The Management of Broodstock Atlantic Halibut (*Hippoglossus hippoglossus)* and the Influence of Nutrition, Holding Conditions and Hormonal Manipulation of Spawning on Gamete Quality.

Thesis presented for the degree of

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by

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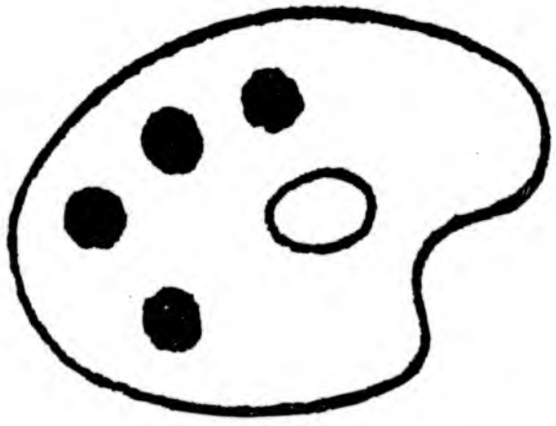

Declaration

Except where specifically mentioned, the work in this thesis was conducted independently and entirely by the author. Sections in Chapter IV were carried out in collaboration with three MSc students from the Institute of aquaculture: Nigel Jordan (1997), John Rees (1998) and Nikolaos Papanikos (1999). Sections in Chapter VI were carried out in collaboration with one MSc student from the Institute of Aquaculture, Andrew Davie, and with Dr. E. Vermeirssen from the CEFAS Laboratory in Lowestoft. This work has not been submitted for any other degree.

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Experiment 2 in Chapter VI was carried out in collaboration with Andrew Davie, MSc student at the Institute of Aquaculture, University of Stirling (1999-2000). Experiments 3 and 4 in the same chapter were performed in collaboration with Dr. E. Vermeirssen from the MAFF laboratory at Lowestoft, to whom I am most indebted for his guidance.

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A mi familia.

Abstract

The Atlantic halibut (*Hippoglossus hippoglossus*) is an important marine species for aquaculture in higher latitudes. Its excellent growth rate in relatively cold waters, high market price and the decreases in wild captures, have provided the incentive for an expansion in interest for the farming of halibut. However, much work remains to be carried out to identify the nutritional, husbandry and environmental requirements of broodstock in order to establish a reliable supply of good quality gametes and larvae.

This thesis describes the dynamics of oocyte growth and ovarian development of Atlantic halibut; the development of pelleted broodstock diets and the influence of levels of essential fatty acids in these diets on egg and larval quality; the effects of stocking density and recirculation technology on egg quality; and the uses of gonadotropin-releasing hormone analogue (GnRHa) implantation for the manipulation of gamete production.

Histology, stereology, electron microscopy and ultrasound scanning were used to study ovarian and oocyte development. Oocyte growth followed the same general processes described for many teleosts, including primary growth, folliculogenesis, cortical alveoli formation, vitellogenesis, and final maturation. Vitellogenesis started from late September, continued during the spawning season, and accounted for some 13% of the final egg size. Final oocyte maturation included yolk coalescence and hydration; this was responsible for some 80% of the final egg size and produced a transparent, buoyant egg. GSI began to increase from late September, from a minimum value of 2.1 in pre-vitellogenic fish (September), to average values of 15.0 in pre-spawning fish (March). The maximum GSI value recorded for spawning fish (March) was 21.6. Ultrasound was used extensively for sexing mature and immature fish, studying ovarian growth throughout the

year and monitoring final maturation of discrete batches of eggs. It provided a discriminating non-intrusive tool for the study of ovarian development.

Pelleted broodstock feeds provided a similar performance, as far as egg and larval quality were concerned, as the wet-feed widely used by commercial farms. Atlantic halibut eggs selectively accumulated docosahexaenoic acid (DHA) and arachidonic acid (AA). Improved fertilisation rates, egg quality scores and hatching rates were achieved at concentrations of 16% DHA and 1.8% AA in the lipid fraction of the diets. In addition, relative concentrations of DHA and AA with respect to eicosapentaenoic acid (EPA) were important. DHA:EPA and EPA:AA ratios of 2 and 4 respectively appeared to provide improved egg and larval quality.

No differences were found in the spawning performance and egg quality of broodstock Atlantic halibut maintained at stocking densities of 2.5 kg/m^2 and 5.21 kg/m^2 respectively. Recirculation of chilled water in Atlantic halibut broodstock tanks had no deleterious effect on spawning performance or egg quality.

Implants of 10 μg GnRHa/kg of body weight were effective in improving the spawning performance of previously unsuccessful female Atlantic halibut. Free oestradiol, sulphated 11-deoxycortisol, free androstenedione and sulphated 3β, 17, 20β-P-5β were the main reproductive steroids present in plasma of pre-spawning and spawning females.

GnRHa implants were successful in reducing the spermatocrit (viscosity) of milt from male Atlantic halibut. This improved milt motility and fertilisation success. 17, 20β-P is suspected to play a major role in spermatogenesis of male Atlantic halibut. The role of 17-20-α-P is unclear and further research is required on its possible participation in spermiation and sperm motility acquisition. Implants of 25 μg GnRHa/kg body weight

towards the end of the spawning season of male Atlantic halibut, once spermatocrit values are above 90%, are recommended to enhance milt availability and quality.

Key words: Atlantic halibut; oocyte growth; ovarian development; ultrasound; broodstock diets; DHA; AA; EPA; stocking density; recirculation; GnRHa implantation; sex steroids.

Chapter I: General Introduction.

The Atlantic halibut (*Hippoglossus hippoglossus*) is an important marine species for aquaculture in higher latitudes. Its good growth rate in relatively cold waters, its high market price and the stable decrease in wild captures, provided the incentive for an intense research effort during the last decade and a half. Today, there is an expanding halibut farming industry in countries like Norway, Iceland, Canada, Chile and the United Kingdom. However, much work remains to be carried out to identify the optimum nutritional, husbandry and environmental requirements of broodstock, establishing a reliable supply of good quality gametes, improving the survival of larvae through the yolk sac and first feeding stages and defining appropriate husbandry techniques for the on-growing of juvenile fish.

The appropriate management of the broodstock fish is one of the essential elements for the successful development of the industry. The following is a brief overview of current practises of broodstock husbandry among Atlantic halibut hatchery operators.

1.1. Taxonomy and distribution

The Atlantic halibut is the largest member of the family Pleuronectidae. It was first described by Linnaeus in 1758 *as Pleuronectes hippoglossus*, to be later classified in a new genus and named *Hippoglossus hippoglossus* by Cuvier in 1885.

The area of distribution of the Atlantic halibut extends from the North of the Atlantic Ocean (and as north as parts of the Arctic Ocean), along the coasts of Norway, Iceland, southern Greenland and the East Coast of Canada, to as far South as, occasionally, the Bay of Biscay and New York. Wild mature halibut gather every year in defined spawning grounds in deep waters (100-700 meters). Spawning takes place over several months, from November-December to March-April, depending on the regions and at temperatures between 5 and 8°C (Haug and Kjørsvik 1989, Haug 1990, Neilson *et al.* 1993).

There is a large variation on age and size at first sexual maturity in different areas. Studies of wild populations appear to link sexual maturity to size and growth rate more directly than to age (Haug 1990). Males appear to reach sexual maturity in the wild at smaller size and age - 4.5 years, 55 cm and 1.7 kg in Faeroes waters - than females - 7 years, 110-115 cm and about 18kg in the same region (Haug 1990). A similar pattern can be observed in farmed fish (Björnsson 1995). Female halibut exceeding 300 kg have been captured, whereas males rarely reach more than 50 kg.

Rønnestad and Rødseth (1989) described how halibut broodstock was collected from the wild at their traditional fishing grounds with long lines and then brought ashore and acclimatised to land based facilities. With the development of the halibut farming industry, more hatchery-produced fish are joining the broodstock, with a spawning performance equivalent to that of the wild fish (Shields *et al.* 1999).

1.2. Holding conditions

Broodstock holding facilities in the hatcheries vary in shape and dimensions, from concrete ponds to fibreglass circular tanks. Most extended among U.K. operators are circular tanks 4-10 m in diameter and 1-2 m deep. A net is placed around the tank, or a

dome on top of it, to avoid losses of fish by jumping and protect the fish from direct sunlight.

In captivity halibut expend some 90% of their time resting on the bottom of the tanks. Rounded gravel is sometimes added to the bottom of the tank to avoid lesions on the blind side of the fish. A different solution is the provision of fixed rugged substrates, like mesh nets, rough concrete or blistered concrete slabs, which do not interfere with the self-cleaning of the tank.

Water quality is certainly important, and some sites relay on deep sea water pumped ashore as a source of stable good quality water. Salinity values of 33-34‰ are regarded as optimal. Oxygen requirements for halibut broodstock are not published, but levels of 6 ppm at the tank outlet, similar to those of turbot, are considered sufficient (Ronnestad and Rodseth 1989). Flow rates and water exchange in the tanks should be as high as possible, with turnover values of 15-17 per day in 7 m diameter tanks being cited in the literature. However, this is sometimes limited by the cost or the capacity for temperature control (see below). Flow rates of 20 l per minute in 4 m diameter tanks, that is 2.4 water changes per day, have appeared to be sufficient in some systems (Shields *et al.* 1999).

Although generally a deep water fish, light appears to be important for growth and maturation of Atlantic Halibut. Subdued ambient light or low intensity artificial lights are used in the Broodstock tanks. The manipulation of the photoperiod regime allows the change of the time of spawning (Smith *et al.* 1991). Thus, different populations of broodstock can be maintained in different photoperiods, and the egg production spread throughout the year.

Temperature and temperature regime exert a direct effect on the quality of the eggs produced (Brown *et al.* 1995). Best results are obtained with water temperatures during the

spawning season of around 5-6°C. There is a detrimental effect in the quality and fertilisation success of the eggs produced at temperatures above 8°C (Brown *et al.* 1995). Thus, temperature control systems are in use in regions or times of the year where the ambient water temperatures are not optimal.

1.3. Nutrition

Broodstock nutrition responds to wider considerations than that of juvenile fish. Broodstock diets are important not only for the health and well being of the brood-fish, but also for the quality of their egg production. Generally, the fish are fed ad libitum two or three times a week and any left over food taken out of the tank as soon as possible. Feeding behaviour changes throughout the year, with a minimum feeding response during the spawning season. The decrease of feeding during spawning, together with the energy demand of reproduction, makes it essential to ensure the nutritional recovery shortly after spawning. Also during autumn, when the transference of nutrients from the female fish into the future eggs takes place (Haug and Gulliksen 1988 a).

Commercial hatcheries moved from the use of whole trash fish (herring, capelin, whiting...), to mixtures of minced trash fish, squid and fish roe blended with fish oils and vitamin additives and prepared into sausage skins. The later makes possible some control over the nutritional value of the feeding stuff. Although juvenile halibut seem to have a higher protein demand than plaice or salmonids (Hjertnes and Opstvedt 1990), the lipid fraction is of special importance for the broodstock fish, and in particular the relative levels of polyunsaturated fatty acids (PUFA).

1.4. Health management

Early handling experience has shown that captive halibut are very susceptible to eye damage. Williams *et al.* (1995) studied the occurrence of gas-filled cysts and cataracts in

captive halibut. There is no clear explanation of why the eye damage appears, but handling is the most likely reason. The condition frequently progresses until the total lost of vision from the affected eye. Some fish become blind of both eyes, with the associated difficulties on feeding and detrimental effect on spawning condition.

In some instances females fail to release hydrated eggs kept in the ovarian lumen, even when assisted by stripping (see *Gamete production and collection* below) becoming "egg bound". Egg bounded females can be injected intramuscularly with antibiotics (Amoxiciline, Oxytetracycline), causing the dead eggs to be released after a few days.

Parasitic infections can appear in the broodstock fish. Among the most common are the "halibut louse" (*Lepeophtheirus hippoglossi*), *Trichodina spp* and Costia (*Ichtyobodo necator*). Ronnestad *et al.* (1989) describes these infestations and its treatment with formaldehyde or freshwater baths.

1.5. Gamete production and collection

Atlantic Halibut is a batch spawner. Each female produces discrete batches of eggs through several weeks during the spawning season. Halibut females of 20-60 kg can be expected to produce 6-16 batches of eggs during one spawning season of around $10x10^3$-$200x10^3$ eggs, with mean inter-ovulation intervals of 70-90 hours (Holmefjord 1991, Norberg *et al.* 1991).

Methven at al. (1992) reported that plasma levels of Vitelogenin, Oestradiol-17ß and Testosterone increased gradually in female halibut up to the onset of spawning, and then oscillated between batches. Attempts to manipulate egg production through hormonal treatment (GnRH agonists) had until now failed.

Female halibut are able to release their eggs naturally in the tanks. Holmefjord and Lein (1990) reported fertilisation rates of over 90% from natural releases in a 10 m

diameter pond in Norway. Still, it is difficult to obtain fertilised eggs from natural releases in smaller tanks. Rabben (1987) describe a method of obtaining the gametes by stripping. In order to obtain both male and female gametes "dry", the stripping operation takes place out of the water by guiding the fish onto an abatable "table" taken into the tank. The eggs are stripped from the females by gentle pressure of the gonads along the abdominal side. Milt is obtained from the males in the same fashion. Due to the big size of the fish, the stripping operation is normally carried out by at least two people, one holding a jug or bucket that receives the gametes and another applying the pressure while holding the fish, or, depending on the size of the fish, a third person might be needed to hold the fish. Best fertilisation results have been reported following the "wet fertilisation" procedure (Basavaraja 1991).

Timing of stripping is of great relevance to the quantity and quality of the eggs produced, and on their fertilisation and hatching rates (Basavaraja 1991, Holmefjord 1991, Norberg *et al.* 1991). Egg viability undergoes rapid decline six hours after ovulation (Bromage *et al.* 1994). The individual ovulatory cycle of each female has to be accurately determined before attempting the stripping. The female is allowed to naturally release the eggs two or three times in the tank, and the average interval between releases is used as the estimated ovulatory rhythm for the rest of her individual season. This timing can vary with changing temperature and with batch number, the inter ovulation time of the last batch being often shorter than the rest. Excessive handling of the female can also alter its ovulatory rhythm. Shields *et al.* (1993) tested ultrasound scanning as a non-invasive technique for assessing ovulatory state of female halibut.

The production of a regular supply of good quality eggs is of primary importance for the development of the culture of any aquaculture species. The optimisation of husbandry

techniques and nutritional parameters for broodstock would allow a better control on the quality of the gametes produced.

1.6. Thesis aims

- To describe the oocyte growth and ovarian development of Atlantic halibut.
- To develop a fully fabricated pelleted diet for Atlantic halibut broodstock and assess the influence of levels of essential fatty acids in the diets on egg and larval quality.
- To study the effects of stocking density of Atlantic halibut broodstock on egg quality.
- To assess the use of recirculation technology for maintaining Atlantic halibut broodstock, and its effects on egg quality.
- To develop workable protocols for the manipulation of egg and milt production of Atlantic halibut with the use of gonadotropin-releasing hormone agonists implantation.

Chapter II: General Materials and Methods.

2.1. Introduction:

The experiments in this thesis were conducted either at the facilities of Seafish Aquaculture at Ardtoe, or at the facilities of the Institute of Aquaculture in the Machrihanish Environmental Marine Laboratory, both in the west coast of Scotland. Histology and lipid analyses were carried out at the Institute of Aquaculture in Stirling, Scotland. Steroid analysis was fulfilled at the CEFAS laboratory at Lowestoft, England.

The methods described below are those used during the completion of the different experiments described in this thesis. Particular chapters will refer to sections in this chapter for description of common practices to avoid repetition.

2.2. General husbandry procedures:

The general husbandry procedures of the fish used in the experiments were similar to those carried out in commercial hatcheries in the U.K.

2.2.1. Collection of gametes:

The ovulatory cycle of each gravid female was monitored in order to establish the optimum time to artificially strip (typically 60-90 hours between egg releases). Females entering the spawning cycle were easily recognised by an increasingly swollen abdomen.

The females were allowed to release their first three batches of eggs in the tank water. These were collected and provided an estimation of the inter-ovulatory period for subsequent batches of each individual female. At the predicted time of ovulation, the water in the tank was drained to an adequate level (50-90 cm). A stripping table with adjustable legs was lowered into the tank. The female was guided gently onto the table. Two people performed the stripping, one handling the female and the other assisting with the jugs and pots used to collect the gametes (figure 2.1). With gentle to hard pressure applied to the swollen abdomen, from back to front, eggs were stripped into a dry 2 l plastic jug. Batch volume was assessed and the jug was put into a cold box to protect the eggs from excessive light and temperature. This procedure was repeated with two males from the same tank, this time drying the gonopore with a paper tissue before collecting the milt into 400 ml plastic beakers, to avoid milt activation by sea water.

2.2.2. Sperm motility assessment:

Atlantic halibut milt is activated by contact with sea water. Sperm cells remained active for approximately 90 seconds after contact with sea water. A drop of milt was placed on a microscope slide. A drop of water was added to the milt and the mixture spread as a thin film with the aid of a second slide, diluting the milt. The milt was observed under the microscope (x10 / x40 Meiji Labax Co. Ltd., Japan), and the percentage of motile sperm cells estimated. This operation was repeated three times and the mean value recorded as sperm motility.

2.2.3. Measurement of spermatocrit:

Spermatocrit is the percentage of packed cell volume of milt. High spermatocrit values correspond to very viscous milt, with little seminal fluid. Spermatocrit was one of the parameters used in the assessment of the effects of GnRHa implantation on male

Figure 2.1: Stripping of ovulated eggs from a female Atlantic halibut. A stripping table was lowered into the tank and the female was guided gently onto the table. With gentle to hard pressure applied to the swollen abdomen, from back to front, eggs were stripped into a dry 2 l plastic jug.

Atlantic halibut (Chapter VI). Milt samples for assessment of spermatocrit were obtained by collecting a small volume of milt (approx. 1ml) into a plastic Pasteur pipette from the male fish. Attention was paid not to mix the milt with any urine, which sometimes was also released by the stripping action. Sub-samples of the milt were drawn out into glass straws (0.7 mm diameter), sealed at one end with Cristaseal (Hawksley and Sons Ltd., U.K.) and centrifuged in a micro-haematocrit centrifuge (Hawksley and Sons Ltd., U.K.) for 30 min. A Hawksley reader was used to assess the percentage of packed cell volume, measured at the boundary between the transparent seminal fluid and the mass of white packed cells.

2.2.4. Fertilisation:

Fertilisation was carried out in two different ways, depending on the facilities available and the nature of the experiment. The manner of fertilisation is specified in the materials and methods section of each experiment in the subsequent chapters. All operations were carried out in temperature controlled rooms (5.5-6.5°C) and under dimmed light filtered through a yellow/red photographic filter.

2.2.4.1 Hatchery procedure:

Milt from two different males were diluted into 1 l of uv sterilised, 5 µm filtered seawater and added immediately to the eggs (500 ml. of eggs : 1 ml. of milt : 500 ml. of water). After 20 minutes, when the eggs had water hardened, they were transferred to a soft net and rinsed with 2 l of clean water. The eggs were then put into an 80 l cylindro-conical black polyethylene tank filled with clean uv sterilised, 1 µm filtered, 34-35ppt salinity sea water at 6°C.

2.2.4.2 Experimental procedure:

Three 10ml samples of eggs were taken from each batch with a plastic measuring scoop, and placed in three plastic specimen pots. With a clean plastic Pasteur pipette, 0.25

ml of milt were taken from two different males and placed in three separate specimen pots. Each milt pot was filled with 60 ml of uv sterilised, 5 μm filtered, 34-36ppt salinity seawater and added immediately to each sub-sample of eggs. The mixture of eggs, water and milt was allowed to stand for 20 minutes. After 20 minutes the eggs from each pot were poured gently through a soft mess net, rinsed with water and returned to a new pot filled with 60 ml of clean water. The pots were stored in an incubator at 6°C until fertilisation assessment took place (see below).

During the fertilisation capability assessment of the milt from the male hormonal manipulation experiments (Chapter VI), the same procedure was used with each milt sample. However, due to the small amounts of milt per sample in experiments 4 and 5, a variation on the addition of the milt was implemented. Milt was diluted in a milt extender (see composition of milt extender in table 2.1) provided by Dr. Etienne Vermeirssen, according to spermatocrit, to achieve the same concentration in all samples. An equivalent volume of diluted milt was pipetted into a pipette tip, and the milt mixed with uv sterilised, 5 μm filtered seawater by injecting the water through the pipette with a 2 ml plastic syringe into a plastic fix pot. The specimen pot was topped up with seawater and added immediately to the eggs.

Table 2.1: Composition of milt extender (information provided by Dr. E. Vermeirssen). The working solution was made by adding BSA and glucose to the stock solution. The working solution was filtered to 0.2 µm and used to make 1/1000 dilutions of sperm. Based on Suquet *et al.* (1998), with an increased glucose concentration.

Stock solution	
NaCl	70 mM
KCl	1.5 mM
$CaCl_2$	2.7 mM
$MgCl_2$	6.1 mM
$NaHCO_3$	25 mM

Working solution	
Bovine serum	1 g / 100 ml
Glucose	3.6 g / 100 ml

2.3. Assessment of spawning performance:

2.3.1. Fecundity:

Fecundity was determined by calculating the total number of eggs shed by each female during a spawning season. The number of eggs per ml obtained by stripping were calculated by repeated counts of eggs in a given sub-sample of eggs of known volume. In the eggs fertilised by the experimental method (section 2.2.4.2), this was achieved by counting all the eggs in the pots where fertilisation took place at the time of the assessment of the fertilisation rate and calculating the mean number of eggs per ml. In the case of the hatchery fertilisation procedure (2.2.4.1), three sub-samples of 2.5 ml of eggs were taken from discrete egg batches from each female, after water hardening, with a plastic measuring scoop. The numbers of eggs in the sub-samples were counted and the mean number of eggs per ml calculated.

Eggs allowed to be released freely into the tank were recovered at an egg collector placed at the outlet of each tank. The egg collectors were made of a wooden frame holding

a 2 mm polyamide mesh, or a piece of plastic pipe (30 cm diameter) fitted with a similar mesh. Water coming from the tank passed through the mesh of the egg collector, that retained any eggs shed into the tank water. The eggs were transferred to a sieve and excess water was removed with tissue paper and then weighted. Three sub-samples of at least 3 g of eggs were counted. The total number of eggs produced by each female was calculated by combining the total sum of eggs obtained by direct stripping and the eggs shed in the tank water.

Relative fecundity for each female was calculated using the formula:

$$RFc = \text{No. of eggs} / \text{kg female}$$

RFc = relative fecundity.

No. of eggs = sum of number of stripped eggs and eggs released in the tank water.

Kg female = wet weight of the female in kg.

2.3.2. Fertilisation rate:

Fertilisation rate was assessed at the 8 cell stage, 96° h after fertilisation. Depending on the fertilisation method, standard hatchery procedure or experimental procedure, a slightly different protocol was followed.

After the eggs were fertilised following the standard hatchery procedure (see section 2.2.4.1), the batch of eggs was kept in an 80 l cylindro-conical black polyethylene tank. Sub-samples of eggs were taken from the floating fraction of the eggs in the tank and the numbers of developing (fertilised) and non-developing (considered unfertilised) eggs determined by the presence of dividing cells. Any drop-out eggs (eggs sank to the bottom of the tank) were collected and weighted and the number of eggs estimated from the

number of eggs in three sub-samples of at least 1 g of eggs. Relative fertilisation was calculated by the formula:

$$RF = [No.\ dv.e. / (No.\ dv.e. + No.\ ndv.e)] \times 100$$

RF = relative fertilisation.

No. dv.e. = number of developing eggs.

No. ndv.e = number of non developing eggs.

Total fertilisation rate was calculated using the formula:

$$F = [No.\ dv.e. / (No.\ dv.e. + No.\ ndv.e + No.\ do.e.)] \times 100$$

F = total fertilisation rate.

No. dv.e. = number of developing eggs.

No. ndv.e = number of non developing eggs.

No. do.e. = number of eggs in drop-out.

When the experimental procedure for fertilisation was used, total number of developing, non-developing and drop-out eggs were counted from each specimen pot and RF and F calculated using the same formulas.

2.3.3. Egg quality assessment:

Egg quality can be defined as the characteristics of the egg which determine its capacity to survive (Bromage 1995) and hatch into a viable larvae. The methods to determine egg quality must be simple to perform and provide an early assessment of their viability. This provides the commercial hatcheries with a useful protocol to decide whether to invest rearing effort in a particular batch of eggs. Shields *et al.* (1997) and Brown

(1998), describe a method for assessing egg quality in Atlantic halibut based on the analysis of the blastomere morphology at the eight-cell stage of development. This protocol, which was used throughout this thesis for the assessment of the egg quality of every batch of eggs obtained with a fertilisation rate over 10%, is described below.

A sterile 96 well microtitre plate (Micro-Pltte; Greiner Labortechnik. Germany) was prepared by filling each well with 200 µl of filtered UV sterilised 6°C sea water. Randomly sampled fertilised eggs at the 8 cell stage (96° h after fertilisation) were deposited in the plate wells (one egg per well). Each egg in the plate was observed under a dissection microscope (Olympus SZ 60, Olympus Optical Co.Ltd., U.K.) at low magnification and the lowest possible illumination, and given a score from 1 to 4 (abnormal to normal) for each of the following parameters (figure 2.2):

Symmetry: bilateral symmetry about the axes of the 8 blastomeres.

Uniformity: uniformity of cell size.

Adhesion: contact between adjacent blastomeres.

Margins: discreteness of cell margins.

Inclusions: vacuoles between blastomeres.

The egg quality score of the batch of eggs was the mean of the sum of the scores for each parameter for each egg (0-20). The plate was then sealed with tape and stored in a cool incubator at 6°C until hatch.

2.3.4. Hatch rate:

At 75 °D (12.5 days at 6°C) the plate was inspected for hatched eggs under the dissection microscope. If any live embryo was found not to have hatched yet, the plate was

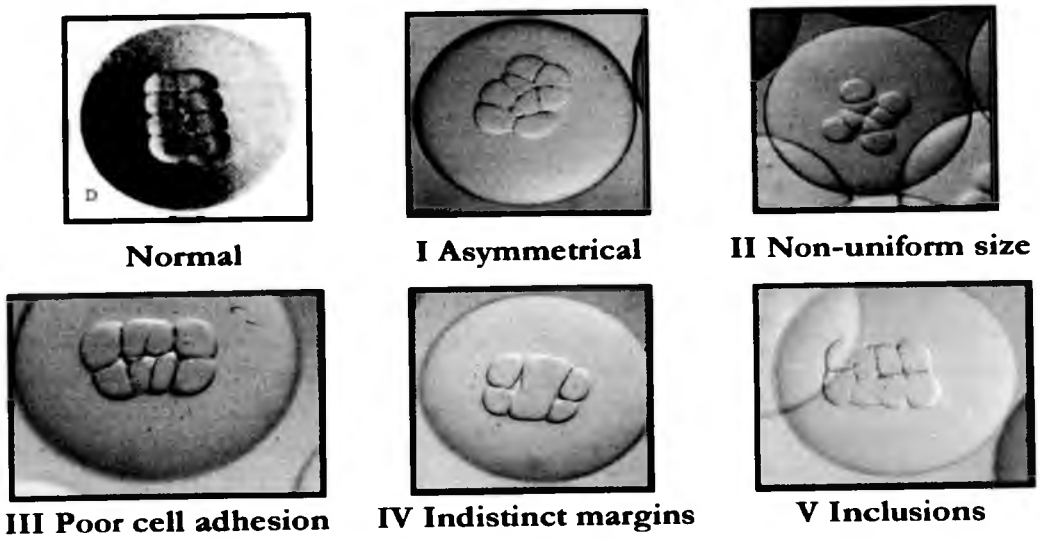


Figure 2.2: Blastomere morphology of eggs of Atlantic halibut at the 8 cell stage (96° h after fertilisation) and different deviations from normal cleavage used in the assessment of egg quality. (Reproduced with permission of N. Brown).

put back into the incubator for later inspection. Hatching rate (HR) was calculated according to the formula:

$$HR = [\text{No. hatched eggs} / \text{No. fertilised eggs}] \times 100$$

2.4. Histology of ovarian tissue:

2.4.1. Biopsy of ovarian tissue:

Ovarian tissue was obtained from anaesthetised females by cannulation for assessment of oocyte diameter *in vivo* and for sampling of oocytes during final maturation for electron microscopical examination (Garcia 1989). Cannulation was only possible shortly (i.e., a few days) before the start of the spawning season of the female.

A piece of silicon plastic tubing (3 mm internal diameter and approx. 20 cm length), attached to a 2.5 ml hypodermic syringe, was used for cannulation. Anaesthetised females were placed horizontally on a table. The free end of the tubing was carefully inserted in the gonopore of the female. Once the tubing was inside the front part of the ovary, gentle suction was applied with the syringe and the tubing was turned on its axis and removed from the gonopore. The sample was transferred into a Petri dish for measurement of oocyte size under a dissection microscope and stored in electron microscopy fixative (see below).

2.4.2. Light microscopy:

Some of the samples analysed in this thesis were collected by Dr. Robin Shields and processed in the CEFAS laboratory at Lowestoft. Most of the material, however, was obtained by the author from mortalities among the broodstock of different hatcheries and from fish harvested from ongrowing tanks. Cuboidal sections of the ovary were cut with a

scalpel, and smaller sections of these (approx. 1 cm^3) fixed and processed for histology and electron microscopy analyses.

2.4.2.1. Fixation:

Sections of ovary were stored in at least 10 times their volume of buffered formalin during 24 h. After 24 h the buffered formalin was renewed, and the sample stored in a cool place until processing. Buffered formalin was prepared as follows:

Na_2HPO_4	6.5 g
Na H_2PO_4	3.5 g
Formalin (40% formaldehyde)	100 ml
Deionised water	900 ml

2.4.2.2. Dehydration, infiltration, embedding and mounting:

Infiltration, embedding and mounting of the tissue samples were carried out according to the instructions of the manufacturer of the Technovit 7100 (Heraeus Kulzer GmdH, Germany), a cold curing resin based on hydroxy-ethyl-methacrylate. However, some adjustments of the recommended infiltration protocol were necessary, due to the size and resistance to infiltration of yolky halibut eggs. The protocol followed was a variation of that used by Coward (1997). The tissue samples were cut to a thickness of 0.5 cm and submitted to dehydration in a series of ethanol solutions of increasing strength, followed by slow infiltration at 4°C according to the following protocol:

70% ethanol	4 days
80% ethanol	2 days
90% ethanol	2 days
95% ethanol	2 days
50:50 (infiltration solution:95% ethanol)	7 days
75:25 (infiltration solution:95% ethanol)	7 days
100% infiltration solution	14 days

After infiltration, samples were placed individually in historesin moulds and covered with embedding medium and allowed to polymerise overnight at room temperature. Fresh mounting media was then added to the moulds and plastic mounting blocks pressed into the media. The mounting media was allowed to harden at room temperature overnight.

2.4.2.3. Sectioning:

Samples mounted and embedded in resin were placed in a dessicator containing self-indicating silica-gel at least one week prior to sectioning. The blocks were sectioned at a thickness of 4-5 μm with a glass knife (Ultramicrotome Glass) mounted on a 2050 Supercut automatic retracting microtome (Reichert-Jung, Cambridge Instruments Gmbh, Germany) at an angle of incidence of 5°.

Selected sections were floated in a water bath (Raymond A. Lamb, U.K.) at 35 °C and extended on Polysine™ microscope slides (BDH Laboratory Supplies, U.K.). Samples were dried on a hot plate at 40 °C for 5 h before staining.

2.4.2.4. Cryostat sectioning of fixed material:

Some material was sectioned using a cryostat (OTF Cryostat, Bright Instruments Co. Ltd., U.K.). This allowed for rapid sample preparation and staining of slides to identify stages of maturation of particular ovaries. Sub-samples of fixed material were placed on a metal mounting piece or chuck and embedded in cryostat embedding material (Cryo-M-Bed embedding compound, Bright Instruments Co. Ltd., U.K.). The embedding material solidified in 3-4 minutes at –40°C inside the cryostat. The sample was then sectioned at –19°C with a stainless steel knife (at 14° incidence) at a thickness of 3-5 μm. Sections were then extended on Polysine™ microscope slides.

2.4.2.5. Staining:

Sections of ovarian tissue mounted on microscope slides were stained with haematoxylin and eosin. Some of the slides prepared at the CEFAS laboratory in Lowestoft were stained with haematoxylin and eosin or toluidine blue.

Slides of tissue sections in historesin were stained with haematoxylin an eosin following the following procedure:

Absolute alcohol	2 min
Methylated spirit	1.5 min
Wash in tap water	
Haematoxylin	5 min
Wash in tap water	
Acid alcohol	3 quick dips
Wash in tap water	
Scott's tap water	1 min

Wash in tap water	
Eosin	5 min
Wash in tap water	
Methylated spirit	30 s
Absolute alcohol	2 min
Absolute alcohol	1.5 min

Slides of tissue sections in cryostat embedding material were stained with haematoxylin an eosin following a slightly different procedure:

Brief water wash	
Haematoxylin	45 s
Water rinse	
Acid alcohol	3 quick dips
Wash in water	
Scott's tap water	30 s
Eosin	15 s
Water rinse	

In both cases, slides were kept in xylene until a coverslip was mounted using Pertex.

2.4.2.6. Image acquisition:

Samples were observed through an Olympus BH-2 binocular microscope (Olympus Optical Co. Ltd., U.K.) connected to a digital camera (Vista Protos DSP; Norbain SDL Ltd., U.K.) and, through a Sony trinitron PVM-1440QM (13 inch screen) colour monitor, to a computer. A x 2.5 lens was fitted in the neck piece connecting the camera to the

microscope. An Image Pro® Plus image analysis software package was used to acquire all images (Data Cell Ltd., U.K.).

2.4.3. Stereological analysis of volume fractions of different oocyte stages.

This method was first applied to fish by Emerson *et al.* (1990), to estimate fecundity in herring, Dover sole and mackerel. The method is based on the Delesse principle (Delesse 1847, cited in Emerson *et al.* 1990), which states that the fractional volume (V_i) of a component (i) in a tissue is proportional to its fractional cross-sectional area (A_i). In this thesis, the method is applied to compare the volume fraction of different oocyte stages (V_i) along the ovary, and to assess the relative presence of oocyte stages in the ovary of Atlantic halibut through the year. The methodology employed was a modification of that of Emerson *et al.* (1990) and the protocol used by Coward and Bromage for tilapia (1998). Validation of the technique can be found in the same sources. The proportional area (A_i), and therefore the proportional volume (V_i), occupied by different oocyte stages was determined by a point counting technique (p_i), using a random grid point procedure. The number of points overlaying a particular oocyte class was divided by the total number of points on the grid (p_t).

Ovarian sections prepared for light microscopy as described in section 2.4.2 were observed under an Olympus BH-2 binocular microscope (Olympus Optical Co. Ltd., U.K.) connected to a digital camera (Vista Protos DSP; Norbain SDL Ltd., U.K) and, through a Sony trinitron PVM-1440QM (13 inch screen) colour monitor, to a computer. A x4 objective was used in the binocular microscope and a x2.5 lens, fitted with a Weibel-2 GW3 stereology graticule (Graticules Ltd., U.K.), was placed in the neck piece connecting the microscope to the camera. The graticule was thus projected onto the image of the tissue preparation recorded on the monitor (see figure 2.3). Total number of points of the graticule appearing on the monitor was recorded and set to 39 for all the image analyses. A

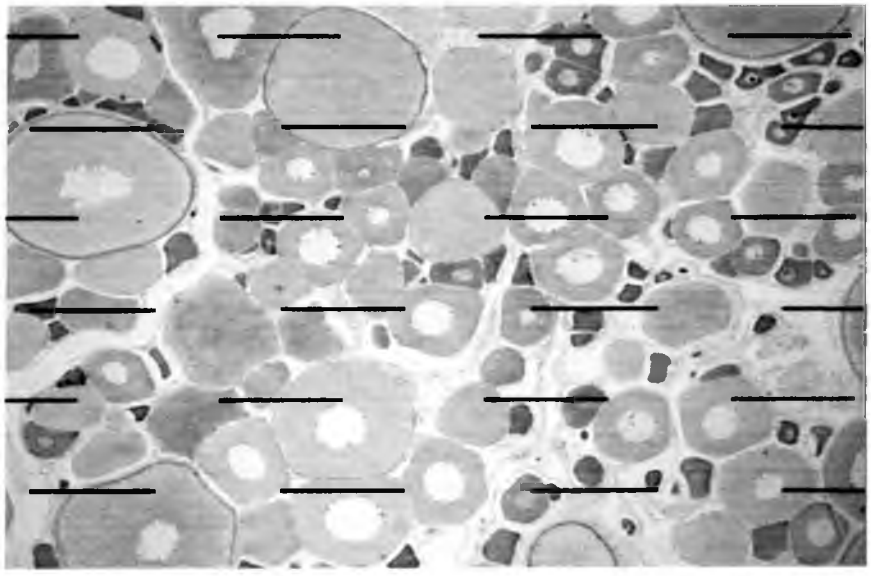


Figure 2.3: Weibel graticule projected onto a photomicrograph of a section of an ovary to illustrate the stereological method. For demonstrative purposes, the number of grid points (end points of horizontal bars) in the field above is 42. For calculation of volume fractions, the number of grid points per field was set to 39. The number of grid points intersecting a particular oocyte stage was counted, and divided by the total number of grid points in the field view. The product of this was the volume fraction of that oocyte stage in that particular field.

protocol was established by which 10 randomly selected fields of each slide were analysed. Series of three slides from the same tissue sample, of a distance in between them of no less than 400 μm, where studied. The number of points of the grid overlaying different oocyte classes was recorded for each field. The mean number of points for each oocyte class in the ten different fields and the three different sections was calculated. The area fraction (A_i), and hence the volume fraction (V_i), of each oocyte class was calculated according to the formula:

$$V_i = A_i = p_i / p_t$$

2.4.4. Electron microscopy:

Samples for electron microscopy were obtained by ovarian biopsy of gravid females. Many of the substances used for sample preparation are carcinogenic or highly irritant, and extreme care has to be taken during the operations, as well as for the disposal of any residues.

2.4.4.1. Fixation, embedding and sectioning:

The tissue obtained was embedded in Spurr's resin (Spurr 1969) using the following protocol:

Fixation: 2.5% glutaraldehyde in 0.1 M Sodium cacodilate plus 3% sucrose at pH 7.2 for a week.

Storage/Rinsing: 0.1% M sodium cacodylate plus 5% sucrose for three months, changing storing medium every month.

Post-fixation: 1% osmium in 0.1 M sodium cacodylate plus 3% sucrose for 2 h.

Staining: 2% uranyl acetate in 30% acetone for 1 h in the dark.

Dehydration: 60% acetone for 1 h.

90% acetone for 1 h.

100% acetone for 1 h.

100% acetone for 1 h.

Infiltration: infiltration was carried out with Spurr's resin using a sample rotator.

Acetone : Spurr's 1:1 overnight.

Spurr's for 24 h.

Tissues were embedded in Spurr's resistant moulds and allowed to polymerise in an oven at 60°C for 48 h.

Polymerised blocks were trimmed manually with a blade to allow the least possible surface area for sectioning. Sections were cut with a glass knife mounted on an ultra-microtome (Reichert OMU3, Austria) at an incidence angle of 4°. Sections were cut at "gold colour" (approx. 90-100 nm). Suitable sections were placed on electron microscopy copper grids (Agar, U.K.).

2.4.4.2. Staining:

Suitable sections were stained with lead citrate and uranyl acetate (in ethanol 50% saturated). Both chemicals were first centrifuged at 3000 rpm for 5 min to get rid of any precipitate. As many droplets of stain as sections were to be processed at a time were put on a square piece of paraffin film, on a filter paper soaked in distilled water, inside a Petri dish. Two Petri dishes were prepared like this, one with uranyl acetate and another with lead citrate. This allowed staining several grids in a single batch, following the protocol below:

Uranyl acetate	3 min in the dark.
Wash in three baths of 50% ethanol	20 dips each.
Wash in 25% ethanol	20 dips.
Wash in distilled water	20 dips.
Lead citrate	8 min. Sodium hydroxyde pellets are added at this stage to absorb CO_2 .

Wash in three baths of distilled water and allow to dry.

Samples were observed and photographed using a Philips 301 transmission electron microscope (FEI U.K. Ltd., U.K.).

2.5. Anaesthesia:

Anaesthesia was used during any protocol that was considered would stress the fish in excess, and is specified in the materials and methods section of each experiment in this thesis. Fish were anaesthetised by immersion in a 1:1000 dilution of 2-phenoxyethanol (Sigma chemicals, U.K.) during 2-4 minutes. After the procedure fish were returned to the holding tank and recovered after a few minutes.

2.6. Fish tagging:

All the fish used in the experiments were tagged with passive subcutaneous transponding – PIT tags (AVID, Labtrac Ltd., U.K.). The fish were anaesthetised and the PIT tags implanted with the use of a plunger or an implantation gun (AVID) in the dorsal area behind the head.

2.7. GnRHa implantation:

In all GnRHa experiments, ethylene-vinyl acetate copolymer pellets (approx. 1mm diameter, 3mm length) containing D-Ala^6,Pro-*N*-ethylamide-luteinising hormone releasing hormone (available commercially in 1996 as Reproboost^{TM} , Aquapharm Ltd. , Columbia, MD, USA, or otherwise provided by Dr. D. Mylonas and Dr. Y. Zohar as a gift to N.Bromage), were used. These implants provide a slow release of the GnRH agonist over an extended period of time (Zohar *et al.* 1995). Pellets were implanted as described for the PIT tags in the previous section (figure 2.4).

2.8. Blood sampling and plasma separation:

Samples of 1-1.5 ml of blood were obtained from the caudal ventral aorta using 21 G sterile hypodermic needles and sterilised 2 ml syringes (Becton Dickinson S.A., Spain). The needles were previously rinsed with heparin (Sigma-Aldrich Co. Ltd, U.K.) to avoid blood clotting. Blood samples were transferred to clean Eppendorf centrifuge tubes (Eppendorf, Netheler-Hinz-GmbH, Hamburg, Germany) and centrifuged at 13,000 rpm for 10 min in a micro-centrifuge (Biofuge, Heraeus Sepatech, Germany). Separated plasma was then transferred to clean Eppendorf tubes and stored at -20°C until analysis.

2.9. Steroid analysis:

Steroid analysis was carried out at the CEFAS laboratory in Lowestoft, under the instructions of Dr. A. Scott and following methods described in Moses Inbaraj *et al.* (1997), Scott *et al.* (1982, 1984 and 1997), Canario and Scott (1990), Moore *et al.* (2000) and Tveiten (2000).

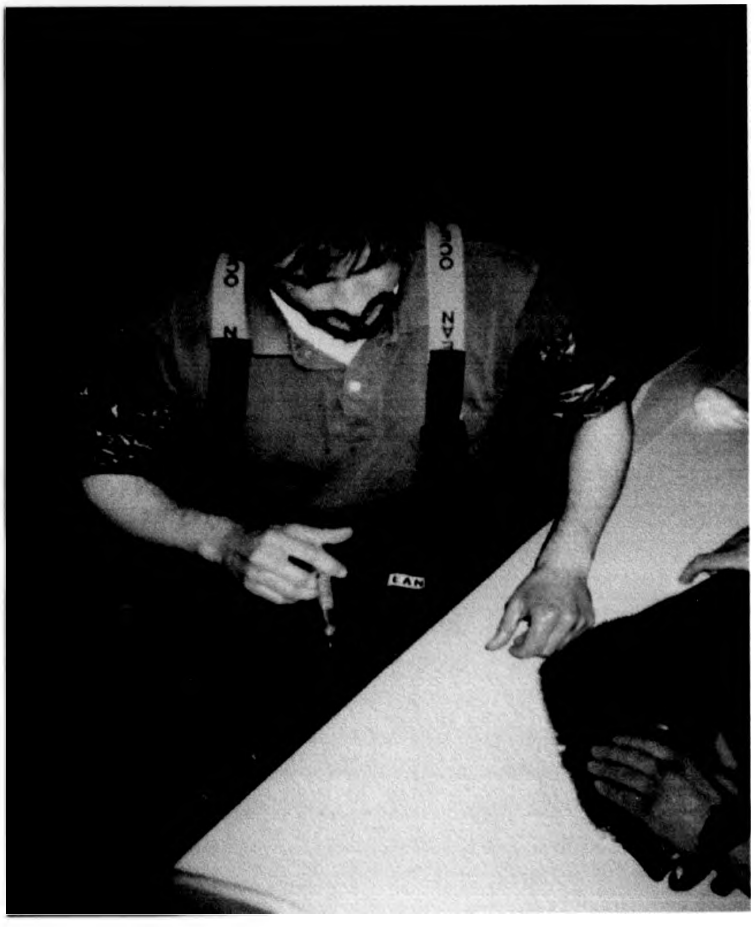


Figure 2.4: Implantation of a male Atlantic halibut with GnRHa pellets with the use of a plunger. The same procedure was used for the implantation of PIT tag.

2.9.1. Extraction of free and sulphated steroids from plasma:

Sub-samples of 100 µl of plasma were transferred to glass tubes (16x100 mm) and 4 ml of diethyl ether was added. Tubes were shaken for 4 min, left to settle and then frozen in liquid nitrogen. The ether from the tubes was then poured into new tubes (12x75 mm), keeping the residue for extraction of sulphated steroids (see below). The ether was evaporated in a water bath at 45°C for approximately 50 min. The free steroids thus extracted were re-dissolved in 1 ml of assay buffer (see table 2.2), the tubes were warmed, shaken and then stored at -20°C.

The residue from the extraction of the free steroids was blown dry under nitrogen for 2 min. Once dry, they were re-dissolved 1 ml of ethanol, vortex and then centrifuged at 2500 rpm for 20 min at 4°C in a cooled centrifuge. The supernatant was poured into new tubes and dried under nitrogen. Once dried, 20 µl of distilled water and 4 ml of a mixture of trifluoroacetic acid and ethyl acetate (100 ml ethyl acetate + 1.4 ml trifluoroacetic acid) was added. The tubes were gently shaken, covered with parafilm and a glass sheet placed over all the tubes. The samples were heated overnight (for at least 15 h) in a water bath at 45°C. The following day the tubes were dried under nitrogen. 200 µl of distilled water and 4 ml of diethyl ether were added to the tubes and these were shaken for 4 min, frozen in liquid nitrogen, and the ether poured into new tubes. The ether was then evaporated in a water bath at 45°C (approx. 50 min). The remaining sulphated steroids were re-dissolved in 1 ml assay buffer, the tube warmed, then shaken and stored at -20°C.

Table 2.2: Composition of assay buffer used in the steroid extraction and analysis. The 0.5 M PO_4 buffer was made and frozen until use. The assay buffer was made as required and never stored for more than 2 weeks.

0.5 M PO4 buffer	
Na_2HPO_4 (anhydric)	115 g
$NaH_2PO_42H_2O$	29.6 g
Distilled water	2 l

Assay buffer	
0.5 M PO_4 buffer	50 ml
Water	450 ml
Bovine albumin	1 g
Sodium chloride	4 g
EDTA acid	0.15 g
Sodium azide	0.05 g

2.9.2. Radioimmuno assays (RIA) of plasma steroids:

RIAs of plasma steroids were carried out in borosilicate glass tubes (10 mm x 75 mm), placed in an aluminium rack. To explain the protocol of the assays, the protocol for 17, 20β-P is described.

100 µl of assay buffer were added to 12 duplicated tubes (labelled s1, s2....s9, max, blank and total). Doubling dilutions of a standard solution of 17, 20β-P were made by transferring aliquots, starting with a concentration of 10 ng 17, 20β-P/ml of assay buffer. This provided a range of 9 standard concentrations from 2-500 pg/100 µl. Once the standards were prepared, 100 µl of each sample was added to duplicated borosilicate tubes.

15 µl of radioactive label (10 µCi/ml in ethanol) were diluted in 12 ml of assay buffer in a polythene scintillation vial. 100 µl of this solution was added to the duplicate "blank" tubes. Then 36 µl of a 1/100 antiserum (sheep antibody) dilution (100 µl of antiserum in 10 ml of 0.05 M PO_4 buffer plus 0.05 g of Sodium azide stored at 4°C) was added to the radioactive label solution in the scintillation vial. The solution of radiolabel

and antiserum was added to all the remaining borosilicate tubes (100 µl per tube). The tubes were then stored at 4°C overnight in the aluminium rack.

A suspension of charcoal was used to wash away the unbound radioactive label. 0.5 g of gelatine powder was dissolved in a minimum amount of warm distilled water in a glass flask. Once dissolved, 50 ml of 0.5 M PO_4 buffer was added, and the solution diluted to 500 ml with distilled water. 2.5 g of Norit charcoal and 0.25 g of dextran T70 were added to the solution, the flask was shaken by hand and then stored at 4°C.

The following morning, the rack of tubes was embedded in crushed ice and 1 ml of chilled charcoal suspension was added to all tubes from a dispenser, except the "totals". This operation was carried out as rapidly as possible, shaking the dispenser regularly to keep the charcoal suspended, and avoiding shaking the tubes (which would encourage the charcoal to pull the radiolabel off the antibody). The "totals" tubes were given 1 ml of assay buffer. After 12 min, the tubes were loaded into centrifuge racks and spun at 2000 rpm for a further 12 min in a refrigerated centrifuge. As rapidly as possible, the liquid from each tube was poured into polythene scintillation vials. To each scintillation vial, 7 ml of Optiphase (Hisafe II, Pharmacia LKB) was added. The vials were then counted in a particle counter and the radioactivity counts compared to the standard curve to measure the steroid concentration in plasma.

The same procedure was used for all steroid RIAs, with the only changes being the volumes of antiserum and radiolabel added to the assay buffer, and the volume of buffer (table 2.3). These were influenced by the concentration of the antisera and the original radioactivity of the radiolabel.

The levels of cross-reaction of the different antibodies with different steroids were taken into consideration (see table 2.4 and literature cited)

Table 2.3: Volumes of assay buffer, radiolabel and antibody used in each steroid RIA. Volumes of radiolabel were calculated to give a final radioactivity count per tube of approximately 4000-5000 dpm. Volumes of antibody were calculated to give a maximum binding of 50%. In parenthesis are the antibody used for each steroid.

Steroid	Assay buffer	Radio label	Antibody
Androstenedione	13 ml	14 µl	21 µl (G3/1)
17, 20 α	12 ml	30 µl	120 µl (255/1)
17, 20 β, 21-P	13 ml	7 µl	54 µl (216/4)
Testosterone	10 ml	15 µl	100 µl (125/6)
11-ketotestosterone	10 ml	14 µl	60 µl (J5)
11 deoxycortisol	12 ml	36 µl	90 µl (ACCMO)
17, 20β-P	12 ml	15 µl	36 µl (Sheep)
Oestradiol	11 ml	44 µl	50 µl (510/5)
5β, 3α, 17, 20β	10 ml	15 µl	72 µl (Sheep)

Table 2.4: Percentage cross reactivity of various steroids with the different antisera.

Steroids	Antisera								
	Rabbit 255/1	Rabbit 216/4	510/5	125/6	J5	G3/1	ACCMO	Sheep	201/3
Testosterone	<0.1	<0.1	<0.01	100	0.4	0.6	-	<0.001	-
11-ketotestosterone	<0.1	<0.1	<0.01	2.4	100	0.05	-	<0.001	-
5α-Dihydrotestosterone	-	-	0.06	31	0.4	2	-	-	-
11β-OH-Testosterone	-	-	<0.01	8.9	2.0	-	-	-	-
Androstenedione	-	-	<0.01	0.4	0.1	100	-	-	-
Androsterone	-	-	<0.01	0.04	<0.01	-	-	-	-
E2	<0.1	<0.1	100	0.05	<0.01	<0.3	-	<0.001	-
Progesterone	-	-	<0.01	<0.01	<0.1	0.02	-	<0.003	-
20β-OH Progesterone	-	-	<0.01	<0.01	-	-	-	0.13	-
17α-OH-Pregnenolone	-	-	-	<0.01	-	-	-	-	-
Estrone	-	-	6	0.01	-	<0.3	-	-	-
Corticosterone	-	-	<0.01	-	-	-	-	<0.001	-
11-Deoxycorticosterone	-	-	<0.01	<0.01	<0.1	<0.02	-	-	-
Cortisol	<0.1	<0.1	<0.01	<0.01	<0.01	<0.3	-	0.05	-
Cortisone	<0.1	<0.1	<0.01	-	-	-	-	0.003	-
21-deoxycortisol	<0.1	-	-	-	-	-	-	-	-
11-deoxycortisol	<0.1	<0.1	-	-	-	<0.02	100	-	-
5β-pregnane-3β,17,20α-triol	0.7	-	-	-	-	-	-	-	-
5β-pregnane-3α,17,20α-triol	-	-	-	-	-	-	-	-	44.5
5β-pregnane-3α,17,20β-triol	<0.1	<0.1	-	-	-	-	-	-	Stdard
5β-pregnane-3α,17,20β-triol 20-sulphate	-	-	-	-	-	-	-	-	65.9
5β-pregnane-3α,17,20β,21-tetrol	<0.1	0.4	-	-	-	-	-	-	100
3α,17,20β,21-tetrahydroxy-5β-pregnan-11-one	<0.1	-	-	-	-	-	-	-	3.4
3α,17-Dihydroxy-5β-pregnan-20-one	-	-	-	-	-	-	-	-	96.1
3α,17,21-Trihydroxy-5β-pregnan-20-one	-	-	-	-	-	-	-	-	64.1
17,20β-P	0.3	0.5	-	-	-	-	<0.01	100	<0.02
17-hydroxy-4-pregnen-3-one	0.2	<0.1	-	-	-	-	-	-	-
17α-OH-Progesterone	-	-	<0.01	<0.01	<0.01	<0.03	-	0.025	-
17α-OH,20β Dehydroprogesterone	-	-	<0.01	<0.01	<0.01	<0.02	-	-	-
17α-Hydroxy-4-pregnene-3,20-dione	-	-	-	-	-	-	2.2	-	-
17α,21-Dihydroxy-5β-pregnane-3,20-dione	-	-	-	-	-	-	10.3	-	-
17,20β,21-trihydroxy-4-pregnen-3-one	<0.1	<0.1	-	-	-	-	-	-	-
21-Hydroxy-4-pregnene-3,20-dione	-	-	-	-	-	-	6.7	-	-

2.9.3. Analysis of GnRHa in plasma:

The analysis of GnRHa in plasma was carried out in duplicated plastic tubes. 100 µl of assay buffer were added to 14 duplicated tubes (labelled s1, s2....s12, max, blank). Doubling dilutions of a standard solution of GnRHa were made by transferring aliquots, starting with a concentration of 2.5 ng GnRHa in 100 µl. Once the standards were prepared, 50 µl of each plasma sample and 50 µl of assay buffer were added to duplicate plastic tubes. 100 µl of antibody (at a concentration of 1/8000 in assay buffer) was added to every tube, except the "blanks" that received 100 µl of assay buffer. Tubes were incubated overnight at 4°C. The following morning, 50 µl of radioactive label was added to all the tubes. One further duplicate of tubes, the "totals", was filled solely with 250 µl of radiolabel. The tubes were incubated for 5 h at 4°C. 100 µl of SAC-CEL (Anti-Rabbit:Second Antibody Cellulose) were then added to all tubes. The SAC-CEL captures the bound antibody. The tubes were vortexed and left to settle for 30 min before adding 1 ml of distilled water and centrifuging at 2500 rpm for 10 min. After centrifugation, the water was discarded and the pellet at the bottom of the tube was retained. The tubes were then counted for radioactive particles and radioactivity counts compared to the standard curve to measure GnRHa concentration in plasma.

2.10. Egg lipid analysis:

Three sub-samples of 50 fertilised eggs were randomly collected from each batch of eggs that exhibited fertilisation rates higher than 10% from the broodstock nutrition experiments (chapter IV). Samples of eggs were stored in chloroform-methanol (2:1 volume) in 10 ml glass bottles. Chloroform-methanol extracts most of the lipids from the eggs, so care was taken to avoid any leakage from the sample bottles. The samples were stored in a chest freezer (-20°C) until analysis of the lipid content.

2.10.1. Extraction of total lipids from eggs:

Lipid extraction from eggs was carried out following a Folch extraction (Folch *et al.*, 1957). Eggs and chloroform-methanol from each sub-sample were homogenised in crushed ice with an Ultra Turrax homogeniser (Janke & Kunnel Ika Labortechnik, Germany). The resulting suspension was filtered through a paper filter (rinsing the filter three times with chloroform-methanol (2:1)) , evaporated to dryness with nitrogen, and re-dissolved in 4 ml of chloroform-methanol (2:1) and 1 ml of potassium chloride (0.25%). The samples were then centrifuged at 3000 rpm (Centaur2 MSE Sanyo) for one second. The top layer was discarded and the bottom layer was poured into a pre-weighed glass bottle and evaporated completely. The amount of lipids remaining in the bottle was weighed, re-dissolved in chloroform-methanol, aerated with nitrogen and stored at -20°C.

2.10.2. Eggs fatty acid analysis:

Fatty acid methyl esters were prepared by acid catalysed transmethylation. 1 mg of the extracted lipids was added to 2 ml of H_2SO_4 (1% in methanol) and 1 ml of toluene, and incubated at 50°C overnight.

The next morning, the samples were allowed to cool down. Methyl esters were extracted first by adding 3 ml of distilled water and 3 ml of diethylether:hexane (1:1). The samples were centrifuged at 3000 rpm, reserving the top layer (organic layer). 3 ml of diethylether:hexane was added to the bottom layer and the samples were re-centrifuged. The top layer from the second centrifugation was added to the organic fraction. 3 ml of 2% $KHCO_3$ were added to the organic fraction and the mixture centrifuged at 3000 rpm. The bottom layer was discarded and the top layer was evaporated gently with nitrogen. The methyl esters were resuspended in hexane with butylated hydroxytoluene (BHT) as an antioxidant (0.01%). Methyl esters were purified by TLC as described in Tocher *et al.*

(1985) before separation and quantification by GLC (Fisons 8000, Thermo Separation Products Ltd., U.K.) using a 30 m x 0.32 mm capillary column with 2β wax coating (Phenomenex Chrompack, Chrompack Ltd, U.K.) and hydrogen as a carrier gas. Individual methyl esters were identified by comparison to known standards.

2.11. Diet preparation:

2.11.1. Hot extrusion:

The High DHA and Krill diets were manufactured at the EWOS Technology Centre in Livingston, Scotland, U.K. Diets were extruded using a Wenger X20 single screw extruder (Wenger, Sabetha, U.S.A.). The extrusion conditions were as follows: 90°C conditioning temperature, 30 s transit time in conditioner and 15% moisture addition. The pellet size was 9 mm in diameter.

2.11.2. Cold extrusion:

The Standard and High AA diets were manufactured at the Institute of Aquaculture. Diets ingredients were mixed in 50 kg batches in a Mini 150 concrete mixer (Belle, U.K.). The ingredients were put through a Hobart A 200 mincer (Hobart Ltd., U.K.) calibrated to a pellet size of 4 mm in diameter.

2.11.3. Diet Proximal analysis:

The diets utilised in Chapter IV were analysed using standard procedures of the Nutrition Laboratory at the Institute of Aquaculture on triplicate samples.

Moisture content was analysed by weighing each sample, oven drying it at 105°C for 12 h and then re-weighing it. Moisture content was calculated by the formula:

$$\% \text{ moisture} = [(\text{wet weight} - \text{dry weight}) / \text{wet weight}] \times 100$$

Protein content was determined by establishing the nitrogen content using the Kjeldal technique. Approximately 200 mg of sample were transferred to a Kjeldal digestion tube. Two mercury kjeltabs (BDH Chemicals Ltd., U.K.) and 5 ml of concentrated sulphuric acid were added to each digestion tube. The samples were placed in a digestion block at 420°C for 1 h and then allowed to cool. 20 ml of deionised water and 5 ml of sodium thiosulphate solution was added to the samples. The samples were then distilled and tritrated using a Kjeltec Auto 1030 Analyser. (Tecator, Hoganas, Sweden). Protein content was calculated according to the formula:

%protein = [(sample titre – blank titre) x 1750.875] / sample weight

Lipid content was determined by the same procedure described for the egg samples in section 2.10.1.

Ash content was determined by ashing the samples in a muffle furnace over 24 h at 500°C, and reweighting the remnants. The percentage ash content of the dry samples was calculated by the equation:

%ash = (ash weight / dry weight) x 100

Fibre content was analysed by using the following protocol. Approximately 1 g of lipid-free sample was weighted into scintiglass crucible. The crucible was placed into a Fibretec Systems M 1020 Hot extractor (Tecator) and 150 ml of boiling 1.25% sulphuric acid solution was added to the sample. 10 drops of octanol was added to each sample. The sample was boiled for 30 min and then washed with 150 ml of boiling deionised water. The sample was then boiled for another 30 min in 150 ml 1.25% sodium hydroxide and 10 drops of octanol. The sample was washed with 150 ml of boiling deionised water. The crucible was placed in an oven at 110°C for 12 h., allowed to cool and reweighed. The

crucible was then ashed at 550°C in a muffle furnace for 2 h., allowed to cool and reweighed. The fibre content was calculated by the formula:

%fibre = [(dried crucible weight – ashed crucible weight)/ sample weight] x 100

2.12. Ultrasound:

The identification of sex and stage of maturity of fish are essential for hatchery operators and reproduction research. Ultrasonography is a non-invasive technique that avoids damage and minimises the stress to valuable broodstock. The study of gonadal development and final maturation in Chapter II in this thesis made extensive use of this technique.

Martin *et al.* (1983) offered a thorough description of the main principles of the use of ultrasound as a tool for sex determination of fish. In essence, a beam of ultrasound is radiated from a directional transducer, which also acts as a receiver. The ultrasound penetrates the animal and is scattered, absorbed or reflected depending on the properties of the different tissues, and the relative changes of these properties between them. The transducer receives the ultrasound echo and a cross-sectional image of the tissue interfaces is generated. The main parameters responsible for the acoustic properties of tissue are density and compressibility. A bright spot in the image represents a large change in one or both of these properties; grey tones indicate smaller changes, and dark structures are areas where no changes take place (Martin *et al.* 1983). The frequency of ultrasound used influences the resolution and the penetration of the beam, with higher frequencies offering better resolution at the cost of decreased penetration.

The ultrasound machines used in this thesis were an Aloka echo camera SSD-210DX11 and a Concept\MLV Dynamic Imaging LTD, both portable units. The

transducers used with each machine were both T-shaped 7.5 MHz linear transducers, which provided ideal resolution and penetration. No animal was culled in the learning process of identification and interpretation of the ultrasound images, and this knowledge was acquired from the very limited literature available and the study of relative changes of structures of Atlantic halibut of known and unknown sex and stage of maturity.

2.13. Statistical analysis:

Data were manipulated using Excel 97 (Microsoft) All the statistical analyses were carried out using GraphPad InStat version 3.01 for Windows 95/NT, GraphPad Software, San Diego California USA, www.graphpad.com. Normality was generally assessed using the method of Kolmogorov and Smirnov and homogeneity of variances using Bartlett's test. Percentage values were arcsine transformed before being analysed by ANOVA. Comparisons between means were tested using Tukey-Kramer analysis, unless otherwise specified. Significance was set at $p = 0.05$.

Data are normally presented as means ± standard error of the mean (s.e.m.). Error bars on graphs are s.e.m.

Chapter III: Oocyte growth and ovarian development.

3.1. Introduction:

The understanding of oocyte growth and ovarian development is an important prerequisite for the better management of seed production in commercial species. This chapter describes the morphology and ultrastructural aspects of oocyte growth in Atlantic halibut, and the dynamics of its gonadal development. The extensive use of ultrasound scanning highlights this technique as a non-invasive method for studying diverse aspects of gonadal development, including sexing, maturation status and final maturation of discrete batches of eggs.

3.1.1. Oocyte growth

The development of female germinal cells is probably the longest physiological process to take place in any given vertebrate – with the exception of ageing. The sequence of events that constitute oocyte development in fish, i.e. the transformation of germinal cells from oogonia into oocytes and, finally, eggs, has been the subject of extensive reviews and studies by many authors over the last two decades (Nagahama 1983; de Vlaming 1983; Dodd and Sumpter 1984; Wallace *et al.* 1987; Bromage and Cumaranatunga 1988; Kjesbu and Kryvi 1989; Wallace and Selman 1990; West 1990; Selman *et al.* 1993; Tyler and Sumpter 1996; Coward and Bromage 1998, 2000). Whatever the diverse oocyte staging and classifications proposed, the same general major events appear to be consistent in all teleost species studied, namely, oogenesis, primary oocyte

growth, cortical alveolus stage, vitellogenesis, maturation and ovulation (Bromage and Cumaranatunga 1988). In any case, one must be aware that any attempt to classify this process into specific categories is only a working tool, the boundaries between one phase and the next may often overlap.

Colonisation of the genital ridge by a finite number of germinal cells occurs early during embryogenesis in vertebrates. A primordial gonad is formed, consisting of somatic and germinal cells. In females, after a phase of proliferation of the germinal cells, a number of oogonia are established. In bony fishes, as opposed to mammals, these cells remain present in the adult female, still entering meiosis. This long meiosis, which is delayed probably for years during the life of the individual and at different stages, constitutes the complex process of oogenesis and egg production. When the development of the ovary is complete, it is constituted by oogonia, oocytes at different stages of development (present or not at the same time in the same ovary. See below), follicular cells, stroma or supportive tissue - often extending towards the centre of the ovary forming folds or trabeculae – and nervous and vascular tissue.

Oocyte development can be described according to processes taking place at nuclear, cytoplasmic, membrane and follicular level. The classification of oocyte stages below follows that of Bromage and Cumaranatunga (1988)

3.1.1.1 Primary growth:

Oogonia are rounded cells of small size, with a high nucleus to cytoplasm ratio, which are frequently grouped in nests or solitary in the stroma of the ovigerous folds (de Vlaming 1983). They can sometimes be seen in the periphery of the folds or close to developing oocytes. Oogonia multiply by mitosis, until they enter the first meiotic division. During this phase, the nucleus is very active, with unpaired chromosomal threads visible

after DNA replication (leptotene), to later organise into pairs of homologous chromosomes (zygotene) that form synaptonemal complexes (pachytene). Chromosomes then become unpaired producing lampbrush structures during diplotene. Meiosis is arrested at this stage in the prophase of the first meiotic division. Wallace and Selman (1990) provide an analysis of the ultrastructural aspects of chromosome development during these events. Leptotene of the first meiotic division is described as the chromatin nucleolar stage or primary oocyte. The appearance of a conspicuous nucleolus associated with chromatin threads characterises this stage. Squamous follicular cells start to surround an oocyte with a central nucleus and a thin cytoplasmic layer. Oocyte and follicular cells together constitute the follicle, which abandons its primitive location in the stroma.

There is now a general increase in oocyte volume, particularly of the cytoplasm, and a reduction of the nucleus to cytoplasm ratio. A number of nucleoli appear in the periphery of the nuclear envelope, giving its name to this phase– the early perinucleolar stage or secondary oocyte (zygotene). In some species a cumulus of basophilic material may appear first in the perinuclear cytoplasm, the Balbiani body. This is composed of different cytoplasmic organelles, including mitochondria, multivesicular bodies, endoplasmic reticulum and Golgi complex. During the late perinucleolar stage, or stage 3, the Balbiani body, when present, migrates to the periphery of the cytoplasm, where it disperses (Nagahama 1983). A reduction in the basophilia of the ooplasm and a further reduction in the nucleus/cytoplasm ratio accompany cytoplasmic growth. This phase corresponds to the diplotene of the first meiotic division. During primary growth, the oocytes grow from a diameter of 10 – 20 μm at leptotene to a diameter ranging from 100 to 200 μm (Nagahama 1983, Wallace and Selman 1990).

Concomitant with the cytoplasmic growth, there is a development of the follicular layer. The follicle cells increase in number, forming first a single layer adjacent to the

oocyte, the granulosa layer. This layer is invested by a basal lamina and a highly vascularised theca containing fibroblast-like cells. The granulosa cells project processes towards the oocyte, which in turn is covered in microvilli. These serve as communication channels between granulosa and oocyte. In between these projections, dense material starts to accumulate to form the zona radiata or chorion.

3.1.1.2 Secondary growth:

The appearance of cortical alveoli signals the start of the secondary growth of the oocyte. These are PAS + vesicular structures that in most cases contain mucopolysaccharide or glycoproteins. Some authors refer to them as yolk vesicles. However, their endogenous nature (they are synthesised by the oocyte), together with the fact that they are displaced to the periphery of the oocyte with the intake of vitellogenin to release their contents into the perivitelline space after fertilisation, thus playing no role in the nutrition of the embrio, make the reference to "yolk" in this later name confusing and inappropriate. The contents of the cortical alveoli serve to harden the vitelline envelope and prevent polyspermy when discharged into the perivitelline space during the cortical reaction (Yamamoto 1961 cited in Tyler and Sumpter 1996; Selman *et al.* 1988). Most of the growth of the oocyte during this phase is due to these structures.

In some species, especially marine species, lipid bodies can appear during the cortical alveoli stage (de Vlaming 1983). These smaller droplets generally coalesce into bigger droplets towards the end of vitellogenesis or during oocyte maturation.

Stage 4 is also characterised by the development of the zona radiata (zona pellucida, vitelline envelope or chorion) between the oocyte cytoplasm and the granulosa layer (Bromage and Cumaranatunga 1988). Pore canals, in which microvillus extensions of both granulosa cells and oocyte can be found, penetrate this layer, which increases in

thickness during the rest of development. The nuclear membrane at this stage presents an irregular shape with numerous gaps in light microscopy preparations.

The next major event in oocyte growth is vitellogenesis. Pituitary gonadotropin is released into the bloodstream stimulating the hepatic synthesis and secretion of vitellogenin (VTG) in a process mediated by estradiol-17β synthesised by the ovary (Elliot *et al.* 1979). Vitellogenin, a large molecular weight (160 kDa monomer weight in Atlantic halibut, according to Norberg 1995) glycolipophosphoprotein, is sequestered by oocytes by receptor-mediated endocytosis, and is the major precursor of yolk proteins. VTG is taken up by oocytes that have reached a certain critical size, possibly after the development of patency, i.e., the opening of intercellular channels through the follicular tissues to the oocyte surface where the highly specific receptors are located (Nagahama 1983). Wallace and Selman (1990) provide an extensive review of the ultrastructural aspects of the aggregation of vitellogenin to yolk granules.

The first yolk granules are formed in the periphery of the oocyte (peripheral yolk granular stage or stage 5 oocytes). The nucleus is at the centre of the oocyte during this stage. The yolk granules begin to accumulate in the cytoplasm in a centripetal fashion, until most of it is occupied. At the same time, the nucleus (germinal vesicle) starts to migrate to the periphery of the oocyte (stage 6 oocytes) towards the animal pole, where the micropyle is situated.

Vitellogenesis can account for as much as 95% of the final size of the egg (Tyler 1991). However, in pelagic eggs the contribution of vitellogenesis is much less (between 11% and 40%), due to the hydration phase which occurs prior to ovulation.

3.1.1.3 Maturation:

When the oocytes reach a critical species-specific size, the sequestration of VTG ceases. When the nucleus reaches the periphery of the oocyte it is known as a stage 7 oocyte (Bromage and Cumaranatunga 1988). In a process mediated by GTH and MIH (maturation inducing hormone), meiosis resumes with the break down of the germinal vesicle envelope (GVBD) (de Vlaming 1983; Nagahama 1983; Nagahama *et al.* 1995). The first meiotic division is finally completed and the first polar body is expelled. Yolk globules fuse together and in many species, especially those producing pelagic eggs, hydration of the oocyte takes place.

3.1.1.4 Ovulation:

During maturation, or after maturation is complete, oocytes are ovulated into the ovarian lumen. Follicular cells begin to dissociate, and the oocyte, together with the chorion, is released into the lumen. Granulosa and theca stay within the ovary, forming the post ovulatory follicle (POF), which eventually degenerates. Ovulated eggs continue meiosis up to the second meiotic metaphase, when development is arrested until fertilisation.

3.1.2 Patterns of ovarian development.

Ovarian development in teleost has been classified as synchronous, group synchronous or asynchronous (Wallace and Selman 1981). Patterns of oocyte development can be characterised by plotting the size distribution of the oocytes in the ovary.

In synchronous ovaries all oocytes develop at the same time, and the size-frequency distribution is unimodal, although there is often a size range during stage 5. This is typical in species that spawn once in their lives and then die, as in the coho salmon and the sockeye salmon.

In group-synchronous ovaries there are at least two cohorts of developing oocytes at any one time. The leading clutch of oocytes, normally more homogeneous, will constitute the eggs to be spawned during the present season, while the other clutch is arrested at an earlier stage of development and will develop in the subsequent spawning seasons. The oocyte size-frequency distribution is multimodal.

In asynchronous ovaries, oocytes at all stages of development can be found at any time. Size-frequency distribution is continuous except at spawning, when there could be a clear separation between the ripe and yolked oocytes.

As a consequence of oocyte growth, and particularly of vitellogenesis, the Gonado-Somatic Index (GSI) of teleost females increases 50 to 100-fold [GSI = {gonad weight / (fish weight – gonad weight)} x 100] from resting to pre-spawning ovaries (Tyler and Sumpter 1996). In synchronous spawners, GSI of mature females ranges between 18 and 25, although it may reach 40. Group synchronous spawners that produce several batches of eggs during the spawning season, normally have lower GSI. However, it can increase and decrease repeatedly during the spawning season due to the hydration of successive batches of eggs.

3.1.3 Ultrasound scanning.

There are several examples in the literature of the use of ultrasound scanning for sexing of different species of fish (coho salmon, Martin *et al.* 1983; Atlantic salmon, Mattson 1991; cod, Karlsen and Holm 1994; Goddard 1995; yellowtail flounder, winter flounder and Atlantic halibut, Martin-Robichaud *et al.* 1998). It has also been used to monitor final maturation (Shields *et al.* 1993). The use of ultrasound offers a non-intrusive and non-destructive technique for studying the dynamics of gonadal development and maturation, while minimising the manipulation of the fish.

The following sections of this chapter describe the oocyte morphology and gonadal development of Atlantic halibut.

3.2. Description of oocyte growth and GSI development.

3.2.1 Materials and methods.

Samples were obtained from ovaries of fish slaughtered for harvesting or lost to disease. Cube shaped portions of tissue, 3-4 g in weight, were removed from the anterior, middle and posterior portion of the top and bottom lobes of the ovary of one mature post spawning fish to asses the distribution of oocyte stages between the lobes. Similar samples were taken from the anterior, medium and posterior portion of the top lobe of three more females, one of them previtellogenic and two at different stages of vitellogenesis, to further assess the distribution of oocyte stages along the ovarian lobe. The material obtained from the females was fixed in buffered formalin and then embedded in Historesin® after progressive dehydration in increasing gradients of alcohol. The material was then sectioned and mounted on microscopy slides, and stained with either haematoxylin and eosin or toluidine blue, for light microscopy analysis (see Chapter II, section 2.4 for a detailed description of the histological procedures).

The volume fraction of each oocyte stage in each portion of the sampled ovaries was calculated using stereology (see Chapter II, section 2.4.3). Volume fractions were arcsine transformed and one way ANOVA was used to test the homogeneous distribution of the different oocyte stages.

A similar cube shape sample of the front portion of the top ovary was removed from 27 more females at different stages in the season to assess maturation status. Eleven immature fish (hatchery produced 4-year-old females) were sampled in a similar way.

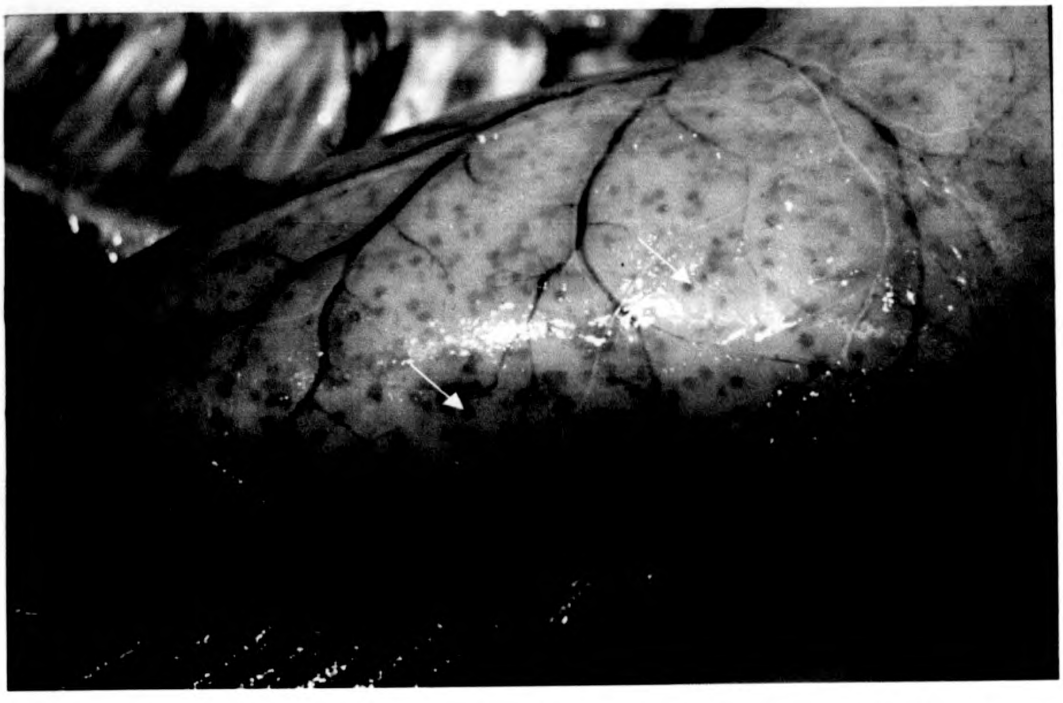
Whenever possible, fish were weighed before slaughter and again after the whole ovary had been removed. Ovary weights were also recorded. Ovarian biopsies were only possible in live fish during, or very close to, spawning. Only three samples were collected in this manner from three different fish (see Chapter II section 2.4.1 for a description of the biopsy technique). Gonadal material was fixed in buffered formalin and embedded in cryo-M-Bed® embedding compound. Sections were obtained using a cryostat, mounted on slides, and stained with haematoxylin and eosin for optical microscopy (see Chapter II, section 2.4.2.4). The material obtained from one of the biopsied fish was fixed in glutaraldehyde and sodium cacodylate for one week and then stored in Sodium cacodylate until processed for transmission electron microscopy (see Chapter II, section 2.4.4).

Images of representative stages of oocyte development were captured as described in Chapter II, with the use of image analysis software (Image-Pro® Plus, Data Cell Ltd.). The diameter of oocytes in different stages was measured using an Olympus BH-2 binocular microscope (Olympus Optical Co. Ltd., U.K.) fitted with a calibrated eyepiece graticule. As most of the oocyte stages were ovoid in shape, diameter was expressed as the mean of long and short axes. Only those oocytes sectioned through the nucleus were measured. At least 30 oocytes of each stage were measured in this way.

3.2.2 Results.

The ovary of Atlantic halibut presented two lobules, which extended laterally along the body cavity (picture 3.1). The wall of the lobules appeared well vascularised (picture 3.2). Both lobules were interconnected at their frontal part, sharing a common oviduct. The ovarian wall extended to the lumen of the ovary in numerous ovigerous folds. Oocyte stages, at least from stage 2 oocytes onwards, were distributed randomly along the ovigerous folds.

Picture 3.1: Dissection of a female Atlantic halibut, showing the top lobe of the ovary extending laterally along the caudal body cavity. The bottom lobe runs parallel to the top one, under the wall formed by the ventral bones. Both lobules were interconnected at their frontal part, sharing a common oviduct (arrow).



Picture 3.2: Detail of the ovary of a female Atlantic halibut showing the vascularised wall of the lobules. Some partially hydrated (hyaline) oocytes can also be seen (arrows).

3.2.2.1 Primary growth.

No oogonia or oocyte 1 stage could be unequivocally identified. Figure 3.1 shows the mean diameters (± s.e.m.) of the different stages of oocyte development.

a. Stage 2 oocytes (early perinucleolar stage).

Stage 2 oocytes ranged from 40 μm to 150 μm in diameter (average = 96.5 ± 2.6 μm). Most of the oocytes at this stage were ovoid in shape. The cytoplasm was highly basophilic and stained dark purple with haematoxylin and eosin (plates 3.1 to 3.5). Through the development of this stage, basophilia of the cytoplasm decreased as its size increased. Areas of increased basophilia appeared close to the nucleus of some oocytes. These, however, did not resemble the Balbiani bodies reported by Bromage and Cumaranatunga (1988) for the rainbow trout.

The nuclear membrane of most stage 2 oocytes was perfectly spherical. Small nucleoli appeared in the periphery of the nucleus. Chromatin strands could be seen in the central area of the nuclear space in some oocytes. The size of the nucleus increased through this phase, but the cytoplasm/nucleus ratio decreased.

b. Stage 3 oocytes (late perinucleolar stage)

Stage 3 oocytes ranged from 130 μm to 400 μm in diameter. The growing cytoplasm decreased in basophilia and its colour faded to a less intense purple when stained with haematoxylin and eosin. The nucleus also increased in size, and many small nucleoli could be seen in the proximity of the nuclear envelope. Stage 3 oocytes could be sub-divided in two sub-stages (see plates 3.6 to 3.9). Stage 3a oocytes presented spherical and continuous nuclear envelope and ranged between 130 μm and 280 μm (mean = 204.2±

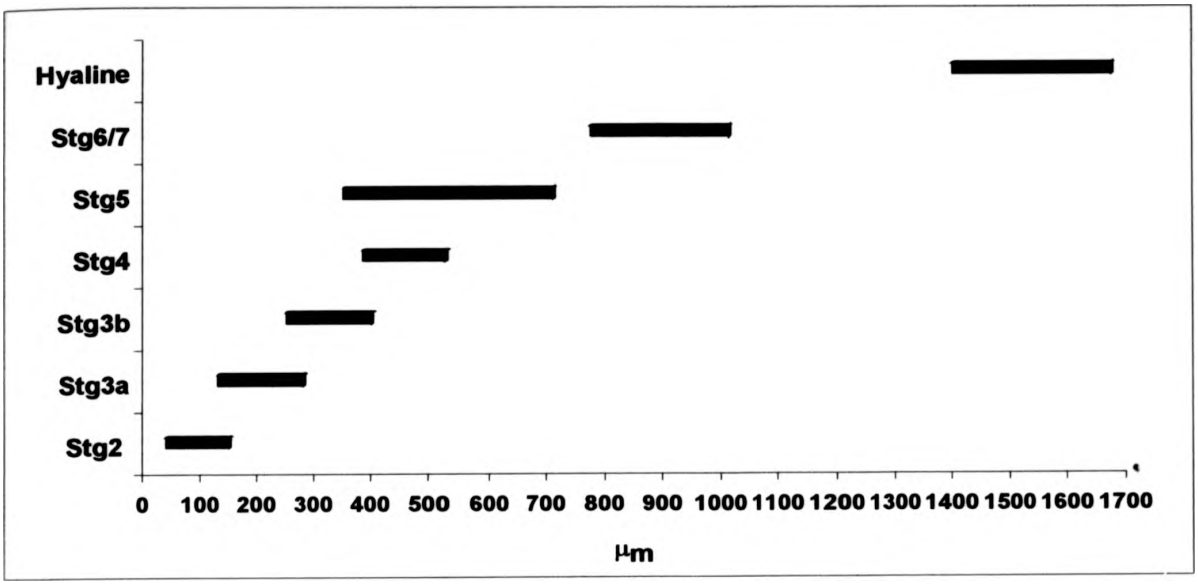


Figure 3.1: Diameter ranges of the different stages of oocyte development of Atlantic halibut. Diameter is expressed in µm. as the mean of long and short axes. Only those oocytes sectioned through the nucleus were measured. At least 30 oocytes of each stage were measured in this way. Stage 2 oocytes ranged from 40 µm to 150 µm in diameter (average = 96.5 ± 2.6 µm). Stage 3a ranged between 130 µm and 280 µm (mean = 204.2 +- 4.1 µm) in diameter. Stage 3b oocytes ranged from 250 µm to 400 µm (average = 329.3 +- 5 µm) in diameter. Stage 4 oocytes ranged from 385 µm to 525 µm (average = 454.5 +- 8.8 µm). Stage 5 oocytes ranged from 350 µm to 710 µm (mean = 559 +- 15.5 µm). Stage 6 and 7 oocytes ranged from 775 µm to 1012 µm (mean = 918.5 +- 12.3 µm). Hyaline oocytes ranged between 1400 µm and 1675 µm (mean 1507.3 +- 23.8 µm) in diameter.

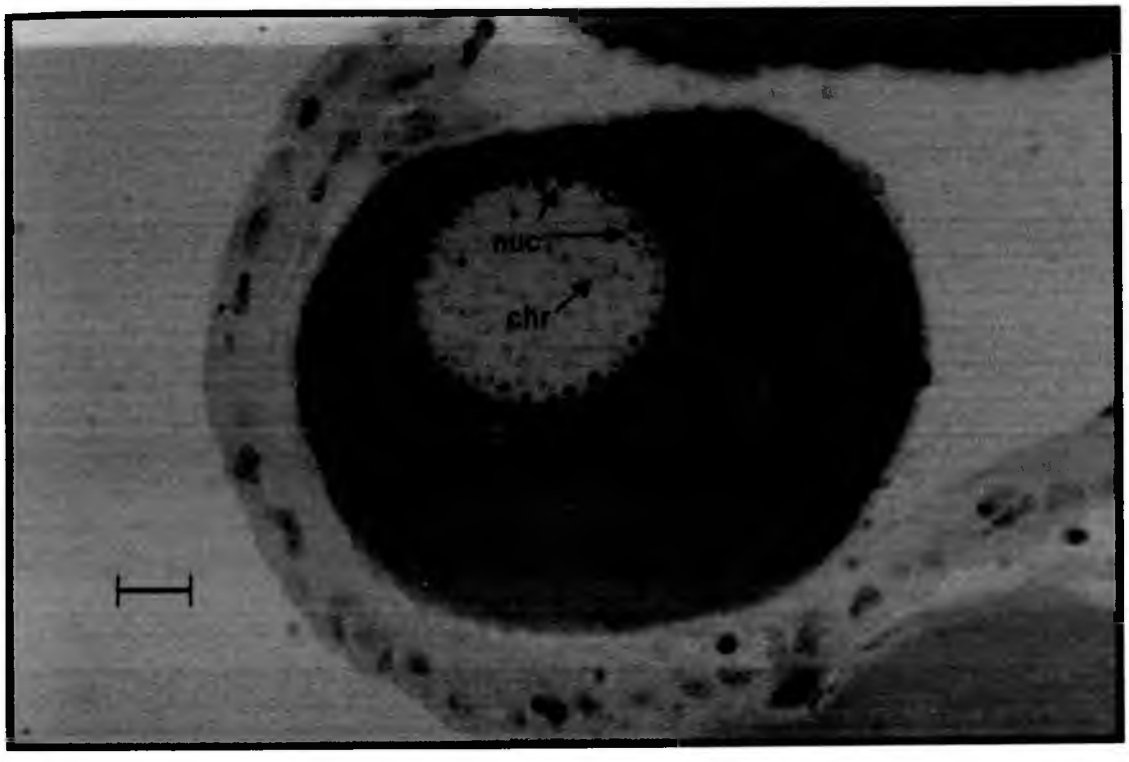


Plate 3.1: Photomicrograph of a 4 μm section of a halibut ovary showing a stage 2 oocyte. Notice the nucleoli in the periphery of the nucleus, and the chromatin strands. Stained with haematoxylin and eosin. Chr, chromatin strands; nuc, nucleoli. Magnification 40 x 3.3. Scale bar = 10 μm.

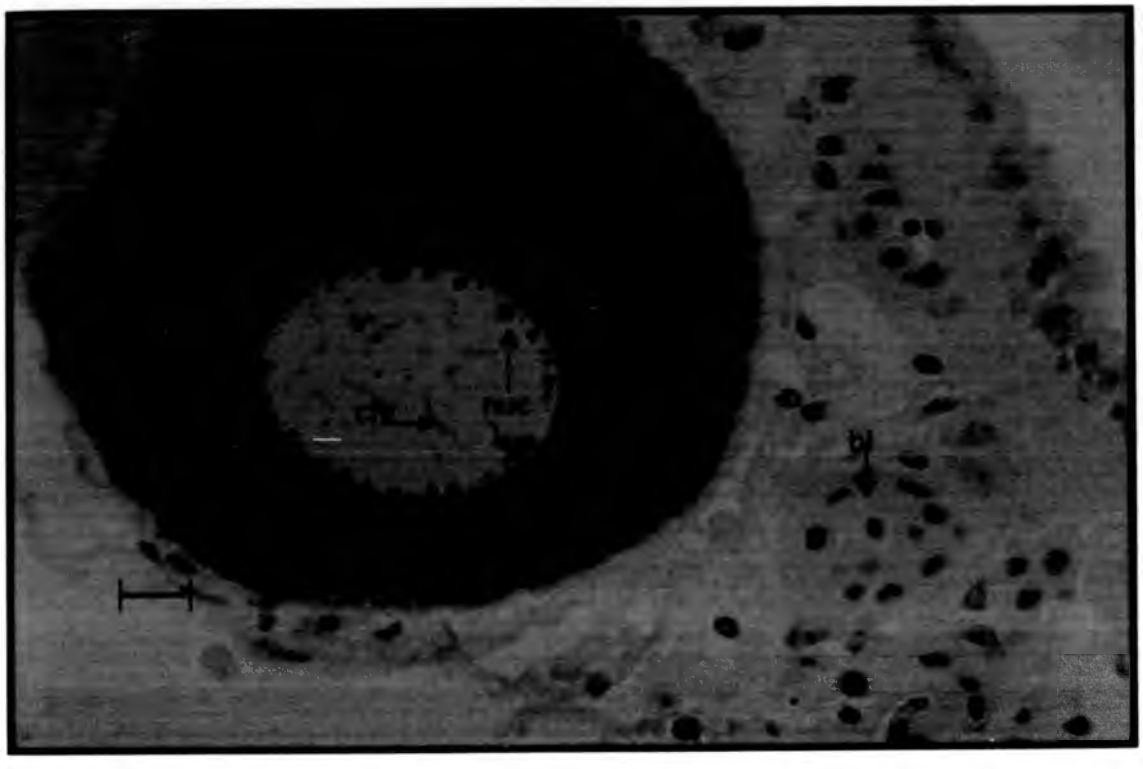


Plate 3.2: Photomicrograph of a 4 μm section of an ovary showing a stage 2 oocyte. Chr, chromatin strands; nuc, nucleoli; bl, blood vessels. Stained with haematoxylin and eosin. Magnification 40 x 3.3. Scale bar = 10 μm.

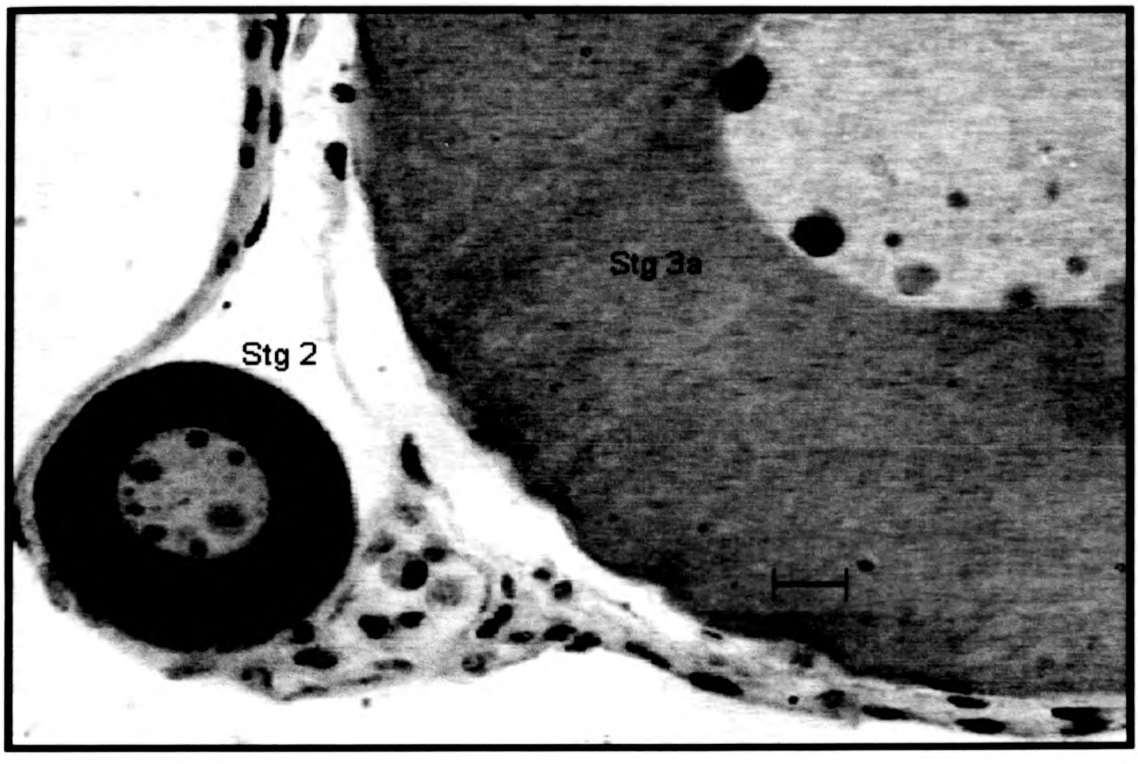


Plate 3.3: Photomicrograph of a 4 μm section of an ovary showing an early stage 2 oocyte (stg 2) close to a stage 3a oocyte (stg 3a). Stained with haematoxylin and eosin. Magnification 40 x 3.3. Scale bar = 10 μm.

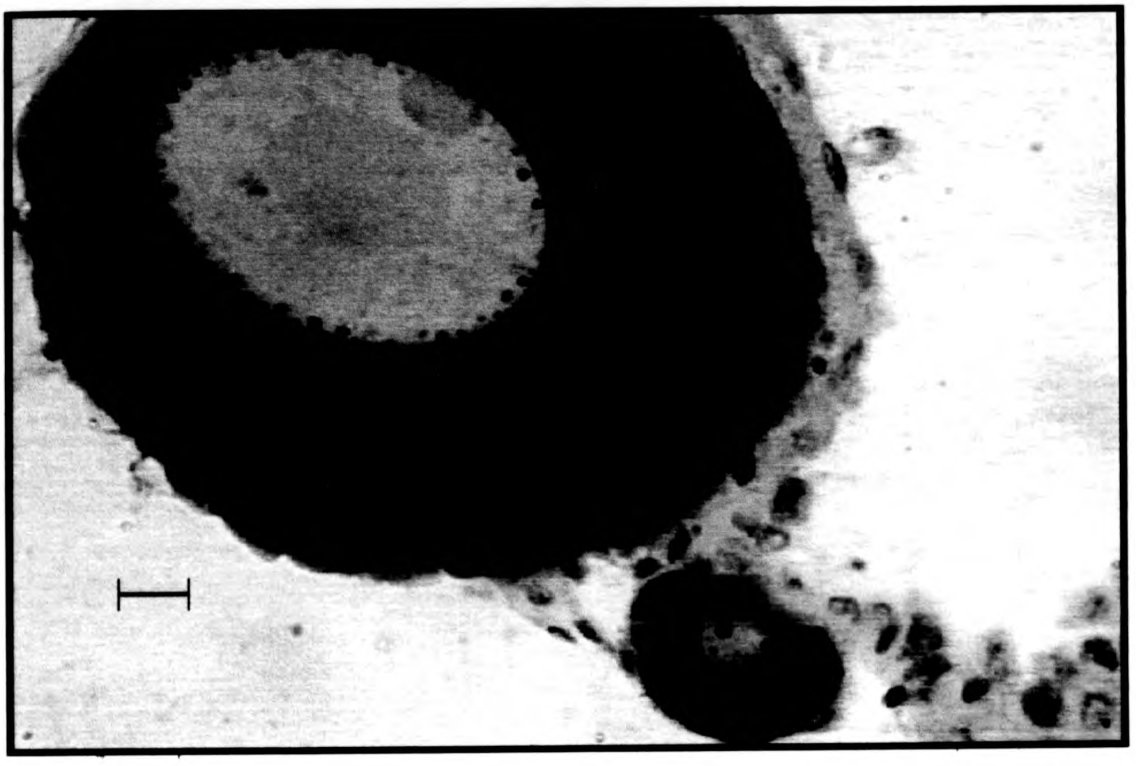


Plate 3.4: Photomicrograph of a 4 μm section of an ovary showing an early stage 2 oocyte (bottom) close to a late stage 2 oocyte (top). Stained with haematoxylin and eosin. Magnification 40 x 3.3. Scale bar = 10 μm.

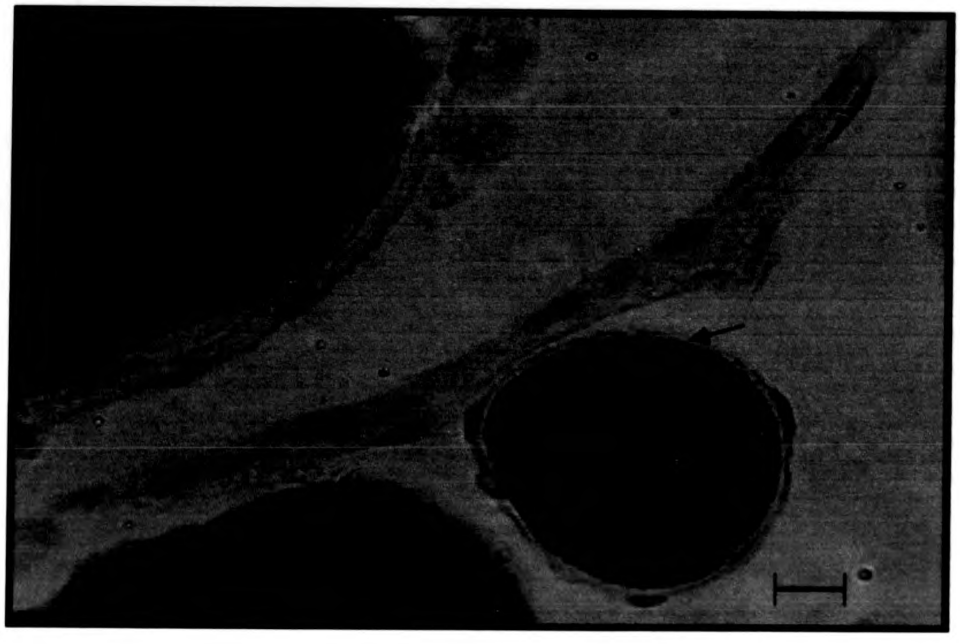


Plate 3.5: Photomicrograph of a 4 μm section of an ovary showing an early stage 2 oocyte with primary follicular cells surrounding it (arrows). Stained with haematoxylin and eosin. Magnification 40 x 3.3. Scale bar = 10 μm.

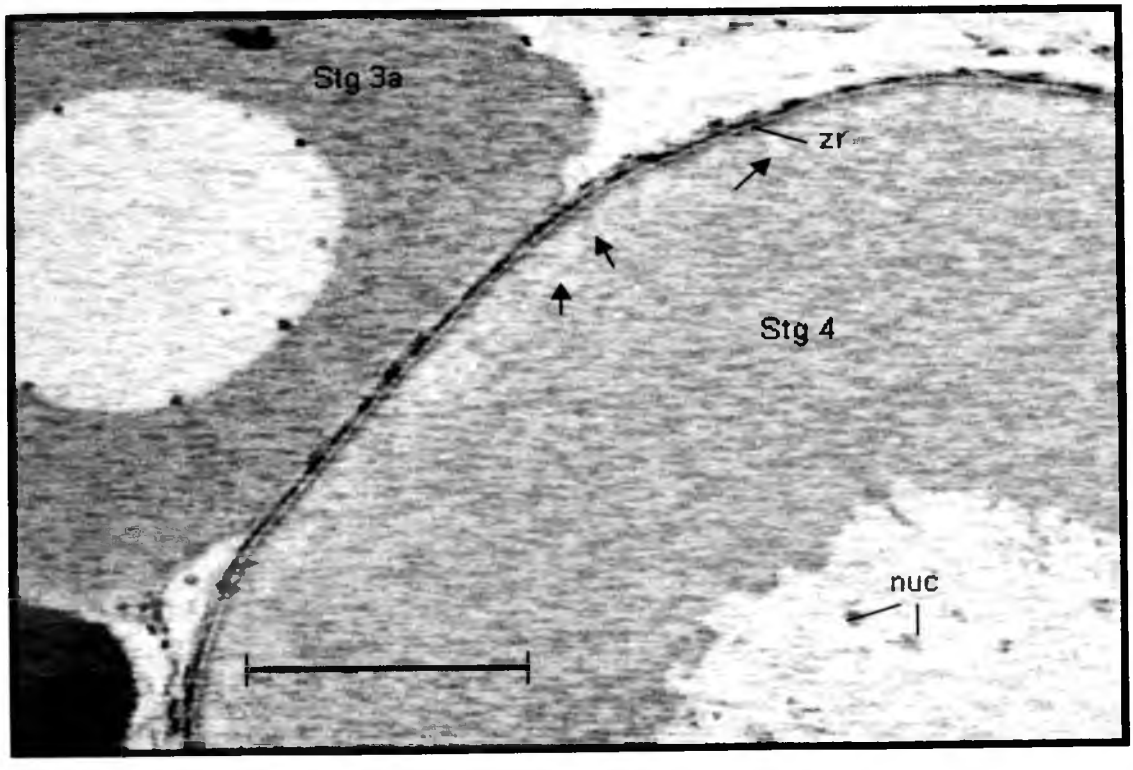


Plate 3.6: Photomicrograph of a 4 μm section of an ovary showing a stage 3a oocyte (stg 3a) close to a stage 4 oocyte (stg 4). Notice the more complex follicular layer in stg4, with a band of dense material that is the forming zona radiata (zr). Cortical alveoli (arrows) can be seen in the periphery of the cytoplasm. Nucleoli (nuc) can be seen away from the periphery of the nucleus. Stained with toluidine blue. Magnification 20 x 3.3. Scale bar = 100 μm.

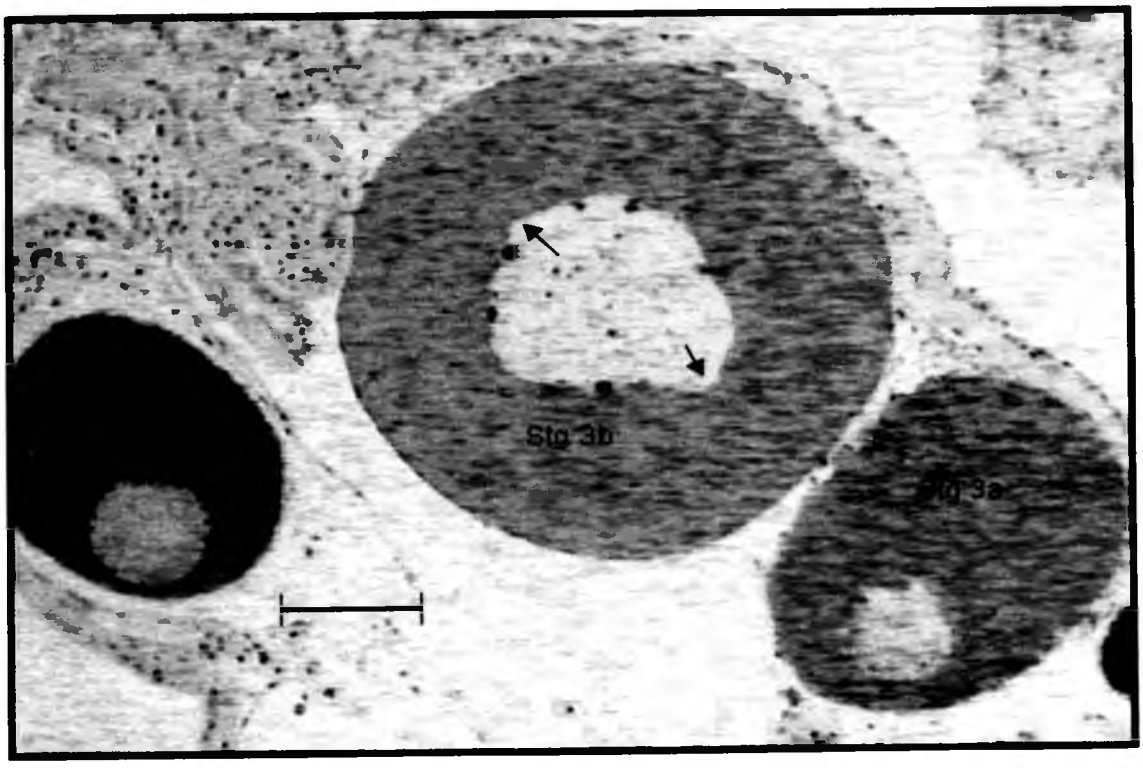


Plate 3.7: Photomicrograph of a 4 μm section of an ovary showing a stage 3b oocyte (Stg 3b) and two stage 3a oocytes (Stg 3a).Notice the difference in the shape of the nucleus, and the numerous folds in the nuclear envelope of stage 3b (arrows). Some nucleoli can also be seen away from the periphery of the nucleus of 3b oocytes. No cortical alveoli are present. Stained with haematoxylin and eosin. Magnification 10 x 3.3. Scale bar = 100 μm.

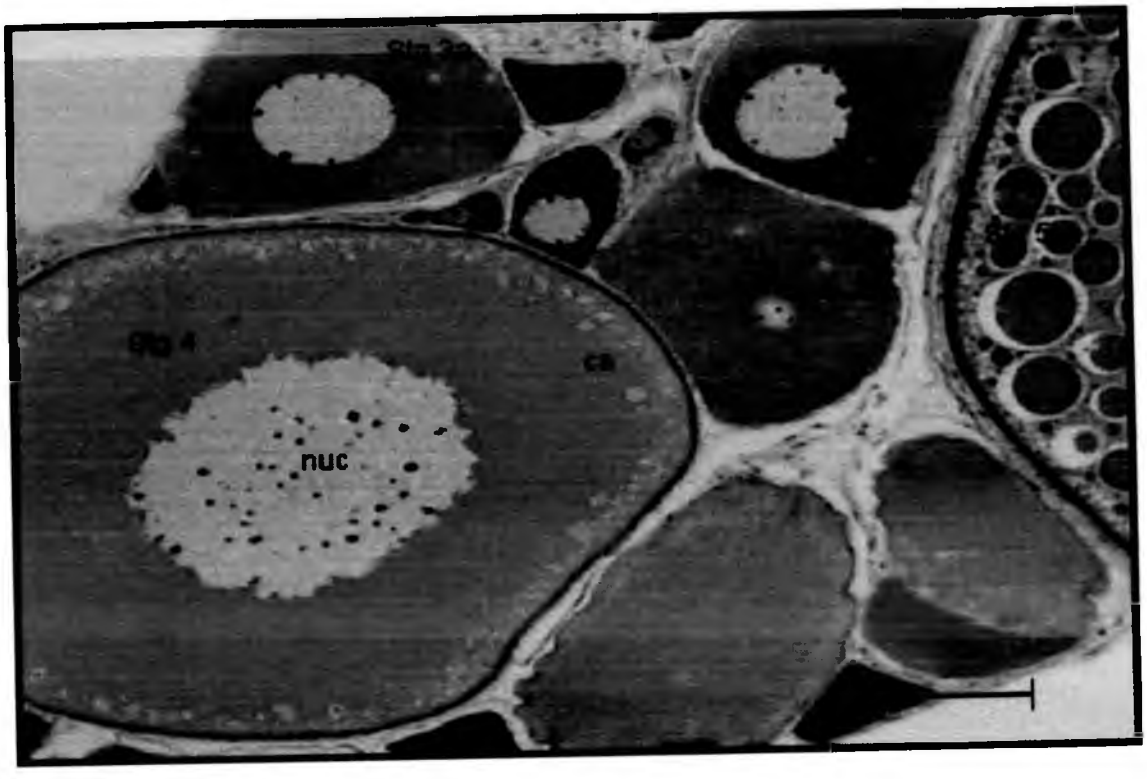


Plate 3.8: Photomicrograph of a 4 μm section of an ovary showing stages 2, 3a, 4 and stage 5 oocytes. ca, cortical alveoli; nuc, nucleoli. Stained with toluidine blue. Magnification 10 x 3.3. Scale bar = 100 μm.

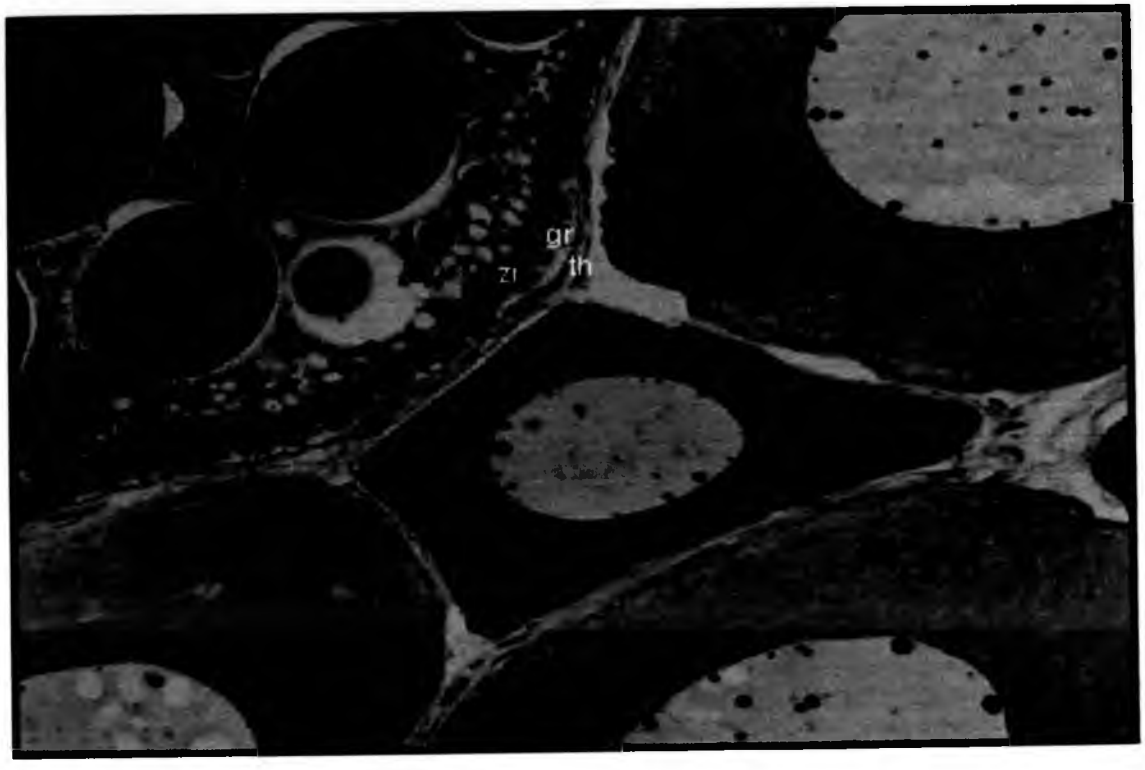


Plate 3.9: Photomicrograph of a 4 μm section of an ovary showing stages 2, 3a and stage 5 oocytes. Yg, yolk globules; arrows are cortical alveoli; zr, zona radiata; gr, granulosa; th, theca. Stained with toluidine blue. Magnification 20 x 3.3. Scale bar = 100 μm.

4.1 μm) in diameter. The nucleus of stage 3b oocytes was more irregular in shape and the nuclear envelope appeared to be discontinuous. The size of Stage 3b oocytes ranged from 250 μm to 400 μm (average = 329.3 ± 5 μm) in diameter.

Plates 3.10, 3.11 and 3.12 show the changes in the follicular layers during primary growth, using transmission electron microscopy (TEM). A single layer of follicular cells rich in endoplasmic reticulum, the granulosa layer, surrounded the homogeneous cytoplasm. Overlaying the granulosa, and separated from it by a basal lamina, appeared a primordial theca. Microvilli from the oocyte and processes from the granulosa cells projected into the sub-follicular space. Electron-dense material accumulated between the microvilli in the sub-follicular space between the oocyte and the granulosa cells. This material developed to form the chorion layer, and increased in thickness and complexity at later stages.

3.2.2.2 Secondary growth.

a. Stage 4 oocytes (cortical alveoli stage).

There was a very limited number of oocytes at this stage in the ovaries of the females analysed. The diameter of stage 4 oocytes ranged from 385 μm to 525 μm (average = 454.5 ± 8.8 μm). Mostly peripheral cortical alveoli were present, appearing as empty ovoid to circular vesicle-like structures close to the cytoplasmic membrane (plates 3.6, 3.8 and 3.13).

The nucleus continued to grow and the nuclear membrane presented numerous folds. Nucleoli appeared as abundant basophilic bodies, not only in the periphery of the nucleus, but also distributed along its entire volume. Chromatin threads could still be observed in the interior of the nucleus.

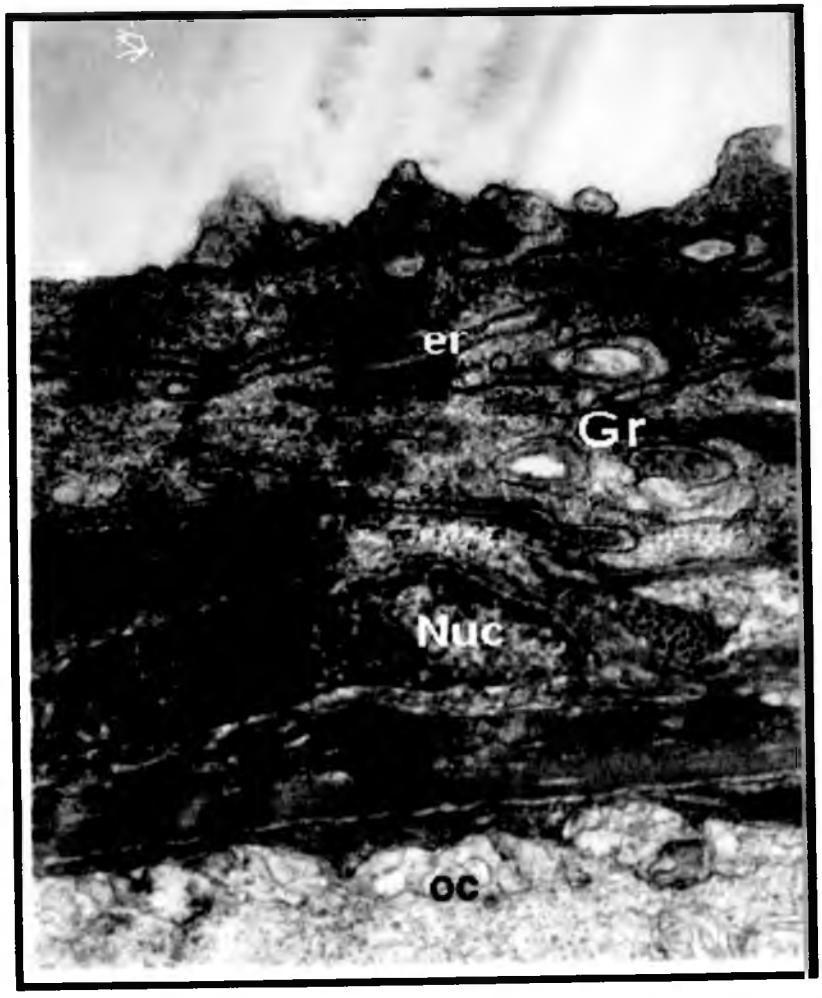


Plate 3.10: Electron photomicrograph of a follicle during primary growth. The oocyte is surrounded by a single layer of follicular cells (granulosa cells) rich in endoplasmic reticulum. Gr, granulosa cell; Nuc, nucleus of granulosa cell; er, endoplasmic reticulum; oc, oocyte cytoplasm. Stained with uranyl acetate and lead citrate Magnification x 13000 x 1.7.

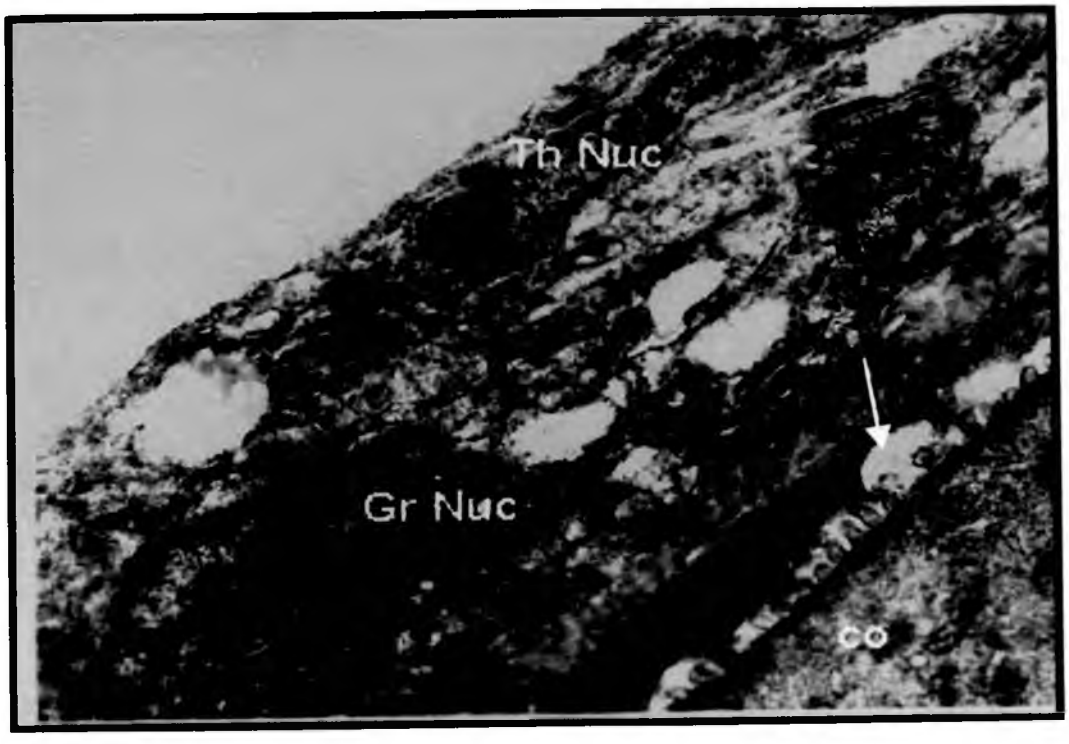


Plate 3.11: Electron photomicrograph of a follicle during primary growth. The oocyte is invested of a granulosa layer with squamous cells, and an outer thecal layer. Sub-follicular spaces (arrow) appear between the granulosa and the oocyte, where projections of the granulosa meet with microvilli from the oocyte. In between these microvilli, dense material starts to accumulate. Microvilli and dense material will form part of the zona radiata. Stained with uranyl acetate and lead citrate Magnification x 9800 x 1.6.

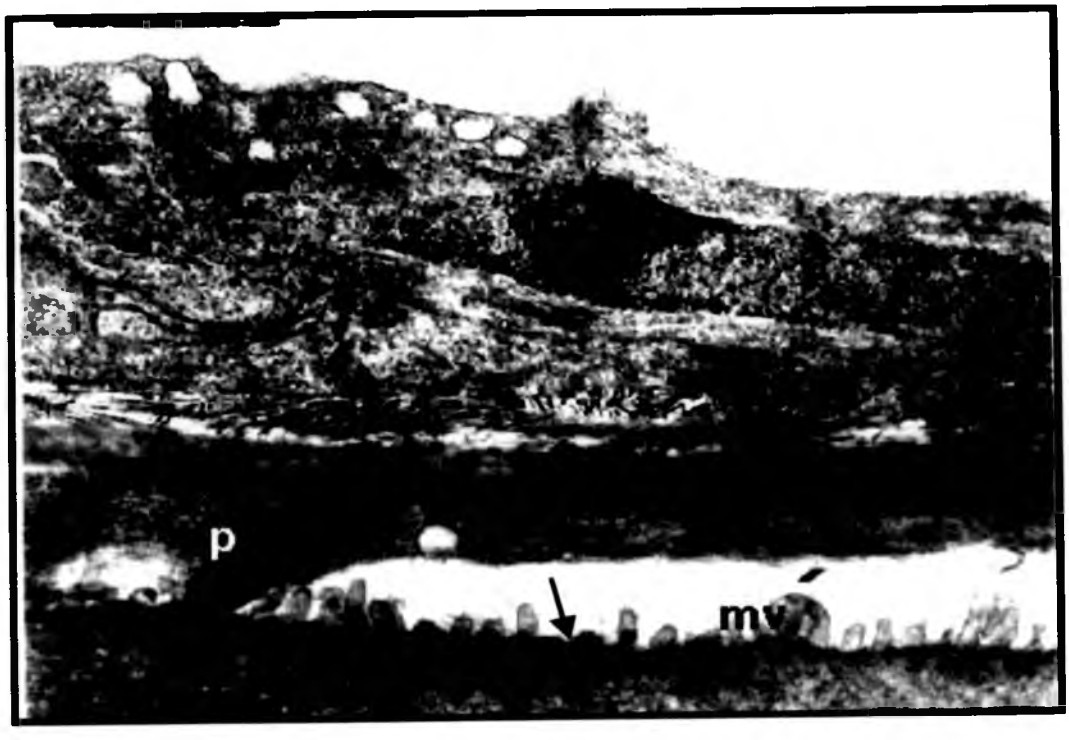


Plate 3.12: Detail of the sub-follicular space showing the microvilli from the oocyte (mv) and processes from the granulosa (p). Dense material (arrows) accumulating between the microvilli will form the zona radiata at a later stage. Stained with uranyl acetate and lead citrate Magnification x 13000 x 1.6.

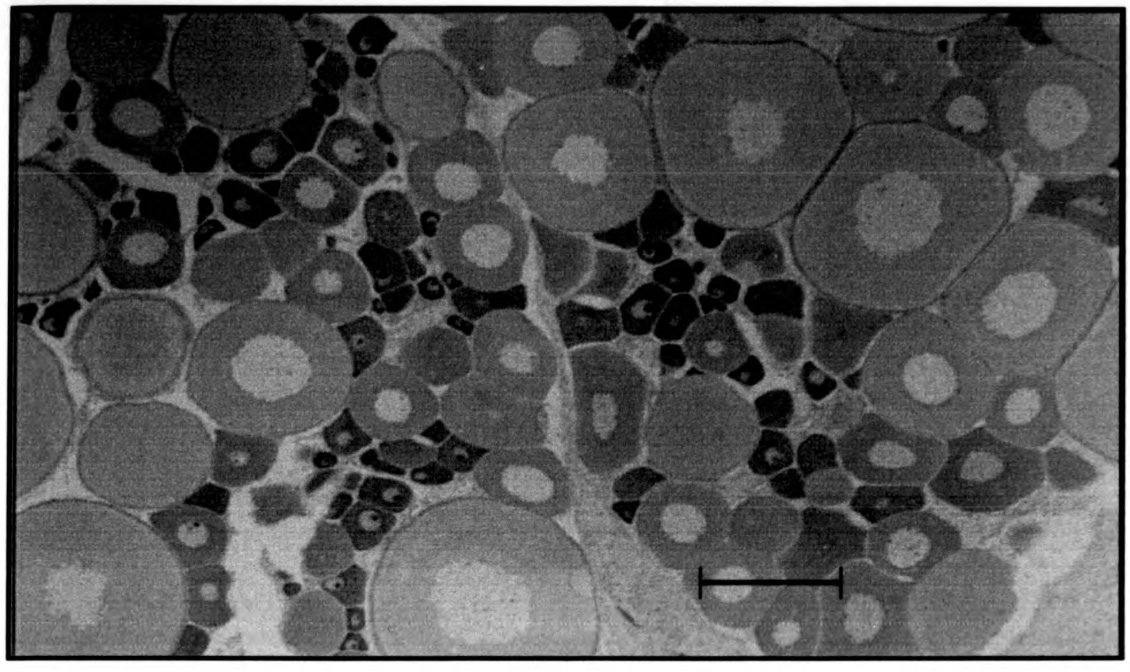


Plate 3.13: Photomicrograph of a 4 μm section of a pre-vitellogenic ovary showing stages stg2, stg3, stg4 oocytes. Stained with haematoxylin and eosin Magnification 4 x 3.3. Scale bar = 200 μm.

Examination of the follicular layers of the oocyte by TEM revealed a developed zona radiata or chorion, perforated by numerous helicoid canals penetrated by microvilli from the oocyte (plate 3.14, 3.15 and 3.16). This is thought to provide a mechanism for the active exchange of information and/or material between the oocyte and the blood. A thin thecal layer surrounded the granulosa layer, separated by a basal membrane. Granulosa cells were squamous in shape.

b. Stage 5 oocytes (vitellogenesis stage)

Stage 5 oocytes ranged from 350 μm to 710 μm (mean = 559 ± 15.5 μm). Early examples of this stage appeared purple in colour, with a band of deep pink yolk granules in the periphery of the cytoplasm, when stained with haematoxylin and eosin. As the process of vitellogenesis progressed, yolk granules aggregated into yolk globules, and increased in number centripetally. (see plates 3.17 and 3.18). Cortical alveoli remained in the periphery of the oocyte, subjacent to the membrane. In later stages of this phase, yolk globules were distributed through the entirety of the ooplasm, with a narrow ring of cytoplasm surrounding the nucleus.

The three layers of the follicle envelope, theca, granulosa and zona radiata, were easily visible in light microscopy preparations (see plates 3.18 and 3.19). The follicular cells of the granulosa acquired a cuboidal shape through this stage, and thin layers of amorphous basophilic material appeared in the intercellular spaces. The zona radiata increased in thickness as yolk sequestration advanced.

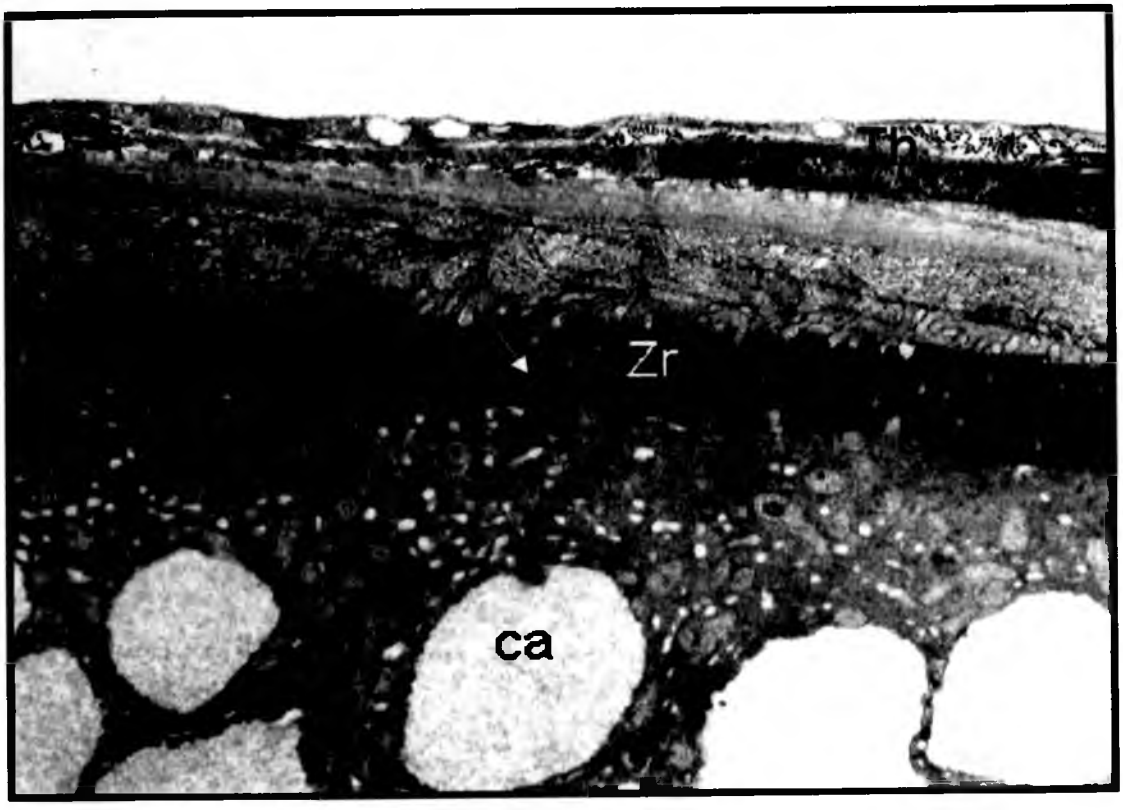


Plate 3.14: Electron photomicrograph of a stage 4 follicle, at the start of secondary growth. Granulosa cells (Gr) are squamous in shape. The zona radiata (zr) is increasing in thickness and pore canals (arrow) transverse this layer in an helicoid fashion. Cortical alveoli (ca) are situated in the periphery of the oocyte cytoplasm (oc). Stained with uranyl acetate and lead citrate Magnification x 5900 x 2.

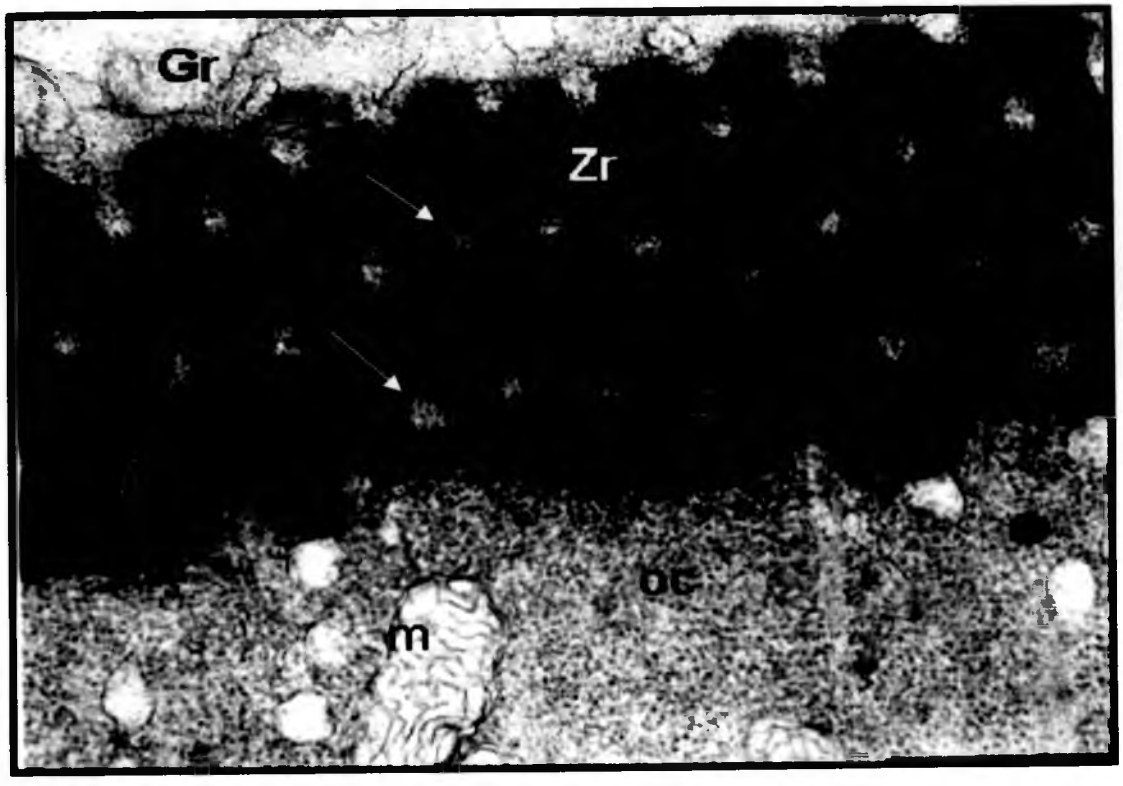


Plate 3.15: Detail of the zona radiata of a stage 4 follicle showing the pore canals (arrows) providing communication between the oocyte and the granulosa layer. Gr, granulosa; Zr, zona radiata; oc, oocyte cytoplasm; m, mitochondria. Stained with uranyl acetate and lead citrate Magnification x 22000 x 1.7.

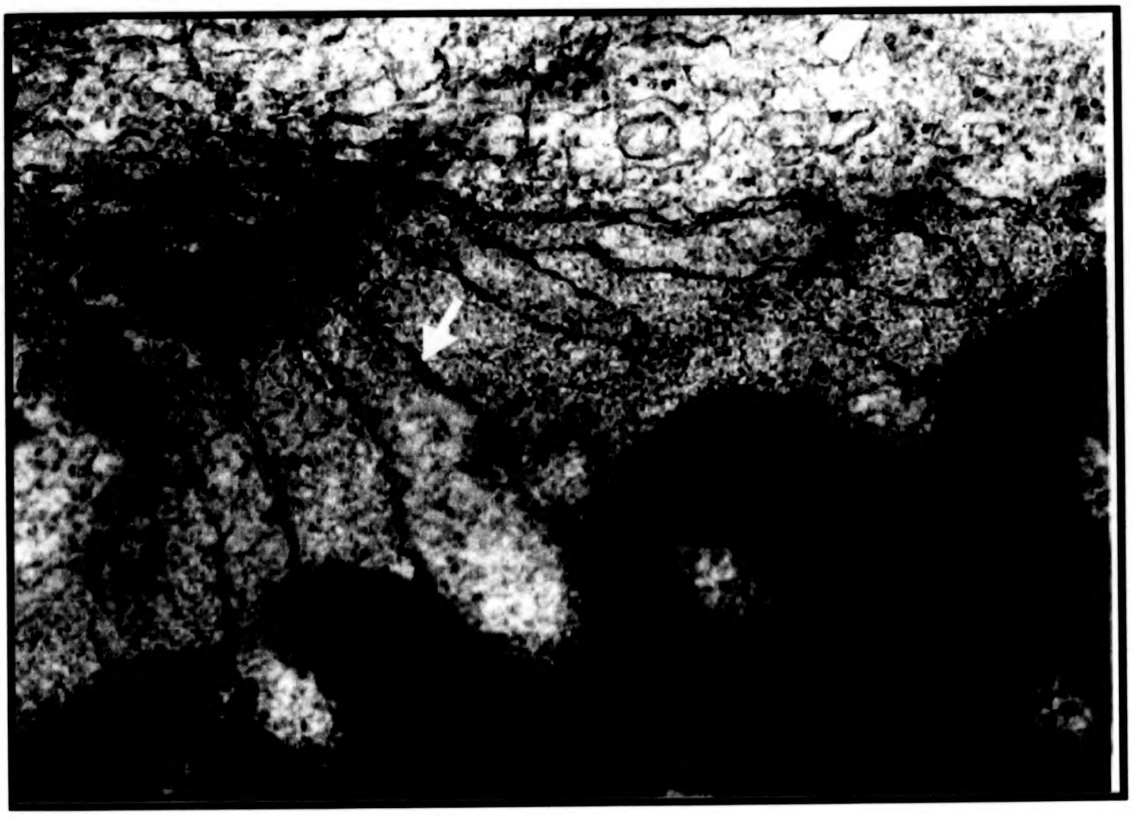


Plate 3.16: Detail of the canals transversing the zona radiata from the granulosa side, in a stage 4 follicle. Notice the microvilli (arrow) concurring to a same area in a granulosa cell. Stained with uranyl acetate and lead citrate Magnification x 43000 x 2.

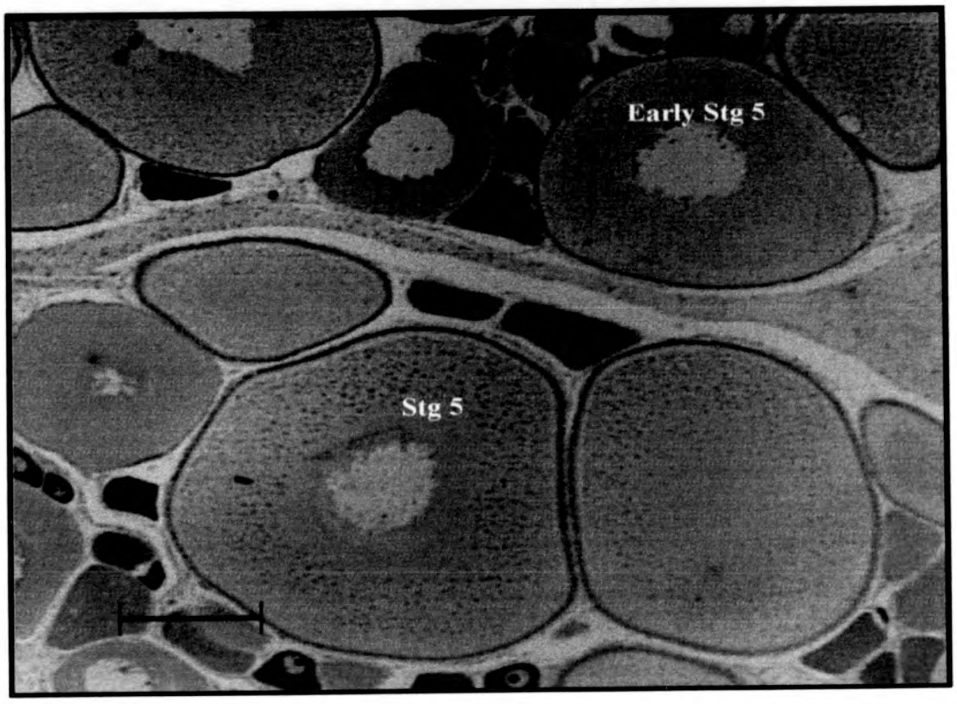


Plate 3.17: Photomicrograph of a 4 μm section of a pre-vitellogenic ovary showing early and late stage 5 oocytes. Yolk granules are bright pink in colour. Stained with haematoxylin and eosin Magnification 4 x 3.3. Scale bar = 200 μm.

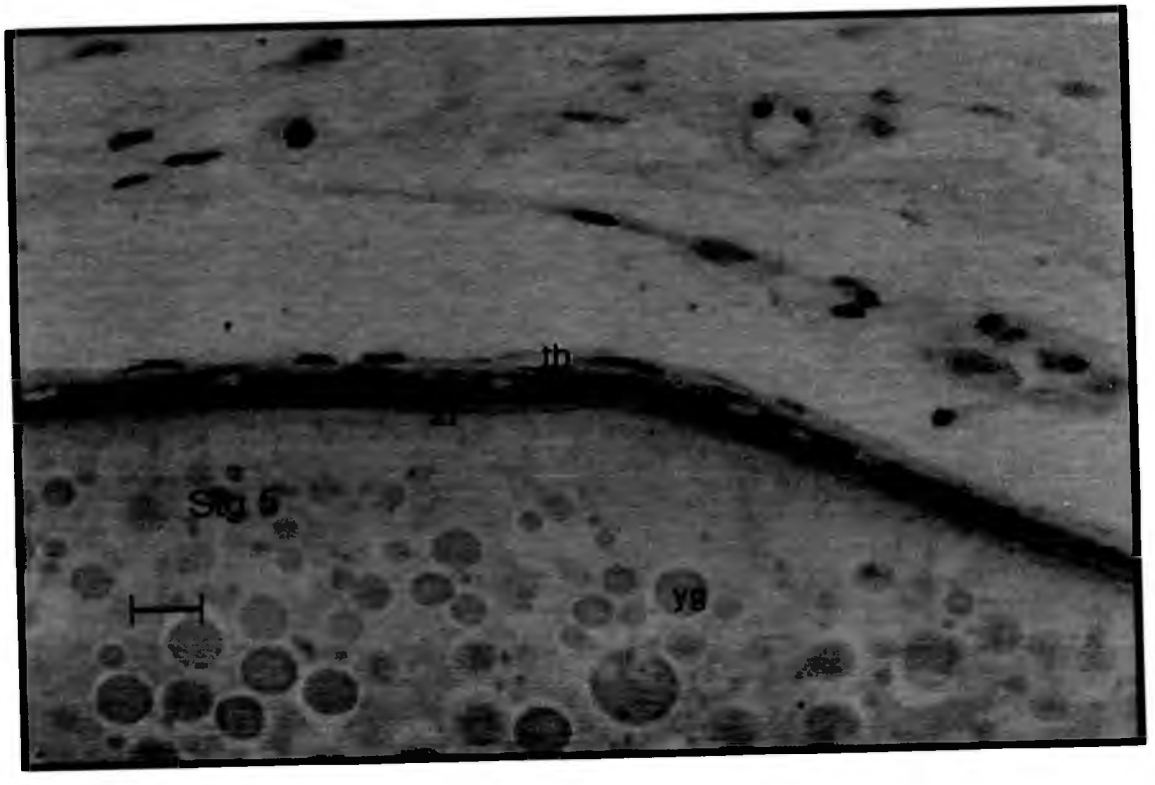


Plate 3.18: Photomicrograph of a 4 μm section of an ovary showing detail of an early stage 5 follicle. Gr, Squamous follicular cells of the granulosa layer; Th, thecal layer; Zr, zona radiata; yg, yolk granule. Stained with haematoxylin and eosin. Magnification 40 x 3.3. Scale bar = 10 μm.

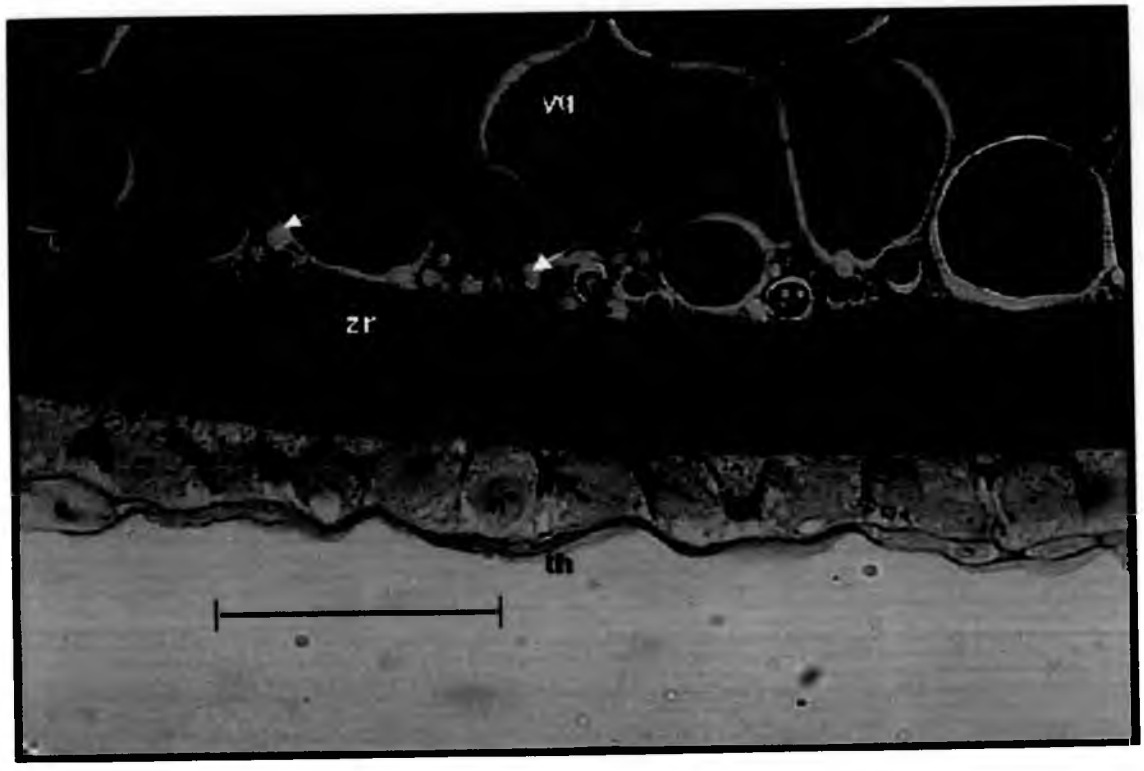


Plate 3.19: Photomicrograph of a 4 μm section of ovary showing detail of a stage 5 follicle and the increased complexity of the follicular layers. Granulosa cells (gr) have turn cuboidal in shape, and amorphous material can be seen between them (black arrows). The zona radiata (zr) increases in thickness. Yg, yolk globules; White arrows, cortical alveoli in the periphery of the oocyte; n, nucleus of a granulosa cell; th, thecal layer. Stained with toluidine blue. Magnification 40 x 2.5. Scale bar = 50 μm.

c. Stage 6 and 7 oocytes (late vitellogenesis and germinal vesicle migration)

Stage 6 and 7 oocytes ranged from 775 μm to 1012 μm (mean = 918.5 ± 12.3 μm). In stage 6 oocytes, large yolk globules occupied the entire ooplasm, with thin interspersed layers of cytoplasm (plate 3.20). The nucleus of stage 6 oocytes was still central. The nucleus of stage 7 oocytes, or germinal vesicle, had migrated to the animal pole (plates 3.21, 3.25 and 3.26).

Plates 3.22, 3.23, 3.24 and 3.28 show the general aspect of the follicular envelope during active vitellogenesis. The zona radiata was thickened considerably and composed of three different layers (plate 3.29). One outer layer, closer to the granulosa layer; a second intermediate layer, the homogeneous layer; and a third, thick, architecturally more complex layer comprised of 13 lamellae, called the multilamellar layer. The pore canals transversed the whole chorion. A pair of microvilli was present inside each pore canal, one originating from the oocyte and one from the granulosa (plate 3.29, 3.30 and 3.31). At a later stage, the multilamellar layer increased in thickness, and over 25 lamellae could be present (plate 3.32). Endocytotic vesicles were visible in the periphery of the ooplasm, as well as cortical alveoli (plate 3.33). Exogenous material in the vesicles was incorporated into yolk granules, which in turn aggregated into yolk globules (plates 3.33 and 3.34).

At some point during this stage, after germinal vesicle migration and vitellogenin sequestration had ended, the yolk globules started to coalesce and oocytes became translucent (plates 3.26 and 3.27). Translucent oocytes were difficult to measure, as G.V.B.D. had started, and it was difficult to find oocytes sectioned through the nucleus.

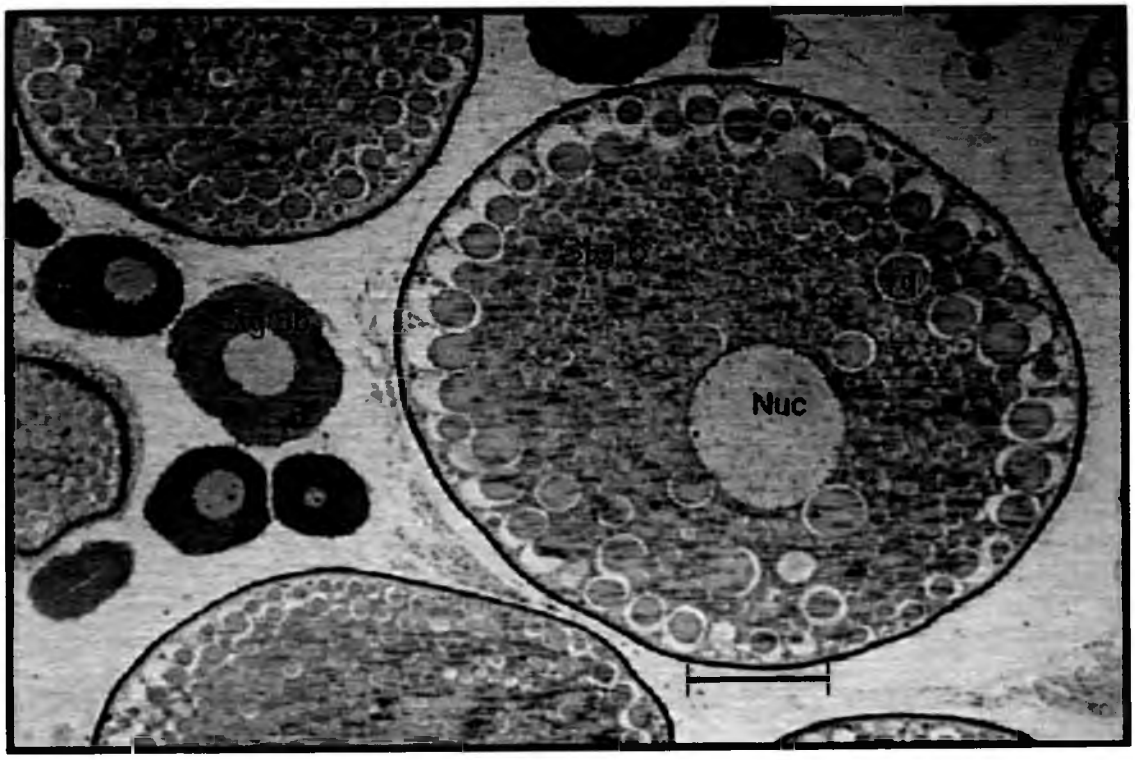


Plate 3.20: Photomicrograph of a 4 μm section of the ovary of a prespawning female showing oocytes at different stages of development, stage 2, stage 3a, stage 3b and stage 6. Nuc, nucleus; yg, yolk globule. Stained with haematoxylin and eosin. Magnification 4 x 3.3. Scale bar = 200 μm.

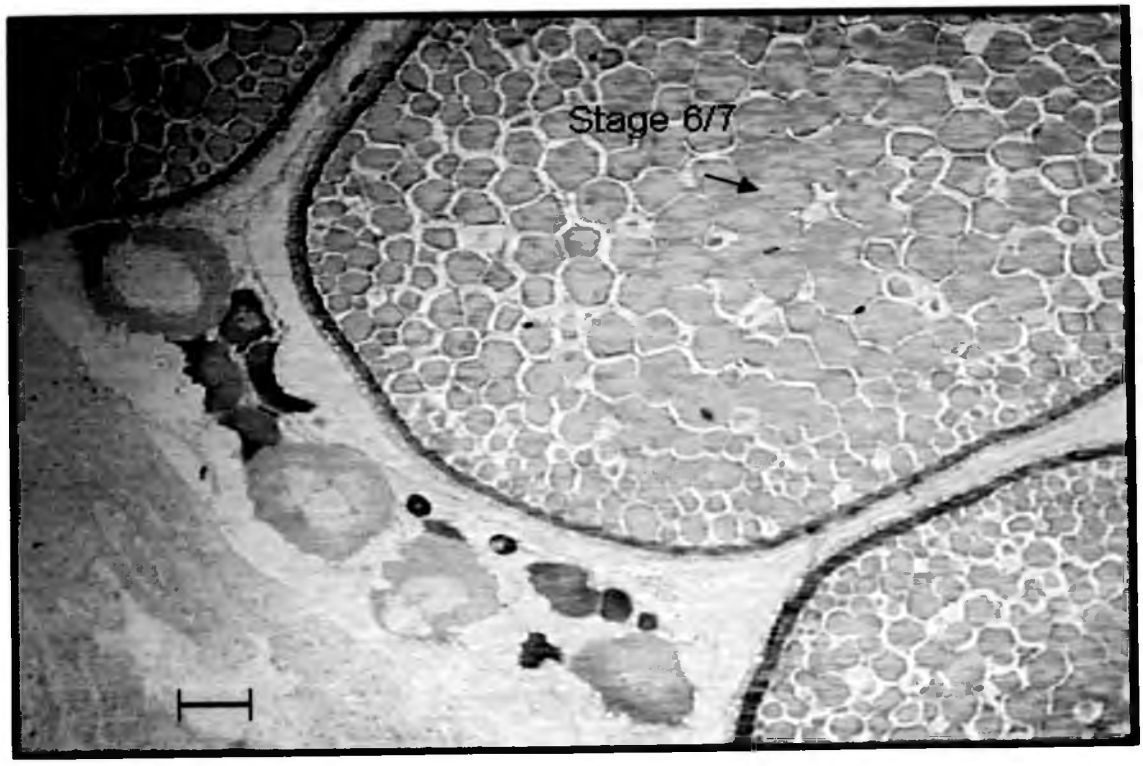


Plate 3.21: Photomicrograph of a 4 μm section of the ovary of a spawning female showing stage 6/7 oocytes presenting signs of initial coalescence of yolk globules (arrow). Stained with haematoxylin and eosin. Magnification 4 x 2.5. Scale bar = 100 μm.

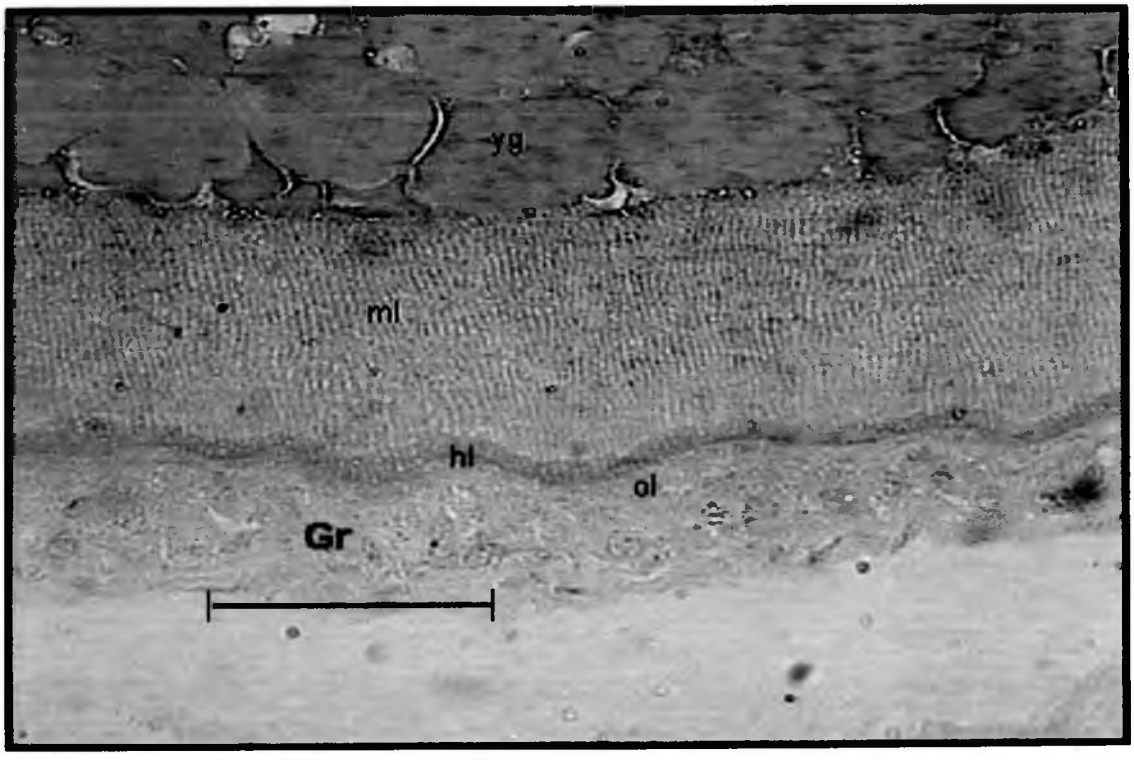


Plate 3.22: Detail of a stage 6/7 follicle showing the three regions of the zona radiata, the outer layer (ol), the homogeneous layer (hl) and the multillamelar layer (ml). Stained with haematoxylin and eosin. Magnification 40 x 2.5. Scale bar = 50 μm.

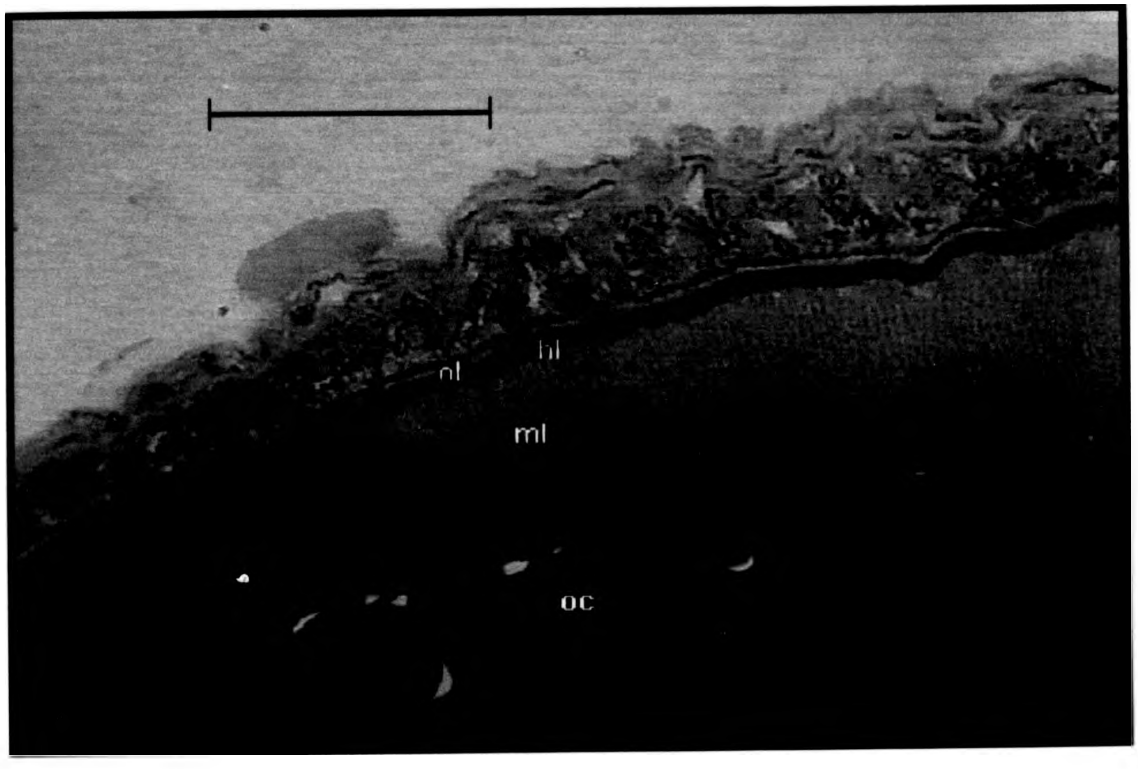


Plate 3.23: Detail of a stage 6/7 follicle as in plate 3.24, showing the three regions of the zona radiata, the outer layer (ol), the homogeneous layer (hl) and the multillamelar layer (ml), surrounding the oocyte cytoplasm (oc). Stained with toluidine blue. Magnification 40 x 2.5. Scale bar = 50 μm.

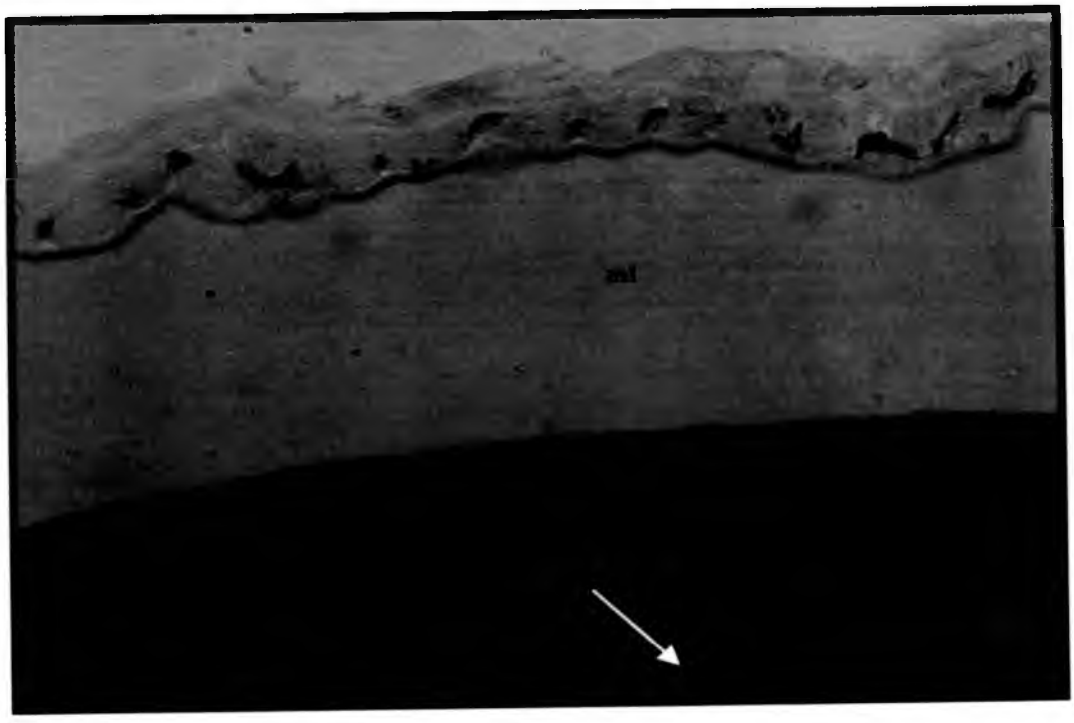


Plate 3.24: Detail of a late stage 6/7 follicle, showing the three regions of the zona radiata, the outer layer (ol), the homogeneous layer (hl) and the increase in size of the multillamelar layer (ml). Arrow shows an area of start of coalescence of yolk globules. Stained with toluidine blue. Magnification 40 x 2.5. Scale bar = 50 μm

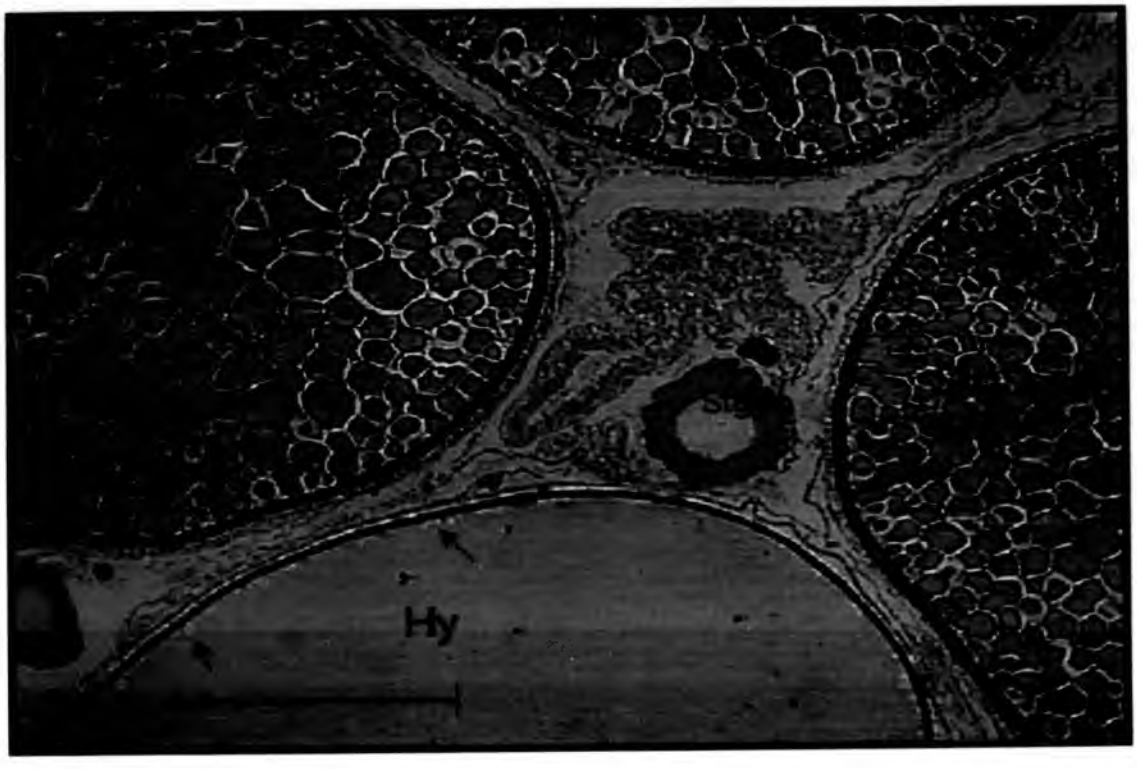


Plate 3.25: Photomicrograph of a 4 μm section of the ovary of a spawning female showing stage 6/7 oocytes with signs of coalescence of yolk globules, and a translucent oocyte (Hy) in which the yolk is homogeneous after the total coalescence of the yolk globules. Vesicles (arrows) are present in the periphery of the cytoplasm (cortical alveoli). Stained with haematoxylin and eosin. Magnification 4 x 2.5. Scale bar = 500 μm.

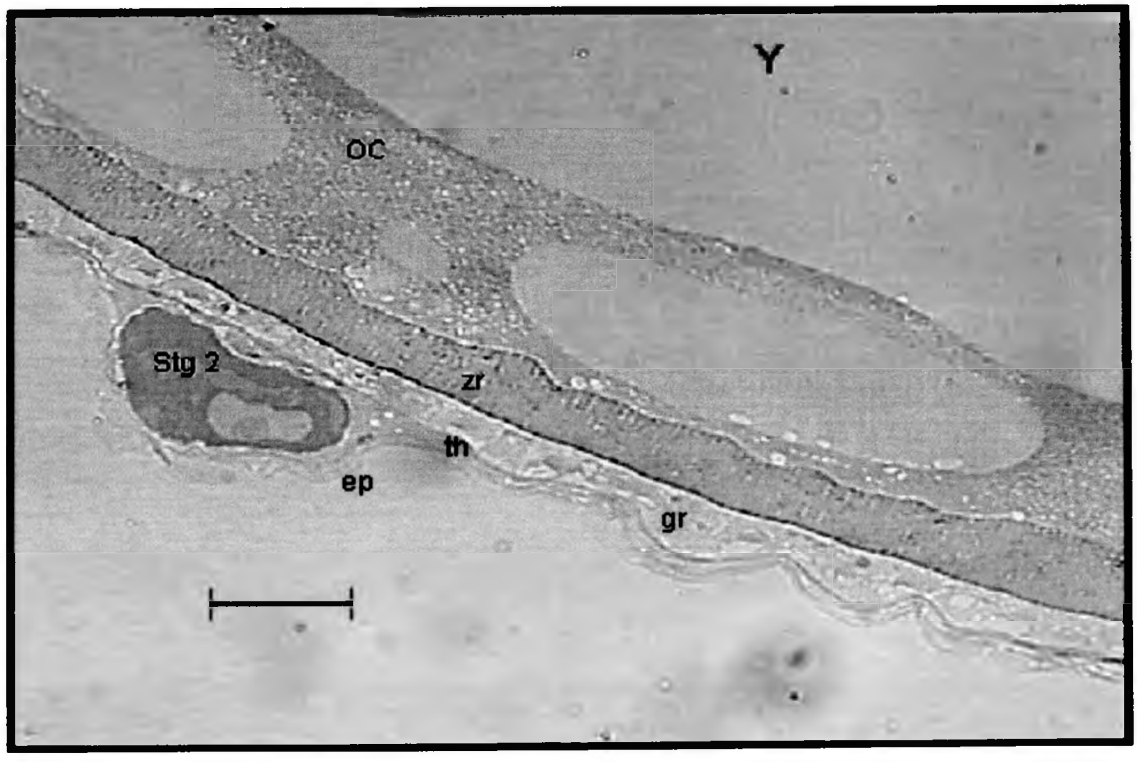


Plate 3.26: Detail of the section of a stage 7 follicle, showing marginal ring of ooplasm and coalescence of the yolk (Y). zr, zona radiata; gr, granulosa layer; th, thecal layer; ep, external epithelium. Stained with haematoxylin and eosin. Magnification 20 x 3.3. Scale bar = 50 μm.

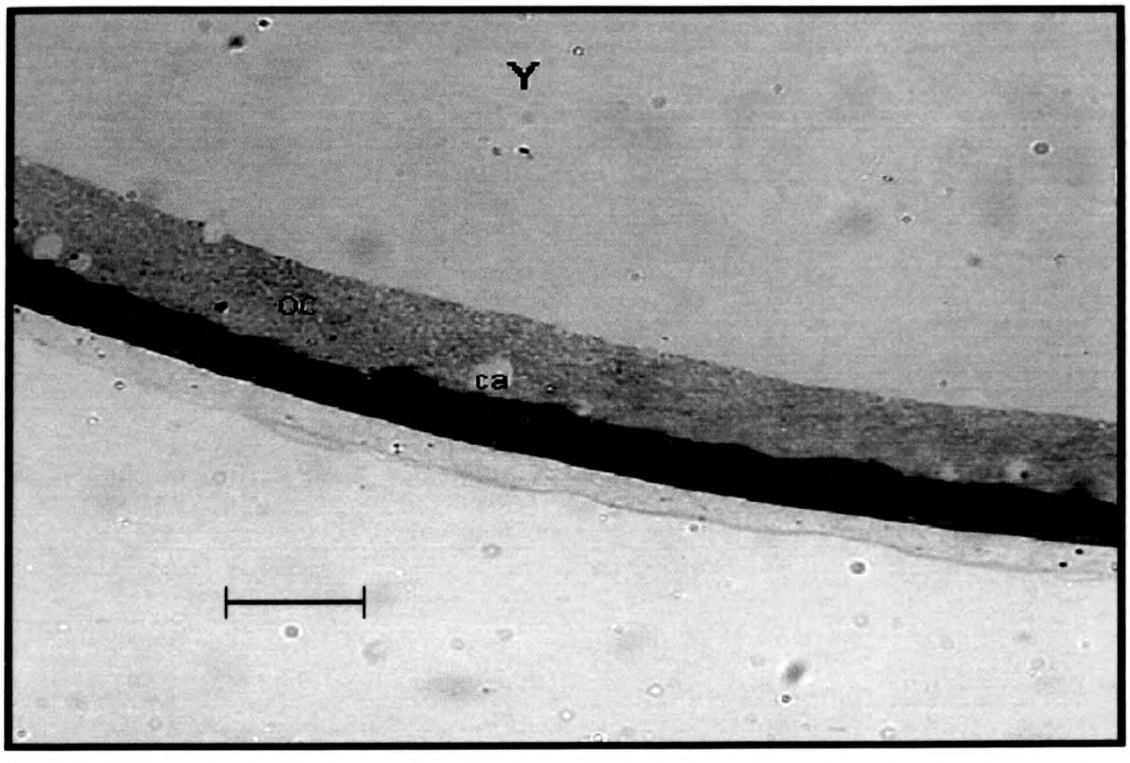


Plate 3.27: Detail of the section of a hyaline oocyte close to ovulation, showing a thin layer of cytoplasm between the zona radiata and the yolk of the oocyte. Y, yolk; ca, cortical alveolus; oc, oocyte cytoplasm; zr, zona radiata. Stained with toluidine blue. Magnification 40 x 2.5. Scale bar = 50 μm.

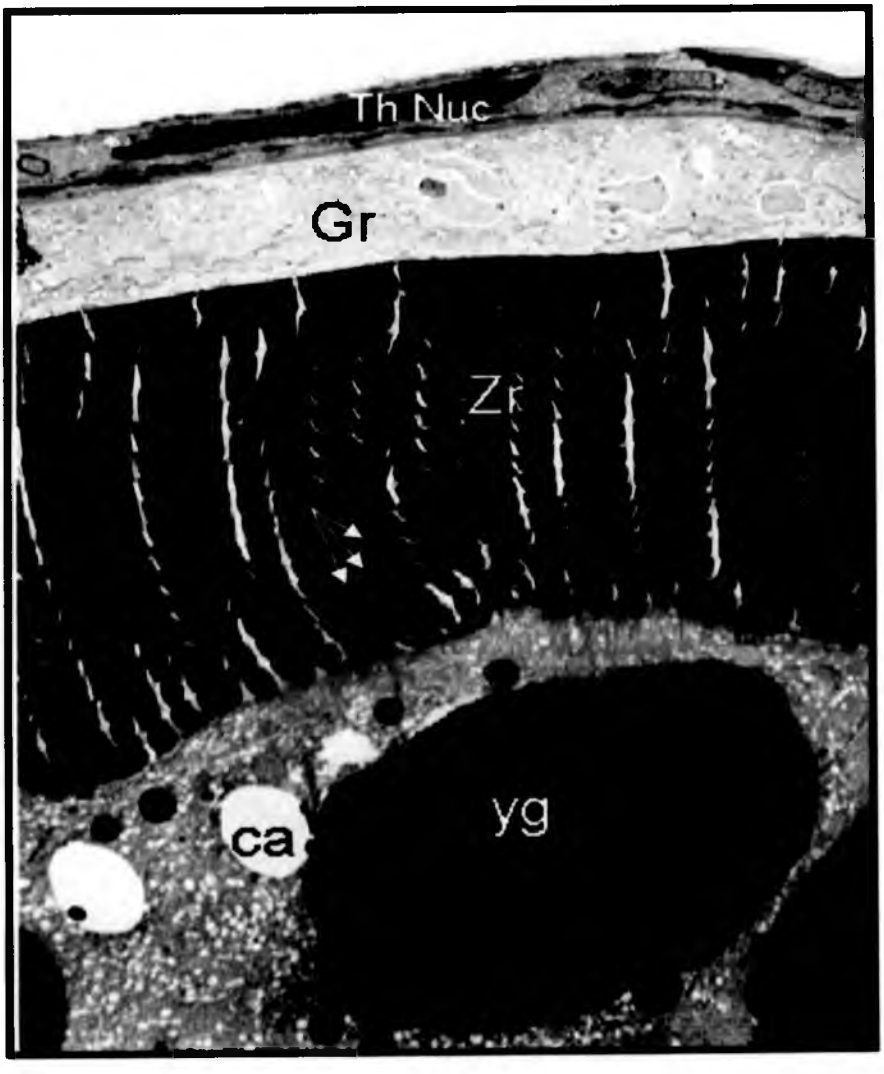


Plate 3.28: Electron photomicrograph of a follicle at the beginning yolk sequestration. Notice the increase in thickness and complexity of the zona radiata (zr) with respect to previous stages. Thirteen lamellae (arrows) constitute the multilamellar layer of the zona radiata. The pore canals transverse the whole layer. Th Nuc, nucleus of thecal cell; Gr, granulosa cell; yg, yolk globule; ca, cortical alveolus; zr, zona radiata. Stained with uranyl acetate and lead citrate Magnification x 13000 x 2.2.

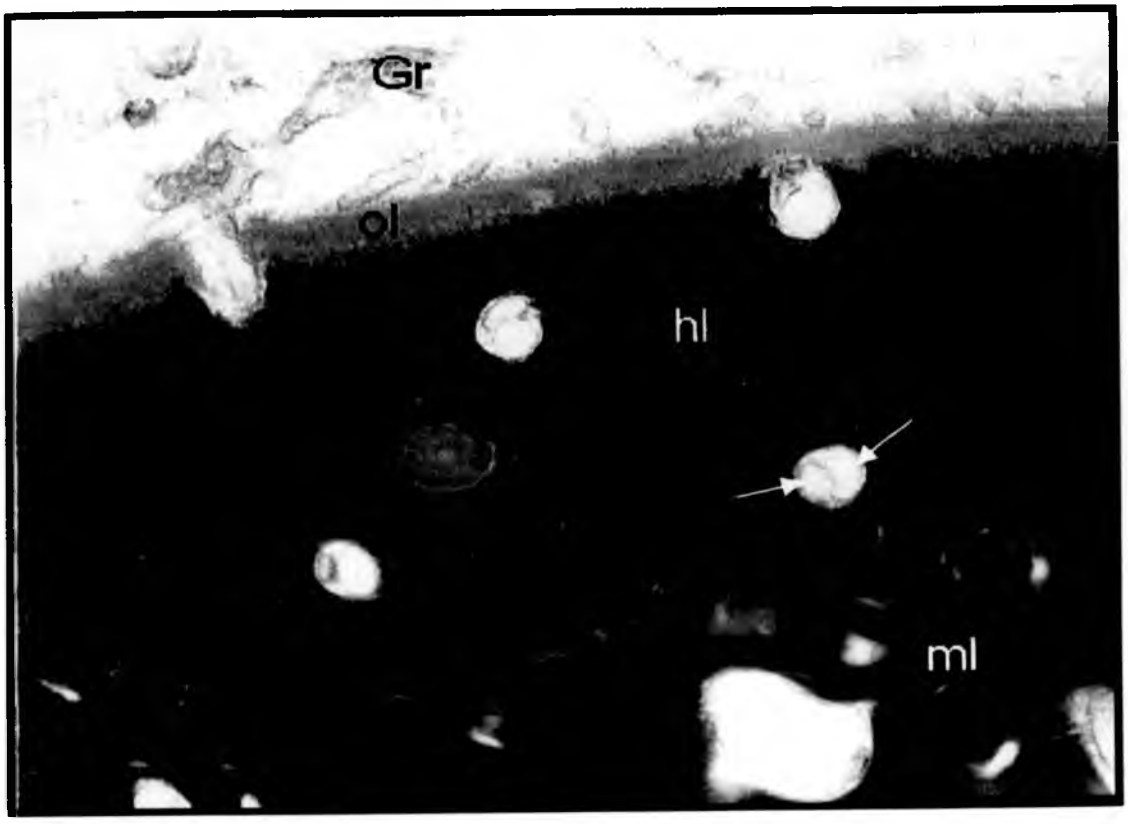


Plate 3.29: Detail electron photomicrograph of the three layers of the zona radiata during vitellogenesis. Notice also the pair of microvilli present inside each pore canal (arrows). Gr, granulosa; ol, outer layer of the zona radiata; hl, homogeneous layer; ml, multilamellar layer. Stained with uranyl acetate and lead citrate Magnification x 13000 x 2.

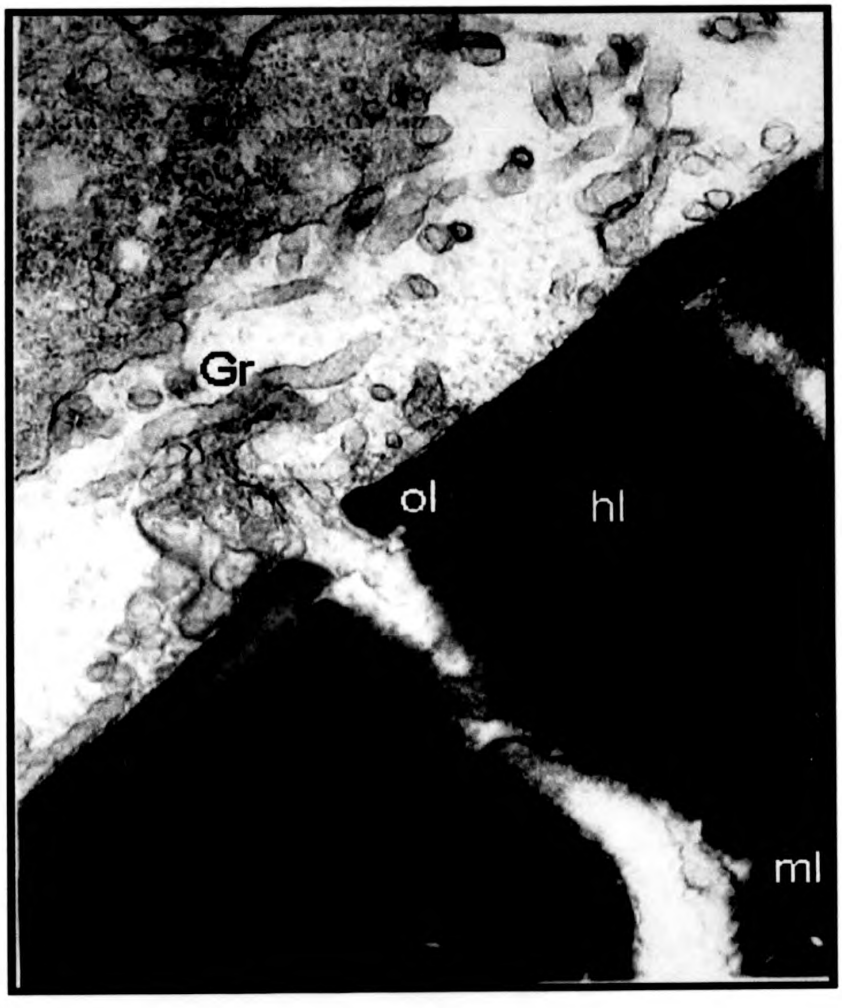


Plate 3.30: Detail electron photomicrograph of the pore canals on the granulosa side. Gr, granulosa; ol, outer layer of the zona radiata; hl, homogeneous layer; ml, multilamellar layer. Stained with uranyl acetate and lead citrate Magnification x 18000 x 2.

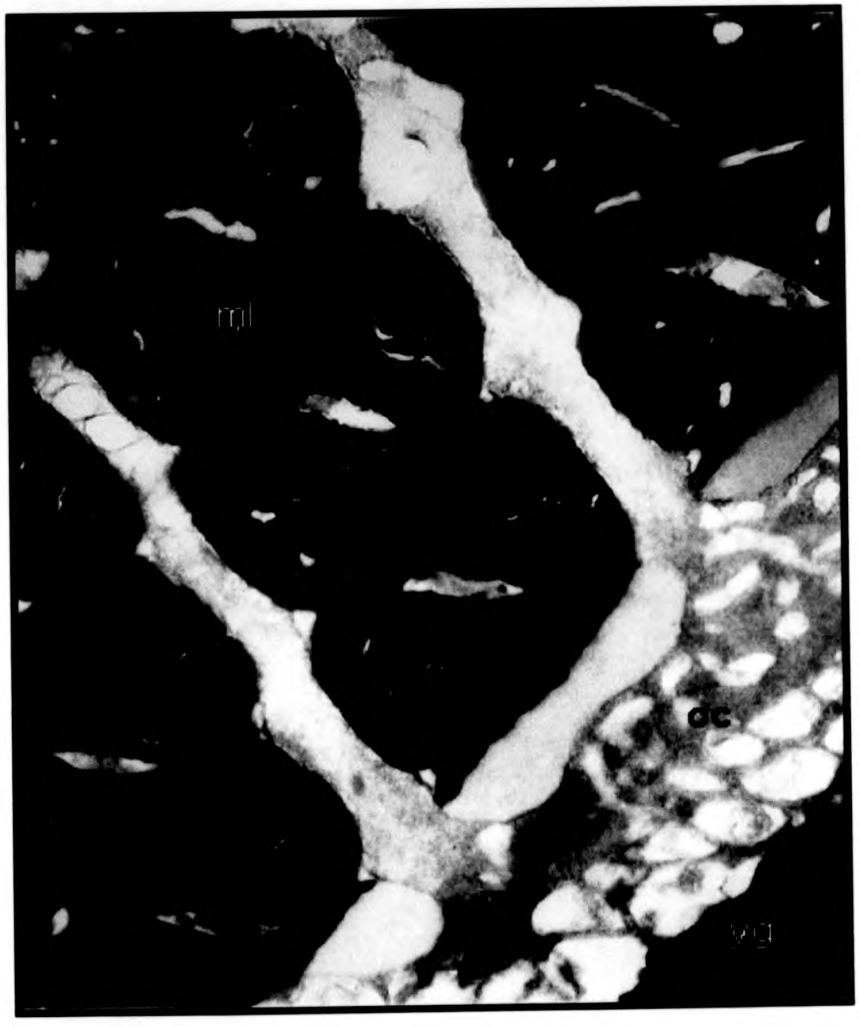


Plate 3.31: Detail electron photomicrograph of the pore canals on the oocyte side. ml, multilamellar layer; oc, oocyte cytoplasm; yg, yolk globule. Stained with uranyl acetate and lead citrate Magnification x 9800 x 1.8.

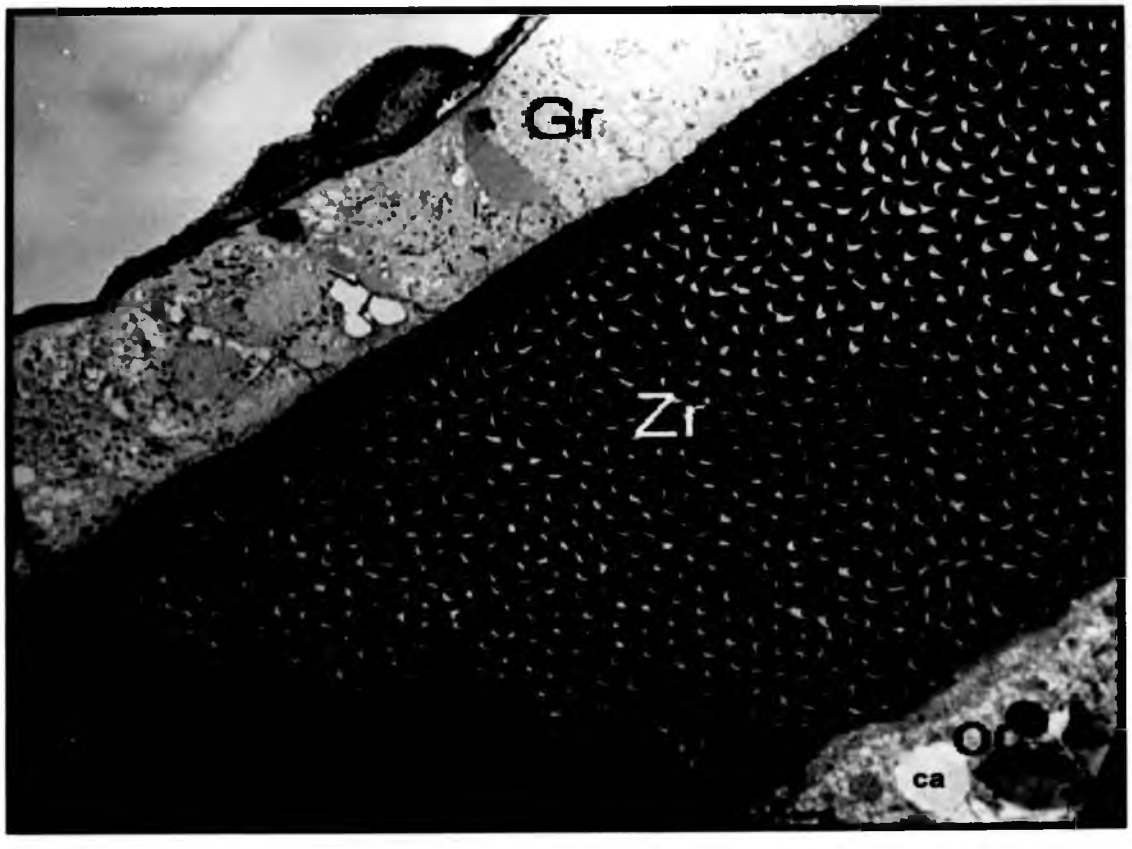


Plate 3.32: Electron micrograph of a follicle at the end of yolk sequestration. Notice the further increase in thickness of the zona radiata (zr), especially the multilamellar layer, consisting now of up to 25 lamellae. Gr, granulosa layer; zr, zona radiata; oc, oocyte cytoplasm; ca, cortical alveolus. Stained with uranyl acetate and lead citrate Magnification x 980 x 2.3.

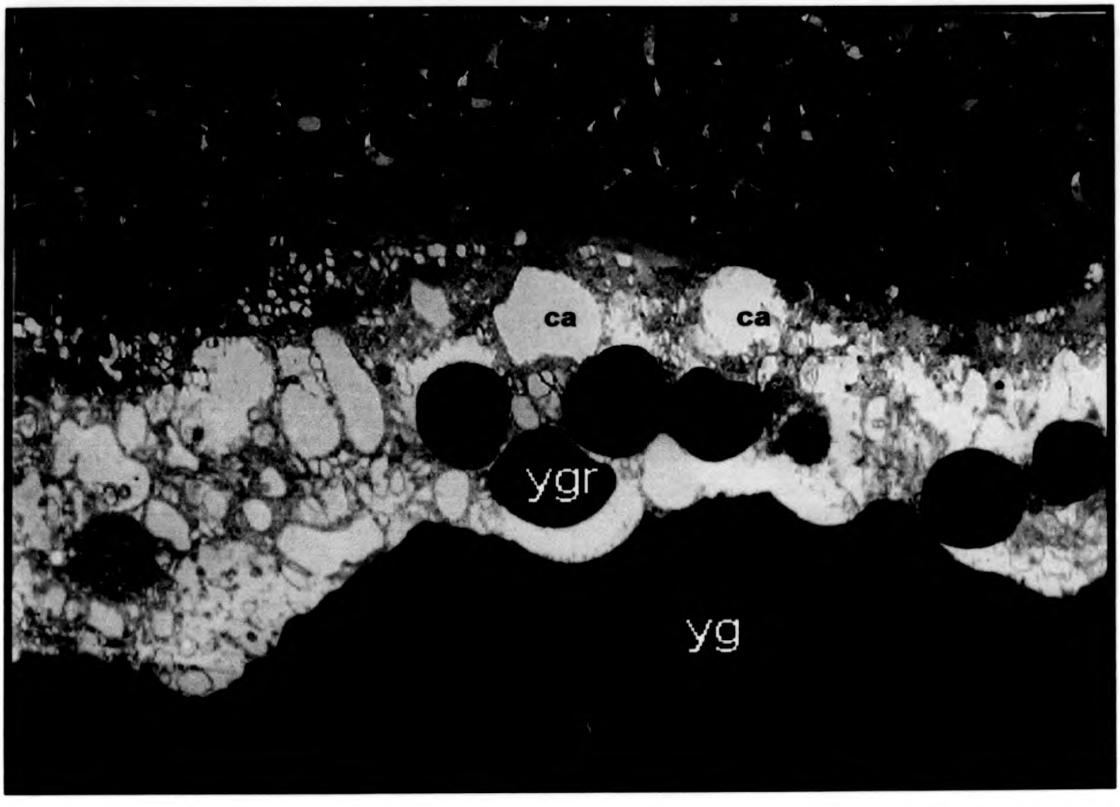


Plate 3.33: Electron photomicrograph of the same preparation as in plate 3.32, showing the aggregation of yolk granules (ygr) into yolk globules (yg); ca, cortical alveoli. Stained with uranyl acetate and lead citrate Magnification x 2800 x 1.8.

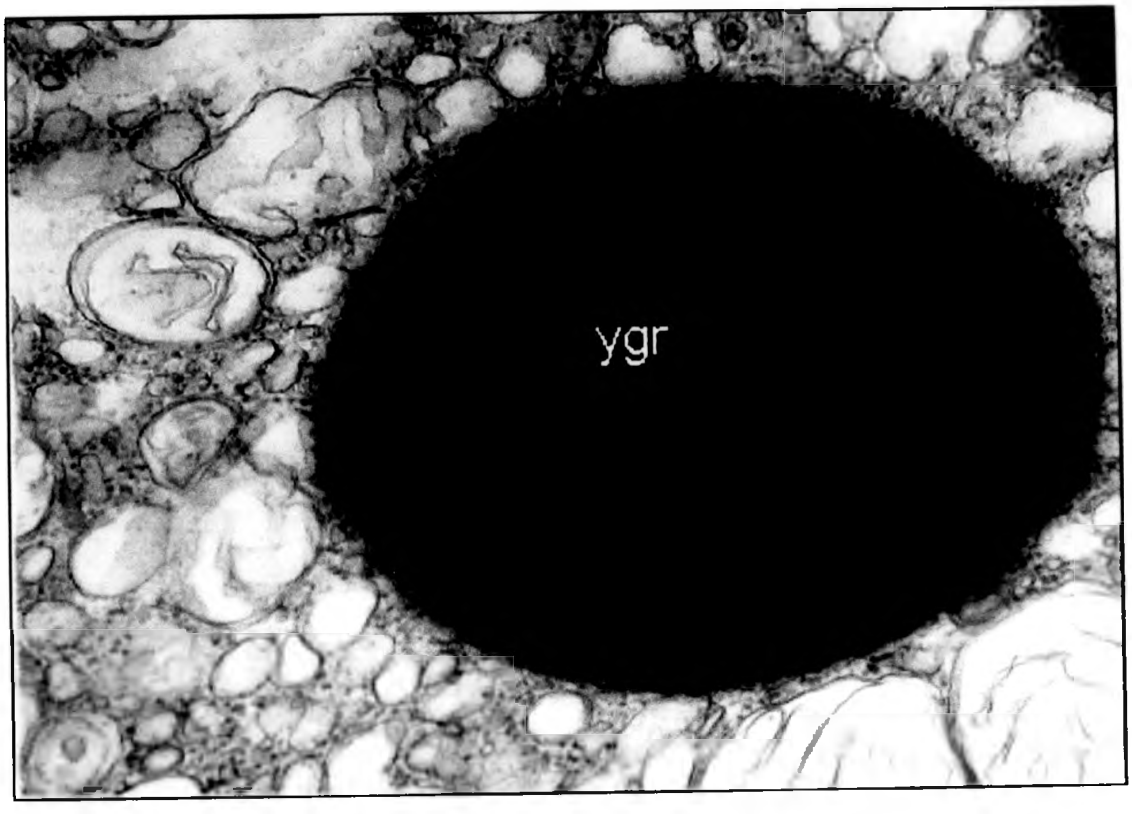


Plate 3.34: Electron photomicrograph of the same preparation as in plate 3.32, showing a yolk granule (ygr) surrounded by vesicles. Stained with uranyl acetate and lead citrate Magnification x 22000 x 2.

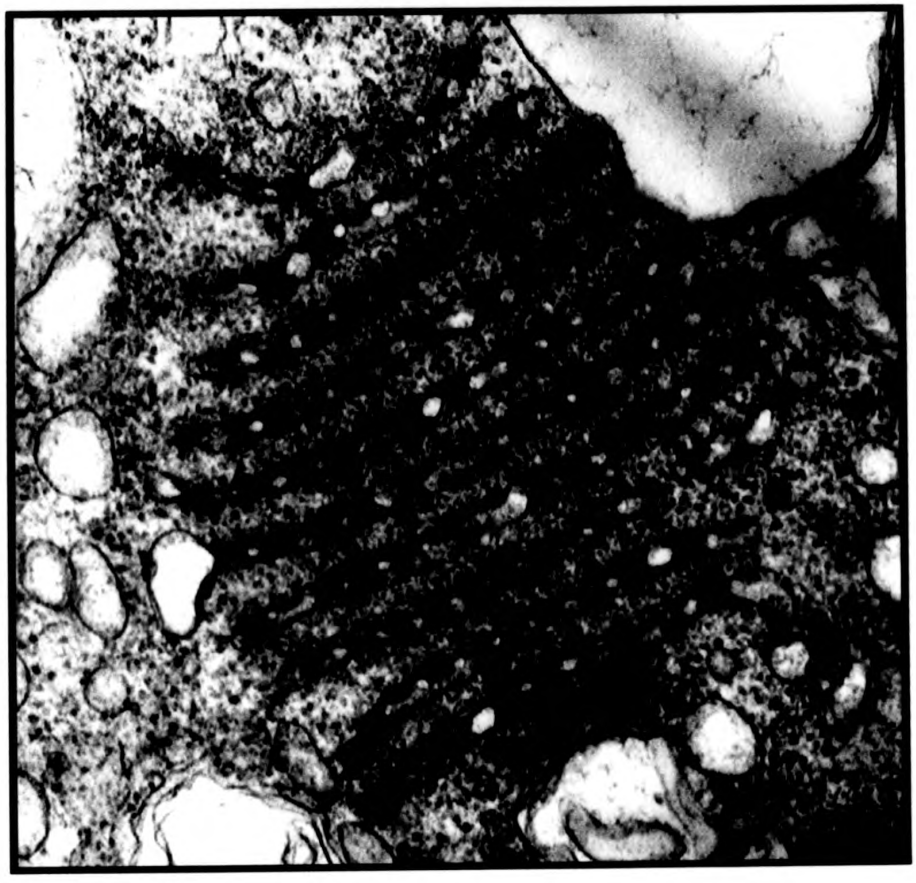


Plate 3.35: Electron photomicrograph showing annulate lamellae in the cytoplasm of a late vitellogenic oocyte. Stained with uranyl acetate and lead citrate Magnification x18000 x 3.

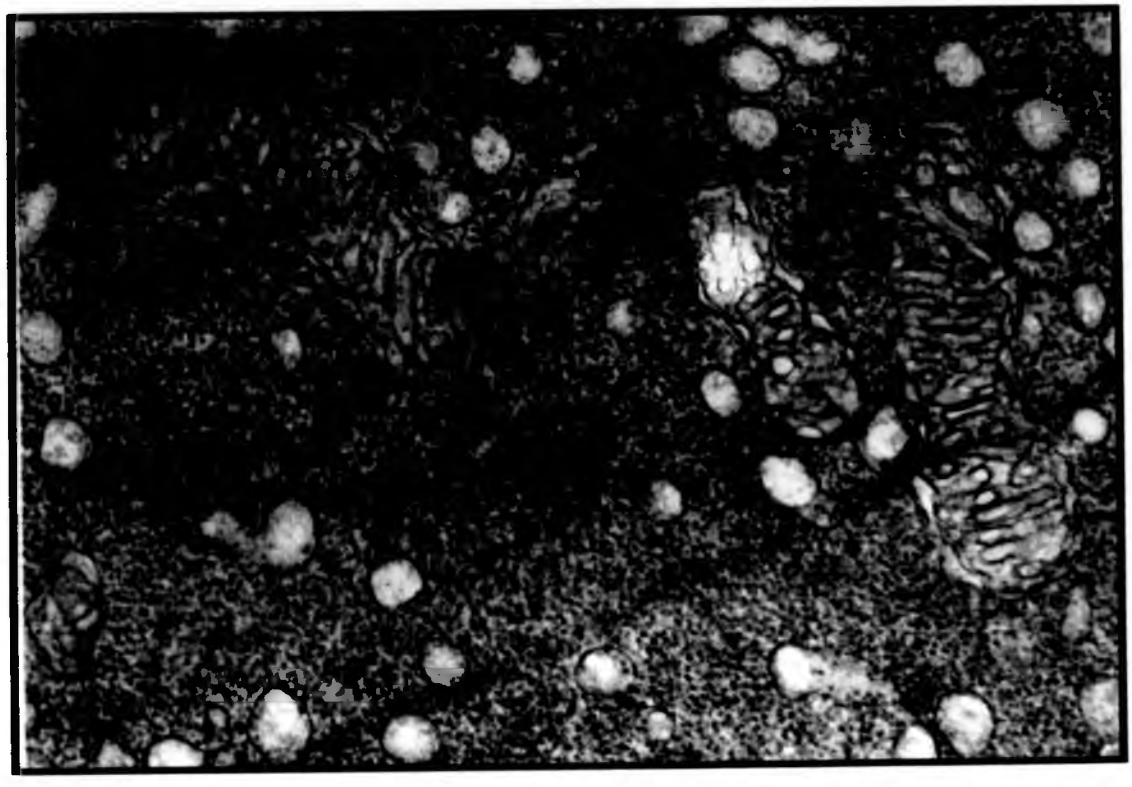


Plate 3.36: Electron micrograph showing mitochondria in the cytoplasm of a late vitellogenic oocyte. Stained with uranyl acetate and lead citrate Magnification x 22000 x 1.7.

However, oocytes measured ranged between 1400 μm and 1675 μm (average 1507.3 +- 23.8 μm) in diameter.

d. Post-ovulatory follicles and atretic oocytes

Only a few atretic oocytes were found, of stage 5 and 6 oocytes, during early vitellogenesis and post-spawned ovaries. The granulosa layer became detached from the oocyte, cytoplasmic material appeared to degrade and yolk had begun to liquefy. At later stages, hypertrophied granulosa cells intruded in the cytoplasm (plate 3.37).

Post-ovulatory follicles (POF) were irregular structures consisting of a hypertrophied granulosa layer and a thecal layer, both highly in-folded (plate 3.38).

3.2.2.3 Volume fractions of oocyte stages.

Volume fractions of the different oocyte stages were calculated using stereology as described in the materials and methods section. Stereology provides a measurement of the proportions of different oocyte developmental stages from histological slides prepared from fish ovaries.

There were no significant differences in the volume fraction of stage 2, 3a and 3b oocytes along the ovary or between the two ovarian lobes in post-spawned ovary (figure 3.2 and 3). POF distribution along the ovary and between the two lobes (figure 3.4) presented significant differences when analysed by ANOVA ($p = 0.0005$). Table 3.1 presents the Tukey-Kramer Multiple Comparisons Test of the POF volume fractions in the different sections of the ovary. The source of difference in distribution appeared to be the higher values of the anterior top lobe and the medium bottom lobe.

No differences in volume fraction distributions of the different oocyte stages were found along the top lobe of the ovary of pre-vitellogenic (figure 3.5 and 6) and vitellogenic

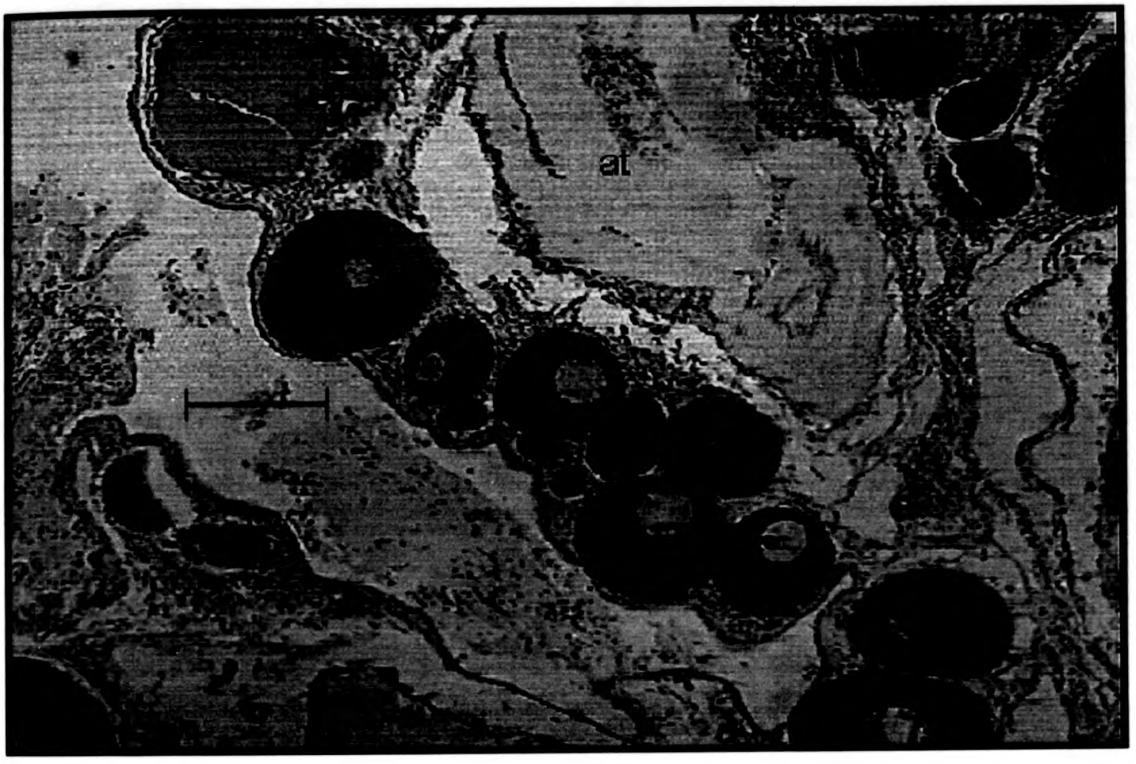


Plate 3.37: Photomicrograph of a 4 μm section of a post-spawned ovary showing a follicle in the final stages of atresia (at). Stained with haematoxylin and eosin. Magnification 4 x 3.3. Scale bar = 200 μm.

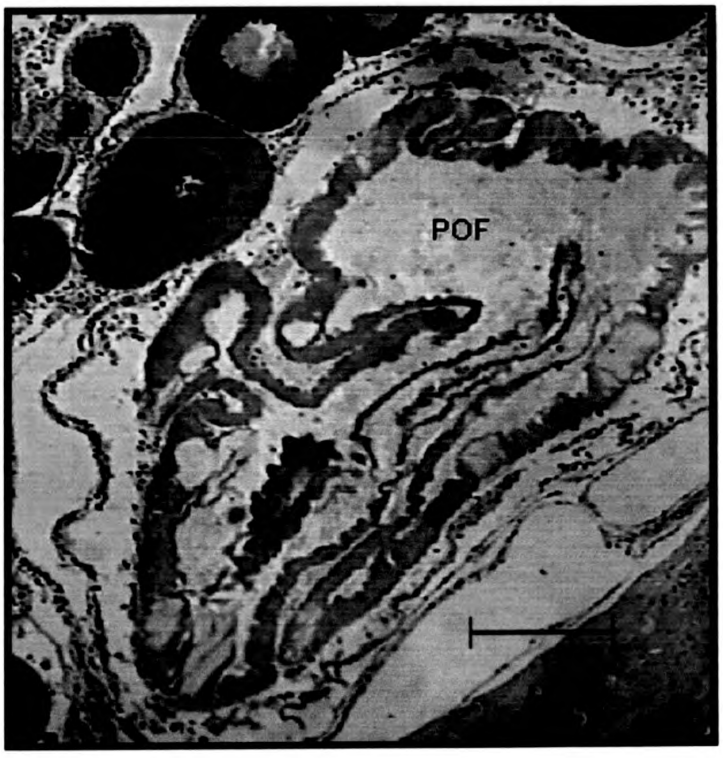


Plate 3.38: Photomicrograph of a 4 μm section of a post-spawned ovary showing a post ovulatory follicle (POF). Stained with haematoxylin and eosin. Magnification 4 x 3.3. Scale bar = 200 μm.

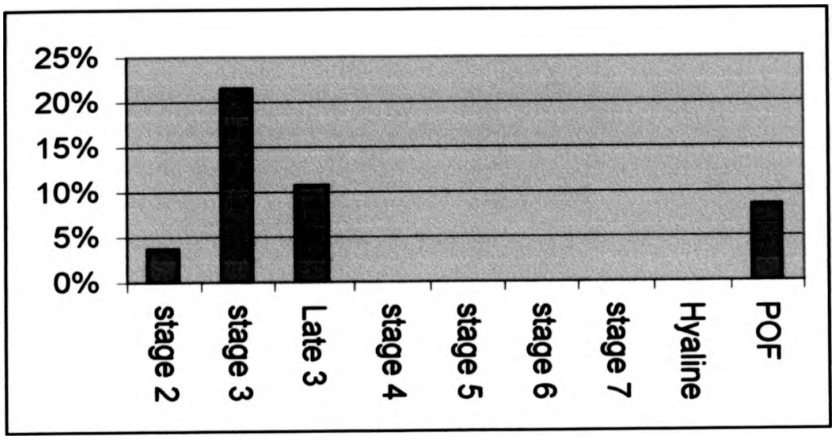


Figure 3.2: Volumetric fractions of the oocyte stages found in post-spawning ovaries.

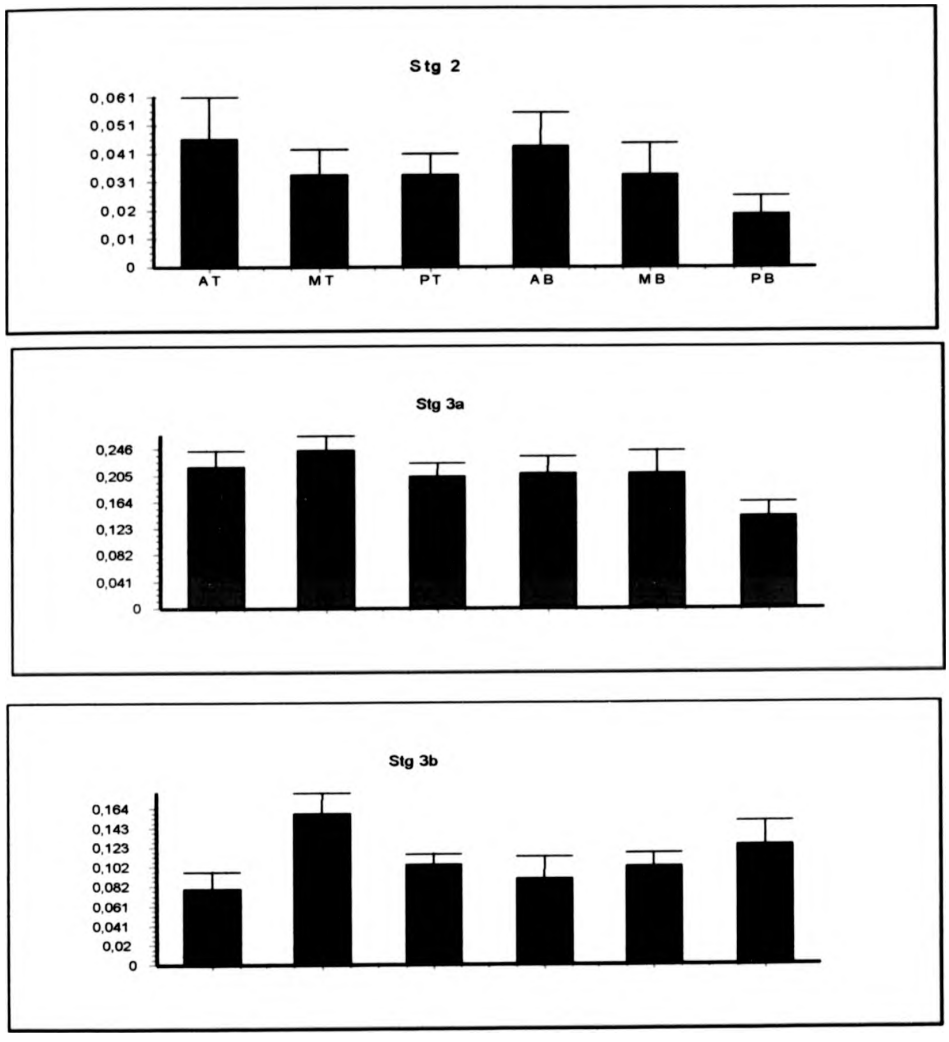


Figure 3.3: Volume fractions (mean with standard error) of Stage 2, 3a and 3b oocytes along the ovary of post-spawning female Atlantic halibut. AT, anterior portion of top lobe of the ovary; MT, medium top lobe; PT, posterior top lobe; AB, anterior bottom lobe; MB, medium bottom lobe; PB, posterior bottom lobe.

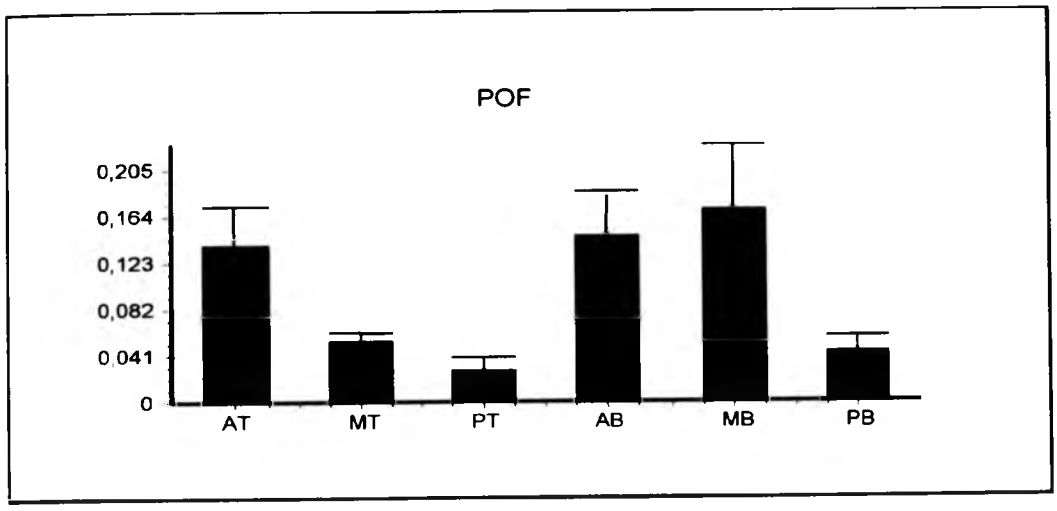


Figure 3.4: Volumetric fractions (mean with standard error) of post ovulatory follicles along the ovary of post-spawning female Atlantic halibut. Significant differences when analysed by ANOVA ($p = 0.0005$) appeared to be due to the higher values of the anterior top lobe and the medium bottom lobe. AT, anterior portion of top lobe of the ovary; MT, medium top lobe; PT, posterior top lobe; AB, anterior bottom lobe; MB, medium bottom lobe; PB, posterior bottom lobe.

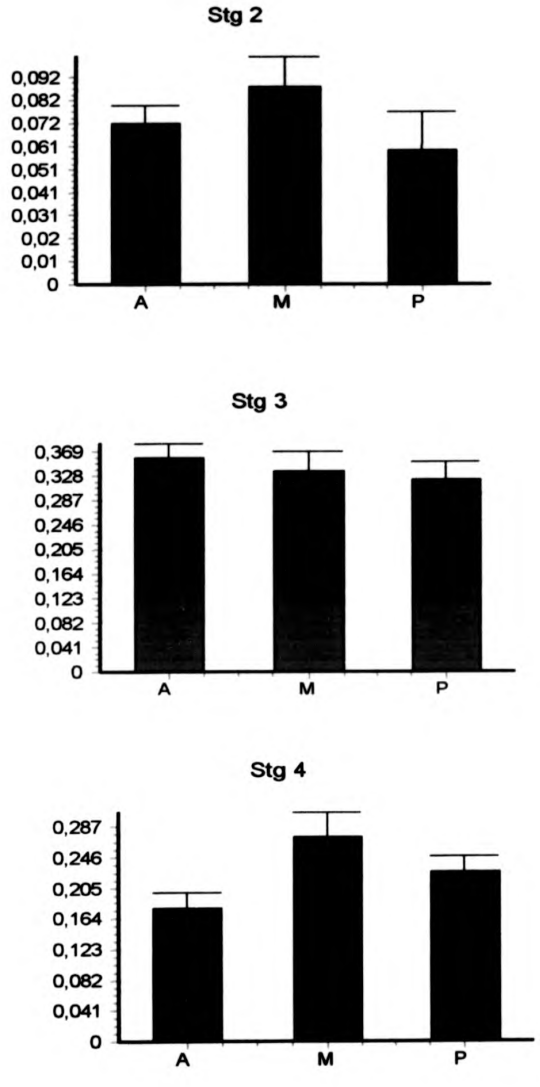


Figure 3.5: Volumetric fractions (average with standard error) of Stage 2, 3 and 4 oocytes along the top lobe of the ovary of pre-vitellogenic female Atlantic halibut. A, anterior portion of top lobe of the ovary; M, medium; P, posterior.

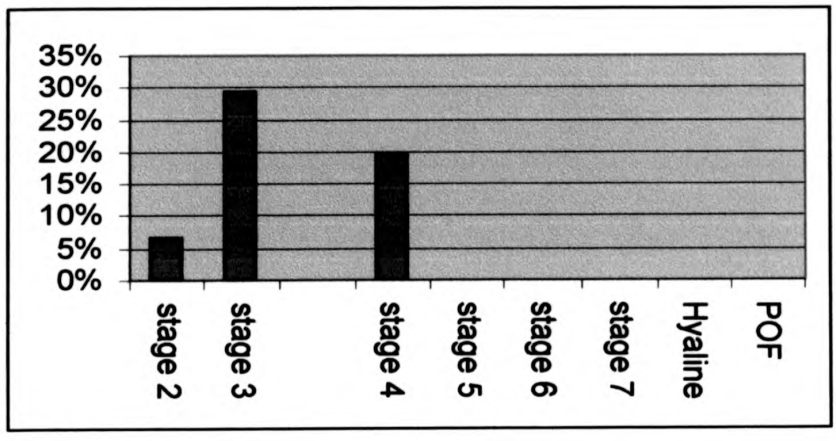


Figure 3.6: Volumetric fractions of oocyte stages present in previtellogenic ovaries. Stages 3a and 3b are both included in stage 3.

(figure 3.7 and 8) females. However, in spawning females, the volume fraction of Stage 6/7 oocytes (figure 3.9 and 10) was significantly smaller in the medium portion of the ovary (p=0.0054).

Table 3.1: Tukey-Kramer Multiple Comparisons Test of POF distribution along the ovary of a post-spawned ovary. If the value of q is greater than 4.225 then the p value is less than 0.05. (at, anterior top lobe; mt, middle top lobe; pt, posterior top lobe; ab, anterior bottom lobe; mb, middle bottom lobe; pb, posterior bottom lobe)

Comparison	Difference	q	Significance	P value
at vs mt	0.2005	4.858	*	P<0.05
at vs pt	0.2098	4.940	*	P<0.05
at vs ab	0.04974	1.206	ns	P>0.05
at vs mb	0.01878	0.4274	ns	P>0.05
at vs pb	0.2042	4.646	*	P<0.05
mt vs pt	0.009301	0.2254	ns	P>0.05
mt vs ab	-0.1507	3.765	ns	P>0.05
mt vs mb	-0.1817	4.245	*	P<0.05
mt vs pb	0.003712	0.08675	ns	P>0.05
pt vs ab	-0.1600	3.878	ns	P>0.05
pt vs mb	-0.1910	4.345	*	P<0.05
pt vs pb	-0.005588	0.1272	ns	P>0.05
ab vs mb	-0.03096	0.7235	ns	P>0.05
ab vs pb	0.1544	3.608	ns	P>0.05
mb vs pb	0.1854	4.084	ns	P>0.05

3.2.2.4 GSI development.

Table 3.2 presents the GSI of the different fish sampled. Immature fish presented a low GSI (mean = 0.5 ± 0.04). GSI of mature fish increased from a minimum value of 2.1 during previtellogenesis (September) to an average of 15.0 ± 1.4 in prespawning individuals (March). Spawning individuals presented an average value of 16.5 ± 1.4. The maximum value recorded from spawning individuals was 21.6, and the minimum was 11.8.

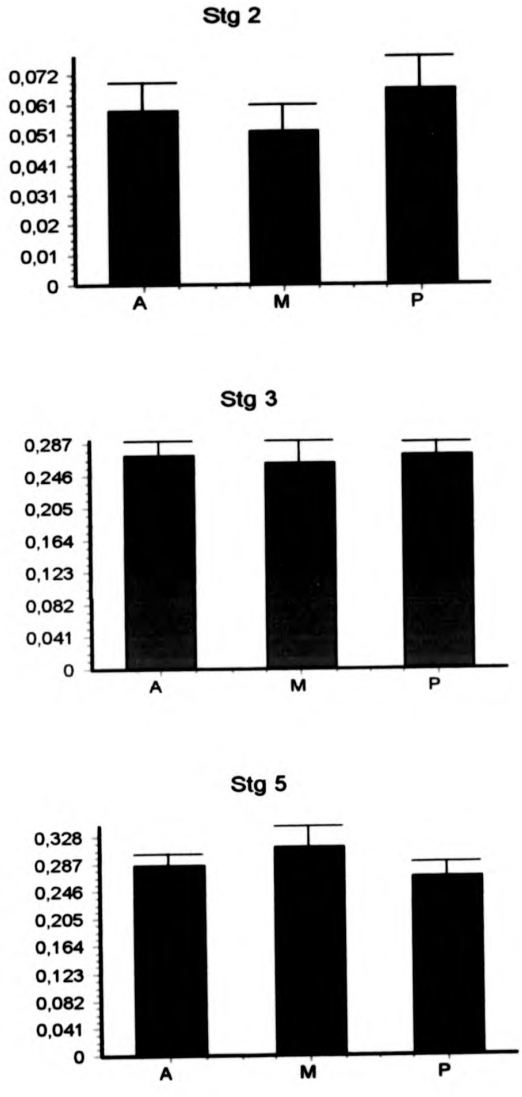


Figure 3.7: Volumetric fractions (average with standard error) of Stage 2, 3 and 5 oocytes along the top lobe of the ovary of vitellogenic female Atlantic halibut. A, anterior portion of top lobe of the ovary; M, medium; P, posterior.

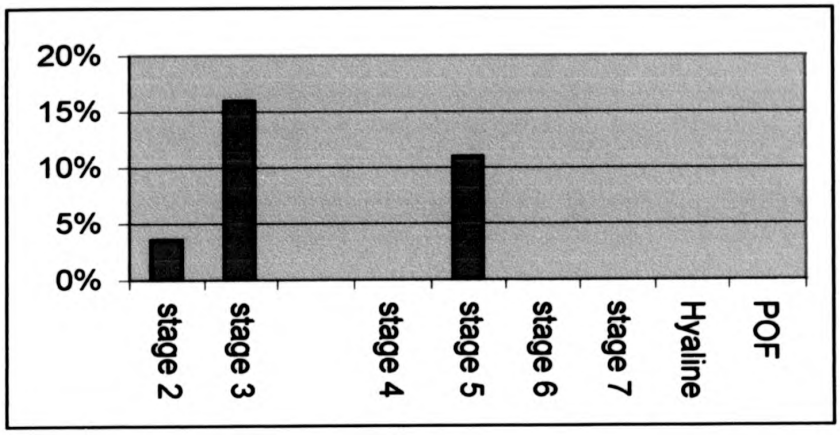


Figure 3.8: Volumetric fractions of oocyte stages present in early vitellogenic ovaries. Stages 3a and 3b are both included in Stage 3.

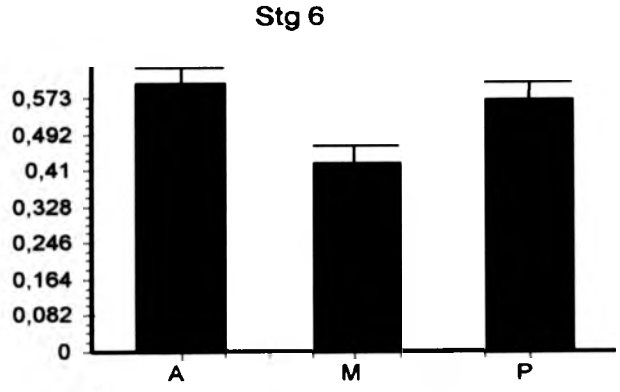


Figure 3.9: Volumetric fraction (average with standard error) of Stage 6 oocytes along the top lobe of the ovary of spawning female Atlantic halibut. The volumetric fraction was significantly smaller in the medium portion of the ovary (P=0.0054). A, anterior portion of top lobe of the ovary; M, medium; P, posterior.

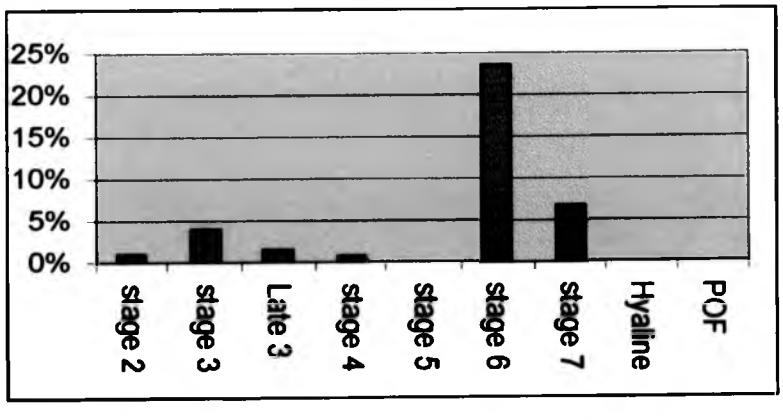


Figure 3.10: Volumetric fraction of the different oocyte stages present in ovaries of spawning female.

Table 3.2: GSI values [{Ovary weight / (body weight - ovary weight)} x 100] of female halibut at different times in ovarian development.

Date	Fish Code	Ovary weight (g)	Fish weight (g)	GSI	Comments
February	1	840	9600	**9.6**	Vitellogenic
March	2	1812	14000	**14.9**	Prespawning
March	3	882	10900	**8.8**	Prespawning
March	4	1282	10400	**14.1**	Prespawning
March	5	1837	12500	**17.2**	Prespawning
March	6	2171	14400	**17.8**	Prespawning
March	7	1793	12400	**16.9**	Prespawning
March	8	1968	12200	**19.2**	Spawning
March	9	2170	12200	**21.6**	Spawning
March	10	1464	13300	**12.4**	Spawning
April	11	1270	12000	**11.8**	Spawning
April	12	1848	12300	**17.7**	Spawning
April	13	1343	10500	**14.7**	Spawning
April	14	1328	11100	**13.6**	Spawning
April	15	20	4200	**0.5**	Immature
April	16	27	6300	**0.4**	Immature
April	17	20	4100	**0.5**	Immature
April	18	21	4800	**0.4**	Immature
April	19	30	3400	**0.9**	Immature
April	20	18	2700	**0.7**	Immature
April	21	17	4600	**0.4**	Immature
April	22	19	4300	**0.4**	Immature
April	23	19	4100	**0.4**	Immature
April	24	18	4700	**0.4**	Immature
April	25	27	5500	**0.5**	Immature
April	26	19	4400	**0.5**	Immature
June	27	3586	21000	**20.6**	Spawning
September	28	212	10500	**2.1**	Previtellogenic
September	29	219	9500	**2.4**	Previtellogenic
September	30	298	11000	**2.8**	Vitellogenic
December	31	1095	16700	**7.0**	Vitellogenic
December	32	520	10700	**5.1**	Vitellogenic

3.3 Sexing of Atlantic halibut with the use of ultrasound scanning

3.3.1 Materials and methods

A stock of 100 hatchery-produced 5 year old Atlantic halibut was maintained in a 10 m diameter tank. On the day of sampling the water in the tank was drained to a level of approximately 30 cm to facilitate the movement of the operators inside the tank and to help with the handling of the fish. Fish were anaesthetised and guided onto a working table. An ultrasound scanner (Aloka echo camera SSD-210DX11) fitted with a linear T-shaped 7.5 MHz transducer (UST – 5511TU Scanning width = 56 mm. Diagnostic range = 98 mm.), was used to capture an image of the gonad of the fish at two different points (see figure 3.11), some five centimeters behind the pectoral fin and just behind the first spinal arc. The mucus of the skin of the fish acted as a coupling medium. A diagnosis of the sex of the fish was made at that point. The images were stored with a videotape recorder connected to the ultrasound machine for later analysis. Descriptions of the external features of the fish were recorded to allow later identification. Fish were distributed into several production tanks and monitored during the following spawning season for confirmation of their sexes.

3.3.2 Results.

Plate 3.39 shows the appearance of the female gonad in the ultrasound image. Mature females were easily identifiable as the ovary appeared to occupy a large portion of the body cavity. The ovary wall could be seen as a strong, well defined structure, overlaying a thin layer of grainy material, the developing oocytes. Ovaries at this stage appeared very opaque to the ultrasound, and penetration was reduced. A number of the females presented hydrating or ovulated eggs, which allowed for more penetration of the ultrasound due to the liquid surrounding them (ovarian fluid), providing a more in-depth view of the ovary.

Figure 3.11: Positions where the linear T-shaped 7.5 MHz transducer was applied to capture the ultrasound images of the gonad of the fish. Some 5 cm behind the pectoral fin and just behind the first spinal arc. The mucus of the skin of the fish acted as a coupling medium.

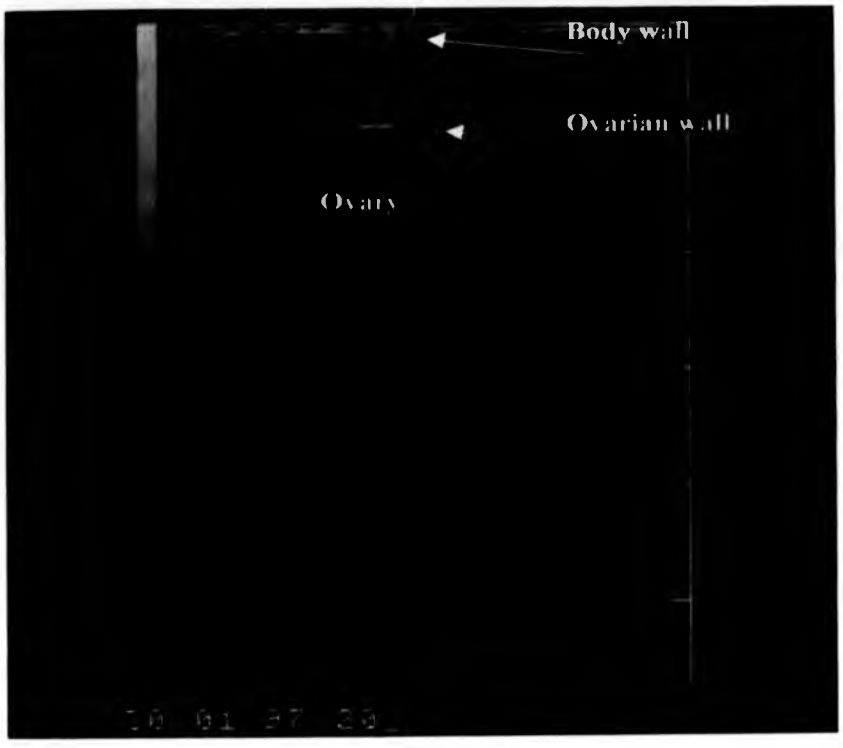


Plate 3.39: The image of the female ovary through the Aloka echo camera SSD-210DX11 fitted with a linear T-shaped 7.5 MHz transducer (UST – 5511TU Scanning width = 56 mm. Diagnostic range = 98 mm.). The ovary wall can be seen as a well defined structure, overlaying a thin layer of grainy material, the developing oocytes. Ovaries at this stage appeared very opaque to the ultrasound, and penetration was reduced.

Some of the fish identified as females had smaller gonads, with ovarian walls enclosing a solid mass of grainy material. Such fish were classified as immature females.

Testicular material was difficult to locate, and male fish were generally identified by exclusion, i.e., "not female". However, some males that were expressing milt at the time of sampling, presented a clearer image of their gonad in the ultrasound scan. These appeared as an oval white / grey, structure surrounded by fluid (dark echo). Unfortunately, the images stored were of poor quality and are not presented here.

3.4 Assessment of the gonadal growth of a stock of hatchery produced Atlantic halibut from pre-maturity to spawning with the use of ultrasound scanning.

3.4.1. Materials and methods.

The gonadal development of eighteen hatchery produced Atlantic halibut was monitored using ultrasound scanning. At the time of sampling, fish were anaesthetised in a seawater bath with phenoxyethanol, guided onto a table and scanned with an ultrasound machine (Concept\MLV Dynamic Imaging LTD) fitted with a linear T-shaped 7.5 MHz transducer set for a penetration of 9 cm. (see figure 3.11 for standard location of scans at the first spinal arch). This machine provided a better quality image and better penetration than the Aloka machine. Fish were in this way sexed and their gonadal development assessed over two spawning seasons. At the beginning of the experiment the fish were 5 years old and females had not presented any signs of gamete production in previous seasons.

3.4.1 Results.

Plates 3.40 to 3.48 show different images of the gonadal development of males and females obtained with the use of the ultrasound scanner. Ovaries were easily identifiable from the beginning of the experiment, and the development of different egg batches during spawning season and the recovery of the ovary post-spawned was simple to monitor. Males were more difficult to identify in the first stages of the study, and were not easy to differentiate from immature females; nonetheless, cumulative experience made the task easier. Normally, both lobules of the testis were visible when scans were taken at the level of the first spinal arc, or just behind this.

The gonad of immature females (plate 3.40) appeared as a double lobed oval structure in the peritoneal cavity. The ovarian wall returned a strong echo and appeared as a clear white line, enclosing a grey-coloured area of grainy texture. This grainy appearance distinguished immature females from males, with a similar shape but darker interior (see plate 3.47 below). The ovary also extended further behind in the body cavity than testis.

Before the start of vitellogenesis (August), ovaries could not be seen when scans were taken anterior to the first spinal arch (plate 3.41). However, as vitellogenesis progressed (November), the ovary increased in volume and started to grow into the body cavity (plate 3.42), gradually displacing and compressing the alimentary canal. The increase in volume of the ovary continued up to the start of the spawning season (February). During the spawning season (February, March and April), batches of eggs were released (plate 3.43) by all females. Eggs were released into the lumen of the ovary, concentrating mainly at the front of the ovary behind the gonoduct (plate 3.44). Ovaries remained distended through the spawning season, still occupying most of the body cavity (plate 3.45). Towards the end of the season, and when most of the egg batches had been released, some of the ovarian trabeculae were distinctly recognisable in the scans (plate

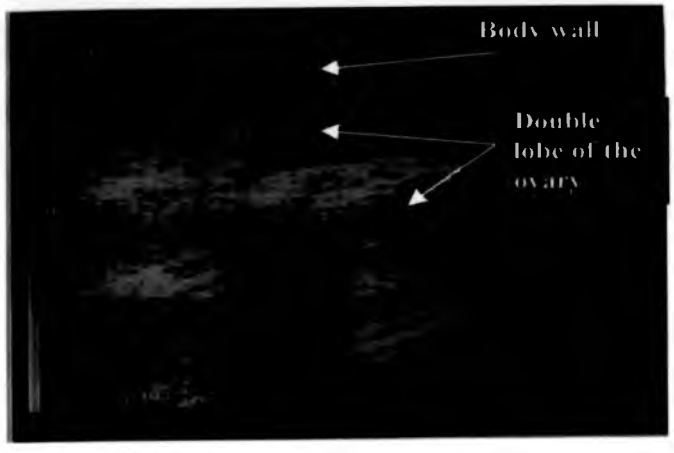


Plate 3.40: Ultrasound image of the ovary of immature female. The double lobed oval structure of the ovary can be seen in the peritoneal cavity. The ovarian wall returned a strong echo and appeared as a clear white line, enclosing a grey-coloured area of grainy texture.

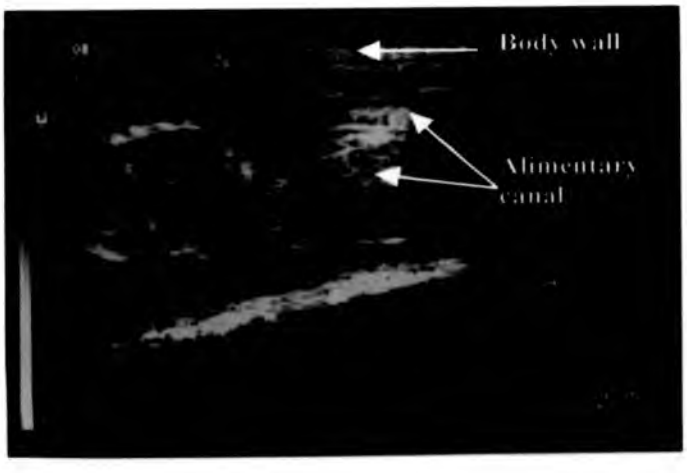


Plate 3.41: Ultrasound image of the body cavity in front of the first spinal arc of a halibut female before the start of vitellogenesis. The peritoneal cavity is completely occupied by the alimentary canal and other viscera. The ovary is reduced in size and situated behind the first spinal arc.

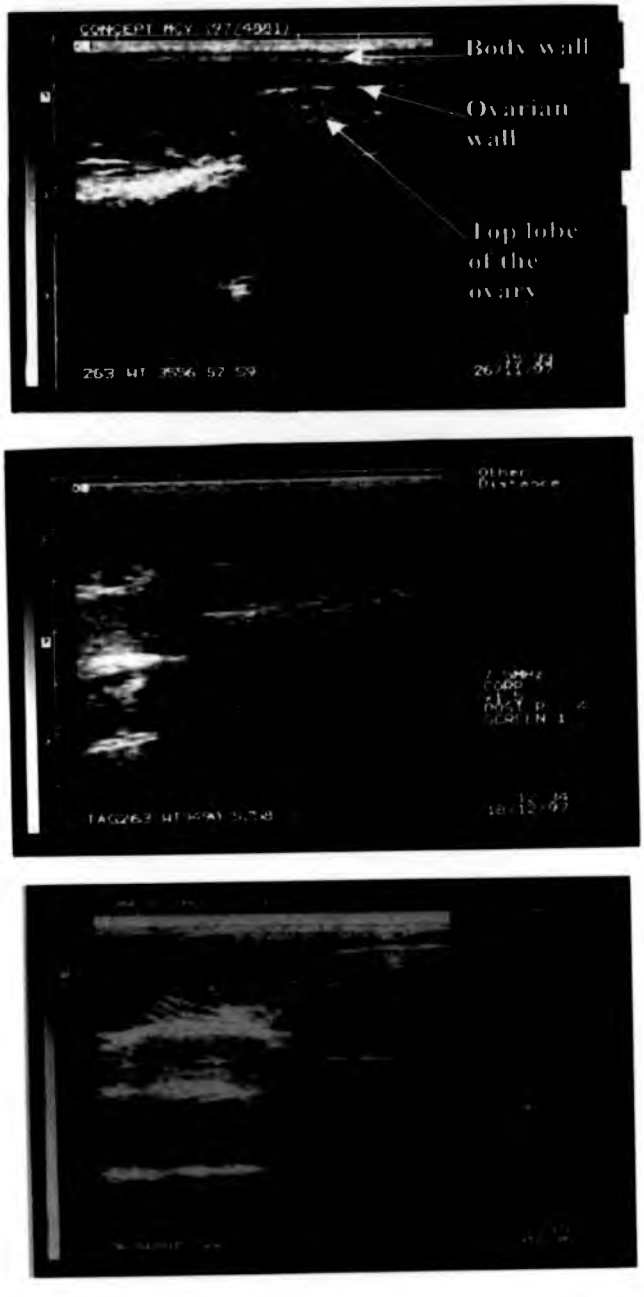


Plate 3.42: Ultrasound image of the vitellogenic ovary in November, December and February. As vitellogenesis progressed the ovary increased in volume and started to grow into the body cavity displacing and compressing the alimentary canal.

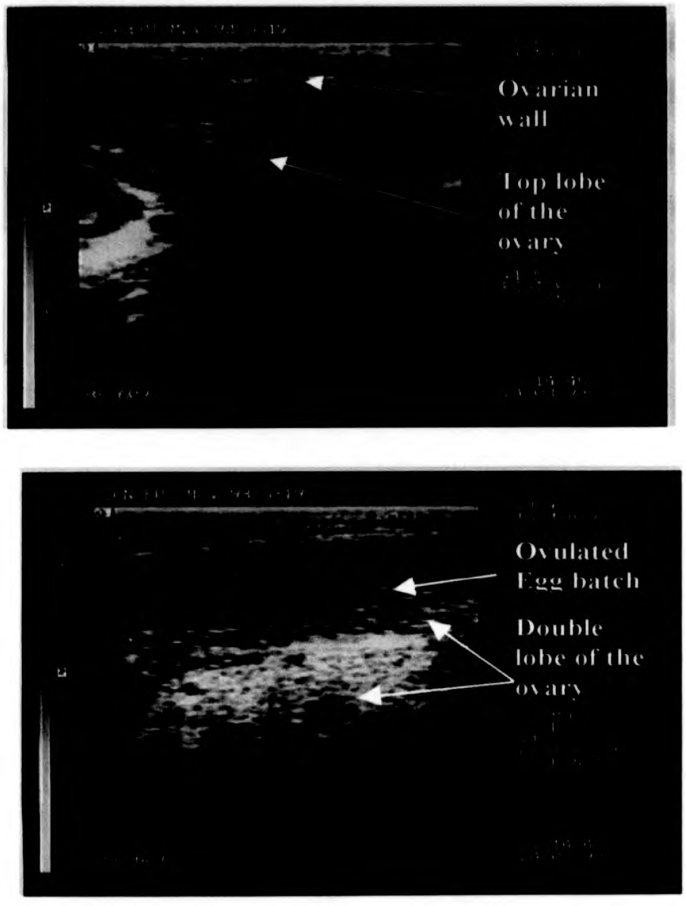


Plate 3.43: Ultrasound image of the ovary of a spawning female at the level of the first spinal arc. The appearance of discrete egg batches could be monitored (see text). The presence of an ovulated egg batch increased the penetration of the ultrasound.

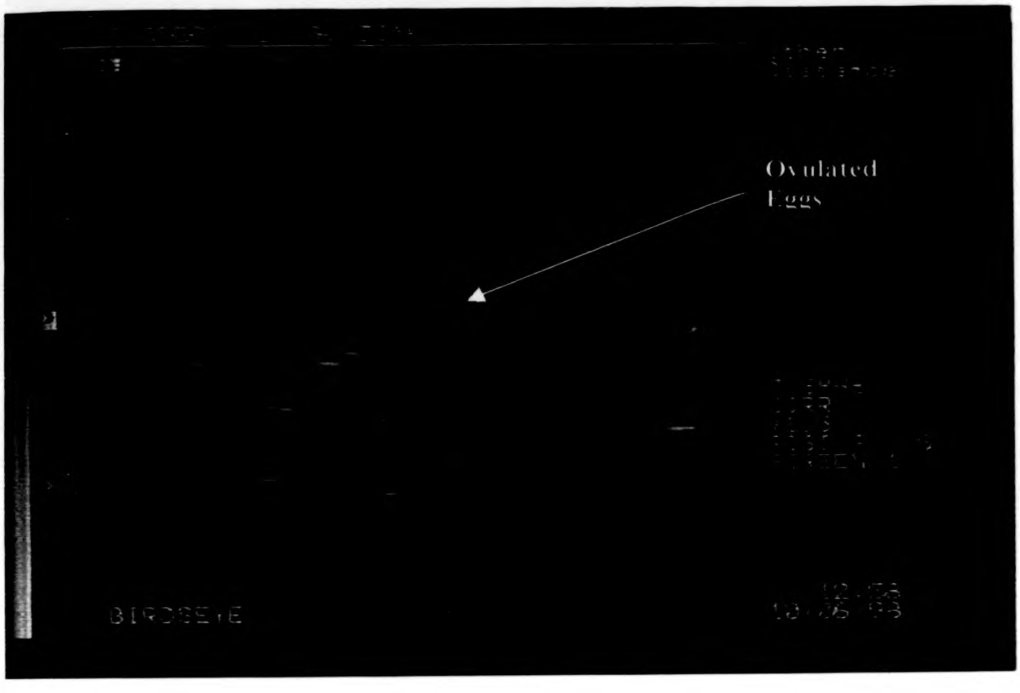


Plate 3.44: Ovulated eggs concentrated at the front of the ovary, close to the gonopore.

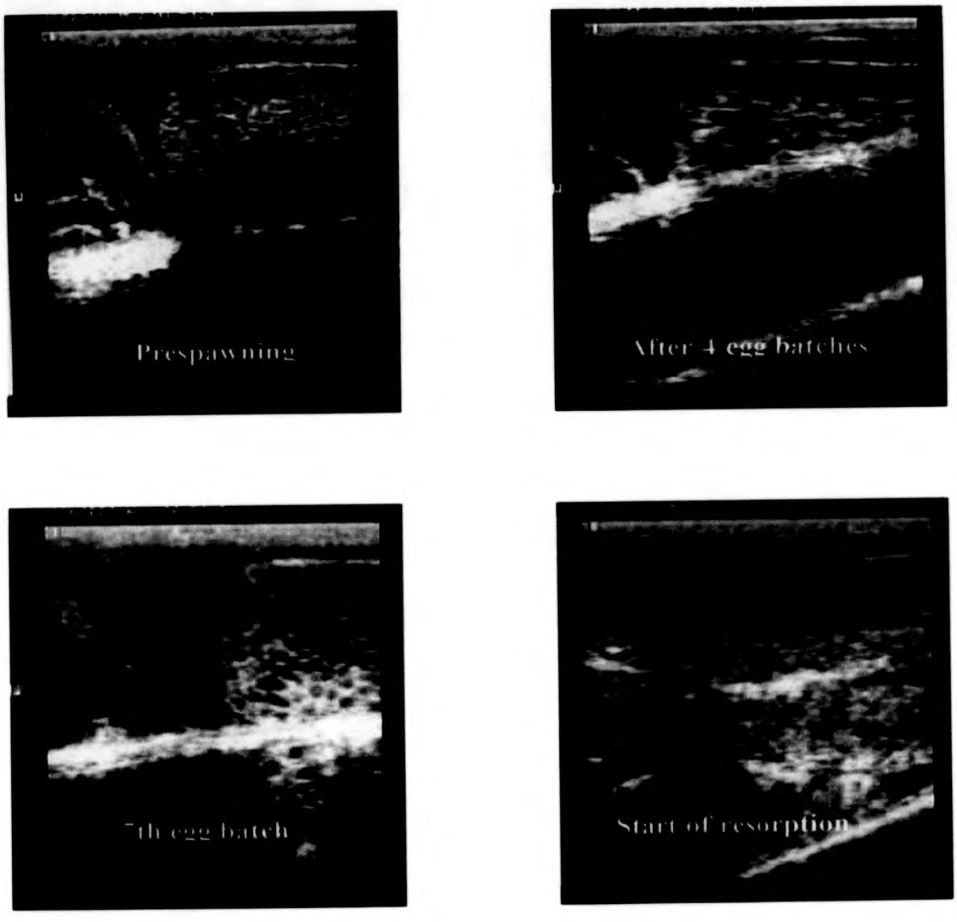


Plate 3.45: Through the spawning season gonadal material is released but ovary remains distended. Pictures show the gonad of the same female at different points in time over the season.

3.46) and the gonadal material appeared less dense to ultrasound. Ovaries remained in this state for several weeks after the last egg batch. A reduction in volume followed, with a concomitant increase in ultrasound density. Ovaries of post spawning females (May to September) showed similar scanned images to those at the start of vitellogenesis.

Testis were more difficult to locate, especially in non-spermiating individuals (September). Testis were located by carefully shifting the position of the transducer along the area immediately behind the first spinal arch (plate 3.47). The shape of the male gonad was more obviously oval and smaller than mature females, with the wall enclosing some white/grey material of undefined shape, surrounded by a dark echo (possibly fluid) underlying the testis wall. As the spawning season approaches, fluid content in the testis increases, and the white area inside the gonad contracts into a line parallel to the testis wall (November, December). Males started expressing milt before females commenced spawning (5 to 7 weeks in advance). The scans of spermiating males presented as a similar structure to those of prespawning individuals. However, the gonad increased in volume due to the accumulation of seminal fluid (plate 3.48). Nevertheless, the increase in volume was never as acute as that of females, and did not interfere with the alimentary canal to the same extent.

3.5 Ultrasound scanning and biopsy study of final maturation.

3.5.1. Materials and methods.

Ultrasound images (Concept\MLV Dynamic Imaging LTD fitted with a linear T-shaped 7.5 MHz transducer set for a penetration of 9 cm) of the ovary and biopsies of ovarian tissue were taken from one female Atlantic halibut throughout an interovulation cycle (see Chapter II, section 2.4.1 for details of ovarian biopsy). The first sample was

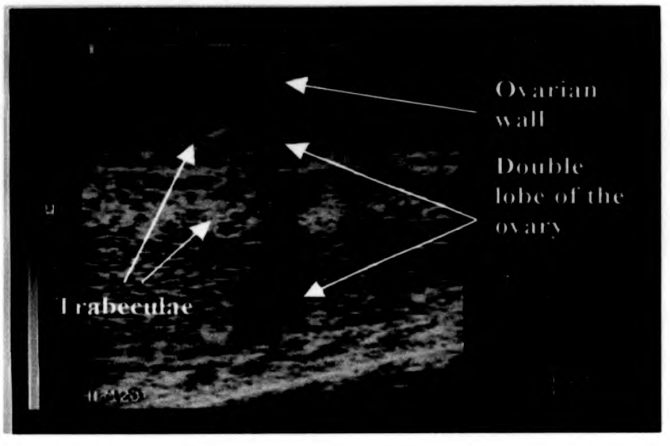


Plate 3.46: Ultrasound images of ovaries of Atlantic halibut towards the end of the spawning season. Some of the ovarian trabeculae are distinctly recognisable and the gonadal material appears less dense to ultrasound.

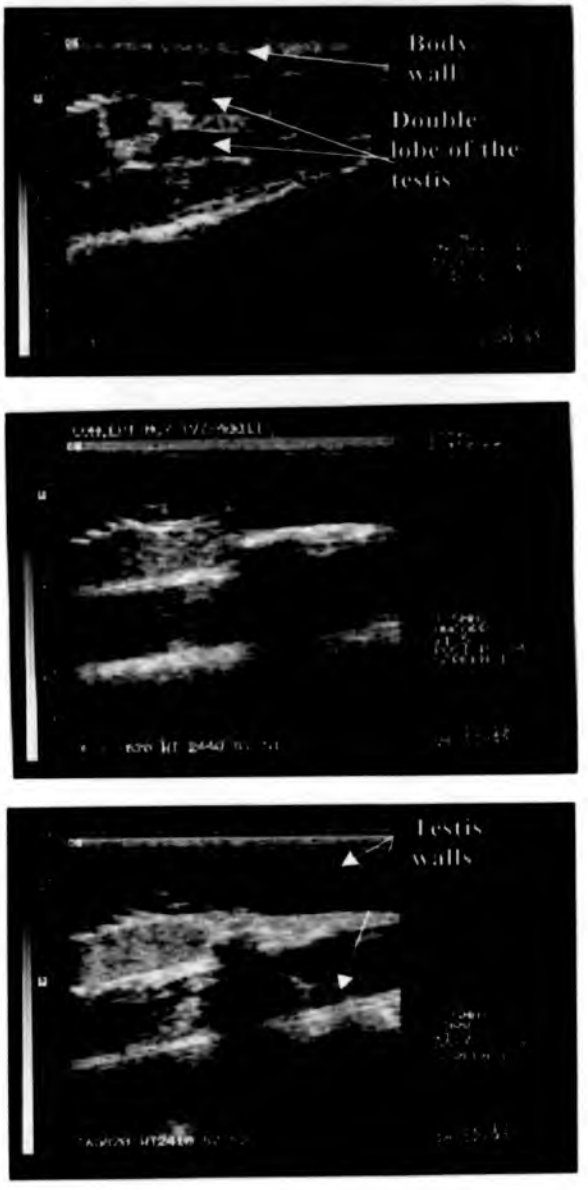


Plate 3.47: The shape of the male gonad in the ultrasound scans is oval and smaller than mature females, with the wall enclosing some white/grey material of undefined shape, surrounded by a dark echo (possibly fluid) underlying the testis wall. As the spawning season approaches (bottom pictures), fluid content in the testis increases, and the white area inside the gonad contracts into a line parallel to the testis wall.

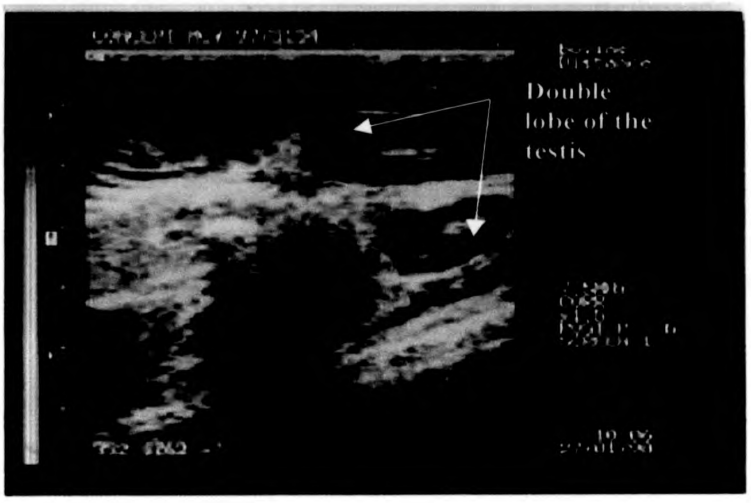


Plate 3.48: Ultrasound image of spermiating male Atlantic halibut. The gonad increased in volume with respect to prespawning individuals due to the accumulation of seminal fluid.

taken after stripping of a batch of eggs at the predicted time of spawning, and further samples were obtained at 24h, 36h, 48h, 60h, 84h and 108h after stripping. A further sample was taken two hours after the last stripping (110h.). No anaesthesia was required during manipulation of the fish. After the 108 h sampling the batch of eggs was considered ready for fertilisation and was released from the female by stripping. The egg batch was fertilised with fresh milt, following the standard hatchery procedure explained in Chapter II, section 2.2.4.1, and fertilisation rate was assessed following the same protocol.

3.5.1 Results.

The first batch of eggs obtained from the female at the predicted time for stripping (0 h) exhibited a very low fertilisation rate (Total fertilisation = 20%). Ultrasound images before stripping showed the presence of a space between the ovary wall and the grainy portion of the gonad, the expanded lumen with ovarian fluid (see plate 3.49). Translucent and hydrated eggs could be seen in the ovarian biopsy, together with opaque, vitellogenic oocytes. After stripping, the ovarian lumen was reduced, but still apparent. Biopsy revealed the presence of translucent and opaque oocytes.

During the sampling at 24h (plate 3.50), ovulated eggs were present in scan and biopsy, and a second batch of eggs was stripped from the female. Biopsies and ultrasound scans were taken immediately before and after stripping. The distinction between ovarian lumen and the rest of the gonad was very apparent before stripping, and ovulated eggs could be seen floating in ovarian fluid along the ovary. Many transparent eggs were present in the biopsy, together with opaque and very few translucent oocytes.

After stripping, the lumen was still visible but surrounded by grainy material. Only a few translucent eggs accompanied the opaque oocytes in the biopsy. The egg batch was fertilised following the hatchery procedure, and fertilisation assessed (total fertilisation =

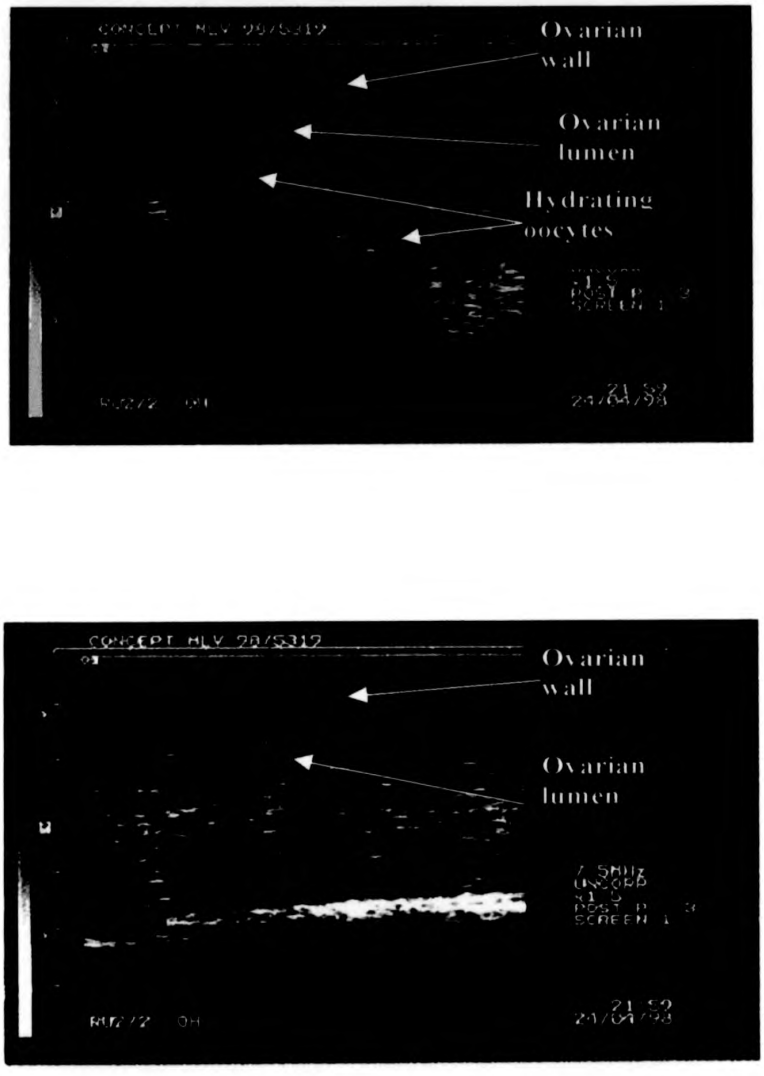


Plate 3.49: Ultrasound images of female Atlantic halibut before stripping (top) -showing the presence of a space between the ovary wall and the grainy portion of the gonad, the expanded lumen with ovarian fluid - and after (bottom) stripping (time 0h).

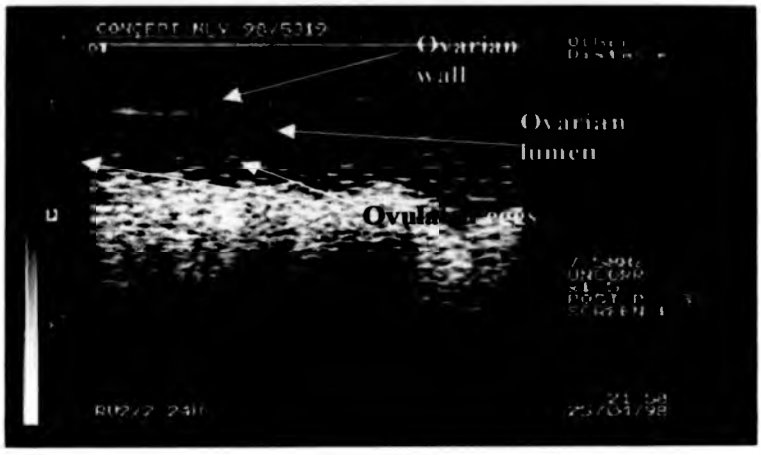


Plate 3.50: Ultrasound image before and after stripping (time 24h). Notice the distinction between ovarian lumen and the rest of the gonad before stripping (top). Ovulated eggs can be seen floating in ovarian fluid in the ovary. After stripping (bottom), the lumen was still visible but surrounded by grainy material.

74.4%). At 36 h. and 48 h. after the first stripping, the ovary presented a compact, grainy picture in ultrasound images (plate 3.51). However, some hydrated eggs were present in the ovarian biopsy at 48 h.

At 60 h., the ultrasound image of the ovary was still compact, but some dark ovoid small echoes could be seen embedded in the grainy structure (see plate 3.52). The biopsy at 72 h. showed translucent eggs turning increasingly transparent. This was more so at 84 h. when the number of dark ovoid shapes had also increased in the scans. The dark space of the lumen started to be perceptible in the 96 h. sampling, and some more discernible eggs were present in the front of the ovary, immediate to the oviduct. 108 h. after the first stripping, i.e. 84 hours after the second stripping, ultrasound scans revealed a fully developed space between the ovarian wall and the rest of the gonad (see plate 3.53), where ovulated eggs could be seen floating freely in ovarian fluid. Numerous hydrated eggs were present in the biopsy. The egg batch obtained at this stage presented a total fertilisation rate of 96%. The final sampling, two hours after this stripping, showed a compact image of the ovary in the ultrasound scan, corresponding with opaque oocytes and a few translucent eggs in the biopsied tissue.

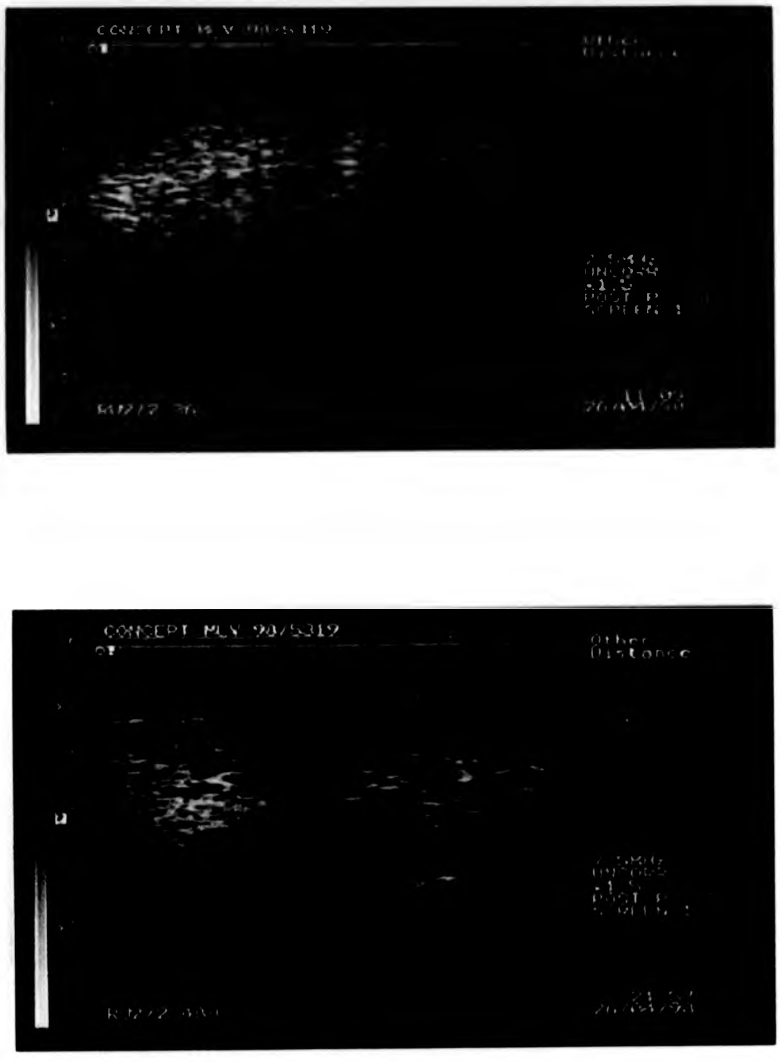


Plate 3.51: Ultrasound images of ovary at time 36 h. (top) and 48 h. (bottom) after first stripping.

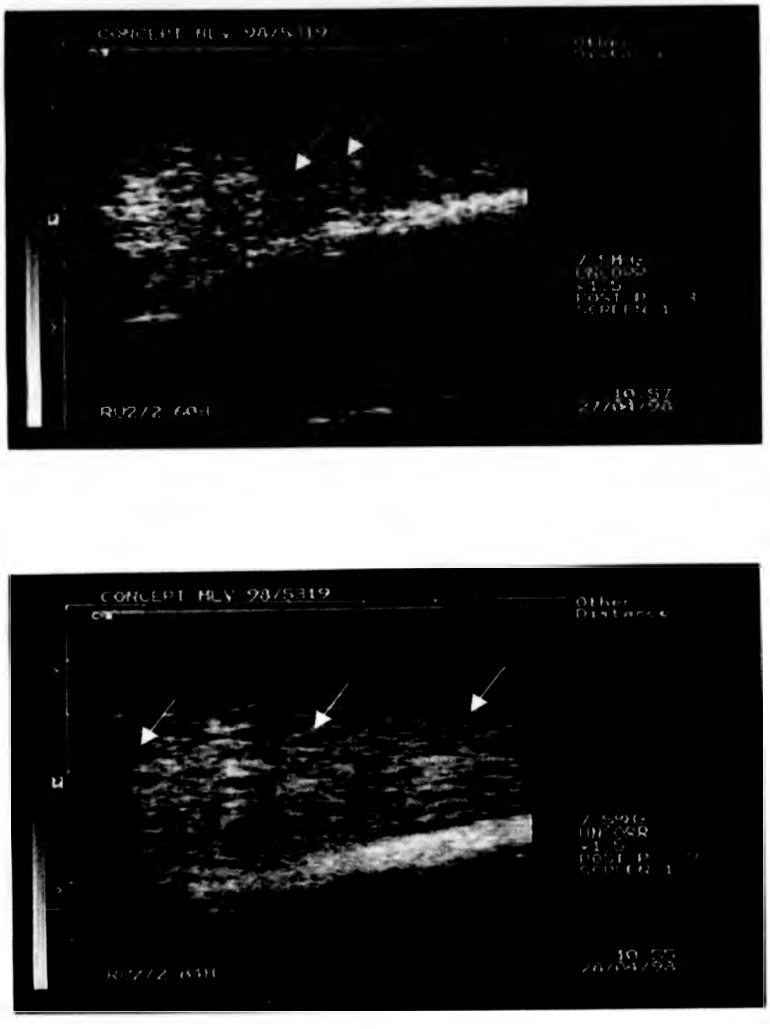


Plate 3.52: Ultrasound image of the ovary at 60 h. (top) and 84 h. (bottom) after first stripping. The ultrasound image of the ovary is still compact and grainy, but some dark ovoid small echoes (arrows) can be seen embedded in the grainy structure.

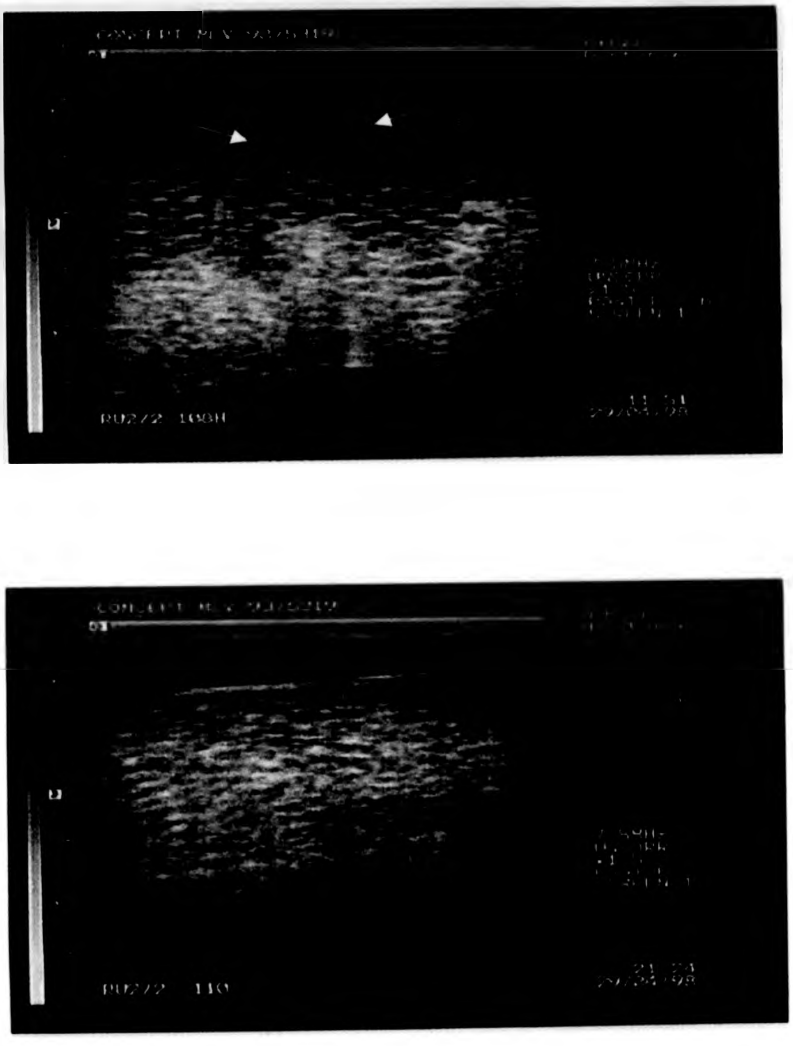


Plate 3.53: Ultrasound image 108 h. after first stripping (top), showing a batch of ovulated eggs floating freely in the ovarian lumen (arrows). At 110 h., after stripping that last batch, the ovary shows again a compact, grainy texture.

3.6 Discussion

Oocyte growth in Atlantic halibut followed the same basic processes described for most teleosts. The steps leading to the transformation of oogonia into stage 1 oocytes could not be described, due to the difficulty encountered in unequivocally identifying these primordial stages. However, the main events of primary growth, folliculogenesis, cortical alveoli formation, vitellogenesis, and final maturation (including yolk coalescence) were documented by both light and transmission electron microscopy.

During the last two thirds of primary growth, oocyte average diameter increased from a minimum of 40 μm in early stage 2 to maxima of 400 μm in late stage 3b oocytes. This growth was accompanied by a decrease in the basophilia of the cytoplasm. Cumaranatunga (1985) described the development of an area of increased basophilia around the nucleus of stage 2 rainbow trout oocytes; Kjesbu and Kryvi (1989) report the appearance of a similar formation in the late perinucleolar stage of cod oocytes. Both formations, identified as Balbiani bodies by Cumaranatunga, and circumnuclear ring by Kjesbu and Kryvi, appeared to be composed of diverse cytoplasm organelles. The Balbiani body has been described for several species of teleosts and amphibians, and equivalent associations of organelles have been reported in a variety of animals (Wallace and Selman 1990), consisting of heterogeneous groups of organelles such as (depending on the animal) mitochondria, Golgi complex, endoplasmic reticulum and vesicular bodies. The function of these associations is not clear. No similar arrangement was observed in Atlantic halibut oocytes. Coward and Bromage (1998) also mention the absence of these formations in tilapia (*Tilapia zillii*). The lack of Balbiani bodies in halibut is of unknown significance.

Numerous peripheral nucleoli and chromatin strands could be seen in the oocyte nucleus during primary growth, one or two of the nucleoli markedly bigger than the rest. Wallace and Selman (1990) provide a study of the ultrastructure of nucleoli and "lampbrush chromosomes". Copies of genes encoding ribosomal RNA are packaged into nucleoli. Similarly, lateral loops in DNA strands in the nucleus are areas of transcription of heterogeneous RNA for the synthesis of poly (A)-containing RNA (maternal message mRNA). This intense nuclear and synthetic activity is responsible for the increase in size of the oocytes during this phase.

In the present study, two discrete sub-stages are described for stage 3 oocytes in halibut. Despite the increase in size and reduction in basophilia of stage 3a oocytes, the nuclear membrane or envelope of stage 2 and stage 3a oocytes is spherical. In contrast, at the end of perinucleolar stage the nuclear envelope presents numerous foldings and an irregular shape. The nucleoli, however, stay mostly in the periphery of the nucleus. This appears to be a separate event from the next step, marked by the production of cortical alveoli. The presence of this stage, stage 3b, in post spawned ovaries, with no presence of stage 4 oocytes in the same ovaries, supports the idea of a separate event taking place at this stage, probably in preparation for the formation of cortical alveoli.

The appearance of cortical alveoli marked the beginning of stage 4. Cortical alveoli were found in the periphery of the oocyte. This, together with the few numbers of stage 4 oocytes encountered, and the fact that they were only present in some numbers in previtellogenic ovaries – and only occasionally in spawning ovaries - is probably an indication that this is a rapid phase in Atlantic halibut. The irregular shape of the nuclear envelope of stage 3b oocytes, became even more pronounced in stage 4 oocytes. The nucleoli increased in numbers and could be seen distributed through the nucleus. Numerous mitochondria were present in the ooplasm. Cortical alveoli could be seen mostly

as empty vesicles close to the periphery of the ooplasm. Wallace and Selman (1990) note that the cortical alveoli stain for protein and carbohydrate, but as they increase in size it is difficult to preserve their contents. In electron photomicrographs, some cortical alveoli of halibut appeared filled with some light material. Cortical alveoli are known to contain a polysialoglycoprotein of high molecular weight (see Tyler and Sumpter 1996), which is synthesised by the oocyte. This endogenous origin, and the fact that their contents are released to the perivitelline space at the time of fertilisation to harden the vitelline envelope (cortical reaction) having no part in the nutrition of the future embrion, renders the use of the terms "endogenous yolk" and "yolk vesicles", still used by some authors, inappropriate to refer to this organelles and the materials inside them.

Follicular cells were observed surrounding early stage 2 oocytes, as a primordial monolayer of a few cells. With the increase in oocyte size, the number of follicular cells also increased, arranged in different layers. The TEM study of stage 3 oocytes showed the development of a granulosa layer of squamous cells, close to the oocyte. Distal to this layer, the thecal cells contained increasing quantities of endoplasmic reticulum that could also be observed in cells of the granulosa. Thecal and granulosa cells play an important role in the regulation of oocyte growth and maturation, synthesising different steroids in response to GTH stimulation (Nagahama 1983, 1994; Dodd & Sumpter 1984; Nagahama *et al.* 1995; Schultz *et al.* 1999). A well developed endoplasmic reticulum and mitochondria population are characteristic of these type of secretory/synthesising cells. At the end of primary growth, the oocyte commenced to increase its surface area by projecting numerous microvilli into sub-follicular spaces. Granulosa cells also developed projections towards the oocyte, although less numerous. At the same time, electron dense material began to accumulate in stage 3 oocytes between the microvilli, and with them formed the zona radiata, which was easily identifiable in electron micrographs of stage 4 oocytes.

Cumaranatunga (1985) and Bromage and Cumaranatunga (1988) described a similar process in rainbow trout, although in light microscopy preparations microvilli could not be observed until late in stage 4 oocytes. In the halibut, microvilli could not be seen in light microscopy until well into stage 5, although they started to be produced during stage 3. The microvilli from the oocyte and granulosa increased in length during stage 4, as the zona radiata thickened, and seemed to converge in groups of several microvilli at particular points, probably in intercellular spaces of the granulosa layer.

At the start of true vitellogenesis, small yolk granules could be seen in the periphery of the ooplasm. During vitellogenesis, vitellogenin (VTG) is synthesised in the liver and is sequestered, processed and packaged into oocytes. VTG reaches the oocyte from the blood stream via an intercellular route through the follicular layers, and is transported after pinnocytosis by endosomes and multivesicular bodies to forming yolk granules (see Wallace and Selman 1990). This is in accordance with the proposal that the distal extreme of microvilli from the oocyte appeared to converge towards intercellular spaces in the granulosa. The study of the ooplasm of stage 5 and 6/7 oocytes with transmission electron microscopy showed numerous mitochondria, in addition to vesicles that concentrated mainly in the periphery of the cytoplasm and around forming yolk granules.

In many species of teleosts, yolk granules aggregate into crystalline yolk platelets (Rainbow trout, Cumaranatunga 1985, Bromage and Cumaranatunga 1988; cod, Kjesbu and Kryvi 1989). However, Wallace and Selman (1981, 1990) report that the yolk proteins of many marine teleost are not found in crystalline form, but in fluid-filled yolk spheres. Such spheres fuse centripetally in some species, e.g. marine pelagic egg spawners, forming a continuos mass. This appears to be the case in Atlantic halibut, where yolk globules do not show polygonal contours but a predominantly spherical shape. Electron micrographs of yolk globules show no crystalline arrangement of proteins.

Vitellogenin sequestration progressed centripetally until yolk spheres occupied most of the ooplasm, with narrow areas of cytoplasm between yolk globules – or "spheres" – and in the region adjacent to the oocyte membrane. Cortical alveoli were also located in this area. There were no signs of major lipidic droplets in the cytoplasm of vitellogenic oocytes.

Stage 5 oocytes had an average diameter of 559 ± 15.5 μm, but minimum diameters of 350 μm. were recorded at this stage in samples taken at the beginning of the autumn (i.e. September). Late stage 7 oocytes, where yolk coalescence had started and yolk sequestration was over, had diameters of around 1507 μm. This means that some 13% of the volume of the ovulated egg (approx. 3 mm) is due to vitellogenesis. These figures are somewhat lower than those presented in Bromage *et al.* (1994) for halibut. In any case, they are consistent with the fact that in marine pelagic eggs, the intense process of hydration before ovulation represents a major contribution to final egg size (Wallace and Selman 1981). In the case of halibut, hydration would appear to represent some 85% of the final egg size. Similarly, Haug and Gulliksen (1988 a) reported that the fluid intake of oocytes of gill-netted female halibut appeared to start in ovaries with mean mature (but still opaque) oocyte diameters of 1.70-2.05 mm.

Besides the general increase in numbers of follicular cells that invest the oocyte, two major structural events take place in follicular layers during yolk acquisition. One is that granulosa cells acquire a cuboidal shape during stage 5 that they maintain through stages 6 and 7. The other major event is the increase in size and complexity of the zona radiata through vitellogenesis. Hyllner *et al.* (1994), reported that the two major vitelline envelope (zona radiata) proteins in halibut were first detected in plasma of females sampled in October, i.e. during vitellogenesis, and that their synthesis was stimulated by oestradiol. The architectural complexity of the zona radiata has been described in several species (cod,

Kjesbu and Kryvi 1989; zebrafish, Selman *et al.* 1993). In the present study, light microscopy showed an increase in thickness of the zona radiata as vitellogenesis progressed, and the development of microvilli from oocyte and granulosa. TEM preparations allowed for the detailed view of three different areas in the zona radiata. An outer layer, sometimes referred to as the porous layer by some authors, a middle layer named homogeneous layer, and the inner layer, the multilamellar layer. The multilamellar layer is the one responsible for most of the size increase in the zona radiata during stages 5, 6 and 7. The name "multilamellar" comes from the stratified structure of this layer. In halibut, some 12 "lamellae" are formed during stage 5, and this is increased to 25 or more during stage 6/7. Cumaranatunga (1985) found no stratification in the zona radiata of rainbow trout oocytes. Kjesbu and Kryvi (1983), reported 5 or 6 in the same material from cod, depending on the orientation of the sectioning, and concluded that the occurrence of lamellae was a function of the orientation of the micrifibrilles of the multilamellar layer and the plane of sectioning, and consequently "not caused by alternating chemical arrangements". In any case, the zona radiata was transversed by numerous pore canals, with two microvilli per canal, one from the oocyte and another from the granulosa cells.

At the end of vitellogenesis, the oocyte nucleus or germinal vesicle migrated to the vicinity of the oocyte membrane. It was difficult to follow this migration as most of the stage 6/7 oocytes were not cut through the nucleus. At some stage of this migration, or most probably when this was over, yolk globules started to coalesce. As mentioned earlier, the coalescence of yolk globules or spheres is characteristic of marine teleost producing pelagic (buoyant) eggs. Mylonas *et al.* (1997) described the coalescence of yolk globules, or "clearing", during final oocyte maturation of striped bass. In this species, coalescence commenced before GVBD, but this followed soon after the start of coalescence. There are reports of proteolysis of yolk proteins in some species, for instance cod (Kjesbu and Kryvi

1989) and *Fundulus heteroclitus* (see Wallace and Selman 1981), associated with hydration. The increase in free amino acid concentration would trigger the influx of water. From the light and TEM study of Atlantic halibut oocytes in late stage 6/7 and translucent oocytes, it is not clear whether the completion of yolk coalescence is previous to the start of GVBD, or even if VTG sequestration is over before clearing starts. Some sections revealed signs of early coalescence when vitellogenesis appeared to still be taking place (plate 3.24 and 3.26). It is, however, likely that vitellogenesis is over by this time, and probably well before yolk coalescence is complete. Thus, light microscopy of translucent oocytes (see plate 3.27) showed a zona radiata with a largely reduced number of microvilli. The increase in size of translucent oocytes with respect to vitellogenic oocytes is attributed to the influx of water.

The combined study of GSI, histology and volume fraction of oocyte stages showed the changes of the female halibut ovaries through development. In immature individuals, mean GSI was 0.5, and the oocytes found in these ovaries were never further developed than the perinucleolar stage (stage 3). Mature, previtellogenic individuals, sampled during early September, showed GSI values of around 2 – 2.5. Stage 4 oocytes were already present in these ovaries. Methven *et al.* (1992) and Norberg (1994) reported that levels of VTG in plasma of female Atlantic halibut from Newfoundland started to increase slowly from July to December. It has been reported before (see Tyler and Sumpter 1996) that plasma levels of VTG increase before the oocytes are ready to start vitellogenin uptake. In any case, the onset of spawning of different stocks of female halibut can differ in months (see for instance Haug 1990; also personal observations), and it is likely that the onset of vitellogenesis could vary in a similar fashion around autumn. In the present study, the first stage 5 oocytes appeared in ovaries sampled in late September, with a GSI value of 2.8. During the following months, GSI increased until prespawning, when individuals had

values around 15 in March. At this point, the volume fraction of stage 6 oocytes was the most important in the ovaries. The maximum GSI value recorded among spawning females was 21.6, and the minimum was 11.8. These are high values for batch spawners (see Tyler and Sumpter 1996), considering that, at the time of sampling the main component of oocyte growth, hydration, was not complete. Batch spawners frequently have lower GSI values than other teleosts. However, the periodic hydration of a batch of eggs prior to release, contributes to much higher "cumulative" GSI values. Over-stimulation of FOM by hormonal treatment, inducing massive hydration of oocytes, can have very negative effects on individual fish, and in fact can cause death, as the volume increase surpasses the physical capacity of the gonad (see Chapter VI, section 6.4.1 in this thesis). In addition to this, vitellogenesis continues during spawning, in clutches of eggs in preparation for being released (see Methven *et al.* 1992; own observations in this thesis). The net result of this strategy is to increase the potential fecundity of the individual.

All stages of this development were easily monitored with the use of ultrasound scanning. There are several examples in the literature of the use of this technique for sexing of different species of fish (coho salmon, Martin *et al.* 1983; Atlantic salmon, Mattson 1991; cod, Karlsen and Holm 1994; Goddard 1995; yellowtail flounder, winter flounder, and Atlantic halibut, Martin-Robichaud *et al.* 1998) and to monitor final maturation (Shields *et al.* 1993). However, the use of ultrasound scanning was never before applied to the extent described in the present work. Immature females were readily identified by the texture and the size of the gonad image. Sexing of young individuals early in development is of major importance in commercial operations of species that, like halibut, present different ages of maturity between the sexes. Male halibut mature earlier in their life history than females, investing energy in milt production at the expense of growth. Also, early selection of potential broodstock and establishment of breeding

populations, provides a better conditioning of the broodstock by tailoring environmental conditions (environmental delay of spawning) or diet (see chapter IV of this thesis).

Another point arising from the study of maturation of the halibut population in section 3.4, was the size at first maturity of the individuals studied. Haug (1990) reported on the differences in size and age at first maturity between wild male and female Atlantic halibut, and between different populations. In halibut captured in Faroese waters, for example, the average (50% level) ages, lengths and weights at maturity were respectively 4.5 years, 55cm and 1.7 kg in males, and 7 years, 110-115 cm and about 18 kg in females. The hatchery produced males in the present study were mature after 5 years, and weighed 1.4 kg, and were 43 cm in length. Females were mature at 6 years, 4.5 kg and 60.8 cm. The fact that these were hatchery produced animals is an important difference with the wild fish studied by Haug, but at the same time the small size of mature females represent an important issue to be addressed by the producers, in terms of energy investment diverted from growth, and of the size of individual broodstock and of broodstock populations.

The use of ultrasound scanning proved to be a valuable tool for the study of final maturation. Sixty hours after stripping, the new batch of eggs could be seen beginning hydration before ovulation. Ovulated eggs could be seen freely floating in the ovarian lumen, and concentrating in the area around the gonopore. Once the egg batch was fully ovulated, eggs were distributed all along the ovarian lumen. This practice is easily applied from the exterior of the tank, thus minimising the stress imposed to the animal. Ultrasound monitoring of ovulation was routinely used at the Machrihanish Environmental Marine Laboratory during the last season of experiments, providing not only a better assessment of the time of ovulation, but also an improved supervision of the remaining gonadal material, post-spawned resorption and of the health status of the production females.

3.7 Conclusions

Atlantic halibut oocytes followed the same general processes described for the oocyte maturation in the majority of teleosts, including primary growth, folliculogenesis, cortical alveoli formation, vitellogenesis, and final maturation. Vitellogenesis started from late September, continued during the spawning season, and accounted for some 13% of the final egg size. Final oocyte maturation included yolk coalescence and hydration, responsible of some 80% of the final egg size, producing a transparent, buoyant egg.

GSI began to increase during autumm, with the commencement of vitellogenesis. High GSI values of around 16 were achieved during spawning, although the periodical final maturation and hydration of several batches of eggs, allowed for the increase of relative fecundity.

Ultrasound scanning was a powerful, non-intrusive tool for the study of maturation, assessment of gonadal development throughout the year, hydration and final maturation of egg batches, and resorption of gonadal material after spawning, with numerous applications in research and hatchery operations.

Chapter IV: Dietary enhancement of spawning performance and egg quality.

4.1. Introduction.

In the previous chapter, the ovarian development and oocyte growth of Atlantic halibut have been described. During vitellogenesis, maternal reserves are mobilised and transported into the oocytes. This provides the energy and the building blocks for the development of the embryo and the yolk sac larvae until the start of exogenous feeding. This means, in a relatively slow developing species like halibut, a period of 252°D during which the individual is dependant for its survival on the maternal nutritional input during vitellogenesis. Thus, the quality of the broodstock nutrition is an important determinant of egg and larval quality.

In this chapter, the influence of maternal nutrition on egg quality of Atlantic halibut is studied. In particular, the influence of the essential fatty acids (EFA) docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA). The replacement of trash fish diets with pelleted diets would benefit the prospective halibut industry, as well as those of other marine fish, as wet diets are variable in composition and carry a risk of introducing pathogens, such as VHS (viral haemorrahagic septicaemia), nodavirus, etc.

Nutrient intake and health status would be more fully controlled using pelleted diets with comparable spawning performance to that obtained from moist-trash fish diets.

The nutritional status of broodstock fish can influence its fecundity. Horwood *et al.* (1989) found that when feeding mature plaice on a deficient ration (initially 0.5% and later 1.8% of wet body weight per day), 39% of the subject fish produced no granular oocytes, whereas they were produced by all fish on a higher ration (2-2.3% of wet body weight per day). Of the fish producing granular oocytes, those on a higher ration had 59% more granular oocytes. The authors proposed that the lack of granular oocytes was due to an early decision not to proceed with gonad development, suggesting a new mechanism for regulation of fecundity in plaice. Similarly, in the Baltic herring individual maturation cycles and timing of spawning can be determined by the feeding conditions prior to spawning (Rajasilta 1992). Cerdá *et al.* (1994) described reduced plasma levels of 17β-oestradiol in captive sea bass fed a ration of 0.45% body weight per day, when compared to fish fed a ration of 1.04%. This had no effect on vitellogenin (VTG) plasma levels. However, vitellogenic oocytes appeared in the ovary of fish fed the lower ration one month later than the fish on the higher ration, and subsequently showed a delay in spawning time.

In vertebrates, lipids are the source of essential materials in cell and tissue membranes, exercise control over metabolism and are used for energy store. In fish, lipids are also the main source of metabolic energy. Dietary lipids play also an important role as the source of essential fatty acids (EFA), and as carriers of certain non-fat nutrients, such as the fat-soluble vitamins A, D and K (see reviews by Watanabe 1982 and Sargent 1995). Lipids can be divided into two categories, namely polar lipids and neutral lipids. Polar lipids play mainly a structural role, and are of major importance as constituents of cellular membranes. They comprise a glycerol backbone to which two fatty acid chains and a functional group are attached (figure 4.1). Polar lipids are sphingomyelin (SM), cardiolipin

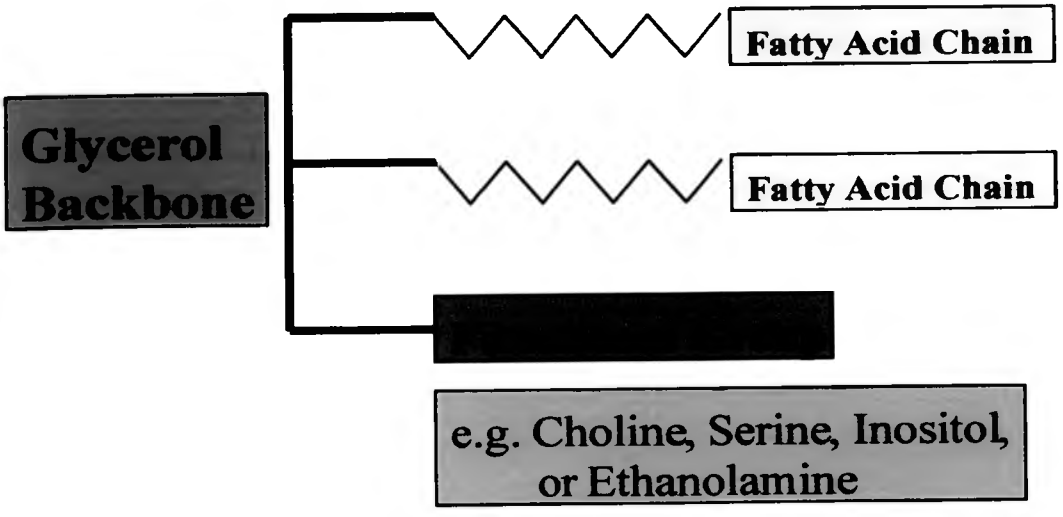


Figure 4.1: Diagrammatic representation of the structure of phospholipids. They comprise a glycerol backbone to which two fatty acid chains and a functional group are attached. Phospholipids are phosphatidylchholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and phophatidylserine (PS). Modified from Bruce (1998).

or phosphatidic acid (CL or PA), phosphatidylchholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and phophatidylserine (PS), in reference to their functional group. Of these, PC, PI, PE and PS are phospholipids, which are a major component of vitellogenin (64% of the lipid content of Atlantic halibut VTG, according to Norberg 1995). Neutral lipids are used mainly for energy storage and consist of a backbone of glycerol to which three fatty acid chains are attached. They are triacylglycerol (TAG), esterol esters (SE) or wax esters (WE).

Fatty acids are chains of carbon molecules with a methyl group and a carboxyl group at the two ends, and varying numbers of double bonds between carbon molecules (figure 4.2). There is a dietary requirement for particular polyunsaturated fatty acids (fatty acids with two or more double bonds or PUFA) in all vertebrate species studied. These are known as essential fatty acids (EFA), and include members of the (n-3) and (n-6) series (see figure 4.2 for an explanation of fatty acid nomenclature). In particular, linolenic acid (18:3 n-3), linoleic acid (18:2 n-6), and their derivatives, 22:6 n-3 docosahexaenoic acid (DHA) and 20:5 n-3 eicosapentaenoic acid (EPA), and 20:4 n-6 arachidonic acid (AA) (Sargent 1995). The requirements of EFA in fish have been reviewed on many occasions (see for instance Sargent *et al.* 1995 and 1999, Izquierdo 1996). Watanabe (1982) presented data from previous work by different authors on rainbow trout, carp, eel and chum salmon showing their requirement for linoleic and linolenic acids as EFA. However, marine species like red sea bream, plaice and yellowtail required adequate levels of DHA and EPA, and freshwater fish also benefited from the addition of this fatty acids in the diet. The inclusion of pollock liver oil in the diet of rainbow trout increased growth, due to its high content of n-3 PUFAs, in particular DHA and EPA, and to the proportion of these two fatty acids in the oil.

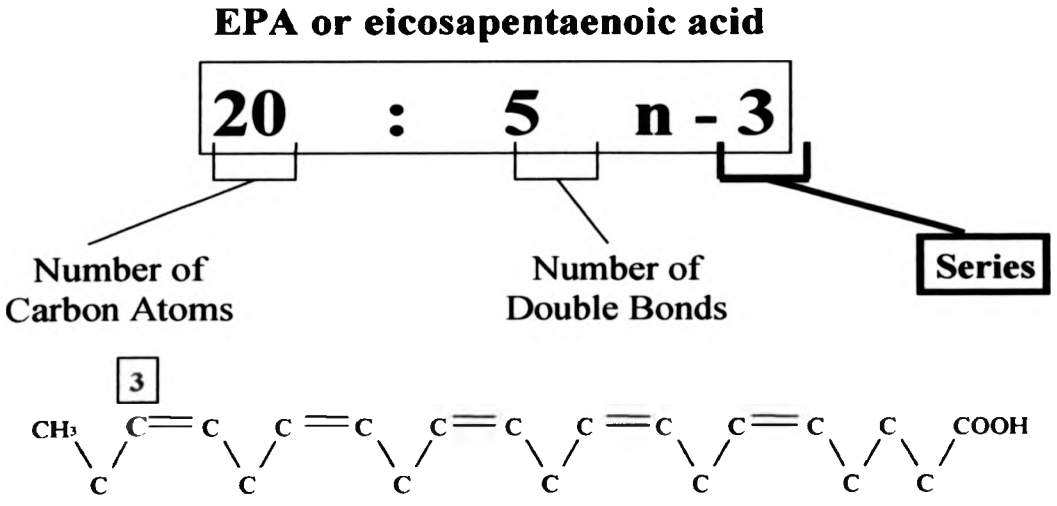


Figure 4.2: Diagrammatic representation of EPA, an essential fatty acid in marine fish. Fatty acids are chains of carbon molecules with a methyl group and a carboxyl group at the two ends, and varying numbers of double bonds between carbon molecules. Modified from Bruce (1998).

Figure 4.3 shows the production pathways for DHA, EPA and AA in freshwater and seawater fish. In freshwater fish, linolenic acid is transformed into EPA and this to DHA. In a similar fashion, linoleic acid is transformed into AA. In marine species, however, there is a deficiency of the Δ5-desaturase enzyme. This enzyme is required for the conversion of α-linolenic acid to EPA, and 18:3 n-6 to AA (Sargent *et al.* 1995). Marine species are unable to convert linolenic and linoleic into DHA/EPA and AA respectively. Thus, in freshwater fish linoleic and linolenic are EFA, whereas in marine species, DHA, EPA and AA are the EFA.

Halibut larvae fed copepods (31% DHA, 7% EPA expressed as percentage of total lipids) had higher survival than larvae fed Artemia enriched with two different products (21% and 23% DHA and 13% and 8% EPA respectively) (Sargent *et al.* 1999). They also had higher rod to cone ratio in retina. Similarly, Arnaiz *et al.* (1993) showed the relation of a "critical period" in turbot larval development with the larval content of DHA. In their experiment, they found that the DHA content in the larvae that did not survive that critical period was lower than in the survivors. EPA and DHA constitute 50% of the fatty acids in phospholipids in fish, occurring in a DHA:EPA proportion of around 2:1 (Sargent 1995). AA is much less abundant and can account for only 1-2% of the fatty acids in phospholipids, except in PI where it can be the major PUFA (Sargent 1995). DHA and EPA are the main PUFAs present in cell membranes in fish. Neural tissues are particularly rich in DHA.

EPA and AA are both precursors of eicosanoids. Eicosanoids are hydroxylated derivatives from C_{20} PUFAs, in particular dihomo γ linoleic acid, AA or EPA. They are short-lived molecules of high biological activity, present in almost all tissues; they act at very low concentrations and for short periods of time in their immediate environment (Vas

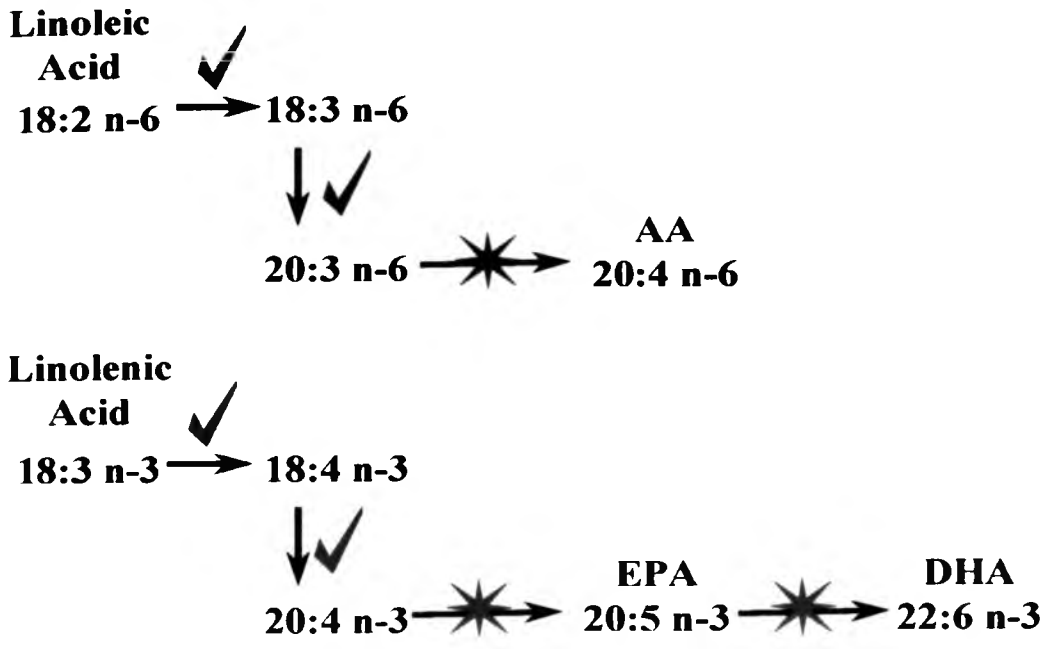


Figure 4.3: Diagrammatic representation of the production pathways for DHA, EPA and AA. In freshwater fish, linolenic acid is transformed into EPA and this to DHA. In a similar fashion, linoleic acid is transformed into AA. In marine species there is a deficiency of the Δ5-desaturase enzyme required for the conversion of α-linolenic acid to EPA, and 18:3 n-6 to AA, at the steps marked with a *. Thus, in freshwater fish linoleic and linolenic are EFA, whereas in marine species, DHA, EPA and AA are the EFA.

Dias 1995). There are over 20 different eicosanoids, which can be divided into prostanoids (PGs), derived from C_{20} PUFAs via cyclo-oxygenase activity, and leukotrienes (LTs) and lipoxins (LXs), via lipo-oxygenase. PGs play an important role in physiological and pathophysiological processes such as the modulation of the release of hypothalamic and pituitary hormones (Lands 1992), contraction of smooth muscle, release of digestive acid in the stomach and blood clotting. Leukotrienes are involved in defence systems (leukocytes and macrophages) (Stanley-Samuelson 1987). Meijer *et al.* (1986) found contrasting effects of fatty acids on the oocyte maturation of several starfish species, with some being induced to mature with small concentrations of AA and EPA and others not being affected. Eicosanoids seem to be involved in the hatching of eggs of barnacle *Balanus balanoides* (Clare *et al.* 1985) and the increase of egg production in the fresh water snail *Helisoma durgi* (Kunigelis and Saleuddin 1986 cited in Stanley-Samuelson 1987).

The functions of the eicosanoids derived from both AA and EPA are similar. However, AA derived eicosanoids are much more bioactive. For instance, eicosanoids derived from (n-3) fatty acids satisfied the requirements for fertilisation, implantation growth, gestation and development, but not parturition, in rats (Leat and Northrop 1981 cited in Lands 1992). These authors found that only (n-6) fatty acids "provided the intensity of eicosanoid formation and function for this process". AA and EPA compete for the same enzymes in the formation of eicosanoids. The supply of free AA from phospholipids is generally regarded as the rate–limiting step for prostaglandin synthesis by tissues (see Norman and Poyser. 2000). Two enzymes involved in AA uptake into, and release from, phospholipids are acyl-CoA:liposophospholipid acyltransferase (ACLAT) and phospholipase A(2) (PLA(2)), respectively. PGF (2α) induces luteolysis in several species including guinea-pigs. The study by Norman and Poyser showed that the continual

production of PGF(2α) by guinea-pig endometrium is not only dependent upon the activity of PLA, for releasing free AA for PGF(2α) synthesis, but also on the incorporation of AA into the phospholipid pool by the activity of ACLAT. Levels of EPA can attenuate the rate of AA eicosanoid formation. EPA eicosanoid production presents a less intense positive feedback of hydroperoxide (first step to the formation of PG), and provides a less vigorous response when bound to specific eicosanoid receptors (in Lands 1992). Alteration of concentration and ratio of (n-6) and (n-3) PUFAs in feeds can influence PG synthesis and metabolism in a number of species (Abayasekara and Wathes 1999), alter macrophage eicosanoid synthesis in rats (Palombo *et al.* 1999), change leucocyte fatty acid composition and plasma eicosanoid concentration in European sea bass (Farndale *et al.* 1999), and modify membrane fatty acid composition and eicosanoid production of Atlantic salmon gill and kidney (Bell *et al.* 1996). Yet, the balance is important. If the formation of eicosanoids is too rapid, for example because of high levels of AA, eicosanoid activity tends to be excessive, which can cause pathophysiology. Thus, Ishizaki *et al.* (1998) found that feeding yellowtail larvae with Artemia containing 4% AA had a negative effect on growth, and Estevez *et al.* (1999) found that increased levels of dietary AA content resulted in poorer pigmentation in turbot.

All this evidence points to the conclusion that not only the total contents of DHA, EPA and AA in the diet are important, but also the relative proportions of these EFAs. Generally in fish the ratio of (n-3)/(n-6) is 3:1, whereas in humans the ratio is 1:5 (Sargent 1995).

During gonadal development in fish, lipids provide two functions: firstly to meet the energy demands of reproduction, and secondly to be deposited in the oocytes in the form of VTG. These lipids are derived from the dietary fatty acids, mobilised from fatty acid reserves or maybe synthesised *de novo* (Wiegand 1996). There is a close correlation

between the fatty acid composition of the broodstock diet and that of the eggs, for instance in rainbow trout (Corraze *et al.* 1991), sea bass and halibut (Bruce *et al.* 1999; Sargent *et al.* 1999). Henderson *et al.* (1984) studied the changes in lipid composition of capelin during gonadal development. Capelin can accumulate 10-20% of their body weight in the form of lipids. In this study, it was determined that over 70% of the lipid content of the muscle was mobilised during ovarian recrudescence. Of this, 38% was deposited in the ovary, and the rest was catabolised to provide metabolic energy for gonadal growth. Similar energy costs are associated with gonadal development in plaice and perch (see Weigand 1996).

Lipoprotein yolk lipids in the eggs are predominantly polar lipids (in particular PC and PE), rich in (n-3) PUFA (mainly DHA). Bruce *et al.* (1993) reported that the most abundant fatty acids in halibut eggs were DHA (26%), palmitic acid (17%) and EPA (12%). There is in fact a selective incorporation of DHA into oocytes (Sargent 1995), and probably also of AA, although the minimal amounts needed make this more difficult to appreciate (Weigand 1996). Also in humans, the placenta selectively extracts AA and DHA from the mother and enriches the foetal circulation (Crawford 2000).

In summary, the manipulation of diets has a direct effect on the parental energy status and on the composition of the eggs being produced. Giving that the growing embryo and the yolk sac larvae depend largely on the parental nutritional input for their development, broodstock nutrition, in particular the EFAs, would be expected to have profound effects on egg and larval quality. Hence, improved control of the quality and quantity of the nutrients delivered to the broodstock would improve the quality of the eggs and larvae being produced.

This chapter describes the substitution of trash-fish based diets with more controllable pelleted diets, and the effects of the manipulation of levels and ratios of DHA, EPA and AA on egg and larval quality of Atlantic halibut.

4.2. Spawning performance and egg quality of Atlantic halibut broodstock fed pelleted diets.

Halibut broodstock in the U.K. have traditionally been fed on trash fish or moist diets. The replacement of moist diets is a high priority for the emergent halibut industry as they are variable in composition and inevitably represent a risk of introducing pathogens, such as VHS (viral haemorrhagic septicaemia), nodavirus and others, which are thought to be transferred via wet fish diets. Therefore, the aims of this experiment were to investigate:

a. The suitability and effectiveness of pelleted diets for halibut broodstock

b. The influence of diet composition on egg quality.

4.2.1. Materials and Methods.

The halibut were maintained at the Institute of Aquaculture's Marine Research Station at Machrihanish on the west coast of Scotland. A total of 14 female and 6 male wild caught (Icelandic) adult halibut were partitioned between three tanks (table 4.1), and maintained under ambient photoperiod in tanks of 4m in diameter ($12m^3$ volume). The water supply was finely filtered (65 μm) and, from October 1996 (after the first spawning season monitored), temperature controlled to match the temperature regimes used successfully by the SFIA Marine Farming Unit at Ardtoe (figure 4.4).

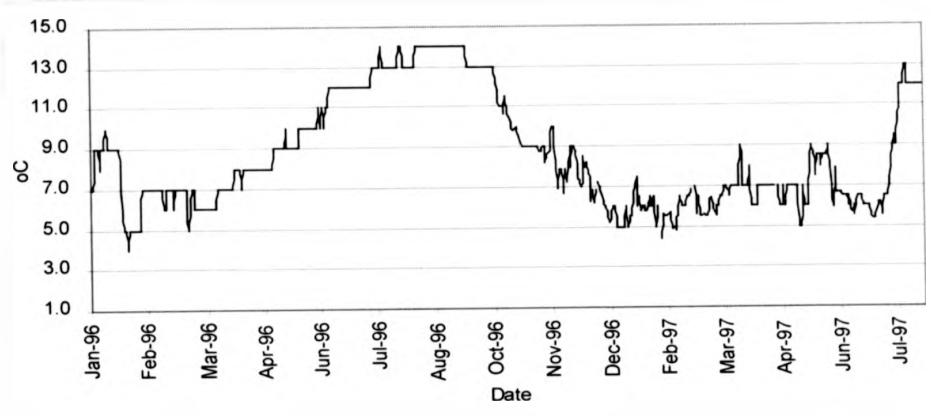


Figure 4.4: Temperature profile of halibut broodstock during this experiment

During the spawning season of 1996, the egg quality and spawning performance of the three groups of halibut broodstock was monitored, and this provided a baseline for future work. After spawning, in May, the 3 groups of halibut broodstock were placed under the following dietary regimes:

Control: moist diet containing wet fish components.

Krill: pellet diet using a mixture of Antarctic krill meal and Northern hemisphere fish meal plus Northern hemisphere fish oil.

High DHA: pellet diet using a Northern hemisphere fish meal plus tuna orbital oil (Ropufa 30, Roche).

The pelleted diets (Krill and High DHA) were produced at the EWOS Technology Centre, EWOS Ltd., Livingstone, Scotland, UK (see Chapter II, section 2.11 for a description of the fabrication of the feeds). The Control diet consisted of a moist sausage, containing wet fish components (see table 4.2 for composition), which had proven to be the most successful broodstock diet to date and used by a number of UK, Canadian, Icelandic and Norwegian halibut concerns. The biochemical analysis of the different diets is

presented in table 4.3. Pelleted and moist diets were presented to the fish in food grade sausage casings (Devro Fresh, U.K.), in portions of suitable size (around 10-15 cm in length and 5 cm diameter).

Table 4.1: weights and length of the fish at the start of the experiment (June 1996). K fish were offered the Krill diet, C fish were fed on the Control moist diet and DHA fish were fed on the High DHA diet.

Control

Code	Sex	Weight (kg)	Length (cm)
C 1	M	9.2	77
C 2	M	9.6	77
C 3	F	16.4	91
C 4	F	15.8	94
C 5	F	15.8	95
C 6	F	27.2	113

Krill

Code	Sex	Weight (kg)	Length (cm)
K 1	F	12.6	81
K 2	F	19.2	103
K 3	F	19.4	96
K 4	F	16.8	91
K 5	M	7.1	69
K 6	M	8.6	66
K 7	F	19.2	97

High DHA

Code	Sex	Weight (kg)	Length
DHA 1	F	22.7	96
DHA 2	M	8.2	72
DHA 3	F	19.2	88
DHA 4	F	19.2	93
DHA 5	F	16.8	96
DHA 6	F	19.8	103
DHA 7	M	7.8	72

Table 4.2: Composition of the Control moist diet.

Components	% weight
Herring (fresh)	34
Squid (fresh)	17
Cod roe (fresh)	17
Herring fish meal (LT94, Norsmeal U.K.)	28
Fish oil (Seamaid 30% PUFA, United Fish Industry, U.K.)	1.7
Standard salmon vitamin premix (Trouw U.K. Ltd.)	1.1
Binder: Guar Gum (Cesalpina U.K. Ltd.)	0.6
Response Booster Supplement (Vit C enhancer, Trouw U.K. Ltd.)	0.6

Table 4.3: Proximate and fatty acid composition of the different diets offered to halibut broodstock during the experiment.

Composition	Control	Krill	High DHA
Proximate (%dry weight)			
Protein	70.1	50.5	51.4
Lipid	10.3	23	20.3
NFE*	7.7	14.7	17
Ash	13.2	9.5	10.2
Fibre	0.4	2.3	1.2
Fatty Acids (% Fatty Acids)			
18:2 n-6	1.4	4.3	4.9
20:4 n-6	1.0	0. 5	1.4
20:5 n-3	9.9	7.4	5.6
22:6 n-3	16.4	7.9	19.5
Saturates	25.3	21.9	28.3
Monoenes	34.2	47.9	30.0
Dienes	2.0	4.9	5.4
PUFA**	35.1	26.4	37.4
n-3	32.0	21.0	29.3
n-6	2.8	5.0	7.8
n-9	19.2	24.6	17.9
n-3/n-6	11.5	4.2	3.8
EPA/AA	9.9	14.8	4
DHA/EPA	1.7	1.07	3.5

* Nitrogen Free Extract

** Polyunsaturated fatty acids

The broodstock were fed these diets for a period of 8 months previous to spawning, coinciding with the recovery of appetite at the end of the previous spawning season. The fish were fed to satiation 3 times a week. At each feeding period, the total weight of diet consumed by each individual was recorded.

During the spawning seasons of 1996 and 1997, batches of eggs were collected by stripping and both eggs and milt were transferred in the dark to an egg handling room. All the subsequent egg handling was carried out under red light, to minimise damage to the eggs by ambient daylight or white light. First, the quality of the collected milt was assessed by checking its motility under a microscope. The milt was then diluted (1ml per male/l) in 5 μm filtered, uv sterilised seawater and mixed with the eggs (1:1 v/v) (see Chapter II section 2.2.4.1 for details of fertilisation procedures). After standing for 20 minutes inside an incubator set at $6^{O}C$, triplicate sub-samples of 50 eggs were stored in chloroform/methanol (2:1 v/v) for lipid analysis (see Chapter II section 2.10 for details on lipid analysis). The remaining eggs were then rinsed in uv sterilised, 5 μm filtered seawater.

During spawning'96, the eggs were then transferred to a small cylindrical container (approx. 10 l) with a conical base and tap, containing 5 l of filtered, uv sterilised seawater (36ppt, $6^{O}C$). The eggs were allowed to stand in complete darkness for a further 20 minutes, so that the initial dropout of eggs could be measured and removed. The floating eggs remained in the incubator ($6^{O}C$) for a further 16 hours ($4^{o}D$), at which time egg quality assessment was carried out by analysis of the blastomere morphology as described in Chapter II section 2.3.3.

During the second spawning season (1997), a slightly different procedure was followed. Eggs were transferred to 80l cylindro-conical tanks, rather than the smaller 10 l ones, with similar water quality. The assessment and incubation were carried out on small

randomly sampled fractions of each egg batch stocked into 96 well microtitre plates: one egg per well each of which contained 200µl of u.v. sterilised, 5 µm filtered seawater as described in Chapter II section 2.3.3. Egg quality was assessed and the microtitre plate sealed, placed in an incubator (6^{O}C), and left undisturbed for 75^{o}D, when the eggs were checked for their hatching performance.

Egg quality, total fertilisation rates, hatching rates and relative fecundities, as well as feed intake from the different diets were evaluated during the spawning seasons of 1996 and 1997. Only batches of eggs with total fertilisation values higher than 10% were assessed, in order to avoid the problems of over-ripening due to late collection of eggs. EFA concentrations in the eggs from the different diets were compared. Percentage values were arcsine transformed and data analysed by ANOVA.

4.2.2. Results.

a. Spawning performance.

The spawning performance of the broodstock during the 1996 season was irregular, and two of the females only produced one batch of eggs each. The quality of milt throughout the spawning season was judged satisfactory, as shown by its good motility. However, all fertilisation rates were poor (mean value 14%). The egg quality was never higher than 6 of a maximum possible score of 20. No hatching took place in the microtitre plates.

From the total number of 14 females, only seven spawned during 1996 compared to eleven in 1997 (4 from both the Control and Krill treatments, and 3 from the High DHA treatment). From the females considered non-spawners in 1997, one of them (on the High DHA treatment) died due to unknown reasons during the spawning season. The autopsy revealed fully matured gonads with hydrated oocytes ready to be spawned. This female did

not spawn in 1996. The relative fecundity of the spawning females did also improve from 4873 ±1256.6 eggs/kg of female (mean±sem) in 1996 to 10637 ±1427.2 eggs/kg of female in 1997, increasing total egg production more than threefold in 1997 with respect to 1996. The average fertilisation rate during spawning in 1997 also improved from 1996. Egg quality scores, assessed at 4°D also showed a major improvement, from an average value of 3.4/20 in 1996 to 15/20 in 1997. The general improvement in spawning performance should be attributed to the management of the temperature profile starting in October 1996.

Figure 4.5 shows the feeding behaviour of the fish. The three diets were equally accepted, although the feeding rate of the fish on the Control diet was generally higher. Feed consumption peaked during the autumn and decreased during the spawning season.

Table 4.4 shows the values of relative fecundity (number of eggs per kilogram of female), fertilisation rates and egg quality (maximum possible score being 20) for the three treatments. Figures 4.6, 7 and 8 compare the relative fecundity, egg quality score and fertilisation rates from each dietary treatment. No significant differences were found between treatments on any of these parameters. Unfortunately, no hatching took place in the microtitre plates, presumably for the lack of temperature control in the room dedicated for the egg handling operations.

Table 4.4: Spawning parameters (mean ± sem) of Atlantic halibut fed Control, Krill and High DHA.

	Relative fecundity	Fertilisation rate	Blastomere quality
Control	11782 (± 1873)	41 (± 4.0)	15.4 (± 0.5)
Krill	9557 (± 3462)	40 (± 6.4)	14.5 (± 0.3)
High DHA	10551 (± 2166)	40 (± 9.1)	15.6 (± 0.7)

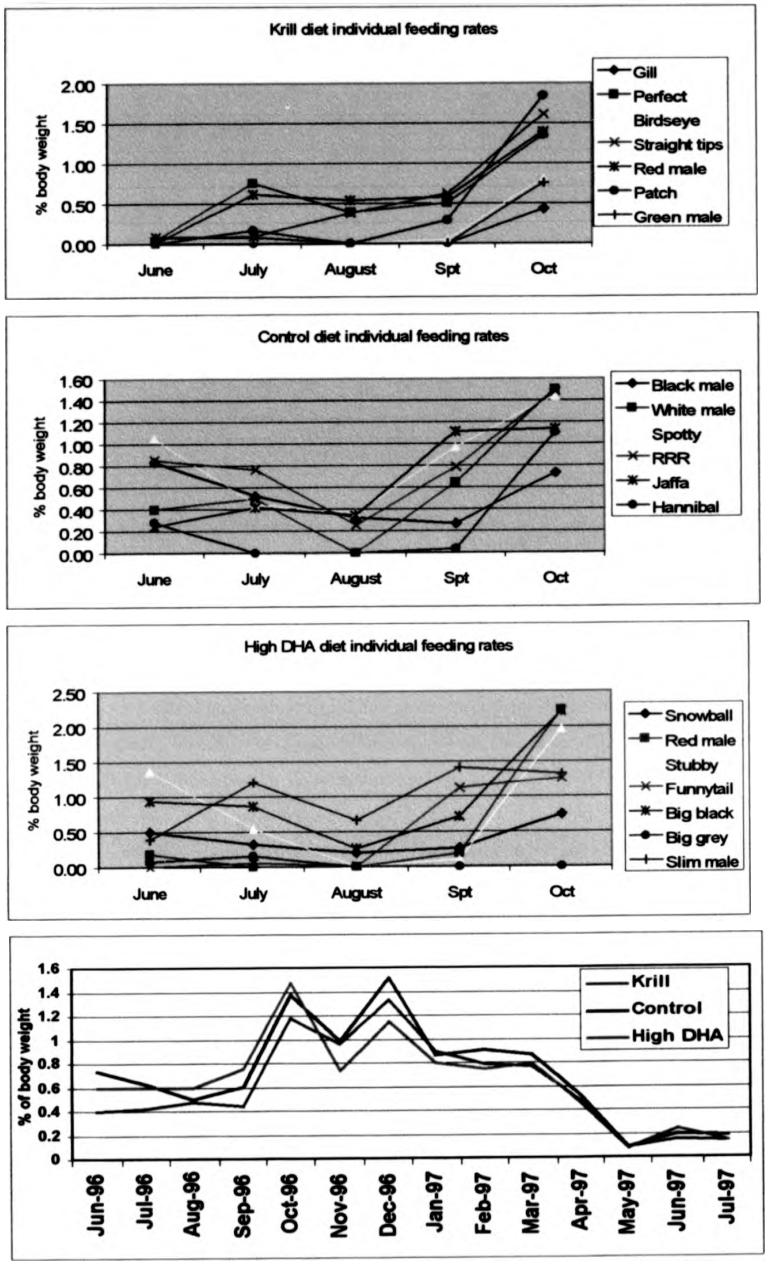


Figure 4.5: Feed consumption per week of Atlantic halibut fed three times a week on the Control, Krill and High DHA diets. The top three graphs show the individual consumption per fish until the end of October 1996 (legends are fish names; males are indicated). Bottom graph shows average consumption per tank. Feeding rate peaked during the autumn and winter, and decreased during spawning (March – May).

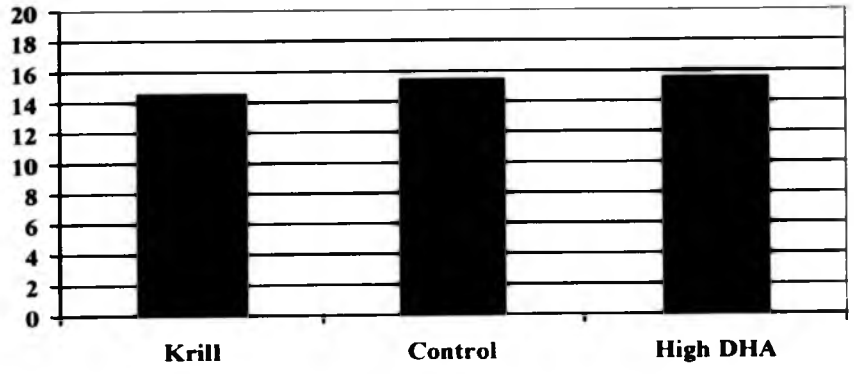


Figure 4.6: Mean egg quality scores (s.e.m. bars were too small to be shown) from Atlantic halibut broodstock fed on the Control, Krill and High DHA diets for 8 months prior to spawning

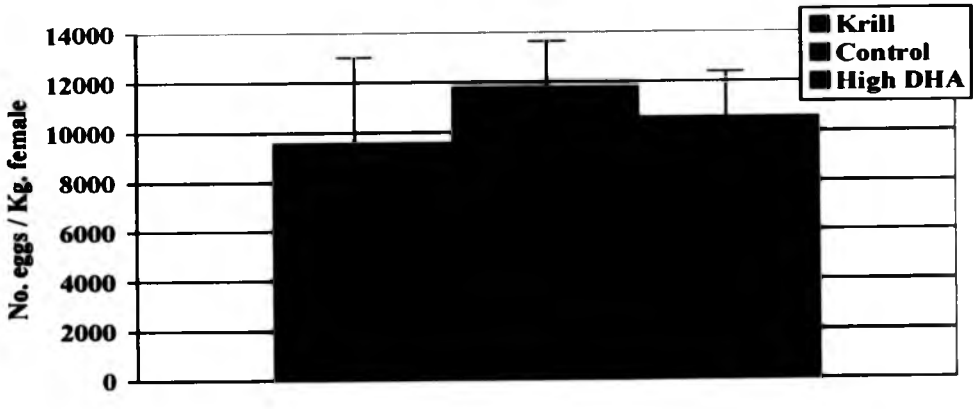


Figure 4.7: Relative fecundities (no. of eggs/kg of female) of female Atlantic halibut broodstock fed on the Control, Krill and High DHA diets for 8 months prior to spawning.

Figure 4.8: Total fertilisation (mean ± sem) of batches of eggs from Atlantic halibut broodstock fed on the Control, Krill and High DHA diets.

b. Egg lipid analysis.

Figures 4.9 and 10 show the percentages of EFA of eggs from broodstock on the 3 different diets, and the ratios DHA:EPA and EPA:AA. There were no significant differences in the egg content of DHA between treatments, which were around 30%. EPA content was significantly lower ($p=0.009$) in eggs from fish on the High DHA diet (11%), when compared to those from the ones on the Control diet (15%), but not with those on the Krill diet (14%). Content of AA in eggs from the fish on the High DHA diet (3%) was significantly higher ($p=0.02$) than the content in eggs from those on the Krill diet (1.6%), but not from the AA content in the eggs from Control fish (2%). Ratios of DHA:EPA were significantly higher ($p=0.01$) in eggs from fish on the High DHA diet (2.9) than in those from fish fed the Control (2.0) and Krill (2.0) diets. Ratios of EPA:AA in eggs from fish on the High DHA diet (3.8) were significantly lower than those in eggs from those on the Control (7.2, $p<0.001$) and Krill (8.9, $p<0.0001$)) diets.

4.3. Spawning performance and egg quality of Atlantic halibut broodstock fed pelleted diets with different levels of arachidonic acid.

The general objectives of this study were defined as follows:

a. To investigate further the suitability and effectiveness of pelleted diets for halibut broodstock.

b. To investigate the effects of enhanced levels of arachidonic acid in the broodstock diets on egg and larval quality.

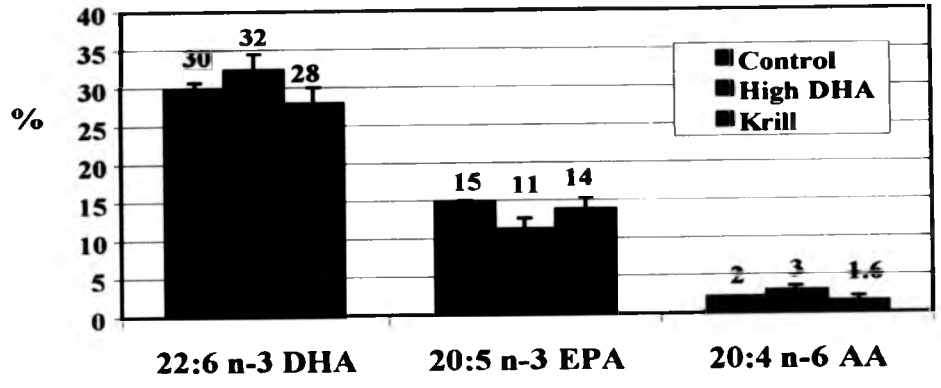


Figure 4.9: Percentage of essential fatty acids (mean ± sem) in lipids of eggs from Atlantic halibut fed on the Control, Krill and High DHA diets. Figures above the bars are bar's values.

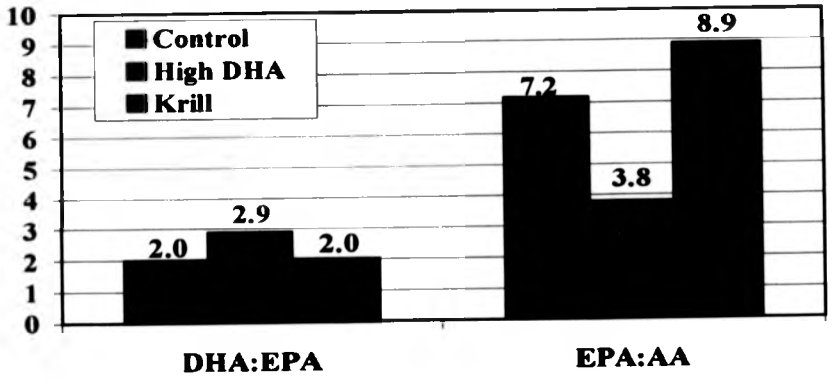


Figure 4.10: Mean EFA ratios in eggs from Atlantic halibut fed on the Control, Krill and High DHA diets. Figures above the bars are bar's values.

4.3.1. Materials and Methods.

A mixed population of hatchery produced (91 year class) and wild caught (Icelandic) Atlantic halibut broodfish (table 4.5) were maintained in two 4m diameter, $12m^3$ tanks under ambient photoperiod at the Machrihanish Marine Laboratory in Scotland, UK. The water supply was finely filtered (65 μm) and the water temperature in the tanks controlled to provide an adequate temperature profile (figure 4.11). Halibut broodstock were fed on two experimental diets (table 4.6) from October 1997:

Standard diet: pelleted diet made of good quality Herring fish meal and oil.

Enhanced AA: Standard diet enhanced with AA rich oil.

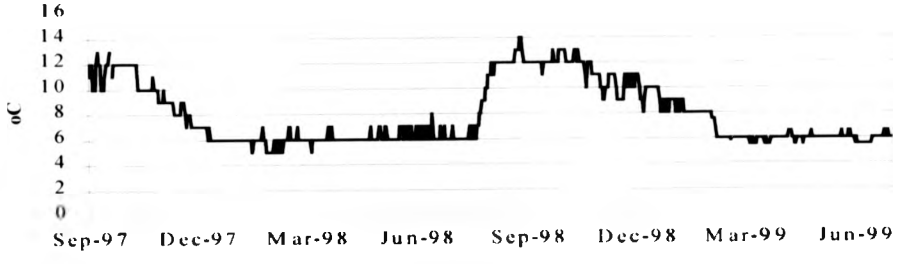


Figure 4.11: Seawater temperature in the tanks during the experiment.

Table 4.5: Fish populations in the experimental tanks. In November 1997, a 14 kg. wild female died in tank 2.

Diet	No. Fish (biomass)	No. females	No. of wild females	No. of farmed females
Standard (tank 1)	16 (134.6kg)	8	5	3
Enhanced AA (tank 2)	16 (120 kg)	6	4	2

Table 4.6. Proximate analysis (% of weight) and EFA composition (% of fatty acids) of Standard and Enhanced AA broodstock diets. The content of AA and the ratios of AA:EPA and n-3:n-6 were significantly different between the 2 diets.

	Standard	Enhanced AA
Lipid	16.4	16.8
Protein	61.7	61.1
Ash	10.9	11.0
Fibre	0.4	0.7
Moisture	6.6	6.3
AA	0.4	1.8
EPA	8.6	8.3
DHA	11.0	10.8
n-3:n-6	6.87	4.31
EPA:AA	21.5	4.61
DHA:EPA	1.28	1.30

The fish were fed on the 2 different diets for two consecutive spawning seasons. During both spawning seasons, egg batches were collected by stripping. Triplicate sub-samples were fertilised following the experimental procedure described in Chapter II section 2.2.4.2. Fertilisation and egg quality were assessed at 16 h post fertilisation in triplicate samples of eggs. At the same time, triplicate samples of 20 eggs per batch were stored in chloroform-methanol (2:1, v:v) for lipid analysis. Every one of these operations took place under red subdued light and, during the second year of the experiment (1999), in a temperature-controlled room at 4-6°C. Batch volume, relative fecundities, fertilisation and hatching rates from each treatment were compared. Only batches of eggs with total fertilisation values higher than 10% were assessed, to minimise the effect of over-ripening due to late collection of eggs. Fertilisation rates, quality scores and hatch rates from the various egg batches from the different females in each treatment were pooled for statistical analysis. The performance of some batches of eggs were assessed until the end of the yolk sac phase, with the collaboration of a commercial hatchery (Otter Ferry Seafish, UK). EFA

concentrations in the eggs from the different diets were compared. Percentage values were arcsine transformed and data analysed by ANOVA.

4.3.2. Results.

a. Spawning performance.

Figure 4.12 shows the spawning dates of the wild fish in tank 1 and 2 during 1997 and the dates of spawning during 1998 of the wild and the hatchery produced fish. Wild females 7 and 4 in tank 1, and 5 and 4 in tank 2 had been first and second spawners respectively in their tanks in previous years. During spawning '98 however, the spawning of the hatchery produced fish overlapped with the recorded spawning dates of these fish in previous seasons. Females Wild/7 and 4 in tank 1, and Wild/4 in tank 2 suffered a delay in their spawning with respect to previous seasons, with erratic spawning intervals and poor rithmithity. In the case of Wild/7 in tank 1, this caused the female to become egg bound, and the release of the gonadal material was induced by injection of antibiotics (Amoxicilin 0.1 ml/kg of female intramuscular). Female Wild/2 in tank 1, and Wild/6 in tank 2 (historically the last spawner of the season), did not spawn in 1998, although ultrasonographic inspection revealed the presence of pre-spawning material in the gonad. The rest of the females resumed normal development, although the timing of the batch production was difficult to follow. This largely affected the results of spawning '98.

Figure 4.13 shows the feeding response of the fish. Both diets were readily accepted. Feeding peaked during the autumn and decreased during the spawning seasons. Figures 4.14 and 4.15 show the results of fecundity and fertilisation rates for the two treatments during the first season of spawning (1998). Total Fertilisation rates of eggs from the fish on the Enhanced AA diet (41%) was significantly higher ($p = 0.009$) than those of eggs from the fish on the Standard diet (14%). There was no statistical difference between treatment

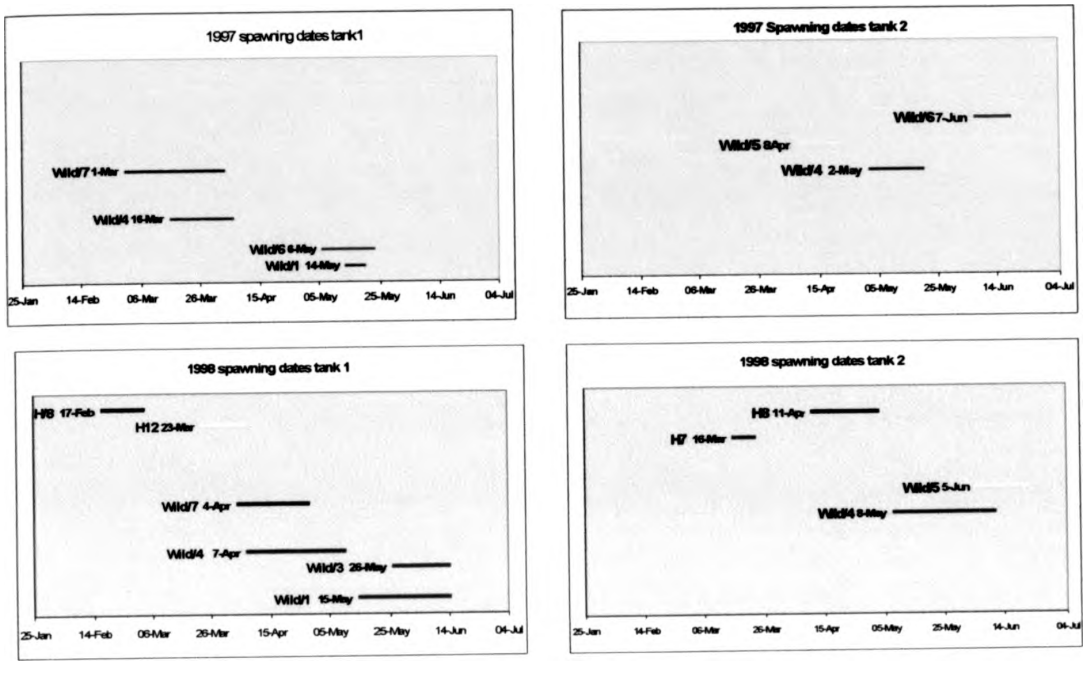


Figure 4.12: Spawning period of the females in tanks 1 and 2, corresponding to Standard and Enhanced AA diets respectively, over two consecutive seasons (1997, previous to the start of the experiment – top graphs - and 1998 – bottom graphs - the first spawning season of the experiment). The graphs show the spawning period (bars), adjacent to the identification of the individual fish (origin and number code), followed by the date of its first egg release. During the second season (bottom graphs), hatchery produced fish (H) were introduced in the tanks where wild caught broodstock (Wild) had been established for several years. The spawning of the hatchery produced fish overlapped with the recorded spawning dates of the wild fish in previous seasons. Females Wild/7 and 4 in tank 1, and Wild/4 in tank 2 suffered a delay in their spawning with respect to previous seasons. Female Wild/2 in tank 1, and Wild/6 in tank 2 (historically the last spawner of the season), did not spawn in 1998.

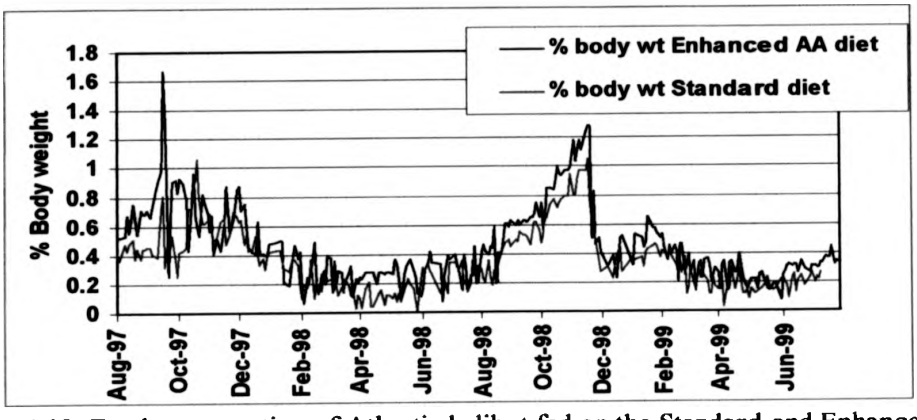


Figure 4.13: Feed consumption of Atlantic halibut fed on the Standard and Enhanced AA diets. Feeding rate peaked during the autumn, and decreased during spawning (March – June).

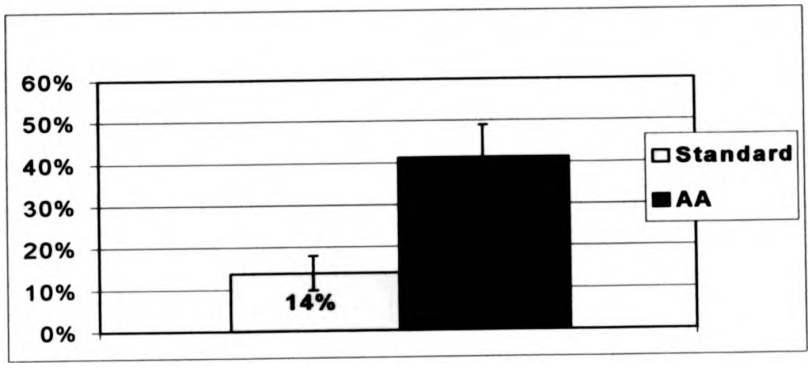


Figure 4.14: Total fertilisation rates (mean ±sem) of egg batches from Atlantic halibut fed on the Standard and Enhanced AA diets during the first spawning season of the experiment.

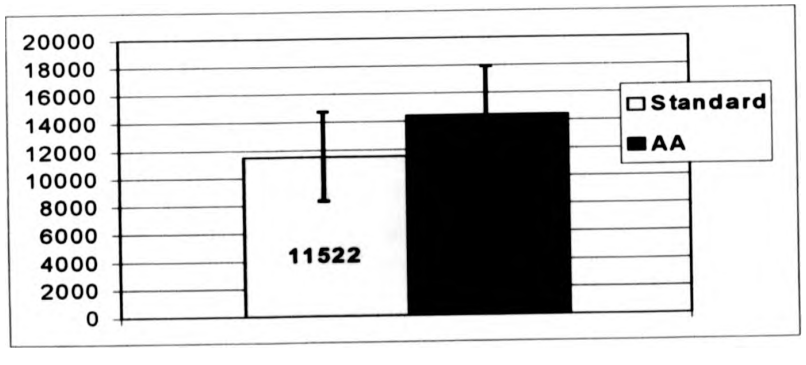


Figure 4.15: Relative fecundity (mean ±sem number of eggs/kg of fish) of female Atlantic halibut fed on the Standard and Enhanced AA diets during the first spawning season of the experiment.

in relative fecundity. Hatch rates were poor for both treatments possibly because of the lack of temperature control of the egg handling room. Mean values from the Enhanced AA diet (13.5%) were significantly higher ($p<0.05$) than those from the Standard diet (5.5%).

Six and four females in the Standard and Enhanced AA groups respectively released eggs during the second spawning season (1999). Figures 4.16, 17, 18 and 19 show the spawning performance (mean ± sem) of the two experimental groups during spawning '99. Females on the Enhanced AA diet exhibited a significantly better performance than those on the Standard diet. There was no significant difference in relative fecundity between treatments ($p = 0.072$). Fertilisation rates from fish fed on the Enhanced AA diet (59%) were significantly higher ($p = 0.0015$) than those of fish on the Standard diet (31%). Egg quality scores were significantly higher ($p = 0.0032$) in the eggs from the fish on the Enhanced AA diet (14) than in those from fish fed on the Standard diet (13). Hatch rates from the fish on the Enhanced AA diet (51%) were significantly higher ($p = 0.02$) than those from fish on the Standard diet (28%).

Eleven batches (10.5 l. of eggs) from the best performing females of each treatment were transferred to a commercial hatchery (Otter Ferry Seafish, U.K.) for further development and quality assessment. Survivals for the batches transported from hatching to the end of the yolk sac phase were 23% and 27%, and to the end of first feeding were 1% and 6% for the larvae derived from fish fed on the Standard and Enhanced AA respectively. These differences were not statistically significant. However, the survivals during the first feeding phase were significantly higher ($p = 0.0026$) for the larvae from fish on the Enhanced AA diet (22.9%) than for those from fish on the Standard diet (5.5%). Nevertheless, these results should be taken cautiously, as the production priorities of the commercial unit could have influenced the hatchery protocols applied to the different batches of larvae.

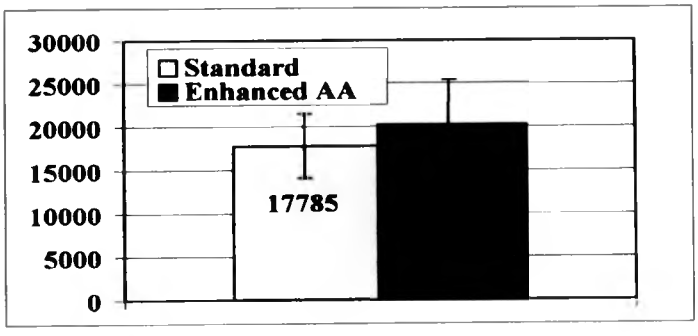


Figure 4.16: Relative fecundity (mean ± sem; no. of eggs/kg of female) from Atlantic halibut fed on the Standard and Enhanced AA diets, during the second season of the experiment.

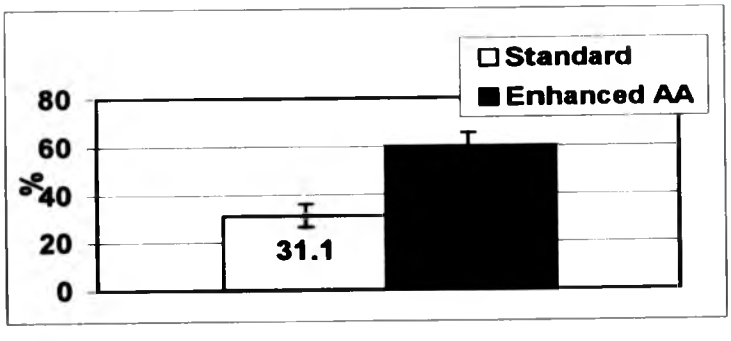


Figure 4.17: Total fertilisation rates (mean ± sem) of batches of eggs from Atlantic halibut fed on the Standard and Enhanced AA diets, during the second season of the experiment.

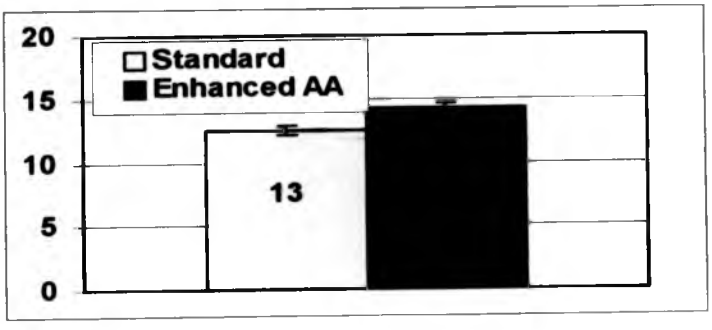


Figure 4.18: Egg quality scores (mean ± sem) from Atlantic halibut on the Standard and Enhanced AA diets, during the second season of the experiment.

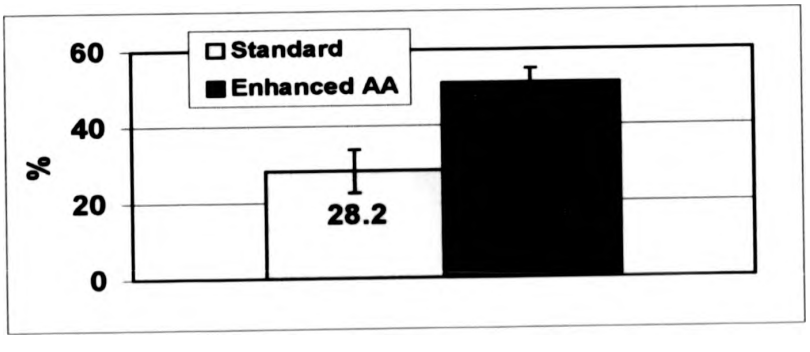


Figure 4.19: Hatching rates (mean ± sem) from Atlantic halibut fed on the Standard and Enhanced AA diets during the second season of the experiment.

b. Egg lipid analysis.

Figures 4.20 and 21 show the content of DHA, EPA and AA in halibut eggs from the two consecutive spawning seasons included in the trial. The levels of DHA sequestered by the eggs remained between 28-31%, with no significant difference between treatments. There was, however, a significant reduction of DHA content in both treatments during the second year of the experiment ($p<0.01$). By contrast, AA concentrations were consistently and significantly higher ($p < 0.001$) in the eggs from fish on the Enhanced AA diet. There was an apparent increase in the concentration of AA in the eggs from broodfish on the Enhanced AA diet from the first year of the experiment to the second, although it was not statistically significant. Ratios of DHA:EPA were not significantly different between diets. Ratios of EPA:AA were significantly lower ($p<0.001$) in eggs from fish on the Enhanced AA diet.

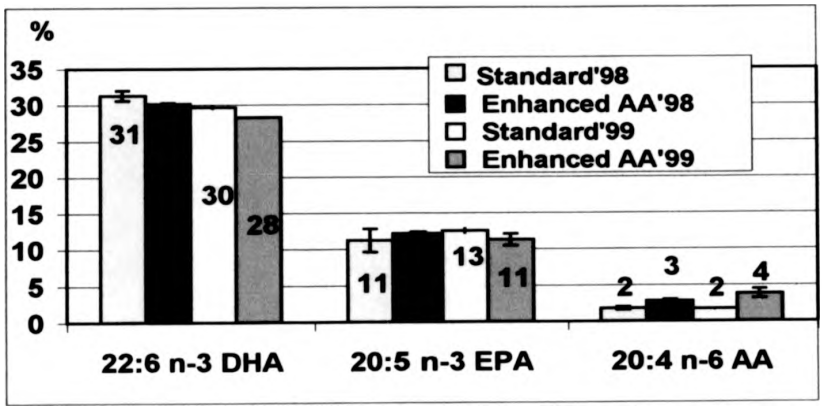


Figure 4.20: Percentage (mean ± sem) of EFA in the lipid fraction of eggs from Atlantic halibut fed on the Standard and Enhanced AA diets over two consecutive spawning seasons (1998 and 1999). In histograms without apparent error bars, the s.e.m. were too small to be shown.

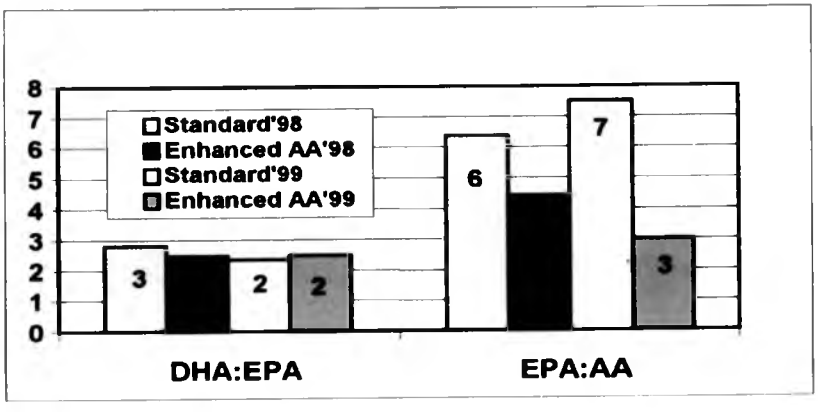


Figure 4.21: Ratios of EFA in the lipid fraction of eggs from Atlantic halibut fed on the Standard and Enhanced AA diets over two consecutive spawning seasons (1998 and 1999).

4.4. Discussion.

The experiments described above show how Atlantic halibut broodstock can be fed on pelleted diets with comparable results regarding spawning performance and egg quality to those obtained from broodstock fed trash-fish based moist diets. They also provide valuable information on the selective delivery of EFA into the eggs, and the consequences of this on egg quality.

The improvement in spawning performance between the spawning seasons in 1996 and 1997 is explained by the introduction of temperature control. Brown *et al.* (1995) reported improved egg viability and fecundity in Atlantic halibut receiving 6°C water during spawning. Personal observations confirm reductions in fertilisation rates with temperatures above 7°C, these being significant at temperatures above 9°C. Water temperature in the broodstock tanks during the spawning season of 1996 ranged between 8°C and 11°C, whereas temperatures during the 1997 spawning season were maintained below 6°C.

Navas *et al.* (1993) reported poor egg quality and spawning performance in sea bass fed artificial diets, with these effects worsening over a two-year period. These authors found no difference in spawning quality with varying lipid levels in the broodstock diets, and suggested that nutritional deficiencies in the artificial diets were the cause of the impaired performance. In contrast, Ramos *et al.* (1993) obtained improved fertilisation and hatching rates in sea bass fed artificial diets produced with fish oil (high in EFA), when compared to those from fish fed artificial diets containing maize oil (low in EFA). The spawning performance of the halibut fed the two pelleted diets in the first experiment was

comparable to those fed the trash fish based moist diet. No significant differences were found in relative fecundity, fertilisation rates or egg quality scores.

The concentrations of EFA in the diets offered to the broodstock were very closely related to their concentrations in the eggs produced. Similar results were reported for sea bass by Bruce *et al.* (1999) and for the rainbow trout by Corraze *et al.* (1991). Although the Krill, and High DHA diets had significantly different DHA contents, there was no significant difference in the DHA concentration in the eggs. There appears to be a positive selective accumulation of DHA to maintain certain levels in the eggs, irrespective of the levels in the diet source. Similar results were reported in the second experiment, where much lower DHA content in the diets produced concentrations in the eggs similar to the ones from the High DHA diet used in the first experiment. In humans, the placenta extracts AA and DHA from the mother into the foetal circulation, and an inadequate supply of AA and DHA during the period of high demand from rapid vascular and brain growth could lead to fragility, leakage and membrane breakdown (Crawford 2000). Henderson and Altamar (1989) found that DHA and EPA were incorporated into the eggs of herring at higher levels than those present in the carcass of the broodfish. Czesny and Dabrowski (1998) reported a lower concentration of lipids in eggs from domesticated walleye salmon than in those from wild stocks. However, concentrations of DHA were the same in eggs from both populations. In the present work, it is possible that the lower levels of DHA in the Standard and Enhanced AA diets were satisfactory. There was, nevertheless, a significant decline in the DHA content of the eggs during the second year of the experiment. This suggests that there was a cumulative effect over time when the nutritional levels of DHA for transfer to the eggs were not met. There is a necessity to provide consistent levels of DHA in the broodstock diet in order to maintain the demands of the broodfish physiology and the egg formation.

The ratios of DHA:EPA in eggs from fish on the Control, Krill and High DHA diets were also linked to those in the diets. However, there was a trend towards an increase in these ratios in the eggs from fish on the Krill and Control diets when compared to the ratios in the diets. By contrast, this trend was the opposite in eggs from the High DHA diet, which in fact had a lower DHA:EPA ratio than that in the diet (2.9 in eggs as compared to 3.5 in diet), although significantly higher than the ratio in eggs from fish fed on the Control and Krill diets. Bruce *et al.* (1993) found ratios of DHA:EPA of around 2.2 in eggs of Atlantic halibut fed trash fish based diets. This corresponds to similar values in eggs from the Control diet in the first experiment. Ratios of 2:1 DHA to EPA are found in phospholipids of eggs of most species studied (Sargent 1995). This suggests that the ratio of DHA:EPA in the High DHA diet was probably higher than necessary. However, there are reports of higher ratios in species like the trout cod (5.4:1) and Murray cod (7.3:1) (Gunasekera *et al.* 1999).

EPA:AA ratios in the eggs were reduced from those in the diets in the Control and Krill groups. By contrast, the ratio in the eggs from fish fed on the High DHA diet was not significantly different from that in the diets, and was significantly lower than the ratio in the eggs from the other diets. At the same time, concentration of AA in eggs from fish fed on the High DHA diet was significantly higher than in those from fish on the Krill diet. There are reports of positive selective incorporation of AA into eggs of fish (Thrush *et al.* 1993; Bell *et al.* 1997; Sargent *et al.* 1999; Bruce *et al.* 1999) and embryos of mammals (Crawford 2000). The relationship between AA and EPA with eicosanoid production has recently focused attention on the importance of the ratio of EPA:AA in addition to their absolute contents (Sargent *et al.* 1999). The results of the second experiment provide support for this hypothesis. By contrast, AA had been related in the past with stress and impaired performance of fish larvae. Estevez *et al.* (1999) referred to dietary induced stress

and malpigmentation of turbot larvae fed rotifers and Artemia enriched to levels of 15% AA. Similarly, Ishizaki *et al.* (1998) reported that levels of 4% AA in Artemia had negative effects on growth of yellowtail larvae. By contrast, dietary fatty acids of the n-3 family reduce ovarian and endometrial synthesis of PG F-2 alpha, decrease ovulation rate in rats and delayed parturition in sheep and humans (see Mattos *et al.* 2000). According to this authors, PUFA such as linoleic, linolenic, EPA and DHA may inhibit PGF-2alpha synthesis through mechanisms such as decreased availability of its precursor AA, an increased competition by these fatty acids with AA for binding to prostaglandin H synthetase, and inhibition of prostaglandin synthetase synthesis activity. Recent studies on early metabolism of Atlantic halibut embryos showed that phospholipase A2 activity, which regulates membrane lipid modification, was undetectable at fertilisation but increased to 230 pmol/mg/h in 10 day old embryos. Phospholipase A2 levels continued to increase to 120% at hatching (Evans *et al.* 1998). The authors suggest that AA removed from PE could serve as precursor for biosynthesis of PG_2 which can play an important role in the development of the embryo. At the same time, as commented in the introduction of this chapter, there is an involvement of eicosanoids in several processes of reproduction, including ovulation. This suggests different requirements for AA during ovarian and embryonic development, from that in first feeding larvae. There was a marked positive effect on egg quality scores, fertilisation and hatching rates of increasing levels of AA in the eggs from fish fed on the Enhanced AA diet. There was also a trend towards increasing levels of AA in the eggs of the fish on the Enhanced AA diet over the two years of the experiment. This, together with the behaviour of the AA and EPA levels in eggs and diets explained above, suggests that a ratio of 3-4:1 would be an adequate EPA:AA ratio for Atlantic halibut broodstock diets.

The significant improvement in survival observed during first feeding in the larvae from the Enhanced AA diet is to be viewed with caution. However, if the benefits of AA enhanced levels in the eggs produce better quality larvae, these would be better prepared for the potentially stressful transition to exogenous feeding.

4.5. Conclusions.

During the present experiments, the suitability of pelleted diets for Atlantic halibut broodstock was demonstrated. Nutrient intake and health status can be controlled more fully using pelleted diets with comparable spawning performance to that obtained from moist-trash fish diets.

Atlantic halibut eggs selectively accumulated DHA to an optimal value around 30% of the total fatty acids. Levels of DHA in the diets should be around 16% of the to avoid depletion of reserves for egg yolk formation.

AA was also selectively accumulated in the eggs. Levels of 1.8% of the total fatty acids in the diets produced improved fertilisation rates, egg quality scores and hatching rates. This could translate in better quality larvae capable of surviving stressful periods like the initiation of exogenous feeding.

DHA:EPA ratios of 3.5 in diets seemed to be excessive, and ratios closer to 2 are recommended. EPA:AA ratios around 4 appeared to be optimal for Atlantic halibut broodstock.

Chapter V: Holding conditions and spawning: recirculation and stocking density.

5.1. Introduction.

The design of rearing systems and protocols for aquaculture has to combine the provision of favourable conditions for fish performance and well being, and the cost effectiveness necessary in a commercial situation. In this chapter, the influence of stocking density on egg quality of Atlantic halibut is investigated. In addition, the use of recirculation technology for holding broodstock, which allows for a more cost effective temperature control, is studied.

Holding conditions and environmental factors are crucial when reproduction in captivity is to be achieved. Water quality, diets and feeding regime, stocking density and other factors can generate stress in captive fish (Bromage 1995). Despite the fact that adult fish are generally more tolerant to stressors, reproduction appears to have a lower threshold, and chronic stress produced by inappropriate holding conditions can have a detrimental effect in spawning performance (Campbell *et al.* 1992, 1994, Bromage 1995) .Yet, there is little information of the conditions of Atlantic halibut in the wild, due to their deep-water habitat. The effects of photoperiod manipulation on spawning have been investigated (Smith *et al.* 1991). There are some reports on the influence of temperature conditions on spawning (Brown *et al.* 1995), and all of the hatchery operations in the UK

are forced to use water chilling to control the water temperature of spawning stocks. However, most of the current husbandry practices are based on observation of perceived best practice (Ronnestad and Rodseth 1989; Shields *et al.* 1998; Bromage *et al.* 2000). This includes the sex ratio and the stocking densities of broodstock fish in the tanks, which will influence the sexual and social interaction of the fish.

Sex steroid production and reproduction of fish are often impaired in captivity (Woods and Sullivan 1993; Linhart and Billard 1994; Clearwater and Crim 1995; Berlinsky *et al.* 1996; Larsson *et al.* 1997; Mylonas *et al.*. 1996; Fortuny *et al.* 1998; Vermeirssen *et al.* 2000). The metabolic products of several sexual steroid, as well as some specifically synthesised peptides, are known to act as pheromones in teleost fish (Irvine and Sorensen 1993; Scott and Sorensen 1994; Stacey *et al.* 1994; Ogata 1994; Goos and Richter 1996; Pottinger and Moore 1997; Kime and Ebrahimi 1997). Sex pheromones in fish are involved in oocyte maturation and ovulation, spermiation, and the onset of reproductive behaviour, including attraction of mates and triggering of gamete release (Vanweerd and Richter 1991). Vanweerd *et al.* (1991) found that when pubertal vitellogenic and previtellogenic female African catfish were exposed to holding water from a mixed-sex population of adult catfish, ovarian growth was accelerated. Analysis of the holding water from the adult population revealed the presence of several steroids and glucuronide derivatives known to be produced in adult prespawning fish. Cardwell *et al.* (1995) found that androgen treatment of catfish increased the magnitude and sensitivity of electro-olfactogram response to prostaglandins without affecting responses to other odours, and juvenile fish exhibited sex behaviour in the presence of prostaglandin-injected fish. The sensitivity of the carp olfactory epithelium to putative pheromones is apparently influenced by gonadal maturity and hormonal condition (Irvin and Sorensen 1993).

Berg *et al.* (1996) studied the influence of stocking density in tanks on the sexual maturation of Atlantic salmon, and found that the highest percentage of mature males happened in the tanks with lower stocking densities. There is evidence of stimulatory roles of multiple hypothalamic and pituitary peptides in the activation of the hypothalamus-pituitary-interrenal axis under crowding conditions in sea bream, inducing chronic stress (Rotllant *et al.* 2000). Msiska and Costa Pierce (1997) found that the population density of broodstock in ponds of *Oreochromis karongae* was critical for fry production. Coward *et al.* (1998) reported that substrate-spawning *Tilapia zilii* failed to spawn in crowded tanks, but exhibited a tendency to spawn soon after transfer to individually partitioned aquaria.

The stocking density in the broodstock tanks will influence the pheromonal and social interactions among the fish, and consequently their spawning performance. In the Atlantic halibut farming industry, there has been a tendency to maintain low stocking densities in the broodstock tanks to minimise stress and facilitate the monitoring of spawning. However, low density populations are costly to maintain, when considering both space and chilling expenditures. Therefore two experiments were set up at the Seafish Industry Authority facilities at Ardtoe, to study the performance of Atlantic halibut broodstock under two different stocking densities, and to validate the use of recirculation technology in broodstock holding tanks to reduce the costs associated with temperature control. This chapter reports on the influence of both experiments on egg quality and spawning performance.

5.2. The influence of stocking density on spawning performance and egg quality of Atlantic halibut broodstock.

The spawning performance and egg quality of two experimental groups of Atlantic halibut broodstock held at different stocking densities was analysed.

5.2.1. Materials and Methods.

Five experimental tanks were stocked with hatchery produced Atlantic halibut broodstock (7 year old, mean weight females 11 ± 0.4 kg, males 3.5 ± 0.2 kg; 1:1 female to male). Two of the tanks, of surface area 16.6 m^2 and 4.6 m diameter, were stocked with six pairs of fish to a mean initial biomass stocking density of 5.21 ± 0.01 kg/m^2. These constituted the high density group. The remaining three tanks (two of 20.6 m^2 and 5.3 m diameter and one of 16.6 m^2 and 4.6 m diameter), were stocked with three pairs of fish each, to a density of 2.5 ± 0.55 kg/m^2 (these tanks also acted as the control group of the "Recirculation experiment" below, section 5.3.1.). The experimental populations were set up in March 1997, and their spawning performance and egg quality monitored during the spawning season of 1998.

Broodstock fish were fed to appetite twice a week with the standard moist diet described in Chapter IV section 4.2.1. An ambient photoperiod was maintained until daylight reached 16 h, when it was fixed at 16h light:8h dark during the spawning season. Water depth in the tanks was maintained at 90 cm during the spawning season.

Eggs were collected, and fertilised using the hatchery procedure (Chapter II, section 2.2.4.1). Data on duration of spawning, relative fecundity, fertilisation rates, egg quality (assessed by blastomere morphology; see Chapter II, section 2.3.3) and hatching rates were collected. Percentage values were arcsine transformed and data from each treatment were compared using ANOVA.

5.2.2. Results.

Figure 5.1 shows the spawning dates of females in both high density and low density treatments. There was no significant difference in the duration of spawning per female between the high density group (23 days ± 2.1) and low density group (23 days ± 3.4).

There was no significant differences in relative fecundity (29000 ± 850 and 24700 ± 1140 eggs/kg of female), fertilisation rate (66% ± 8 and 48% ± 10) and egg quality scores (13.6 ± 0.5 and 14.1 ± 0.6) from the high and low density groups respectively (figures 5.2, 3 and 4). Hatching rates were generally poor, but there was no significant difference between the high (15% ± 5.7) and low density (17% ± 9.5) treatments (figure 5.5).

5.3. The influence of water recirculation on spawning performance and egg quality of Atlantic halibut broodstock.

Atlantic halibut broodstock spawning performance is greatly improved by maintaining water temperature at 5-6°C during prespawning and spawning (Brown *et al.* 1995). The chilling costs associated with this temperature control are high, especially with photperiod-manipulated stock spawning out of season. Chilling would be more cost efficient if the chilled water could be recirculated through the system. An experiment was carried out to validate this technology, and to investigate the effects on egg quality and spawning performance.

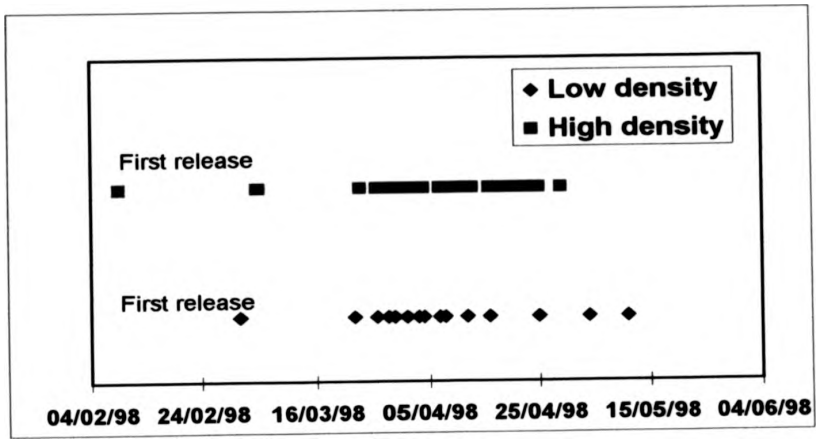


Figure 5.1: Spawning dates of the Atlantic halibut broodstock held at high and low stocking density.

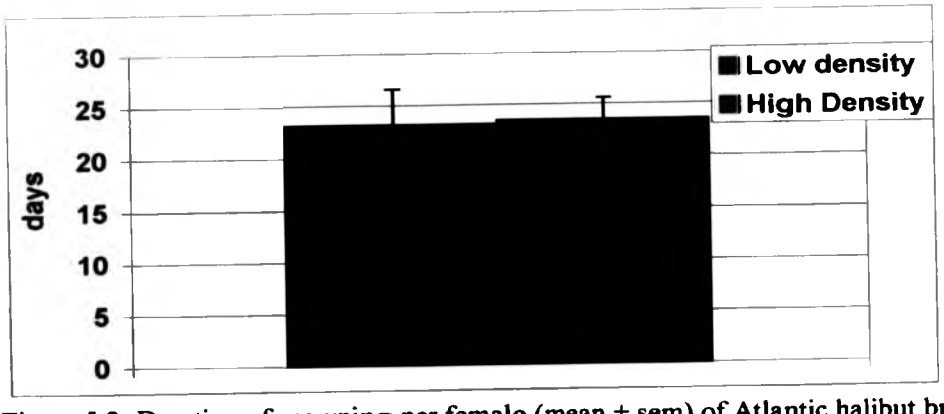


Figure 5.2: Duration of spawning per female (mean ± sem) of Atlantic halibut broodstock held at high and low stocking density.

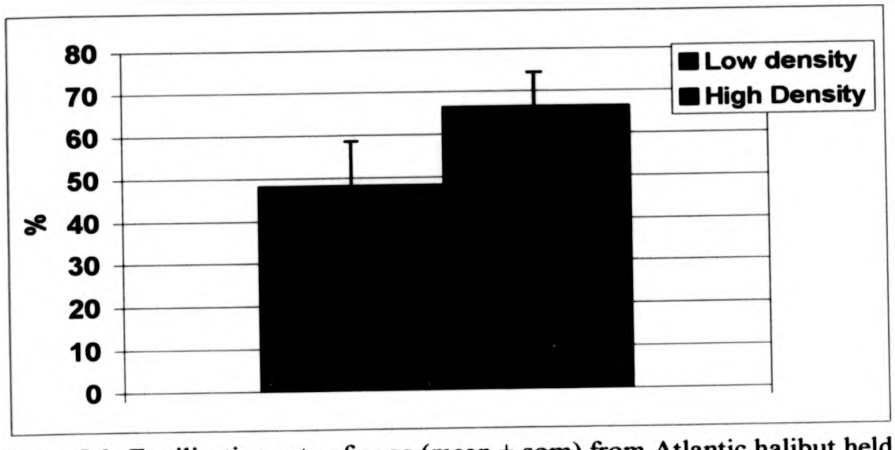


Figure5.3: Fertilisation rate of eggs (mean ± sem) from Atlantic halibut held at high and low stocking density.

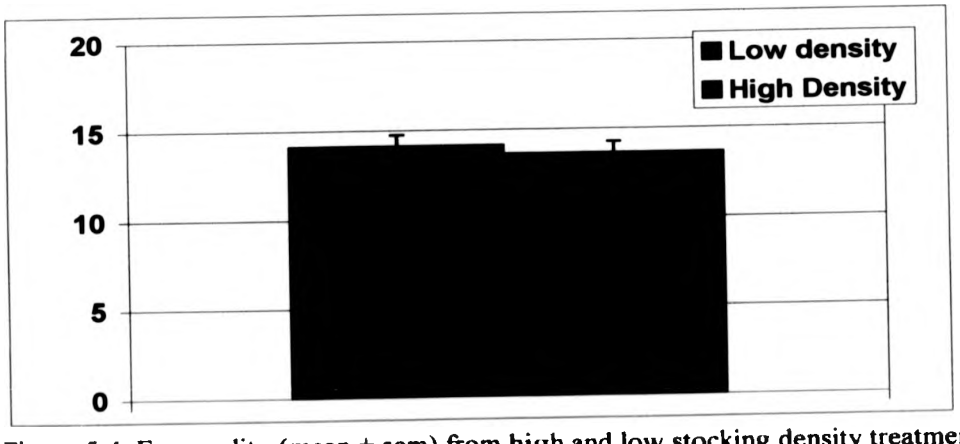


Figure 5.4: Egg quality (mean ± sem) from high and low stocking density treatments.

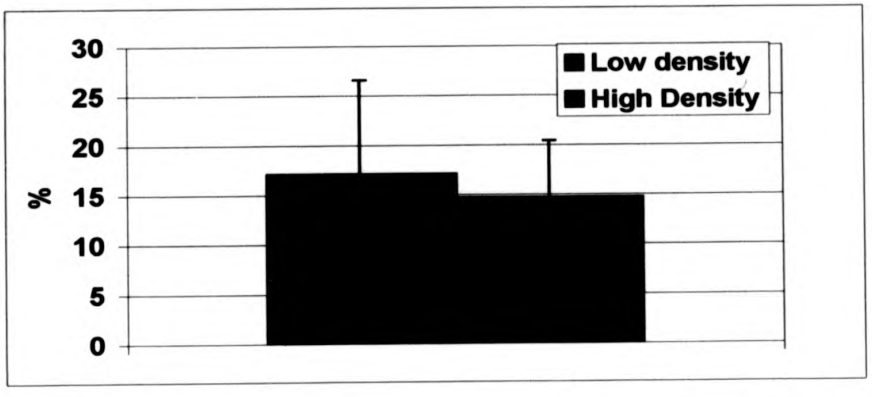


Figure 5.5: Hatching rate (mean ± sem) of eggs from the high and low stocking density treatments.

5.3.1. Materials and Methods.

Hatchery reared Atlantic halibut broodstock (7 year old, mean weight females 9 ± 0.8 kg, males 3.8 ± 0.2 kg) were distributed in two sets of three tanks all with the same characteristics as described for the "low density" group in the stocking density experiment (see section 5.2.1), three pairs of fish per tank (1:1 female to male). One set of 3 tanks was connected to a recirculation system consisting of a sump, a foam fractionation column, and a biological filter (see figure 5.6 for system description). The other set of tanks received each a flow-through water supply (filtered at 15 µm, 33 ppt salinity). Water was chilled through the year to provide a suitable temperature (the same for all tanks). Total ammonia nitrogen (TAN) was monitored by Ardtoe staff (figure 5.7).

Broodstock fish were fed to appetite twice a week with the standard moist diet described in Chapter IV section 4.2.1. Fish were maintained on an ambient photoperiod until daylight reached 16 h. They were then held at this photoperiod over the spawning season. Water depth in the tanks was maintained at 90 cm during the spawning season.

Eggs were collected and fertilised using the hatchery procedure described in Chapter II section 2.2.4.1. Data on duration of spawning per female, relative fecundity, fertilisation rates, egg quality (assessed by blastomere morphology; see Chapter II, section 2.3.3) and hatching rates were collected. Percentage values were arcsine transformed and data from each treatment were compared using ANOVA.

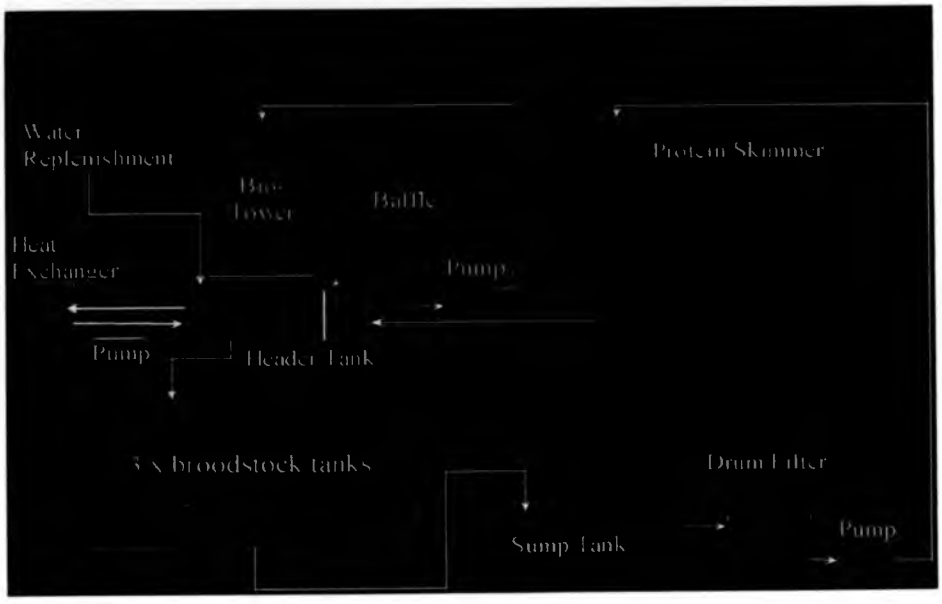


Figure 5.6: Diagrammatic representation of the main elements of the recirculation system for Atlantic halibut broodstock. The drum filter after the sump was not operational during the trial.

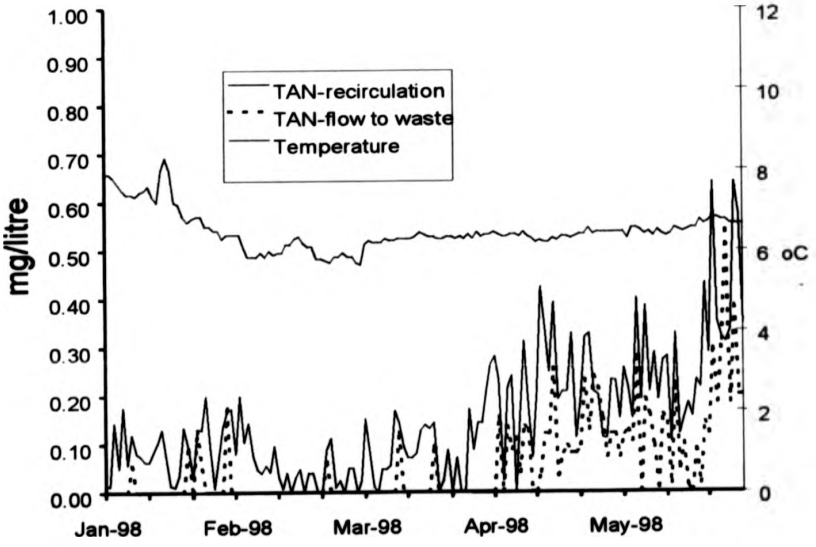


Figure 5.7: Temperature and total ammonia nitrogen (TAN) in the recirculation and flow through tanks.

5.3.2. Results.

Figure 5.8 shows the spawning dates of females in both high density and low density treatments. There was no significant difference in the duration of spawning per female between the recirculation group (21 days ± 2.9) and the flow-through group (23 days ± 3.4).

There was no significant differences in relative fecundity (29000 ± 850 and 24700 ± 1140 eggs/kg of female), fertilisation rate (62% ± 7 and 48% ± 10) and egg quality scores (13.2 ± 0.5 and 14.1 ± 0.6) from the recirculation and flow through groups respectively (figures 5.9 and 10). Hatching rates were generally poor, but there was no significant difference between the recirculation (27% ± 7.1) and flow through (17% ± 9.5) treatments (figure 5.11).

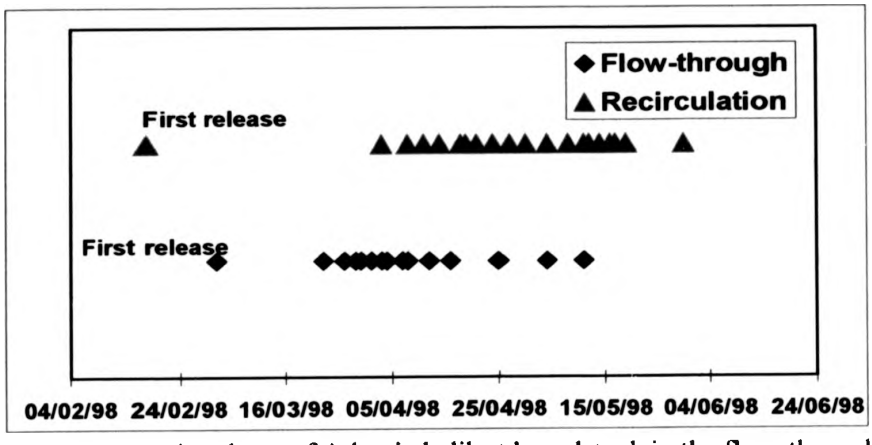


Figure 5.8: Spawning dates of Atlantic halibut broodstock in the flow-through and recirculation tanks.

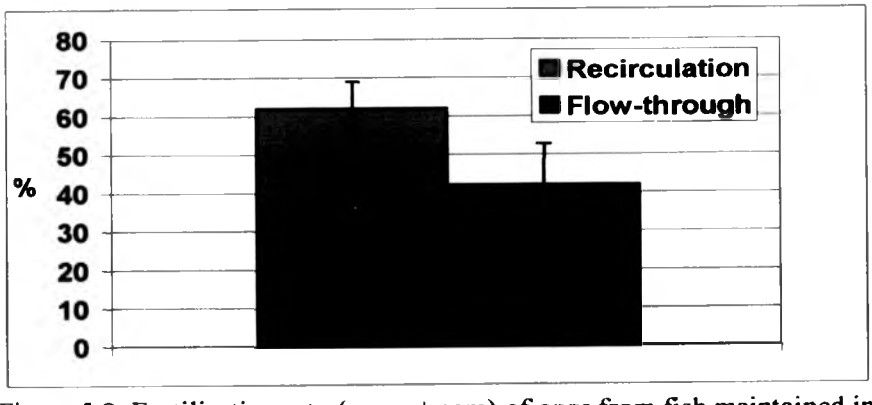


Figure 5.9: Fertilisation rate (mean ± sem) of eggs from fish maintained in the recirculation and flow-through tanks.

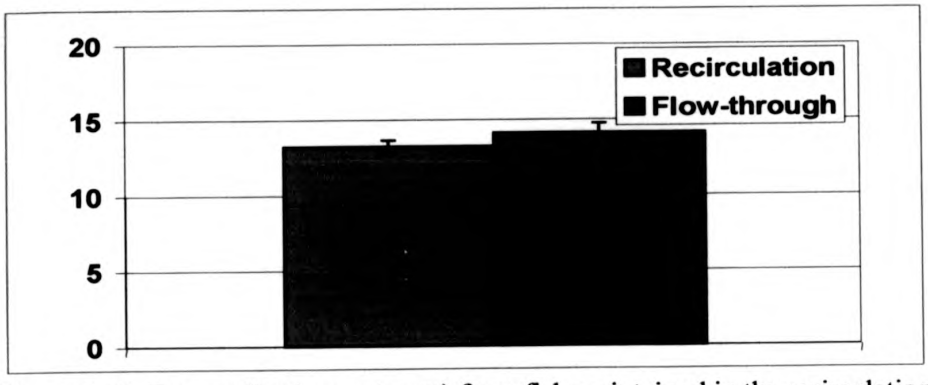


Figure 5.10: Egg quality (mean ± sem) from fish maintained in the recirculation and flow-through tanks.

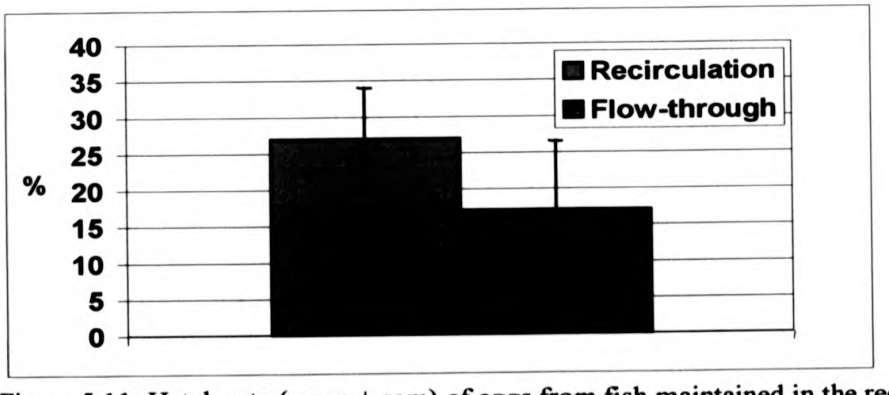


Figure 5.11: Hatch rate (mean ± sem) of eggs from fish maintained in the recirculation and flow-through tanks.

5.4. Discussion.

No significant differences were found in any of the spawning parameters between the treatments.

It has been reported that crowding and confinement stress provokes increase in cortisol levels in plasma of many teleost species; for instance in brook charr (Vijayan and Leatherland 1990), sea bream (Arends *et al.* 1999) and sockeye salmon (Kubokawa *et al.* 1999). The increase in cortisol levels provoked by acute or chronic stress is normally accompanied by a decrease in sex steroid concentrations in plasma (Kubokawa *et al.* 1999). Cortisol implantation has deleterious effects on reproduction of brown and rainbow trout (Carragher *et al.* 1989). Also, periods of chronic confinement stress during the final stages of reproductive development disrupt the reproductive endocrinology and the survival rates of progeny from rainbow and brown trout (Campbell *et al.* 1994). The stocking density in the high density treatment was double the one in the low density tanks (5.21 kg/m^2 and 2.5 kg/m^2 respectively). However, stocking density in broodstock tanks of some UK operators is as high as 11 kg/m^2, with no apparent detrimental effects (Shields *et al.* 1998), and even higher densities in broodstock tanks are normal practice in other species, for instance sea bream (10-15 kg/m^3) (Zohar *et al.* 1995).

The use of recirculation technology had no detrimental effect on the spawning performance of Atlantic halibut broodstock. Recirculation technology has been used for rearing larvae of a number of farmed species, for instance turbot (Dhert *et al.* 1991) and sea bass (Corneillie 1989). It is also used at the nursery stage to accelerate the growth of juvenile fish, for instance before being put into cages,, in species like turbot, seabass and sea bream. There is no previous literature, to the author's knowledge, describing the use of

recirculation for holding broodstock of a cold water marine species. Analysis of the economic advantages of recirculating the chilled water in the broodstock tanks is out-with of the objectives of this research. However, the fact that there were no deleterious effects on the spawning performance of the fish in the recirculation system opens the subject for economic consideration and should be of importance for commercial operators.

5.5. Conclusions.

No differences were found in the spawning performance and egg quality of broodstock Atlantic halibut maintained at stocking densities of 5.21 kg/m^2 and 2.5 kg/m^2 respectively.

Recirculation of chilled water in Atlantic halibut broodstock tanks had no deleterious effect on their spawning performance or egg quality.

Chapter VI: Manipulation of gamete production by GnRH-agonist implantation.

6.1. Introduction

a. Endocrine regulation of gamete production.

The endocrine regulation of gametogenesis in fish has been extensively reviewed in recent years (Nagahama 1983, 1994; Dodd and Sumpter 1984; Nagahama *et al.* 1995; Schultz *et al.* 1999). It is now well accepted that the response of the individual to a range of environmental, seasonal, behavioural and physiological cues is mediated by the interaction of several chemical substances with hormonal function produced at different levels in the hypothalamus–pituitary–gonad axis and acting on different target tissues.

The onset and maintenance of reproductive behaviour is regulated by annual cycles in gonadal steroids under the control of gonadotropin secreted by the pituitary (Goetz 1983; Berlinsky and Specker 1990; Methven *et al.* 1992). In response to an array of specific cues, neurosecretory cells in the hypothalamus produce gonadotropin releasing hormones (GnRH) that stimulate gonadotropes in the pituitary. According to Dellovade *et al.* (1998), three aspects of GnRH neurobiology may be universal among vertebrates. First, the GnRH neuronal migration from the olfactory placode into the basal forebrain appears to hold true for forms ranging from fish to humans. Second, for proper agonist activity in

the anterior pituitary, GnRH must be released in a pulsatile fashion. Third, there are remarkable effects of social context on GnRH expression in fish and mammals.

Teleost GnRHs have been found to be peptides similar to GnRH in reptiles and birds (King and Millar 1990), and with structural and functional parallels with mammalian luteinizing hormone releasing hormone (LHRH). Sherwood *et al.* (1983, 1984) described two forms of GnRH in chum salmon. One was a decapeptide homologous to mammalian LHRH, referred to as salmon-I-GnRH (sGnRH-I). The second was thought to be identical to chicken-II-GnRH (cGnRH-II). Two similar forms of GnRH have been described in several species of teleosts (Sherwood *et al.* 1984; Sherwood 1987). A third form (sbGnRH) was characterised by Powell *et al.* (1994) from brains of sea bream.

In response to GnRH, gonadotropes in the *pars distalis* of the adenohypophysis, produce gonadotropic hormone (GTH). Gonadotropins are glycoproteins consisting of two subunits (α and β chains), both approximately 100 amino acid residues long. Two forms of GTH (GTH I and GTH II) were first described in chum salmon (Suzuki *et al.*, 1988). They are functionally and structurally similar to mammalian follicle stimulating hormones (FSH) and luteinizing (LH). Weltzien *et al.* (2000) recently identified FSH and LH from pituitary glands of Atlantic halibut, consisting of 117 and 124 amino acids respectively.

Once released into the bloodstream, the GTHs act on the Leydig and Sertoli cells of the testis, or the thecal cells of the oocyte follicle. Steroids mediate the regulation of gametogenesis by GTH. Steroid hormones are small hydrophobic molecules derived from cholesterol. In vertebrates, the main types of steroids according to their functions are oestrogens, progestagens, androgens, glucocorticoids and mineralocorticoids. The effects of steroids are varied and include, among others, sexual differentiation and regulation of membrane permeability. Some end products of steroid metabolism are thought to have pheromonal actions, see for instance 17, 20α-P and 3β,17,20α-P5β in dab in Canario and

Scott 1990c and 5β-reduced and conjugated steroids in plaice in Scott and Canario 1990; also see review by Stacey *et al.* 1987. In a number of male teleosts, 11-ketotestosterone and not testosterone seems to be the most important androgen (Scott *et al.* 1980; Fostier *et al.* 1982). Levels of 11-ketotestosterone are high at the time of spermatogenesis, and decrease after the onset of spermiation (Nagahama 1999, 1994). This is associated with the beginning of milt production and the expression of secondary sex characters that are concern with breeding activity. In females, 17-β oestradiol concentrations in plasma peak in pre-spawning individuals, to fall in most species just prior to spawning (Atlantic halibut Methven *et al.* 1992; goldfish, Volkoff and Peter 1999; North Sea plaice, Scott *et al.* 1999).

The process of sperm production, spermatogenesis, consists of two proliferative steps (stem cell renewal and mitotic proliferation of spermatogonia) followed by two differentiation steps (meiosis and spermiogenesis), taking place within testicular cysts formed by Sertoli cells (Grier 1981; Billard *et al.* 1982; Callard 1991). At the end of the first differentiation step, meiosis, the spermatogonia emerge as haploid rounded spermatids. During spermiogenesis, spermatids are transformed into motile cells specialising in the "stable transfection" of female germ cells, the flagellated spermatozoa, through a process of cellular restructuring (Schultz 1999). When spermatogenesis is finished, Sertoli cells release the spermatozoa (spermiation), to which they had been attached for the duration of spermatogenesis. Once the spermatozoa are released to the efferent ducts, a process of capacitation has to take place for it to become fertile.

According to the model described by Nagahama for the hormonal regulation of milt production in male fish (Nagahama 1994), gonadotropin binds to membrane receptors in the Leydig cells. In response, these cells synthesise and secrete 11-ketotestosterone, which crosses the basal membrane and enters the Sertoli cells that surround the clusters of spermatogonia. The Sertoli cells synthesise activin B, which binds to receptors in the

membrane of the spermatogonia, initiating spermatogenesis (see figure 6.1). At a different point in development, gonadotropin binds to membrane receptors in the Sertoli cells in the testis, inducing the synthesis of 17α-hydroxyprogesterone. The 17α-hydroxyprogesterone produced by the Sertoli cells is used as a precursor in the spermatozoa to produce 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP), in the presence of 20β-hydroxysteroid dehydrogenase (20β-HSD). 17α, 20β-DP acts to produce an increase of pH in the sperm duct with a concomitant increase in intrasperm cAMP, allowing for the acquisition of sperm motility (Miura *et al.* 1992).

Similarly, in the model proposed for endocrine regulation of oocyte development (Nagahama 1994), FSH acts on the thecal cells, which change cholesterol into testosterone in a cAMP mediated process (see figures 6.2 and 6.3). Testosterone is transformed into oestradiol-17β in granulosa cells, in the presence of P-450 aromatase, which is released into the blood stream and stimulates the production of vitellogenin in the liver. Vitellogenin binds to membrane receptors in the oocyte and is incorporated into yolk granules in the cytoplasm. A shift in steroid production from C18 to C21 steroids in thecal and granulosa cells occurs at the end of oocyte growth. Thecal cells produce 17α-hydroxyprogesterone in response to LH stimulation. This is metabolised by the granulosa cells to 17α, 20β-DP by 20β-HSD. 17α, 20β-DP binds to receptors in the membrane of the oocyte, forming a maturation-promoting factor (MPF) that acts on the germinal vesicle to stimulate final maturation.

b. Artificial control of gamete production.

The control of seed production is of primary importance in aquaculture; therefore diverse techniques are applied to promote the gonadal maturation of the broodstock. Artificial alterations in light cycles and temperature are used to influence seed quality,

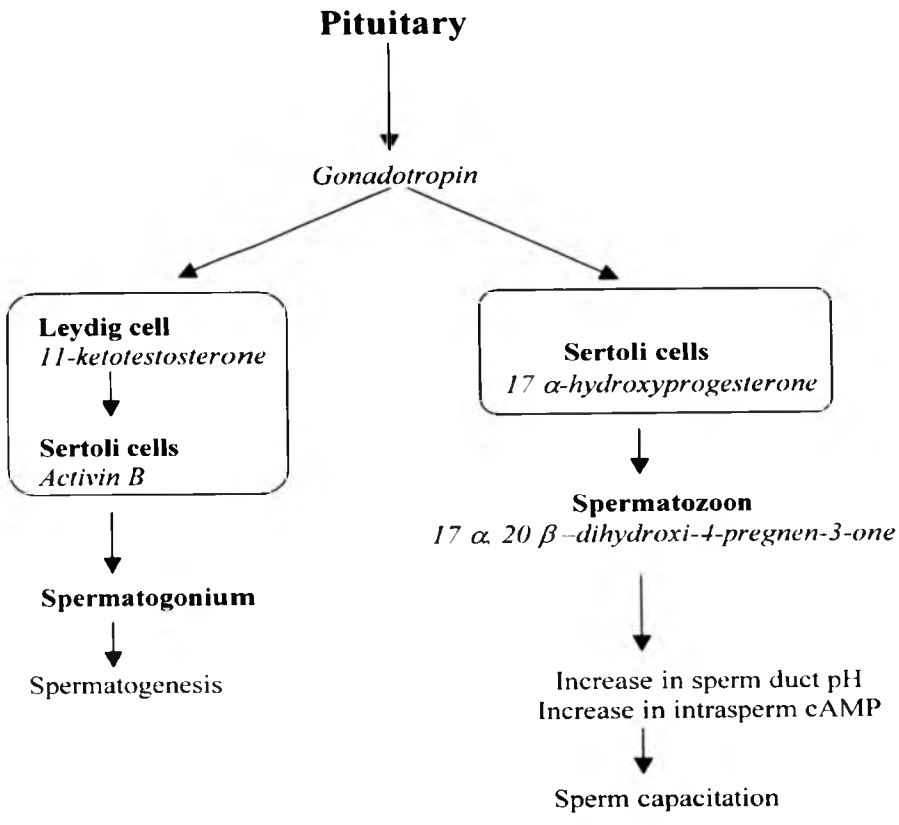


Figure 6.1: Hormonal regulation of testis development and sperm production in fish (modified from Nagahama 1994). GTH acts on Leydig cells inducing the synthesis of 11-ketotestosterone. This stimulates Sertoli cells to produce activin B that acts on the spermatogonium attached to the Sertoli cells, iniciating spermatogenesis. A different form of GTH stimulates Sertoli cells to synthesise 17 α-hydroxyprogesterone. This in turn stimulates the production of 17 α, 20 β –dihydroxi-4-pregnen-3-one in the spermatozoon, responsible of the increase in sperm duct pH and in intrasperm cAMP.

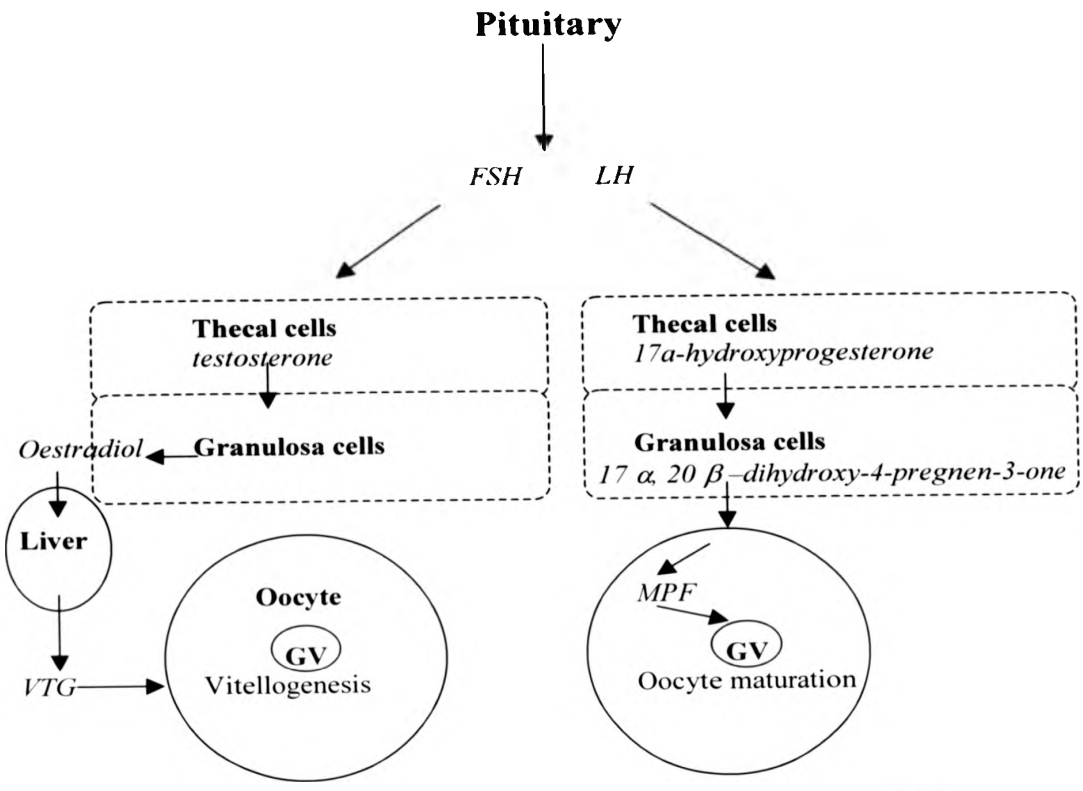


Figure 6.2: Hormonal regulation of vitellogenesis and oocyte maturation in fish (modified from Nagahama 1994). GV: germinal vesicle; MPF: maturation promotion factor

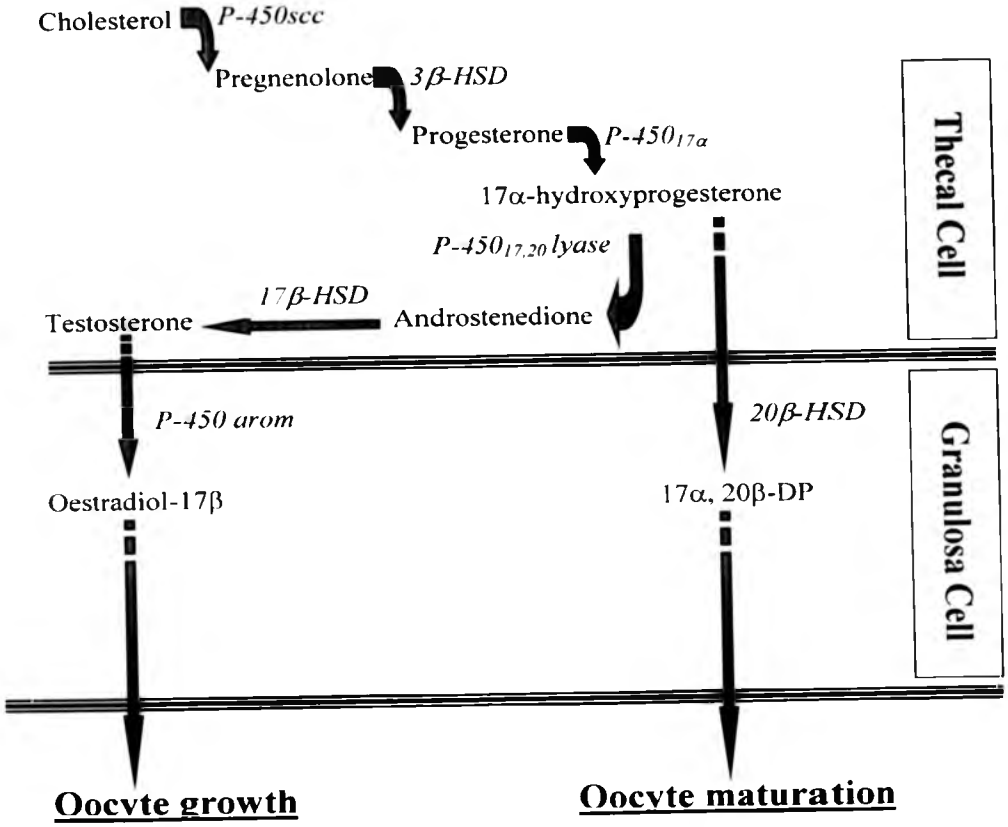


Figure 6.3: Steroid biosynthesis in the ovarian follicle of salmonids (modified from Nagahama, 1994). 17α, 20β-DP, 17α, 20β-dihydroxy-4-pregnen-3-one; P-450scc, Cholesterol side-chain clevage cytochrome P-450; 3β-HSD, 3β-hydroxyesteroid dehydrogenase-isomerase; P-450$_{17\alpha}$, P-450 17α-hydroxylase; P-450$_{17,20}$ lyase, P-450 17,20-lyase; 17β-HSD, 17β-hydroxy-steroid dehydrogenase; P-450 arom, aromatase cytochrome P-450; 20β-HSD, 20β-hydroxysteroid dehydrogenase.

trigger gonadal maturation, and to advance or delay the spawning season (Bromage and Cumaranatunga 1988; Zohar 1989; Imsland *et al.* 1997; Davies *et al.* 1999; Brown *et al.* 1995), thus spreading production throughout the year by managing different stocks of breeding fish. There are, however, species currently cultured in which reproduction in captivity does not occur naturally, such as the sábalo (Fortuny *et al.* 1998) and the striped bass (Woods and Sullivan 1993). In batch spawners, and particularly in flatfish, the rhythmicity of ovulation can be impaired in captivity and egg quality diminished, as in the Southern flounder (Berlinsky *et al.* 1996) or the yellowtail flounder (Larsson *et al.* 1997). Milt production can also be impaired in captivity. Low milt production has been reported for cultured stocks of white bass (Mylonas *et al.*. 1996), yellowtail (Clearwater and Crim 1995) and European catfish (Linhart and Billard 1994) among others.

The implantation of adult fish with GnRH analogues (GnRHa), inducing the release of LH by the pituitary, is proven to be a good tool for improving spawning performance. Egg production and quality has been enhanced in this way in several batch-spawning species, including sea bass (Almendras *et al.* 1988), gilthead sea bream (Zohar *et al.* 1995), white bass (Mylonas *et al.* 1997), American shad (Mylonas *et al.* 1995), southern flounder (Berlinsky *et al.* 1996), turbot (Prat *et al.* 1993; Mugnier *et al.* 2000) and yellowtail flounder (Larsson *et al.* 1997). GnRHa implantation has also been used in male broodstock to induce spermiation or improve milt quality in pacu (Rosa e Silva *et al.* 1988), sturgeon (Faulkner and Moberg 1997), white bass (Mylonas *et al.* 1996) and plaice (Vermeirssen *et al.* 1998).

The control of gamete production in Atlantic halibut presents similar problems to the ones explained above. Cultivation of halibut relies on the collection of gametes by stripping. Monitoring of female ovulatory rhythms is required to prevent post-ovulatory egg quality deterioration (Bromage *et al.* 1994; Norberg *et al.* 1991); however, irregular

spawning intervals can occur, resulting in poor quality eggs. Often females present impaired rhythmicity and erratic ovulation. There have been unpublished reports of mortalities and mass oocyte hydration following GnRHa treatment, although these were uncontrolled commercial trials. In captive male Atlantic halibut broodfish, production of abnormally viscous milt with a high spermatocrit is a common problem, resulting in low sperm motility values with detrimental effects on fertilisation rates (Vermeirssen *et al.* 2000). Reduction of milt production at the end of the spawning season is also a problem as males start producing milt earlier in the season than females. This results, in commercial operations, in a lack of functional milt towards the end of the spawning season while females are still producing egg batches.

In this chapter, the influence of GnRHa implantation on spawning performance and steroid levels of female Atlantic halibut is described. Similarly, the effects of GnRHa treatment in male spermiation is studied with regard to milt quality, time and dose of implantation and induced steroid concentration in plasma. In all experiments, ethylene-vinyl acetate copolymer pellets containing D-Ala^{6},Pro-*N*-ethylamide-luetinising hormone releasing hormone (available commercially in 1996 as Reproboost^{TM} , Aquapharm Ltd., Columbia, MD, USA, or otherwise provided by Dr. D. Mylonas and Dr. Y. Zohar as a gift to N. Bromage), were used. These implants provide a slow release of the GnRH agonist over an extended period of time (Zohar *et al.* 1995).

Five experiments were carried out. Two experiments addressed the effects of GnRHa implantation on egg production, and three experiments described the effects on milt production. The materials and methods and results of each series of experiments are described individually in the following sections.

6.2. Effects of GnRHa implantation on egg production

Two experiments were carried out. Experiment 1 compared the spawning performance and steroids concentrations in plasma of non-implanted and GnRHa implanted females. Experiment 2 investigated the influence in spawning performance of different doses of GnRHa implantation.

6.2.1 Comparison of spawning performance and plasma steroid concentrations between GnRHa implanted and sham implanted females. Experiment 1.

6.2.1.1 Materials and Methods

Two groups of four hatchery produced Atlantic halibut females (5.5 years old, 7.2 kg ± 1.5) were maintained in a $12m^3$ (4 m diameter, 1 m deep) tank supplied with flow-to-waste sea water chilled to 6°C, together with the same number of males. The fish were anaesthetised with phenoxyethanol and implanted either with an implant containing 2µg GnRHa /kg of fish or a sham implant with no GnRHa. The fish were implanted on March 24 1999, and an ovarian biopsy removed by cannulation, where possible, to establish the stage of maturation (see Chapter II section 2.4.1). Ovarian development was monitored by ultrasonography. Spawning performance (relative fecundity, no. of batches per female, no. of eggs per ml, total fertilisation rate and relative hatch rate) was recorded. Fish were anaesthetised and blood samples collected with the use of 5 cm heparinised needles at weekly intervals. Plasma was separated by centrifugation for 10 minutes at 13000 RPM in a micro centrifuge and the plasma fraction decanted into a 1.5 ml eppendorf and frozen at –20 °C for later hormonal analysis. GnRHa and steroid concentrations in plasma were analysed by RIA as described in Chapter II, section 2.9. Pools of plasma from the GnRHa implanted fish from days 0, 5 and 12 post treatment were analysed for concentrations of

free and conjugated androstenedione (4-androstene-3,17-dione), 17, 20α-P (17α, 20α-dihydroxy-4-pregnen-3-one), triol or 17, 20β, 21-P (17α, 20β, 21-trihydroxy-4-pregnen-3-one), testosterone (17β-hydroxy-4-androsten-3-one), 11-ketotestosterone, 11-deoxycortisol (17, 21-dihydroxy-4-pregnene-3, 20-dione), 17, 20β-P (17α, 20β-dihydroxy-4-pregnen-3-one), Oestradiol-17β, and 3β, 17, 20β-P-5β (3β, 17α, 20β-trihydroxy-5β-pregnane). Of these, free oestradiol, sulphated 11-deoxycortisol, free androstenedione and sulphated 3β, 17, 20β-P-5β presented significant fluctuations and were consequently analysed for each individual at each sampling point.

6.2.1.2 Results

a. Spawning performance

At the time of implantation it was only possible to obtain ovarian biopsies from two females. One of them was already producing its second egg batch. The second fish presented two clear size classes of oocytes: the first 0.3 mm (± 0.02) in diameter and the second class 1.9 mm (± 0.04) diameter. The rest of the females appeared to have collapsed oviducts that made it impossible, or otherwise too traumatic, to introduce the catheter for sampling.

Table 6.1 shows the averaged spawning parameters (mean ± sem) of the two experimental groups in experiment 1. GnRHa implanted females started spawning 17 (±8) days after treatment - not considering one female that was already spawning at the time of implantation - while the sham implanted fish did not begin spawning until 28 (±5.2) days. One of the sham controls did not spawn during the season. No mortality or mass oocyte hydration was found in the implanted fish, which exhibited similar spawning performances to the controls. The GnRHa treated fish appeared to have a slightly higher relative

fecundity and somewhat smaller eggs. However, there was no significant difference in any of the spawning performance parameters assessed between treatments.

Table 6.1: Effects of GnRHa implantation on spawning performance (mean ± sem) in experiment 1.

Treatment	Relative fecundity No eggs/kg female	Batches per female	Eggs/ml	Fertilisation rate (%)	Hatch rate (%)
GnRHa	26408 (±6778)	6 (±0.6)	64 (±2.2)	67 (±3)	65 (±4.8)
Sham	19160 (±2693)	5 (±0.9)	61 (±5.3)	84 (±5)	59 (±10)

b. GnRHa and steroids in plasma

GnRHa levels in plasma of control fish dropped significantly (p=0.0026) with respect to GnRHa implanted fish after day 12 (figure 6.4).

Mean plasma oestradiol-17β (E_2) levels in GnRHa implanted fish (figure 6.5) decreased with respect to initial values (significantly from day 19, p = 0.009). By day 26 concentrations were significantly lower than in sham implanted fish (p = 0.04). In general, levels of E_2 in implanted fish increased after treatment to values around 10 ng/ml, including in female 609 which was already spawning at the time of implant (see figure 6.6). Plasma E_2 concentrations decreased just before (female120) or shortly after (female 299 and 311) the release of the first batches of eggs, to levels around 1 ng/ml and remained at these levels until the end of spawning for each fish. In sham implanted fish, E_2 remained high (around 10 ng/ml) for a longer period of time after treatment, until the release of the first batch of eggs; subsequently concentration decreased to values of about 1.5-2.5 ng/ml which remained until the end of spawning. The highest concentration of E_2 was measured

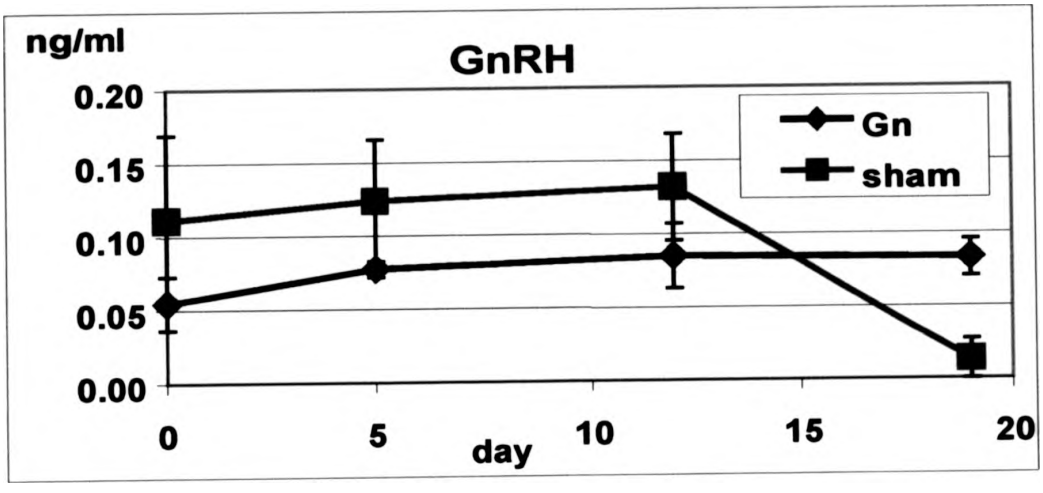


Figure 6.4: GnRH plasma concentration (mean +-sem) in GnRHa implanted (at time 0) and sham implanted Atlantic halibut females. (experiment 1).

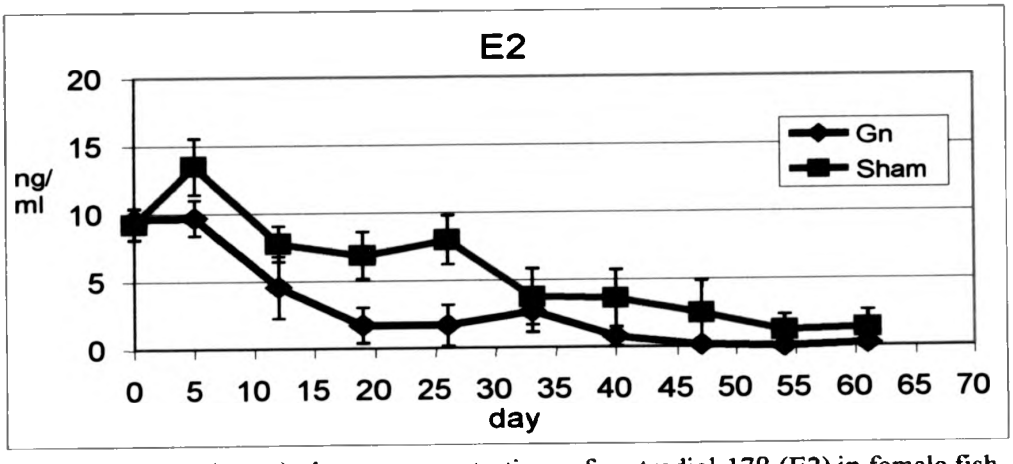
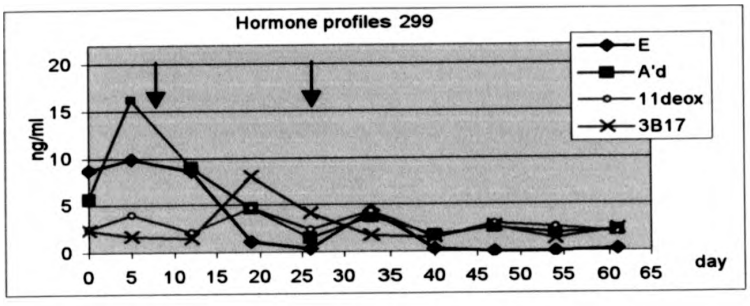


Figure 6.5: Mean (±sem) plasma concentrations of oestradiol-17β (E2) in female fish following GnRHa or sham implantation (experiment 1).

Figure 6.6: Plasma concentrations of free oestradiol (E_2), sulphated 11-deoxycortisol (11deox), free androstenodione (A'd) and sulphated 3β, 17, 20β-P-5β (3B17) in GnRHa implanted (opposite page) and Control sham-implanted (second page) Atlantic halibut females during experiment 1. Arrows correspond with the first and last batches of eggs respectively. Day 0 corresponds to the day of GnRHa implantation.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E
A'd
11deox
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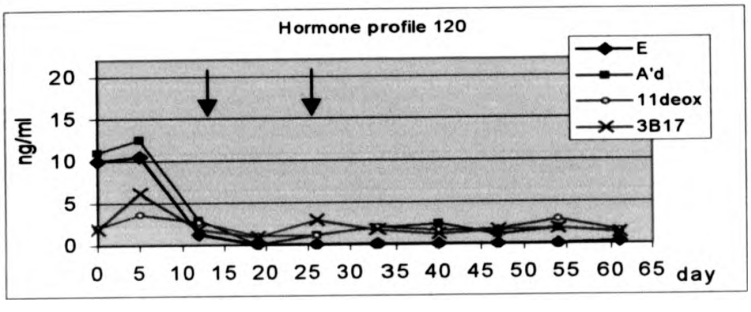
Hormone profile 120
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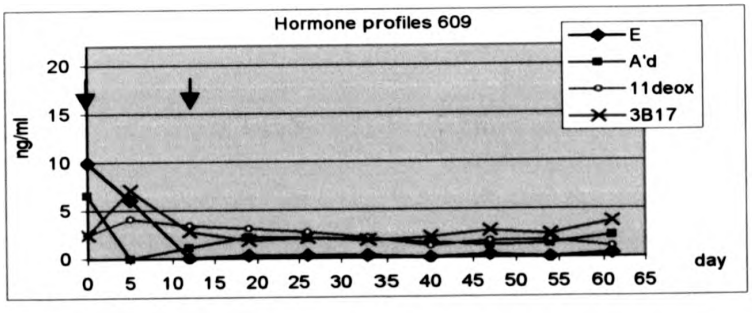
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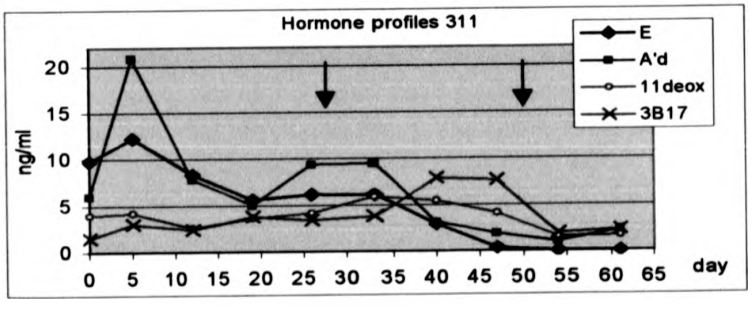
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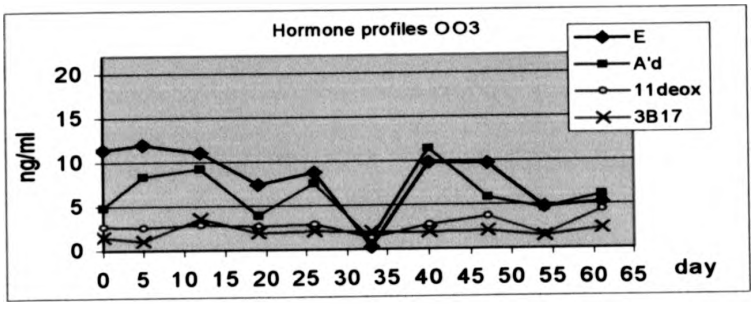
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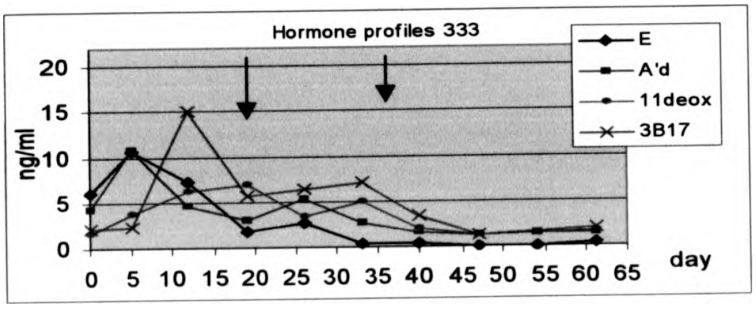
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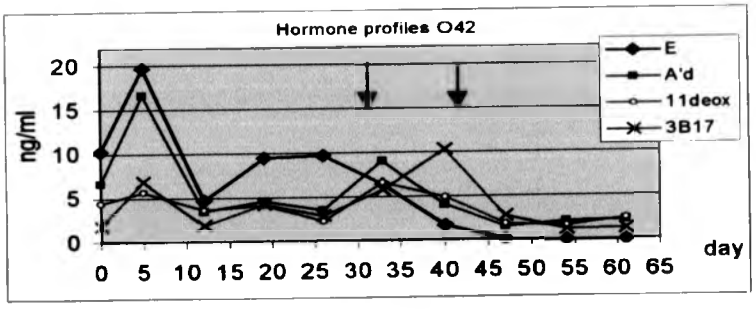
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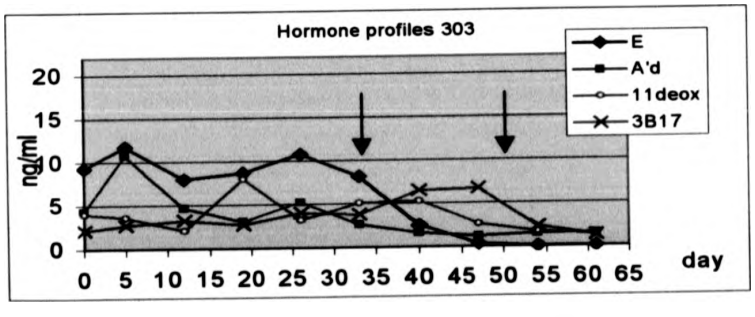
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day

in female 042 at 19.6 ng/ml on day 5. E_2 values in female 003, which failed to spawn, remained around 10 ng/ml to fall to 5 ng/ml at the end of the sampling period.

Plasma androstenedione concentrations behaved in a very similar fashion to E_2. In implanted females, concentration remained higher than E_2 (max. = 16.2 ng/ml). In sham implanted fish, however, androstenedione concentrations were lower than that of E_2, with maximum values in female 042 of 16.7 ng/ml on day 5.

Sulphated 11-deoxycortisol plasma concentration of implanted fish increased from initial values to values around 4 ng/ml in all fish; except in female 311 that rose from initial values of 4 ng/ml to 6 ng/ml on day 33 after treatment, after the release of its first batch of eggs. Overall, sulphated 11-deoxycortisol concentrations increased during spawning, to decrease towards the end of the spawning season of each female.

Concentrations of sulphated 3β, 17,20β-trihydroxy-5β-pregnane 20 (3β, 17,20β -P-5β-S) in plasma reached high values during spawning in most females (max. = 10.3 ng/ml in female 042 on day 40 after the start of the experiment). Values fell after the release of the last batch of eggs. Concentration of 3β, 17,20β -P-5β-S in female 120 decreased to a minimum of 0.9 ng/ml after the release of the first egg batch, but increased to 2.9 ng/ml before the end of spawning, to fall again after the last batch of eggs was released.

6.2.2 Effect of different doses of GnRHa implantation on spawning performance and plasma steroid concentrations. Experiment 2.

6.2.2.1 Materials and Methods

Three female broodfish of wild origin (Icelandic sea) maintained at the Machrihanish Marine Laboratory (Machrihanish, Argyll, Scotland), were implanted with GnRHa slow release pellets as described above (see table 6.2 for fish details). Fish were held in two 4 m diameter, 90 cm deep fibreglass tanks, supplied with 60 μm filtered sea water maintained at 5.5 – 6 °C. Other mature fish were held in the same tanks (Total numbers, tank 1: 7 females at 13.6 kg ± 0.8 and 7 males at 3.9 kg ± 0.7; tank 2: 4 females at 13.1 kg ± 5.8 and 9 males at 4.7 kg ± 1.0). Fish were monitored for external signs of maturation, and eventually with the use of ultrasound scanning (Dynamic Images™ fitted with a linear T shaped transducer) from the outside of the tank to minimise stress to the fish (see Chapter II section 2.12 and Chapter III for details of ultrasound operation). At the time of implant, fish were anaesthetised by immersion in an inflatable dingy that was floated inside the tank and filled with fresh sea water dosed with phenoxyethanol, and then ushered onto a low working table. GnRHa pellets were implanted deep into the muscle of the anterior-dorsal area, behind the head of the fish, with the use of a plunger. Fish 2 and 1 were implanted with 10 μg GnRHa / kg of fish before the first batch of eggs was released, whereas fish 3 was implanted after the first egg release with a dose of 5 μg GnRHa / kg of fish. Blood was collected (1.5 ml) using 5 cm heparinised needles attached to plastic 2 ml syringes, and plasma was separated as described above. Further plasma samples were obtained from anaesthetised fish on day 3 after implantation and after the release of their last batch of eggs. Each batch of eggs was fertilised following the experimental procedure, and samples

of eggs stocked in microtitre plates for assessment of fertilisation rates and blastomere morphology following the procedure explained in Chapter II section 2.3.3.

The spawning performance of implanted females was recorded and compared to the performance of non-implanted females from the same stock (fish 4 and 5). Relative fecundity data were compared to historical records of the same females. To account for the small number of individuals and the influence of individual performance and previous history of each female, a multivariate analysis of the results was performed by compiling performance criteria into a similarity matrix. Mean ovulation time from implant (hours), spawning interval (hours), total number of ovulations, mean number of eggs per ml, mean batch volume (ml), mean relative batch fecundity (eggs/kgfemale / batch number.), total fecundity (eggs/kg female), total numbers of eggs released, mean fertilisation rate, mean rate of undividing eggs, mean dropout rate, mean blastomere symmetry, mean blastomere uniformity, mean blastomere margin score, mean blastomere adhesion, mean blastomere inclusion score and mean total blastomere morphology score were the assessment criteria utilised. Cluster analysis on the measured variables was performed by a similarity matrix, using Spearman Coefficient, which automatically ranks data, to standardise the matrix and allow comparison. The results were displayed in an Unweighted Paired Group Method for arithmetic Averaging (UPGMA). This analysis was performed by Andrew Davie, using MVSP for Windows 3.1 (Kovach Computer Services 1998).

Table 6.2: Experimental fish used in experiment 2. * are implanted fish.

Experimental females in tank 1.

Fish code	Weight (kg)	Length (cm)
2*	12.5	84
3*	20.5	94
4	16	98

Experimental females in tank 2.

Fish code	Weight (kg)	Length (cm)
5	13.3	92
1*	29.5	113

6.2.2.2 Results

Fish 1 and 2, implanted with the high dose of GnRHa, ovulated within 117 hours and 71 hours respectively after implant. Fish 3, on the low dose, ovulated within 38 hours. Both fish 1 and 2 exhibited a prolonged first spawning interval (fish 1 = 89.5 hours, fish 2 = 168.5 hours) followed by a subsequent series of relatively shorter spawning intervals (average fish 1 = 34.1 h ± 6.4 (n=10), average fish 2 = 39.3 h ± 6.7 (n=6)). This pattern was not apparent with fish 3,4 and 5. The average inter-spawning interval for fish 1 in year 2000 was 39h ± 7.6 (n=10) compared to 97 ± 18.4h in earlier seasons (n=3) [Mean ± sem (n = number of intervals)]. The significance of this difference could not be confirmed due to the few historical data available. Fish 2 presented no significant difference of mean spawning interval when compared to previous seasons (95 h ± 10.2 (n=7)). However when comparing the mean spawning interval excluding the first spawning (39 h ± 6.7 (n=7)), the difference was significant ($p = 0.001$). Similar comparisons for fish 3-5, including and excluding the first spawning interval, were not significant.

Figure 6.7 presents the relative fecundity of each female compared to its performance in previous seasons. Fish 1, 2 and 3 showed higher fecundity than in previous years, with increases of 62%, 75% and 72% respectively. Fish 4 and 5 also exhibited an increase in fecundity compared with historical maximums but of only 6% and 40% respectively.

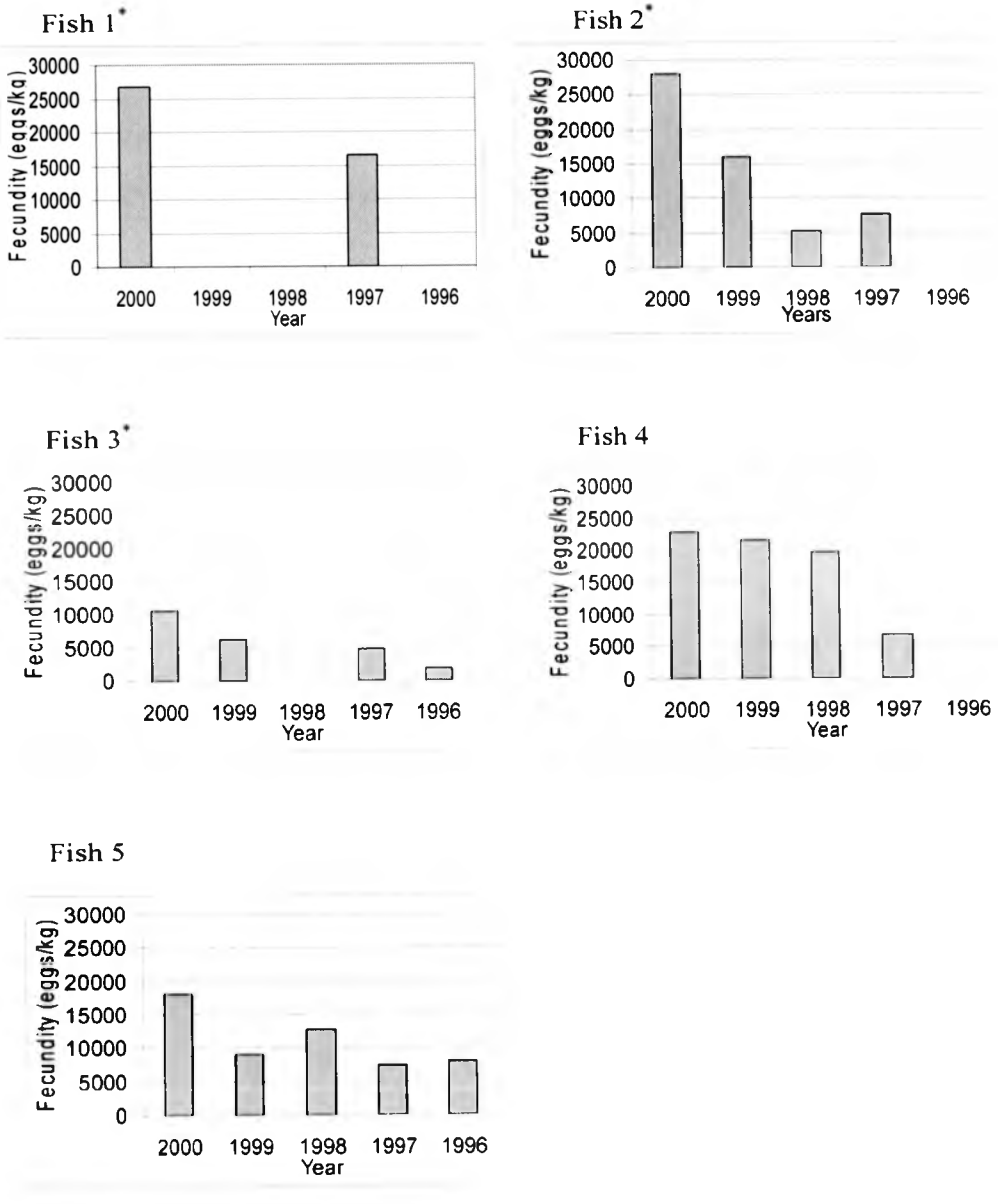

Figure 6.7: Effects of GnRHa on female relative fecundity (eggs/kg) in experiment 2 (year 2000) compared to historic records 1996-1999 for individual fish. Fish marked * were implanted during the experiment in 2000.

Table 6.3: Effects of two doses of GnRHa implantation on the spawning parameters of females in experiment 2. Spawning intervals are average (±s.e.m)

Female	Treatment	Relative fecundity No eggs/kg female	Batches/ female	Spawning interval (h)	Eggs/ml
1	10 µg /kg	26683	12	39 (±7.6)	51
2	10 µg /kg	28068	8	58 (±19.4)	52.8
3	5 µg /kg	10719	9	59(±8.6)	41.5
4	-	22725	7	72(±6.4)	49.5
5	-	17982	5	85(±20.7)	61

Figure 6.8 displays the Unweighted Paired Group Method for arithmetic Averaging (UPGMA) representation for the spawning parameters compared in experiment 2. This clearly shows, when considering the ranked data for spawning criteria, two distinct groups. Group 1 constituted the control fish (fish 4 and 5) while group 2 constituted the implanted fish (fish 1-3). Within the second group there was a further separation. Fish 1 and 2 exhibited a closer correlation to each other than they did with fish 3.

6.3. Effects of GnRHa implantation on sperm production of male halibut.

6.3.1. Effects of GnRHa implantation on spermatocrit. Experiment 3.

6.3.1.1. Materials and Methods.

Eleven hatchery reared mature male halibut (average weight 3.16 ±0.25 kg) were maintained in a 10m diameter, 2m deep tank, at ambient temperature (6-9° C) and photoperiod. Other 40 broodstock halibut, including spawning females, were kept in the same tank. Five fish were implanted with pellets containing GnRHa, at a dose of 25µg/kg of fish (7 March 1997). Six fish were implanted with a sham pellet with no GnRHa content (see table 6.4). All fish were producing milt at the time of implantation. Milt samples were collected on days 0, 3,

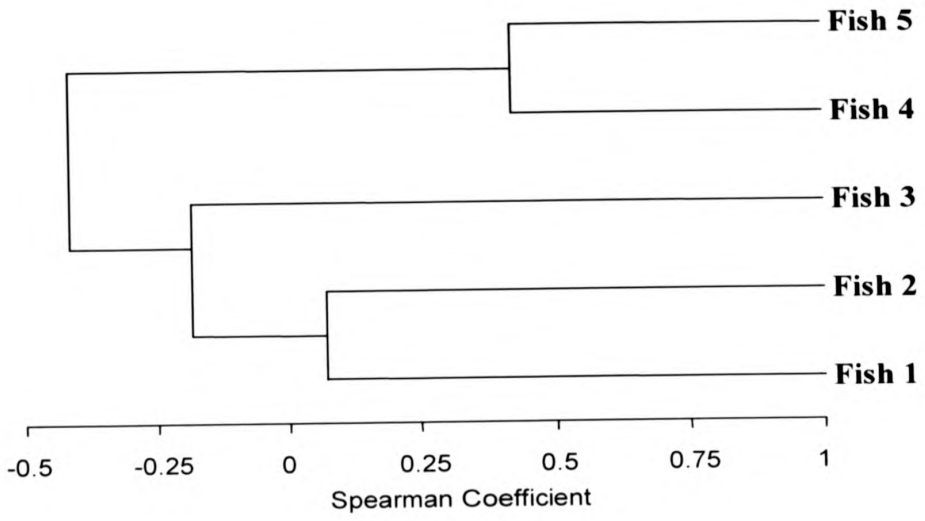

Figure 6.8: Unweighted Paired Group Method for arithmetic Averaging analysis of ranked spawning performance from fish 1-5 during experiment 2.

6, 10, 20, 31, 34, 47 and 62 and spermatocrit measured (see Chapter II section 2.2.3 for details of spermatocrit measurement).

On day 47 following treatment, the fertilisation capability of milt pooled from the GnRHa implanted fish was compared to that of the milt pooled from the sham implanted.

Table 6.4: weight and GnRHa dose of the fish used in the experiment.

Male	Weight	Dose GnRHa
1	3.6	-
2	2.5	-
3	2.8	-
4	3.8	-
5	2.6	-
6	2.8	-
7	4.2	23.8 µg/kg
8	4	25 µg/kg
9	3	25 µg/kg
10	3.9	25.6 µg/kg
11	1.6	31.6 µg/kg

Two replicates of 50 ml of eggs per individual milt were fertilised following the standard hatchery procedure (see Chapter II section 2.2.4.1) in 1000 ml beakers, with 1 ml of milt diluted in 500 ml of seawater. Eggs were rinsed after 30 minutes with filtered, u.v. sterilised seawater and left to stand in clean seawater inside an incubator at 6° C. At 16 h post fertilisation, the fertilisation rate relative to the floating fraction of eggs was recorded. A third sub-sample of eggs was fertilised at the same time using milt pooled from male halibut from established production broodstock, and used for comparison as a form of control. Fertilisation values of the experimental groups were expressed as a percentage of this "control" fertilisation. Relative fertilisation was preferred in this instance to total fertilisation, to avoid as much as possible the influence of varying egg quality.

6.3.1.2. Results.

Figure 6.9 shows the spermatocrit values (mean ± sem) of the two treatments for the duration of the experiment. There was an initial reduction of spermatocrit from initial levels in both groups on day three after treatment. This reduction was only significant in the GnRHa treated fish ($P < 0.0005$). The spermatocrit of implanted fish fell from 47% (± 8.5) on day 0 to 22% (± 4.3) on day 34. On day 62 only two implanted males expressed milt. From day 3 onwards, the spermatocrit of control fish increased from a mean value of 37% (± 2.0) to 79% (± 5.5) on day 62.

Relative fertilisation values from implanted and non-implanted fish on day 47 after treatment were 111% and 108% respectively of the value of the "control" fertilisation (Absolute values, Implanted = 36%, Non-implanted = 35%, Control fertilisation = 32%). There was no apparent difference in fertilisation capability between implanted and non-implanted fish.

6.3.2. Effects of two different doses of GnRHa implantation on spermatocrit and milt quality. Experiment 4.

6.3.2.1. Materials and Methods.

Twenty-one hatchery reared mature male halibut (3,4 ± 0.11 kg) were tagged with PIT tags and maintained in a 10m diameter, 2m deep tank, at ambient temperature (6-9° C) and photoperiod. Other 40 broodstock halibut, including spawning females, were kept in the same tank. Fish were randomly allocated to three different treatment groups (see table 6.5) as follows:

- High dose GnRHa implant (Target dose of 25 µg / kg of fish).
- Low dose implant (Target dose 5 µg / kg of fish).
- Control with no form of implant.

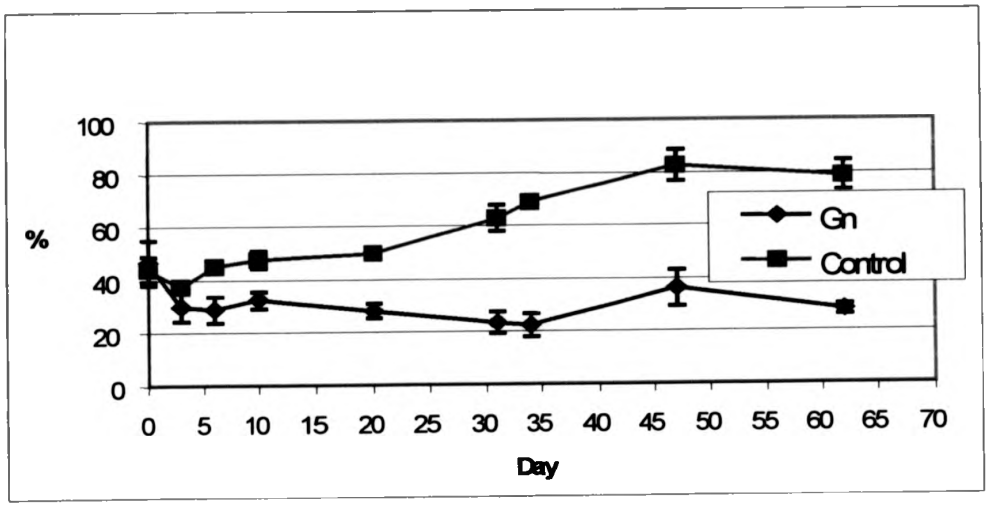

Figure 6.9: Mean spermatocrit (% ± sem of packed cell volume in milt) of GnRHa implanted and non-implanted control male Atlantic halibut (experiment 3).

Control, non-implanted males, were subjected to the same procedures as implanted fish. On day 0, 10, 20 and 40, blood (1 ml) and milt samples (0.5-1 ml) were collected and plasma separated as in experiment 1. On day 68 all milt was stripped from each male On day 82 males were sampled for milt.

Table 6.5: Weights (kg) of fish and GnRHa dose used in experiment 4. HD is high dose treatment; LD is low dose treatment; C is control treatment.

Male	Weight	Treatment	Dose GnRHa
1	2.7	C	-
2	2.7	C	-
3	3.1	C	-
4	3.2	C	-
5	3.5	C	-
6	4.0	C	-
7	4.4	C	-
8	2.9	HD	25.86 µg/kg
9	3.0	HD	25.00 µg/kg
10	3.1	HD	24.19 µg/kg
11	3.4	HD	22.39 µg/kg
12	3.5	HD	21.43 µg/kg
13	3.9	HD	25.64 µg/kg
14	4.0	HD	25.00 µg/kg
15	2.5	LD	5.00 µg/kg
16	3.3	LD	3.85 µg/kg
17	3.0	LD	4.17 µg/kg
18	3.5	LD	3.62 µg/kg
19	3.5	LD	7.14 µg/kg
20	3.7	LD	6.76 µg/kg
21	4.0	LD	6.25 µg/kg

On days 0, 10, 20 and 68 after treatment, single batches of eggs were fertilised using the experimental procedure described in Chapter II section 2.2.4.2. Milt from the individual fish was dissolved in milt extender according to spermatocrit, to achieve similar cell concentrations (see Chapter II section 2.2.4.2). Sub-samples from single batches of

eggs were used for each fertilisation experiment. Fertilisation capability of the milt from the fish in the different groups was assessed by comparing the relative fertilisation obtained from these experiments. Relative fertilisation was preferred in this instance to total fertilisation, to avoid as far as possible the influence of egg quality.

6.3.2.2. Results

Figure 6.10 shows the spermatocrit of the different treatment groups for the duration of the experiment. Low dose and High dose treatments both produced a drop in spermatocrit by day 10. However, at day 40 post-treatment, the spermatocrit of the low dose treatment had increased over the initial values at day 0, and they were not significantly different from the values of the control group. On day 82 only two fish from the low dose group were still producing milt, with spermatocrit values of 20% and 72%. The spermatocrit of the high dose implanted fish remained significantly lower than that of the controls until the end of the experiment on day 82, when all the males in this group except two were still expressing milt.

Table 6.6 shows the relative fertilisation rates (average ± sem.) of the different treatment groups at different days post treatment. Fertilisation rates were similar for all the treatments on day 0, 10 and 20. On day 68, relative fertilisation values from the low dose treatment were significantly lower than those of the high dose treatment ($p < 0.05$).

Figure 6.11 presents the distribution of spermatocrit versus fertilisation on day 68. Very high values of spermatocrit (99-100%) produced very poor fertilisation. However, spermatocrit values of 95% to 3% related to similar good fertilisation rates.

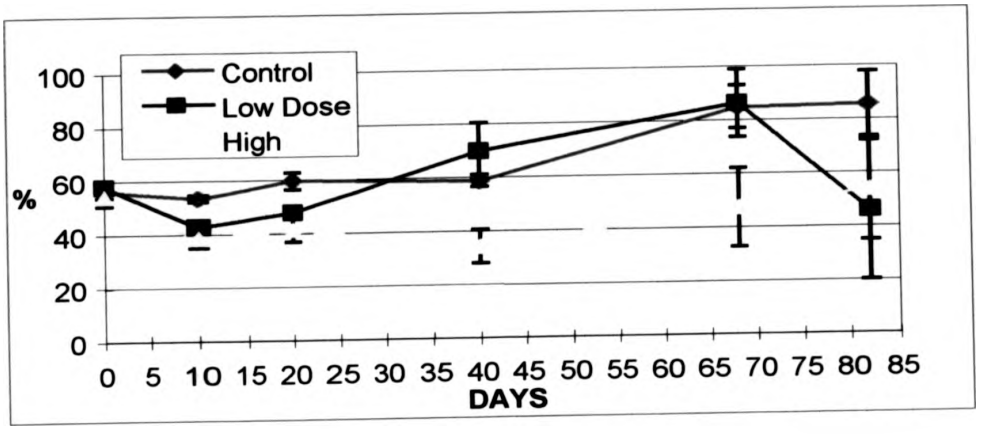

Figure 6.10: Spermatocrit (mean +-sem) of high dose, low dose and non-implanted control male halibut during experiment 4.

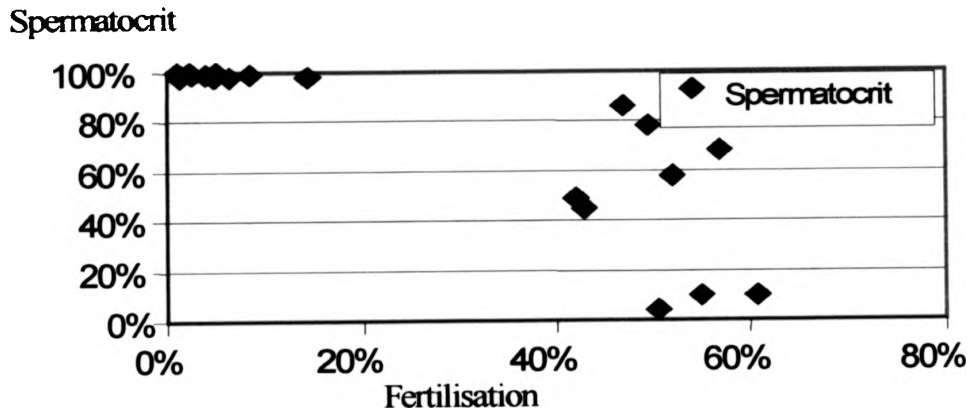

Figure 6.11: Distribution of spermatocrit vs. fertilisation on day 68 after treatment in all groups of experiment 4.

Table 6.6: Relative fertilisation rates (mean +-sem) of control fish and those implanted with high and low doses of GnRHa.

Fertilisation	Day 0		Day 10		Day 20		Day 68	
		SEM		SEM		SEM		SEM
Control	43%	2.0	77%	0.5	88%	0.6	27%	8.8
Low dose	43%	2.2	77%	0.8	89%	0.5	11%	7.3
High dose	42%	2.4	76%	1.4	89%	0.8	44%	8.7

6.3.3. Effects of end-of-season GnRHa implantation on spermiation. Experiment 5.

6.3.3.1. Materials and Methods.

Three groups of 6 male Atlantic halibut were maintained in a 10 m diameter, 2m deep tank, at ambient temperature (6-9° C) and photoperiod. Other 40 broodstock halibut, including spawning females, were kept in the same tank. Milt samples were collected weekly for measurement of spermatocrit. Once the spermatocrit of each fish reached 100% the fish from two of the groups were implanted either with a pellet containing 25µg GnRHa / kg of fish (Gn group), or a sham pellet with no GnRHa. Fish in the third group received no implant (intact controls). From the day of implantation onwards, blood and milt samples were collected weekly and fertilisation trials conducted from fish in all three groups, following the experimental method explained in Chapter II section 2.2.4.2. Sperm motility was estimated for each individual on each sampling day, as the percentage of spermatozoa actively moving after activation with sea water (in three sub-samples of milt per individual per sampling). Single batches of eggs were used for each fertilisation experiment. The amount of milt used in each fertilisation was calculated to maintain equal numbers of sperm from each male. Fertilisation capability of the milt from the fish in the different groups was assessed by comparing the relative fertilisation obtained from these

experiments. Relative fertilisation was preferred in this instance to total fertilisation, to avoid as far as possible the influence of varying egg quality.

GnRHa and steroid concentrations in plasma were analysed by RIA (Chapter II section 2.9). To assess which steroids presented significant concentrations, pools of plasma from the GnRHa implanted fish from days 0, 7 and 14 post treatment were analysed for concentrations of androstenedione (4-androstene-3,17-dione), 17, 20α-P (17α, 20α-dihydroxy-4-pregnen-3-one), triol or 17, 20β, 21-P (17α, 20β, 21-trihydroxy-4-pregnen-3-one), testosterone (17β-hydroxy-4-androsten-3-one), 11-ketotestosterone, 11-deoxycortisol (17, 21-dihydroxy-4-pregnene-3, 20-dione), 17, 20β-P (17α, 20β-dihydroxy-4-pregnen-3-one), oestradiol-17β, and 3β, 17, 20β-P-5β (3β, 17α, 20β-trihydroxy-5β-pregnane). Of these, only 17, 20α-P and 3β, 17, 20β-P-5β exhibited significant changes and were consequently analysed for each individual at each sampling point.

6.3.3.2. Results.

a. Milt production and quality

Figure 6.12 shows the changes on spermatocrit values of the different treatments during the experiment. In all three groups, spermatocrit value increased throughout the spawning season until it reached 100%, i.e., extremely viscous milt with virtually no seminal fluid. Two fish in the control group and one in the Gn group never reached 100% spermatocrit. The spermatocrit of the GnRHa-treated fish dropped from 100% to averages of 63% and 32% on days 7 and 14 respectively, decreasing to 1% on day 40. Two males in this group had spermatocrit values of 0% on days 42 and 49 respectively, (i.e. only seminal fluid could be expressed from the testis). Fish from the other groups maintained high values until they stopped producing milt, with the exception of one fish, the last in the control group to produce any milt, which presented an unusually low value on day 42. On

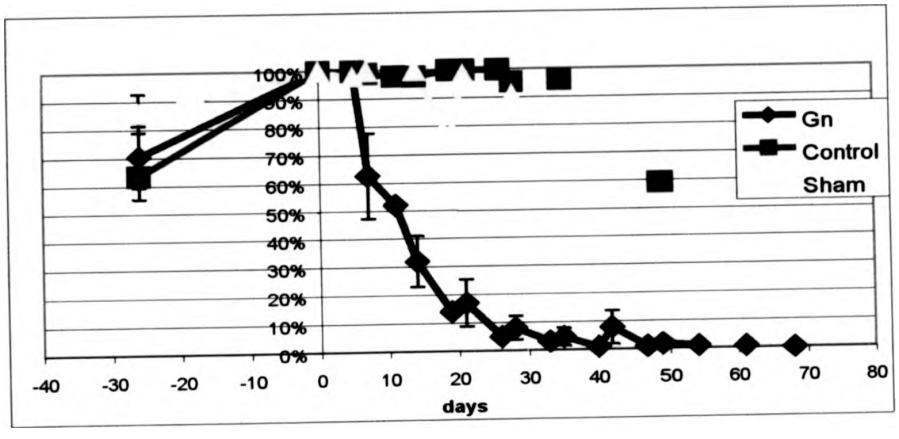

Figure 6.12: Changes in mean (± sem) spermatocrit values with time of the GnRHa and sham implanted fish and intact controls during experiment 5. Fish were monitored during spawning and treated when their spermatocrit reached 100% (day 0).

day 33 all the GnRHa treated fish were still running, while only one control fish was producing milt. On day 49 after treatment, all GnRHa treated fish but one were still running

Sperm motility of the GnRHa treated fish was significantly different from that of the other two groups from day 7 and generally increased after implantation (figure 6.13). Control and sham controls showed no significant difference between them, and presented low values after reaching 100% spermatocrit value.

Fertilisation trials showed that the milt from the GnRHa implanted fish produced good levels of fertilisation (average 76%±10 of a control fertilisation) until spermatocrit fell below 1% (figure 6.14). Sham and control groups produced lower values once they reached 100% spermatocrit (average 36%±15 and 12%±4 respectively).

b. GnRHa and steroids in plasma.

Figure 6.15 shows the plasma concentrations of GnRHa in the different treatment groups during the experiment. There was a significant difference between the GnRHa concentrations in the treated males and those of the fish in the other groups up to day 5. Levels fell below detectable levels on days 58 and 61 for shams and controls and on day 96 in GnRHa implanted fish. Of the steroids initially analysed, only free and sulphated 17, 20α-P showed noticeable variations during the experiment (figures 6.16 and 6.17), with concentrations substantially higher in the GnRHa implanted group for up to 60 days. Free 17, 20α-P showed the best correlation to spermatocrit and sperm motility (two tailed correlation p value of logarithmically transformed data = 0.0008). Concentrations of sulphated 3β,17, 20βP-5β, did not show any significant difference between treatments in the present study (figure 6.16).

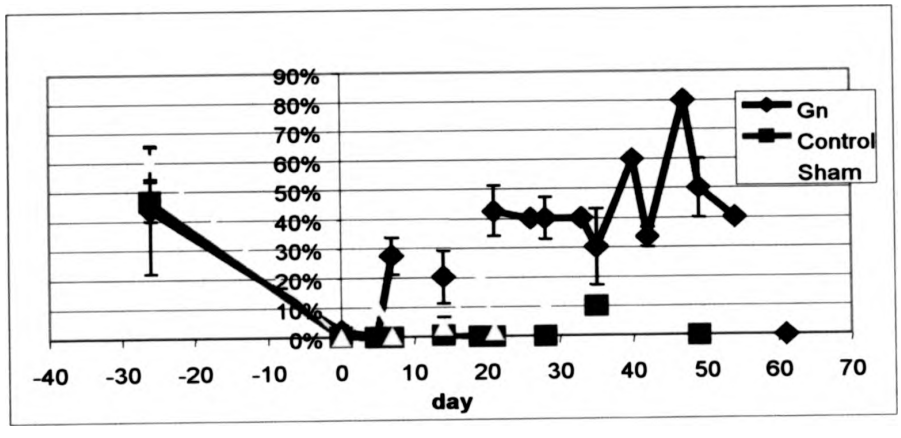

Figure 6.13: Changes in mean (± sem) sperm motility values with time of the GnRHa and sham implanted fish and intact controls until day 60 of experiment 5.

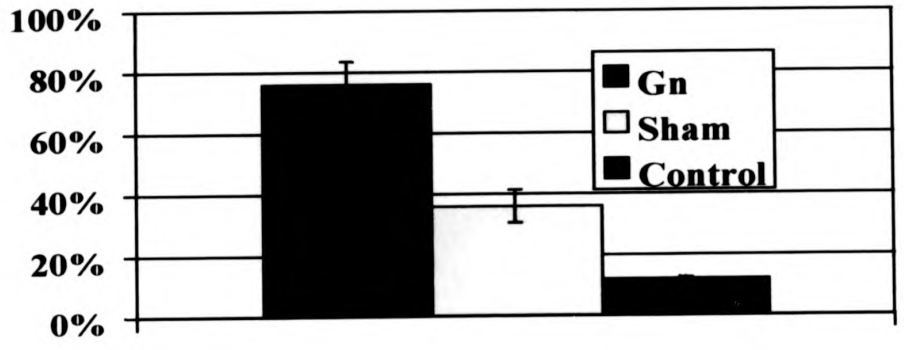

Figure 6.14: Mean relative fertilisation rates (± sem) from the GnRHa and sham implanted fish and intact controls in experiment 5.

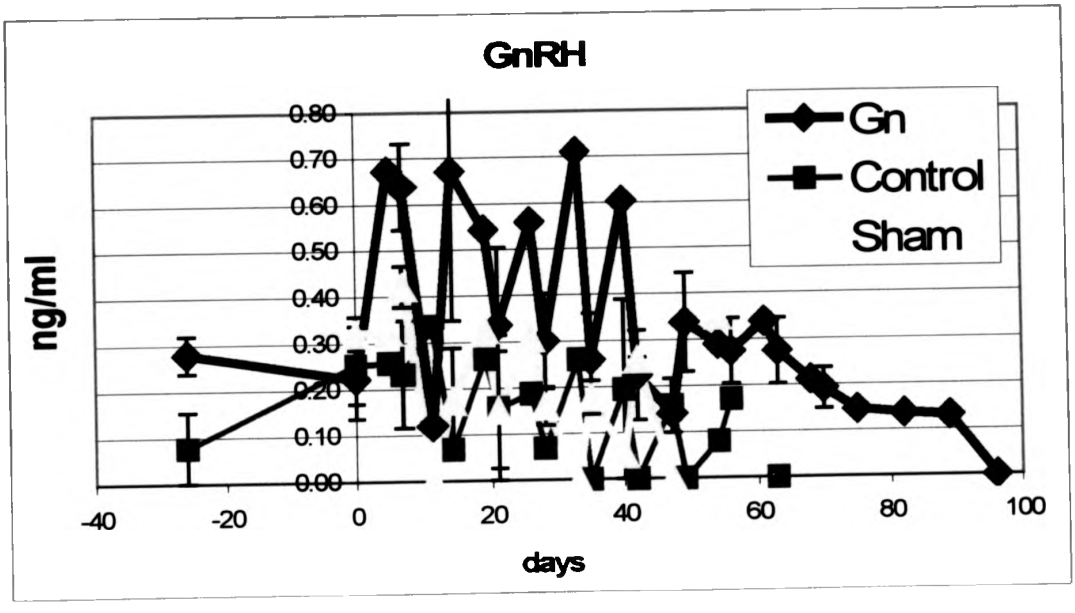

Figure 6.15: Changes in mean (± sem) plasma GnRH concentrations with time of males from the GnRHa and sham implanted fish and intact controls during experiment 5.

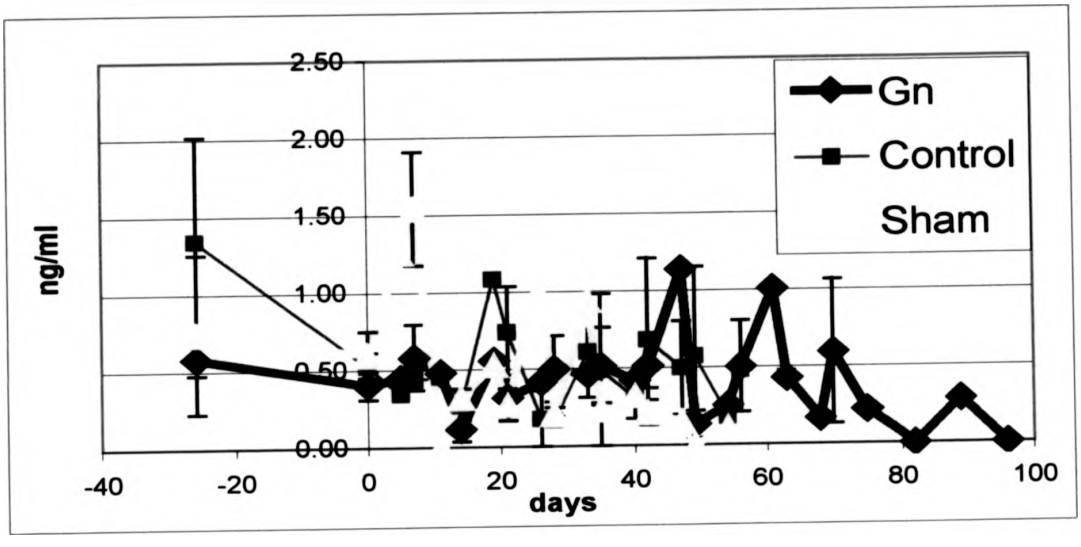

Figure 6.16: Changes in mean (± SEM) concentrations with time of sulphated 3β, 17, 20βP-5β in plasma of males from the GnRHa and sham implanted fish and intact controls during experiment 5.

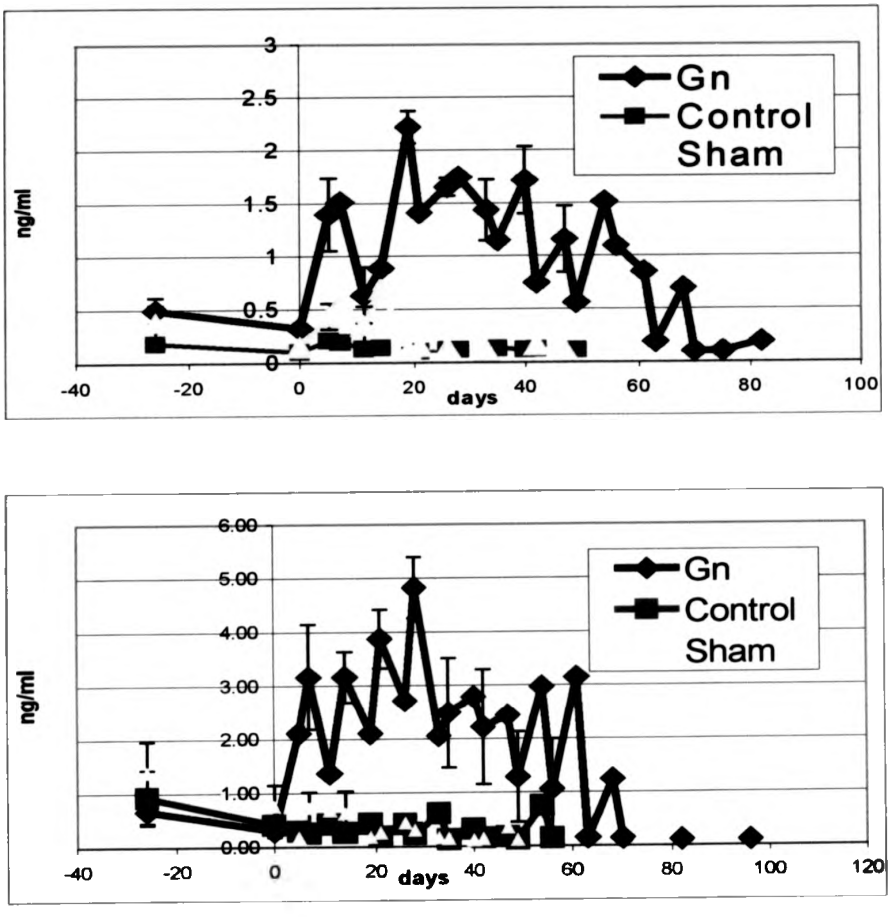

Figure 6.17: Changes in mean (± SEM) concentrations with time of free (top graph) and sulphated (bottom graph) 17-20-α-P in plasma of males from the GnRHa and sham implanted fish and intact controls during experiment 5.

6.4. Discussion.

6.4.1. Female implantation.

This work provides the first experimental data regarding the effects of GnRHa implants on the spawning performance of broodstock halibut. These preliminary data also suggest the doses of GnRHa treatment that have influences over spawning performance. Although the dose of GnRHa used in experiment 1 did not have a significant effect on the spawning parameters assessed, the analysis of steroid levels in plasma provided valuable information. Multivariate statistical analysis of the spawning performance of females in experiment 2 identified trends that suggest GnRHa implantation of female Atlantic halibut had influences on the spawning performance. The analysis clearly showed the difference between high dose treated, low dose treated and untreated individuals when considering a series of ranked spawning parameters.

Spawning induction:

Two out of three females implanted with GnRHa before the release of their first batch of eggs in experiment 1 began spawning well before the sham-implanted fish. All implanted fish in experiment 2 ovulated within 117 hours of implantation of a GnRHa pellet. Hormonal treatment has been successfully used for induction of ovulation and synchronisation of spawning in several flatfish species. Canario and Scott (1990c) found that Human Chorionic Gonadotropin (HCG) injections stimulated final maturation within 5 days of treatment in dab, and oocyte final maturation (but not ovulation) in plaice (Scott and Canario 1990). The time to first ovulation after hormonal treatment has been related to oocyte diameter in other marine batch spawners (e.g turbot, Mugneir *et al.* 1999; yellowtail

flounder, Larsson *et al.* 1997; and milkfish, Tamaru *et al.*.1988). It was impossible to obtain ovarian biopsies from females until very close to their first egg release. There were two distinct egg size classes in the biopsy obtained from fish 299 in experiment 1; one averaging 0.3 mm (± 0.02.) in diameter and the other with an average 1.9 mm (± 0.04.) diameter. Berlinsky *et al.* (1996) found that all female Southern flounder implanted with GnRHa ovulated synchronously, provided their oocyte diameter was equal to or over 500 µm. Similarly, Prat *et al.* (1993) found that turbot hormonally treated at oocyte diameters of around 300 µm, ovulated later than those with oocyte diameters of around 370 µm at the time of treatment. It is likely that the distension of the oviduct days before the first egg release in Atlantic halibut could be taken as an indication of the susceptibility of the oocytes to respond to the treatment, with larger oocytes present in this individuals. However, cannulation is a stressful procedure and may damage the oviduct of the females. Thus, in experiment 2 implantation was delayed until the females were assumed to be mature from their external appearance and ultrasound imagery. In a similar way, Alvariño and Peleteiro (1993) found that, when implanting female turbot according to the apparent development of the ovary lobes, there was a difference in the response of females with no apparent development of either of the two lobes, and females with different degrees of development of the individual lobes. In experiment 2, the delay between fish 1 and fish 2 suggests that they were implanted at different stages of maturation, with fish 1 being less advanced than fish 2. One implanted female had only spawned once previously in captivity. However, after implantation it released 7 batches of eggs, with a 67% increase in total fecundity compared to its previous spawning event. Similarly, wild-caught female winter flounder are reported not to release eggs unless treated with GnRHa implants, (Harmin *et al.* 1992). Wild caught halibut broodstock could be induced to spawn where the stress of handling and capture had inhibited this process.

Length of Inter-ovulation Interval:

During experiment 2, fish implanted with the higher dose of GnRHa exhibited a prolonged first spawning interval. However, this interval was greatly reduced in consecutive egg batches. Female 2 (dose 10 µg GnRHa/kg), exhibited a significant reduction in inter-ovulation time when compared to historical records. Fish 1 receiving the same treatment appeared to present the same trend, but this could not be statistically tested. The 5 µg/kg dose did not appear to produce a significant change in mean inter-ovulation interval. This indicates that the minimal effective treatment dose should be between 5 and 10 µg GnRHa/kg body weight. Treatment dose can influence the quality of the eggs produced. Gillet *et al.* (1996) suggested that the plasma GTH levels, stimulated by GnRHa treatment of female broodstock Arctic charr were negatively related to egg batch survival. Over-stimulation is also related to oocyte mass hydration that could lead to the death of the fish (see next section).

Fecundity:

In general, implanted fish exhibited an increase in fecundity when compared to unimplanted fish. This is also true in experiment 2 when comparing relative fecundities with data of previous seasons. Larsson *et al.* (1997) reported a two fold increase in egg production in yellowtail flounder treated with GnRHa, while Barbaro *et al.* (1997) reported a significantly higher production after GnRHa treatment of gilthead seabream. However, too high doses of GnRHa can induce massive hydration and over-stimulation of fecundity. Previous attempts to treat female Atlantic halibut using a hundred fold higher doses produced the death of the fish (J. McDonald, Orkney Marine Hatcheries, Scotland,

personal communication). Prat *et al.* (1993) reported that female turbot injected or implanted with LHRHa died after the second ovulation. Similarly, two female halibut implanted with slow release GnRHa analogous to the ones used in our experiments at a dose of 2 µg. GnRHa / kg body weight, but maintained at temperatures around 8-9°C, presented signs of over-stimulation (P. Smith, Seafish Aquaculture, U.K. personal communication). Each female produced only a single batch of eggs, and ovarian follicles were present among the ovulated eggs. Alvariño and Peleteiro (1993) observed pieces of ovary and blood clots in 45% of the egg batches stripped from female turbot implanted with 5µg.LHRHa/kg body weight, and in consequence recommended a lower dose treatment. No signs of over-stimulation were observed during experiments 1 and 2. Although doses were similar or higher than those reported by Smith, the temperature in the tanks was lower during the present experiments. It is possible that the difference in temperature affected the release pattern of the GnRHa. This highlights the importance not only of the choice of an appropriate treatment dose, but also of the control of environmental conditions during hormonal treatment.

Steroid concentrations in plasma:

Unfortunately it was not possible to measure GnRHa and steroid levels in plasma of the fish involved in experiment 2. However, the analysis of steroid levels in plasma of the females in experiment 1 provided valuable information.

In general, levels of oestradiol-17β in implanted fish increased after treatment to values around 10 ng/ml, decreasing shortly after the release of the first batch of eggs or just prior to it to levels around 1 ng/ml and remaining low until the end of spawning for each fish. Oestradiol-17β levels reported for a number of species show a wide range of

concentrations. Lowest levels around 1 ng/ml have been reported for the orange roughy, blue cod and bluefish (MacGregor *et al.* 1981; Pankhurst and Conroy 1987 and 1988). By contrast, salmonids have much higher levels. The highest, over 50 ng/ml, are found in brook trout and rainbow trout (Scott *et al.* 1983; Tam *et al.* 1986). The maximum levels found in this study (19.6 ng/ml) are in agreement with data published by Methven *et al.* (1992) for Atlantic halibut. These authors described how oestradiol increased from concentrations of around 3 ng/ml in July-August (previtellogenesis), to values of 20 ng/ml at the peak of vitellogenesis, falling to about 4 ng/ml at the start of ovulation. Minimum levels in the present study were circa 1 ng/ml before, or shortly after the first batch of eggs was released. Methven *et al.* (1992) also describe the appearance of multiple peaks of oestradiol during the spawning season. Similarly, Canario and Scott (1990c) found that concentration of oestradiol-17β in plasma of dab injected with HCG decreased 24h after treatment, to increase to 8.2 ng/ml at ovulation. Both works explain these as fluctuations corresponding to the development of different oocyte clutches and the involvement of oestradiol in vitellogenesis. No cyclic peaks were found in the present study, but this was probably due to the sampling interval. The range of concentration of E_2 was similar in implanted and non-implanted fish, however the timing of fluctuation was earlier in the season in GnRHa implanted fish. Scott and Canario (1990) found no significant difference in E_2 concentrations between plaice injected with HCG and non-treated controls, although they found a similar time delay related to the onset of spawning.

Androstenedione concentration in plasma behaved in a very similar fashion to oestradiol (E_2). In implanted females, concentration remained higher than E_2 (max. = 16.2 ng/ml). In sham implanted fish, however, androstenedione concentration was lower than that of E_2, with maximum values in female 042 at 16.7 ng/ml on day 5. 17α-hydroxyprogesterone is a common precursor of both 17α, 20β-DP (17α, 20β-dihydroxy-4-

pregnen-3-one) and androstenedione, which in turn is a precursor of oestradiol-17β (figure 6.3). The decrease in E_2 production in granulosa cells is related to a decline in aromatase activity and a GTH-induced increase in transcription of 20β-HSD mRNA (see review by Nagahama 1994). The reduction of androstenedione concentration is presumably due to the increase in 20β-HSD activity transforming 17α-hydroxyprogesterone into 17α, 20β-DP rather than androstenedione.

Overall, sulphated 11-deoxycortisol concentrations increased in the plasma of all females to values around 4 ng/ml at the time of first release of eggs; this then decreased towards the end of the spawning season. Scott and Canario (1990) found that concentrations of both free and conjugated 11-deoxycortisol rose significantly in plasma of HCG-implanted female plaice, decreasing later from peak levels to concentrations no different from non-implanted controls 88 h after treatment. However, ovulation was not induced in this experiment. In a similar experiment with dab the same authors (Canario and Scott, 1990) reported a transient increase in 11-deoxycortisol in both HCG-injected females and untreated controls, but found no evidence of it being synthesised at the time of oocyte final maturation and ovulation. The results of the present study seem to suggest a more important role of 11-deoxycortisol during ovulation.

Concentrations of 3β, 17,20β-trihydroxy-5β-pregnane 20-sulphate (3β, 17,20β -P-5β-S) in plasma reached high values during spawning in most females (maximum value 10.3 ng/ml). Values dropped after the release of the last batch of eggs. 3β, 17,20β -P-5β has been found in a number of teleost species, including the ayu (Suzuki *et al.* 1981), amago salmon (Nagahama *et al.*, 1983) and rainbow trout (Jalabert and Finet, 1986). At the time of final maturation in all teleost species studied there is a shift in steroid production from C18 to C21 steroids in ovarian follicles. In many of the species so far examined, 17α,

20β-dihydroxy-4-pregnen-3-one (17, 20β-P) produced from 17α-hydroxyprogesterone in the presence of 20β-HSD is the most potent C21 steroid in the induction of final maturation. However, in some teleosts 17, 20β-P does not seem to be involved, presenting only very low or undetectable concentrations in the plasma (see Fostier *et al.*, 1983). Canario and Scott (1989) point out that most of these species are batch spawners "in which only a fraction of the oocyte are matured and ovulated at any one time". It is suggested that despite low levels in some species, for instance the dab, 17, 20β-P would be both synthesised and utilised in the follicle and would not enter the blood stream. Once formed, 17, 20β-P can undergo 5β-reduction, 3α-OH or 3β-OH reduction and sulphation and the products of these reactions can be detected in plasma. 17, 20β-P was not detected in plasma of the female Atlantic halibut included in the present study. However, the highly significant levels of sulphated 3β, 17,20β -P-5β could be the result of the reduction of 17, 20β-P in the follicle. Further research should be directed to elucidate the relative role of 17, 20β-P, sulphated 3β, 17,20β -P-5β in Atlantic halibut final maturation.

6.4.2. Male implantation.

The spermatocrit of milt produced by captive Atlantic halibut progressively increases throughout the spawning season until it reaches values close to 100. The sperm motility in such highly viscous milt is dramatically reduced, and with that its fertilisation capability. This, together with the fact that males normally start producing milt earlier in the season than females, can bring about a shortage or lack of milt during the last weeks of the spawning season in commercial hatcheries.

The implantation of male halibut with GnRHa produced a significant reduction in spermatocrit of the milt produced. This suggests that there was a major stimulation of seminal fluid production and spermiation, independently of the possible stimulation of

spermatogenesis *de novo*. The progressive reduction of spermatocrit to very low values in experiment 4, where only seminal fluid was being expressed from the testis, seem to further support this view. There was no indication of new spermiogenesis being stimulated by GnRHa implantation in this last experiment. Mylonas *et al.* (1996) reported no reduction in sperm density in white bass after treatment with GnRHa embedded microspheres, and demonstrated that sustained GnRHa release increased spermatozoa production as well as seminal fluid. This is also true for many other species of teleosts (Yueh *et al.* 1990; Sorbera *et al.* 1996). However, Takashima *et al.* (1984), Garcia (1991, 1993) and Vermeirssen *et al.* (1998) reported reductions of sperm density after single injections of GnRHa in male carp, rabbitfish and slow release implants in plaice respectively.

Fertilisation capability was not affected by the treatment in experiments 3 (on the effect of GnRHa treatment on spermatocrit) and 4 (GnRHa dose experiment). It was only when spermatocrit closely approached 100% in untreated fish and motility was drastically reduced, that fertilisation rates decreased. Given that sperm numbers during the fertilisation experiments were the same for all individuals irrespective of spermatocrit, the explanation for the difference in fertilisation capability of 100% spermatocrit milt has to be physiological. The same pattern was observed in experiment 5, where sperm motility and fertilisation rates from non-implanted fish with spermatocrit values around 100% were severely reduced. However, GnRHa implantation was able to decrease spermatocrit, thus recovering the fertilisation capability of the milt and extending the availability of viable milt for over 50 days. Spermatocrit values closer to 0% also corresponded to low motility and fertilisation values.

It is well reported that progestagens play an important role in the regulation of spermiogenesis (Bromage and Cumaranatunga 1988). Vermeirssen *et al.* (2000) reported

increased concentrations of 17, 20β-P after GnRHa implantation in halibut males implanted with GnRHa in the middle of the spawning season. However, there was no such increase when fish were implanted after reaching 100% spermatocrit in experiment 5. This could probably be explained by a change in the physiological/enzymatic machinery at the end of spermiogenesis. Vermeirsen *et al.* (1999) also reported a 5% reduction in spermatocrit of male halibut from 100% to 95% 5 days after implantation with 17, 20β-P; however, there was no further sampling and the duration or final degree of this reduction was not reported. GnRHa implantation induced a conspicuous increase in plasma 17-20-α-P concentrations during experiment 5. Lee *et al.* (1998) described how sperm cells were able to metabolise 17-hydroxy-4-pregnen-3, 20 dione to 17-20-α-P. However, the progressive reduction in spermatocrit, and consequently of sperm numbers, makes it unlikely that the increase of 17-20-α-P after implantation can be explained by this. The concentrations of 17-20-α-P in experiment 5 presented a very significant correlation with sperm motility. Also, the reduction in motility of sperm from GnRHa treated fish after day 58 coincides with a marked decrease in 17-20-α-P concentration in plasma. The role of 17-20-α-P is not clear and further research on the effects of this steroid on cell preparations *in vitro* would probably help elucidating its possible participation in spermiation and sperm motility acquisition.

6.5. Conclusions.

GnRHa implantation was used for the first time on female Atlantic halibut with no detrimental effect on the performance of the fish. Implantation with doses of 10 μg. GnRHa / kg of body weight was effective in improving the spawning performance of

previously unsuccessful female Atlantic halibut. Holding conditions, and particularly temperature, should be carefully controlled when treating with GnRHa.

Free oestradiol, sulphated 11-deoxycortisol, free androstenedione and sulphated 3β, 17, 20β-P-5β were the main steroids present in plasma of pre-spawning and spawning females. 17, 20β-P, was not detected in plasma, but 3β, 17, 20β-P-5β seemed to be associated with spawning Levels of oestradiol-17β in implanted fish increased after treatment to values around 10 ng/ml, decreasing shortly after the release of the first batch of eggs or just prior to it to levels around 1 ng/ml and remaining low until the end of spawning. Androstenedione concentration in plasma behaved in a very similar fashion to oestradiol. Sulphated 11-deoxycortisol concentration increased in plasma of all females to values around 4 ng/ml at the time of first batch release, to decrease towards the end of the spawning season.

GnRHa implants were successful in reducing the spermatocrit (viscosity) of milt from male Atlantic halibut. Implantation at the end of the season, when spermatocrit is close to 100%, produced a major stimulation of seminal fluid production and spermiation, independently of the possible stimulation of spermatogenesis *de novo*. This improved milt motility and quality, and greatly increased fertilisation success. 17, 20β-P is suspected to play a major role in spermatogenesis of male Atlantic halibut. However, the role of 17-20-α-P is not clear and further research on the effects of this steroid on cell preparations in vitro would probably help elucidating its possible participation in spermiation and sperm motility acquisition.

A dose of 25 μg GnRHa per kg body weight towards the end of the spawning season of male Atlantic halibut, once spermatocrit values are above 90%, is recommended to enhance milt availability and quality.

Chapter VII: General Conclusions.

Atlantic halibut oocytes followed the same general processes described for the oocyte maturation in the majority of teleosts, including primary growth, folliculogenesis, cortical alveoli formation, vitellogenesis, and final maturation. Vitellogenesis started from late September, continued during the spawning season, and accounted for some 13% of the final egg size. Final oocyte maturation included yolk coalescence and hydration, responsible of some 80% of the final egg size, producing a transparent, buoyant egg.

GSI began to increase during autumn, with the commencement of vitellogenesis. High GSI values of around 16 were achieved during spawning, although the periodical final maturation and hydration of several batches of eggs, allowed for the increase of relative fecundity.

Ultrasound scanning was a powerful, non-intrusive tool for the study of maturation, assessment of gonadal development throughout the year, hydration and final maturation of egg batches, and resorption of gonadal material after spawning, with numerous applications in research and hatchery operations.

Artificially pelleted diets of adequate composition were suitable for Atlantic halibut broodstock. Nutrient intake and health status can be controlled more fully using fabricated diets with comparable spawning performance to that obtained from moist-trash fish diets.

Atlantic halibut eggs selectively accumulated DHA to an optimal value around 30% of the total fatty acids. Levels of DHA in the diets should be around 16% to avoid depletion of reserves for egg yolk formation.

AA was also selectively accumulated in the eggs. Levels of 1.8% of the total fatty acids in the diets produced improved fertilisation rates, egg quality scores and hatching rates. This could translate in better quality larvae capable of surviving stressful periods like the initiation of exogenous feeding.

DHA:EPA ratios of 3.5 in diets seemed to be excessive, and ratios closer to 2 are recommended. EPA:AA ratios around 4 appeared to be optimal for Atlantic halibut broodstock.

No differences were found in the spawning performance and egg quality of broodstock Atlantic halibut maintained at stocking densities of 5.21 kg/m^2 and 2.5 kg/m^2 respectively.

Recirculation of chilled water in Atlantic halibut broodstock tanks had no deleterious effect on spawning performance or egg quality.

GnRHa implantation was used for the first time on female Atlantic halibut with no detrimental effect on the fish performance. Implantation with doses of 10 μg. GnRHa / kg of body weight was effective in improving the spawning performance of previously unsuccessful female Atlantic halibut. Holding conditions, and particularly temperature, should be carefully controlled when treating with GnRHa.

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treatment to values around 10 ng/ml, decreasing shortly after the release of the first batch of eggs or just prior to it to levels around 1 ng/ml and remaining low until the end of spawning. Androstenedione concentration in plasma behaved in a very similar fashion to oestradiol. Sulphated 11-deoxycortisol concentration increased in plasma of all females to values around 4 ng/ml at the time of first batch release, to decrease towards the end of the spawning season.

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Glossary of common and scientific names of fish species mentioned in this thesis.

African catfish	*Clarias gariepinus*
American shad	*Alosa sapidissima*
Atlantic halibut	*Hippoglossus hippoglossus*
Atlantic salmon	*Salmo salar*
Arctic charr	*Salvelinus alpinus*
Ayu	*Plecoglossus altivelis*
Bluefish	*Pomatomus saltator*
Brook charr	*Salvelinus fontinalis*
Brown trout	*Salmo trutta*
Capelin	*Mallotus villosus*
Carp	*Cyprinus carpio*
Chum salmon	*Oncorhynchus keta*
Cod	*Gadus morhua*
Coho salmon	*Oncorhynchus kisutch*
Dab	*Limanda limanda*
Dover sole	*Solea solea*
European catfish	*Silurus glanis*
Gilthead sea bream	*Sparus aurata*
Goldfish	*Carassius auratus*
Herring	*Clupea harengus*
Japanese flounder	*Paralicthys olivaceus*
King Mackerel	*Scomberomorus cavalla*
Mackerel	*Scomber scombrus*

Milkfish,	*Chanos chanos*
Murray cod	*Maccullochella peelii peelii*
Pacu	*Piractus mesopotamicus*
Plaice	*Pleuronectes platessa*
Rabbitfish	*Siganus gattatus*
Rainbow trout	*Oncorinchus mikiss*
Sábalo	*Prochilodus platensis*
Sea bass	*Dicentrarchus labrax*
Sea bream or red sea bream	*Pagrus major*
Sockeye salmon	*Oncorhynchus nerka*
Southern flounder	*Paralichthys lethostigma*
Striped bass	*Morone saxatilis*
Sturgeon	*Acipenser transmontanus*
Tilapia	*Tilapia zillii*
Trout cod	*Maccullochella macquariensis*
Turbot	*Scophthalmus maximus*
Walleye	*Stizostedion vitreum*
Winter flounder	*Pseudopleuronectes americanus*
White bass	*Morone chrysops*
Whitefish	*Coregonus lavaraetus*
Yellowtail flounder	*Limanda ferruginea*
Zebrafish	*Brachydanio rerio*