fertilization of the eggs before the eggs could be transferred into seawater for incubation. Larvae hatched from *in vivo* artificially inseminated eggs.

Studies of copulation and spawning behaviours of the ocean pout showed that males developed a papilla (protrusion of the genital pore) and fish copulated through direct genital contact for internal fertilization of eggs. Females spawned spontaneously in captivity, 6-17 hours after copulation. A complete spawning process was determined to consist of 4 successive steps, including (1) oviposition (spawning), (2) wiping the eggs with skin mucus, (3) wrapping herself around the eggs and (4) guarding the egg-mass. Wiping the eggs with skin mucus could be an effective means for preventing leech and fungal infection of the eggs. Parental care and fanning increased water flow to the eggs, which is important for egg survival during a lengthy period (3 months) of incubation.

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CHAPTER 1

GENERAL INTRODUCTION

1.1. A GENERAL REVIEW OF TELEOST REPRODUCTIVE BIOLOGY

Reproduction in most teleosts is seasonal, following the seasonal changes in photoperiod, water temperature and food availability. Artificially changing the environmental factors, especially photoperiod cycle, has been successful in altering the reproductive cycle in some fish (Bromage et al., 1982,1984). The photoperiod acts through the "third eye" or the pineal gland in the brain epithalamus to alter the secretion of melatonin which in turn influences the reproductive process of fish through blocking the release of gonadotropic hormone-releasing hormone (GnRH) from the hypothalamus or by suppressing the response of gonad tissues to gonadotropic hormone (GtH) from the pituitary (Reiter 1978; Relkin 1983).

Reproduction in fish and other vertebrates is an integrated process and involves the activities of various hormones and tissues including the hypothalamus (brain), hypophysis (pituitary) and gonad, which form the brain-pituitary-gonadal axis (Fig.1) . The regulatory centre for reproduction is located in the hypothalamus, particularly the anterior proventricular areas where "nuclei" (aggregations of a group of neurons of similar function), such as the suprachiasmatic nucleus, the proventricular preoptic nucleus, the ventromedial nucleus, the medial preoptic area and the arcuate nucleus are distributed. These nuclei produce GnRH, or other regulatory factors (Johnes and Ingleton 1987), which control the synthesis and release of GtH from the pituitary (Sherwood 1987).

After being synthesized in the cell, GnRH is transported directly (via axons) in fish or indirectly (via a portal system in the median eminence) in mammals to the surface of

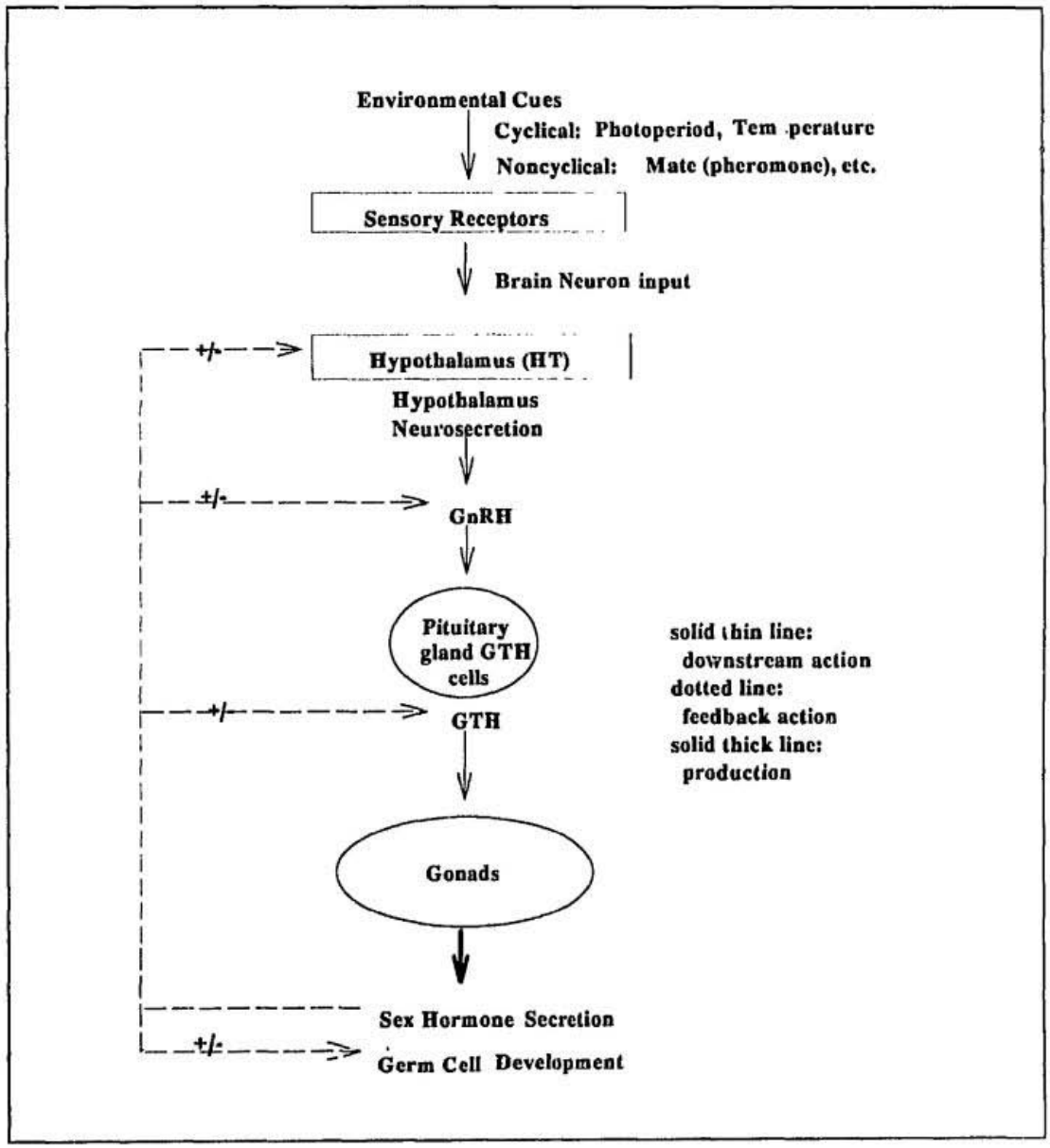

Figure 1. A model of the hypothalamo-hypophysial-gonadal axis.

pituitary gonadotropes (or gonadotropic hormone producing cells) to control the synthesis and release of GtH from the pituitary (Lam et al., 1976; Sherwood 1987). The GtH in turn controls gonadal development (Crim et al., 1983; Breton et al., 1990; Pankhurst and Carragher 1992).

The gonads have a dual function, i.e., producing sperm or eggs (gametogenesis) and steroid hormones (steroidogenesis) including testosterone, 17ß-estradiol and 17α,20ß-dihydroxy-4-pregnen-3-one (17α,20ß-P) in females or testosterone, 11-ketotestosterone (particularly in fish) and 17α,20ß-P in males. Steroids are produced from the follicle layer (thecal cells and granulosa cells) in the ovary of females or from the interstitial tissues (Leydig cells) in the testes of males (Hoar et al., 1983).

A majority of fish are oviparous (egg layer). Growth and development of the ovary involves uptake and deposition of vitellogenin, a yolk precursor protein of hepatic origin, from the plasma into eggs. This egg yolk will serve as an energy source and endogenous food of the young (Ng and Idler 1983; Mommsen and Walsh 1988). Synthesis of vitellogenin in the liver is initiated and primarily controlled by 17ß-estradiol from the ovary (Tata 1987; Lim et al., 1991) but is also affected by other hormones, such as prolactin (antagonistic) and growth hormone (synergistic) and by water temperature (Olin et al., 1989). Uptake of vitellogenin into the eggs requires stimulation of GtH from the pituitary (Fig.2) (Tyler et al., 1991; Nagahama et al., 1993) and results in growth of the oocytes (Wallace and Selman 1985).

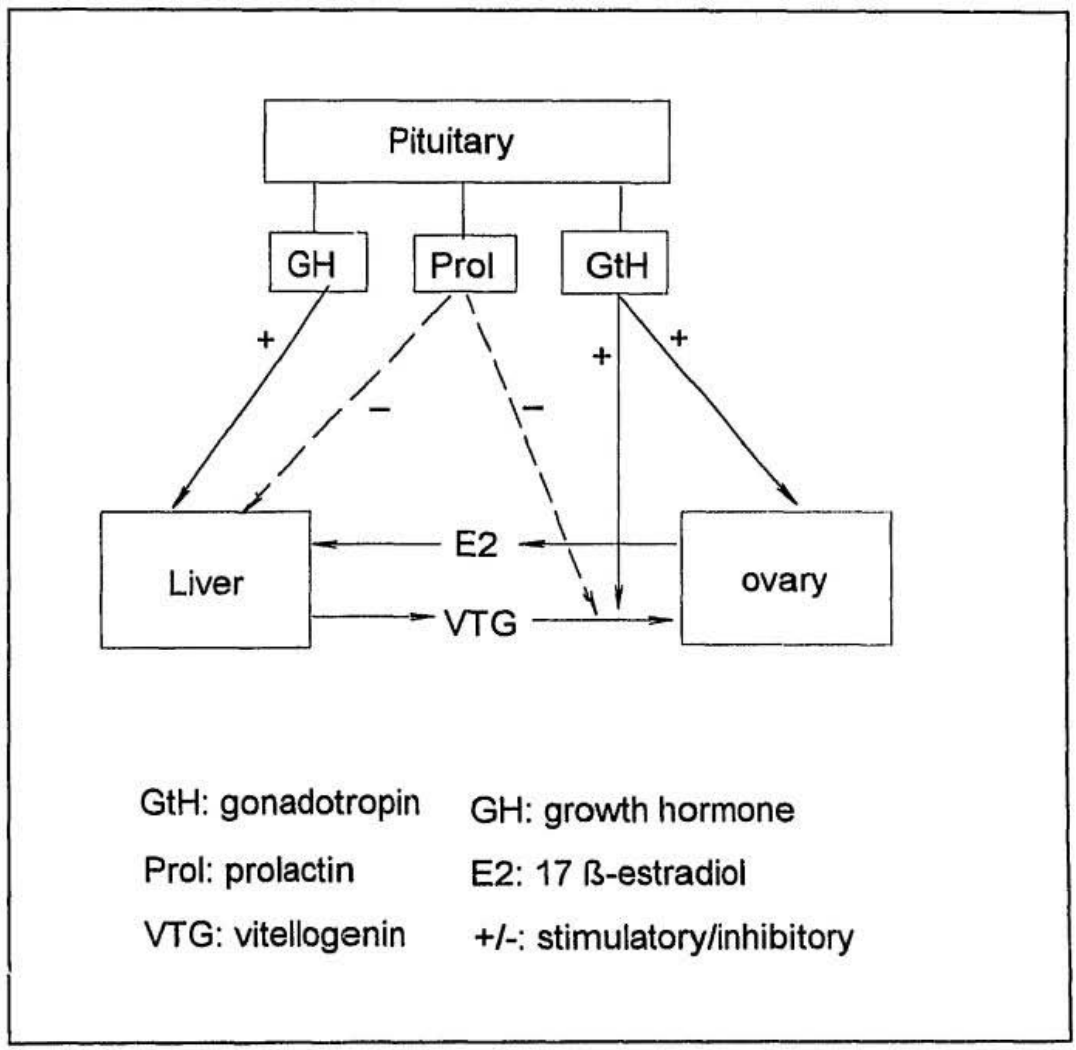

Figure 2. A model of the hormonal regulation of hepatic vitellogenin synthesis.

Steroidogenesis and gametogenesis are closely associated and proceed simultaneously. Plasma testosterone and 17ß-estradiol levels increase in parallel with the development of the ovaries in early reproductive stages and decline in the later stages (Fitzpatrick et al., 1986) as the plasma level of oocyte maturational inducing hormone of 17α,20ß-P (a steroid hormone produced from the follicular cells and stimulating the maturation of oocytes) increases and the oocytes undergo maturation (Nagahama et al., 1993). Besides the direct actions on gonads, sex steroids also feedback (+/-) to the brain and pituitary to affect the release of GnRH or GtH (Goos 1987) (Fig.1).

The sex steroids induce the appearance of the secondary sex characteristics including nuptial colours and sex behaviours. Conjugated or glucuronidated steroids and 17α,20ß-P may act as pheromones to advance gonad growth or initiate mating behaviour (Weed and Richter 1991). In goldfish, the preovulatory female releases 17α,20ß-P into the water to induce a rapid increase in plasma GtH and milt volume of males, and initiate the mating behaviours of both males and females, thus synchronizing the spawning of the two sexes (Hontela and Stacey 1990) (Fig.3).

Most fish display external fertilization. Since the gametes have a short life span, i.e., the sperm survive from 30 seconds to a few minutes after activation by water (Billard 1990; Aas et al., 1991; Suquet et al., 1992) and fertility is lost beyond this period of time, a synchronous release of gametes from both parents is essential for fertilization. In the case of internal fertilization, males develop accessory structures such as papillae, modified fins or scale sheaths to assist with the internal insemination, and the processes of egg insemination and oviposition can be dissociated in timing (Pusey and Stewart 1989).

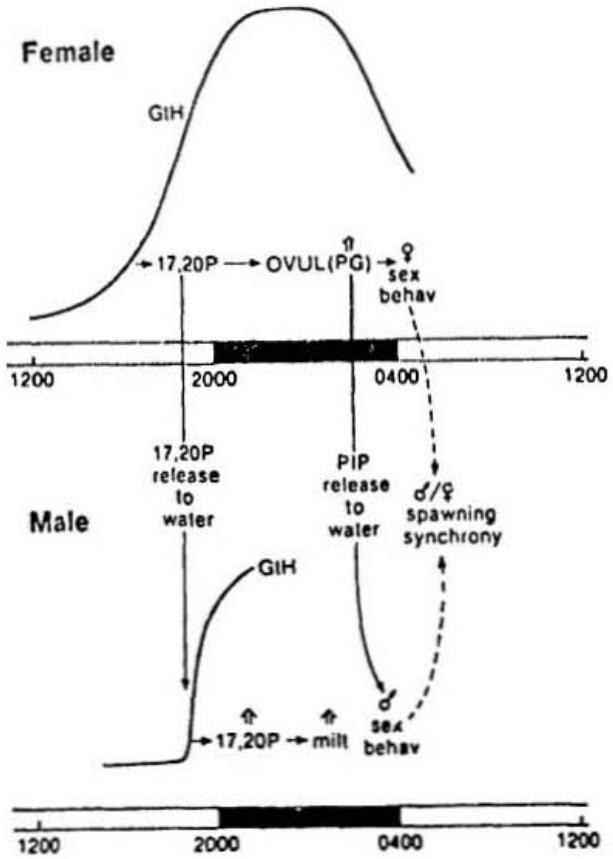

Figure 3. A model of the hormonal and pheromonal synchronization of gonadal maturation and spawning behaviour in goldfish. 17,20-P: 17α,20ß-dihydroxy-4-pregnen-3-one; ovul: ovulation; PG: prostaglandins; PIP: prostaglandins-induced pheromone. Vertical bars indicate the time schedule in hours (Hontela and Stacey 1990).

Fish are poikilothermic animals. Their metabolism is influenced by water temperature. Water temperature alters protein synthesis, enzyme concentration and activity, and the viscosity of the cell membranes (Bols et al., 1992). In terms of reproduction, the changes in water temperature might affect pituitary GtH secretion (Crim 1982) or steroidogenesis, e.g. activating 5ß-reductase, which blocks the synthesis of testosterone and 17ß-estradiol in ovary. The changes in temperature might also alter the catecholamine content in the hypothalamic preoptic nucleus thus affecting GnRH release (Johnes and Ingleton 1987). However, the response of fish to the changes in water temperature and photoperiod varies among species.

1.2. A REVIEW OF THE BIOLOGY OF THE OCEAN POUT

Taxonomy and Description: The ocean pout (*Macrozoarces americanus*) is a marine fish in the Family Zoarcidae, Order Gadiformes, Suborder Zoarcoidei (Nelson 1976). The ocean pout has a broad head, a terminal mouth with large fleshy lips and an elongate and moderately compressed body which tapers to a caudal point (Fig.4). The eyes are small and high on the head. Both dorsal and anal fins extend posteriorly to the base of the small caudal fin. The pelvic fins are short and small and located in front of the large pectoral fins. The scaleless skin is smooth and covered with mucus. The colour of the dorsal skin varies from muddy yellow to reddish brown mottled with grey colour in contrast to a white to dull yellow colour on the belly.

Common names for this species include eelpout, congo eel (or conger eel), lamper eel and mutton-fish (Olsen and Merriman 1946; Scott and Scott 1988). Although these

names are often used, the **ocean pout** seems to be the most accurate one for this species (Olsen and Merriman 1946; Scott and Scott 1988) and is used in this thesis.

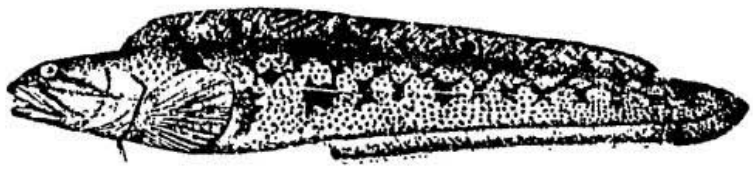

Figure 4. The ocean pout, *Macrozoarces americanus* L. (Scott and Scott 1988)

Distribution: The ocean pout has been reported to occur on both sides of the North Atlantic Ocean (Olsen and Merriman 1946; Keats et al., 1985). In the Western North Atlantic Ocean, it appears in the North from Battle Harbour (Labrador), the Gulf of St. Lawrence, off Newfoundland and along the coasts of the Maritime provinces, the offshore banks to New Jersey, the Bay of Fundy (and Passamaquoddy Bay and Minas Basin) and to Delaware and off North Carolina (Scott and Scott 1988).

The ocean pout is a benthic species occupying a variety of depths from the inter-tidal zone to over 183 meters over all types of substrate. The fish prefers a water temperature in a range of 6-9°C but may well adapt to a temperature range from 0-16°C (Olsen and Merriman 1946).

Abundance of the ocean pout in the coastal waters varies seasonally and depends on geographic locations (Olsen and Merriman 1946; Shenouda et al., 1979). In the Gulf

of Maine, Passamaquoddy Bay and off Newfoundland, the fish become more abundant from April to late autumn when the water temperature is relatively high, but disappear from these areas in winter when the water becomes too cold, suggesting an inshore-offshore migration of the fish in the Spring and Autumn (Olsen and Merriman 1946; Keats et al., 1985). In Southern New England, ocean pout become a resident fish and the majority of fish do not migrate regularly inshore and offshore probably due to a warmer year-round water temperature in these localities (Olsen and Merriman 1946; Sheehy et al., 1977).

Feeding and Growth: The ocean pout is not fully piscivorous (Olsen and Merriman 1946), but feeds on various invertebrates including marine worms, sea urchins, brittle stars, sand dollars, crabs, shrimp, amphipods, mussels, scallops and many other molluscs and sea squirts (Keats et al., 1987; Scott and Scott 1988). The presence of strong teeth enables the fish to crush molluscs to obtain meat (MacDonald and Green 1986). Occasionally, small fish such as herring, capelin and smelt are also found in the stomach of ocean pout (Hacunda 1981; MacDonald et al., 1982; Keats et al., 1987). The diet range reflects the feeding behaviour of fish, and laboratory observations indicate that ocean pout feed from a resting position on the substrate, scooping sediment by mouth from which food is extracted (Macdonald 1983). On the other hand, ocean pout, especially the young individuals, are preyed upon by a variety of species including the barndoor skate, longhorn sculpin, sea raven, cod and harbor seals (Olsen and Merriman 1946).

The growth rate of the ocean pout differs widely between different geographic locations and is probably related to water temperature and the abundance of food. The

growth rate is slow in the North and increases southward. For example, in the Bay of Fundy the fish body length is only 31 cm at 5 yrs, 55-58 cm at 12-13 yrs and 64-68 cm at 16-18 yrs. In the Gulf of Maine and New England, where the water temperature is warmer, the ocean pout reach 31 cm by 3 yrs, 48 cm by 5 yrs, 61 cm by 6-7 yrs and 81 cm by 10 yrs (Olsen and Merriman 1946). When fed with artificially formulated diet (wet pellet) in captivity, the growth rate is even higher (Brown et al., 1992).

Reproduction: Age at maturation differs between males and females. The smallest fish reported to mature are 39 cm in body length for males (4-5 yrs) and 45 cm for females (5-6 yrs). All fish are mature at 65 cm. In contrast to its European counter-part, the eel pout *Zoarces viviparus*, which is viviparous and produces live larvae, the ocean pout is oviparous (laying eggs) and produces 1300 (intermediate size fish of 55-60 cm) to 4200 eggs in large individuals (80-90 cm) (Olsen and Merriman 1946). The gonado-somatic index of females (percentage of the body weight represented by the ovary) also tends to increase in larger fish.

Spawning of the fish is seasonal and occurs in late Autumn from mid-August to September (Northern waters) or October (Southern waters) (Keats et al., 1985). Olsen and Merriman (1946) suggest that water temperature might be an important cue for induction of spawning of the ocean pout because the fish always spawns in a water temperature range of 9-10°C irrespective of spawning locality. Gonad development of the fish is also seasonal. A rapid increase in GSI occurs from June to October and the GSI drops to base levels in October-November as a consequence of spawning (Olsen and Merriman 1946). To date, reproductive physiology and endocrinology and the influence of environmental

factors on seasonal reproduction of the ocean pout are unknown.

Although spawning of the ocean pout is thought to occur in crevices or holes under large boulders in the wild (Olsen and Merriman 1946; Keats et al., 1985), direct observations of fish spawning have not been reported and the biology of egg fertilization is unknown. It was not even known whether the ocean pout eggs are fertilized in the water or within the ovary (internal fertilization) as is the case in its European counterpart eel pout *Zoarces viviparus*. Since fertilized eggs were never produced from captive fish, studies of embryonic and larval development of the ocean pout had to rely on fertilized eggs collected from the wild (Methven and Brown 1991; Brown et al., 1992).

Aquaculture Interest: It is reported that the ocean pout flesh is very palatable (Olsen and Merriman 1946). Chemical analysis and taste panel evaluation demonstrate that the ocean pout is a lean fish of low cholesterol content (Sheehy et al., 1977). Its nutritional quality of protein is higher than a casein reference standard (Jhaveri et al., 1985). Market surveys have shown that this fish is acceptable to American as well as Canadian consumers (Sheehy et al., 1977; Brown et al., 1992).

Biochemical and physiological studies show that the plasma and tissues of ocean pout contain high levels of multiple sets of antifreeze proteins (Li et al., 1985; Hew et al., 1988) all year-round (Fletcher et al., 1985). The presence of the AFPs depresses and prevents the formation of ice crystals in the tissues during the cold winter and enables the ocean pout to survive at temperatures down to -1.7°C (Kao et al., 1986; King et al., 1989). The tolerance of fish to cold temperatures, the good nutritional value and good consumer acceptability makes the ocean pout a very good aquaculture species in Eastern

Canada (Brown et al., 1989; Brown et al., 1992), where the severe cold winter water temperatures and the long winter season of 4-5 months have limited the development of aquaculture for other commercially attractive species such as the salmonid species (King et al., 1989).

Other traits of the ocean pout include large eggs and large larvae at hatching (Methven and Brown 1992). Ocean pout eggs are about 8.5 mm in diameter and it takes 2.5-3.5 months for the fertilized eggs to hatch. The fish are hatched as "juveniles" (adult-like in appearance), at about 30-40 mm in length, with functional eyes and mouth (teeth), well ossified and pigmented (Methven and Brown 1991). Egg yolk is rapidly absorbed in 24 hr and larvae start to feed exogenously at two days post-hatch in subzero temperatures (-0.5 to -1.0°C). Since there is no pelagic stage or metamorphosis, survival rates for the first two months of exogenous feeding are high (>90%) and slightly reduced to 75-80% in the second year (Methven and Brown 1991; Brown et al., 1992).

However, domestication of the species requires supply of the young fish in large quantities, which has not been available and this becomes a "bottle neck" for development of the aquaculture of the ocean pout. The capability to produce gametes in large quantity by artificial propagation is highly desirable. On the other hand, since the ocean pout shares similarities in reproductive biology with the wolffish (*Anarhichus lupus*), which is another marine fish of great economic value for aquaculture (Tilseth 1990), the study of the ocean pout reproduction might provide information for better understanding the reproduction of wolffish and for developing techniques for the hatchery production of wolffish fingerlings (Methven and Brown 1989).

1.3. OBJECTIVES FOR THE PRESENT RESEARCH PROJECT

With a clear understanding of the aquaculture potential and the problems encountered for domestication of this species in Newfoundland, the current research project was initiated in 1990. The primary objectives for this study were:

a) to determine the husbandry, feeding and reconditioning of the post-spawned fish for repeat maturation and spawning in captivity.

b) to examine the seasonal reproductive cycle of the ocean pout in relation to endocrine changes.

c) to determine the effect of water temperature and photoperiod and hormonal regulation on feeding, growth and reproduction of the fish in captivity.

d) to develop artificial fertilization techniques for the production of fertilized eggs and larvae from captive fish.

1.4. EXPERIMENTAL APPROACHES

The project was approached in 3 steps:

Step 1: This step focused on biochemistry of the egg yolk precursor protein or vitellogenin including (i) the hormonal induction of vitellogenin synthesis, (ii) the isolation and purification of vitellogenin from fish plasma, (iii) biochemical characterization of vitellogenin and (iv) the development of radio-immunoassay for the vitellogenin for the studies of the seasonal changes of plasma vitellogenin.

Step 2: This step focused on the description of the annual reproductive cycle of

the fish, and the regulation of the seasonal reproductive cycle by alteration of water temperature and photoperiod or by application of exogenous hormone such as the gonadotropic hormone-releasing hormone analog. The husbandry and the effect of water temperature on fish feeding and growth were also studied.

Step 3: This step focused on the biology of the fish egg fertilization, including the studies of *in vitro* and *in vivo* artificial insemination and the behavioral observations of the fish courtship, spawning and parental care, which would provide information for management of artificial incubation of eggs.

1.5. OUTLINE OF THE THESIS

The investigations, which form the basis of this thesis, cover broad topics related to (a) the biochemical nature of yolk precursor protein, (b) the development of biotechnology for the study of the yolk precursor protein, (c) reproductive endocrinology in association with the environmental and hormonal regulation and (d) the biology of egg fertilization of the ocean pout, and are described in the subsequent chapters as summarized below:

Chap.2. This chapter describes the induction of hepatic vitellogenin synthesis by 17ß-estradiol, the isolation, purification and characterization of the vitellogenins from the ocean pout (*Macrozoarces americanus*), Atlantic cod (*Gadus morhua*), Atlantic lumpfish (*Cyclopterus lumpus*) and the Arctic charr (*Salvelinus alpinus*).

Chap.3. In this chapter, procedures for development of radioimmunoassay (RIA) and enzyme-linked immunosorbent assay for these vitellogenins are described.

Chap.4. This chapter describes the detection of vitellogenin in skin mucus of the adult female ocean pout. By vitellogenin RIA, the sex of fish can be determined from the mucus.

Chap.5. This chapter describes the seasonal reproductive cycle and effect of water temperature and photoperiod on seasonal reproduction, and the induction of spawning with gonadotropic hormone-releasing hormone analog.

Chap.6. The biology of egg fertilization is reported in this chapter which indicates the possibility of internal fertilization of eggs in the ocean pout.

Chap.7. Copulation, spawning and parental care behaviour of the ocean pout are described in this chapter providing evidence for the ocean pout internal fertilization hypothesis.

Chap.8. This chapter describes the *in vitro* artificial insemination of ocean pout eggs.

Chap.9. This chapter summaries the overall results of this research project and discusses the findings in a broader context. Possible directions for future study of the ocean pout reproduction are discussed.

CHAPTER 2

A Comparative Study of Fish Vitellogenins: Isolation and Biochemical Characterization of Vitellogenins from the ocean pout (*Macrozoarces americanus*), lumpfish (*Cyclopterus lumpus*), Atlantic cod (*Gadus morhua*) and the Arctic charr (*Salvelinus alpinus*)

2.1. ABSTRACT

Vitellogenesis, including hepatic vitellogenesis, was induced by 17ß-estradiol in three marine fish: the ocean pout (*Macrozoarces americanus*), the lumpfish (*Cyclopterus lumpus*) and Atlantic cod (*Gadus morhua*). Vitellogenins were isolated from the plasma of these three species and one other species, the Arctic charr (*Salvelinus alpinus*), using a combined procedure of gel Sephacryl-S300 filtration, ultrafiltration and DEAE-Sephacel ion-exchange chromatography at low temperatures (4-9°C). The purified vitellogenins were characterized according to their molecular weights, total lipid and total phosphorus contents, and amino acid compositions. Molecular weights of vitellogenin for the three marine species were similar (485-630 KD) while the Arctic charr vitellogenin had a lower molecular weight (444 KD). The lipid content of vitellogenin was similar (17.92-21.34%) in the three marine species, but was lower in the Arctic charr vitellogenin (9.93%). Total phosphorus and lipid-bound phosphorus contents of vitellogenins varied from 1.11% to 3.56% and from 0.43% to 2.86%, respectively. However, protein-bound phosphorus content (0.62-0.72%) and amino acid composition of the four vitellogenins were similar. All four vitellogenins contained high levels of the essential amino acids including arginine (6.38-5.74%), lysine (8.16-8.74%), isoleucine (5.60-6.82%), leucine (9.61-11.39%) and valine (6.76-7.23%). The methionine (1.51-3.38%) and histidine (2.27-2.72%) levels were low, while the levels of threonine (4.69-6.09%) and phenylalanine (3.38-5.04%) varied between species. Among non-essential amino acids, aspartic acid (8.60-9.68%), serine (6.09-7.68%), glutamic acid (11.45-11.97%) and alanine (6.49-7.79%) were predominant.

This study suggests that vitellogenin from different species has a similar core protein structure but variable lipid-bound phosphorus content.

2.2. INTRODUCTION

Hepatic vitellogenin synthesis has been studied in several teleosts (Ng and Idler 1983; Wallace and Selman 1985; Mommsen and Walsh 1988). Synthesis of vitellogenin occurs in rough endoplasmic reticulum of the liver under the control of 17ß-estradiol produced by the ovary (Lazier et al., 1985). After translation, the vitellogenin protein undergoes post-translational modulations which include the phosphorylation, glycosylation and lipidation in the Golgi apparatus yielding a glycolipophosphoprotein molecule. It is then released into circulation and transported to the ovary for incorporation into the growing oocytes (Wallace and Selman 1985). Therefore, vitellogenin is an egg yolk precursor protein produced in adult females. The presence of vitellogenin in plasma may be used to distinguish the sex of fish (Craik and Harvey 1984). Since previous studies have indicated that the liver of both male and immature female may respond to 17ß-estradiol treatment and produce vitellogenin, the male or immature fish are often used for the studies of hormonal controlled vitellogenesis (Lim et al., 1991).

To date, vitellogenin has been characterized in only a few fish species, including the goldfish *Carassius auratus* (de Vlaming et al., 1980), rainbow trout *Oncorhynchus mykiss* (Campbell and Idler 1980; Norberg and Haux 1985; Fremont and Riazi 1988), sea

trout *Salmo trutta* (Norberg and Haux 1985), carp *Cyprinus carpio* (Tyler and Sumpter 1990) and tilapia *Oreochromis aureus* (Lim et al., 1991). These studies show that the amino acid composition and lipid (18.0-21.0%) and protein-bound phosphorus (0.58-0.79%) content of vitellogenin are similar between species. However, it is not clear if the characterization of vitellogenin established from these freshwater species adequately represents the vitellogenin structure from marine species on which few studies have been conducted. Because marine species are becoming increasingly important in aquaculture, a thorough understanding of their reproduction is desirable.

Since vitellogenin is an egg yolk protein precursor and plays a key role in fish reproduction, comparative studies of vitellogenin should provide useful information. In addition, information about the amino acid and lipid composition of vitellogenin might provide useful guidance for fish starter diet formulation (Ketola 1982). The objective of this study was to isolate and compare vitellogenins from marine and freshwater fish including the ocean pout, lumpfish (*Cyclopterus lumpus*), Atlantic cod (*Gadus morhua*) and the Arctic charr (*Salvelinus alpinus*).

2.3. MATERIALS AND METHODS

2.3.1. Fish

Immature ocean pout (399 $\pm$ 157 g, body weight $\pm$ std), lumpfish (159 $\pm$ 12 g) and Atlantic cod (802 $\pm$ 225 g) were kept in separate tanks. Experiments with the ocean pout

and lumpfish were conducted in December-January 1990 on fish held in indoor tanks (ca. 400 l) supplied with aerated flow-through seawater (3-4°C). All fish were exposed to a simulated natural photoperiod cycle (40W lamp 50 cm above water surface). Experiments on Atlantic cod were conducted in May-June 1991 on fish held in outdoor tanks (ca. 5000 L) supplied with ambient sea water (6-7°C) and natural photoperiod cycle. All fish were fed chopped capelin to satiation twice a week.

2.3.2. Hormonal induction of vitellogenin synthesis

Synthesis of vitellogenin in the fish was induced by 17ß-estradiol, which was dissolved in a small volume of absolute ethanol and mixed with an equal volume of saline (0.9% NaCl solution) to achieve a concentration of 10 mg/ml (Tyler and Sumpter 1990). The 17ß-estradiol was injected (10 mg/kg body weight) intraperitoneally (ip) weekly into the ocean pout (N=3), or biweekly into the lumpfish and cod (N=3, for each species). Control fish (N=3 of each species) received blank treatments of the ethanol-saline mixture following the same injection schedules (total 3 treatments).

A blood sample (0.5-1.0 ml) was taken from both treated and control fish each week via the caudal vein using chilled syringes and immediately centrifuged at 15,000 g for 3 minutes. The plasma was collected and analyzed by native polyacrylamide gel electrophoresis (PAGE) (So et al., 1985). The gels were stained with Coomassie Brilliant Blue G-250 (BIO-RAD) and acetylated Sudan Black B (BDH) to identify the 17ß-estradiol-induced new lipoprotein or vitellogenin in the plasma (So et al., 1985, Labbe and Loir 1991) (details in Appendix I). Total protein in the plasma was determined according

to Lowry et al. (1951). The plasmas were extracted with dimethylether and 17ß-estradiol and testosterone in the ether fraction were measured by radioimmunoassay (RIA), from which the steroid levels in fish plasma were calculated (details in Chapter 5 and Appendix II).

One week after the last hormone treatment, a larger volume (up to 10 ml) of blood was withdrawn from every fish into chilled vials containing the protease enzyme inhibitor aprotinin (20 trypsin inhibitor unit (TIU)/ml) and centrifuged for collection of plasma. The plasma was stored frozen at -20°C until use for isolation of vitellogenin. Plasma of adult male and female Arctic charr was provided by the Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, from cultured broodstock and stored at -20°C without addition of aprotinin before purification of vitellogenin.

2.3.3. Purification of vitellogenin

Vitellogenin was purified from the plasma of the 17ß-estradiol treated fish (ocean pout, lumpfish and cod) or adult females (Arctic charr) by successive Sephacryl-S300 gel filtration, ultrafiltration and DEAE-Sephacel ion-exchange chromatography. The superfine gel Sephacryl S300 was packed into two columns (90x1.5 and 70x1.5 cm) and equilibrated with a large volume of vitellogenin buffer (50 mM Tris, 0.5M NaCl, 10 mM EDTA, 100 TIU/l aprotinin, pH 8.0; So et al., 1985) (see Appendix III). Two connected columns (90x1.5 and 70x1.5 cm) were used at a flow rate of 12 ml/hr to increase the resolving efficiency of gel filtration. Before loading onto columns, the plasma was equilibrated with vitellogenin buffer (1:1, v/v), centrifuged (15,000 g, 3 minutes) and the

supernatant retained for use. Fractions (1.2 ml/tube) were collected and monitored by optical density (O.D) at 280 nm. Plasma from 17ß-estradiol treated and control fish, or from adult males and females, was individually chromatographed and their elution patterns compared. Fractions containing vitellogenin proteins were retained and equilibrated with a large volume (1:10, v/v) of Tris buffer (50 mM Tris, 100 TIU aprotinin/l, pH 8.0) in the Amicon ultrafiltration apparatus (membrane XM 100) and further chromatographed with DEAE-Sephacel (30x1.5 cm column) by NaCl step-gradients (0.00-0.30 M) in Tris buffer (Norberg and Haux 1985; Tyler and Sumpter 1990). Purity of vitellogenin was assessed by native PAGE and the vitellogenin content of the eluent was estimated according to Lowry et al. (1951). Since the elution volume of vitellogenin from DEAE column was often large, the vitellogenin was concentrated by ultrafiltration on the Amicon apparatus fitted with XM 100 membrane. All procedures were conducted in a cold room (4-9°C) to reduce proteolysis of the vitellogenin.

2.3.4. Characterization of Vitellogenin

Molecular weights of the vitellogenins were estimated by gel filtration by comparing the elution volume (peak position) of vitellogenins to that of proteins of known molecular weight, including thyroglobulin (669 KD); catalase (232 KD); bovine serum albumin (67 KD), chymotrypsinogen A (25 KD) and blue Dextran 2000 as leading dye (Pharmacia).

Lipid and phosphorus contents of vitellogenin were determined from the purified vitellogenin. Before lipid determination, a 50 µl aliquot of vitellogenin solution (2 mg/ml)

were extracted (1:80, v/v) twice with a methanol:chloroform:0.9% NaCl (2:1:1) solution modified from Norberg and Haux (1985). The chloroform extracts were retained, pooled and dried under N_2 at low heat (35°-40°C). Total lipid and lipid-bound phosphorus contents of vitellogenin were determined from the chloroform extracts (Bartlett 1959; Marsh and Weinstein 1966). Total phosphorus content of vitellogenin was determined directly from non-extracted vitellogenin solution according to the method of Bartlett (1959). Protein-bound phosphorus content of vitellogenin was calculated by subtracting the lipid-bound phosphorus from the total phosphorus content of vitellogenin. For each assay, the same volumes (50 µl) of Tris buffer (in 0.2 M NaCl) were subjected to the same measurement as control (detailed procedures are given in Appendix IV) .

Amino acid composition of the vitellogenins were determined (at the laboratory of amino acid analysis, Department of Biochemistry, Memorial University of Newfoundland) from the purified vitellogenin. The samples were dialysed in distilled water (1:1000, v/v) for 48 hr at 4°C (one change of the distilled water) and freeze-dried. The vitellogenin samples were then hydrolysed at 110°C in 6.0 N HCl for 24 hr. The amino acids were separated, identified and quantified according to the methods of Blackburn (1968). In this assay, cysteine and methionine were measured as cysteic acid and methionine sulphone, respectively, from the samples oxidized by performic acid prior to the hydrolysis in 6.0 N HCl. The content of each amino acid was expressed as the percentage of total amino acid content in vitellogenins.

2.4. RESULTS

One week after 17ß-estradiol treatment, both 17ß-estradiol and total protein in the plasma of ocean pout increased significantly and continued to increase progressively following repeated 17ß-estradiol treatment, while testosterone level remained unchanged except in one fish in which the testosterone level was increased on day 24 (after 3 treatments) giving an increased group mean and greater standard error. In contrast, plasma 17ß-estradiol, testosterone and total protein in the control fish remained unchanged throughout this study (Table 1).

Electrophoresis revealed the appearance of a new large molecular weight protein in the plasma one week after 17ß-estradiol treatment, with the quantity of this new protein continuing to increase following repeated 17ß-estradiol treatment (Fig. 1). This new protein reacted with Sudan Black B, indicating its lipoprotein nature (So et al., 1985, Labbe and Loir 1991). In contrast, this lipoprotein was not present in fish either before 17ß-estradiol treatment or in control fish throughout the experiment (Fig. 1). Similar results were seen in both lumpfish and Atlantic cod following 17ß-estradiol treatments.

Four distinct peaks were resolved by Sephacryl S300 gel filtration from 17ß-estradiol treated and adult female ocean pout plasma (Fig.2A,B). In the control fish (non-treated immature fish, males or females) and adult males, the second peak (PK II) was not present. Since the PK II protein(s) also stained with Sudan Black B and could bind to Affi-Gel Con-A (data not shown), this indicates that the 17ß-estradiol induced or the female specific PK II protein(s) is a glycolipoprotein (Idler and Ng 1979). Further

chromatography of PK II fractions on a DEAE-Sephacel column yielded a sharp peak at 0.20-0.21 M NaCl in Tris buffer (Fig.2C), containing a single vitellogenin protein band, as was confirmed by native PAGE (Fig.3). Following the same procedures, vitellogenin of lumpfish, Atlantic cod and Arctic charr were also isolated and purified.

The biochemical characteristics of the vitellogenin, including the molecular weight, lipid and phosphorus contents and amino acid compositions from the four species, are shown in Tables 2 and 3. Vitellogenins of the ocean pout, lumpfish and Atlantic cod were similar in terms of the molecular weight (485-630 KD) and lipid content (17.9-21.4% whereas vitellogenin of Arctic charr had a lower molecular weight (444 KD) and lipid content (9.93%). Total phosphorus content and the lipid-bound phosphorus content of vitellogenin were more variable, ranging from 1.11% to 3.56% and from 0.43% to 2.86%, respectively, with the highest value in the ocean pout and the lowest value in Arctic charr (Table 2). All four vitellogenins were similar with regard to protein-bound phosphorus content and amino acid composition, i.e., all containing relatively high levels of the essential amino acids arginine (6.38-5.74%), lysine (8.16-8.74%), isoleucine (5.60-6.82%), leucine (9.61-11.39%) and valine (6.76-7.23%), but low levels of methionine (1.51-3.38%) and histidine (2.27-2.72%). The levels of threonine (4.69-6.09%) and phenylalanine (3.38-5.04%) varied between species. Among the non-essential amino acids, the levels of aspartic acid (8.60-9.68%), serine (6.09-7.68%), glutamic acid (11.45-11.97%) and alanine (6.49-7.79%) were also relatively high in the four vitellogenins (Table 3).

2.5. DISCUSSION

This study demonstrated that hepatic vitellogenesis is inducible by 17ß-estradiol treatment in the ocean pout, Atlantic cod and lumpfish, in which a new protein appears in plasma following 17ß-estradiol treatment. The Sudan Black B staining (for lipoprotein), the phosphorus determination and the Con-A Affi-Gel affinity test (for glycoprotein) confirmed that this 17ß-estradiol-induced new protein is a glycolipophosphoprotein. Because this protein has an identical elution position to that of the female specific protein on Sephacryl-S300 gel filtration, suggesting an identical molecular weight, it is defined as vitellogenin. Although 17ß-estradiol treatment induced hepatic vitellogenin synthesis, the plasma level of testosterone remained unchanged following the 17ß-estradiol treatment. The induction of hepatic vitellogenin synthesis by 17ß-estradiol in the three marine species agrees with previous studies on other teleost species (Campbell and Idler 1980; Norberg and Haux 1985; Bradley and Grizzle 1989; Olin et al., 1989; Flett and Leatherland 1989; Lim et al., 1991).

Although various procedures have been adopted for the isolation of vitellogenin from fish plasma, gel chromatography using Sepharose 6B was most frequently employed in earlier studies (Wiley et al., 1979; Norberg and Haux 1985; Tyler and Sumpter 1990). So et al. (1985) improved the resolution of vitellogenin (in Atlantic salmon) using superfine Sephacryl-S300 and the purification was completed in a single step of gel filtration. In the present study, a combination of Sephacryl-S300 gel filtration, ultra-filtration (XM 100) and DEAE-Sephacel ion-exchange (So et al., 1985; Norberg and Haux

1985; Tyler and Sumpter 1990) proved effective and was standardized for the isolation of vitellogenins from the ocean pout, lumpfish, Atlantic cod and Arctic charr. In these procedures, ultrafiltration in an Amicon apparatus removed the salt NaCl (0.5 M, in buffer) and other contaminating smaller proteins (molecular weight $\leq$ 100 KD) from the VTG eluent, consequently increasing the resolving efficiency of the subsequent DEAE-Sephacel ion-exchange chromatography. NaCl Tris buffer (Norberg and Haux 1985) was also used in this study replacing the phosphate ($H_2PO_4^-/HPO_4^{2-}$) Tris buffer for DEAE-Sephacel ion-exchange (Tyler and Sumpter 1990). This eliminates the interference of phosphate salts from buffer with the determination of vitellogenin phosphorus content. Previous studies on amphibia *Xenopus laevis* (Wiley et al., 1979), rainbow trout *Oncorhynchus mykiss*, brown trout *Salmo trutta* and Atlantic salmon *Salmo salar* (Campbell and Idler 1980; Norberg and Haux 1985) have shown that distilled water may precipitate vitellogenin, thus resolving vitellogenin from other plasma proteins. However, trials with this method for the ocean pout vitellogenin were not successful.

Chemical characterizations of vitellogenins from the four species show a large variation in molecular weight ranging from 440 KD to 630 KD. Large variations in vitellogenin molecular weight were also noted in other species, such as the Atlantic salmon (520 KD, So et al., 1985), sea trout (440 KD, Norberg and Haux 1985), white-spotted charr *Salvelinus leucomaenis* (540 KD, Kwon et al., 1990), carp (390 KD, Tyler and Sumpter 1990) and tilapia (500 KD, Lim et al., 1991). In rainbow trout, the vitellogenin molecular weight was estimated to be 440 or 560 KD, respectively, by Norberg and Haux (1985) and Fremont and Riazi (1988) using different methods. In this

study, isolation of vitellogenin and determination of the vitellogenin molecular weight from the different species were conducted following a standardized procedure, which might provide a better comparison of the molecular weight of vitellogenin between species.

The methods of Marsh and Weinstein (1966) and Bartlett (1959) have been generally adopted in the previous studies for the measurement of lipid and phosphorus content of fish vitellogenin. With the same methods, a similar lipid level in vitellogenins of the three marine species was noted. However, levels of the lipid-bound phosphorus or phospholipid of these vitellogenins differed greatly, indicating a different lipid phosphorylation of vitellogenin between species. In rainbow trout, over 70% of total lipid in vitellogenin is phospholipid (Norberg and Haux 1985; Fremont and Riazi 1988). This phospholipid(s) plays a key role in the surface structure of the vitellogenin molecule (Fremont and Riazi 1988). Since incorporation of vitellogenin into eggs is a receptor-mediated process (Stifani et al., 1990; Chan et al., 1991), the phospholipid content in vitellogenin might affect the interaction between the vitellogenin complex and the receptors in the oocyte membrane, hence modulating the oocyte incorporation of vitellogenin (Stifani et al., 1990). Phospholipids in vitellogenin also seem to be the important vehicles for transporting various substances into eggs. These substance include the essential fatty acids or polyunsaturated fatty acids (Fremont et al., 1984; Fremont and Riazi 1988), thyroxine (T_4) and triiodo-thyronine (T_3) (Babin 1992), and some ions such as Zn^{++} (Olsen et al., 1989), Ca^{++} and Mg^{++} (Bjornsson and Haux 1985; Carragher and Sumpter 1991). The importance of phospholipids for embryonic development has been

partially demonstrated in the Atlantic halibut (*Hippoglossus hippoglossus*), Atlantic cod, plaice (*Pleuronectes platessa*) and turbot (*Scophthalmus maximus*), e.g., the phospholipids are essential for the biomembranes of embryos (Rainuzzo et al., 1992). Since vitellogenin phospholipid is important for fish reproduction (Daniel et al., 1993), perhaps the difference in the phospholipid contents of vitellogenins relates to different reproductive patterns of fish. To better understand this aspect, more studies are required.

In contrast to the consistent lipid content (ca.20%) in vitellogenin of the three marine species, the lipid contents (9.93%) of vitellogenin from the Arctic charr are unreasonably low. Perhaps this low level was caused by a breakdown of the vitellogenin resulting from storage (Silversand et al., 1993) since the plasma of Arctic charr for the vitellogenin isolation was sampled at Prince Edward Island and stored without addition of the protease enzyme inhibitor aprotinin. Delipidation or dephosphorylation of the vitellogenin might have occurred before vitellogenin isolation.

In previous studies, only protein-bound phosphorus of the vitellogenin was reported (de Vlaming et al., 1980; Campbell and Idler 1980; Norberg and Haux 1985). In this study, lipids were extracted from vitellogenin in the chloroform phase. Presumably, the protein-bound phosphorus should be measured in the water phase (Norberg and Haux 1985). However, it was difficult to dry the large volume of the water phase and the large amount of NaCl precipitate also interfered with the determination of phosphorus. Therefore the protein-bound phosphorus content of vitellogenins was determined by subtracting the lipid-bound phosphorus from the total phosphorus content of vitellogenin, giving a comparable protein-bound phosphorus level (0.62-0.72%) in the four

vitellogenins. These figures agree with those reported from the previous studies (de Vlaming et al., 1980; Campbell and Idler 1980; Norberg and Haux 1985). Previous studies have shown that the protein-bound phosphorus of vitellogenin represents the level of amino acid phosphorylation, particularly the level of serine in the form of phosphoserine, in vitellogenin (Campbell and Idler 1980; de Vlaming et al., 1980; Fremont and Riazi 1988). Indeed, serine contents of the four vitellogenins are comparable.

The amino acid composition of the four vitellogenins is similar and is in agreement to that reported in previous studies from rainbow trout (Hara and Hirai 1978; Campbell and Idler 1980), goldfish (de Vlaming et al., 1980), Atlantic salmon (Ketola 1982), carp (Tyler and Sumpter 1990) and to a lesser degree in the amphibian *Xenopus laevis* (Wiley et al., 1979; de Vlaming et al., 1980). This similarity seems to support the hypothesis that vitellogenin genes of non-mammalian vertebrates are highly conserved (Shapiro et al., 1987; Tata 1987; Lee et al., 1992).

It is known that vitellogenin, after being taken up and deposited into eggs, provides both energy and amino acids for embryonic development. However, the role of each individual amino acid of vitellogenin in the embryonic development is not known. In trout and salmon, lysine is essential for growth and for prevention of fin rot and mortality (Ketola 1982). Deficiency of methionine in fish diets causes the development of bilateral lens cataracts and reduces growth. Deficiencies of some of these essential amino acids can be spared by some non-essential amino acids. For example, cystine spares part of the methionine requirement in rainbow trout, while tyrosine spares phenylalanine. A better understanding of the amino acid composition of fish vitellogenin

might provide useful information for the understanding of fish nutritional requirements, especially that of the larval fish. It might also provide guidance for the formulation of starter diet of fish in the future and for supplementation of the amino acids in fish feed to promote better growth and survival (Ketola 1982).

Table 1.Plasma levels of 17ß-estradiol, testosterone and protein in the ocean pout with or without 17ß-estradiol treatment.

days of sampling	No. of inj.	17ß-estradiol ng/ml	testosterone ng/ml	protein mg/ml
	treated group			
0	0	5.62 (5.79)	0.53 (0.21)	37.5 (5.66)
7	1	*206.12 (72.64)	0.17 (0.04)	*75.84 (21.5)
14	2	*280.56 (60.34)	0.70 (0.21)	*91.61 (10.61)
24	3	*213.76 (62.52)	1.42 (1.64)	*128.0 (6.83)
34		* 41.19 (17.99)	0.49 (0.02)	*89.02 (5.10)
	control group			
0	0	2.15 (0.27)	0.53 (0.21)	37.51 (5.66)
7	1	3.99 (1.35)	0.22 (0.01)	42.83 (12.91)
14	2	4.72 (1.94)	0.93 (1.18)	53.16 (5.06)
24	3	2.49 (3.16)	0.72 (0.52)	58.85 (13.10)
34		7.01 (1.36)	0.49 (0.40)	59.76 (3.67)

data are expressed as mean ± standard error (in bracket), N=3.
* significant difference ($P<0.05$) from day 0 values.

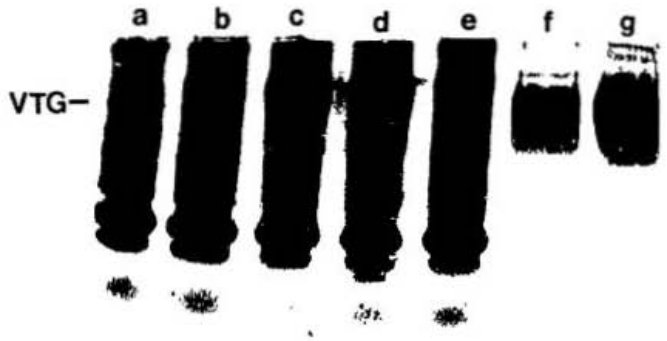

Figure 1. Electrophoretic results of 5 µl plasma from the control and 17ß-estradiol treated immature ocean pout. The first column on the left (column **a**) shows a plasma from the fish before receiving 17ß-estradiol treatment; columns **b**, **c** and **d** show the plasmas sampled from the fish after 1, 2 & 3 17ß-estradiol treatments, respectively; column **e** shows a plasma from the control fish after 3 repeat injections of blank solution (no hormone). Columns **f** and **g** show the vitellogenin fractions (in PK II) from Sephacryl-S300 gel filtration. VTG: vitellogenin.

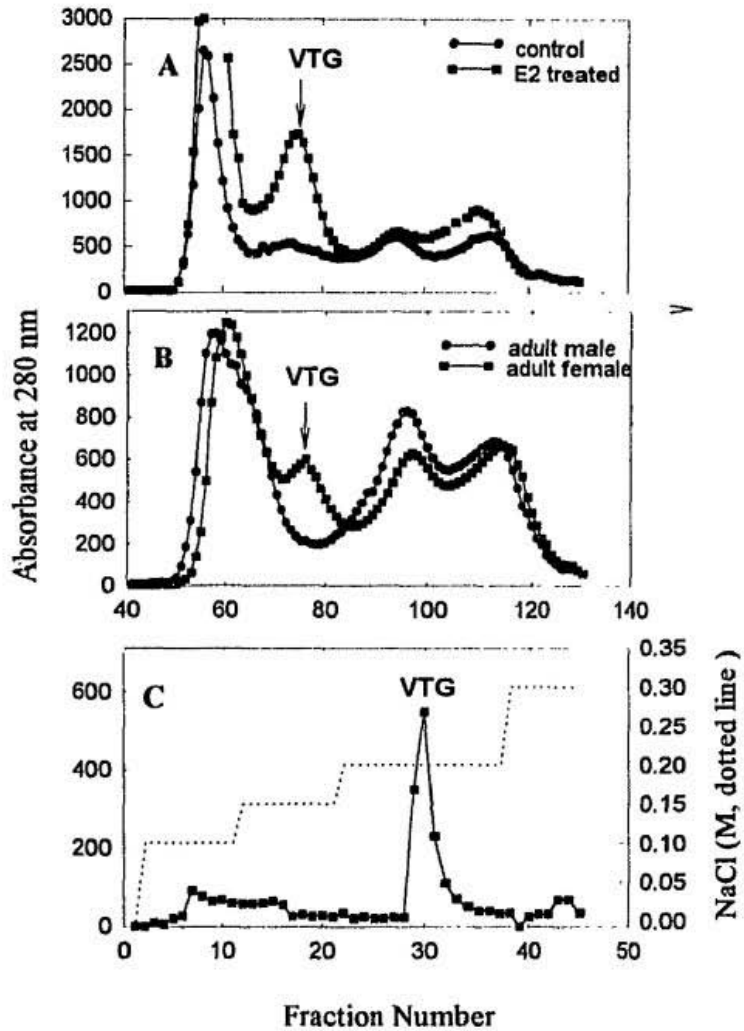

Figure 2. Resolution profiles of ocean pout vitellogenin (arrow) by gel Sephacryl-S300 filtration on plasmas from (A) control and 17ß-estradiol treated ocean pout; (B) mature male and mature female and (C) by DEAE-Sephacel ion-exchange purification of vitellogenin from the partially purified vitellogenin in the factions of #75-77 (peak II) of 17ß-estradiol treated fish plasma (A). In panels A, B & C, the Y-axis indicates the absorbance (optical density at 280 nm), the X-axis indicates the fraction number (1.2 ml/fraction in A & B; 5 ml/fraction in C) from column elution. In panel C, the solid line with open triangles indicates the vitellogenin resolution profile, the dotted line indicates the concentration gradient of NaCl (M) in Tris buffer. VTG:vitellogenin.

Figure 3. Electrophoretic results of vitellogenin elutions from Sephacryl-S300 gel filtration (column **a** and **b**) and DEAE-Sephacel ion-exchange chromatography (columns **c** and **d**). VTG: vitellogenin.

Table 2. Chemical composition of vitellogenins from ocean pout (*M. americanus*), lumpfish (*C. lumpus*), Atlantic cod (*G. morhua*) and Arctic charr (*S. alpinus*).

items (%)	ocean pout	lumpfish	cod	charr
total lipid	17.92	21.18	21.34	9.93
total phosphorus	3.56	2.45	2.15	1.11
lipid-bound P	2.86	1.73	1.51	0.43
protein-bound P	0.68	0.72	0.62	0.68
MW (KD)	577.8	630.5	485.2	444.7

P: phosphorus; MW:molecular weight

Table 3. Amino acid composition of vitellogenins from ocean pout (*M. americanus*), lumpfish (*C. lumpus*), Atlantic cod (*G. morhua*) and Arctic charr (*S. alpinus*).

Amino acid (%)*	ocean pout	lumpfish	cod	charr
aspartic acid	8.60	9.68	8.79	9.23
threonine	4.69	6.09	5.14	5.10
serine	6.88	7.68	6.09	6.28
proline	4.13	3.91	4.13	4.44
glutamic acid	11.97	11.86	11.45	11.96
glycine	2.80	2.65	3.02	2.83
alanine	7.57	6.49	7.79	7.19
cystine	1.32	0.90	1.69	1.64
cysteic acid	0.47	0.14	-	-
valine	6.83	6.99	6.76	7.23
methionine	3.38	1.51	2.48	2.35
cystathionine	0.10	0.11	0.13	0.12
isoleucine	5.60	6.82	6.58	6.01
leucine	9.61	11.39	10.99	9.74
tyrosine	3.58	3.09	4.21	4.15
phenylalanine	4.41	3.38	3.97	5.04
ethanolamine	0.14	0.16	0.20	-
ornithine	0.07	0.07	0.12	0.07
lysine	8.74	8.44	8.71	8.16
histidine	2.59	2.27	2.67	2.72
arginine	6.54	6.38	5.08	5.74

* all values are expressed as percentage (%) of total amino acids

-: absent or not detectable.

CHAPTER 3

Development of Radioimmunoassays and Enzyme-linked Immunosorbent Assay for the Vitellogenins of the ocean pout (*Macrozoarces americanus*), Atlantic cod (*Gadus morhua*) and Arctic charr (*Salvelinus alpinus*).

3.1. ABSTRACT

Homologous vitellogenin radioimmunoassays were developed for vitellogenins of the ocean pout (*Macrozoarces americanus*), the Atlantic cod (*Gadus morhua*) and the Arctic charr (*Salvelinus alpinus*) using isolated vitellogenins as standard, I^{125} labelled vitellogenin (I^{125}vitellogenin) as tracer and vitellogenin antisera. The vitellogenins were iodinated with NaI^{125} in iodogen (1,3,4,6,tetrachloro-3α,6α-diphenyl-glycoluril). The I^{125}vitellogenin was separated from free I^{125} on a disposable PD10 Sephadex G-25M column. Vitellogenin antibody was prepared in New Zealand rabbits by repeated challenge of the animals with vitellogenin emulsified in either Freund's complete or incomplete adjuvants. These radioimmunoassays are highly sensitive (ranging 1-675 ng/ml) and specific, and the cross reaction between species is low. An enzyme-linked immunosorbent assay with a range of 3.1-200 ng/ml was developed for vitellogenin of the ocean pout using titration plates.

3.2. INTRODUCTION

Vitellogenin is an egg yolk precursor protein found in the plasma of adult female fish. It is synthesized in the liver within the rough endoplasmic reticulum under the control of 17ß-estradiol from the ovary. After release into circulation, vitellogenin is transported to the ovary for incorporation into the oocytes (Ng and Idler 1983; Wallace and Selman 1985; Mommsen and Walsh 1988). Since the level of vitellogenin in fish plasma reflects the ovarian activity and it changes seasonally following the seasonal reproductive cycle (Copeland and Thomas 1988; Carnevali et al., 1991; Methven et al., 1992), knowledge of the plasma vitellogenin level assists with understanding of fish reproductive cycles. On the other hand, since vitellogenin is egg yolk protein precursor and female specific and appears in plasma at least a year before sexual maturation and spawning (Craik and Harvey 1984), detection of vitellogenin in fish plasma allows sex determination in premature fish.

Reliable detection of plasma vitellogenin requires sensitive assay methods. To date, several techniques have been adopted for the detection and quantification of vitellogenin in fish blood including an indirect method by measuring the plasma calcium or protein-bound phosphorus content (Craik and Harvey 1984) and direct methods of immuno-diffusion, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), which measure the vitellogenin level in plasma using specific antibodies (So et al., 1985; Chan et al., 1991; Kwon et al., 1990,1993; Maisse et al., 1991; Kishida et al., 1992). Among these techniques, RIA and ELISA are widely used due to their high sensitivity

and specificity. However, because of the high specificity or low reactivity of the vitellogenin antibodies to heterologous vitellogenin from the other species, these immunological techniques (either RIA or ELISA) have to be developed individually for the vitellogenin of each fish species.

Because the reproductive biology of ocean pout, Atlantic cod and Arctic charr are currently being investigated in our laboratory, which required a sensitive and reliable technique to measure the plasma vitellogenin, the current study was conducted to develop RIAs for these fish species. RIA requires the use of a high dose of isotope (for labelling the vitellogenin), which is an apparent disadvantage of this technique. Compared with RIA, the ELISA seems to have some advantages such as having comparable sensitivity and specificity but not using any radioactive isotope. Therefore, an ELISA was also developed for the ocean pout vitellogenin and tested for its sensitivity.

3.3. MATERIALS AND METHODS

3.3.1. Isolation of fish vitellogenin

Vitellogenins were isolated and purified from the plasma of 17ß-estradiol treated ocean pout and Atlantic cod, and from adult female Arctic charr, respectively, by a combined procedure of gel filtration, ultrafiltration and ion-exchange chromatography.

3.3.2. Production of fish vitellogenin antisera

1.0 mg purified ocean pout vitellogenin was emulsified with Freund's complete adjuvant and injected subcutaneously into New Zealand rabbits (BW 2-3 kg) to raise vitellogenin antibodies. The Freund's adjuvant enhances the immune response to vitellogenin, thus promoting a better antibody production in the rabbit. One month after the primary challenge, the rabbits were boosted twice at 1-1.5 month intervals by the purified ocean pout vitellogenin (1.0 mg) emulsified in Freund's incomplete adjuvant. Five months after the initial immunization, a large volume of blood (50 ml) was withdrawn from the immunized rabbits via the ear vein into large culture tubes and placed in a fridge (4.0°C) over-night. The antisera were subsequently collected, aliquoted and stored frozen at -20°C until use. For production of the Arctic charr and Atlantic cod vitellogenin antibodies, rabbits were injected with the charr or cod vitellogenin at a dose of 250 µg per injection following the same procedures and schedules as that for the ocean pout.

3.3.3. Development of vitellogenin RIA

Development of vitellogenin RIA requires labelled vitellogenin (isotope) as tracer and appropriately diluted vitellogenin antiserum. The procedures for the RIA are summarized as follows:

Preparation of labelled vitellogenin: Iodine NaI^{125} was used to label the vitellogenin in iodogen (1,3,4,6,tetrachloro-3α,6α-diphenyl-glycoluril) (So et al., 1985). The iodogen was dissolved in dichloromethane (1.0 mg/ ml) and 5 µl of this solution was pipetted into a 6x50 mm disposable tube and air dried. To start the reaction, 10 µg

vitellogenin (in 5-10 µl Tris buffer, pH 8.0) and 0.5 mCi fresh NaI^{125} (New England Nuclear) were added to the iodogen tube and gently shaken. The reaction was terminated 12 minutes later by addition of 600 µl 0.05 M phosphate buffer and the content of the reaction tube was pipetted into a PD10 Sephadex G-25M column (Fisher Scientific Ltd.), which was pre-saturated by 1 ml 5% bovine serum albumin (BSA) in 0.05 M phosphate buffer. The column was eluted with 0.05 M phosphate buffer and fractions (1.0 ml/fraction) were collected, separating iodinated vitellogenin (I^{125}vitellogenin) from free iodine (I^{125}) (see Appendix V). Before use for RIA, the I^{125}vitellogenin fraction from the PD10 column was re-chromatographed on a small column of Sephacryl S300 (20x0.5 cm) to increase the purity of the I^{125}vitellogenin.

Titration of vitellogenin antisera: An appropriate vitellogenin antibody concentration is important for optimizing the sensitivity of the RIA. For the vitellogenin antisera titration, 200 µl barbitol buffer (80 mM barbitol, 0.5% BSA, 0.01% thimerosal, pH 8.6), 200µl I^{125}vitellogenin solution (about 25,000 cpm) and 200 µl serially diluted vitellogenin antiserum were added to duplicate 12x74 mm polypropylene tubes (Fisher Scientific Ltd.). After gently shaking, the tubes were incubated at room temperature for 3 hr before adding 100 µl diluted (1:80) normal rabbit serum and 100 µl diluted (1:20) goat anti-rabbit γ-globulin. Following a further 6-12 hr of incubation at room temperature, the tubes were centrifuged at 4,000 x g for 15 minutes to separate the bound I^{125}vitellogenin-antibody complex from free I^{125}vitellogenin. The supernatant (containing free I^{125}vitellogenin) was carefully aspirated and radioactivity in the precipitates (containing the I^{125}vitellogenin-antibody complex) was determined in a Packard Auto-

Gamma Counter. Percentage of binding (%), i.e., the count (CPM, count per minute) of I^{125}vitellogenin-antibody complex as a percentage of total count of I^{125}vitellogenin added into the tube, was calculated for each dilution of the vitellogenin antiserum. The dilutions which yielded 50% binding of the total I^{125}vitellogenin count were chosen as the antisera titre for RIA.

Optimization of the RIA: High non-specific binding of I^{125}vitellogenin, i.e., the I^{125}vitellogenin sticking to the tube, was often encountered in the vitellogenin RIA, which reduced the sensitivity of the RIA. To reduce the non-specific binding and optimize the RIA conditions, various combinations of normal rabbit serum (at 1:10, 1:20, 1:40 and 1:80 dilutions) and goat anti-rabbit γ-globulin (at 1:10, 1:20, 1:40 and 1:80 dilutions), and the use of borosilicate and polypropylene tubes, were tested. For RIA, 100 µl vitellogenin standard solution (range 1-675 ng/ml) or diluted plasma samples were added to tubes. With a pre-determined antiserum concentration (titre) and amount of I^{125}vitellogenin in the RIA tubes, addition of the unlabelled vitellogenin to the tubes will reduce the binding of the I^{125}vitellogenin to antisera because the unlabelled vitellogenin competes with the I^{125}vitellogenin for antibodies. If no unlabelled vitellogenin is added, all antibodies in the tubes will bind to the I^{125}vitellogenin yielding a maximum binding (Bo) between I^{125}vitellogenin and antibody. However, when the unlabelled vitellogenin is added to the RIA tubes, competition between labelled (I^{125}vitellogenin) and unlabelled vitellogenin to the antibodies occurs. Increasing the amount of the unlabelled vitellogenin in the RIA tubes will decrease the amount of binding of the labelled vitellogenin (I^{125}vitellogenin) to the antibodies (B). Therefore, the percentage of I^{125}vitellogenin bound to antisera (B/Bo,

also called displacement) will decrease progressively with the increase in the amount of the unlabelled vitellogenin, producing a standard curve. Similarly, since vitellogenin is present in female plasma, serial dilution of the plasma should produce a parallel curve to the vitellogenin standard curve in the RIA. A higher level of vitellogenin in plasma would produce a greater displacement. The vitellogenin level in the plasma is estimated by comparing the displacement (B/Bo) of the plasma sample to that in the standard curve.

Since the binding between antibody and vitellogenin is highly specific, the vitellogenin antibody will not react with heterologous vitellogenin (from other fish species). Therefore plasma dilutions from other species will not show a parallel curve to the standard vitellogenin curve, or will not show any displacement at all. In this study, plasma from the same or different species were serially diluted and tested for each RIA (detailed protocol is given in Appendix VI). The reliability of the RIA is indicated by its reproducibility, which is tested by setting a standard (containing a known level of vitellogenin) for repeated measurement by the RIA. The intra- and inter-assay variations are determined by comparing the deviation between measurements of several standards within an assay (intraassay) or between several assays (interassay). The coefficient of variation (CV) is calculated from the mean and the standard error of mean of these measurements (CV = standard error/mean).

3.3.4. Development of vitellogenin ELISA

ELISA is another sensitive technique for the measurement of plasma hormone substances and vitellogenins. The principle of ELISA is based on an enzyme-dependant

colour development, from which the concentration of protein (or hormone) in the reactant is estimated. The ELISA is normally performed on titration plates (containing 12x8=96 wells). A protein standard (e.g. hormone or vitellogenin, etc) is coated onto the wall of each well (coating) on the titration plate. The antisera (antibody made in rabbit against this protein) and the protein standard or samples are then added into the wells to initiate the reaction between the antisera and the protein. Because the newly added free proteins (standard or sample) compete with the coated proteins for the antibody, the more free proteins are added into the wells, the less antibody will react with and bind to the coated protein. After reaction, the antibodies that do not bind to the coated protein on the wall are removed by washing and a second antibody (sheep anti-rabbit γ-globulin) linked to a peroxidase enzyme is added into the wells. The second antibody will react with the first antibody which is initially bound onto the wall through the coated protein. The non-bound or free second antibody is then removed by washing and a substrate (H_2O_2) of the enzyme (peroxidase) is added into the wells. A yellowish colour will develop from this enzymatic reaction. The extent of the colour reaction depends on the amount of enzyme present in the wells, which is initially determined by the amount of protein added into the wells, and the colour reaction decreases with the increased amount of protein added into the wells. Based on this correlation, a standard curve can be established. By comparing with the standard curve, the amount of the same protein (hormones) in samples can be estimated according to its colour reaction. In the present study, an ELISA for ocean pout vitellogenin was developed following the procedures of Maisse et al. (1991). The procedure of the ELISA includes:

Plate coating: 150 µl purified vitellogenin solution, equilibrated in sodium bicarbonate buffer (50 mM $NaHCO_3$, pH 9.6) containing gentamycin (5.0 mg/l), was pipetted into each well. Eight coating levels (0, 0.47, 0.94, 1.87, 3.75, 7.50, 15 and 30 ng vitellogenin/well) were tested. After addition of the 150 µl vitellogenin solution into each well on the plate, the plates were incubated at 37°C for 3 hr before discarding the contents of the well and washing the wells 5 times (200 µl/well/washing) with Tris buffer (TBS-T) (10 mM Tris-HCl, 0.15 M NaCl, pH 7.5, 0.1% Tween and 5.0 mg/l gentamycin). Next, these wells were further saturated with 200 µl Tris buffer containing 2.0% goat serum (TBS-T-GS) for 30 minutes. These coated plates were ready for immediate ELISA or they could be stored at -20°C for later use.

Titration of vitellogenin antisera: 100 µl of diluted vitellogenin antisera (from 1:200 to 1:25600, diluted in TBS-T-GS) and 50 µl TBS-T-GS buffer were added in triplicate to vitellogenin coated wells. After gently shaking for 3 minutes on a Titer Plate Shaker (Lab-Line Instrument, Inc.), the plate was incubated at 20°C for 16 hr. At the end of this incubation period, the well contents were discarded and the wells washed 5 times with the TBS-T buffer, before adding with 150 µl of diluted sheep anti-rabbit γ-globulin (1:2000, second antibody). After another 2 hr incubation at 37°C, the well contents were discarded and the wells washed 5 times again with TBS-T buffer before adding 150 µl OPD solution (see Appendix VII) of 0.05% 1,2-phenylene diamine in 50 mM ammonium acetate-citric acid (pH 5.0) containing 0.5 ml/l of 30% hydrogen peroxide (H_2O_2). The plate was placed in the dark at 20°C (room temperature) for one hour for colour development. After 30 minutes, the colour reaction was stopped by addition of 50 µl 5.0

M sulphuric acid and the colour in each well on the plate was measured by absorbency at dual wave-lengths of 490 and 630 nm in a Microplate Autoreader. Titration of the antisera was conducted in each vitellogenin coating level (0--30 ng vitellogenin/well). The effect of various concentrations of the second antibody (sheep anti-rabbit γ-globulin) on colour development in the wells was tested.

ELISA for the vitellogenin: ELISA procedures for vitellogenin were similar to that for the antisera titration except that 50 µl of vitellogenin standard solution (3.1-200 ng/ml) or of diluted samples was added into the vitellogenin coated wells. Titre of the vitellogenin antisera for the ELISA was 1:25600. The ELISA procedures are summarized as a protocol in Appendix VII).

A modified procedure of the ELISA: Since the regular procedure was too complicated, it was simplified by combining the steps of additions of the vitellogenin antisera and the sheep anti-rabbit γ-globulin (second antibody) into one step, i.e., both antisera were added simultaneously into the ELISA wells. The protocol for the modified procedures is given in Appendix VII.

Effect of plate type on ELISA: It has been suggested that the plate type affects the accuracy of the ELISA. Two types of titration plates, the Greiner and Nunc plates, were compared for ELISA following the above procedures..

3.4. RESULTS

Preparation of I^{125}vitellogenin: Purified vitellogenins of the ocean pout, Atlantic cod and Arctic charr were labelled successfully by the iodogen method adopted from So et al. (1985). The I^{125}vitellogenin was separated from free NaI^{125} by PD10 Sephadex chromatography in peak I or fraction #4 (Figs. 1A-3A). A small peak (PK II) appeared in fraction #9 (Fig.1A), which was perhaps NaI^{125} since it (peak II) disappeared with the reduction of the dose of NaI^{125} (i.e., less free I^{125} was left after reaction).

I^{125}vitellogenin from the PD10 Sephadex column was further purified using a small Sephacryl-S300 column yielding one or two peaks depending on the freshness of the preparation. A single radioactive peak was found from freshly labelled vitellogenin (Figs. 1-3B), while storage for 2-weeks (old I^{125}vitellogenin) yielded two peaks (Fig.1B), indicating that the labelled vitellogenin had started degrading during storage, since peak II did not react with the vitellogenin antisera.

Titration of vitellogenin antisera: Titration of ocean pout vitellogenin antisera was conducted using the isotope from peak I. It showed a maximum binding of approximately 88% at low antisera dilutions (10^3x) and the binding decreased with the increase of the antisera dilution (Fig.1C-3C). Repeated immunization increased the antiserum titre (determined by the dilution which produced 50% binding) from 1:1600 to 1:25600 after 2 and 3 immunizations in the ocean pout (Fig.1C). For Atlantic cod, the binding of cod-I^{125}vitellogenin to the cod-vitellogenin antisera was maximum (94.2%) at 1:800 antisera dilution and decreased with the increased antisera dilution. After the third

immunization, the antisera titre increased from 1:25600 to 1:51200 (Fig.2C). Titration of charr vitellogenin antisera showed a maximum binding of 89% at the antisera dilution of 1:1600. The antisera titre shifted from 1:6400 to 1:25600 after the third immunization (Fig.3C).

Radioimmunoassay: In the vitellogenin RIA for all three species, a combination of goat anti-rabbit γ-globulin (1:20 dilution) and normal rabbit serum (1:80) yielded lowest non-specific binding but highest antigen-antibody binding. In general, the vitellogenin RIA performed better in Polypropylene tubes compared to the borosilica tubes, i.e., having a lower non-specific binding and higher antigen-antibody binding thus having a higher assay sensitivity (data not shown). In the ocean pout vitellogenin RIA, the competitive displacement was well reproducible over a range of 1.3-675 ng/ml and the intraassay and interassay CVs were 1.64% (N=6) and 1.76% (N=3), respectively. Addition of plasmas from the adult female or E2 treated ocean pout also yielded a competitive displacement parallel to the standard curve. However, plasma of males did not react with the vitellogenin antisera (Fig.4A), indicating that vitellogenin was absent in the plasma of males. Addition of the plasma of male and female Atlantic cod, Arctic charr and lumpfish did not show any cross-reaction with the ocean pout vitellogenin antisera (Fig.4B), indicating that the ocean pout vitellogenin RIA is highly specific.

For Atlantic cod, a reproducible displacement curve was displayed within a range of 0.96-976 ng/ml vitellogenin. The dilution of plasma from adult females and 17ß-estradiol treated fish was parallel to the standard vitellogenin displacement curve, while plasma of adult males and control fish (not treated by 17ß-estradiol) did not react with

the vitellogenin antisera thus not producing any parallel displacement to the standard curve (Fig.5). This indicates a high specificity of the vitellogenin antisera to vitellogenin protein (for cod). Similar results were shown for the vitellogenin RIA of Arctic charr, and the range of sensitivity of the RIA was 10-512 ng/ml. The standard vitellogenin RIA curve was parallel to the dilution curves of plasma from premature and mature females but not to that from males (Fig.6).

The ocean pout vitellogenin ELISA: The extent of colour reaction (measured by absorbency) developed in each well on the ELISA plate represents the extent of antiserum reaction to the coated vitellogenin on the plate wall. This reaction depends on the amount of antiserum added into the wells and the amount of vitellogenin coated to the well. The colour increases with the increase in antisera titre as well as the amount of vitellogenin coated to the well (Fig.7). Hence, an appropriate level (titre) of antisera and vitellogenin coating level should be chosen. An antiserum dilution of 1:25600 at a vitellogenin coating level of 3.75 ng/well produced 50% optical absorption. This antiserum dilution (titre) and vitellogenin coating level were adopted for the ELISA. On the other hand, the colour reaction is also affected by the concentration of the second antibody (sheep anti-rabbit γ-globulin) in each well (Fig.8). Increasing the second antibody dilution decreased the colour development. The dilution level of 1:2000 of the second antiserum produced a higher optical absorption.

With the above assay conditions, e.g., the 1:25600 vitellogenin antibody (first antibody), 1:2000 sheep anti-rabbit γ-globulin (second antibody), and the vitellogenin coating level of either 3.75 or 7.4 ng/well, addition of purified vitellogenin reduced the

colour reaction in the wells (the added vitellogenin competed with the coated vitellogenin for antibody and reduced the amount of antibody bound to the coated vitellogenin). The colour reaction decreased with increasing vitellogenin level in the wells yielding a reproducible standard curve in a range of 3.1-200 ng/ml

ELISA run on Greiner and Nunc plates did not show much difference (Fig.9). Standard curves from normal and modified procedures were comparable at a vitellogenin coating level of 3.75 ng/tube, while at the coating level of 7.5 ng/well the standard curve of the modified procedure flattened off below 25 ng/ml (Fig.10), suggesting a reduction in sensitivity.

3.5. DISCUSSION

Both RIA and ELISA are sensitive, specific and accurate methods for the measurement of fish vitellogenin that have been developed for the measurement of fish plasma vitellogenin in previous studies (So et al., 1985; Norberg and Haux 1988; Tyler and Sumpter 1990; Maisse et al., 1991). In this study, RIA for the vitellogenins of the ocean pout, Atlantic cod and Arctic charr were developed following the methods of So et al. (1985). The RIA for the ocean pout vitellogenin is useful for sex determination (Chapter 4) and for the studies of seasonal vitellogenesis in the ocean pout (Chapter 5).

Preparation of labelled vitellogenin is a key step for RIA. To date, both the I^{131} and the I^{125} have been used to label fish vitellogenin (Norberg and Haux 1985; So et al ,

1985; Tyler and Sumpter 1990). In this study, I^{125} and iodogen were used to label the vitellogenin. After PD10 column separation, the I^{125}vitellogenin was usually further purified on a Sephacryl-S300 column before its use for the RIA. This step proved necessary especially when the preparation was old, since degradation of I^{125}vitellogenin occurred during storage. By removal of degraded isotope, the non-specific binding remained low for at least 2 weeks.

Although RIA are reliable, high non-specific binding values are often encountered in the vitellogenin RIA, which reduces the assay sensitivity. Reduction of non-specific binding and increase of total binding may be achieved by adjusting the amount of goat anti-rabbit γ-globulin and normal rabbit serum added to the tubes and by the use of polypropylene tubes instead of borosilicate tubes. Dilutions of the goat anti-rabbit γ-globulin and the normal rabbit serum at 1:20 and 1:80, respectively, minimized the non-specific binding of the isotope to the tube wall thus optimizing the RIA conditions.

With these vitellogenin RIAs, the presence of vitellogenin was clearly demonstrated in the plasma of females and 17ß-estradiol treated fish and not in the males of the three species, supporting the earlier conclusion that vitellogenin is a female specific, 17ß-estradiol inducible protein (Wallace and Selman 1985). Since vitellogenin is an egg yolk precursor protein incorporated into growing oocytes (Tyler and Sumpter 1991), it is actively synthesized and appears in relative high levels only in mature females during the reproductive season. In an immature female, the ovary is inactive (or at low activity levels) producing low levels of 17ß-estradiol in plasma and, consequently, there are low levels of vitellogenin synthesis in the liver. In contrast, the male fish does not

produce 17ß-estradiol thus not synthesizing vitellogenin in the liver. Cross reaction of vitellogenin antibodies to heterologous vitellogenins is generally very low, as was also confirmed by this study. Because of this high specificity of vitellogenin antibodies, the vitellogenin RIA has to be developed individually for each species.

Since RIA requires use of isotopes, which is often a limiting factor for its use, ELISA (which does not require radioactive isotope and is supposedly more convenient) was developed and tested. Although the ocean pout vitellogenin ELISA was valid for vitellogenin measurement and the standard curve was reproducible over a consistent range of vitellogenin concentration, it had a lower sensitivity (higher detecting limit, i.e. 3.1 ng/ml in ELISA vs 0.96-1.3 ng/ml in RIA) and a smaller detecting range (3.1-200 ng/ml in ELISA vs 0.96-675 ng/ml in RIA), and was therefore not employed in the subsequent studies for the measurement of plasma vitellogenin.

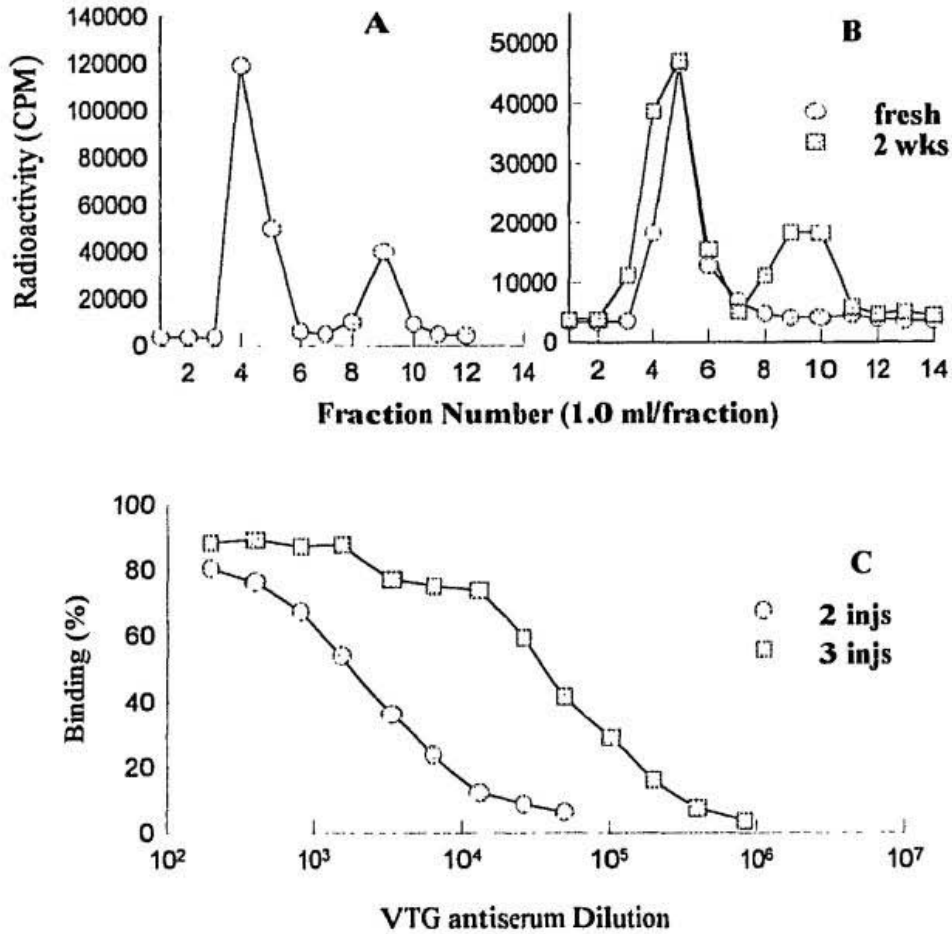

Figure 1. Preparation of isotope and titration of antisera for the ocean pout vitellogenin. A) PD10-Sephadex separation of I^{125}vitellogenin (peak I) from free I^{125} (peak II). B) Sephacryl purification of the I^{125}vitellogenin from peak I (of A) of the fresh (dotted circle) or stored preparation (dotted square, stored at 4°C for 2 weeks); C) Titration of the vitellogenin antisera sampled from rabbit after 2 (dotted circle) and 3 (dotted square) immunizations at monthly intervals. Y-axis in panel A & B shows the radioactivity (count per minute, CPM) of fractions resolved from PD10-Sephadex (A) or Sephacryl-S300 (B) and X-axis shows the fraction numbers from the column. In panel C, the Y-axis indicates the total binding of I^{125}vitellogenin to the antisera (in percentage of the total I^{125}vitellogenin added into the RIA tube) and X-axis indicates the dilution of vitellogenin antisera (log in scale).

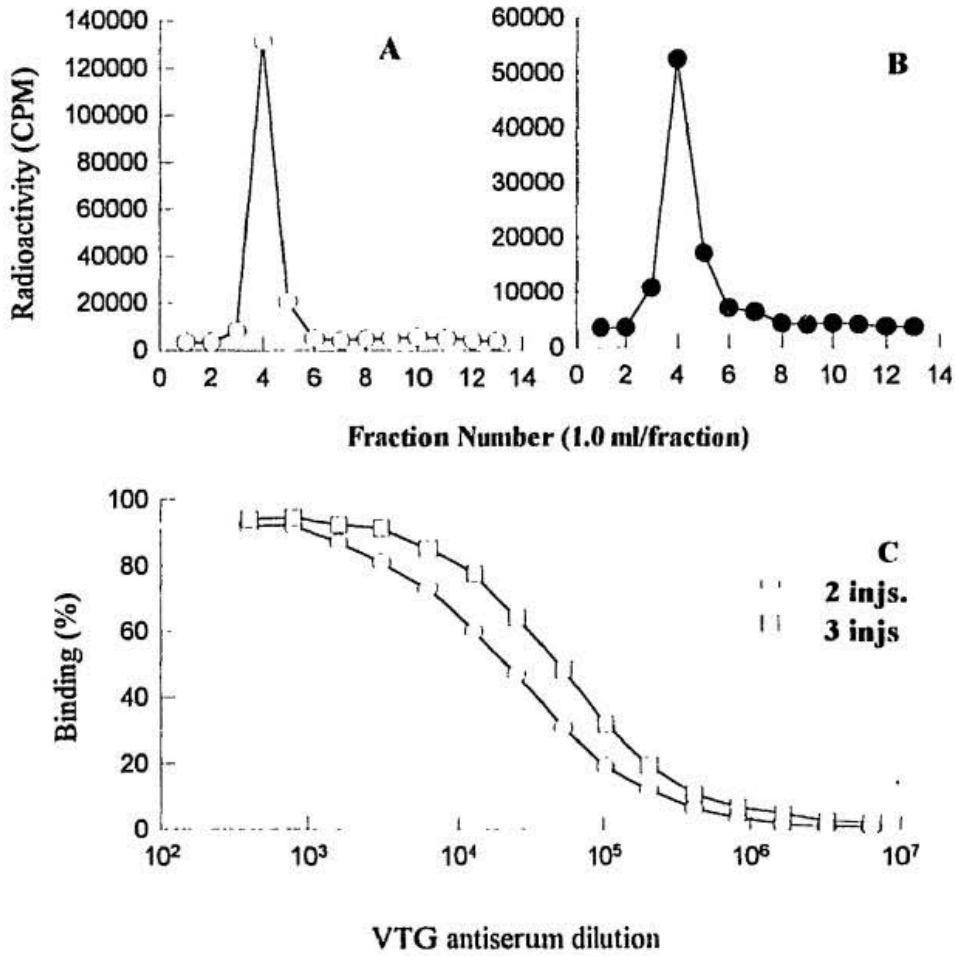

Figure 2. Preparation of isotope and titration of antisera for the Atlantic cod vitellogenin. A) PD10-Sephadex separation of I^{125}vitellogenin (in the peak) from free I^{125} (not appeared); B) Sephacryl-S300 purification of the I^{125}vitellogenin from fresh preparation; C) Titration of the vitellogenin antisera sampled from rabbit after 2 (open circle) and 3 (open square) immunizations at monthly intervals. (For explanation of the Y,X-axes, see Fig.1).

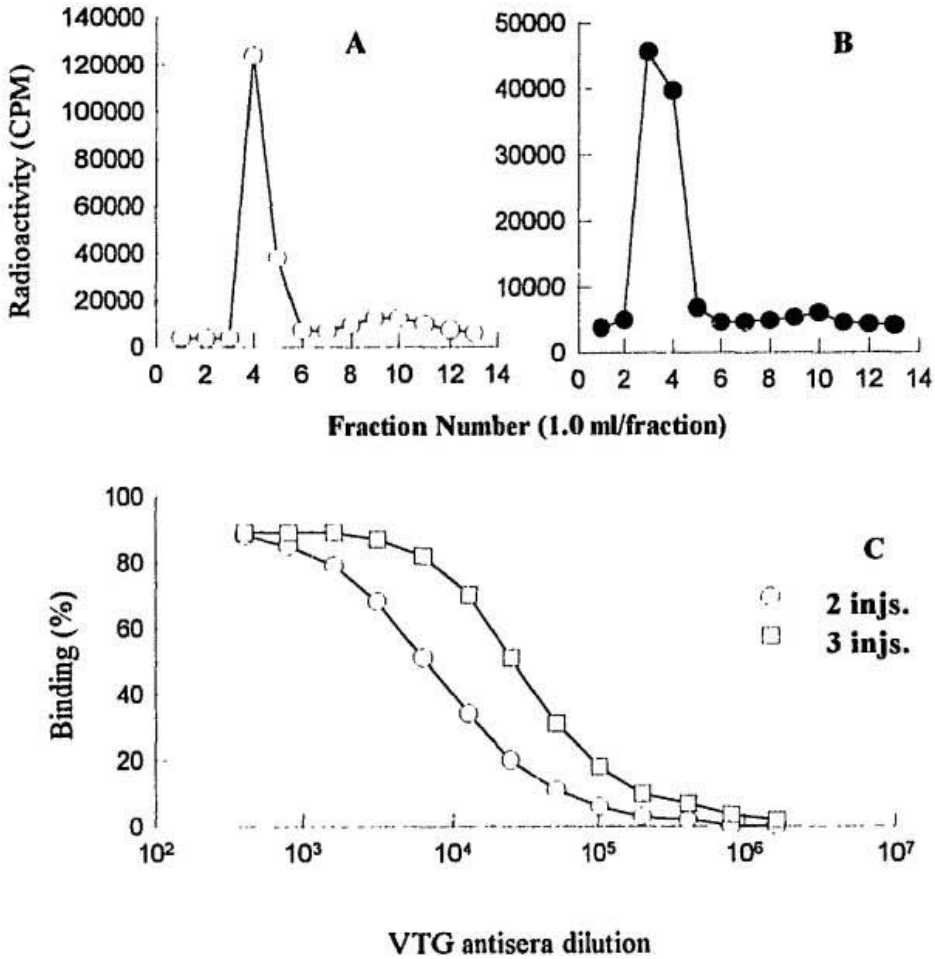

Figure 3. Preparation of isotope and titration of antisera for the Arctic charr vitellogenin. A) PD10-Sephadex separation of I^{125}vitellogenin (peak) from free I^{125} (not appeared). B) Sephacryl-S300 purification of the I^{125}vitellogenin from fresh preparation; C) Titration of the vitellogenin antisera sampled from rabbit after 2 (open circle) and 3 (open square) immunizations at monthly intervals (Y,X-axes, see Fig.1).

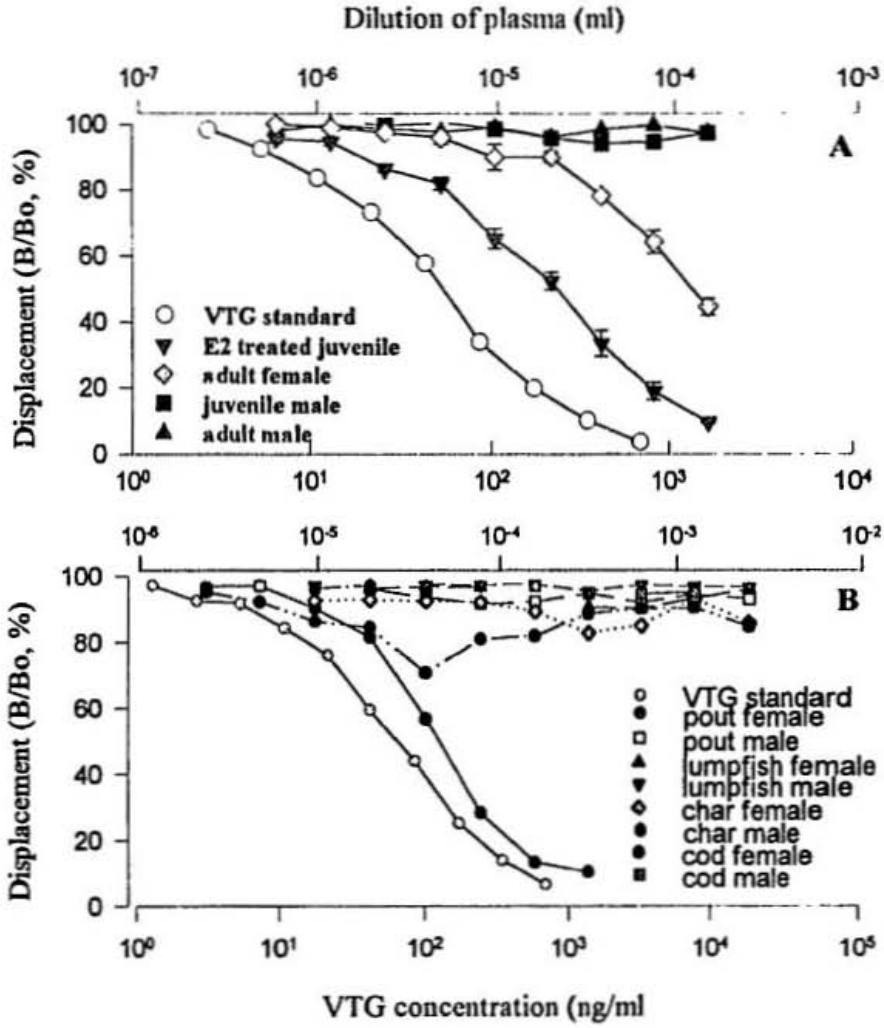

Figure 4. Results of the specificity test of the ocean pout vitellogenin RIA using (A) plasma of adult male and female, and 17ß-estradiol treated or non-treated ocean pout; (B) plasma of adult ocean pout, Atlantic cod, Arctic charr and lumpfish. Symboles in graph indicate the fish from which the plasma was sampled.. The Y-axes indicate the % of displacement (the antibody bound I^{125}vitellogenin (B) to total I^{125}vitellogenin (Bo) added to tube, or B/Bo), the upper X-axis indicates the dilutions of plasma; the lower X-axis indicates the concentration of vitellogenin (ng/ml), both are in log scale.

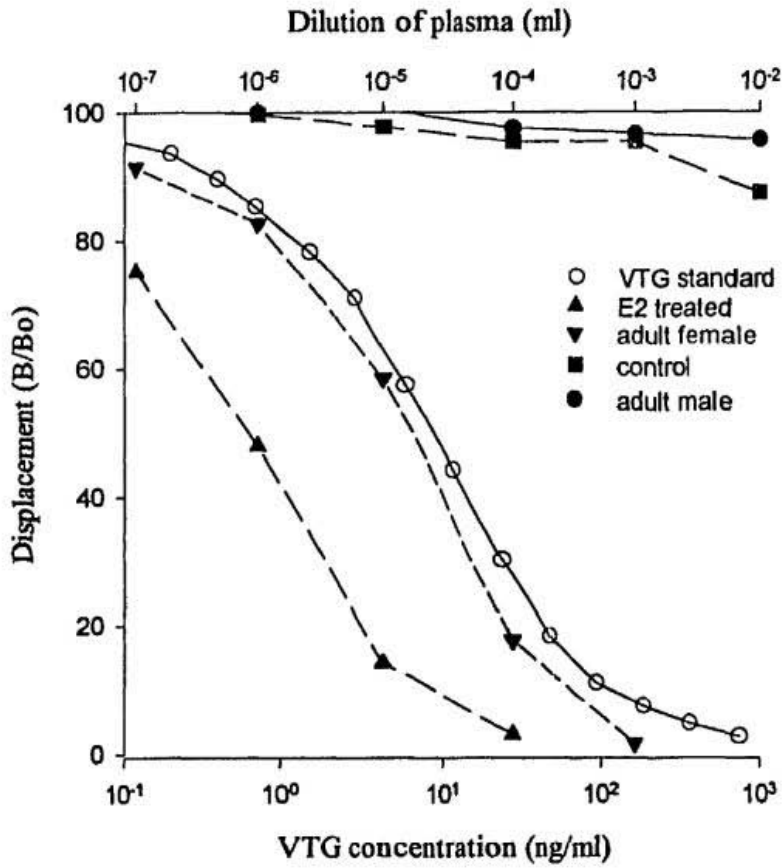

Figure 5. Results of the specificity test of the Atlantic cod vitellogenin RIA using plasma of adult males and females, 17ß-estradiol treated and non-treated (control) fish. Symboles in graph indicate the fish from which the plasma was sampled (For explanation of the Y and X-axes, see Fig.4).

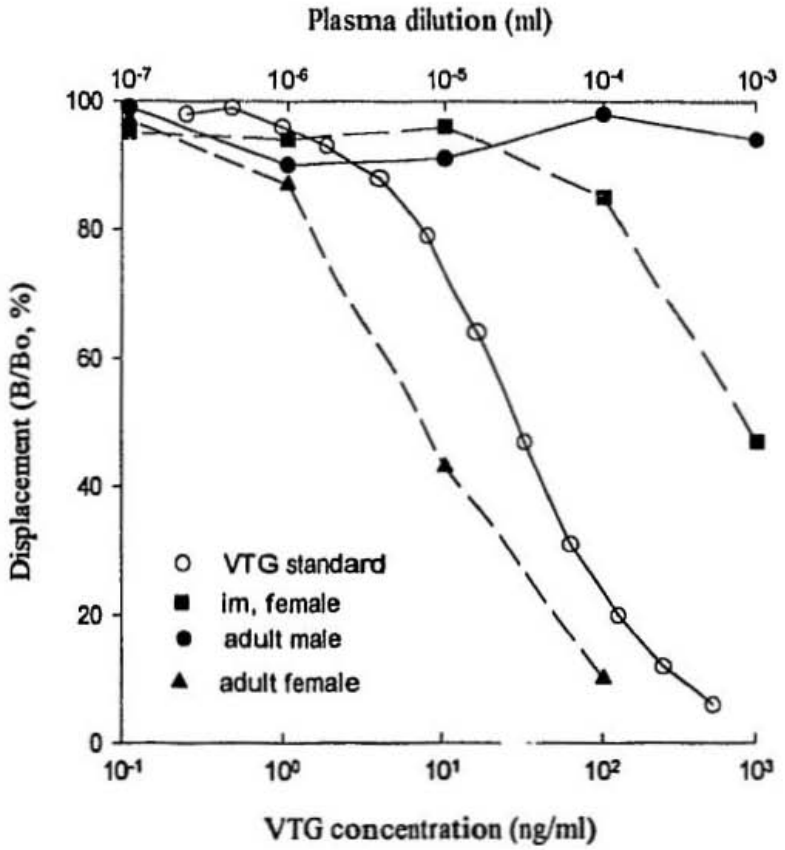

Figure 6. Results of specificity test of the Arctic charr vitellogenin RIA using plasma of an adult male, an immature and adult females. standard curve. Symboles in graph indicate the fish from which the plasma was sampled. (For explanation of the Y and X-axes, see Fig.4).

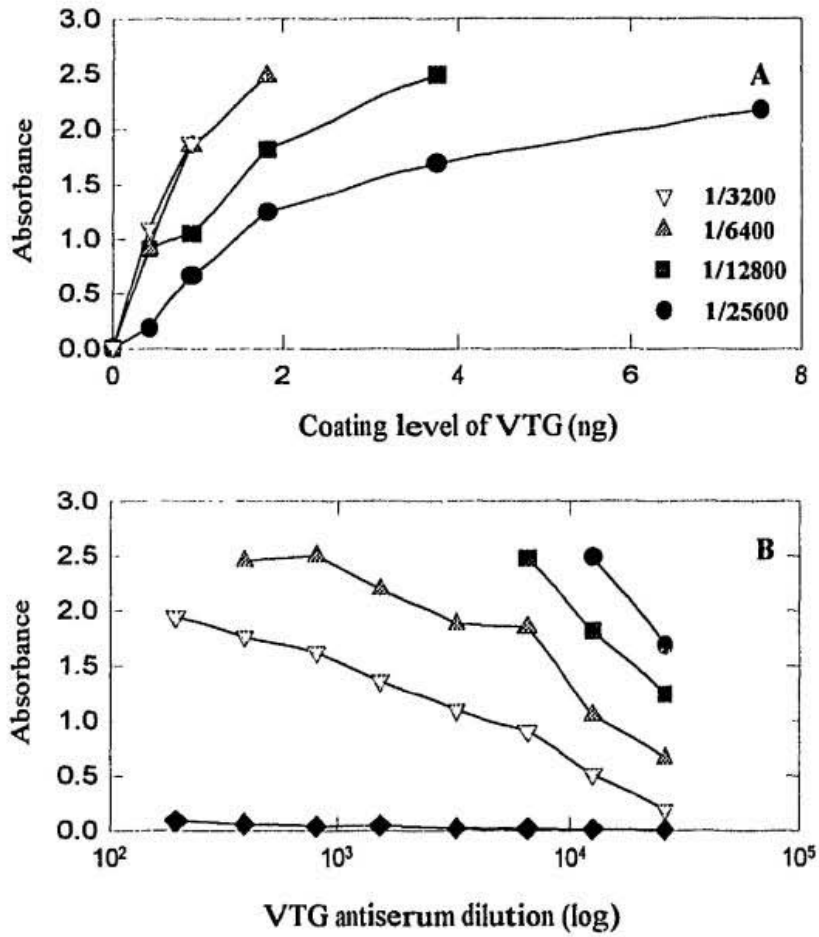

Figure 7. A) Determination of vitellogenin coating level for ELISA plate at various vitellogenin antisera dilutions from 1:3200 - 1:25600 (see symbles in graphe); B) titration of the vitellogenin antiserum at various vitellogenin coating levels (dotted ◇:0.0ng; dotted ▽:0.047; dotted △:0.94ng; dotted □:1.87ng; ●3.75 ng vitellogenin/well; 7.5 ng/well appeared out of the detecting range of absorbance). The Y-axes indicate the absorbance (optical density) of reactant in the wells at 490 & 630 nm, and the X-axis shows the vitellogenin coating level (ng/well) in panel A and vitellogenin antiserum dilution (in log scale) in panel B.

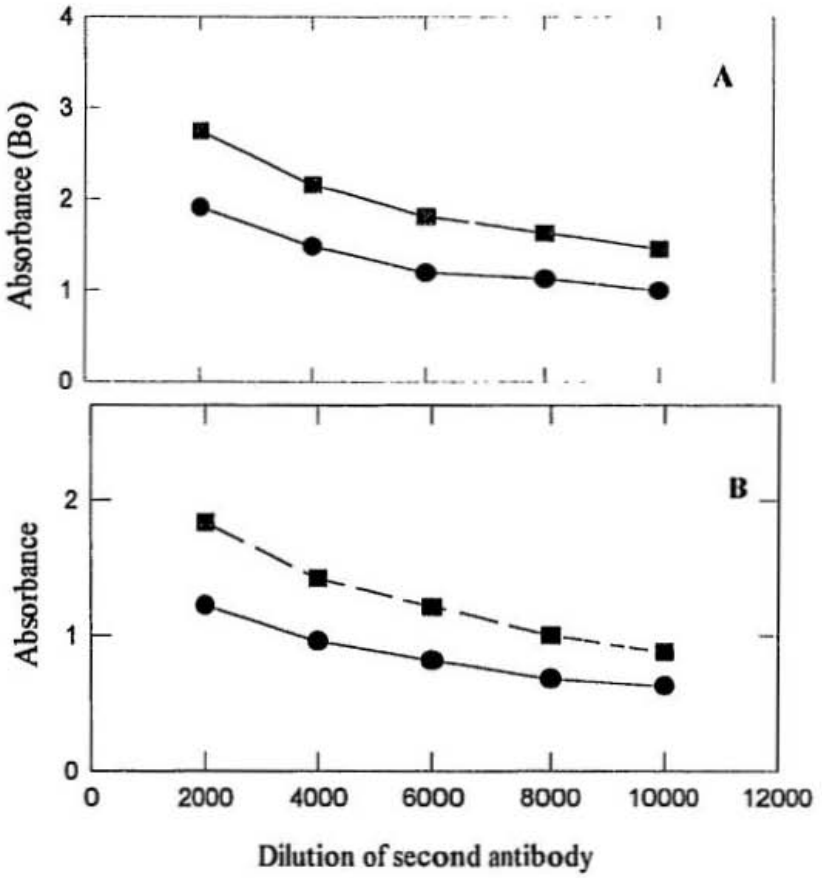

Figure 8. Titration of the second antiserum (sheep anti-rabbit γ-globulin) at two vitellogenin coating levels (●:3.75 ng/well; dotted □:7.5 ng/well) in Greiner (A) and Nunc (B) plates. Y-axis shows the absorbanceof reactant in the wells at 490 & 630 nm, and X-axis shows the dilution of the second antisera (sheep anti-rabbit γ-globulin) in log scale.

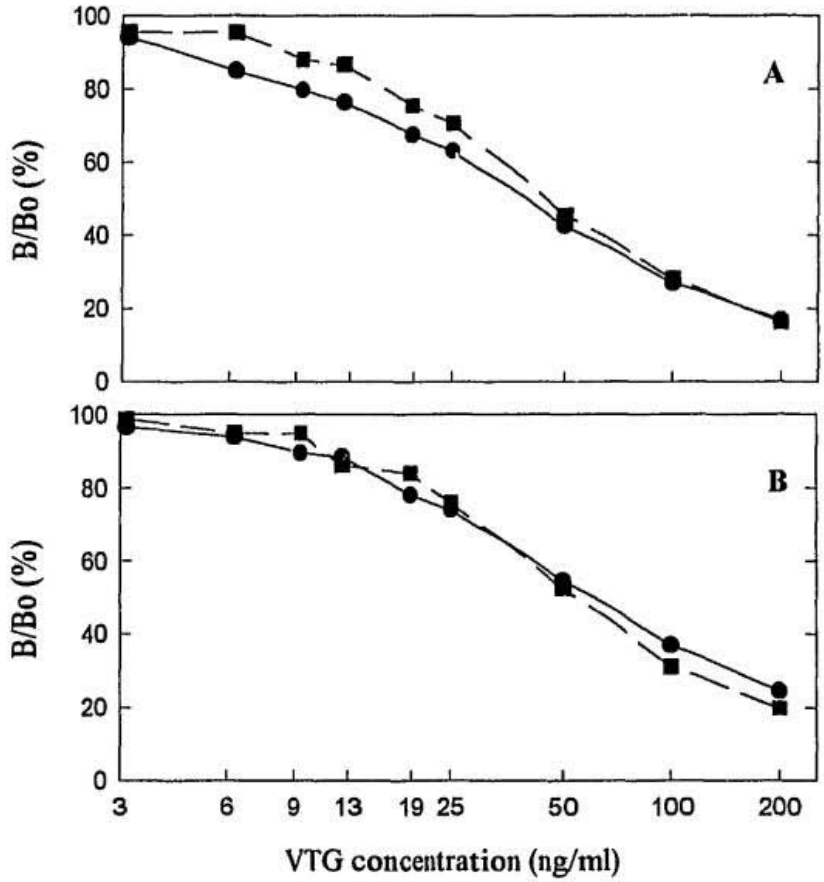

Figure 9. Comparison of the vitellogenin standard curves at a vitellogenin coating level of (A) 3.75 ng/well and (B) 7.5 ng/well) in (●) Greiner plate or in (■) Nunc plate. Y-axis shows the displacement (B/Bo, %), i.e., the absorbance of the reactant in percentage of total absorbance at 490 & 630 nm, and X-axis shows the concentrations of vitellogenin (in log scale).

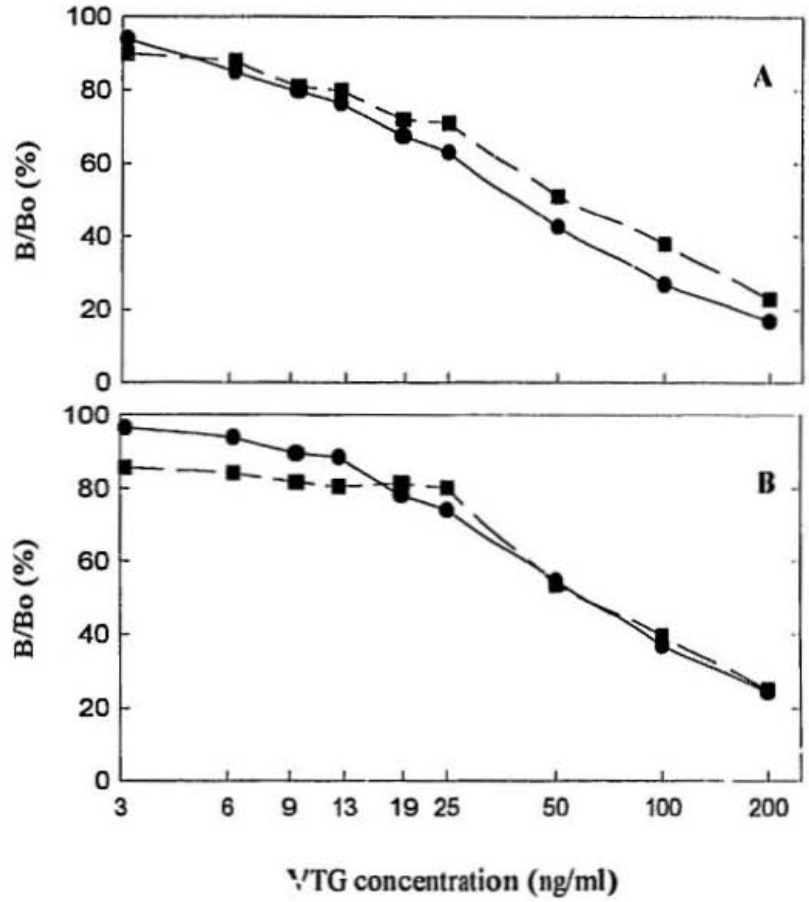

Figure 10. Comparison of the vitellogenin standard curves made from (●) normal or (□) modified procedures at a vitellogenin coating level of (A) 3.75 ng/well and (B) 7.5 ng/well). Y-axis shows the displacement (B/Bo, %) and X-axis shows the concentrations of vitellogenin (in log scale, see Fig.9).

CHAPTER 4

Sex determination from skin mucus in the ocean pout (*Macrozoarces americanus* L.)

4.1. ABSTRACT

The current study demonstrated that the sex of fish could be determined through analysis of skin mucus. Skin mucus can be obtained easily from the ocean pout (*Macrozoarces americanus* L.) causing minimal stress to fish. Vitellogenin or vitellogenin-like substance was detected by radioimmunoassay in the skin mucus of adult females but not in males or juveniles.

4.2. INTRODUCTION

Knowing the sex of fish is important for broodstock management. However in most fish species, sexual determination is often difficult due to the absence of clear sexual dimorphism. Direct examination of the gonads through autopsy is accurate but involves sacrificing the animals, which is often impractical. Sex steroids such as 17ß-estradiol and 11-ketotestosterone together with the female specific egg yolk precursor protein (or vitellogenin) in plasma have also been used as indicators for fish sexual determination (Craik and Harvey 1984). However, blood sampling requires well trained human resources and the fish are stressed chemically (by anaesthetics) and physically (by handling), which is deleterious to fish, particularly broodstock. Recent studies have shown the presence of vitellogenin in fish mucus (Gordon *et al.*, 1984; Kishida *et al.*, 1992; Kishida and Specker 1994). This suggests that the sex of fish can be determined by measuring the

female specific protein of vitellogenin in fish skin mucus.The current study was conducted in a marine fish, the ocean pout (*Macrozoarces americanus* L.), and attempted to develop an easy method for determination of fish sex.

4.3. MATERIALS AND METHODS

4.3.1 Fish

Adult and immature ocean pout were collected by SCUBA divers in Conception Bay, Newfoundland in the Autumn of 1991 and 1992 and acclimated to captive conditions in 2x2x0.5 m tanks. The fish were supplied with flow-through ambient sea water in a simulated natural photoperiod and fed chopped capelin or moist pellets.

4.3.2 Sampling and vitellogenin measurement

The study was conducted in August. Epidermal mucus of the fish was sampled from post-spawned females (1808 $\pm$ 92 g, N=5), spermiating males (3292 $\pm$ 105 N=3) and immature female fish (341 $\pm$ 45 g, N=3) by gently scraping the fish's dorsal skin with a microscope slide. The mucus was homogenized (1:1, w/v) in chilled Tris buffer (50 mM Tris, 0.5 M NaCl, 10 mM EDTA, 100 TIU/l aprotinin, pH 8, So et al., 1985) and centrifuged at 15,000 x g for 3 minutes. Supernatant was retained and diluted for the measurement of vitellogenin by RIA (Chapter 3).

4.4 RESULTS AND DISCUSSION

Radioimmunoassay of mucus from adult females yielded a dilution curve parallel to the vitellogenin standard curve (Fig.1A), indicating the presence of vitellogenin in the mucus. Levels of vitellogenin in the mucus was estimated to be approximately 9.28 ± 2.37 μg/g (Fig.1B). In contrast, mucus of male and immature ocean pout did not react with the vitellogenin antisera (no parallel dilution curve present) indicating that vitellogenin is not present in mucus of male and immature fish.

Vitellogenin is a yolk precursor protein synthesized in the liver and deposited into oocytes to form the principle nutritive reserve of eggs (Wallace and Selman 1985). This nutritive reserve constitutes the food supply for developing embryos and larvae before they are capable of feeding (Heming and Buddington 1988). The presence of vitellogenin or vitellogenin-like substance in plasma or in eggs has been reported in many studies (So et al., 1985), but the presence of vitellogenin in skin mucus was only reported in the adult female coho salmon (*Oncorhynchus kisutch*) (Gordon *et al.*, 1984), the female striped bass (*Morone saxatilis*) (Kishida and Specker 1992) and the tilapia (*Oreochromis mossambicus*) (Kishida *et al.*, 1994). It is not clear why vitellogenin should appear in the mucus although Kishida and Specker (1994) suggested that vitellogenin in the mucus of adult females might serve as food for young larvae since they had also detected the vitellogenin components in the gut extracts of young larvae of tilapia by both ELISA and western blot. Gordon *et al.* (1984) reported that the timing of the appearance of vitellogenin in skin mucus was correlated to certain degrees with the timing of ovarian

recrudescence in coho salmon. This suggests that the mucosal vitellogenin could be an indicator for seasonal ovarian development. Since vitellogenin is a female specific protein in fish, the presence of vitellogenin in mucus can be used for sex determination of fish. An added advantage of this method is that the skin mucus is more easily accessible compared with blood sampling and it does not cause stress to the animals.

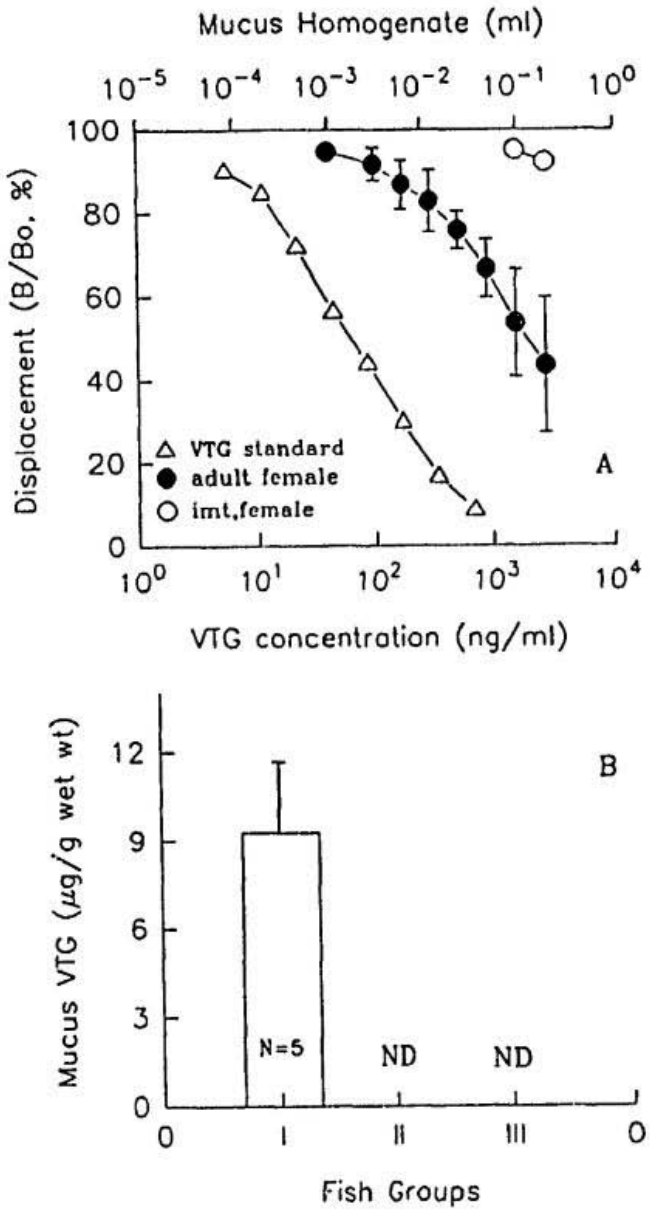

Figure 1. (A) Dilution curves of the homogenate of skin mucus made from adult females (●, N=5), immature females (○, N=3) and adult males of ocean pout (no cross-reaction occured, therefore no dilution curves were shown on the graph, N=3);
(B). Vitellogenin levels (μg/g) in the mucus of adult females (I, N=5), immature females (II, N=3) and adult males (III, N=3) of the ocean pout. ND:not detectable. bar indicates the standard error of mean, N=5.

CHAPTER 5

The seasonal Reproductive Cycle and Regulation of the Reproduction of captive ocean pout (*Macrozoarces americanus* L.)

5.1. ABSTRACT

The seasonal reproductive cycle, including gonadal development and the plasma sex steroids levels, of the ocean pout *(Macrozoarces americanus* L.) in both ambient and heated seawater under various photoperiod conditions was investigated. The effects of gonadotropic hormone-releasing hormone analog (GnRH-A) on the induction of spawning of the female ocean pout were studied. In ambient seawater, the seasonal reproductive cycle of female ocean pout could be organized into four phases according to the ovarian development and the plasma profiles of sex steroids testosterone, 17ß-estradiol and egg yolk protein precursor or vitellogenin. These phases were: (1) a quiescent phase (from post spawning in August to February); (2) the preparatory phase (March-May); (3) the rapid ovarian growth phase (June-August) and (4) ovulation and spawning (August-September). Two classes of oocytes (previtellogenic and vitellogenic oocytes) were present in the ovary throughout the year, but only the vitellogenic oocytes developed annually to maturation and ovulation. The female ocean pout was an annual single batch spawner. In males, the seasonal reproductive cycle was composed of 2 phases, a quiescent phase (October-May) and an active growth phase (June-September). Spermiation started coincident with a rapid testicular development following a dramatic increase of plasma testosterone and 11-ketotestosterone. Raising and maintaining the winter water temperatures at 5°C increased fish feeding activity by two fold compared with fish held in cold ambient seawater (-0.5 to 1°C). However, the growth rate, the spawning season and the plasma steroid and vitellogenin profiles were similar with the two temperature

treatments. Photoperiod cycle affects the seasonal reproductive cycle of the ocean pout. Increasing day length in the winter stimulated steroidogenesis and vitellogenesis and advanced the timing of ovarian recrudescence and spawning. In contrast, short day lengths in the winter and spring inhibited steroidogenesis and postponed ovarian recrudescence and spawning for 2-5 weeks compared with those held in a long photoperiod in the winter. The captive ocean pout spawned sporadically during the spawning season from the beginning of August to mid-September. A single injection of 30 μg/kg BW of GnRH-A (D-Ala^{6},Pro^{9}-ethylamide GnRH) in the spawning season induced the oocyte maturation and hydration and synchronized spawning. Starting from days 7 following the GnRH-A treatment, 86% of the hormone treated fish spawned within 3 days in contrast to just 22% spawning in control females. The GnRH-A treatment also accelerated the prespawning decline of plasma testosterone and 17ß-estradiol. 17α,20ß-dihydroxy-progesterone, a common oocyte maturation inducing hormone in teleosts, was never detected in the plasma of the spawning ocean pout.

5.2. INTRODUCTION

Generally speaking, reproduction of vertebrate animals is controlled by an integrated action of various hormones including GnRH, pituitary GtH and gonadal steroid hormones such as testosterone, 17ß-estradiol, 11-ketotestosterone, and 17α,20ß-dihydroxy-progesterone (17α,20ß-P), etc., which form a hypothalamo-hypophysial-gonadal axis (HHG) (Crim and Idler 1978). In fish, activities of the HHG are affected by environmental factors such as water temperature and photoperiod and are actually entrained by these environmental factors leading to a cyclic reproduction in many fish species. With a basic understanding of the seasonal reproductive cycle in relation to the hormonal controls and environmental changes, it may be possible to develop methods for the control of fish reproduction.

Fish are poikilothermic animals. Alteration of water temperature changes enzyme activity, metabolic rate and hormone production (Bols et al., 1992). It also affects GtH secretion and the response of endocrine organs to hormone stimulation (Crim 1982) thus affecting spawning (Davies and Hanyu 1986). Photoperiod is another major environmental factor regulating the fish reproductive cycle. In rainbow trout *Oncorhynchus mykiss* compressing the natural photoperiod cycle from 12-months into a 6- or 9-month cycle in ambient water temperature advances the spawning of fish by 12 and 6 weeks, respectively (MacQuarrie et al., 1979; Bromage et al., 1982).

Reproduction of the ocean pout is distinctly seasonal. Adult fish migrate inshore to shallow waters in the spring and spawn in late summer (Keats et al., 1985). However,

to date, the cycle of gonadal recrudescence and development, and how hormonal and environmental factors regulate seasonal reproduction, all remain unknown in the ocean pout. The objectives of this study were to investigate 1) the seasonal cycle of gonadal development and plasma sex steroids levels under ambient conditions, 2) the effect of water temperature on seasonal reproduction and growth, 3) the effect of photoperiod on seasonal reproduction and 4) the hormonal induction of spawning by GnRH-A in captive ocean pout.

5.3. MATERIALS AND METHODS

5.3.1. Fish

Adult ocean pout were collected from Conception Bay, Newfoundland, by SCUBA divers in the Autumn of 1990, 1991 and 1992. The fish were housed in 2x2x0.5 m tanks with flow-through ambient seawater. Fish were fed with chopped capelin to satiation 2-3 times/week. The following four experiments were conducted from 1990-1993.

5.3.2. Experiment I: Seasonal reproductive cycle at ambient temperatures

The fish were housed in a 2x2x0.5 m tank supplied with ambient seawater in the period from November 1990 to December 1991. Fish (N=3~8 males or females) were killed at 2-3 month intervals for examination of gonadal development. Prior to autopsy, blood was taken from these fish via caudal vein into heparinized and chilled syringes and

kept on ice until centrifugation at 15,000 g for 3 minutes. Plasma was carefully collected after centrifugation and stored at -20°C until assay for the steroid hormones including testosterone, 17ß-estradiol and 11-ketotestosterone. After autopsy, the gonad and liver were weighed and gonad development expressed as gonado-somatic index (GSI % = [gonad weight x 100]/body weight). Ovaries were fixed in Gilson's solution (Appendix VIII) and size frequency distribution of oocytes in the ovary was examined under a binocular microscope fitted with a micrometer. The hepato-somatic index (HSI % = [liver weight x 100]/body weight) of the fish was also calculated.

5.3.3. Experiment II: The effect of water temperature on the seasonal growth and reproductive cycle.

In November 1990, 16 adult fish of similar body weight (male 2573 ± 341 g; female 1598 ± 265 g) were randomly divided into two groups consisting of 4 males and 4 females and placed into two separate 1x1x0.5 m tanks under simulated natural photoperiod cycle. Three PVC pipes (30x25 cm) were placed into each tank to simulate the natural habitat in the wild (Keats et al., 1985). From November 1990 to December 1991, one group of fish was supplied and maintained in ambient seawater (flow rate 14-15 l/min) while the second group was supplied with heated seawater (ca.5°C) from December to May. The fish were fed chopped capelin and food consumption was recorded.

Once a month, the fish were anaesthetized in 2-phenoxyethanol (100 ppm, Sigma Chemical Co.) and their body weight (BW) and length were measured (to 1.0 g and 0.5 cm) for calculation of growth, which is expressed as relative BW increment (Growth %

= ([BWt - BWo]/BWo) x 100, where BWt is the BW at time t while BWo is the BW at the beginning (t0) of the experiment). Before returning the fish to their tanks, a 1.0 ml blood sample was withdrawn from each fish, the plasma was retained after centrifugation (15,000 x g, 3 inutes) and stored at -20°C until the measurement of plasma sex steroid hormone and vitellogenin levels. Spawning time of the fish was monitored.

5.3.4. Experiment III: The Effect of Photoperiod on Seasonal Reproductive Cycle

In November 1992, 50 fish of similar BW were randomly divided into five groups of 10 fish and kept in five separate 1x1x0.5 m tanks supplied with aerated ambient seawater (15 l/min). Three PVC pipes were placed in each tank. The tanks were covered by black plastic boards and five photoperiod treatments, controlled by electronic timers, were established using a 40W light, 50 cm above the water. The natural photoperiod (day length) at the time when the experiment started was approximately 8 hr light and 16 hr dark (8L:16D).

Tank A: These fish were exposed to a 7 hr light-on and 17 hr light-off (7L:17D) cycle in November, shifted to 8L:16D in December, shifted to 9L:15D in January, to 10L:14D in February and March, and to 13L:11D in April until spawning.

Tank B: These fish were exposed to a long photoperiod (18L:6D) from November to spawning in August.

Tank C: These fish were exposed to a short photoperiod (6L:18D) from November to January and shifted to a long photoperiod (18L:6D) from 1 February to

spawning.

Tank D: These fish were exposed to a short photoperiod (6L:18D) from November to March and shifted to a long photoperiod (18L:6D) from 1 April to spawning.

Tank E: These fish were exposed to a short photoperiod (6L:18D) from November to August (a constant short photoperiod).

The lights-on phase in all tanks started daily at 08:00 hr. Feeding was carried out during the lights-on period and food consumption was recorded. Body weights of fish were measured biweekly for determination of growth. Blood sample (ca. 1.0 ml) was taken from the females monthly for the measurement of sex steroids and vitellogenin levels.

5.3.5. Experiment IV: Hormonal induction of spawning with GnRH-A

At the beginning of spawning season in August 1992, two groups of mature females, the control (N=9) and the hormone treated (N=7) groups, were held with adult males in ambient seawater tanks. Fish in the control group (N=9) received an intraperitoneal (ip) injection of saline (113 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM Na_2HPO_4 , 1 mM $NaHCO_3$, 1 mM sodium pyruvate, 5 mM glucose, 5 mM Hepes, pH 7.5). Fish in the hormone treatment group (N=7) received an ip injection of GnRH-A (D-Ala^6,Pro^9-ethylamide GnRH) (30 µg/kg BW) dissolved in the saline solution. Body weight changes and spawning of fish were monitored daily following the

treatments. Blood samples (1.0 ml) were collected from each fish beginning with day one (prior to hormone treatment) to day 12 at 1-4 day intervals for the measurement of plasma sex steroid hormone levels.

5.3.6. Measurement of plasma sex steroid hormones

Plasma levels of the sex steroids testosterone, 17ß-estradiol, 11-ketotestosterone and 17α,20ß-P were measured by radioimmunoassay (RIA) from dimethylether extract of the plasma following the procedures described previously by Harmin and Crim (1993). In brief, 0.1 ml plasma and 10 µl ethanol containing 1,000 CPM of tritiated testosterone, 17ß-estradiol or 11-ketotestosterone, 17α,20ß-P (depending on the measurement), were added into borosilicate disposable culture tubes and incubated 1-2 hr at 20°C (room temperature). The mixtures were extracted twice with 2 ml dimethylether and aqueous phases were discarded after freezing over solid CO_2 . The two ether extracts were retained, pooled and evaporated under nitrogen with gentle heating (40°C). The resultant plasma residues were redissolved in 1.0 ml absolute ethanol and allowed to equilibrate overnight at 4°C before performing the RIA. 100 µl of the ethanol equilibrated plasma was pipetted into scintillation vials containing 10 ml scintillation cocktail and subjected for the determination of the recovery (%) of the tritiated steroids added to the plasma in a beta counter (Minaxi ß, Tris Carb 4000 series). This recovery (%) represents the extraction efficiency of the sex steroid from the plasma.

For RIA of testosterone and 17ß-estradiol, a portion (0.1 ml) of the ethanol reconstituted extract of plasma or standard steroids in ethanol (1-1,000 pg/tube) were

pipetted into duplicate borosilicate disposable culture tubes (12x75 mm) and evaporated under nitrogen. Testosterone was determined by bringing the assay tubes to 0.4 ml with assay buffer (50 mM Na_2HPO_4 , 100 mM NaCl, 0.1% NaN_3 , 0.1% gelatin, pH 7.4) containing diluted testosterone antiserum and 10,000 CPM of iodinated testosterone. After incubation for 1 hr at 37°C, 1.0 ml separating reagent was added into the tubes and the tubes were centrifuged 25 minutes later at 1,000 x g for 15 minutes to separate the antibody bound steroids from free steroids. Following centrifugation, the supernatant (containing free steroid) was discarded by careful aspiration and radioactivity in the precipitates was determined in a Packard Auto-Gamma Counter. A standard displacement curve was built from the standard solution, from which steroid levels in the tubes were calculated. Plasma 17ß-estradiol in the ether extracts was assayed following the same procedures using 17ß-estradiol antisera and iodinated 17ß-estradiol (see protocol in Appendix II).

For 11-ketotestosterone and 17α,20ß-P measurement, 0.1 ml ether extract of plasma was pipetted into duplicate tubes and dried by nitrogen. The tubes were brought to 0.3 ml with PBS buffer (30 mM $NaH_2PO_4 \cdot H_2O$, 60 mM Na_2HPO_4 , 150 mM NaCl, 0.1% gelatin, pH 7.0) containing 11-ketotestosterone antiserum (1:70,000 dilution) and 10,000 cpm of tritiated 11-ketotestosterone (for 11-ketotestosterone assay) or 17α,20ß-P antiserum and tritiated 17α,20ß-P (for 17α,20ß-P assay). Following an overnight incubation at 4°C, 0.6 ml dextran-charcoal suspension (1.25 g charcoal, 0.25 g dextran T-70 in 500 ml PBS buffer) was added and the incubation was continued for another 1 hr at 4°C. The tubes were centrifuged at 1,000 x g for 15 min and supernatant was

decanted into vials containing 10 ml scintillation cocktail and subjected for determination of bound isotope in a β-counter. All assay values were corrected with recovery rate of extraction (Harmin and Crim 1993) (a protocol is given in Appendix IX).

5.3.7. Measurement of plasma vitellogenin

Vitellogenin levels in the plasma were determined by RIA (details,see Chapter 3).

5.3.8. Statistical analysis

Data are expressed as mean ± standard error (SEM). Significance ($P<0.05$) of the treatments (e.g., water temperature, photoperiod and GnRH-A) on seasonal growth, plasma sex steroid hormone and vitellogenin levels was tested using one-way ANOVA followed by the measurement of protected least significant difference (protected LSD or PSD) (Snedecor and Cochran 1980). All statistic procedures were run using SAS software in VAX/VMS Version V5.5-1.

5.4. RESULTS

Experiment I investigated the sequence of seasonal development of the gonads in the ocean pout to spawning. The female ocean pout had a single ovary with a very short oviduct, which appeared round or oval in shape and located posteriorly in body cavity. Premature or post-spawned females had a small ovary enveloped by a thick wall. GSI in

the post-spawned fish in the fall (October-November) was low (1.21 ± 0.25, N=9). It increased by April (4.61 ± 2 34, N=6) and reached its peak prior to spawning in August (16.76 ± 6.54, N=8) before returning again to basal values (1.96 ± 1.0, N=4) after spawning (Fig. 1A). Initiation of the ovarian development by spring (April) was confirmed by the analysis of oocyte size-class frequency distribution (Fig.2). Whereas only small previtellogenic oocytes (ca. 1 mm in diameter) were present in the ovaries in November, a class of developing (vitellogenic) oocytes (4-5 mm in diameter) was clearly demonstrated by April. These vitellogenic oocytes grew and increased in size towards the centre of ovary, while the previtellogenic oocytes remained at the periphery of ovary, lining along the inner wall of the ovary. Continuation of the vitellogenic development of oocytes was evident by the growth of oocytes to approximately 8 mm in diameter prior to spawning in August. By the time of spawning, the female ovaries were big (also indicated by high GSI), the ovarian wall was highly stretched and became very thin. The matured or ovulated eggs remained at the centre of the ovaries in association with thick gelatinous slime. Seasonal changes also occurred in HSI which peaked in May (Fig.1B) in conjunction with the onset of ovarian development. Overlapping with the increases in GSI and HSI, plasma levels of testosterone and 17ß-estradiol began to rise in parallel in April and peaked in July before returning to low levels in August after spawning (Fig.1C).

The males had an unevenly paired lobules of testis (one is bigger than the other), which were small and hard in immature males (or in non-reproductive season) but were big and soft (hydrated) in mature males in August and milt containing motile spermatozoa flew out of the testis when the lobules were dissected. However, compared with females,

the GSI of males were remarkably low and the range of seasonal changes in GSI was clearly more limited (ranges between 0.3-2.0%), yet still evident. Although the GSI started rising between May and July, it peaked in August-September coincident with the occurrence of spermiation (Fig.3A). Unlike the HSI changes observed in females, significant seasonal HSI variation was not noted in males (range from 2.77-4.58%). Plasma testosterone and 11-ketotestosterone levels in males remained relatively stable throughout the year except in June when both testosterone and 11-ketotestosterone increased significantly (Fig.3B).

Experiment II examined the influence of water temperature on feeding, growth and reproduction. Water temperature for both groups of fish started at 5°C in November. During the winter, fish in the heated water group experienced a stable water temperature around 5-6°C. In contrast, fish held in ambient water were exposed to declining temperatures to below zero from January to May. By June, fish in the heated water group were also transferred to ambient seawater, for which the temperatures gradually rose from 4-5°C in June to 10-11°C in August (Fig. 4A). Health of the fish under both temperature regimes was good as just one mortality occurred in each group.

Feeding activity varied considerably between the two temperature treatments (Fig.4B). Feeding activity was relatively high in December and remained high in the heated water group although it tended to decline with the approach of spawning. In contrast, feeding activity of fish exposed to ambient seawater declined rapidly in the winter although feeding never completely stopped even at cold temperatures below -0.5°C. Overall, the total food consumption for fish reared at 5°C in the winter was approximately

two-fold greater than fish exposed to cold ambient seawater.

Despite markedly different food consumptions, growth remained comparable for the two groups of fish ($P>0.05$) with the males and females gaining approximately 30% and 50% of their initial BW, respectively, by the time of spawning in August (Fig.5). Body length of the fish did not increase much during the experimental period (data not shown). All surviving females (N=6) in the two groups spawned spontaneously although the spawning occurred sporadically from mid-August to mid-September.

Plasma steroid and vitellogenin level were measured monthly and since no significant difference ($P>0.05$) was found between the two groups, the data were combined (Fig.6). A similar seasonal profile to that in Experiment I (Fig.1C) was found, i.e., the levels of 17ß-estradiol and testosterone were low in December but increased in January, peaked in June-July before spawning (Fig.6A). In conjunction with the increase of 17ß-estradiol, significant elevations in plasma vitellogenin level were observed by February and the high levels were maintained until August before returning to basal levels after spawning (Fig.6B). In males, significant elevations of plasma testosterone and 11-ketotestosterone occurred by June before the onset of spermiation from July to September (Fig.7A,B). The maturation inducing hormone 17α,20ß-P was not detected in either females or males.

Experiment III examined the effect of photoperiod on the seasonal reproductive cycle of the ocean pout in ambient seawater. The seasonal profile of ambient seawater temperature in 1992-1993 was similar to that of 1990-1991 (Expt.II). However, the ambient seawater was colder in the winter of 1993 when the water temperature dropped

to nearly -1.6°C from January to April before returning to >0°C by May (Fig.8A). Feeding activity, which decreased and increased seasonally matching the seasonal changes in water temperature with minimal food intake in March, was not obviously affected by photoperiod (Fig.8B). However, growth rate was affected by photoperiod, and fish in long photoperiod had a higher growth rate than fish in short photoperiod. For example, the fish in tank B, where the photoperiod was advanced in November, had significant ($P<0.05$) higher growth rates in April, May and June than fish from other tanks, while fish in tank E (constant short photoperiod) had generally lower monthly growth rate throughout the experimental period. Accordingly, long photoperiod advanced the spawning season of fish. The first spawning in tank B occurred on 2 August, about 1 week before that of tank A or 2-5 weeks before tank C, D & E, in which the fish began to spawn on 9 August, 17 August, 12 September and 14 August, respectively. Spawning season of the fish in tank D, where the increase of photoperiod was delayed to April, was delayed for 5 weeks. Difference in the size of eggs spawned by fish from different tanks was not noted (data not shown). However, health condition of fish was poorer in short photoperiod than in long photoperiod and more fish developed a head skin ulcer, particularly in tank E.

Plasma profiles of 17ß-estradiol and vitellogenin also changed in response to the increase of photoperiod (Fig.10). Plasma levels of 17ß-estradiol were low in fish exposed to short photoperiod but were significantly increased following the increases of photoperiod in tank A, B, C and D fish. This increase in plasma 17ß-estradiol and vitellogenin was greater in warmer water, e.g., in tank B where the increase of photoperiod occurred in November when the ambient seawater was still relatively warm

(ca. 5°C), than in cold temperature (tank C and D). Nevertheless, the elevation of plasma 17ß-estradiol and vitellogenin was not inhibited by cold ambient water temperature in the winter. The magnitude of increase of 17ß-estradiol and vitellogenin correlated with the magnitude of increase in photoperiod since the increase of 17ß-estradiol and vitellogenin in tank B fish (photoperiod shifted from 6 hr light to 18 hr light) was apparently more pronounced than that in tank A fish (day length increased 1 hr/month).

The females spawned sporadically in the spawning season from August to September (Expts II and III). Experiment IV was conducted to determine whether this sporadic spawning could be synchronized by hormone treatment with GnRH-A. Following GnRH-A treatment, body weights increased steadily and peaked by day 4 before dropping to a lower level by day 8-10 after spawning (Fig.11A). After initiation of spawning which started 7 days after hormone treatment, 86% of the hormone treated fish (6 of 7) spawned within 3 days (Fig.11B). By contrast, little change was noted in the body weight of control fish and just 22% of (2 of 9) of control fish spawned in the same period (Fig.11A,B). Clearly, the GnRH-A treatment synchronized the spawning of the ocean pout. Plasma levels of testosterone and 17ß-estradiol in spawning fish, either treated or not treated by GnRH-A, dropped rapidly prior to and during spawning, but declined slowly in non-spawned fish (Fig.12).

5.5. DISCUSSION

The present study described the seasonal reproductive cycle of the ocean pout under various water temperature and photoperiod conditions and the regulation of spawning with GnRH-A. The seasonal changes in GSI and oocyte growth of the ocean pout reported in this study (Expt.I) agree with the previous observations of Olsen and Merriman (1946) and suggest that the annual cycle of ovarian growth in female ocean pout can be organized into four phases of reproduction: 1) a quiescent phase following spawning from September to January, in which the inactive ovaries are dominated by previtellogenic oocytes coincident with low levels of sex steroids in plasma; 2) a preparatory phase (March-May) in which the slowly growing ovaries consist of both previtellogenic and small vitellogenic oocytes and plasma levels of the sex steroids and vitellogenin begin to rise; 3) a rapid ovarian growth phase (June-August) where both GSI and size of the vitellogenic oocytes increase dramatically matching the rapid rise of sex steroids levels in plasma and 4) ovulation and spawning (August-September) when fish spawn and the plasma sex steroids decline to basal levels.

Although two classes of oocytes are present in the ovary, only the vitellogenic class of oocytes is active and grow to the spawning size (8-9 mm in diameter) while previtellogenic oocytes remain small and inactive throughout the current year. This observation matches the previous field observations of spawning and the conclusion that the ocean pout spawn just once per year producing a single batch of mature eggs (Keats et al., 1985).

As was reported previously for other species (Crim and Idler 1978), an integrated activity of the liver and gonads was also noted in the ocean pout, indicated by the increase of GSI matching the increases of HSI, and the plasma 17ß-estradiol and testosterone (a precursor of 17ß-estradiol). In fish, vitellogenin (egg yolk precursor protein) is synthesized in the liver in response to the stimulation of 17ß-estradiol from follicular granulosa cells (Chapter 2). Synthesis of vitellogenin always occurs in conjunction with a hypertrophy of the liver (Haux and Norberg 1985; Olin et al., 1989; Lim et al., 1991) leading to a parallel rise of HSI to the increase of 17ß-estradiol and vitellogenin in the plasma.

Although synthesis of vitellogenin in the ocean pout started in winter, ovarian growth remained slow suggesting that uptake of vitellogenin into oocytes is not active in the winter and neither testosterone nor 17ß-estradiol stimulate the vitellogenin incorporation into the oocytes. In fish, uptake of vitellogenin into oocytes usually requires stimulation by gonadotropic hormone (GtH) from the pituitary (Sundararaj et al., 1982; Tyler et al., 1991; Nagahama et al., 1993). Currently there is no method for measurement of GtH of ocean pout (requiring a sensitive and specific assay), and hence the factors regulating vitellogenin uptake in the ocean pout remain unknown.

Oocyte maturation and ovulation requires GtHII and oocyte maturation-inducing hormones such as 17α,20ß-P (Scott and Canario 1987; Nagahama et al., 1993). In some fish species, a preovulatory surge of GtH has been detected (Kobayashi et al., 1987; Pankhurst and Carragher 1992) and this GtH induces a shift in the steroidogenic activity of key enzymes to the production of the 17α,20ß-P (Nagahama et al., 1993). In ocean

pout, a rapid decline of testosterone and 17ß-estradiol was detected prior to and during fish spawning, indicating the possibility of a shift in the steroidogenesis of the ocean pout. However, the expected increase of 17α,20ß-P during spawning was not detected in the ocean pout. Therefore, factors regulating oocyte maturation and ovulation in the ocean pout remain unclear.

In male ocean pout, the rapid increase in GSI from July to September matches the spermiation season and testes development was apparently related to the steroid hormones testosterone and 11-ketotestosterone since the GSI increased in July following significant elevations of testosterone and 11-ketotestosterone in the plasma in June. Previous studies in other species have indicated that testosterone induces spermatogenesis while 11-ketotestosterone accelerates the formation of spermatozoa and spermiation (Idler et al., 1971; Billard et al., 1983).

It is generally believed that temperature and photoperiod are the two major environmental cues entraining the seasonal reproductive cycle of fish (Duston and Bromage 1987,1988). Alteration of one or both of these two factors has been successful in changing fish reproductive cycles (Bromage et al., 1982; Razani et al., 1989; Asahina and Hanyu 1991). In determining how increased water temperatures in the winter affect the feeding, growth and reproduction of ocean pout, it was found that warmer winter water temperature increased feeding activity but did not change the hormone and vitellogenin profiles nor the time of subsequent spawning. This suggests that steroidogenesis and vitellogenesis in the ocean pout were not affected by water temperature. Fletcher et al. (1985) have reported the presence of high levels of antifreeze

protein in the plasma of ocean pout. The continued production of the steroid hormones in the ocean pout during the winter in the cold ambient seawater might be associated with the presence of antifreeze protein in the plasma, which enables the fish to continue the reproductive process in the cold ambient water temperature.

Photoperiod demonstrates a clear influence on growth, spawning, steroidogenesis and vitellogenesis of the ocean pout. Advanced increases of photoperiod in the winter induced a greater body weight increase in the spring (April-June), which perhaps implies a quicker ovarian growth in a longer photoperiod, since the increase in body weight in April matches the timing of ovarian recrudescence in the ocean pout (which normally starts in the spring). As a consequence, the spawning season of fish in longer photoperiod (tank B) was advanced.

Although similar studies of photoperiod regulation of reproduction have been reported in other fish species, the mechanism of the photoperiod regulation remains poorly known. In lower vertebrates including fish, it has been suggested (Garg 1987; Skene et al., 1991; Tabata et al., 1991) that both the eyes and the pineal gland in the epithalamus are important photoreceptors which mediate the photoperiod changes (external) and the internal hormonal regulation and reproduction. In the pineal gland of the European minnow *Phoxinus phoxinus*, two types of cells are reported which react differently, i.e., either depolarized or hyperpolarized, to light (Nakamura et al., 1986). Melatonin is released from the pineal gland during the dark period (Tabata et al., 1991) inhibits GnRH release and GtH secretion, or suppresses the response of gonadal tissues to GtH, thus inhibiting the gonad development (Reiter 1978; Relkin 1983; Garg 1987). Shorter

photoperiod (or longer dark-phase) should stimulate more melatonin release and consequently slow down the gonad development and delay fish spawning. In contrast, in day time or experimental light, melatonin is rapidly metabolized to 5-methoxytryptamine, which is subsequently deaminated to the inactive products of 5-methoxyindoleacetic acid and 5-methoxytrypophol (Skene et al., 1991). Therefore, advanced gonadal recrudescence and/or earlier spawning could be expected in long photoperiod, as was demonstrated in the fish *Mendia audens*, *Brachydanio rerio* and *Repomucenus beniteguri* (Zhu et al., 1991a,b). In the ocean pout, the plasma 17ß-estradiol and vitellogenin levels were low in short experimental photoperiod but increased when exposed to a longer photoperiod. Delaying the increase of photoperiod restrains the increase in plasma 17ß-estradiol and vitellogenin, suggesting the involvement of the melatonin regulation in the hypothalamo-hypophysial-gonadal axis of the ocean pout resulting in inhibition of steroidogenesis and vitellogenesis.

In rainbow trout and the barbel *Barbus barbus* (Cyprinidae), abrupt changes of experimental photoperiod appear more effective than the magnitude of photoperiod changes for alteration of spawning time. The "changing" photoperiod serves as a "zeitgeber" or translator to entrain the endogenous rhythms of fish (Duston and Bromage 1987,1988; Poncin 1989). While under constant dark or light conditions, a zeitgeber is not present and the ovarian activity free runs with circannual periodicity (Bromage et al., 1984; Zhu et al., 1991a,b). A circadian oscillator generating rhythmic release of melatonin under dark conditions was reported in the pineal gland of pike *Esox lucius* and goldfish *Carassius auratus* (Tabata et al., 1991). The occurrence of zeitgeber entrainment for the

endogenous rhythmicity of the ocean pout reproduction is indicated by the plasma hormone responses of group A, B, C and D, where an abrupt increase in photoperiod in the winter induced or entrained a rapid elevation of plasma 17ß-estradiol. This "zeitgeber" entrainment seems not to be inhibited by water temperature because the elevation of plasma 17ß-estradiol and vitellogenin was induced at various water temperatures such as in December (group B), March (group C) and June (group D) although warmer water might enhance this "zeitgeber" entrainment (indicated by a higher elevation of plasma 17ß-estradiol in group B). A larger increase in photoperiod seems to produce a stronger "zeitegeber" effect since the magnitude of 17ß-estradiol increase in group B was greater than that in group A (both had a photoperiod increase in November-December but with different magnitudes of increases). It probably can be concluded from these observations that both the abrupt increase and the magnitude of increase in photoperiod are important for entraining the reproductive rhythmicity in the ocean pout. On the other hand, photoperiod in tank E remained short (6L:18D) and a "zeitgeber" supposedly was not present. However, spawning was delayed but not inhibited. This suggests the existence of a free running circannual rhythmicity in the ocea pout.

Vitellogenin is an egg yolk precursor protein. The significant elevation in plasma vitellogenin in the winter indicates an active vitellogenesis. However, the expected rapid incorporation of vitellogenin into oocytes following the increase of plasma vitellogenin did not occur until April-May as indicated by the small body weight changes in the winter. This suggests that cold water might inhibit ovarian uptake of vitellogenin although not steroidogenesis and vitellogenesis in the ocean pout. Perhaps, increasing water

temperature along with increases in photoperiod in the winter might be more effective for advancing the ocean pout reproductive cycle since the increasing water temperature might accelerate the vitellogenin incorporation into oocytes in the winter. This hypothesis should be tested.

Regarding the induction of spawning by GnRH-A, previous studies of other species have shown the presence of GnRH receptors in fish pituitary (Crim et al., 1988; Pagelson and Zohar 1992; Weil et al., 1992). Injection of GnRH-A induces pituitary GtH secretion, which in turn stimulates gonad development and spawning (Marte et al., 1988; Lin et al., 1993). From the hypothalamus of the ocean pout, a GnRH peptide, having a similar molecular structure to the GnRH-A ([D-Ala^6,Pro^9 NHEt] GnRH) used in this study, has been isolated by Shower et al. (1992). This similarity might allow this GnRH to bind to the pituitary gonadotropes and stimulate GtH secretion in the ocean pout. Oocyte maturation in fish is often accompanied by hydration of the oocytes, which will cause a sudden increase in body weight, as was shown by the study of Harmin and Crim (1992) in the winter flounder (*Pseudopleuronectes americanus*). A body weight increase following GnRH-A treatment was also noted in the ocean pout, suggesting that the GnRH-A treatment induced the oocyte maturation. The sporadic spawning of the ocean pout was apparently synchronized. This stimulative effect of GnRH-A for spawning was perhaps achieved through inducing a shift in steroidogenesis since a more rapid and synchronized decline of both testosterone and 17ß-estradiol was noted in the GnRH-A induced spawning females.

The present study has also demonstrated the influence of water temperature and

photoperiod on seasonal body weight increase (growth) in the ocean pout. It demonstrated that increasing water temperature in the winter increased feeding activity but did not enhance growth (Expt.II). In contrast, fish in cold ambient seawater had a comparable growth rate to those in warmer water although they consumed less food (about half of that consumed by their counterpart in warm water). In other words, the efficiency of energy (food) utilization in the cold ambient seawater was higher than that in warm water. Previous studies have demonstrated that growth in fish is determined by metabolic scope (B), which is calculated by an equation: B = C-F-U-R, where C is total energy intake in food and the other components in the equation are the energy losses in faeces (F), in excretory products (U) and in all others (R) including the energy released in the course of metabolism in unfed and resting fish (Rs), the energy required for swimming and other activity (Ra) and for processing food materials (Rd) (R = Rs+Ra+Rd; Elliott 1982). More food intake (C) usually produces a higher metabolic scope (B) or a higher growth rate. This however depends on water temperature. Fish are poikilothermic animals. Raising water temperature causes an increase in the metabolic rate (R) and the swimming activity, thus leading to more energy loss, and accelerates food passage through the digestive tract, which should result in an increase in energy loss in faeces (F) or a reduction of food digestibility (C). In fish, the energy losses in faeces vary from 2% to 31% of the total energy intake (Elliott 1982). Therefore a higher feeding activity in warmer water may not produce a higher metabolic scope or better growth than fish in cooler water. Although lowering water temperature reduces the feeding activity, it also reduces the energy loss in F, U and R. Lowering water temperatures to subzero degrees might be lethal to most

fish species but was not deleterious for the ocean pout which contained a high level of antifreeze protein (Fletcher et al., 1985; King et al., 1989). Perhaps the presence of antifreeze protein in plasma allows the fish to utilize energy more efficiently in cold water than in warm water. In this study (Expt. II), fish in both temperature treatments were fed to satiation. This would allow the fish to produce a maximum metabolic scope and the value might be comparable under both conditions. Since Expt.II indicated that ambient cold seawater did not affect the ocean pout growth and reproduction, Expt.III was also conducted in ambient seawater. However growth rates in the winter of 1993 (Expt. III) were much lower than that 1991 (Expt.II). This lower growth in 1993 was probably due to a much colder temperature (-1.5°C, close to fish freezing limit of -1.7°C) in the winter of 1993 than in 1991 (ca. -0.5 to 1.0 °C).

The tolerance to low temperature and a higher efficiency of food utilization in cold ambient seawater is an advantage for aquaculture. This study also showed that the ocean pout was a very hardy fish, tolerant of cold temperature, insensitive to frequent handling and capable of being kept at high stock densities in captivity, i.e., 33.4 kg/m^1, maintaining good growth and health conditions. These observations, together with the other traits in their early life history, including large adult-like juvenile at hatch, high survival rate and feeding at very low water temperatures (<0°C) (Brown et al., 1992), suggest that the ocean pout might be a good candidate for cold ocean mariculture in Eastern Canada.

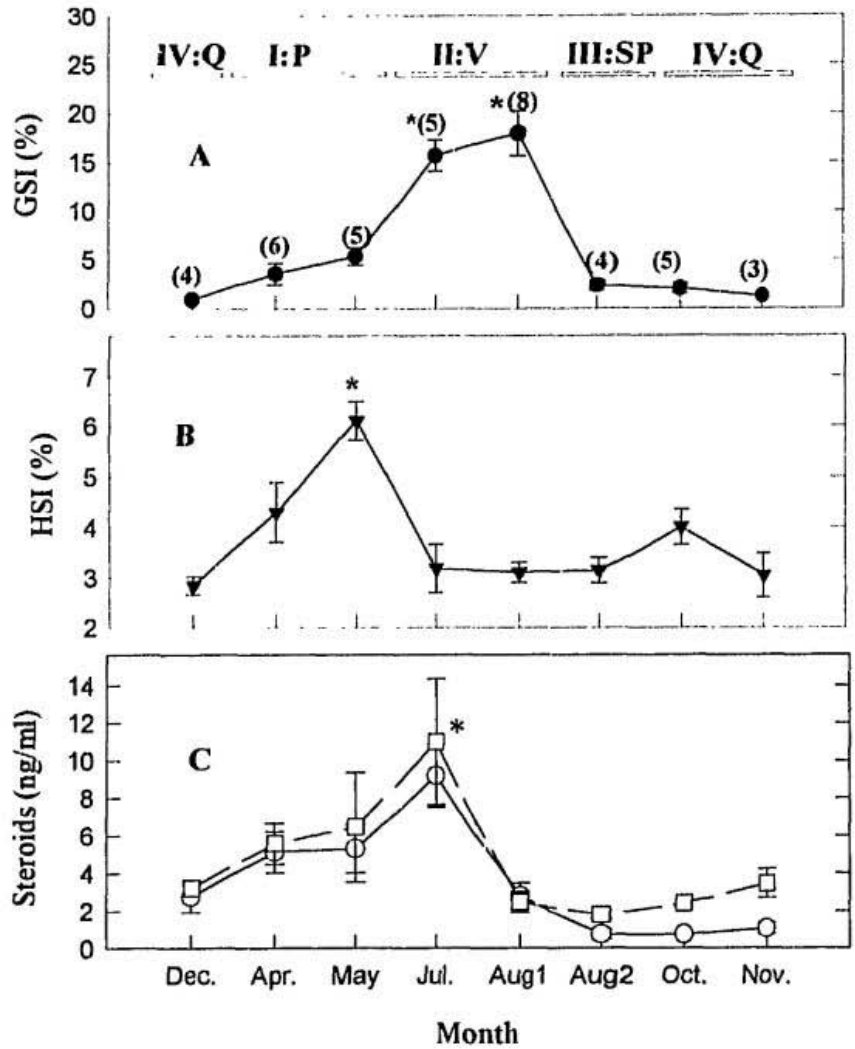

Figure 1. Seasonal changes in (A) GSI, (B) HSI and (C) plasma sex steroids 17ß-estradiol (○) and testosterone (□) of the adult female ocean pout. Error bars indicate standard error, numbers in brackets indicate the number of measurements (fish); **Q**:quiescent phase; **P**:reproductive preparatory or slow gonad development phase; **V**:rapid ovarian growth phase; **SP**:spawning phase; * indicates a significant difference ($P<0.05$) from the values in December. **Aug1** and **Aug2** indicate the prespawning and post-spawned females in August, respectively.

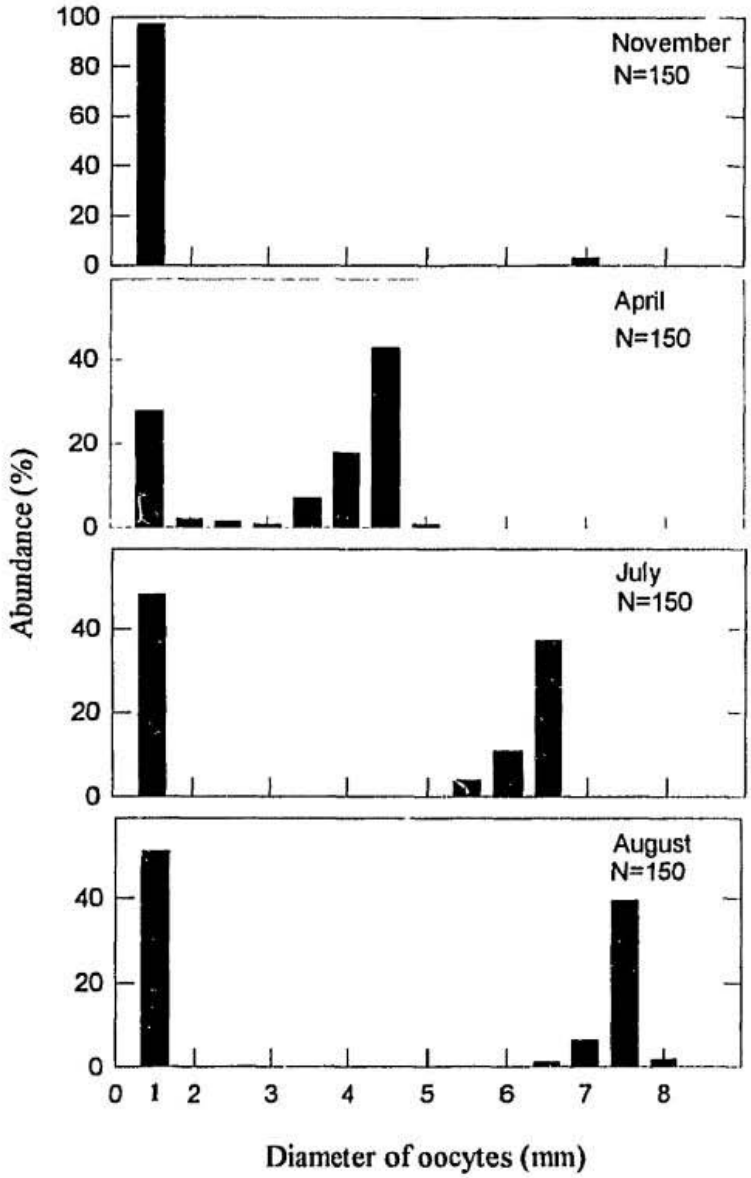

Figure 2. Seasonal changes in size-class frequency of oocytes in ocean pout ovaries. Each panel represents a measurement of 150 eggs. Y-axis indicates the abundance (in % of the 150 eggs) of each oocyte size-class, X-axis indicates the diameter of the eggs. The first and second classes of oocytes are the previtellogenic (1.0 mm in diameter) and vitellogenic oocytes (≥2.0 mm in diameter) oocytes, respectively.

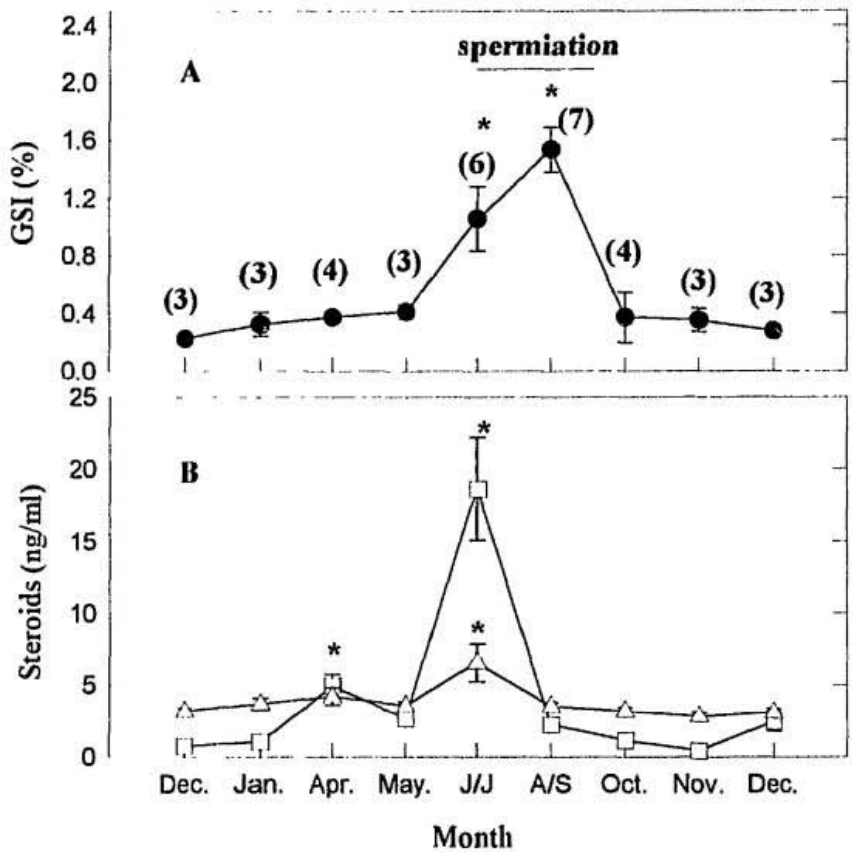

Figure 3. Seasonal changes in (A) GSI and (B) plasma sex steroids testosterone (△) and 11-ketotestosterone (□) of adult male ocean pout. The thin line in panel A indicates the period of spermiation. Values in brackets indicate the number of measurements (fish) (same for A & B). Error bars indicate standard error. *:significant difference ($P<0.05$) from the values in December. J/J:June/July; A/S:August/September.

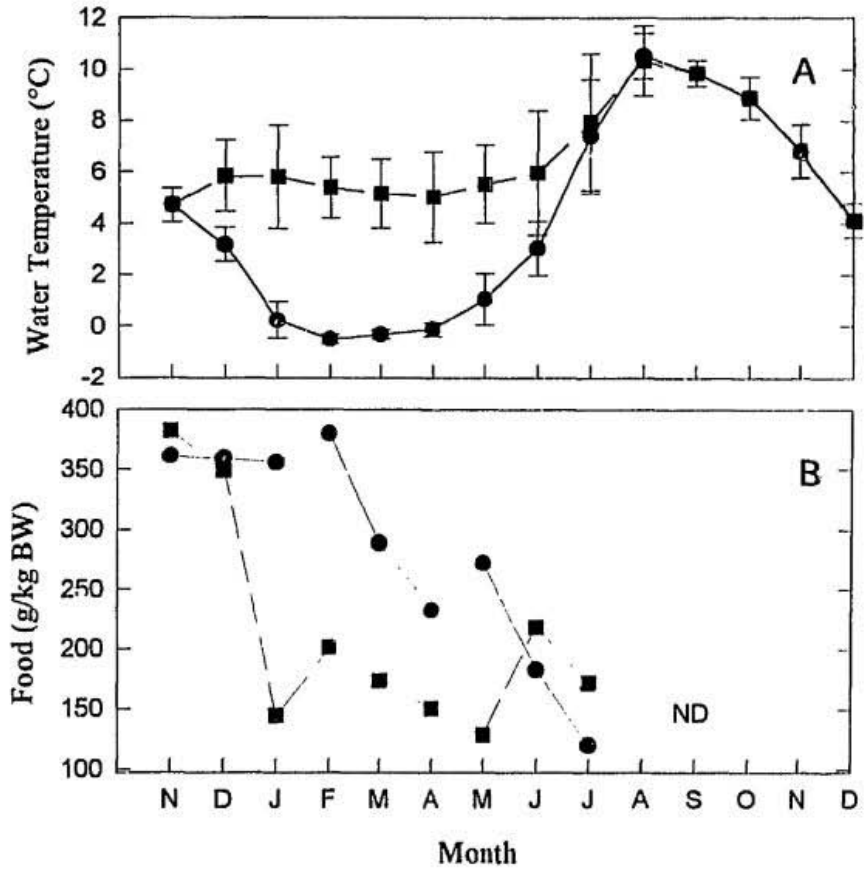

Figure 4. Influence of water temperature (°C) on seasonal feeding of the ocean pout. (A) Seasonal temperature profiles (error bars indicate standard error) and (B) monthly food consumption for fish in (●) ambient or (■) heated seawater. Separate measurements of food consumptions for the two groups were not conducted after July because the two groups of fish were mixed and kept together in same tanks after July..

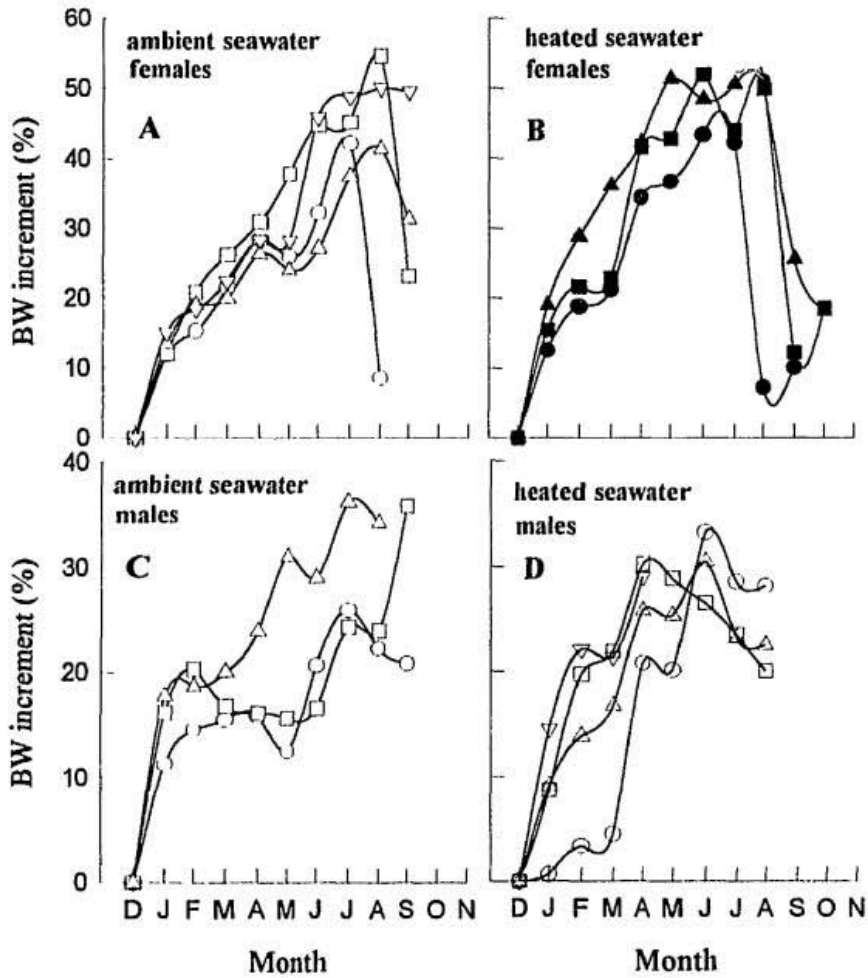

Figure 5. Cumulative growth responses (body weight increment) in individual fish exposed to either (A & C) ambient or (B & D) heated seawater (calculation of the BW increment, see Materials & Methods).

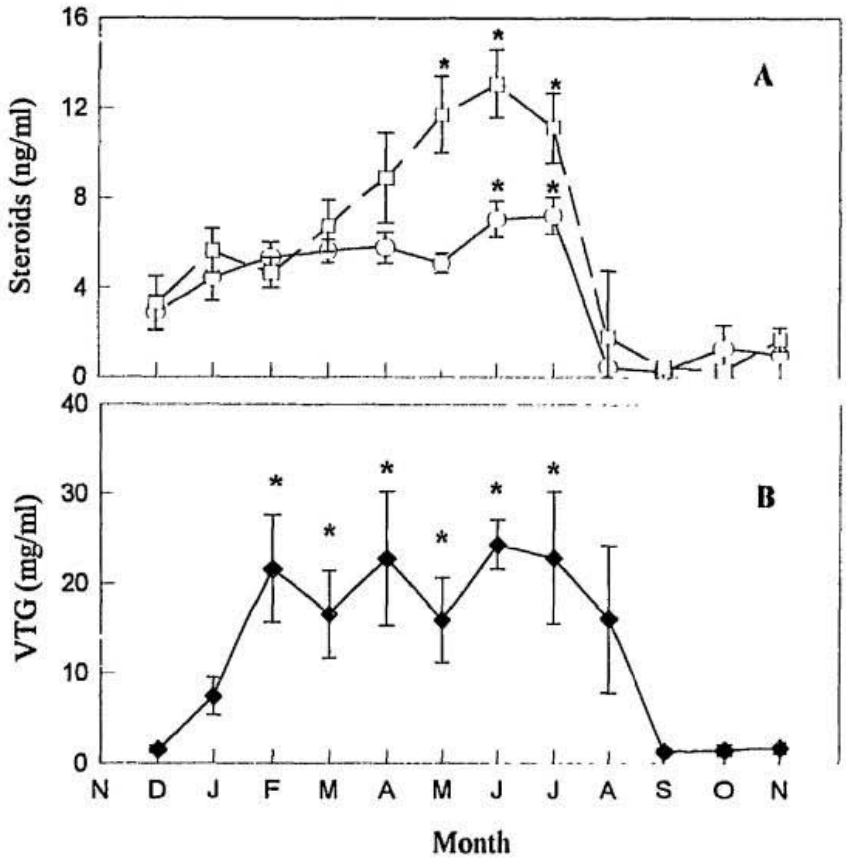

Figure 6. Seasonal profiles of (A) plasma (□) testosterone and (○) 17ß-estradiol, and (B) vitellogenin levels in adult females. * indicates a significant difference ($P<0.05$) compared to the level in December.

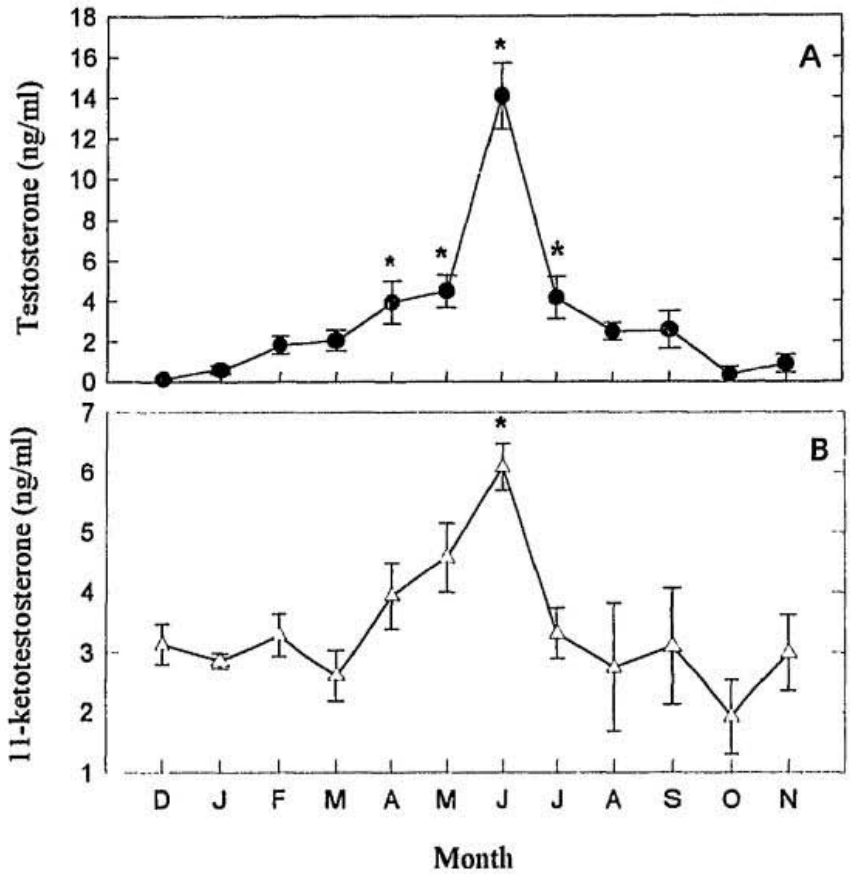

Figure 7. Seasonal profiles of (A) plasma testosterone and (B) 11-ketotestosterone in adult males. * indicates a significant difference ($P<0.05$) from the value in December.

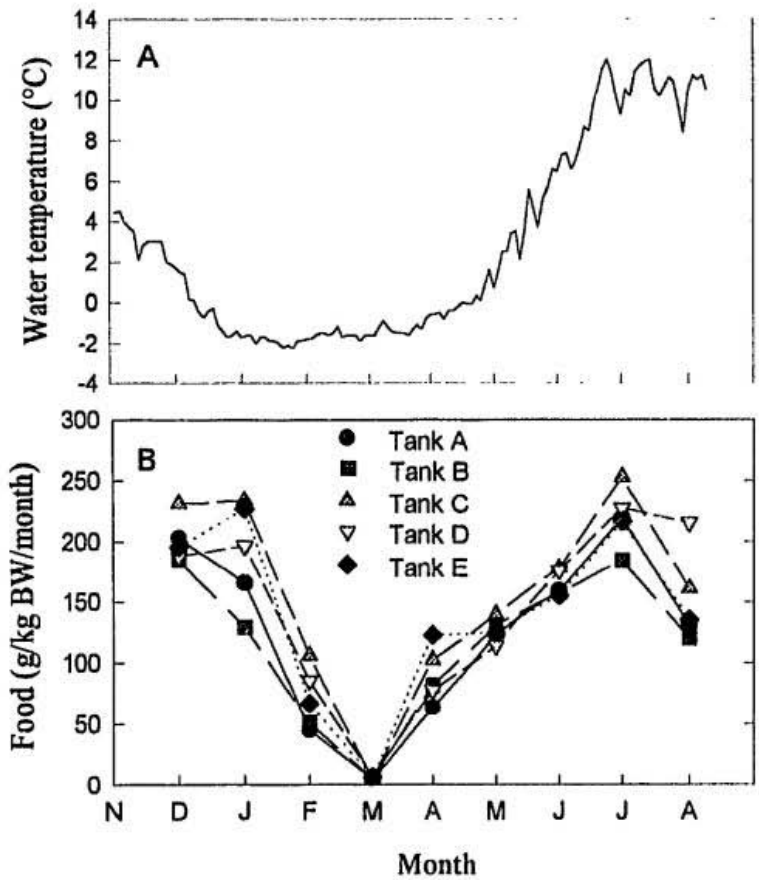

Figure 8. Influence of water temperature and photoperiod on seasonal feeding activity (g/kg BW) of the ocean pout; (A) Seasonal profile of ambient seawater temperatures; (B) seasonal changes in food intake for fish under 5 different photoperiod conditions (For explanation of Tanks A, B, C, D & E, see Material and Methods).

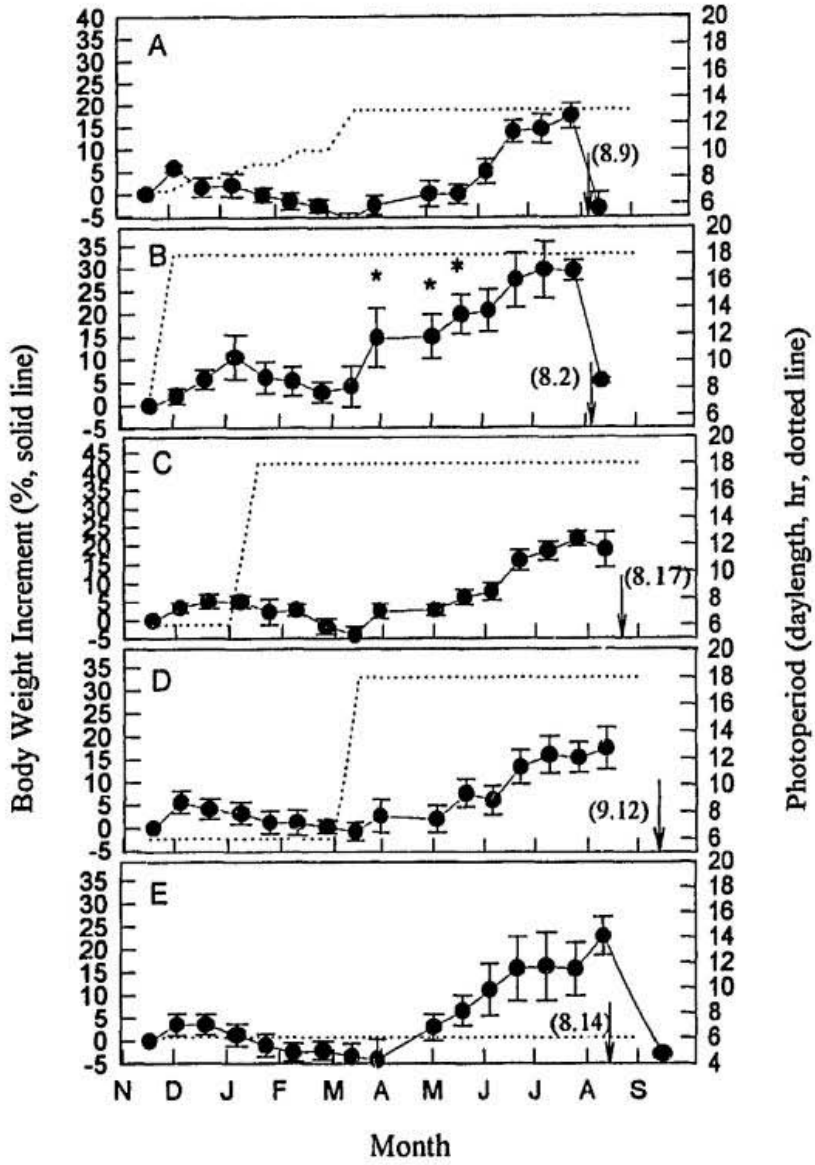

Figure 9. Influence of photoperiod (dotted line) on seasonal growth response (% BW increment, solid line) of adult females. Panels A, B, C, D & E indicate 5 tanks in different photoperiod regimes. The left Y-axes indicate the BW increment (for calculation, see Materials & Methods), the right Y-axes indicate the photoperiod (or day-length in hr). * indicates a significant difference ($P<0.05$) from the other photoperiod treatments. Arrow and values in bracket indicate the date of first spawning (month,day); Error bars indicate standard error.

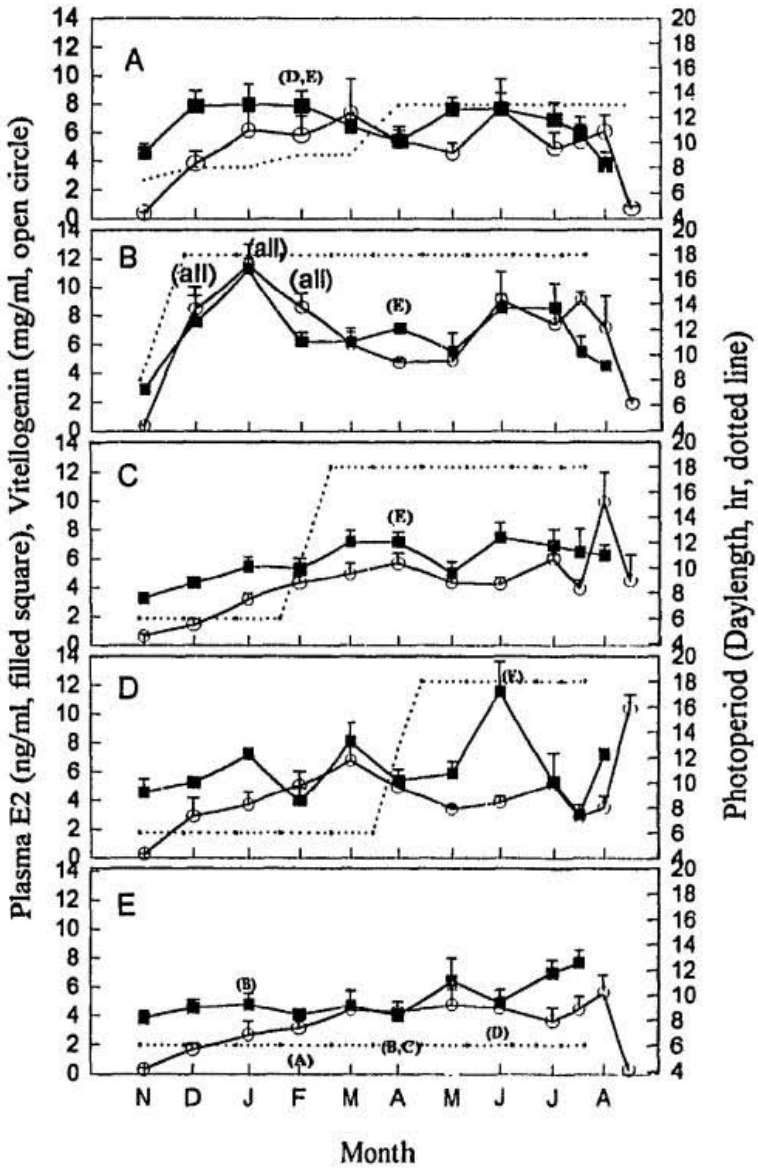

Figure 10. Seasonal changes in plasma levels of 17ß-estradiol (■, ng/ml) and vitellogenin (○, mg/ml) of females under 5 different photoperiod conditions (dotted line) in tank A, B, C, D & E. The left Y-axes indicate the plasma levels of 17ß-estradiol and vitellogenin, and the right Y-axes indicate photoperiod (or day-length in hr). * indicates a significant difference ($P<0.05$) from the other photoperiod treatments shown in the brackets. Error bars indicate standard error.

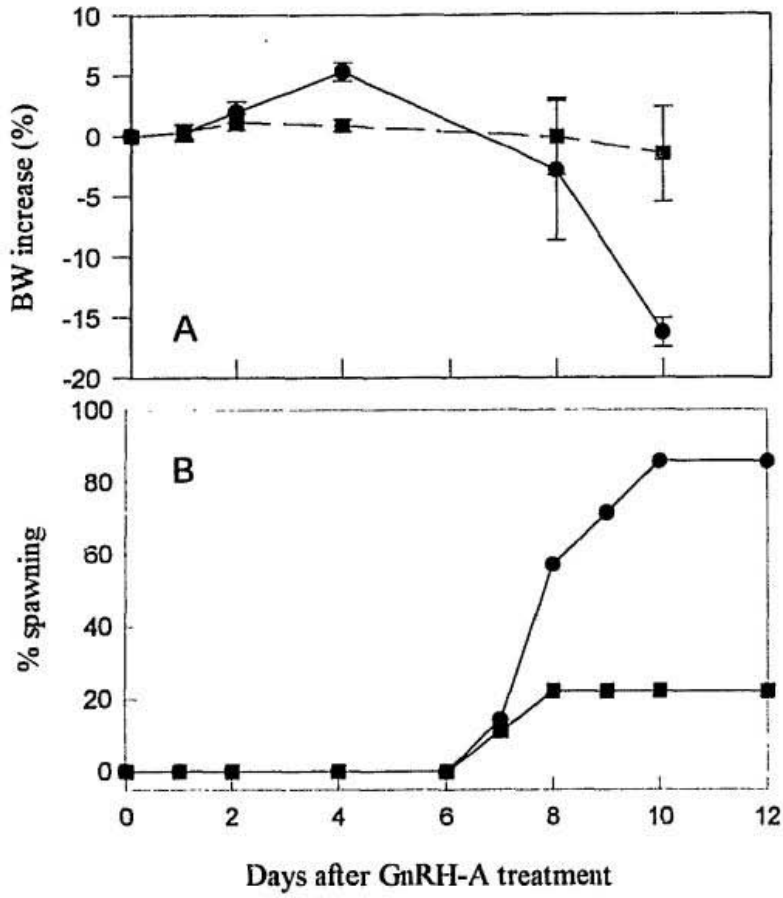

Figure 11. The effect of GnRH-A treatment on (A) body weight changes and (B) the timing of spawning of adult females. ●:GnRH-A treated fish (N=7); ■:control fish (N=9).

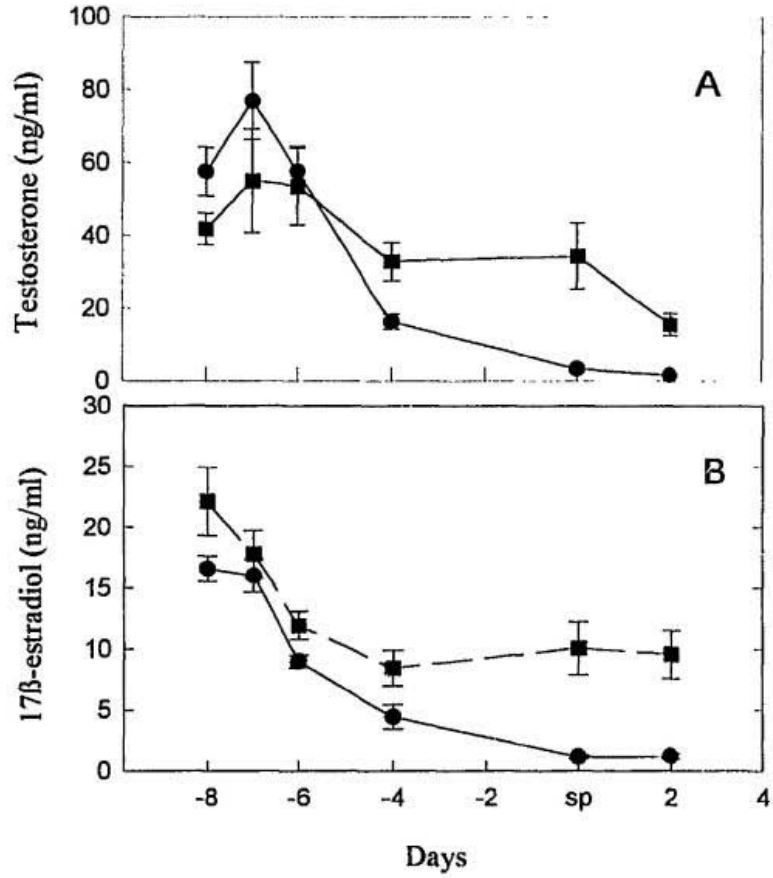

Figure 12. Changes in plasma (A) testosterone and (B) 17ß-estradiol in spawning (●, N=8) and non-spawning (■, N=8) females in August. Error bars indicate standard error *: significant difference ($P<0.05$) from the other group.

CHAPTER 6

Spawning of the ocean pout (*Macrozoarces americanus* L.): Evidence in favour of internal fertilization

6.1. ABSTRACT

Sperm physiology and *in vivo* artificial insemination of the ocean pout (*Macrozoarces americanus* L) were studied. Milt was collected from the reproductive tract of mature males by inserting a catheter into the sperm duct. The fresh milt had a low sperm concentration and contained highly motile spermatozoa. Sperm motility was retained *in vitro* for at least 24 hr at 4°C in both seminal plasma and ovarian slime collected from the oviduct of pre-spawning females. Instead of activating sperm, dilution in seawater instantly immobilized the spermatozoa. pH and osmolarity of the seminal plasma ranged from 7.2-7.5 and 365-406 mOsM, respectively. A study of the ionic composition of the seminal plasma showed the presence of various ions including Na^+, K^+, Ca^{++}, Mg^{++}, and Cl^-, with a lower K^+ concentration compared to that from other fish species. Injections of milt containing motile sperm into the ovaries of prespawning females, which spawned in the absence of males, yielded fertilized eggs, and viable larvae were hatched after 3 months incubation in ambient sea water (9-10°C). It is concluded from this study that the ocean pout is an internal fertilizer. With proper timing of *in vivo* artificial insemination of mature females, the fertilized eggs can be obtained from fish in captivity.

6.2. INTRODUCTION

The ocean pout (*Macrozoarces americanus*), a marine bottom fish (Zoarcidae), occurs in the waters from the intertidal zone to over 183 m in depth along both sides of the Atlantic Ocean (Keats et al., 1985; Scott and Scott 1988). Many traits, including its good tolerance to cold (King et al., 1989), good growth rates at low water temperatures (Brown et al., 1989), good quality flesh of low cholesterol but high protein content and low mortality of the well developed nearly adult-like larvae at the time of hatch (Sheehy et al., 1977; Shenouda et al., 1979; Jhaveri et al., 1985), make the ocean pout a good candidate for aquaculture in Atlantic Canada.

Reproduction in the ocean pout follows a seasonal cycle. In the wild, the fish undergoes an inshore (early spring) and offshore (late autumn) seasonal migration. Spawning of the fish occurs in autumn in rocky holes or crevices and, after spawning, the female guards the egg-masses (Keats et al., 1985). It takes about 3 months of egg incubation before the larvae are hatched in nearly adult form (Methven and Brown 1991). Since to date there is no direct observation of fish spawning and only eyed eggs have been collected from the field, the biology of egg fertilization of ocean pout remains unknown. On the other hand, our recent failures to fertilize ocean pout eggs by artificial insemination *in vitro* suggests that male ocean pout might inseminate females internally.

The objective of this study was to test whether *in vivo* artificial insemination, i.e., injecting sperm directly into the ovary, would produce fertilized eggs.

6.3. MATERIAL AND METHODS

6.3.1. Fish

Adult ocean pout for this study were collected in Conception Bay, Newfoundland, by SCUBA divers in the Autumn 1991 and 1992, and acclimated to captive conditions in indoor 2x2x0.4 m tanks. The tanks were supplied with aerated, flowing sea water at ambient temperature (9-12°C in the summer), under a simulated natural photoperiod. The males and females were kept together and fed chopped capelin and a moist pellet diet to satiation 2-3 times a week.

6.3.2. Obtaining and chemical analysis of the male sex product

Milt was collected from mature male ocean pout (3108 $\pm$ 638 g) from July to September by a catheter (polyethylene tubing, I.D 1.14 mm, O.D 1.57 mm, Clay Adams Co.) inserted into the sperm duct after the fish had been anaesthetized with 2-phenoxyethanol (100-300 ppm, Anachemia). The milt was held in cool glass scintillation vials on crushed ice and immediately subjected to examination for sperm motility and centrifugation for spermatocrit analysis (packed spermatozoa as a proportion of total milt volume, N=4). One drop of milt (20-40 µl) was placed onto a microscopic slide and sperm motility was examined immediately under light microscope before and after addition of 20-40 µl seawater or ovarian fluid from mature females. Sperm morphology was examined and measured by light microscopy (400-1000x) using an ocular lens micrometer. Seminal plasmas from three individual males were analyzed for pH,

osmolarity and ionic composition. The pH and osmolarity were measured by pH indicator strips in a range of pH 6.6-10 (EM Science Inc.) and by a FISKE osmometer, respectively, while the ionic composition was determined using the SYNCHRON CX3 system (a multi-analyte discrete analyzer). Sodium, potassium and chloride were measured by an indirect potentiometry using ion selective electrodes housed in a flow cell. Carbon dioxide was measured using a pH electrode covered with a silicone rubber membrane. Glucose was measured in individual reaction cups by means of a polarographic electrode. Calcium and magnesium were measured spectrophotometrically in a colorimetric reaction.

6.3.3. *In vivo* insemination and spawning of female ocean pout

Three sets of experiments were conducted from 1991 to 1993 to study the spawning performance of female ocean pout. Initially, spawning and the production of fertilized eggs were determined from a group of 6 females held together with mature males (1991). In the second experiment (1992), fertility was studied in two different groups of females held either with (N=6 females) or without (N=8 females) males. Where no males were present, the females received artificial insemination treatment *in vivo*. In 1993, a third experiment tested the spawning performance of artificially inseminated females and the production of fertilized eggs according to the following groups of fish: A) 6 females were housed together with males; B) 21 females were held separate from males. Of these 21 females, 3 fish received no artificial insemination (control) and the other 18 females were artificially inseminated with milt containing motile sperm.

Before artificial insemination *in vivo*, the skin colour and ovipore development of

mature females were checked daily. Following the opening of the ovipore, the fish were removed from the spawning tank (1x1x0.5 m), the genital area blotted dry and 3-4 ml of freshly collected or 24 hr stored milt containing highly motile sperm were injected into the ovary, using a small catheter inserted into the ovipore (Fig.1). After insertion, the catheter was slowly moved back and forth, left and right, to assist sperm dispersal within the ovary. After insemination, a procedure requiring approximately 2 min., the females were returned to the spawning tanks and housed without males.

Following oviposition, the egg masses were either removed from the spawning tank and incubated in pans supplied with flowing ambient (9-12°C) seawater or left in the tank under the care of the spawning female. At regular intervals, either daily during the early period or weekly after the first month, the eggs were examined under a dissecting microscope to assess egg fertilization and embryo development.

6.4. RESULTS

Morphological changes associated with sexual maturation were observed in both sexes of ocean pout during the spawning season. In mature males the genital area became obviously swollen and protruded. Autopsies in August showed that the testes (paired lobules) of mature males were well developed (mean GSI 2.18 $\pm$ 0.25 N=8) and the sperm duct filled with milt. A catheter could easily be inserted into the sperm duct via the genital pore and 5-20 ml of uncontaminated milt were collected per sampling without

application of pressure on the genital area. Each mature male could be sampled for milt 2 - 3 times at intervals of 1 - 2 weeks in one spawning season. Without the catheter, however, no milt could be collected by the traditional method of hand-stripping. In contrast, the genital area in immature males remained flat with the genital pore tightly closed, preventing insertion of a catheter into the sperm duct.

The cloudy, white and watery milt, freshly collected from males, was characterized by a low spermatocrit value and the seminal plasma had a neutral pH and osmolarity of approximately 380 mOsM (Table 1). In freshly collected milt, the spermatozoa were composed of an oval sperm-head (3.0x2.25 μm), a mid-piece (3.0x1.5 μm, length x width) and a long flagellum (81 μm); they were swimming actively. No change in motility was apparent for the first 24 hours with storage at 4°C. Motile sperm were also observed in the fluid collected directly from the testes of mature males at autopsy. When ovarian slime from spawning females was added to the milt, sperm motility was enhanced. By contrast, the motile sperm were immobilized instantly after addition of seawater. Table 1 also shows the ionic composition of ocean pout seminal plasma which consisted of Na^+, K^+, Cl^-, Ca^{++} and Mg^{++}. The ocean pout seminal plasma also contained glucose and CO_2.

By spawning time in August, the females had a greatly enlarged abdomen and had gained approximately 40-55% increment of their post-spawned body weight (Chapter 5). Often, the skin of maturing females changed colour from black to light brown or yellow and the usually invisible ovipore, located posterior to the anus, opened 16-22 hr prior to oviposition and the opening was plugged with thick gelatinous slime. The females were artificially inseminated shortly after the ovipore had opened (Fig. 1).

The results of egg fertilization produced by females spawning in the presence of males or from artificially inseminated females held without males are summarized in Table 2. In a series of three sequential experiments from 1991 to 1993, despite the display of courtship behaviour between males and females, e.g., direct ventral contact (Chapter 7), just 1 of 18 (or 6% in total) females produced fertilized eggs in the presence of mature males. By contrast, 3 of 8 (or 37%) artificially inseminated females were confirmed to produce fertilized eggs in 1992; 5 other females in the group successfully spawned but the eggs were lost prior to determining fertilization due to inadequate egg incubation conditions. In 1993 in the absence of males, a small group of females (N=3) not receiving the insemination treatment spawned infertile eggs. However, following artificial insemination of 18 broodstock fish, 12 females (or 67%) produced fertilized eggs.

Oviposition of females occurred in the presence or the absence of males, usually at night (in the dark period). The newly laid spherical large eggs, averaged 8.2 $\pm$ 0.4 mm (N=40) in diameter, were translucent, light yellow or orange in colour and adhered together in a gelatinous ovarian slime forming an egg-mass, which is often guarded by the post-spawned female (Fig.2). Fecundity, estimated from the total egg mass weight divided by the mean weight of single eggs, ranged from 1200 to 1700 eggs. Microscopic examination of eggs did not show the perivitellin space but revealed the presence of abundant tiny oil droplets centralized in the eggs (Fig.3A). Following 3-5 days of incubation in ambient seawater (9-12°C), non-fertilized eggs became transparent. The oil droplets in eggs fused forming 1-4 large oil globules (Fig.3B) and the eggshell became very fragile promoting fungal growth. In contrast, fertilized eggs remained opaque with

little change in oil droplet distribution (Fig.3B) and primitive embryos appeared in the eggs beginning at 10-14 days (Fig.3C) (ca.100-140 degree-days). Therefore, the success of egg fertilization could be assessed as early as day 3-5 of incubation. By 2.5 months, the embryos were developed in the fertilized eggs (Fig.3D), from which adult-like larvae (total length about 33 mm) with yolk sac were hatched after 3 months incubation in ambient seawater (Fig.4).

Non-fertilized ocean pout eggs were usually found scattered individually throughout tanks where the non-inseminated females were housed and spawned with or without the presence of mature males. In contrast, artificially inseminated females produced fertilized eggs and guarded their egg mass after hardening of the egg membranes (Fig.2). The rate for egg fertilization in viable egg masses seemed high but could only be estimated as attempts to separate the eggs after their membranes had hardened proved difficult and damaged most of the eggs.

6.5. DISCUSSION

In the present study, fertilized ocean pout eggs were produced after *in vivo* artificial insemination of the female ocean pout. There was only one case in which fertilized eggs were produced naturally by females that were maintained along with males. Since the females held without males produced fertilized eggs only if they were *in vivo* inseminated, this insemination treatment was clearly effective and resulted in the

fertilization of the ocean pout eggs in the ovary.

Supportive evidence for internal fertilization of female ocean pout under natural conditions was also drawn from studies on the ocean pout sperm physiology. For example, ocean pout sperm, like those of mammals (also internal fertilizers), are motile before leaving the sperm duct. The motility of sperm is required for fertilization (Levanduski and Cloud 1988). Since dilution of the ocean pout milt with seawater quickly killed the sperm cells, this indicates that ocean pout egg fertilization is unlikely to occur externally in the seawater medium.

In teleosts, fertilization of eggs occurs after the sperm have entered the eggs via the micropyle. Success of egg fertilization depends on the sperm motility and the number of motile sperm surrounding the eggs (Hirai 1988; Billard 1990). In fish with external fertilization, sperm are not motile while residing in the male reproductive tract but they are activated after release into the water medium (Billard and Cosson 1992; Billard et al., 1993). Ordinarily, the life-span of the motile spermatozoa is very short, ranging from 30 seconds to a few minutes following activation (Scott and Baynes 1980; Aas et al., 1991; Billard et al., 1993), hence their movement covers only limited distances, e.g. 2 mm for chum salmon (*Oncorhynchus keta*) sperm (Morizawa et al., 1982; Billard 1990). A short life-span of fish sperm would likely reduce the chances of most sperm, except those in the vicinity of the micropyle, from reaching the egg micropyle, particularly when eggs are large. For such fish, the reproductive strategy is based upon production of large numbers of sperm to ensure a sperm:egg ratio of at least $10\text{-}300\text{x}10^3$ sperm per egg during fertilization (Munkittrick and Moccia 1987; Billard 1990; Aas et al., 1991). In these fish

species, the spermatocrits usually range from 20% to 95% (Billard 1990; Garcia 1993; Aas et al., 1991; Suquet et al., 1992; Harmin and Crim 1993). In contrast, the spermatocrit of ocean pout milt is very low (≤2.0%), indicating a low concentration of spermatozoa in the milt, but these sperm have a steady motility and long life-span in the ovarian slime of females. These features of the ocean pout sperm are apparently related to a mechanism of internal fertilization in the ovary, i.e. 1) the milt of ocean pout is not diluted by a large volume of medium in the ovary thus maintaining a proper ratio of egg:sperm in the ovary, 2) the long life-span and high motility of sperm in ovarian slime should enable the sperm to move long distances in the ovary required to reach the micropyle of large ovulated eggs for fertilization

Fish sperm motility is affected by the ionic composition of seminal plasma (Billard and Cosson 1992). Previous studies have shown that a high K^+ concentration or a low pH of semen strongly inhibits sperm motility within the fish reproductive tract. Dilution of the milt in water might reduce the K^+ concentration and increase the pH of the medium leading to the activation of sperm (Baynes et al., 1981; Morizawa et al., 1983; Goodall et al., 1989; Billard and Cosson 1992; Suquet et al., 1993). In ocean pout milt, this preliminary study of the ionic composition showed a lower concentration of K^+ (10.25 ± 1.73 mM, N=3) than in the milt of rainbow trout *Oncorhynchus mykiss* (25 mM) (Baynes et al., 1981) and in Atlantic salmon *Salmo salar* (27.5 ± 5.3 mM) (Aas et al., 1991). Perhaps this low K^+ concentration is associated with the long-term motility of sperm in the ocean pout sperm duct. Indeed, when looking at the ionic composition of seminal plasma from individual males, it appears that the motility of ocean pout sperm increases

with decreased K^+ concentration in the milt (data not shown). More accurate roles of K^+ and other ions and substances in milt on the regulation of sperm motility, and the mechanism of ocean pout sperm activation in the sperm duct are currently under investigation.

Although motility of sperm is affected by ionic composition in seminal plasma, the life-span of fish sperm largely depends on the energy supplies from mitochondria in the mid-piece (Inoda et al., 1988). In fish with external fertilization, the life-span of sperm is generally short, which likely results from the small number of mitochondria in the short mid-piece and a rapid exhaustion of energy (Billard 1990; Suquet et al., 1993). Addition of exogenous ATP to the medium may revive the motility of the exhausted immotile sperm (Billard et al., 1993). In internally fertilizing fish such as the live-bearing half-beak (*Hemirhamphodon pogonognathus*), a notable extension of mitochondria in the elongated sheath midpiece around the proximal region of the axoneme was shown by electron microscopy (Jamieson 1989), suggesting a rich energy supply for the sperm movements. This rich energy supply is no doubt essential for sperm to travel long distances within the ovary to fertilize eggs. The microscopic investigation (1000x) of the ocean pout sperm also revealed a long midpiece (1:1 to the length of sperm head) and a powerful flagellum (27 times of the sperm head in length), suggesting some similarities in sperm structure between the ocean pout and the half-beak. The energy generation in the midpiece requires substrate supplies, which likely come from the seminal plasma. The large volume of ocean pout seminal plasma, about 98% (v/v) of total milt volume, and the presence of glucose reserves, suggests that ocean pout seminal plasma could serve as a nutritional

medium for the sperm cells, as was suggested by Goodall et al. (1989) for the summer whiting, *Sillago ciliata*. The active respiration for energy generation in the ocean pout sperm was also indicated by the high CO_2 concentration in the milt (Table 1).

Success of artificial *in vivo* fertilization of ocean pout eggs depends on quality of the gametes and timing of the insemination. In this study, only the milt samples containing motile sperm were used for insemination. Insemination with one-day old milt (stored on ice in fridge) containing motile sperm also yielded egg fertilization. For successful insemination, milt must be introduced into the females at a time shortly after the ovipore has opened, which might coincide with the occurrence of ovulation in this fish. The females usually spawned their eggs within 24 hours following insemination. Premature introduction of milt into females prior to the opening of the ovipore proved difficult and did not produce fertilized eggs (data not shown).

The internal fertilization of eggs in the ocean pout might resemble its European counterpart, the eel pout (*Zoarces viviparus* Pisces: Zoarcidae) although the latter is viviparous (Korsgaard 1986). Recent studies indicate that the wolffish (*Anarhichas lupus*) also conducts internal fertilization (Johannessen et al., 1993) and females lay their eggs in masses (Keats et al., 1985) sharing similar reproductive features with the ocean pout. For example, the wolffish milt also contains a low concentration of sperm ($0.07\text{-}4.4\text{x}10^8$ spermatozoa/ml) and the sperm are motile in seminal plasma without seawater activation (Pavlov and Radzikhovskaya 1991). Also, artificial injection of milt into the ovary of female wolffish yielded fertilized eggs (Pavlov and Moksness, 1994). The patterns of internal egg fertilization in these two fish are therefore considered similar.

Although high fertilization rates have been achieved by injecting sperm directly into the female ovaries in both the ocean pout and wolffish, it is not clear how sperm can quickly disperse in the ovaries and locate the micropyle of eggs or whether the sperm entrance into eggs requires the micropyle. For most teleost eggs, the micropyle is the sole avenue for sperm entry since fish sperm generally lacks an acrosome (Billard 1990; Wolenski and Hart 1988; Hirai 1988). However, the presence of an acrosome was proposed in *Lepidogalaxias salamandroides* (Pusey and Stewart 1989), suggesting that the sperm of this fish could penetrate any egg membrane location similar to the fertilization process in mammals. On the other hand, the presence of up to 5 micropyles in the membrane of some wolffish eggs was recently revealed by scanning electron microscopy (Dzerzhinskij et al., 1992). This suggests the possibility of multiple entrances through the egg membrane available for sperm access into wolffish eggs. Either type of the sperm entry, i.e. independence of the micropyle due to the acrosome or multiple micropyles for sperm entry, might be important for the internal fertilization of the large eggs in ocean pout. Currently ultrastructure of both the sperm and the micropyle of eggs in the ocean pout is under investigation by electron microscopy.

No doubt, further studies are needed to understand the biology of egg fertilization in the ocean pout as well as the wolffish.

Table 1. Milt characteristics and chemical composition of seminal plasma in the male ocean pout.

Measurements	Range of values	Composition	mM
Spermatocrit (%)*	0.8-1.8	Na^+	183.6±1.18**
		K^+	10.25±1.73
Osmolarity (mOsM)	365-406	Cl^-	181.9±5.0
		Ca^{++}	1.45 ± 0.17
pH	7.2-7.5	Mg^{++}	0.84 ± 0.13
		Glucose	0.15-0.17
		CO_2	4.70-7.27

* [packed spermatozoa/total milt] x 100 (%, v/v).

** Mean ± standard error, N=3.

Table 2. Number of females (fertile females in brackets) in presence of males or in absence of males and artificially inseminated.

year	total	with males	without males	
			without artificial insemination	with artificial insemination
1991	6	6 (0)	0	0
1992	14	6 (1)	0	8 (3)
1993	27	6 (0)	3 (0)	18 (12)

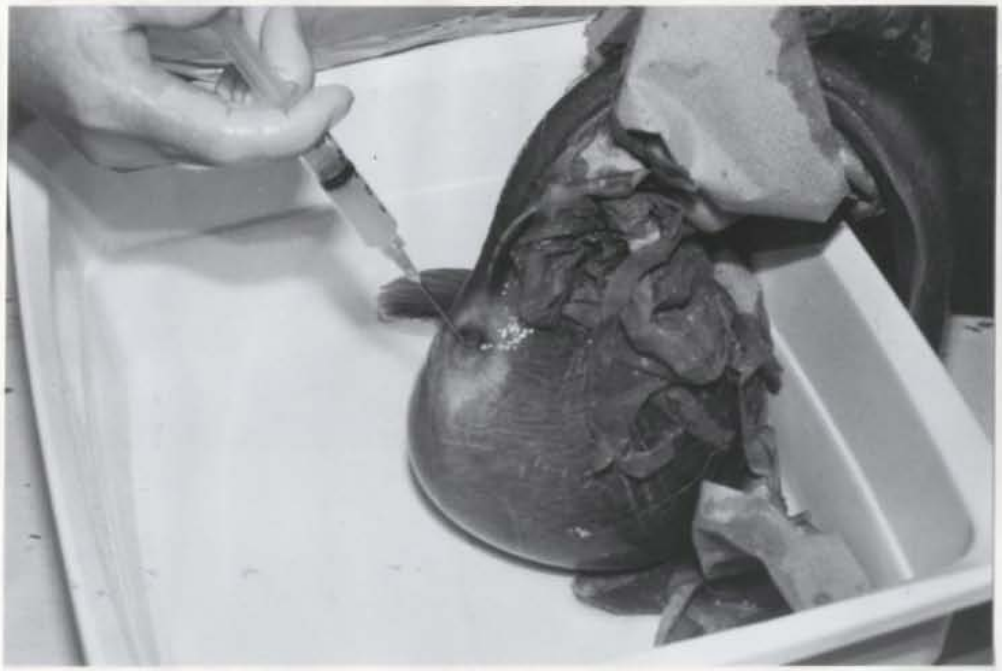

Figure 1. *In vivo* artificial insemination of the prespawning female ocean pout.

Figure 2. A post-spawned female displaying parental care for her fertilized egg-mass.

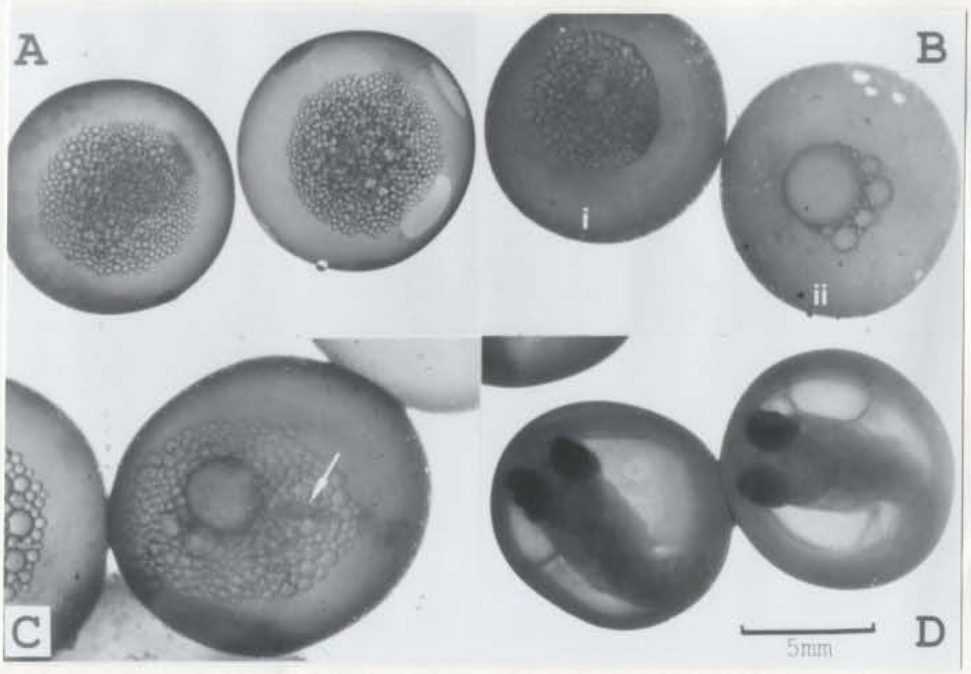

Figure 3. The fertilized ocean pout eggs at different developmental stages. A) newly laid eggs on day 1; B) fertilized (i) and non-fertilized (ii) eggs on day 5; C) primitive embryo (arrow pointed) in fertilized egg on day 14; D) advanced embryos in the fertilized eggs 2.5 months after spawning.

Figure 4. A newly hatched ocean pout larva (total length 33 mm) after 3 months incubation in flowing ambient sea water (9-12°C).

CHAPTER 7

Copulation, spawning and parental care in captive ocean pout

(*Macrozoarces americanus* L.)

7.1. ABSTRACT

Copulation, spawning and parental behaviours of captive ocean pout (*Macrozoarces americanus* L.) were studied through a continuous video surveillance. The ocean pout copulate through direct genital contact for internal fertilization. A complete spawning event consists of copulation, oviposition, and the female wiping and wrapping herself around the eggs. Parental care prevents leech and fungal infections of the eggs.

7.2. INTRODUCTION

In Northwest Atlantic regions, the ocean pout *Macrozoarces americanus* (Pisces: Zoarcidae) spawns in late Autumn. After spawning the female performs parental care by guarding the egg-mass (Keats et al., 1985). From recent studies of sperm physiology and *in vivo* artificial insemination of the ocean pout (Chapter 6), it was concluded that ocean pout is an internal fertilizer resembling its European counterpart eel pout (*Zoarces viviparus*) although the latter is viviparous (Korsgaard 1986). This chapter describes the observations of copulation, spawning and parental care behaviour of the captive ocean pout.

7.3. MATERIALS AND METHODS

7.3.1. Fish

Adult ocean pout were collected by SCUBA divers in Conception Bay, Newfoundland in the Autumn of 1991 and 1992. The fish were kept in 2x2x0.5 m tanks and supplied with flow-through ambient sea water under a simulated natural photoperiod condition and fed chopped capelin or moist pellets.

7.3.2. Observation of fish copulation and spawning

The study was conducted during the spawning season in August of 1992 and 1993. Fish (males and females) were transferred from the large tank (2x2x0.5 m) into small square tanks (1x1x0.5 m) and mature males and prespawning females were kept together. PVC pipes (30x25 cm) were placed in the spawning tank providing fish with a simulated spawning habitat (Keats et al., 1985) and a video camera was fixed above the tank to monitor fish copulation and spawning. The tank was exposed to a dim or red light at night to assist with video surveillance.

7.4. RESULTS

During the spawning season, the genital pore of mature males enlarged and developed into a papilla (Fig.1A) and the ovipore of prespawning female opened 6-17 hr prior to oviposition (Fig.1B). Copulation was first observed at 16.00 hr on 14 August 1992. A male approached the prespawning female in a PVC pipe rolling his body 90-180° to the side or the bottom directing his abdomen towards the female leading to direct genital contact (Fig.2). During copulation, the female remained quiet while the male exhibited slow lateral head movements and quivered occasionally. Copulation was terminated by the female, who swam away from the male. Following initiation, the fish copulated several times in the period from 16.00 hr to 03.00 hr 15 August, each lasting 2-3 minutes, and stopped about 6 hr prior to spawning, which occurred at 09.30 hr. Although there were more than one male and female residents of the tank, only the largest male, who was very aggressive to other individuals, copulated with the prespawning female whose ovipore had opened. These copulation behaviours were repeatedly observed but only one of 18 batches (6%) of eggs was fertilized in the August of 1991 - 1993.

Females spawned spontaneously 6-17 hr following the opening of the ovipore and copulation. The prespawning females usually rested quietly at the bottom of the tank, occasionally elevating the head using their pectoral fins. Spawning occurred in a relaxed manner when the female was kept alone and the fish lay on her belly, arched and waved her tail slowly while discharging eggs, which formed an egg-clump together with a gelatinous ovarian fluid. Oviposition was completed in about 16 minutes (n=2) and the

post-spawned female rested beside her eggs for several minutes before beginning to fan and wipe her skin over the egg-clump for nearly 30 minutes. The fish eventually wrapped herself tightly around the egg-mass (Fig.3). The fertilized eggs usually adhered to each other forming an egg-mass, 10-15 cm in diameter (containing 1400-1700 eggs), after the egg membrane had hardened, while non-fertilized eggs lost their adherent capability and were scattered throughout the tank. While guarding the egg-mass, the females remained quiet, either curving around their eggs or intermittently swimming in a slow circle and fanning the eggs with the pectoral fins to increase water flow. Parental care continued for up to 3 months and eggs guarded by females remained clean. Embryos developed well in these guarded eggs and larvae hatched after three months of incubation in ambient seawater (9-12°C). If the eggs were removed and incubated without parental care, they were soon invaded by the water-borne leeches *Oceanobdella microstoma* or *O. sexoculata* (Khan and Meyer 1976), which destroyed the eggs and promoted fungal infection.

When more than one female were housed together, the prespawning female became aggressive to other females prior to spawning (Fig.4A). The eggs were attacked by other individuals in the tank shortly after deposition (Fig.4B). Although initially the post-spawned female tried to protect her eggs from being eaten (Fig.4C), she ate the remaining eggs after most of them had been eaten by intruders and the parental behaviour stopped.

7.5. DISCUSSION

The present observations showed a direct genital contact between the two sexes of ocean pout during copulation, similar to the copulatory behaviours observed in wild ocean pout (Mercer et al., 1993) and in the wolffish (*Anarhichas lupus*), another marine teleost displaying internal fertilization (Johannessen et al., 1993).

Fish displaying internal fertilization of eggs often utilize accessory copulatory structure(s) to assist with insemination. In the fish *Lepidogalaxias salamandroides*, accessory copulatory structures including the modified anal fins and associated scale sheaths develop in the spawning season (Pusey and Stewart 1989). In adult elkhorn sculpin (*Alcichthys alcicornis*) and wolffish, the uro-genital pores of mature males swell and protrude and serve as papillae for insertion into the female ovipore (Munehara 1988; Johannessen et al., 1993). Because a similar genital protrusion developed in mature male ocean pout and the fish copulated through direct genital contact, it appears that ocean pout also accomplish egg fertilization by internal insemination.

Timing of copulation or insemination is crucial for a successful egg fertilization in most teleosts since life span of fish gametes is generally short (Billard 1990). In ocean pout, the studies of sperm physiology have shown that the sperm lost their motility instantly in seawater (Chapter 6). This short life span of sperm in seawater and delayed spawning of females, which occurred 6-17 hours after copulation, eliminated the possibility of external fertilization of eggs. A prolonged motility of ocean pout sperm was noted in female ovarian slime (Chapter 6). This motility in ovarian slime would ensure

a successful internal fertilization. Copulation occurred after the opening of the female ovipore, an indication of ovulation (Johannessen et al., 1993; Pavlov and Moksness 1994). The low success rate of natural insemination perhaps resulted from the high tank stocking density, which might have increased stress levels and shortened copulations. Since fertilized ocean pout eggs have been successfully produced by artificially injecting milt into the ovaries of mature females (Chapter 6), both the physiological and behavioral evidence supports the hypothesis that ocean pout is an internal fertilizer.

Although the genital area of mature male ocean pout swelled during the spawning season, milt was never stripped by the traditional method of stripping. However, 5-20 ml of milt could be easily collected from adult males by inserting a polyethylene tubing into the sperm duct through the genital pore (Chapter 6). The reason why such a large volume of milt in the sperm duct could not be stripped remains unknown.

The spawning of female ocean pout may be organized into four successive steps: 1) oviposition, 2) wiping their eggs on skin, 3) wrapping their body around the egg-clump and 4) continued curving around the eggs and performing parental care. Step 2 serves to coat the eggs with skin mucus, which might contain bacteriolytic and wormicidal substances (Itami et al., 1987; Rhee et al., 1987; Takahashi et al., 1987; Kusuda et al., 1987) and hence might increase the resistance of eggs to pathogenic infection. Step 3 appears to assist with egg-ball formation when the egg membrane is hardening. Only the fertilized eggs adhered to each other and formed an egg ball after their membranes had hardened, and the egg-balls were always guarded by parental females. In contrast, non-fertilized eggs did not adhere to each other and scattered in the tank. Parental care

behaviours appeared to be important for survival of fertilized eggs because only the eggs guarded by female remained clean and free of fungal and leech infection and survived to hatch. Fac'ors inducing these copulatory behaviours remain unknown.

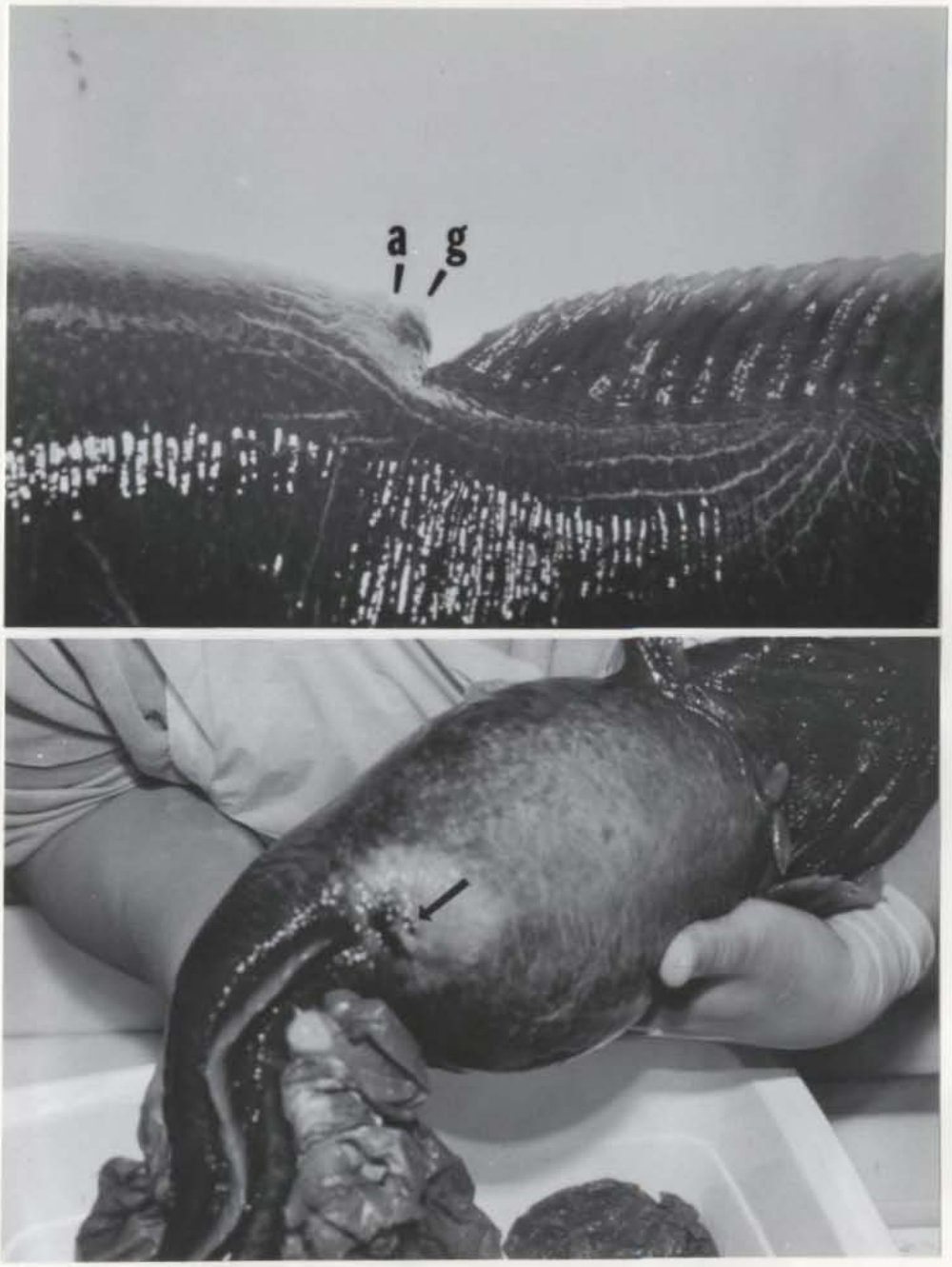

Figure 1. (A) Genital protrusion or papilla (**g**) of mature male; (B) Ovipore opening (arrow) of prespawning female. **a**:anal pore.

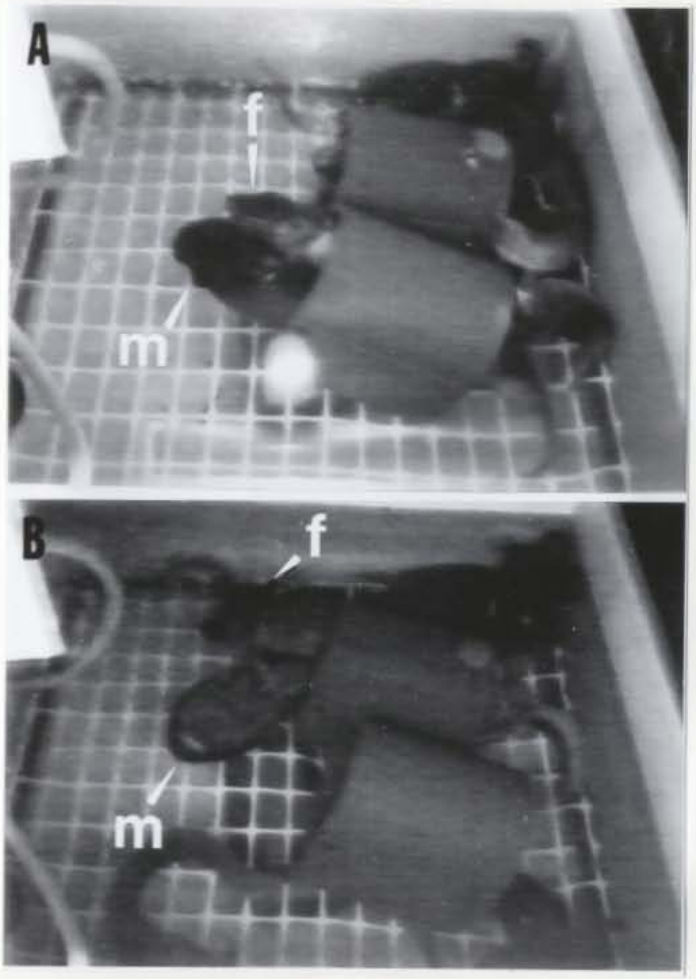

Figure 2. Copulation of captive ocean pout with male (**m**) lying (A) on his side and (B) on his back directing his abdomen towards to the female (**f**). Photos were made from video-recording.

Figure 3. Post-spawned female (**ps**) wrapping herself tightly around the eggs (**e**) to assist with egg-mass formation.

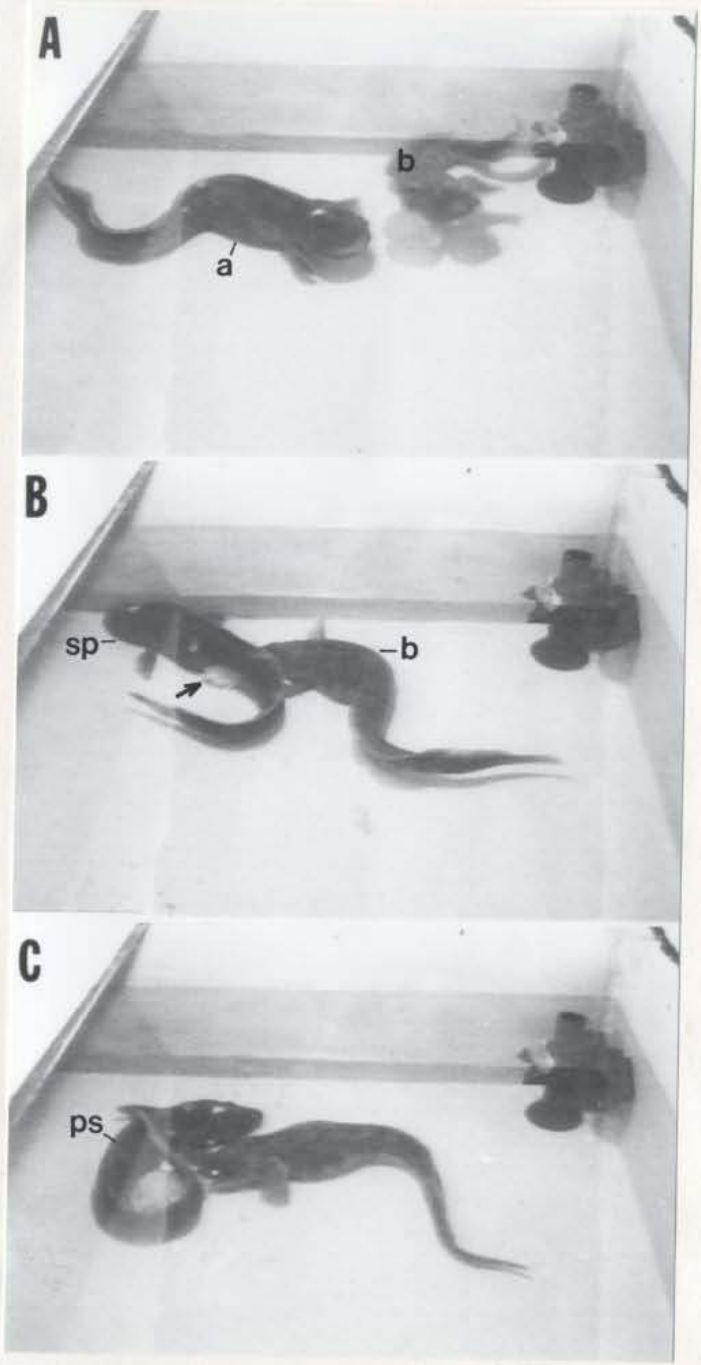

Figure 4. Parental female protecting her eggs. (A) Aggressive prespawning female (**a**) prior to spawning, (B) eggs (**arrow**) were attacked by another individual (**b**) while the spawning female (**sp**) was laying eggs and (C) the post-spawned female (**ps**) protecting her eggs from being attacked.

CHAPTER 8

In vitro Artificial Insemination of eggs from the ocean pout

(*Macrozoarces americanus L.*)

8.1. ABSTRACT

Trials of *in vitro* artificial insemination of the ocean pout (*Macrozoarces americanus* L.) eggs were conducted from 1991-1994. Fresh eggs were stripped from ovulated females and milt was added to the eggs *in vitro*. After a contact period between the eggs and sperm of 5 hours, the eggs were transferred into flowing seawater for incubation. Within 3-5 days, successfully fertilized eggs became opaque in which the primitive embryo appeared by day 7.5-8 at a water temperature of 13-15°C. Since the fertilized eggs appeared to have a high oxygen demand and required a continuous water flow, the up-welling incubator seems most suitable for this purpose. Success of *in vitro* insemination depends on both the egg and milt quality, which in turn might be affected by the timing of stripping. Eggs were only stripped from the ovulated females after the ovipores were completely opened (to 0.5-1.0 cm in diameter). High fertilization rates (87-90%) were achieved using fresh milt. However cool stored milt (2-3 days old, at 2-3°C) or cryopreserved milt also produced limited success after *in vitro* artificial insemination of eggs.

8.2. INTRODUCTION

It was demonstrated previously that the ocean pout (*Macrozoarces americanus L.)* is an internally fertilizing species and fertilized eggs have been produced by *in vivo* artificial insemination (Chapter 6 and 7).

In vitro artificial insemination is a major hatchery technique used to produce fertilized fish eggs for aquaculture (Billard 1990). A recent study of the wolffish (*Anarhichas lupus*) showed that *in vitro* artificial insemination of eggs from the internally fertilizing species is possible (Pavlov and Moksness 1994). Therefore, although *in vivo* insemination of the ocean pout egg is successful (Chapter 6), it is desirable to produce the fertilized ocean pout eggs by the *in vitro* insemination method as well.

This Chapter describes the *in vitro* artificial insemination trials of the ocean pout eggs from 1991 to 1994.

8.3. MATERIALS AND METHODS

8.3.1 Fish

Adult male and female ocean pout for this study were collected in Conception Bay by SCUBA divers and kept in captivity as was described in the previous Chapters.

8.3.2 Experiment 1

The first *in vitro* artificial insemination trial (Trial #1) was conducted in August, 1991 to test if the fish is an internal or external fertilizer. For example, if the fish was an external fertilizer, a successful *in vitro* fertilization would occur soon after additions of milt and seawater to the eggs (in a short contact period between the eggs and sperm, i.e., treatment 2 and 3 in Table 1). Otherwise the successful *in vitro* fertilization should occur after a long contact period between the eggs and sperm before addition of seawater. Table 1 would also assist with the determination of optimal contact period between the eggs and sperm for successful fertilization.

Eggs were stripped from a prespawning female after the ovipore was opened and milt from a mature male following the procedures described in Chapter 6. Both the eggs and milt were retained on ice while conducting *in vitro* artificial insemination. Ten treatments with 3 replicates were established according to Table 1. Each group of approximately 45 eggs (46 $\pm$ 6 eggs) were placed into separate 200 ml beakers and gently stirred with 30 µl fresh milt. At various times after insemination, 100 ml of 9-12°C seawater containing penicillin G (13.3 mg/l) and streptomycin sulphate (20 mg/l) was added to the eggs and the incubation of eggs started (at 9-12°C). Seawater in the beakers was changed and the eggs checked daily under a binocular microscope for signs of fertilization, e.g., cell division to 4- or 8-cells.

Table 1 *In vitro* insemination trial in 1991

Treatment	Milt	Duration of sperm-egg contact
1 (control)	-	0 hr,
2	+	1/12hr (5 min.)
3	+	1/6 hr (10 ..)
4	+	1/2 .. (30 ..)
5	+	1 .. (60 ..)
6	+	4 ..
7	+	8 ..
8	+	16 ..
9	+	32 ..
10	+	72 ..

(-): no milt was added (control); (+): milt was added

8.3.3 Experiment 2

A second trial (Trial #2) was conducted in August, 1993 following a similar protocol to the initial trial (#1). However, these eggs in beakers were immersed in flowing ambient seawater (10-12°C) and checked at days 3 and 10 for evidence of fertilization.

8.3.4 Experiment 3

Three trials of artificial *in vitro* insemination of the ocean pout eggs were conducted in August 1994. A milt diluent (183 mM NaCl, 10 mM $KHCO_3$, 1.45 mM $CaCO_3$, 0.84 mM $MgSO_4$ $\cdot 7H_2O$ and 0.15-0.17 mM glucose, pH 7.2-7.5, osmolarity between 290-310 mOsM) formulated according to the ionic composition of ocean pout seminal plasma previously measured (Chapter 6) was developed for this *in vitro* artificial insemination. Ocean pout sperm showed an increased motility and a longer life span when diluted 2-5 times in this milt diluent.

Eggs were stripped from ovulated females into beakers on crushed ice and divided into aliquots in separate 200 ml beakers. Six insemination treatments with duplication were conducted according to Table 2 by adding the following milt samples to the eggs: (1) no milt was added to eggs (control); (2) fresh milt (containing ≥70% motile sperm) was added to eggs; (3) and (4) fresh milt (at two different volumes, respectively), diluted 1:3 (v/v) in milt diluent; (5) cool stored milt, diluted 1:3 in milt diluent; (6) cryopreserved milt, diluted 1:3 in milt diluent, frozen in liquid nitrogen (-190°C) and thawed (reported separately). The eggs and milt were retained over crushed ice for 5 hr in a covered container and were gently stirred every half hour during the first 1.5 hr before being transferred into ambient (13-15°C) seawater in up-welling incubators (Trials #3.1 & #3.3) or in flow-through pans (Trial #3.2) for incubation. The eggs were checked on days 4, 7-8 under a binocular microscope for evidence of fertilization.

Table 2. *In vitro* insemination trials in 1994

Trial#	female GSI (%)	eggs no. /group	treatments and milt volume (ml), (1)	(2)	(3)	(4)	(5)	(6)
#3.1	31.3	170-180	0	1.5	1.5	0.5	2.0	-
#3.2	30.9	220-230	0	2.0	2.0	-	2.0	2.0
#3.3	32.2	260-270	0	2.5	-	-	-	2.5

8.4. RESULTS

Although eggs were incubated at 9-12°C in 200 ml beakers and the seawater was changed daily in the first trial (Trial #1), no signs of egg fertilization were observed. Egg incubation was terminated after one week because all of the eggs had died.

Again in 1993 a second trial (Trial #2) was not successful since the eggs were incubated in 200 ml beakers and water exchange between the beaker and the tank was poor; all of the eggs died before fertilization could be determined. In 1994, successful *in vitro* artificial insemination of the ocean pout eggs were achieved, particularly with those eggs incubated in the up-welling incubators and supplied with continuous up-flowing seawater (Trial #3.1). The newly stripped eggs were 8.6 $\pm$ 0.3 mm in diameter and contained many centralized tiny oil-granules. After 4 days incubation, the fertilized eggs became opaque with little change in the plasmic distribution of the oil-granules and these eggs adhered to each other forming an egg-mass, while non-fertilized eggs became transparent with the plasmic oil-granules fusing into 1-3 large oil globules (Chapter 6). In the up-welling incubators, the non-fertilized eggs had less buoyancy than the fertilized eggs and flowed in the water column. With time of incubation, the difference between fertilized and non-fertilized eggs became more distinct and non-fertilized eggs eventually collapsed. In the fertilized (opaque) eggs, small primitive embryos started to appear by days 7.5-8 at the 13-15°C incubation conditions. The highest egg fertilization rates, determined on day 4, were obtained with the use of fresh milt and under well-oxygenated incubation conditions found in the up-welling incubators (Table 3).

Table 3. Percentage (%) fertilization rate of *in vitro* artificially inseminated eggs in 1994.

Trial#	treatments (1)	(2)	(3)	(4)	(5)	(6)
#3.1	0	90.6	85.2	88.9	77.2	-
	0	87.4	84.0	83.1	63.4	-
#3.2	0	0	18.7	-	15.8	2.3
	0	20.0	29.5	-	10.1	0
#3.3	0	48.3	-	-	-	32.6
	0	57.9	-	-	-	ND

-: not conducted; ND: not determined; each value (fertilization rate,%) in the table was calculated from an examination of 150-210 eggs.

8.5. DISCUSSION

This chapter describes trials of *in vitro* artificial insemination of ocean pout eggs leading to successful production of fertilized ocean pout eggs in 1994.

It was learned from the trials conducted in 1991 and 1993 that the ocean pout eggs have a high oxygen demand and need a good water flow during incubation. In 1994, new up-welling incubators were developed for the purpose of incubation of the *in vitro* inseminated ocean pout eggs and they provided a continuous water flow and eggs remained clean and viable. The high fertilization rates (80-90%) in Trial #3.1 likely resulted from the high sperm:egg ratio (≤200 eggs/0.5-2.0 ml milt). Fertilization rates were comparable between the treatments of (2), (3) and (4), suggesting that a deleterious

effect of the milt dilution was absent and that a milt volume of 0.5-1.5 ml represented sperm saturation (for fertilization of $\leq$ 200 eggs). Fertilization rate using cool stored milt dropped to 63-77%, indicating a reduction of fertility of the stored sperm. In Trial #3.3 the inseminated eggs were also incubated in the up-welling incubators but fertilization rates were considerably lower (48-58%) even with the use of fresh milt. In Trial #3.3, the stripping might have been applied to the female too early since the eggs were a little more difficult to strip indicating an incomplete ovulation. Plastic flow-through pans were apparently not good for incubating eggs since water exchange through the fine screens was very poor, which might have resulted in the low fertilization rates in these pans.

Cryopreserved milt was used for *in vitro* insemination in two trials (#3.2 and #3.3). Since maximum recovery of motility in cryopreserved milt was $\leq$ 20-25% after thawing (unpublished data), this perhaps explains the reduction in fertilization rates in those trials using cryopreserved milt.

For *in vitro* artificial insemination, the timing of stripping may be crucial since eggs can only be stripped when the ovipore has completely opened (0.5-1.0 cm in diameter). Premature stripping could induce atresia of the eggs in the ovary and the stressed females retained their eggs and did not spawn spontaneously after this premature stripping. In contrast to the *in vitro* artificial insemination, females can be inseminated *in vivo* as soon as the ovipore begins to open and produce fertilized eggs (Chapter 6).

Successful *in vitro* artificial insemination of wolffish eggs was recently reported by Pavlov and Moksness (1994), who demonstrated that (1) a minimum sperm:egg ratio of above 0.2×10^6 spermatozoa per egg is required for a successful *in vitro* artificial

fertilization of the wolffish eggs (2) a contact period of 4-6 hr between eggs and sperm produces maximal fertilization rates and (3) milt diluted 1:3 in a modified Ringer solution (Cobb et al., 1973) improves egg fertilization. These results were consistent with the present study of *in vitro* insemination in the ocean pout, where a good fertilization rate was achieved after a 5 hr contact period between the eggs and sperm. It was noted that dilution of ocean pout milt with the newly developed milt diluent enhanced sperm motility and prolonged life-span of ocean pout sperm. Diluted milt for insemination would presumably assist with the spread of sperm thus increasing the success of fertilization of eggs, particularly when the volume of milt is limited and the eggs are big.

CHAPTER 9

GENERAL DISCUSSION

The ocean pout (*Macrozoarces americanus* L.) has been proposed as an alternative species for cold-water aquaculture in Eastern Canada (Brown et al., 1989). Since extensive knowledge of embryonic development and larval culture of this species has been obtained through studies of fertilized eggs collected in the wild (Methven and Brown 1991; Brown et al., 1992), the capability to spawn the fish in captivity represents a break-through for domestication of this species.

Efforts were made in this study to understand the reproductive physiology and the biology of egg fertilization of this marine species. This included studies of the biochemistry of the egg yolk precursor protein, vitellogenin (Chapter 2), the seasonal reproductive cycle, the environmental and hormonal regulation of the seasonal reproductive cycle (Chapter 5) and the biology of egg fertilization (Chapter 6, 7 and 8).

As in other species, vitellogenesis can effectively be induced by the ovarian steroid, 17ß-estradiol, in the ocean pout. Vitellogenin is a large glycolipophosphoprotein molecule (577 KD) rich in lipid (20-21%) and some essential amino acids (Chapter 2). The similarity of the amino acid composition of vitellogenins from various species, including the ocean pout, seems to support the hypothesis that vitellogenin genes are highly conserved in non-mammalian oviparous animals (Tata 1987; Lee et al., 1992). Differences in the vitellogenins from various species are noted in the phospholipid content of vitellogenin, which indicates a difference in the post-translational modification of the vitellogenins. This perhaps reflects a different reproductive strategy of the different fish species since the phospholipid content of vitellogenin affects the receptor-mediated pinocytosis of vitellogenin into oocytes (Stifani et al., 1990), the biomembrane as well as

the larval development of fish (Rainuzzo et al., 1992). Vitellogenin was also detected in the skin mucus of adult female ocean pout (Chapter 4). This allows a sex determination of the adult ocean pout through detecting the presence of vitellogenin in skin mucus because vitellogenin is a female specific protein. This method of sex determination is easy to conduct and less deleterious to the fish.

This study suggests that the seasonal reproductive cycle of the female ocean pout can be organized into 4 phases according to the seasonal changes in GSI, oocyte growth and the plasma steroids levels (Chapter 5). These phases are 1) a quiescent phase in post-spawned fish lasting from August-September to February, 2) the preparatory phase (March-May), 3) a rapid ovarian growth phase (June-August) and 4) the spawning phase (August-September). The seasonal reproductive cycle of adult males is composed of inactive (October-May) and active (June-September) phases. In the active phase, the plasma testosterone and 11-ketotestosterone increase and peak in June, which is followed by a remarkable increase of GSI and the initiation of spermiation from July-September.

Alteration of water temperature in the winter (5°C, compared with colder ambient temperatures) does not affect the reproductive cycle and the growth rate of fish, although the rate of food consumption is altered (Chapter 5). Perhaps the presence of the antifreeze protein in the plasma (Fletcher et al., 1985; King et al., 1989) allows the fish to utilize energy (food) more efficiently at low water temperatures than at warmer temperatures (5°C). Alterations of the photoperiod cycle clearly affects the spawning season of the ocean pout. Long days (18L:6D) stimulate steroidogenesis and vitellogenesis and advance the spawning season of fish for 2-4 weeks as compared to fish exposed to short days

(Chapter 5). Cold ambient seawater temperatures in the winter were not inhibitory to steroidogenesis and vitellogenesis but seemed inhibitory to ovarian incorporation of vitellogenin thus inhibiting ovarian growth (Chapter 5). A combination of long days and warmer water temperatures in the winter might be able to induce steroidogenesis, vitellogenesis and the ovarian incorporation of vitellogenin and thus more effectively advancing the spawning season of ocean pout. Although little is known about the regulatory mechanism of photoperiod on reproduction of fish, it is suggested that the pineal gland in the epithalamus plays a decisive role in this regulation but more studies are needed.

This study has shown the ocean pout is an internal fertilizer, i.e., eggs are fertilized in the ovary. Supportive evidence includes sperm physiology (Chapter 6), the behavioral observations of fish copulation and spawning (Chapter 7) and a successful artificial insemination of eggs by both *in vivo* (injecting milt into the ovaries of mature females) and *in vitro* inseminations producing fertilized eggs. To produce fertilized eggs, *in vivo* insemination should be conducted shortly after the opening of the female ovipore (Chapter 6), while for artificial insemination *in vitro* eggs and sperm should have a 5-hour contact period before addition of seawater (Chapter 8). Larvae were hatched from *in vivo* artificially inseminated eggs. Behavioral studies have emphasized the importance of parental guarding for prevention of fungal infection and leech infestation of the eggs (Chapter 7). These studies, both the *in vivo* and *in vitro* artificial insemination and the behavioral observations, provide valuable information for hatchery management of the ocean pout reproduction.

In conclusion, an in-depth knowledge of the husbandry, reproductive physiology and endocrinology, and the biology of egg fertilization of the ocean pout has been obtained through the current study. One major contribution of the current study is that it has allowed the completion of the reproductive cycle of the ocean pout in captivity and the production of fertilized eggs and larvae through artificial insemination.

10. APPENDIX

I. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Buffers:

Solution A:	1.0 N HCl	48 ml	96 ml
	Tris (Base)	36.6 ml	73.2 ml
	TEMED*	0.46 ml	0.92 ml
	$D.H_2O$** to	100 ml	200 ml

(*: N.N.N'N'-Tetramethylethylenediamine, ** : distilled water)

		4%	5%	7.5%
Solution B:	Acrylamide	16 g	20g	30g
	BIS	0.43g	0.53g	0.8g
	Potassium ferricyanide	15mg	15mg	15mg
	$D.H_2O$ to	100ml	100ml	100ml

Ammonium per sulfate (APS) (0.14%):

0.014 g in 10 ml $D.H_2O$ *or* 0.07 g in 50 ml $D.H_2O$

Procedures:

5% gel for PAGE	ratio		volume
Solution A	1		4 ml
Solution B	2		8 ml
$D.H_2O$	1		4 ml
APS	4	+	16 ml
			32 ml

N.B. * solutions are mixed and filled into electrophoresis apparatus for polymerization.

** electrophoresis run at 200 Volt

Tank Buffer (stock solution)*:

Tris (base)		6 g	12g
Glycine		28.8g	57.6g
$D.H_2O$	to	1000 ml	2000 ml pH 8.3

* stored in fridge (4°C), dilute 10x for use

Protein stain:

1). Staining solution (1.0%)*:

Coomassie Brilliant Blue R-250	1.0 g
DH_2O	100 ml

* stored in a fridge (4°C), diluted 1:10 with 7% acetic acid for use.

**stain gel over night.

2). Destaining solution*:

Methanol		250 ml
Acetic acid		100 ml
DH_2O	to	1000 ml

*remove gel(s) from the staining solution; add the destaining solution to the gel(s), change several times or until gel is completely destained.

Lipoprotein stain:

1). Staining solution:

Sudan Black B		0.5 g
Acetone		20 ml
Acetic acid		15 ml
$D.H_2O$	to	100 ml

2). Destaining solution:

Acetic acid		150 ml
Acetone		200 ml
$D.H_2O$	to	1000 ml

Procedures:

- dissolve Sudan Black B in acetone before addition of acetic acid and $D.H_2O$, stirred for 30 min and filter the solution,
- stain the gel(s) overnight,
- destain the gel(s) with 3 changes (or more) until the protein band appears clearly.

II. TESTOSTERONE & 17ß-ESTRADIOL RADIOIMMUNOASSAY (RIA)

Assay buffer

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

Na_2HPO_4	6.30 g/l
KH_2PO_4	0.75 g/l
NaCl	5.86 g/l
NaN_3	1.00 g/l
$D.H_2O$	1000 ml, pH 7.4

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

Label

Make the appropriate assay dilution using assay buffer such that there is approximately 10,000 cpm/100µl. **NOTE**: This isotope is stored in buffer not ethanol and does not need to be dried down.

The recovery estimates will be done using the tritiated isotope. Using the dilution of 1000 cpm/10µl.

Antisera
The antibody is stored at -20°C The dilution is made up in assay buffer.

Standard curve
The standard curve is prepared in ethanol. The final volumes are dried down under N_2 in the RIA tube and brought back to 100µl with assay buffer.
Stock solution: 1 mg/10ml ethanol
Prepare: 100x = 100µl stock brought to 10 ml ethanol
10,000x = 100µl (100x) brought to 10 ml ethanol

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

Preparation of samples
Untreated or suspected low level samples: 100µl plasma
Treated or suspected high level samples: 25-50µl plasma.

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

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

Dry down the ether under N_2 and add 1ml of distilled ethanol to the resultant solute. Vortex 1 min. Allow the samples to sit at room temperature for at least 1 hour or overnight at 4° C before doing the recovery estimates. Count 100µl for 20 min.

PROTOCOL

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

NB: all reagents must be at room temperature!!

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

-Add 200µl assay buffer, vortex.

-Add 100µl isotope, 100µl antibody (working dilution), Vortex, let stand 1 hr at room temperature.

-Add 1.0 ml of separating reagent, vortex and let sit @ room temperature for 25min.

-Centrifuge at 1000 x g for 15 minutes.

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

III. PACKING GEL CHROMATOGRAPHIC COLUMN

Procedures: see attached reprint (see page 180-185)

Tris Buffer:	Tris (base)	50 mM
	NaCl	0.5 M
	EDTA	10 mM
	aprotinin	100 TIU/l, pH 8,

IV. PROTEIN PHOSPHORUS AND LIPID CONTENTS ANALYSIS

1. Procedures for total phosphorus analysis

-add 2.0 ml sample (or extract, or standard) in a 12 ml conical centrifuge tube,
-add 0.5 ml 10 N H_2SO_4, vortex,
-heated in oven (150-160°C) for at least 3 hr,
-add 2 drops of 30% H_2O_2, returned to oven (150-160°C) for at least 1.5 hr,
-add 4.6ml 0.22% ammonium molybdate (a.moly),
-add 0.2 ml Fiske-Subbarow reagent, vortex,
-placed in a boiling water bath for 7 min. covered by marbles,
-cool in room, measured at 830 nm (O.D, optical density)

2. Procedures of inorganic orthophosphate determination:

-add 4.4 sample (or 2.0ml sample +2.1ml $D.H_2O$) in a 12ml conical centrifuge tube
-add 0.2 ml 10 N H_2SO_4,
-add 0.2 ml 5% a.moly,
-add 0.2 ml Fiske & SubbaRow regent, vortex,
-keep at room temperature for 7 min, measured at 660 nm (O.D),
-add 4.1 sample (or 2.0 ml sample + 2.1 ml $D.H_2O$) in a 12 ml conical centrifuge tube;
-add 0.5 ml 10 N H_2SO_4
-0.2 ml 5% a.moly,
-0.2 ml Fiske and SubbaRow regent, vortex,
-keep in a boiling water bath for 7 min, cool to room temperature,
-measured at 830 nm (O.D).

a). Fiske-SubbaRow reagent:
0.5 g 1-amino-2-naphthol-4-sulfonic acid (Eastman Organic Chemicals), dissolved in freshly prepared 200 ml 15% sodium bisulphate (anhydrous); add 1.0 g anhydrous sodium sulfate. NB: the solution should be filtered, stored in a dark bottle, and freshly prepared weekly.

b). Standard preparation
Stock phosphorus solutions: 2.1956g KH_2PO_4 in 500 ml 1 N H_2SO_4 (i); pipette 1.0 ml (i) solution to 50 ml $D.H_2O$ (ii, 20 µg P/ml); pipette 1.0 ml (ii) to 20 ml $D.H_2O$ (2 µg P/ml, work solution). Detecting range (standard curve): 0--7 µg phosphorus.

3. Procedures for the measurement of lipid-bound phosphate

Lipid Extraction

-25 µl sample or plasma etc.
-add 5 ml ice-chilled 20% TCA,
-centrifuge at 2000 g for 5 min, precipitate retained,
-add 2.0 ml methanol:chloroform:saline(0.9% NaCl) (2:1:1), vortex,
-chloroform retained (1x),
-add another 2.0 ml methanol:chloroform:saline solution to the water phase, vortex,
-hloroform retained (2x),
-chloroform (1x, 2x) pooled.

Lipid-bound phosphorus determination:

2.0 ml chloroform extract is evaporated to dry,
bring back to 2.0 ml with $D.H_2O$ and
follows the procedures of total phosphate measurement.

4. Procedures for lipid determination (charring method)

-samples (or series of standard concentration)
-N_2 blow at 80-100°C to dryness,
-add 2.0 ml concentrated sulphuric acid for 15 sec.
-keep in 200°C heating block for 15 min,
-placed in water bath at room temperature for 15 sec.,
-placed in ice bath for 15 min,
-add 3 ml $D.H_2O$, vortex,
-returned to ice bath for 10 min (or till all bubbles disappeared),
-measured for absorbency (O.D) at 375 nm

V. IODINATION OF VITELLOGENIN

-iodogen preparation: 1,3,4,6-tetrachloro-3α,6α-diphenul-glucoluril dissolved in dichloromethane, 1mg/ml
-add 5-20 µl iodogen to a disposable tube (6x50mm) or eppendorf tube, air dry.
-add 10 µg vitellogenin (in 25µl sodium phosphate buffer, 0.5 M, pH7.4.
-add 1.5 mCi I^{131} or 1.0 mCi I^{125} (NaI^{125}), react for 12 min.
-add 600 µl of 0.05 M phosphate buffer to terminate the reaction.
-the labelled vitellogenin is separated from free I^{125} on a disposable PD10 Sephadex G-25M column. The column was pre-saturated with bovine serum albumin to reduce non-specific binding of the labelled vitellogenin to the column. Fractions are collected and counted for radioactivity.
-the labelled vitellogenin is stored in fridge (4°C) in short term (1-2 weeks) or in 50% glycerol at -20°C for longer term.
-stability of the labelled vitellogenin is assessed by gel-filtration.

VI. VITELLOGENIN RIA

Buffers:

Barbitol buffer (0.08 M):

barbitol,		10.0 g
sodium acetate		6.5 g
$D.H_2O$	to	2000 ml, pH 8.6

* stored in fridge (4 °C) (stock solution)

Barbitol work solution:

stock solution		250 ml
bovine serum albumin	(0.5%)	1.25 g
thimerosal	(0.01%)	0.025 g

PROTOCOL

tube#	ID		STD#	Vol	label	AB	buffer	NRS/GAR
1,2	CT				200µl	-	-	- / -
3,4	0 AB				"	-	400µl	100/100µl
5,6	0.00	ng			"	200µl	200µl	" / "
7,8	0.07	"	12	100µl	"	"	100µl	" / "
9,10	0.13	"	11	"	"	"	"	" / "
11,12	0.27	"	10	"	"	"	"	" / "
13,14	0.54	"	9	"	"	"	"	" / "
15,16	1.10	"	8	"	"	"	"	" / "
17,18	2.2	"	7	"	"	"	"	" / "
19,20	4.3	"	6	"	"	"	"	" / "
21,22	8.6	"	5	"	"	"	"	" / "
23,24	17.2	"	4	"	"	"	"	" / "
25,26	34.5	"	3	"	"	"	"	" / "
27,28	68.9	"	2	"	"	"	"	" / "
29,30	137.9	"	1	"	"	"	"	" / "
31,32	sample				100µl	"	"	" / "

-add 100 µl vitellogenin (VTG) standard solution or sample to RIA tubes.
-add 200 µl label (I^{125}vitellogenin) (ca.25.000 CPM).
-add 200 µl diluted antiserum (AB, 25600x).
-gently shake the tube to mix the solution, let stand 3 hr to overnight at room temperature (incubation).
-add 100 µl NRS (normal rabbit serum, 80x) and 100 µl GAR (goat anti-rabbit γ- globulin, 20x) to RIA tubes
-gently shake the tube to mix the solution, let stand 6 hr at room temperature.
-centrifuge at 1000 x g, 15 minutes.
-aspirate supernatant carefully, count pellet 1.0 minute in gamma counter.

VII. VITELLOGENIN ELISA

Buffers

Sodium bicarbonate buffer (SBB)

$NaHCO_3$	(50 mM)	4.2006 g	
gentamycin		5 g	
$D.H_2O$		1000 ml	pH 9.6

Tris buffer (TBS-T)

Tris-HCl	(10 mM)	1.211 g	
NaCl	(0.15 M)	8.766 g	
Tween	(0.1 %)	1 ml	
gentamycin		5 mg	
$D.H_2O$		1000 ml	pH 7.5

TBS-T-SG (TBS-T + 2% normal goat serum)

** 10 g goat serum dissolved in 10 ml distilled water, aliquoted, 1 ml/aliquot; 2 g/2 ml/100 ml TBS-T (2%).

50 mM ammonium acetate-citric acid (AACA buffer)

50 mM ammonium acetate: 0.3854 g dissolved in 100ml H_2O (pH 6.68),

50 mM citric acid: 0.5254 g dissolved in 50 ml H_2O (pH 2.13), adjust the pH of ammonium acetate to 5.0 using 50 mM citric acid.

OPD solution (**prepared just before use, fresh !**)

0.5 g/l 1.2-phenylene diamine:	18 mg
0.5 ml/l 30% hydrogen peroxide (H_2O_2):	18 µl
AACA buffer:	36 ml.

N.B: 1,2-phenylene diamine toxic, working on tin-fold, wearing gloves.

Vitellogenin standard curve preparation: Standard curve contains 12 levels:
tube #1-10, VTG standard curve: 200-0.4 ng/ml (or 10-0.02 ng/50 µl/well),
tube #11: 0.0 VTG, antibody added, total count (B),
tube #12: 0.0 VTG, no antibody added, blank count (Bo).

Regular procedure (Maisse et al., 1991)

1.Coating:	-vitellogenin diluted in SBB and added into each well,
	-4.1 ng vitellogenin/150µl/well,
	-incubated at 37°C, 3 hr,
2.Washing:	-washed 5 times with TBS-T buffer, 200µl/washing,
3.Saturation:	-add TBS-T-SG (goat serum), 200µl/well,
	-incubated at 37°C, 30 min.,
	-solution discarded, no washing,

4.First antibody:	-100µl vitellogenin-antibody (1st antibody), 1:25600x in TBS-T-SG, -50µl vitellogenin standard or sample diluted in TBS-T containing 0.5% BSA, -incubated at 20°C, 16 hr,
5.Washing:	-washed 5 times with TBS-T buffer, 200µl/washing,
6.Second antibody:	-150µl sheep anti rabbit γ-globulin, 1:2000 in TBS-T-SG, -incubated at 37 °C, 2 hr,
7.Washing:	-washed 5 times with TBS-T buffer, 200µl/washing,
8.Colouring:	-add 150µl OPD/well -incubated at 20°C, 1 hr, at dark
9.Stop colouring:	-add 50µl 5 M sulphuric acid -shaking on auto-shaker for 10 min.
10. O.D reading*:	-Microplate Autoreader, at double wavelength (490 and 630 nm) *O.D: optical density

Modified Procedure:

1.Coating:	-vitellogenin diluted in SBB and added into each well, -4.0 ng vitellogenin/150µl/well, -incubated at 37°C, 3 hr,
2.Washing:	-washed 5 times with TBS-T buffer, 200µl/washing,
3.Saturation:	-add TBS-T-SG (goat serum), 200µl/well, -incubated at 37°C, 30 min., -solution discarded, no washing,
4.Reaction:	-50µl vitellogenin-antibody (1st antibody), 1:25600, -50µl standard or sample, -50µl sheep anti rabbit γ-globulin (2nd antibody), 1:2000, -incubated at 20°C, 16 hr,
5.Washing:	-washed 5 times with TBS-T buffer, 200µl/washing,
6.Colouring:	-add 150µl OPD/well, -incubated at 20°C, 1 hr, at dark,
7.stop colouring:	-add 50µl 5 M sulphuric acid, -shaking on auto-shaker for 10 min.,
8.O.D reading:	-Microplate Autoreader, at double wavelength (490 and 630 nm).

VIII. GILSON FIXATIVE (for egg fixation)

Reagents

60% ethanol	100 ml
distilled water	880 ml
80% nitric acid (concentrated)	15 ml
glacial acetic acid	18 ml
mercuric chloride	20g (or 20 ml forn alin)

preparation:
-pour distilled water first
-nitric acid
-glacial acetic acid
-alcohol
-mercuric chloride
-put in stirrer/ stirring

IX. 11-KETOTESTOSTERONE

Assay Buffer

$NaH_2PO_4.H_2O$ (28 mM)	3.87g	
Na_2HPO_4 (61 mM)	8.66g	
NaCl (154 mM)	9.00g	
$D.H_2O$	1000 ml,	pH 7.0

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

Label

For recovery:	1000 cpm/10µl
For assay:	5,000 cpm/100µl

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

For example:
10µl stock isotope = 90,000 cpm
for each tube we need 5000~8000 cpm and e a final volume of 100µl/tube.
If we have 200 tubes for this assay, we will need :
200 x (5000~8000) = 1,000,000 ~ 1,600,000 cpms for the entire assay in a final volume of 200 x 100 µl = 20000 µl or 20 ml dilution.
Since we have 90,000 cpm/10 µl stock isotope, we will need:
10 x [1,000,000 ~ 1,600,00]/90,000 = 110 ~ 176 µl of stock isotope.

Antisera
The antibody is stored in the -20°C freezer. It is stored in 50µl aliquot (100x). Presently we are using either 1:60,000 or 1:70,000 dilution for RIA.

Standard curve
(see that for testosterone and estradiol)

Separation

Dextran-Coated Charcoal

Kodak Charcoal RIA Grade (0.25% w/v)	1.25g
Dextran T-70 (0.025% w/v)	0.125g
Assay Buffer	500 ml

Sample extraction and Preparation

(see that for testosterone and estradiol)

PROTOCOL

#	ID	Std#	Volume	Buffer	AB	Isotope	Char.
1,2	CT			800µl	-	100µl	-
3,4	O AB			200µl	-	"	600µl
5,6	O Hormone			100µl	100µl	"	"
7,8	0.98 pg	11	100µl	"	"	"	"
9,10	1.95 pg	10	"	"	"	"	"
11,12	3.9 pg	9	"	"	"	"	"
13,14	7.8 pg	8	"	"	"	"	"
15,16	15.6 pg	7	"	"	"	"	"
17,18	31.2 pg	6	"	"	"	"	"
19,20	62.5 pg	5	"	"	"	"	"
21,22	125.0 pg	4	"	"	"	"	"
23,24	250.0 pg	3	"	"	"	"	"
25,26	500.0 pg	2	"	"	"	"	"
27,28	750.0 pg	1	75µl	"	"	"	"
29,30	1000.0pg	1	100µl	"	"	"	"
31,32	sample		100µl	"	"	"	"

-Add standards and samples to RIA tubes (in ethanol), dry down under N_2

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

-Add 100µl isotope (5,000 cpm/100µl)

-Add 100µl antibody (at appropriate dilution)

-Incubate overnight at 4°C

-Add 600µl charcoal suspension (@ 4°C), vortex - Leave 1 hour on ice.

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

-Centrifuge at 1000 x g for 25 min.

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

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SEPHACRYL® S-200, S-300, S-400 High Resolution

To obtain good resolution in gel filtration, it is important that the top of the column be well packed. An air bubble or a small disturbance in the gel surface disturbs the sample application and the sample zone broadens. This broadening is amplified as the zone migrates down the column. The result is broader peaks and lost resolution.

With traditional packing methods you often get good results. However, a disadvantage is that the gel becomes most tightly packed at the bottom of the column, instead of at the top.

Pharmacia has developed an improved packing method which results in increased resolution. A short version of the packing method follows:

1. Insert an adaptor at the bottom of the column.
2. Pour the gel into the column and pack the column in 2 steps.
3. Insert the bottom piece or a second adaptor at the top.
4. Turn the column upside-down.

The sample will be applied in the most tightly packed zone of the gel, now at the top of the column. The result will be improved resolution.

We recommend you to follow this procedure since it has been shown to give the best results.

Equipment needed

	Laboratory scale		Process scale
Pump	P-1, P-3 or P-500		~1 800 ml/h
Column*	K 16/40 K 16/70 K 16/100	K 26/40 K 26/70 K 26/100	K 50/60 K 50/100
Cross-sectional area of the column	2.0 cm^2	5.3 cm^2	19.6 cm^2
Adaptor	A 16	A 26	A 50
Packing reservoir	R 15/16	R 25/26	R 50

* The first number in the column name refers to the inner diameter of the column in mm. The second number refers to the length of the column in cm.

C and HR columns can also be used. The C columns must be used with the appropriate thermostat jacket.

For preparative chromatography we recommend column K 26/70.

Measuring cylinder
Large beaker
Buffer**
Glass rod
Small spoon or plastic spatula
(Pasteur pipette)

** The buffer may be degassed, but it is usually not necessary.

Pharmacia
Laboratory Separation Division

To prepare the gel suspension

1. Determine the desired packed bed volume by multiplying the cross-sectional area of the column (see table above) by the desired bed height.
2. Gently shake the bottle of Sephacryl HR to make an even slurry.
3. Measure out the required volume of gel slurry, 1.5 × the desired packed gel volume*, using a measuring cylinder and pour it into a beaker.
4. Dilute the gel suspension with eluent buffer to 2 × the desired packed gel volume.
5. Stir with a glass rod to make a homogeneous suspension** free from aggregates. Never use a magnetic stirrer.

* The required volume of settled gel is about 1.1 x the desired packed gel volume.
** The gel suspension may be degassed, but it is usually not necessary.

To pack the column

Note: columns may be packed using either one adaptor and a bottom piece, or two adaptors. The packing methods for these two arrangements differ only in point 10 and 13.

Pack the column at the temperature at which it will be used.

1. Make sure the column is not damaged and that all parts are really clean. It is of special importance that the nets, net fasteners and glass tube are not damaged.
2. Attach the packing reservoir tightly (don't forget the sealing ring) and mount the column vertically on a stand.
3. Wet the adaptor by drawing water through it, making sure no air bubbles are trapped under the net. This is best done by submerging the plunger in a beaker of water and attaching the tubing to a pump (Fig. 1) or a syringe. Close the tubing with a stopper when all air bubbles have been removed.

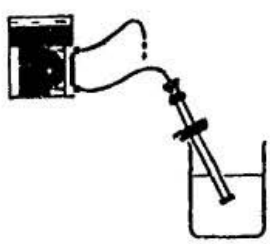


Fig. 1.

4. Insert the adaptor at the bottom of the column far enough to give the desired bed height.
5. Wet the column glass tube with eluent leaving a few centimeters of fluid in the bottom. Make sure the net is completely free from air bubbles.

6. Resuspend the gel and pour the well-mixed gel suspension carefully down the wall of the column using a glass rod (Fig. 2). Pour all the gel in one operation. Fill the reservoir to the top with buffer.

7. Screw on the reservoir cap tightly. Connect it to the pump. Open the outlet (Fig. 3).

Fig. 2.

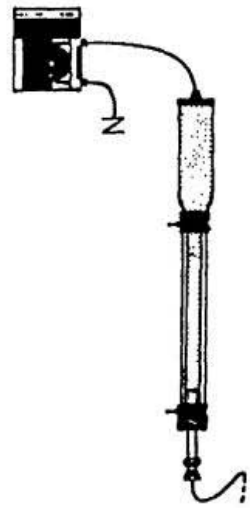


Fig. 3.

8. Pack the column in two steps using the flow rates given in the table below. Pack the gel in STEP 1 for 2 hours or until the gel has reached a constant height. Then increase the flow rate to the value listed for STEP 2 and pack for 60 minutes.

Packing flow rates (ml/h)

	Sephacryl S-200 HR		Sephacryl S-300 HR		Sephacryl S-400 HR	
Column	STEP 1	STEP 2	STEP 1	STEP 2	STEP 1	STEP 2
K 16/40	60	150	60	190	60	250
K 16/70	60	110	60	140	60	180
K 16/100	60	100	60	120	60	160
K 26/40	150	410	240	490	240	650
K 26/70	150	300	240	360	240	480
K 26/100	150	270	180	320	240	430
K 50/60	600	1150	600	1400	600	1800
K 50/100	500	800	600	950	600	1300

9. Stop the pump and close the outlet. Remove the packing reservoir. This is most easily done by first removing the column from the stand and then unscrewing the reservoir over a sink (Fig. 4). For larger columns it may be easier to use a siphon.

10. **Using one adaptor and a bottom piece.** Remove excess gel carefully with a small spoon or a plastic spatula. The bed surface should be about 2 mm below the end of the glass tube. When the bottom piece is inserted in point 13, it will be pressed about 5 mm into the gel. If there is not enough gel in the column it will be necessary to use an adaptor (see below) or to repack the column with excess gel.

    Using two adaptors. Remove excess gel by gently stirring the top of the bed with a glass rod and removing the suspended gel with a Pasteur pipette. Remove enough gel so that the plunger will be visible below the end piece.

11. Mount the column vertically on the stand and fill the column to the top with buffer.

12. Wet the bottom piece (or a second adaptor) as described above (3).

13. **Using one adaptor and a bottom piece.** Insert the bottom piece carefully so that no air bubbles are trapped under the net (Fig. 5). Open the bottom piece outlet (not the outlet from the adaptor at the bottom of the column). Then press down and tighten the bottom piece. Close the bottom piece outlet again.

    Using two adaptors. Insert the second adaptor carefully so that no air bubbles are trapped under the net, as shown for the insertion of the bottom piece in Fig. 5. Remove the stopper from the second adaptor tubing (not from the adaptor at the bottom of the column). Bring the adaptor down into the column and make sure that there are no air bubbles under the net. Bring the adaptor to the gel surface and then a further 5 mm into the gel. Tighten the O-ring above the plunger and lock the adaptor in this position. Close the adaptor tubing with a stopper.

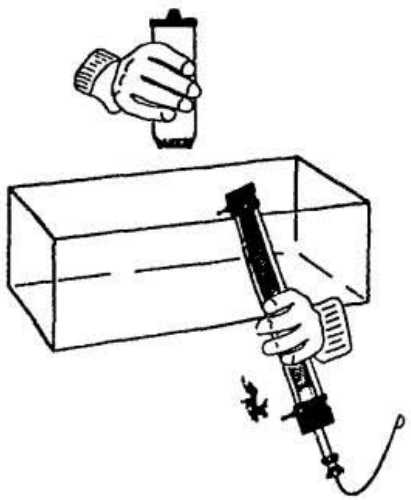


Fig. 4.

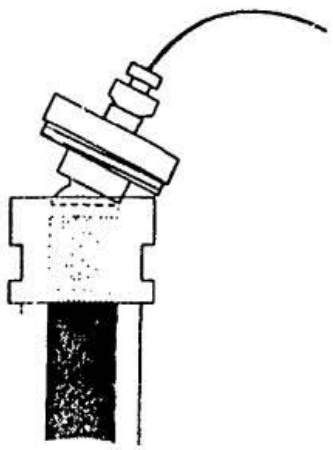


Fig. 5.

14. Run the pump to remove the air from the pump tubing.
15. Remove the stopper from the first adaptor (Fig. 6a). If there is an air bubble in the tubing, remove it by opening the upper outlet for a few seconds. Connect the tubing from the first adaptor to the pump or a valve (Fig. 6a).
16. Turn the column upside-down (Fig. 6) or use it with upward flow.
17. When running the column, do not exceed the flow rate given for STEP 1 in the table above.
18. Equilibrate the column with two bed volumes of start buffer. A larger volume may be required with detergent solutions.

Provided that the packing instruction was followed, you will now have a column with excellent separation capability. In almost all cases you can use the column directly. Unless you have an extremely difficult separation to perform and must use Sephacryl HR to its maximal limit STOP HERE. For exceptional cases requiring optimal packing see below.

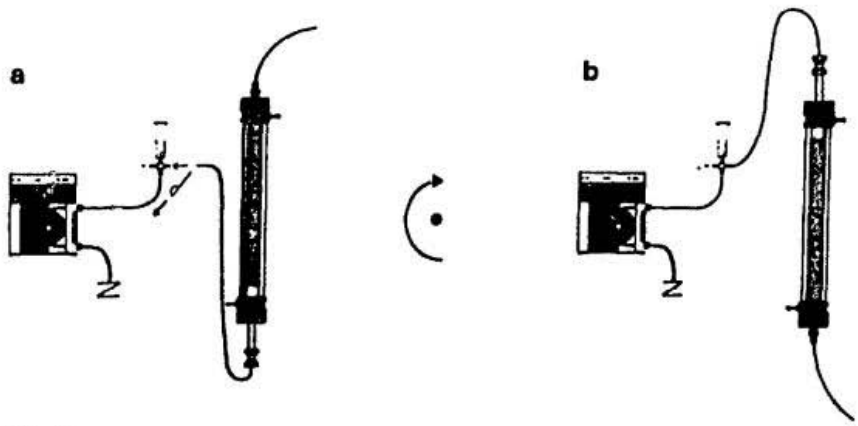


Fig. 6.

Further information

The steps below are special measures only to be carried out for exceptionally difficult separations and are seldom necessary.

Determination of plate number

1. Prepare a sample of acetone 5-10 mg/ml in distilled water or your buffer.

2. Use the test conditions given for the appropriate column in the table below.

Test conditions	Column		
	K 16	K 26	K 50
Sample volume (µl)	200	500	500
Flow rate (ml/h)	60	150	400
Chart speed (cm/h)	30	30	12
Detection (nm)	280	280	280

3. Calculate the plate number (N) according to the formula

$$N = 5.54\left(\frac{V_e}{W_{1/2\,h}}\right)^2 \times \left(\frac{1000}{L}\right)$$

N = Plate number per metre

V_e = Peak elution volume (ml)

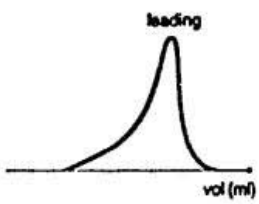
$W_{1/2\,h}$ = Peak width at half peak height (ml)

L = Length of column (mm)

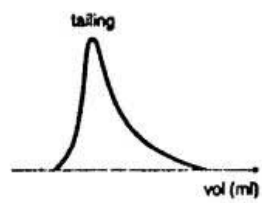
A plate number of 9 000 per metre or more, which corresponds to a reduced plate height of 2.4, is often achieved.

Peak symmetry

For advanced packing the flow rate in STEP 2 can be adjusted depending on the shape of the acetone peak.



When repacking decrease the flow rate in step 2 by 10–20 %



When repacking increase the flow rate in step 2 by 10–20 %

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