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THE OVARIAN CYCLE OF RAINBOW TROUT, WITH SPECIAL REFERENCE TO ATRESIA

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Submitted for the degree of

Doctor of Philosophy

University of Aston in Birmingham

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To the memory of my Father

and

to my Mother

without whose unfailing efforts and guidance

I would not have had the opportunity to

accomplish a successful education.

Declaration

The work described in this thesis was carried out between 1982 and 1985 at the University of Aston in Birmingham. It has been done independently and has not been submitted for any other degree.



P.R.T. Cumaranatunga

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Thesis submitted for the degree of Doctor of Philosophy
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Summary

Light and electron microscopical techniques together with counts of oocytes at the different stages of development were used to study the structural and compositional changes in the ovary of three strains of rainbow trout (Salmo gairdneri) over the first three years of life. Concurrently, the seasonal changes in ovarian histology were correlated with circulating levels of oestradiol-17β and testosterone measured by radioimmunoassay and vitellogenin (as calcium) analysed by fluorimetry.

A methacrylate plastic embedding technique enabled 1-2 μ sections to be produced even of the largest yolky oocytes. Seven stages of oocyte development were identified, together with primary and secondary oogonia and hypertrophic and non-hypertrophic (oolytic) atresia. Oogonia were present throughout the reproductive cycle with the largest numbers occurring immediately after sex differentiation and also during the post-ovulatory period. After the first spawning stage 4 oocytes were present throughout the reproductive cycle indicating that there is no significant phase of ooplasmic vesicle formation. In contrast, for each strain and age-group of trout, vitellogenin incorporation occurred during a specific period of the annual cycle. In 1+ and 2+ year-old fish, vitellogenesis commenced during July/August and April respectively. Vitellogenin incorporation, assessed histologically and also by using horseradish peroxidase as a tracer, began under increasing or long daylengths whereas the later stages of maturation occurred under short or decreasing daylengths.

Atresia was a common phenomenon during vitellogenic phase of oocyte development (stage 4, 5, 6 and 7), with atretic oocytes numbering up to 700, $100g^{-1}$ body weight, i.e. 5-10% of the total numbers. Reductions in the numbers of previtellogenic oocytes were also evident, although clear evidence for atresia was not found in these stages.

Oestradiol-17β administration during vitellogenesis caused a significant increase in the number of oocytes undergoing atresia possibly as a result of the associated reductions in levels of testosterone and/or gonadotropin. Oestradiol although increasing hepatic vitellogenin synthesis, did not affect oogenesis or yolk incorporation.

Reductions in food intake increased the numbers of atretic oocytes, delayed the recruitment of oocytes into stages 5, 6 and 7 and also the time of spawning, reduced fecundity and decreased the sizes of both the developing oocytes and the eggs at ovulation. There was evidence for a critical phase during germinal vesicle migration 1-2 months prior to ovulation after which dietary alterations were without effect.

It is suggested that atresia is the most significant determinant of fecundity in the rainbow trout.

Key words: Oocyte development, atresia, trout, steroids, diet.

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CHAPTER 1

General Introduction

General Introduction

In all vertebrates, including fish, ovarian development involves a complex series of interrelated events whose timing is cued and subsequently modulated by environmental and hormonal mechanisms. In most mammals oocyte maturation is rapid and may only occupy a month or so of development. In contrast, in oviparous vertebrates particularly those where the mature egg contains large quantities of yolk (telolecithal eggs), oocyte maturation may take up to a year or more to complete. Over this extended period the ovary shows marked and successive changes in size and structure.

Although there are differences in ovarian morphology between species and different strains of the same species, development in salmonids and most other seasonally breeding teleosts can be divided into four well-defined phases, as follows: oogonial proliferation, oogenesis and folliculogenesis, endogenous and exogenous vitellogenesis, and maturation and ovulation (Vakaet, 1955; Takano, 1964; Yamamoto et al, 1965; Braekevelt and McMillan, 1967; Tokaz, 1973; Khoo, 1979; Hurk and Peute, 1979, Kagawa et al, 1981; Wallace and Selman, 1981; Nagahama, 1983). In the wild, timing of these phases in relation to the prevailing environmental conditions is important because it enables the production of young to be synchronised with the availability of optimal food supplies. Knowledge of this timing and the mechanisms of control of ovarian development are also important in the farming of fish, for aquaculture often requires that eggs and fry are available outside the normal spawning times in order to maintain a continuity of production.

The structural changes which occur during ovarian development in

the rainbow trout have only been studied on a limited number of occasions (Yamamoto et al, 1965; Hurk and Peute, 1979; Schulz et al, 1981; Schulz, 1984; Sumpter et al, 1984). Yamamoto et al (1965) and Hurk and Peute (1979) both described the different stages of oocyte development, but only Hurk and Peute (1979) included any comment regarding the relationship of these phases to calendar time. Schulz et al, 1981, Schulz (1984) and Sumpter et al, (1984) correlated oocyte development with the timing of changes in serum protein calcium and hormone levels although many of these constituents are related to exogenous vitellogenesis or spawning, these being the most easily defined phases of ovarian maturation. However, these studies provide only limited information about the timing of the other stages of oocyte development, ie. oogonial proliferation, oogenesis, the uptake of exogenous yolk, precursor proteins and oocyte maturation, all of which are equally important in the formation of mature oocytes.

Of all the stages of oocyte development possibly the uptake of yolk precursor proteins is the most important because it is during this stage that much of the oocyte growth takes place. In all oviparous vertebrates, so far studied, the complex yolk precursor proteins, called "vitellogenins" by Pan et al (1969) are synthesised in the liver and then transferred via the blood to oocytes where they are sequestered by a receptor-mediated process (Follett and Redshaw, 1974; Wallace, 1978; Ng and Idler, 1983). The uptake of yolk proteins and their fate within the oocytes have been followed in the sheepshead minnow by Selman and Wallace (1982, 1983) using the large heterologous electron-dense tracer horseradish peroxidase (H.R.P.) as a marker for vitellogenin. This work demonstrated that horseradish

peroxidase (and hopefully other large molecular weight compounds particularly vitellogenin) leaves the perifollicular capillaries via an interendothelial route and then traverses the theca, patent intercellular channels in the follicular epithelium and lastly the pore canals of the vitelline envelope. At the oocyte surface vitellogenin is incorporated by micropinocytotic processes and translocated to growing yolk spheres in the peripheral ooplasm. The present work aimed to trace the path of uptake of large protein molecules in the rainbow trout oocytes using the horseradish peroxidase technique as described by Selman and Wallace (1982, 1983).

The process of yolk deposition is preceded by vesicle formation within the oocytes, with the former generally being referred to as true yolk deposition or exogenous vitellogenesis, and the latter, yolk vesicle formation or endogenous vitellogenesis (Yamamoto, 1956b, 1957; Tokaz, 1978; Hurk and Peute, 1979; Khoo, 1979; Wallace and Selman, 1981; Kagawa, 1981; Nagahama, 1983). Although most of the work cited above suggests that vesicle formation and yolk deposition are interrelated events, with both phases being essential for yolk formation during exogenous vitellogenesis, the interdependence of these two processes remains unproven. It is also not clear whether the categorisation of vesicle formation as a part of the vitellogenic phase of oocyte development is justified. In view of the quite different origins of the endogenous and exogenous yolk, possibly it would be more appropriate if vesicle formation were not referred to as endogenous vitellogenesis or the "yolk vesicle stage". This question is considered further in the present work, where for the first time the structure of all the different stages of oocyte development will be studied.

In addition to the limited histological information available on rainbow trout ovary, none of the studies cited above offers any explanation of the factors which control the numbers of developing oocytes and their recruitment to successive developmental stages. The only data available on oocyte numbers relate to the numbers of ripe eggs produced at ovulation, ie. fecundity (Scott, 1962; Nomura, 1963; Baiz, 1978; Springate and Bromage, 1984a). Although these and other studies (Bromage et al, 1984) have showed that there are differences in the sizes of eggs and the fecundities of different strains and age groups of trout, it is not clear what factors or mechanisms are responsible for these changes. Vladykov (1956), working with brook trout suggested that the fecundity of fish is determined by the levels of atresia and reported that 80 and 45% of the oocytes up to 1mm and 3.5mm in diameter respectively are lost before maturity and attributed these reductions to atresia although no counts of atretic oocytes were made. Levels of 27% atresia were also recorded in the chub sucker (Wagner and Cooper, 1963). In contrast, Henderson (1963b) reported only 6% of atresia in brook trout and suggested that atresia may not occur in natural populations. It is evident from the descriptions included in these publication that the structure of atretic oocytes was not fully understood.

Atresia in teleost ovaries has been the subject of many investigations (Bretschneider and Duyvene de Wit, 1947; Sathyanesan, 1961; Scott, 1962; Henderson, 1963b; Rastogi, 1966; Braekevelt and McMillan, 1967; Chan et al, 1967; Guraya, 1972, 1976; Macer, 1974; Khoo, 1975; Byskov, 1978; Saidapur, 1978; Lang, 1981), although sufficient histological evidence is not provided to describe

different stages of atresia. Some authors have expressed difficulties in identifying atretic oocytes in ovaries which have been separated in Gilson's fluid in preparation for counts of total numbers of oocytes (Macer, 1974). There are also difficulties in fully separating oocytes from the extra-follicular tissues. Consequently, many studies have determined the numbers of oocytes and the levels of atresia solely from histological sections (Macer, 1974; Townshend and Wootton, 1984). This technique may lead to errors in estimating the actual numbers of oocytes at the different stages of development and possibly more importantly the numbers of atretic oocytes. These sources of errors were avoided in the present work by counting the total numbers of intact and atretic oocytes. Other studies have shown this to be the most reliable method (see review by Bagenal, 1968). In addition to the estimation of the proportions of developing and atretic oocytes, comparisons were also made of the proportions of the different oocytes from fish sampled at different times of the year and from fish of different age groups and strains.

In recent years much information has accumulated on the hormonal changes which accompany gonadal development in different teleosts (Fostier et al, 1983; Idler and Ng, 1983; Goetz, 1983) including rainbow trout (Lambert et al, 1979; Whitehead et al, 1978a,b 1983; Elliott et al, 1979a,b 1984; Scott et al, 1980, 1984; Van Boheman and Lambert, 1981; Bromage et al, 1982a; Scott and Sumpter, 1983; Schulz, 1984; Sumpter et al, 1984). These have shown that reproduction is controlled by a cascade of neuroendocrine mechanisms involving the hypothalamus, pituitary gland and gonads (Upadhyay et al, 1978; Wallace and Selman, 1981; Bromage et al, 1982a; Idler and Ng, 1983; Whitehead et al, 1983; Elliott et al, 1984). In the rainbow trout

and probably all other teleosts oestradiol-17β is said to be involved in the control of exogenous vitellogenesis. However, it is the presence of high levels of oestradiol-17β during the mid-cycle of ovarian development (Whitehead et al, 1978b, 1983; Elliott et al, 1979a,b, 1984; Bromage et al, 1982a; Sumpter et al, 1984) and of raised vitellogenin levels in blood after oestradiol-17β administration (Elliott et al, 1979a, 1980) which provide the basis for this proposition. At present there is little evidence to suggest that oestradiol-17β is involved in the uptake of yolk by the oocyte. This question is further examined in the present work by correlating the levels of circulating oestradiol-17β with histological changes in the ovary, and also by studying the structure of the ovary after oestradiol-17β injection.

In most vertebrate groups it is the oestrogens and often oestradiol which provides the most important steroidal influence in ovarian development. However, in fish testosterone is found in higher levels than the oestrogens and in the rainbow trout increases in testosterone closely parallels those of vitellogenin during active vitellogenesis (Elliott et al, 1984, Sumpter et al, 1984). Testosterone is also found in high levels in other female fish (Campbell and Idler, 1976; Wingfield and Grimm, 1976; Stuart-Kregor et al, 1981). Despite the presence of such high levels a role for androgens in ovarian development has not been established. Yamazaki (1972) and Elliott (1984) both suggested that raised levels of testosterone may lead to the degeneration or resorption of ova ie. atresia, a process which may be of importance in the reduction of oocyte numbers. Kagawa et al (1982b), Nagahama et al (1982) and Young et al (1982c) working with amago salmon have suggested that the

changes of testosterone are involved with modifications in steroid synthesis in the ovary during development. In their "two cell type model" Kagawa et al (1982b), Nagahama et al (1982) and Young et al, (1982c), suggested that testosterone is produced within the follicular theca and after transfer to the granulosa, during the active vitellogenic phase it is aromatised to oestradiol-17β. histological studies of the ovary of the rainbow trout were conducted in parallel with measurements of circulating testosterone and oestradiol-17β levels in order to provide further information of the role of testosterone in oocyte development.

Although the gonadal development is under the direct control of the endocrine system, the triggering and subsequent modulation of the endocrine mechanims of control is achieved by a variety of geophysical parameters, eg. photoperiod, temperature, rainfall and salinity (de Vlaming, 1972; Donaldson, 1975; Peter and Hontela, 1978; Whitehead et al, 1978a,b, 1983; Bromage et al, 1982a, b; Elliott et al, 1984). Amongst these factors photoperiod is known to be the most important in the control of salmonid reproduction. Salmonids' dependence on photoperiod for the timing and regulation of reproduction has provided commercial farmers with simple methods for altering times of spawning (Hoover, 1937; Allison, 1951; Hazzard and Eddy, 1951; Nomura, 1962, Henderson, 1963a; Shiraishi and Fukuda, 1966; Breton and Billard, 1977; Whitehead et al, 1978a,b, 1983; McQuarrie et al, 1979; Whitehead and Bromage, 1980; Bromage et al, 1982a, 1984; Scott and Sumpter, 1983; Elliott et al, 1984; Scott et al, 1984). Most of the studies in which alterations in spawning have been induced by photoperiod manipulations have only been related to the timing of stripping or release of ripe eggs after ovulation, with

little consideration given to the involvement of photoperiod cues in the control of the earlier phases of oocyte development. A knowledge of the timing of the different stages of oocyte development and their relationship to the prevailing photoperiod should provide further insight into the nature and importance of daylength cues at times other than at ovulation. This will be of value to aquaculture because the successful farming of fish requires a full control of all aspects of the biology of the species in question with the control of reproduction arguably being the most important. Further knowledge of the control of reproduction requires a more complete understanding of histological changes which occur within the ovary over the year-long reproductive cycle. This may also prevent maturing fish from inadvertently being introduced into artificial photoperiodic conditions at an inappropriate phase of oocyte development.

Aside from the influences of photoperiod, another major factor which influences reproduction is nutrition. Diet restrictions have been said to delay maturation, decrease fecundity and reduce egg size (Townshend and Wootton, 1984; Springate et al, 1985). It has been reported that the reductions in fecundity have resulted from increased atresia (Scott, 1962; Townshend and Wootton, 1984). However, both these studies derived the levels of atresia from estimates of the reductions in either the vitellogenic or total numbers of oocytes (Scott, 1962; Townshend and Wootton, 1984). Neither study is able to provide unequivocal evidence for the absolute numbers of oocytes which have become subject to atresia. Hence in the present investigation the effects of starvation and feeding on low rations were correlated directly with the absolute numbers of atretic and intact oocytes at each of the different stages

of development.

In summary the present work provides an extensive histological study of the ovary of the rainbow trout of three different strains, viz Caribou, Grampian and Whitebrook and age groups. Measurements of concomitant changes in circulating oestradiol-17β, testosterone and vitellogenin (as calcium) have enabled correlations to be made between the structural changes in the ovary and the hormonal and environmental influences responsible for their control.

CHAPTER 2

General Materials and Methods

2.1 Fish maintenance, handling and, ovarian and blood sampling

2.1.1 Fish maintenance

The rainbow trout, Salmo gairdneri (Pisces: Salmonidae) was used throughout this study. It is the major farmed freshwater fish species both in Great Britain and the rest of Europe. Fish of several different stocks, strains and age groups were used, although in the main the strains used were Caribou, Grampian and Whitebrook, which under natural conditions spawned in October/November, November/December and December/January respectively. Further details regarding experimental protocol are included under the descriptions of each experiment. Attention was focused mainly on the histological changes which occur within the ovaries of fish maintained under natural conditions and also after a variety of hormonal treatments and modifications of ration.

The fish used for the monthly ovarian sampling were obtained from the Avon Springs Hatchery, Pewsey, Wiltshire, while those used for experiments carried out at the University of Aston were purchased from several commercial farms including Avon Springs Hatcheries (Pewsey, Wiltshire), Burwarton Trout Farm (Bridgnorth, Shropshire) and the Itchen Valley Trout Farm (Winchester, Hampshire). At Aston all the fish were housed in the Fish Culture Unit of the University. Whenever possible the fish used for each experiment were obtained from the same stock and population to avoid strain differences in ovarian morphology or rate of maturation.

All the fish used for experiments conducted at the University of Aston were maintained under constant 18 hr light : 6 hr dark (long day) conditions in a water recirculating system consisting of three

circular tanks with diameters of 0.9 m (Appendix 1). At the beginning of each experiment the weight and lengths of fish were recorded, and also as required, blood samples were taken from the Cuverian sinus. Fish were fed daily with a commercially available dry trout diet (Mainstream B.P. Nutrition, U.K. Ltd., Witham, U.K.) at rations as specified in the company's feed tables unless otherwise mentioned in experiments including different ration levels.

2.1.2. Identification of fish.

All experimental fish were marked with small numbered tags of appropriate colours (Charles Neal, Finchley Ltd., East Finchley, U.K.). These tags were fixed through the anterior margin of the dorsal fin by plastic pins punched through the holes in the tag and through the fin using a tagging gun. The gun (Kimbal Systems Ltd., Leics., U.K.) is commonly used by the clothing industry for attaching prices and labels.

2.1.3 Anaesthesia

In order to minimise handling stress all fish were anaesthetised before tagging, blood sampling or the injection of hormones and other solutions, using 2-phenoxy ethanol (Sigma Chemicals, Poole, Dorset, England) at a concentration of 1:20,000 in water. After carrying out the various manipulations fish were transferred to a tank with a rapid freshwater flow in order to aid their recovery from anaesthesia and to avoid post-sampling mortalities. Generally, fish recovered within five minutes.

2.1.4 Blood sampling

Blood samples were taken from the Cuverian sinus using Grade 19G and 21G sterile hypodermic needles (Gillette, U.K. Ltd., Middlesex) and Sarstedt Monovettes (Sarstedt Ltd., Beaumont Leys, Leicester, England). The blood was allowed to clot and centrifuged at 2500 rpm for 10 min and the supernatant serum removed and stored at - 20°C until hormonal and Ca^{2+} analysis.

2.1.5 Sacrificing

Ovarian samples were obtained from each fish by sacrificing them in a direct-current electric fish killer.

2.1.6 Ovarian sampling.

Ovaries were removed from all sacrificed fish and the left and right ovaries separately weighed. Right ovaries were fixed in Bouin's Fixative (section 2.2.1.1.a) while the left ones were preserved in Gilson's Fluid (section 2.3.1.a). For electron microscopical studies a small portion of the right ovary was fixed in modified Karnovsky's Fixative (section 2.2.2.1.a). Ovarian samples fixed in Bouin's and Karnovsky's fixatives were later used for histological studies while those preserved in Gilson's Fluid were examined macroscopically and then used for counting and other measurements of the oocytes. By using the body weight and the gonadal weight, the gonadosomatic index (G.S.I.) was calculated as indicated below.

2.1.6.1 Calculation of gonadosomatic index

Body weight of the fish = B g

Weight of the right ovary = R g

Weight of the left ovary = L g

Total ovarian weight = (R + L) g

$$\text{Gonadosomatic index} = \frac{(R + L)}{B} \times 100$$

2.2 Histology

Fixation, embedding and staining for light and electron microscopy were carried out as recommended by Bancroft & Stevens (1982) except where JB-4 plastic and Poly/Bed 812 araldite embedding techniques were used, here methods were as described on data sheets (Nos. 123 and 233) provided by Polysciences Inc. (Polysciences Ltd., Moulton Park, Northampton).

2.2.1 Light microscopy

2.2.1.1 Materials

(a) Fixatives

Bouin's Fixative

(b) Ethanol - Absolute, 95%, 75%, 50%, and 30%

(c) Xylene

(d) Embedding media

(i) Wax - 'Paramat' ('Gurr's', Baird & Tatlock, Romford, England)

(ii) JB-4 Embedding Kit (Polysciences, Inc. Moulton Park, Northampton)

(e) Plastic Embedding moulds

(f) Gelatin capsules (Agar Aids, Stansted, Essex)

(g) Ester wax

(h) Glycerine albumin

(i) Staining

(i) Ehrlich's Haematoxylin and Eosin

(ii) Mallory's Triple stain

(iii) Lee's Methylene blue

(iv) PAS (Periodic Acid Schiff) solution

(j) Acid alcohol (0.5% HCl in 70% Ethanol)

(k) Scott's Tap water

Sodium bicarbonate 3.5 g

Magnesium sulphate 20 g

Tap water 1000 ml

(Dissolve salts separately and mix)

(l) Slides, cover slips etc.

(m) D.P.X. mountant.

2.2.1.2 Methods

a) Fixation

Ovarian samples were cut into blocks with a thickness less than 7.5 mm. before introduction to Bouin's fixative. Fixation was carried out for 6-24 hours depending on the size of the oocytes within the ovarian sample. Oocytes containing large amounts of yolk were allowed to fix for 24 hours. After the fixation was completed the ovarian tissue was rinsed and then stored in 70% ethanol.

b) Embedding

Initially ovarian tissue was embedded in paraffin wax although difficulties were experienced in sectioning due to the hardness of the yolky eggs and incomplete impregnation with wax. Subsequently JB-4 plastic embedding technique was used which helped to overcome the above difficulties.

(i) Wax embedding

Samples fixed in Bouin's fixative and stored in 70% ethanol were dehydrated by passing through 95% ethanol and two to three changes of absolute ethanol. They were kept in each solution from a few minutes

to several hours depending on the size of the oocytes. Ovarian samples with mature or yolky oocytes were kept in each solution for more than 12 hours. After the completion of dehydration samples were cleared in xylene and embedded in paraffin wax (M.P. 55-60°C). For complete impregnation the duration and number of changes in wax varied with the size and consistency of the sample. Samples with yolky oocytes were left in wax for at least three days and within this period wax was changed on at least three occasions. When the impregnation was completed ovarian samples were embedded in wax and stored at 4°C until used for microtomy.

(ii) JB-4 plastic embedding.

During wax embedding, wax did not readily penetrate the chorion of the yolky oocytes and this resulted in holes in the sections. In many cases the yolky contents also became separated from the egg chorion making it impossible to section entire oocytes.

JB-4 infiltration medium

JB-4 solution A	100 ml
JB-4 catalyst	0.9 g

By storing the above solution at 4°C it remained active for several months.

JB-4 embedding medium

JB-4 infiltration medium	17.5 ml
JB-4 solution B	1.0 ml

This medium was prepared just before use. Preparation was carried out at 4°C in order to slow down the rate of polymerisation. It was

stirred by the aid of a magnetic stirrer.

Ovarian samples which were fixed in Bouin's fixative and stored in 70% ethanol were transferred directly into the JB-4 infiltration medium. Complete dehydration was not necessary due to the hydrophilic nature of the infiltration medium. Infiltration was carried out at 4°C, for 1 to 2 weeks, with 2 to 3 changes within that period, depending on the size of the oocytes in the ovarian sample. Large yolky oocytes gave a better penetration when infiltration was carried out over longer periods. When infiltration was complete the specimens became transparent and sank to the bottom of the vial used. At this point samples were transferred into gelatin capsules containing freshly prepared JB-4 embedding medium. Subsequently the capsules were closed and embedding was allowed to become completed at 4°C. At all times precautions were taken to prevent moisture entering the embedding medium, during its preparation and embedding because this produced a softening of the blocks. Soft blocks resulted in difficulties in sectioning.

Temperature was kept at 4°C in order to slow down the rate of polymerisation. Rapid polymerisation prevented adequate penetration of the embedding medium into the oocytes, resulting in incomplete polymerisation and the appearance of holes within the specimen on sectioning. The gelatin capsules were removed from the blocks by cutting them carefully with a razor blade. It was not possible to remove the gelatin capsules by boiling due to the hydrophilic nature of the polymerised JB-4 plastic blocks.

Direct handling of the plastic infiltration and embedding media, was avoided because they cause haemolysis and serious skin

eruptions. Thick rubber gloves were used to protect hands as JB-4 solutions penetrates thin surgical gloves.

(c) Microtomy

Specimens embedded in wax were sectioned at a thickness of 4 to 6 μ using a rotary microtome (Baird & Tatlock, Romford, England) and the sections mounted on glass slides.

Ovarian samples embedded in JB-4 plastic were mounted on wooden blocks using ester wax. Sections were obtained from these blocks at a thickness of 1 to 2 μ using a base-sledge microtome (Measurements and Scientific Equipment Ltd., Crawley, Sussex, England). Sections were floated in a water bath and mounted on clean glass slides. Blocks which were too soft were left in a desiccator containing silica gel for a few days to remove the moisture and thus avoid consequent difficulties in sectioning. Hard and brittle blocks were left in a desiccator with a jar of saturated potassium bicarbonate solution. Difficulties in sectioning hard blocks were overcome by simply breathing on the block before cutting each section. Slides mounted with sections were dried at 50°C and stored in a cool dry place until staining.

(d) Staining

Wax-embedded sections were left in xylene for 2 to 5 min. to remove wax, and hydrated through a series of ethanol (absolute, 90%, 70%, 50% and 30%) before staining. For JB-4 plastic sections hydration was not necessary and slides were transferred directly into staining solutions. Ehrlich's haematoxylin and eosin, Lee's methylene blue-basic fuchsin and PAS stain gave satisfactory staining

in both wax and JB-4 plastic embedded sections, while Mallory's Triple staining technique gave good results only with wax-embedded sections. Mallory's stain's showed poor penetration through JB-4 plastic embedded materials. In order to achieve better stained plastic sections, with Mallory's stains, slides had to be left in staining media for prolonged period (twice the recommended time) although this resulted in significant amounts of background staining. During staining of plastic embedded sections with Ehrlich's haemotoxylin, acid alcohol differentiation could not be accomplished, as it removed the sections from the slides. Consequently, the sections were stained progressively with haemotoxylin and eosin. Stained preparations were mounted under DPX and examined and photographed using a Photo Microscope II (Carl Zeiss, W. Germany).

2.2.2 Electron micrscopy

2.2.2.1 Materials

(a) Modified Karnovsky's Fixative (Karnovsky, 1965)

Paraformaldehyde	2 g
1M sodium hydroxide	1-3 drops
50% gluteraldehyde	5 ml
0.1 M phosphate buffer (pH 7.4)	
Sucrose	2 g

2 g of paraformaldehyde was dissolved in 25 ml of distilled water by stirring and heating to 60-70°C. 1-3 drops of sodium hydroxide was added until the solution became clear. Then the solution was cooled and 5 ml gluteraldehyde and 2 g sucrose were introduced. The solution was made up to 50 ml by adding 0.1 M phosphate buffer (pH 7.4).

(b) 0.1 M phosphate buffer (pH 7.4).

(c) 1% Osmium tetroxide solution in 0.1 M phosphate buffer (pH 7.4).

(d) 4% Sucrose in 0.1 M phosphate buffer (pH 7.4).

(e) Ethanol - Absolute, 95%, 70%, 50% and 30%.

(f) Epoxy-propane (propylene oxide).

(g) Poly/Bed 812 embedding kit (Polysciences Inc., Polysciences Ltd., Moulton Park, Northampton).

(h) Gelatin capsules (Agar Aids, Stanstead, Essex).

(i) Copper grids (300 mesh).

(j) Freshly prepared 50% uranyl acetate in 50% ethanol.

(k) Reynold's lead citrate (Reynold, 1963).

(l) 0.02M sodium hydroxide.

2.2.2.2 Methods

(a) Fixation

Oocyte-containing follicles were dissected out from the ovaries, immediately after the fish were sacrificed. These oocytes were fixed for 1-2 hours. Fixed oocytes were rinsed with 0.1M phosphate buffer containing 4% sucrose and then post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer.

(b) Embedding

Fixed oocytes were dehydrated rapidly through a series of alcohols and soaked in epoxy-propane for 10 minutes (2 changes) at room temperature. They were then left in a mixture of epoxy-propane and embedding medium (1:1 vv) for 60 minutes and then in a 1:2(vv) mixture of the same materials. Subsequently they were transferred into complete embedding mixture and left overnight. After two 1 hour changes in fresh embedding medium they were placed in gelatin capsules, containing embedding mixture, and the air bubbles removed under a vaccum for 10 minutes. Subsequently they were heated in an oven at 60°C for 24-48 hours in order to complete the polymerisation. When the embedding was completed the capsules were removed by boiling in water.

c) Microtomy

Embedded samples were sectioned at 60-90 nm thickness by using an ultramicrotome (LKB Ultrotome).

The sections were mounted on copper grids and left in a dry place before staining.

d) Staining

Grids containing the specimens were washed in 50% alcohol, stained with uranyl acetate for 5 to 15 min. and then re-washed in 50% alcohol. Subseqently sections were counterstained with lead citrate and washed in 0.02 M sodium hydroxide. Dry-stained grids were examined and photographed using a transmission electron microscope (AE1. EMGB).

2.3 Oocyte counting

Oocytes from fish of different age groups and after different treatments were sorted into stages and counted, in order to determine the fecundity and the rates of recruitment from one developmental stage to the next. Some of the methods which are used to count ovarian ova were reveiewed by Bagenal (1968). Several preservation methods are described, of which, the following are the most effective and widely used: boiling (Reibisch, 1899; Mitchell, 1913); 4% or 5% formalin (Scott 1962; Wagner & Cooper, 1963), Gilson's fluid (Franz, 1910) or a modification of this solution (Simpson, 1951). When compared with the former methods modified Gilson's fluid has been found to act satisfactorily with wide range of fish species as not only does this mixture harden the oocytes, but it also helps to liberate them from the remaining interstitial ovarian tissue (see review by Bagenal, 1966). Modified Gilson's fluid was used throughout the present study to preserve the oocytes from the left ovary of each fish sampled.

2.3.1 Materials

(a) Modified Gilson's fluid

Mercuric Chloride	20 g
60% ethanol	100 ml
80% nitric acid	15 ml
Glacial acetic acid	18 ml
Distilled water	880 ml

Mercuric chloride should be completely dissolved before adding the other components into the solution.

(b) 2.5 ml Plunger-sampling-pipette and a 250 ml dilution bottle (Hydro-bios, Apparatebau Gmbtt, Kiel-Holtenau, Fed. Rep. of Germany).

(c) S-shaped glass counting chamber specially designed for the present study (Appendix 2).

(d) Plastic storage jars with plastic lids. This avoids the corrosion problems caused by contact of Gilson's fluid with metal lids.

2.3.2 Methods

2.3.2.1 Preservation

Ovaries were removed immediately after the fish were sacrificed. The left ovary was weighed and its epithelium cut open along the axis of the ovary; it was then turned inside out before transfer into labelled jars containing Gilson's fluid. This enhanced the penetration of preservative into the ovary. Large ovaries were carefully dissected into several small pieces before preservation. Simpson (1959) reported that ovaries were sufficiently preserved after only 24 hours treatment. In contrast Pitt (1964) found that the best results were obtained if the ovaries had been treated in Gilson's fluid for 3 months. During the present study most of the samples were stored for at least 6 months in Modified Gilson's fluid. This gave a far better separation of oocytes and breakdown of ovarian tissue than treatments for shorter periods. During the preservation it is important that the ovarian samples are kept completely covered with preservative. Bagenal (1968) reported that gentle heating in an oven hardened the oocytes in Gilson's fluid, and

thus prevented damage to the oocytes during handling. Vigorous shaking of ovaries stored in Gilson's enhanced penetration and preservation by the fluid.

2.3.2.2 Counting of oocytes

In previous studies counting was carried out by two different methods. Smyly (1957) and, McFadden & Cooper (1964) counted the total number of oocytes in the whole ovary whereas Simpson (1951), Kändler & Pirwitz (1957), Bagenal, (1957, 1966) and Pitt (1964) counted the numbers of eggs in sub-samples. It was suggested that subsampling is a more practical method for counting eggs in more fecund fish species (see review by Bagenal, 1968). Parrish et al, (1960) and Baxter (1963) introduced an automatic fish egg counter although Bagenal (1968) in his review explained the difficulties in using such counters due to the interference of separated follicular and other ovarian tissue present in the preserved oocyte samples.

Before counting the oocytes the Gilson's fluid was drained off and the samples washed in distilled water. Precautions were taken to prevent any losses of small oocytes when draining the solutions. Larger pieces of tissue which had become detached from the oocytes were removed manually.

In earlier studies several sampling techniques were adopted to obtain the samples of oocytes. Simpson (1951) and Kändler and Pirwitz (1957) used a stempel pipette designed for sub sampling marine plankton (Hensen, 1887; Jenkins, 1901). Whereas Bagenal (1957, 1966) used a cylindrical museum jar half-filled with 2 litres of water containing the eggs and this mixture was stirred to obtain a homogenous sample, from which sub-samples were taken quickly with a

small glass or plastic jar attached to a rod. Pitt (1964) used a whirling flask designed for sub sampling plankton (Wiborg, 1951). During the present study a plunger-sampling pipetter of 2.5 ml capacity and a 250 ml dilution bottle was used (Hydro-bios, Kiel-Holtenau, Fed. Rep. of Germany).

2.3.2.2 (a) Method

All the oocytes in the left ovary which had been preserved in Gilson's fluid were separated from the other ovarian tissues and placed in the 250 ml dilution bottle. In ovaries containing large vitellogenic oocytes with diameters greater than 1.5 mm., large oocytes were separated from the rest and counted manually. Only the oocytes below 1.5 mm were introduced into the dilution bottle. Then the sample was made up to 250 ml with distilled water. After placing the plunger-sampling-pipette attached to the stopper of the dilution bottle, the bottle was shaken vigorously in order to distribute oocytes within the sample. Immediately after shaking a sub-sample of 2.5 ml was obtained by lifting the piston of the plunger sampling pipette, and the sample transferred into the s-shaped glass counting chamber specially constructed for this study. The eggs within the sub-sample were counted and their diameters measured using a binocular microscope with a micrometer eye-piece graticule. They were then sorted into the different developmental stages on the basis of their diameters (section 2.3.2.2, Fig. 2.1 & Table 2.1). From each sample at least three sub-samples were used for counting. Intra-sample coefficient of variation was 6.16 $\pm$ 3.68% (mean + SD).

2.3.2.2. (b) Calculations

Total gonadal weight = G_t g

Weight of the right ovary = G_r g

Weight of the left ovary = G_l g

$$G_r + G_l = G_t \text{ g}$$

Volume of the total sample = 250 ml

Volume of a sub-sample = 2.5 ml

Total number of oocytes in a sub-sample = s_t

Total number of vitellogenic oocytes in a sub-sample = s_v

Total number of previtellogenic oocytes in a sub-sample = s_p

Total number of atretic oocytes in a sub-sampled = s_a

$$\therefore \quad s_t = s_v + s_p + s_a$$

Total number of oocytes in the left gonad = S_l

$$S_l = \frac{st}{2.5} \times 250$$

Total number of oocytes in both gonads = S_t

$$S_t = \frac{Sl}{Gl} \times Gt$$

$$= \left(\frac{st}{2.5} \times 250\right) \times \frac{Gt}{Gl}$$

Total number of vitellogenic oocytes in both gonads = S_v

$$S_v = \left(\frac{sv}{2.5} \times 250\right) \times \frac{Gt}{Gl}$$

Total number of previtellogenic oocytes in both gonads = S_p

$$S_p = \left(\frac{sp}{2.5} \times 250\right) \times \frac{Gt}{Gl}$$

Total number of atretic oocytes in both gonads = S_a

$$Sa = \left(\frac{sa}{2.5} \times 250\right) \times \frac{Gt}{Gl}$$

In the same way the number of oocytes at each developmental stage was calculated.

2.3.3 Measurements of oocyte diameters

Oocyte stages were identified in the 2 μ sections of the JB-4 plstic embedded ovarian samples [section 2.2.1.2.b(ii)]. Diameters of oocytes up to stage 5 were measured at X50 magnification while the larger ones were measured at X10 magnification, using a binocular microscope fixed with a micrometer eye-piece graticule. Measurements are given in Table 2.1 Using these data oocytes were grouped into stages according to their diameters.

The lowest diameter which could be measured under X50 magnification was 33 μ and all the cells which had a diameter below 33 μ were categorized as stage 1 oocytes. This may have caused an overestimation of stage oocytes, as oogonia which have a diameter around 17 μ also may have been included. Similarly late stage 1 oocytes may have been included in stage 2. This was due to the difficulty in superficially identifying stage 1 oocytes from oogonia and stage 2 oocytes after preservation in Gilson's fluid.

Table 2.1 Diameters of oocytes at different developmental stages.

Oocyte stage	number n	Mean Oocytes diameter μ	±	S.D.
1	17	36.501	±	7.701
2	46	160.892	±	80.660
3	126	371.806	±	58.636
4	65	726.310	±	193.300
5	6	1058.330	±	65.250
6	13	2313.640	±	608.898
7	15	4260.000	±	646.750

2.4 Radioimmunoassay for oestradiol-17β and testosterone.

Assays for oestradiol-17β and testosterone were carried out according to the method developed by Whitehead (1979) and Elliott (1982) in this laboratory. The materials and procedures are outlined in more detail below.

2.4.1 Materials

(a) Assay buffers

Disodium hydrogen phosphate	8.88 g
Sodium dihydrogen phosphate	5.82 g
Sodium chloride	4.5 g
Sodium azide	0.03 g
Gelatin	0.5 g
Analar water	

All the salts and gelatin were dissolved in analar water and made up to 500 ml.

(b) Ethyl acetate

(c) Labelled Hormones

2,4,6,7 - ^{3}H Oestradiol-17β
1,2,6,7 - ^{3}H testosterone

These labelled hormones were supplied by the Radiochemical Centre, Amersham, Bucks.

supplied as 80 Ci(curie) mM^{-1} ($1Ci=3.7 \times 10^{10}$ dpm)
stock solution 2.22×10^{7} dpm
working solution 20,000 dpm 100 μl^{-1}

To prepare the working solution 100 µl of stock solution which was made up in absolute ethanol was dried down under a N_2 current and reconstituted with 10 ml of assay buffer.

(d) Antiserum

Antiserum for both oestradiol-17β and testosterone (Steranti research, St. Albans, Herts) were provided as freeze dried pellets. To make the stock solutions the dry pellet was dissolved in 1 ml of assay buffer and stored in the freezer in 100 µl aliquots which when used were made up to 10 ml with assay buffer.

(e) Standard

A standard stock solution of 1000 pg 100 μl^{-1} was prepared in ethanol and from this working solutions were made by dilution of 100 µl samples to 1 ml using ethanol. The working solution contained 100 µg 100 μl^{-1}. Using this solution a series of dilutions ranging from 4 to 500 pg were prepared again using assay buffer.

(f) Dextran-coated charcoal tablets ("separex" - Steranti research, St. Albans, Herts).

Each tablet when dissolved in 50 ml of assay buffer to produce a homogenous suspension of dextran-coated charcoal. This solution was made up fresh for each assay and after preparation the temperature was always maintained at 4°C.

(g) Scintillant

Diphenyl oxazole (P.P.O)	20 g
Toluene	2.5 l
Ethoxy-ethanol	2.5 l

The above constituents were mixed to prepare 5 l of scintillant.

(h) Serum samples

2.4.2 Method

(a) 100 µl of each serum sample was pipetted out into separate polypropylene tubes (Hughes & Hughes Ltd, Romford, England).

(b) To each tube 2 ml of ethyl acetate was added and tightly stoppered.

(c) They were attached to a rotary mixer for 1 hour and then centrifuged for 10 minutes at 1500 rpm.

(d) 100 µl and 50 µl aliquots of supernatant solution were transferred to rimless soda assay tubes for oestradiol-17β and testosterone assays respectively. Smaller volumes of extracted steroid were used for testosterone assay because of the very high

(200 ng ml^{-1}) serum levels of testosterone at maturation (Elliott et al, 1984).

(e) A series of dilutions of standard hormones (section 2.4.1.e) were prepared (0-1000 pg 100 μl^{-1}) in duplicate and added to similar assay tubes.

(f) Extracts (section 2.4.2.d) and standards (section 2.4.2.e) were dried in a vacuum oven at less than 35°C.

(g) Dry samples were cooled in a refrigerator at 4°C.

(h) 100 µl of buffered antiserum (section 2.4.1.d) was added to each of the tubes, vortex mixed for 10 seconds and allowed to incubate at 4°C for 30 minutes.

(i) 100 µl of labelled hormone (section 2.4.1.c) was added to each of the tubes, vortex mixed for 10 seconds and allowed to incubate over night at 4°C.

(j) 0.5 ml of a dextran-coated charcoal suspension (section 2.4.1.f) was added to each tube, vortex mixed for 10 seconds and incubated for 10 min. at 4°C.

(k) The above mixture (section 2.4.2.j) was centrifuged at 2000 rpm (at 4°C) and 0.4 ml of the supernatant solution was transferred into scintillation vials containing 10 ml of scintillation fluid (section 2.4.1.g)

(l) 100 µl of labelled hormone (section 2.4.1.c) and 10 ml of scintillation fluid were added to two further scintillation vials. These were used to obtain the total number of counts added to each of the assay tubes.

(m) The level of background counts was obtained by adding 10 ml of scintillation fluid into another scintillation vial.

(n) Each vial (sections 2.4.2.k,l & m) was counted for 5 minutes in a scintillation counter (Packard Tri-carb 2660, M.S.E.)

(o) The amounts of hormone in each tube were calculated according to the method outlined below:-

2.4.3 Calculations

(a) Background counts = B (normally zero counts)

Counts obtained for 100 µl of labelled hormone = T

$\therefore$ Total counts = T - B

Since the background counts = 0

Total counts = T

counts obtained for the standards = s

counts obtained for the unknowns = u

(b) From the 0.7 ml available in each assay tube, only 0.4 ml was transferred into scintillation vials (section 2.4.2.k). Therefore to obtain the total counts for 0.7 ml each standard (s) and unknown (u) values should be multiplied by 0.7/0.4

Corrected counts for standards = $s \times \frac{0.7}{0.4} = S$

Corrected counts for unknowns = $u \times \frac{0.7}{0.4} = U$

(c) % bound calculation

% bound for standards = $\frac{S}{T} \times 100$

% bound for unknowns = $\frac{U}{T} \times 100$

(d) Standard curve

% bound for each standard was plotted against the concentration of steroid in each standard (0 - 1000- pg) in order to obtain a standard curve.

(e) Concentration of steroid (p.g) present in each of the unknown samples was read off the standard curve using the % bound calculated in section 2.4.3.c.

(f) From the p.g of steroid present in 100 µl of serum for oestradiol-17β and in 50 µl of serum for testosterone, calculated the amount of hormone found in 1 ml of trout serum (ng ml^{-1}).

(g) During each assay a serum sample with a known concentration of each steroid was assayed to find the inter-assay and intra assay variations. These were always found to be less than 10%. Cross reaction data of oestradiol-17β antiserum and testosterone antiserum are given in Tables 2.4.1 & 2.4.2.

Table 2.2 Cross reaction data for oestradiol-17β anti-serum with some other steroids (Elliott, 1982)

Steroid	% cross reactivity
Oestradiol-17β	Taken as 100
Oestrone	7.5
Oestriol	12.2
Testosterone	1.6
11-Ketostestosterone	1.0
Androstenedione	1.8
17α Hydroxy-20β-Dihydroprogesterone	< 0.5
Cortisol	< 0.5
Pregnenolone	<0.5
(Non specific)	2.1

Table 2.3 Cross reaction data for testosterone antiserum with some other steroids (Elliott 1982)

Steroid	% Cross reactivity
Testosterone	Taken as 100%
11 Ketotestosterone	34.5%
Dehydroepiandrosterone	20.0%
Oestradiol-17β	5.8%
Oestrone	2.9%
Cortisol	< 0.5%
17α Hydroxy-20β-Dihydroprogesterone	1.66%
17α Hydroxy progesterone	0.5%
Pregnenolone	3.3%
Non specific	1.8%

2.5 Assay of total serum calcium

Total serum calcium was measured in a Corning calcium analyser model 940. Analytical procedure with this analyser was based upon the quenching of the fluorescence from the non dissociative complex formed between calcien (dye) and calcium ions in an alkaline medium, by chelating calcium ions with the titrant EGTA (Ethyleneglycol-bis N.N-tetra acetic acid (Schmidt & Reilley, 1957). Most of the samples were assayed for calcium within a short period of blood sampling and others were stored under -20°C without thawing until used for assay. No significant change in calcium levels have been observed in serum samples stored under such conditions for long periods (Elliott, 1982).

CHAPTER 3

The morphology and the histology of the ovary.

3.1 Introduction.

The basic and most important function of the ovary of all animals is to produce the fertilisable ova necessary for successful reproduction. As a result of sophisticated light and electron microscopical techniques, much of the microscopical anatomy of the ovary has been revealed, although there is considerable controversy concerning some of the histological and cytological changes which occur in the ovary of oviparous vertebrates during oocyte development in particular during vitellogenesis and atresia. These aspects are considered in more detail in the present study, which describes annual cycle of ovarian development in the rainbow trout, Salmo gairdneri.

3.1.1 Anatomy of the ovary.

The ovary of teleosts like that of other vertebrates is found attached to the dorsolateral lining of the peritoneal cavity, with which it is either fully or partially covered. In the ovary which is fully covered by the peritoneum or mesovarium, mature oocytes are released into a central ovarian cavity which is continuous with the posterior oviduct (Mullerian duct); this is described as a cystovarian (closed) condition. The partially covered ovary which releases mature oocytes directly into the body cavity (peritoneal cavity) is referred to as gymnovarian (naked) (Hoar, 1969; Dodd and Sumpter, 1984). Studies on Family-Salmonidae, to which the rainbow trout belongs, have revealed that they possess ovaries of the gymnovarian type (Hoar, 1969). In rainbow trout, ova are discharged into the body cavity, and subsequently enter the oviduct which is a posterior continuation of the peritoneum which partially covers the

ovary. The oviduct is found to open into a urogenital sinus which has an orifice through which oocytes are released to the exterior (Scott, 1962; Hurk and Peute, 1979).

In the rainbow trout ovary, partial transverse septa, arranged along the ovarian axis, have been observed (Scott, 1962; Hurk and Peute, 1979). A similar complex series of folds in the internal lining of the ovary which may almost obliterate the ovarian cavity has been observed in other teleosts; these are known as ovigerous lamellae (Braekevelt and McMillan, 1967; Hoar, 1969; Brummett et al, 1982). The inner epithelium covering the ovigerous lamellae is often referred to as germinal epithelium, since primary oogonia and ovarian follicles are known to develop from/or in association with this tissue (Hoar, 1969; Hurk and Peute, 1979). Ultrastructural studies on germinal epithelium of Fundulus heteroclitus have revealed that these cuboidal epithelial cells contain cellular organelles such as Golgi complexes, endoplasmic reteculum, endocytotic pits and empty cytoplasmic vesicles (Brummett et al, 1982), however, their functional importance is unknown. Several studies have described the histology of the extrafollicular tissue of the ovary of the Fundulus heteroclitus (Mathews, 1938; Brummett et al, 1982) and Eucalia inconstans (Braekevelt and McMillan, 1967), however, it is not known whether the rainbow trout and other salmonid ovaries follow a similar ovarian structure. Earlier studies on rainbow trout ovary have described only some of the structural changes which occur within the oocyte and its follicular tissue (Yamamoto et al, 1965; Hurk and Peute, 1979). Thus in the present work, investigation will also be made of extrafollicular tissue in addition to those concerned with oocyte and follicular development.

3.1.2 Structure of the ovarian tissue.

The outer wall of the teleost ovary which is continuous with the peritoneum is referred to as the tunica albugenia (Mathews, 1938). The tunica albugenia has been found to be more highly vascularized and thicker than the germinal epithelium, due to the presence of smooth muscles and densely packed collagen, elastic and reticular fibres, which are externally lined by squamous to cuboidal epithelial cells containing vacuoles. Among these epithelial cells desmosomes are found. A highly vascularised stroma is found within the ovigerous lamallae between the tunica albugenia and germinal epithelium and it is within this tissue that the developing oocytes are found. Loosely bound strands of collagen, elastic and reticulate fibres known as trabeculae are found to extend more or less perpendicularly from the tunica albugenia into the stroma. In the post-spawned ovaries trabeculae are highly folded while those in ovaries with multiplying oogonia and developing oocytes are stretched and elongated and thus difficult to recognise. Desmosomes are observed among the cells in trabeculae and it is suggested that they provide a structural support for the ovaries and subsequently become distended due to growth of the oocyte (Braeckevelt and McMillan, 1967; Brummett et al, 1982). In the ovary of Fundulus heteroclitus each follicle is surrounded by a basket of reticular fibres which are attached to the trabeculae by a stalk with a central arteriole surrounded by smooth muscles collagen fibres, and occasional nerve fibres (Brummett et al, 1982). During the present study ultra-thin sections of the ovarian tissue will be examined under the light and electron microscopes.

3.1.3 Ovarian development.

In teleosts the ovary may show structural differences according to species and the nature of their reproductive cycle. In addition further changes are found within a species during the different phases of oocyte development (see review by Nagahama, 1983). Three major patterns of oocyte development are recognised, namely synchronous, group-synchronous and asynchronous or metachrome (Wallace and Selman, 1981; de Vlaming, 1983). In synchronous types of ovary all oocytes are found to mature at the same time and fish die after the first and only spawning; this is seen in most anadromous and catadromous fish (eg. Pacific salmon and eel). In group synchronous type the ovary contains oocytes of at least two developmental phases and these fish spawn once a year for a number of years (eg. rainbow trout). Ovaries of asynchronous type contain oocytes at all stages of development and they are known to spawn many times during a prolonged breeding season (eg. turbot). In the rainbow trout, seven stages of ovarian development have been recognised (Yamamoto et al, 1965; Hurk and Peute, 1979), and they are similar to those observed in other teleost species (Vakaet, 1955; Gokhale, 1957; Polder, 1961; Takano, 1964; Abraham et al, 1966; Braekevelt and McMillan, 1967; Hyder, 1969, 1970; Gupta and Yamamoto, 1971; Shakley and King, 1977; Tokaz, 1978; Khoo, 1979; Kagawa et al, 1981; Wallace and Selman, 1981; Forberg, 1982; Matsuyama and Matsuura, 1982). These stages are categorised under four phases, namely oogenesis and folliculogenesis, vitellogenesis, maturation and ovulation (Figs. 3.1 and 3.2). Oogenesis is preceded by oogonial proliferation. Although these stages can be easily identified under the light microscope many of the cytological changes which occur

during development are not fully described. This is especially true of the ultrastructural modifications which accompany endogenous and exogenous vitellogenic stages which occupy a major portion of oocyte development in the salmonids where the eggs are amongst the largest of all teleosts. The structural changes which occur during the degeneration of oocytes, ie. atresia, also poorly understood. During the present work the oocyte development of rainbow trout is studied using light and electron microscopical techniques. Special attention will be focussed on the vitellogenic oocytes and also those undergoing atresia as it is important that the stage or stages of oocyte development in which atresia occurs is more clearly defined.

3.1.4 Sex-differentiation and oogonial proliferation.

In all teleosts oogonial proliferation occurs for the first time, soon after the sexual differentiation (Hann, 1927). The onset of teleost sex differentiation is said to have occurred only when oocytes just enter the first meiotic prophase (Fig. 3.2) (Franchi et al, 1962). As a result of this, distinction between spermatogonia, oogonia and primary oocytes prior to the first meiotic prophase is somewhat arbitrary (Reinboth, 1972). D'Ancona (1956) recognised three patterns of sex differentiation in teleosts. In some cases randomly mixed male and female elements may be recognised within the gonad, with one or the other of these predominating during development. In the second pattern two distinct zones are recognisable within the gonad and both male and female elements may be seen. Sometimes both components ripen to produce a hermaphroditic gonad but more usually one phase may precede the other giving either protandrous or protogamous development. In the third group sexuality is

established early in development although there may be traces of hermaphroditism (Dodd and Sumpter, 1984).

The undifferentiated rainbow trout gonad is found to be a long thin transparent structure containing primordial germ cells, each with one or more nuclei (Hurk and Slof, 1981). As explained above in teleosts oogonia are observed in the ovaries soon after sex differentiation (Braekevelt and McMillan, 1967; de Vlaming, 1972). In rainbow trout sex differentiation begins 45 to 50 days after fertilization, shortly after the completion of yolk-sac resorption and just before the first acceptance of food (Takashima et al, 1980; Hurk and Slof, 1981). The oogonium consist of a large nucleus with a prominent single nucleolus enclosed within an indistinct narrow rim of cytoplasm (Braekevelt and McMillan, 1967; de Vlaming, 1972). In teleosts in general, oogonia are also found to occur singly or more often in small nests with occasional mitotic figures within the nuclei (Mathews, 1938; Barr, 1963; Braekevelt and McMillan, 1967; Moser, 1967; de Vlaming, 1972; Tokaz, 1978; Khoo, 1979). Similar nests of oogonia are also found in the rainbow trout (Yamamoto et al, 1965; Takashima et al, 1980; Hurk and Slof, 1981). Oogonia have been observed in many sexually-mature teleosts and are found to undergo periodic mitotic division to produce primary and secondary oogonia (Fig. 3.2) (Franchi et al, 1962; Khoo, 1979). In the rainbow trout primary and secondary oogonia have been identified and the former are reported to be present throughout the annual reproductive cycle whereas the latter are said to be present just before and after spawning (Hurk and Peute, 1979). The structural differences between primary and secondary oogonia are however not well documented and thus in the present study the primordial germ cells, the primary and

secondary oogonia and the primary oocytes will be described together with their occurrence during the annual reproductive cycle.

3.1.5 Oogenesis.

The transformation of oogonia into oocytes is not well documented for any teleost although it is believed that the secondary oogonia are transformed into oocytes with the commencement of first meiotic prophase (Tokaz, 1978; Nagahama, 1983) (Fig. 3.2). Wiebe (1968) suggested, that only a proportion of the primary oogonia in the ovary of the viviparous sea perch are transformed into secondary oogonia and hence to oocytes, while the rest are reserved for future reproductive seasons. Whether this phenomenon is also found in the rainbow trout, will be investigated during this study. The monthly variations in ovarian development which are discussed in Chapter 4 may provide more information on oogonial proliferation and oogenesis of rainbow trout.

During oogenesis, oogonia are said to acquire an increased amount of cytoplasm (see review by Tokaz, 1978). At the same time follicular cells become associated with the oocyte (Wallace and Selman, 1981). In rainbow trout seven stages of oocyte development are observed, namely the chromatin nucleolar stage, the early perinucleolar stage, the late perinucleolar stage, the endogenous or vesicular yolk stage, the peripheral yolk granular stage, the yolk granule migration stage and the final maturation stage (Fig. 3.2). They are classified according to the size (diameter) of the oocyte; the shape, number and position of the nucleoli within the nucleus; the position of the nucleus and lastly the presence of other organelles within the ooplasm, ie. "Balbiani bodies", vesicles, yolk granules etc. The chromatin nucleolar and, early and late

perinucleolar stages have been classified as previtellogenic oocytes both in the rainbow trout (Yamamoto et al., 1965; Hurk and Peute, 1979) and also other teleost species (Yamamoto, 1956a; Yamamoto and Yamazaki, 1961, Takano, 1964; Braekevelt and McMillan, 1967; Lambert, 1970a; Tokaz, 1978, Khoo, 1979; Wallace and Selman, 1981; Forberg, 1982). Brusle´ (1980) described four stages of previtellogenic oocytes in Mugil (Liza) auratus, on the basis of the structure of the nucleus and the nucleoli and also on the nature and the position of the "Balbiani bodies". Kessel (1981) suggested that the events associated with previtellogenesis in rainbow trout oocytes are sufficiently similar to those described by Brusle´ (1980) although a more comprehensive description has to be made for the rainbow trout before this conclusion can be reached. During the present study the classification suggested by Hurk and Peute (1979) will be used to describe the oocyte stages with the growth phases of the oocyte up to the late perinucleolar stage defined as the primary growth phase (Fig. 3.2) (Wallace and Selman, 1981).

3.1.5.1 Stage 1 - Chromatin nucleolar stage.

Oocytes of the chromatin nucleolar stage are known to be slightly larger than oogonia (Takano, 1964; Yamamoto et al, 1965; Braekevelt and McMillan, 1967; Lambert, 1970a; Tokaz, 1978; Hurk and Peute, 1979; Khoo, 1979; Brusle´, 1980; Kessel, 1981; Forberg, 1982). In these cells the cytoplasm is seen as a narrow weakly-basophilic zone which surrounds a large nucleus, with one large spherical centrally-located nucleolus (Takano, 1964; Yamamoto et al, 1965; Braekevelt and McMillan, 1967; Khoo, 1979; Brusle´, 1980; Kessel, 1981; Forberg, 1982) and also several peripherally-located,

thread-like basophilic nucleoli which are all attached to the inside of the nuclear envelope (Yamamoto, 1956a; Rai, 1967; Tokaz, 1978). Chromatin nucleolar stage oocytes can be further subdivided into the synaptic and post-synaptic stages according to the exact stage of the meiotic prophase. Pre-synaptic oocytes undergo synapsis and form post synaptic oocytes (Tokano, 1964; Yamamoto 1956a). The chromosomes at this stage have been identified as dense clumps of chromatin which become aligned along the inner membrane of the nucleus (Brusle', 1980). These oocytes have been shown to go through the leptotene and zygotene stages of first meiotic prophase before reaching the post-synaptic stage (Yamazaki, 1965; Tokaz, 1978), and the oocytes then enter the perinucleolar stage.

3.1.5.2 Stage 2 - Early Perinucleolar stage.

During the early perinucleolar stage the centrally-located nucleus appears together with several smaller peripheral nucleoli. At the same time the basophilic nature of the nucleus become gradually diminished while the cytoplasmic basophilia reaches a maximum (Takano, 1964; Yamamoto et al, 1965; Abraham et al, 1966; Braekevelt and McMillan, 1967; Lambert, 1970a; Beams and Kessel, 1973; Guraya, 1979; Hurk and Peute, 1979; Khoo, 1979; Brusle', 1980; Kessel, 1981; Wallace and Selman, 1981; Forberg, 1982, Nagahama, 1983). At this time shallow undulations in the nuclear envelope are observed and a basophilic electron-dense substance accumulates in the juxtanuclear region. These are referred to as yolk nuclei or "Balbiani bodies". It has been suggested that these are composed of several different cellular organelles including mitochondria, Golgi complexes, smooth endoplasmic reliculum, polyribosomes,

multivesicular bodies, lipid granules etc. (Dummont, 1972; Beams and Kessel, 1973; Guraya, 1979; Kessel, 1981; Wallace and Selman, 1981). When associated with mitochondria this dense material is refered to as "cement" and when separated from the mitochondrial aggregates it is known as "nuage" (Brusle, 1980). In addition to the above observations annulate lamellae are found in the vicinity of "Balbiani bodies" (Kessel, 1981). Although its function is still not clear, a structural relationship of fine filamentous bundles have been observed. Therefore, it is suggested that these filaments may be involvedin the movement of cellular organelles during oocyte development (Kessel et al, 1984). The more recent observations on the association of annulate lamellae with rough endoplasmic reticulum suggest a role for annulate lamellae in the synthesis of cellular organelles(Kessel, 1985). Guraya (1979) also suggested that the yolk nuclei or "Balbiani bodies" are the centres for the formulation of cellular organelles, ie. endoplasmic reticulum etc., although no evidence has been provided to support this statement. The function of the annulate lamellae and Balbiani bodies remains unclear and requires further investigations.

3.1.5.3 Stage 3 - Late perinucleolar stage.

The late perinucleolar stage is distinguished by the presence of small spherical nucleoli closely situated to the nuclear membrane. During this stage it has been reported that the basophilic nature of the ooplasm becomes reduced and the "Balbiani bodies" first migrate to the periphery and then disappear (Braekevelt and McMillan, 1967; Hurk and Peute, 1979; Brusle, 1980; Nagahama, 1983). A decrease in nucleus/cytoplasmic ratio has also been observed (Brusle 1980). The

significance of these cytological changes has yet to be explained. However, by the end of the late perinucleolar stage a definitive follicle is formed, which consist of a growing zona radiata, a granulosa layer and an outer double celled theca layer (Wallace and Selman, 1981). These changes will be discussed in more detail under folliculogenesis below.

3.1.6 Folliculogenesis.

The follicle is thought to be formed by the epitheloid or prefollicle cells located near the oogonia during the period of oogonial proliferation. Prefollicle cells are smaller than oogonia and found in greater numbers near the oogonial nests (Braekevelt and McMillan, 1967; Tokaz, 1978; Brusle, 1980). It has been suggested that these cells are derived from the germinal epithelium (Mendoza, 1943; Moser, 1967). They are characterised by their flattened shape, irregular outline and the presence of deeply interdigitate membrane folds. Desmosome-like junctions have also been observed between the prefollicle cells. Their cytoplasm has been found to be electron-dense due to the abundance of ribosomes, mitochondria with a dense matrix and short cristae, endoplasmic reticulum and Golgi complexes (Brusle, 1980). During oogenesis primary oogonia have been found to move from their nests to associate with the prefollicle cells (Moser, 1967). At the onset of meiotic prophase, the oocyte includes a follicular layer which is one cell thick (Brusle, 1980). With the growth of the oocyte these cells become increasingly flattened (Takano, 1964; Tokaz, 1978; Brusle, 1980). Additional cells become attached to developing follicle as a result of the mitotic division of existing follicle cells or by the attachment of neighbouring

3.1.7 Vitellogenesis.

3.1.7.1 Endogenous Vitellogenesis (Stage 4).

During this stage vesicle formation occurs within the ooplasm. At the end of the primary growth phase oocytes become arrested at the diplotene stage of the meiotic prophase (Yamazaki, 1965; Wasserman and Smith, 1978; Wallace and Selman, 1981; Forberg, 1982; Nagahama, 1983) (Fig. 3.2). Lampbrush chromosomes are also seen at this stage (Braekevelt and McMillan, 1967) and these persist until the breakdown of the nuclear membrane or germinal vesicle at the end of the secondary growth phase (Bara, 1960; Guraya, 1979). The first structures to appear within the ooplasm during the secondary growth phase are peripherally located vesicles. These have been named yolk vacuoles (Yamamoto, 1956b; Bisht and Joshi, 1975), primary yolk globules (Beach, 1959), lipid vesicles (Moser, 1967), oil vesicles or primary yolk vesicles or granules (Krajhanzl et al, 1984a,b; Nosek, 1984; Nosek et al, 1984) or cortical alveoli (Anderson, 1968). Although most of the above terms indicate the presumed relationship of those vesicles to the yolk formed during the secondary growth phase, their structure, synthesis and functional importance are not yet fully understood. Autoradiographic studies on zebra fish oocytes have shown that the vesicular inclusions are glycoprotein in nature and this material is synthesised within the oocyte (Korfsmeier, 1966). Supportive evidence for such origins have been provided by both electrophoresis (Heesen and Engles, 1973) and immunological studies (Heesen, 1977), of zebra fish oocytes. Earlier studies proposed that those vesicle contained an intravesicular yolk (Marza et al, 1937) whereas other later and more detailed studies have

suggested that this material includes a polysaccharide and a glycosaminoglycans (Yamamoto, 1956b) or is a polysaccharide (Yamamoto, 1956b; Malone and Hisoaka, 1963; Guraya, 1965; Yamazaki, 1965; Anderson, 1968; Khoo, 1979). The vesicular inclusions have also shown to be PAS-positive (Khoo, 1979). These vesicles which are initially found peripherally have also been reported to move centrally and to increase in size and number (Braekevelt and McMillan, 1967; Wallace and Selman, 1981). Several authors have proposed that the vesicles give rise to cortical alveoli at later stages of development (Yamamoto, 1956b; Malone and Hisoaka, 1963; Khoo, 1979), although all the evidence is histological. Recently biochemical and immunofluorescent studies, which have used lectines to bind specifically to carbohydrate groups in the glycoprotein inclusion of the vesicles, have confirmed the peripheral movement of vesicles during oocyte development. These studies have also shown clearly that the vesicles give rise to cortical alveoli later in development (Krajhanzl et al, 1984a, b; Nosek et al, 1984; Nosek, 1984). Vesicles are found close to the oolemma during final stages of oocyte maturation (Monroy, 1965). Ultra-structural studies have shown that the vesicles arise from the Golgi complex of oocytes in goldfish (Yamamoto and Onozato, 1968; Gupta and Yamamoto, 1971). The involvement of endoplasmic reticulum and Golgi complex in the formation of cortical alveoli has been proposed by Anderson (1968). Elliott (1982) observed protein synthetic organelles such as mitochondria with lamellar cristae close to the growing vesicles. Although many studies have been carried out on the formation of the ooplasmic vesicles, they have failed to provide any functional role for the vesicles or explain their relationship with that of true yolk

deposition. Whether the true yolk is derived from a precursor material during the yolk vesicle stage is still unclear. Further investigation of the connection between exogenous and endogenous vitellogenesis are included in the present work although a complete understanding of this question will only be accomplished by a combination of biochemical and ultrastructural investigations.

3.1.7.2 Exogenous vitellogenesis - True yolk deposition.

In all oviparous vertebrates so far studied, including the teleosts, major portion of oocyte growth occurs as a result of the deposition of extra-ovarian yolk proteins within cooplasm (see review by Wallace and Selman, 1981). Yolk proteins in teleosts similar to that of other vertebrates are said to be derived from a hepatically-produced female specific plasma protein (Thurston, 1967; Aida et al, 1973; Heesen and Engles, 1973; Wallace, 1978; Hurk and Peute, 1979; Campbell and Idler, 1980; Wallace and Selman, 1981). These yolk precursor proteins were called "vitellogenins" by Pan et al, (1969), although this study was initially conducted on invertebrates. These precursors are thought to be transferred from the blood serum into the oocytes (Korfsmeier, 1966; Heesen and Engels, 1973; Campbell and Jalabert, 1979). Autoradiographic and electron dense tracer techniques have shown that vitellogenin is transported into the oocyte via perivitelline capillaries and follicular tissues. This yolk is subsequently incorporated into the oocyte via micropinocytotic vesicles at the oocyte surface and that these complexes are then translocated to growing yolk spheres in the peripheral ooplasm. Once internalised it is found to be proteolytically cleaved and converted into the proteins lipovitellin

and phosvitin which are then deposited as crystalline yolk platelets (Selman and Wallace, 1982 and 1983). Vitellogenin incorporation has not been extensively studied in rainbow trout although vitellogenin has been isolated and purified (Sumpter, 1984).

The incorporation of yolk globules is referred to as true vitellogenesis or yolk deposition (Yamamoto, 1956b, 1957; Hurk and Peute, 1979; Khoo, 1979; Wallace and Selman, 1981; Kagawa et al, 1981; Nagahama, 1983). In the rainbow trout, oocytes undergoing tru vitellogenesis are divided into three stages namely peripheral yolk granular stage (stage 5), centrally-migrating yolk granule stage (stage 6) and final maturation (stage 7) (Hurk and Peute, 1979). Similar stages have been identified for other teleost species (Braekevelt and McMillan, 1967; Khoo, 1979; Kagawa et al, 1981; Forberg, 1982). These stages are further characterised according to the position of the nucleus (Wallace and Selman, 1978). The present study involved a more detailed study of the exogenous vitellogenic oocytes

3.1.7.2.1 Stage 5 - Peripheral yolk granuler Stage.

It is during this stage that the eosinophilic peripheral yolk granules are first observed (Hurk and Peute, 1979). These yolk granules are known to stain in deep orange or red colour with Mallory's stain (Khoo, 1979). During stage5, the nucleus is found at the centre of the oocyte (Wallace and Selman, 1978, 1981), whereas during the later stages (6 and 7) it is found to have migrated towards the periphery of the oocyte.

3.1.7.2.2 Stage 6 - Migrating yolk granule stage.

During this stage, in addition to the observation of central migration of yolk granules (Hurk and Peute, 1979), it has also been found that the nucleus also changes its position. This phenomenon referred to as nuclear migration (Wassermann and Smith, 1978) or as germinal vesicle migration (Wallace and Selman, 1978). During the present study above phenomenon is referred to by the name suggested by Wallace and Selman (1978), The germinal vesicle migration is known to occur until it reaches the periphery of the oocyte (Wassermann and Smith, 1978; Wallace and Selman, 1978, 1981). This migration has been studied in vitro in rainbow trout oocytes and the germinal vesicle migration has been shown to coincide with the increases in oocyte sensitivity to 17α hydroxy-20β dihydro-progesterone and maturational gonadotropin (Jalabert and Fostier, 1984). However the physiological role of above hormones during the development of oocytes is still unclear.

3.1.7.2.3 Stage 7 - Final maturation.

Oocytes at this stage are known to reach the end of yolk deposition which is the beginning of tertiary growth phase of the oocyte (Hurk and Peute, 1979; Wallace and Selman, 1981; Nagahama, 1983). The breakdown of the nuclear membrane at this stage is also referred to as germinal vesicle breakdown (Wallace and Selman, 1978). This stage has been superficially observed in in vitro studies on rainbow trout oocytes (Jalabert and Fostier, 1984). Concomitant with germinal vesicle breakdown the nucleoplasm protrude into the yolk mass releasing its nucleoli into the ooplasm amongst the yolk globules (Wassermann and Smith, 1978). Although the

resumption of the first meiotic division is said to occur at the time when the first polar body is extruded. The timing of this change is not well documented for teleosts. Oocytes have been shown to either remain at this stage until the resumption of meiosis in response to sperm penetration or they become resorbed (Wassermann and Smith, 1978). The germinal vesicle migration and breakdown and also the other cytological changes which occur within the rainbow trout oocyte during the later stages of development are studied during the course of this investigation. Resumption of tertiary growth phase is marked by the enlargement of oocytes as a result of hydration (Wallace and Selman, 1978). The mechanisms of hydration or the possible ionic fluctuations which occur at this time are however, not fully understood, although it is known that hydration is not caused by osmotic adjustments to changes in the ovarian fluid in which the follicle is bathed (Wallace and Selman, 1981). *In vitro* studies on *Fundulus heteroclitus* oocytes have shown that the swelling phenomenon and the final size of the oocytes is influenced by hormonal treatment and by the tonicity of the culture media (Wallace and Selman, 1978).

The endocrine processes which control final oocyte maturation, resumption of meiosis and ovulation has been studied in many teleosts including trout (Kirshenblatt, 1959; Goswami and Sundararaj, 1971, 1974; Hirose, 1972; Hirose and Donaldson, 1972; Jalabert *et al*, 1972; Fostier *et al*, 1973; Osanani *et al*, 1973; Iwamatsu, 1974; Jalabert, 1976; Pendergrass and Shroeder, 1976; Sundararaj and Goswami, 1977; Iwamatsu and Katoh, 1978; Wallace and Selman, 1978; Hirose, 1980; Jalabert and Fostier, 1984). In general it would appear that final oocyte maturation and the other changes associated with this process are ultimately under the control of pituitary gonadotropin(s), which

induce the follicle to synthesise specific steroids, which in turn act directly on the oocyte to permit maturation. In addition to this control, it is shown that ovulation can be triggered by catecholamines and also prostaglandin treatment provided calcium ions are present (Jalabert, 1976). The hormonal control of oocyte maturation will be dealt with in more detail in Chapter 5 where the effects of oestradiol-17β treatment on rainbow trout oocyte development are considered.

3.1.8. Follicular growth

Concurrent with the extensive growth of the oocytes of oviparous teleosts many structural changes occur within the follicular tissues during the secondary and tertiary growth phases. The squamous granulosa cells which are organised into a single layer at the end of primary growth become cuboidal in shape as a result of mitotic divisions. Later in development they become more flattened due to the extensive growth of the oocyte (Braekevelt and McMillan, 1967; Kagawa et al, 1981; Forberg, 1982). Intercellular spaces are seen amongst the granulosa cells during development (Flugal, 1967a,b; Kagawa et al, 1981) and they are found to be filled with an amorphous material which forms a net-like structure at all points except those with specialized attachments (Kagawa et al, 1981). The significance of these spaces is not clearly understood but it is suggested that they assist in the passage of vitellogenin from the blood capillaries to the oocytes during the phase of active vitellogenesis (Anderson, 1967; Abraham et al, 1982; Selman and Wallace, 1982). However, this needs further ultrastructural investigation. Ultrastructural studies of granulosa cells have revealed the full complement of organelles

necessary for protein synthesis within the cytoplasm, ie. rough endoplasmic reticulum, mitochondria with lamellar cristae etc. (Hoar and Nagahama, 1978; Kagawa et al, 1981); this suggests that the granulosa cells are responsible for theproduction of proteins which are incorporated during the growth of the zona radiata and deposited between the oolema and the granulosa (Wourms, 1976; Wourms and Sheldon, 1976; Hoar and Nagahama, 1978). Similar observations have been made by Elliott (1982) for the rainbow trout. In addition to the above organelles a prominent golgi complex, lyosomes and electron-dense, membrane-bound secretory granules are found within the golgi field in association with the cellular membrane (Kagawa et al, 1981). This suggests a role for the granulosa in the handling or formation of the phospholipids which are transported into the oocyte during the secondary growth phase (Wourms, 1976; Wourms and Sheldon, 1976; Hoar and Nagahama, 1978). The functional significance of these organelles is, however, still to be revealed. There is good evidence for a steroidogenic function for the granulosa cells. Thus in the African catfish 3β-hydroxysteroid dehydrogenase activity had been demonstrated within the granulosa cells prior to ovulation. In follicles which surround mature oocytes, PAS-positive granules have been observed in the apical cytoplasm of granulosa cells (Yaron, 1971). It is unclear whether these PAS-positive granules are equivalent to the lipid droplets observed by Kagawa et al, (1981). In more recent studies on amago salmon a "two cell type model" has been proposed for the steroidogenic function of follicle (Kagawa et al, 1982b; Nagahama et al, 1982; Young et al, 1982c). In this model testosterone is produced within the thecal cells and it is transported into the granulosa, in which it is aromatised to

oestradiol-17β. This pathway probably changes to produce 17α hydroxy 20β dihydro-progesterone at/or near maturation. The granulosa cells are separated from the rich capillary plexus and flattened fibroblast-like thecal cells, by a basal lamina which is found to be a thick glycoprotein layer containing collagen fibres (Anderson, 1967).

The follicular theca first appears during the primary growth phase (Forberg, 1982) and is characterised by cells of irregular shape and with an elongate nucleus orientated parallel to the oolema. The thin layer of cytoplasm is found to contain many microfilaments which may give strength to the growing oocyte and other cellular organelles (Brusle, 1980). In the follicles surrounding oocytes at later stages of development the theca consists of two cell layers known as the theca interna and externa. Cytochemical studies on teleost follicles, including those of rainbow trout, have revealed high enzyme activity in some thecal cells known as special thecal cells and these are thought to be involved in steroidogenesis (Hoar and Nagahama, 1978; Hurk and Peute, 1979; Kagawa et al, 1982b). In the rainbow trout special thecal cells are found in the theca externa (Hurk and Peute, 1979) whereas in other teleosts they are found in the theca interna (Dodd and Sumpter, 1984). Cytologically these cells have been shown to be similar to the steroidogenic leydig cells of the testis (Dodd and Sumpter, 1984). Unlike the granulosa cells the special thecal cells of the preovulatory follicles have been shown to possess ultrastructural features of steroidogenic cells (Yamamoto and Onozato, 1968; Nicholls and Maple, 1972; Nagahama et al, 1978; Hurk and Peute, 1979; Kagawa et al, 1981). These special thecal cells are known to be the only

cells in the theca which are responsible for steroidogenesis in salmonid fish (Nagahama et al, 1978; Hurk and Peute, 1979). In the rainbow trout steroidogenesis reaches a peak during the maturation and ovulation of oocytes and it is at these times that the special thecal cells also appear within the follicle (Hurk and Peute, 1979). Therefore it is possible that the special thecal cells may play a major role in the ovarian steroidogenesis.

In addition to the changes which occur in the granulosa and theca, many changes occur in the zona radiata which appears for the first time at the end of primary growth phase. The zona radiata has also been referred to as the chorion, the vitelline membrane or the egg membrane of ovulated eggs (Braekevelt and McMillan, 1967; Hurk and Peute, 1979; review by Laale, 1980). Many authors have described the formation of the zona radiata in teleost fish (Flügal, 1964, 1967a, b; Anderson, 1967, 1974; Ulrich, 1969; Busson-Mabillot, 1973; Azevedo, 1974; Wourms and Sheldon, 1976; Flegler, 1977; Shakley and King, 1977; Dumont and Brummett, 1980; Laale, 1980). However, the zona radiata would appear to show many inter-specific variations in development and structure. It may show a mono-, bi- or tri-layered structure (Guraya, 1978) with each layer showing marked histochemical and structural changes during development (See Dodd and Sumpter, 1984). Ludwig (1874) named these layers primary, secondary and tertiary depending on whether they are of ooplasmic, follicular or oviductal origin. Villi projecting from both the oocyte and granulosa cells into the zona radiata have been observed and this provided support for a dual ooplasmic and follicular origin of this layer (Braekevelt and McMillan, 1967). Furthermore at the time when most active growth of zona radiata occurs, protein synthetic

organelles are well shown in the granulosa layer. This supports the follicular origin of the zona radiata (Woums, 1976; Woums and Sheldon, 1976). In oviparous fish in particular, it has been suggested that the extensive development of the zona radiata is related to the physico-chemical properties of the water into which the mature oocytes are shed (Guraya, 1978). However, it is more likely that the radial striations observed in the zona radiata (Forberg, 1982) are the pores through which cytoplasmic microvilli penetrate for metabolic exchange between the oocyte and follicle (Flügal, 1967b).

In mature oocytes a small depression is seen at the animal pole where the micropyle is found (Forberg, 1982). The micropyle has been found to be covered with a single enlarged granulosa cell which remains until the ova are discharged. This is known as the micropylar cell (Yaron, 1971; see review by Nagahama, 1983). The micropyle which is known to appear after completion of yolk deposition within the oocyte, permits entry of only a single sperm into the ovulated egg for fertilization (see review by Wallace and Selman, 1981). The structure of the rainbow trout follicle has been studied on a few occasions and in general these investigations have mainly focussed on the steroidogenic nature of the special thecal cells (Hurk and Peute, 1979, 1985). Therefore the present study will describe light microscopically all the changes within the granulosa, theca and zona radiata of the rainbow trout follicles which occur from the beginning of oogenesis until ovulation.

3.1.8.1 Post-ovulatory follicle.

When final maturation is completed the oocytes are ovulated

still enclosed in the zona radiata and this layer becomes a tough chorion. The granulosa and theca of the follicle are known to be left within the ovary which subsequently forms the post-ovulatory follicle or the post-ovulatory corpora luteum (Hoar, 1969). The granulosa cells of post-ovulatory follicles become hypertrophied and collapsed into the follicular lumen forming an irregular mass attached to the basement membrane (Kagawa et al, 1981). Various stages of degeneration and autophagocytosis have been described within the granulosa (Kagawa et al, 1981; Hurk and Peute, 1985). Steroidogenic activities have been demonstrated in both the granulosa and special thecal cells in the post-ovulatory follicles but it is not clear what functions this steroid production may perform. It may be connected with oogonial proliferation (Hurk and Peute, 1979, 1985; Kagawa et al, 1981). Lam et al, (1978) suggested that the post-ovulatory corpora lutea play a role in maintenance of ovulated eggs in the ovarian cavity through secretion of steroid(s) which in turn stimulate the ovarian epithelium to secrete the bathing fluid of the egg. The 3β-hydroxy-steroid-dehydrogenase activity of thecal and granulosa cells in post-ovulatory follicles reported by many authors (Bara, 1965; Lambert and Van oordt, 1974; Khoo, 1975; Nagahama et al. 1976; Kagawa and Takano, 1979; Hurk and Peute, 1979; Kagawa et al, 1981; Lang, 1981) suggests a resemblance between the post-ovulatory follicle in teleosts and the mammalian corpus luteum (review by Nagahama, 1983). Hoar (1969) suggested that the post-ovulatory follicle of teleosts although it may be treated as a homologous structure to mammalian corpus luteum, secretes oestrogens rather than the progesterones produced by corpora lutea. This aspect needs further careful histochemical attention to decide whether post-

ovulatory follicles in fish are involved in steroidogenesis. Although in most studies ultrastructural changes in the post-ovulatory follicles are investigated, their fate when the ovaries enter the next developmental cycle are poorly understood.

Apart from the development and maturation of teleost oocytes, possibly, one of the most important processes which occur within the ovary is the degeneration or atresia of ovarian ova. This is known to be of common occurrence in all vertebrate groups (Hisaw and Albert, 1947; Brambell, 1956; Guraya, 1973; Byskov, 1978; Saidapur, 1978).

3.1.9. Atresia.

In vertebrates many oocytes initiate growth and development, but only a species-specific number of oocytes reach final maturation and ovulation (Saidapur, 1978). During development, a proportion of oocytes are lost from the ovary due to atresia (Ingram, 1962). Atresia of fish ova has been studied by many authors (Cunningham, 1897; Wallace, 1903; Wheeler, 1924; Hickling, 1930; Mathews, 1938; Jaski, 1939; Fraser and Renton, 1940; Bretschneider and Duyvene de Wit, 1947; Pickford and Atz, 1957; Beach, 1959; Bara, 1960; Dodd, 1960, 1972; Sathyanesan, 1963; Yamamoto and Yamazaki, 1961; Chester Jones and Ball, 1962; Chieffi, 1962; Scott, 1962; Barr, 1963, 1968; Henderson, 1963b; Hisaw and Hisaw, 1960; Nair, 1963; Yamamoto and Yoshika, 1964; Hoar, 1965, 1969; Yamazaki, 1965; Rai, 1966; Rastogi, 1966; Braekevelt and McMillan, 1967; Chan et al, 1967; Moser, 1967; Lehri, 1968; Wiebe, 1968; Lambert, 1970b; Yaron, 1971; Guraya, 1973, 1976; Lofts and Bern, 1972; Macer, 1974; Saidapur and Nadkarni, 1976; Byskov, 1978; Saidapur, 1978; Lang, 1981; Wallace and Selman, 1981; Nagahama, 1983), although in most of these studies little indication

is given of the functional role or the timing of these processes. Many of the changes which occur during the process of oocyte resorption are not well documented. Bretschneider and Duyvene de (1947) identified four successive stages in atresia, within the ovaries of bitterling and guppy, namely α, β, γ and δ. Comparable stages have also been identified in plaice (Barr, 1963), rice fiel eel (Chan et al, 1967), goldfish (Khoo, 1975) and perch (Lang, 1981). Although Hurk and Peute (1979) mentioned the presence of atretic oocytes in rainbow trout ovaries and Scott (1962) attempted to explain the function of atretic oocytes in the brook trout, to date, no detailed description has been made of the atretic oocytes trout.

During the α stage of atresia which is the first stage of oocy degeneration observed by Bretschneider and Duyvene de Wit (1947) th oocytes lose water and shrink. At the same time the oolema and oocyte contents are resorbed as a result of phagocytosis by the granulosa cells. The yolk becomes liquified forming irregular drops and its acidophilic nature diminishes producing basophilic granules. At the same time the nucleus ruptures and releases its contents into the degenerating ooplasm. The β stage is characterize by an invasion and multiplication of follicle cells (Bretschneider and Duyvene de Wit, 1947). It has been proposed that the granulosa layer of post-ovulatory follicles secretes a hormone named oviductin, and that for this reason these cells may be homologous to lutein cells of the mammals (Bretschneider and Duyvene de Wit, 1947). This role has, however, still to be substantiated. Among the multiplying follicles numerous blood vessels have also been observed. It has been suggested that leucocytes from the invading blood capillaries

and theca cells, are responsible for the degeneration of oocyte contents (Barr, 1963; Rai, 1966; Braekevelt and McMillan, 1967; Rastogi, 1966). Lang (1981) concluded that the leucocytes do not have a major role during the atretic process. All secretory functions of the atretic follicles are said to have ceased when they enter the δ stage and the follicular disintegration has been completed. A lutein pigment, which is thought to be the residual tissue from the degenerated follicle cells, also accumulates in the degenerating oocytes. After the δ stage the atretic follicles are known to regress due to the migration of their cells into the surrounding ovarian tissue and also due to further resorption (Bretschneider and Duyvene de Wit, 1947). Ball (1960) reported that the δ stage atretic follicles, unlike the earlier stages, show a Schultz-positive reaction for cholesterol. Khoo (1975) observed a 17β hydroxysteroid dehydrogenase activity in δ stage atretic follicles and again suggested that they were homologous to the corpora lutea of other vertebrates. These observations support a steroidogenic function for the atretic follicles as first suggested by Hoar (1969). However, in other species of fish there have been reports of negative or weak reactions for 3β hydroxysteroid dehydrogenase activity in the atretic tissue (Bara, 1965; Lambert, 1970b; Yaron, 1971; Khoo, 1975; Saidapur and Nadkarni, 1976; Lang, 1981). The atretic follicles also do not have cells containing smooth endoplasmic reticulum mitochondria with lamellar cristae or other cellular organelles commonly associated with steroid synthesis in other vertebrate cells. Possibly some of the apparent steroidogenic characteristics described are due to the metabolism of cholesterol and lipid in the degenerating follicles of atretic oocytes.

The δ stages of atresia are the final stage of atretic process described by Bretschneider and Duyvene de Wit (1947), also referred to as "Brown bodies" because of the presence of brown or black residual bodies within the regressing follicles. These atrophied follicles said to differentiate into irregular bundles of connective tissue and cells (Bretschneider and Duyvene de Wit, 1947). Khoo (1975) described a fifth stage of atresia in goldfish namely the ε stage and suggested that it was involved in oogenesis mainly because oogonia were present in large numbers in post-ovulated ovaries at the same time as this stage of atresia (Yamazaki, 1965; Hickling, 1935; Mathews, 1938; Mendoza, 1943; Barr, 1963; Tokaz, 1978). However, this proposition needs further histological investigations. Lang (1981) has proposed that atretic follicles are merely degenerative structures which exhibit histological changes related to phagocytosis and lysosome formation. Although Scott (1962) suggested a secretory function for atretic follicles in rainbow trout, Hurk and Peute (1979) concluded that they do not have a significant role in ovarian physiology. This conclusion is also supported by obervations on other teleosts by Rastogi (1966); Wallace and Selman (1979, 1981); Lang (1981). These authors suggest that atresia is merely a "mopping up" process which comes into operation at the end of the breeding season especially in those fish which have relatively asynchronous ovaries or multiple batches of eggs (Hickling, 1930; Mathews, 1938; Yamamoto and Yamazaki, 1961; Barr, 1963; Hisaw, 1963).

Two types of atresia were described in Xenentodon concila by Rastogi (1966), namely phagocytotic and hypertrophic atresia the

former is similar to the atretic process described by Bretschneider and Duyvene de Wit, (1947) and other authors. In addition to the term "atretic oocyte" or "atretic follicle" used to describe pre-ovulatory follicles undergoing atresia (Sathyanesan, 1963; Henderson, 1963b; Rastogi, 1966; Chan et al, 1967; Lambert, 1970b; Guraya, 1973; Khoo, 1975; Hurk and Peute, 1979; Wallace and Selman, 1981), different authors refer to atretic follicles by different names; corpora atretica (Ball, 1960; Rai, 1966; Wiebe, 1968; Yaron, 1971; Dodd, 1972; Saidapur, 1978), corpora atreticum (Barr, 1963, 1968), corpus atresia (Yamazaki, 1965) and pre-ovulatory corpora lutea (Bretschneider and Duyvene de Wit, 1947). Throughout this study they will be referred to as atretic oocytes.

Atresia is said to occur at any stage of oocyte development (Rastogi, 1966) although this has to be widely studied. The factors causing atresia in vertebrate follicles at different stages of follicular growth have also received little attention (Guraya, 1973). Factors such as age, stage of reproductive cycle, nutrition, hormonal status etc. have been implicated as possible causes of atresia in some vertebrates (Ingram, 1962). However, in almost all arguments, the evidence is equivocal. Chester Jones and Ball (1962) suggested that any factor which reduces gonadotropin activity and secretion could induce atresia: hypophysectomy (Ball, 1960; Yamazaki, 1965; Saidapur and Nadkarni, 1976), environmental factors (Ball, 1960; Yaron, 1971; De Motelambert et al, 1978), nutritional status (Scott, 1962; Rai, 1966; Springate et al, 1985) are found to be the factors causing atresia in fish. Some of these factors will be dealt in more detail in the chapters which follow.

Jaski (1939) suggested that male fish produce an atresia-

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inducing substance called "copulin" during spawning and that this might be taken up by the female through gills, although there is little proof of its existence. Vladykov (1956) who observed a 40-50% reduction in developing oocytes before ovulation suggested that the space available within the body cavity of fish is not enough to accommodate all the developing oocytes. In general atresia is said to be an unknown event in well fed and physiologically healthy females (Henderson, 1963b; Wallace and Selman, 1981). Atretic oocytes at previtellogenic stages are also known to disappear with very little trace (Byskov, 1978). Further work is required on the timing of atresia, the factors which bring about its occurrence and the dynamics of the resorptive processes particularly in the younger oocytes.

Despite the widely differing reproductive strategies of teleosts the ovarian cycle shows a remarkable controversy. Differences in egg size, the quantities of yolk and the numbers of eggs produced, however, require species differences in the basic ovarian pattern of development. Many of the differences have only been described for a few species. The salmonids in particular are of considerable economic importance and there are many aspects of ovarian development of which we need further information. Amongst them the following will be considered in the present work.

1. A full description of the ovarian stages.
2. Identification of primary and secondary oogonia and primary oocytes.
3. The events associated with previtellogenic development of oocytes.

4. Relationship of endogenous and exogenous vitellogenesis.

5. The histological changes which occur within the follicular and extra-follicular tissue during the oocyte development.

6. Atresia, its occurrence, timing and the dynamics.

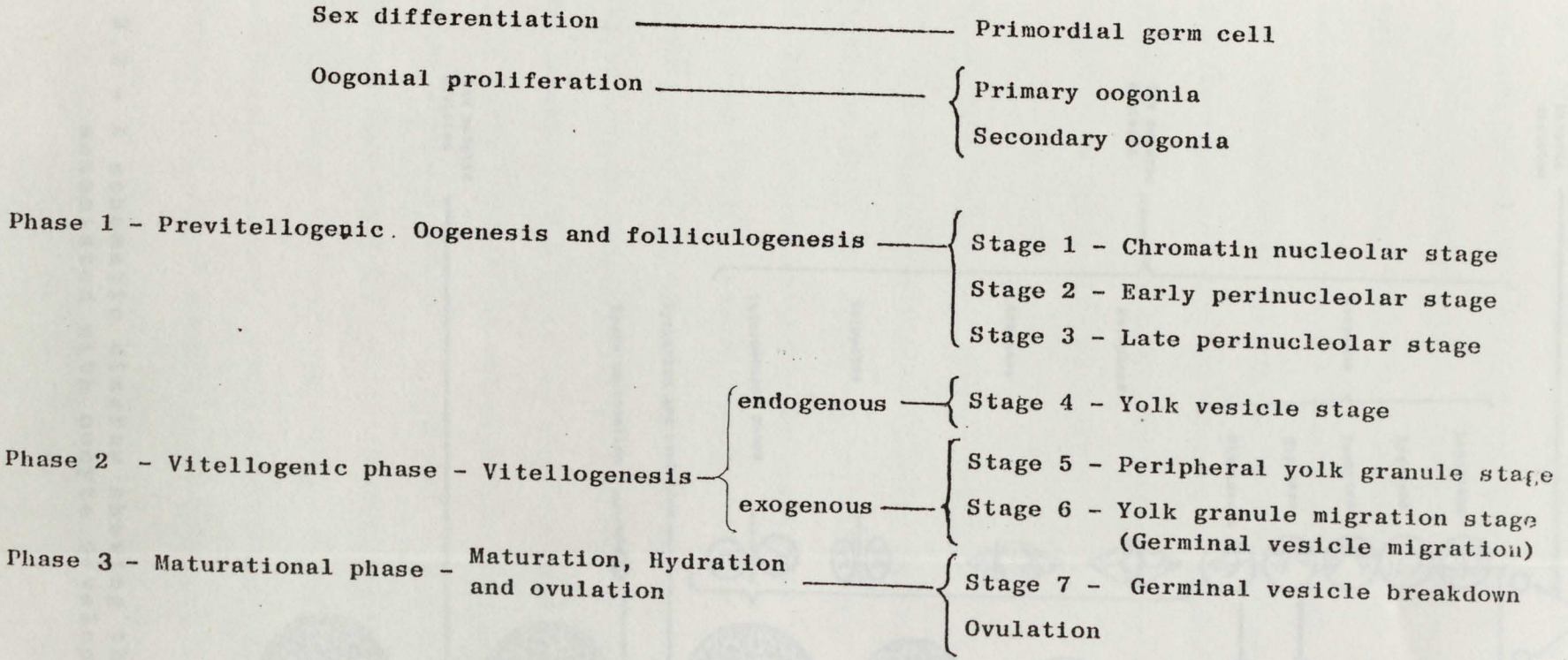


Fig. 3.1 - A schematic diagram showing a summary of events associated with gonadal development of female teleosts in relation to oocyte developmental stages.

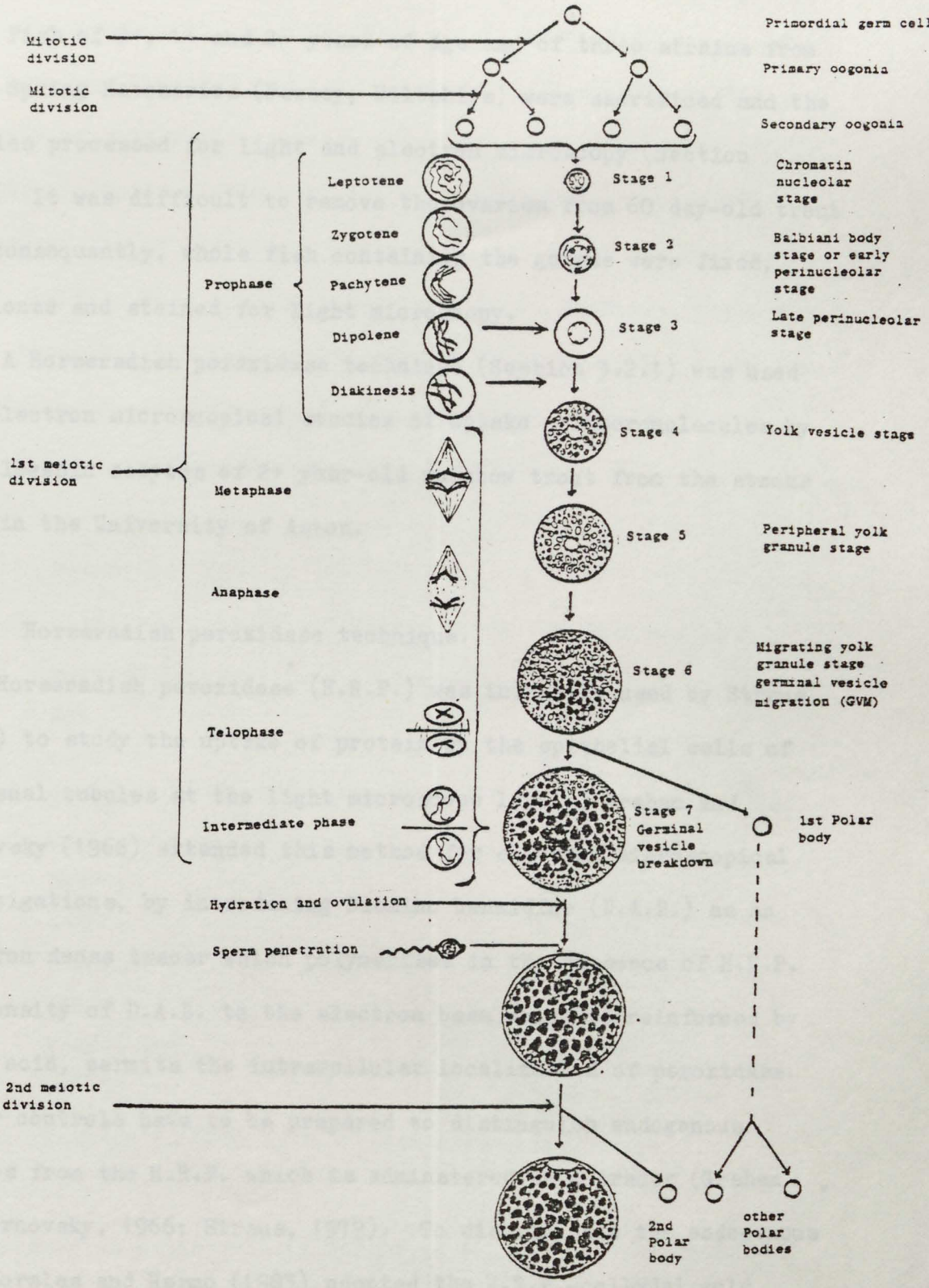


Fig 3.2 - A schematic diagram showing the cytological events associated with oocyte development.

3.2 Materials and Methods

Fish of 0+, 1+ and 2+ years of age and of three strains from Avon Spring Hatcheries (Pewsey, Wiltshire) were sacrificed and the ovaries processed for light and electron microscopy (Section 2.2). It was difficult to remove the ovaries from 60 day-old trout and consequently, whole fish containing the gonads were fixed, sectioned and stained for light microscopy.

A Horseradish peroxidase technique (Section 3.2.1) was used for electron microscopical studies of uptake of macromolecules by vitellogenic oocytes of 2+ year-old rainbow trout from the stocks held in the University of Aston.

3.2.1 Horseradish peroxidase technique.

Horseradish peroxidase (H.R.P.) was initially used by Straus (1957) to study the uptake of protein by the epithelial cells of the renal tubules at the light microscope level. Graham and Karnovsky (1966) extended this method for electron microscopical investigations, by introducing Diamino benzidine (D.A.B.) as an electron dense tracer which polymerises in the presence of H.R.P. The density of D.A.B. to the electron beam which is reinforced by osmic acid, permits the intracellular localization of peroxidase. Proper controls have to be prepared to distinguish endogenous enzymes from the H.R.P. which is adminstered as a tracer (Graham and Karnovsky, 1966; Straus, 1979). To discriminate the endogenous ones Morales and Hermo (1983) adopted the H.R.P.-colloidal gold complex, prepared by Geoghegan and Ackerman (1977); this does not require the use of D.A.B. Since H.R.P. is not absorbed to the plasma membrane this technique was extensively used to study the

fluid phase endocytosis (Steinman and Cohn, 1972; Steinman et al, 1974; Silverstein et al, 1977; Blok et al, 1981; Goud et al, 1981).

The H.R.P. method was used to study the microendocytotic activity of enterocytes in carp (Cyprinus carpio) by Mason (1983) and the micropinocytotic activity of vitellogenic oocytes of sheepshead minnow (Cyrinodon variegatus) by Selman and Wallace (1982, 1983). They used modified Karnovsky's Fixative (Karnovsky, 1965; Section 2.2.2.1.a) as the primary fixative. This study aimed to establish the exogenous nature of vitellogenin and also to distinguish endogenous from exogenous yolk.

3.2.1.1 Materials

a) F.O. Solution (Wallace and Selman, 1978) or fish saline.

NaCl	113 mM
KCl	5 mM
CaCl	2 mM
$MgSO_4$	1 mM
Sodium pyruvate	1 mM
$NaHPO_4$	1 mM
$NaHPO_3$	1 mM
Glucose	5 mM
Hepes	5 mM
Phenol red	0.0001%
NaOH	2.5 mM (for pH 7.5)

Made up to 1000 ml with distilled water.

b) Horseradish peroxidase (H.R.P. type 11; Sigma Chemical Co., Mol. weight - 40,000). A solution of 40 mg ml^{-1} in F.O. solution was prepared

c) Modified Karnovsky's Fixative (Section 2.2.2.1.a)

d) 0.08 M phosphate buffer (pH 7.4)

0.08 M NaH_2PO_4 (1.248 g in 100 ml distilled water)

0.08 M Na_2HPO_4 (1.132 g in 100 ml distilled water)

9.5 ml of NaH_2PO_4 solution was mixed with 40.5 ml of Na_2HPO_4 solution and was made up to 100 ml with distilled water.

e) 0.1 M phosphate buffer (pH 7.4)

0.1 M NaH_2PO_4 (1.56 g in 100 ml of distilled water)

0.1 M Na_2HPO_4 (1.132 g in 100 ml of distilled water)

9.5 ml of NaH_2PO_4 solutionm was mixed with 40.5 ml of Na_2HPO_4 solution and was made up to 100 ml with distilled water.

f) 0.05 M phosphate buffer (pH 7.6)

0.05 M NaH_2PO_4 (0.78 g in 100 ml of distilled water)

0.05 M Na_2HPO_4 (0.8075 g in 100 ml of distilled water)

65 ml of NaH_2PO_4 solution was mixed with 54.5 ml of Na_2HPO_4 solution and was made up to 100 ml with distilled water.

g) 0.05% diamino benzidine (D.A.B.) (Sigma Chemical Co.) containing 0.01% hydrogen peroxide in 0.05 M phosphate buffer (pH 7.6).

h) 1% O_sO_4 in 0.1 M phosphate buffer (7.4) for electron microscopy.

i) Uranyl acetate and lead acetate stains for electron microscopy (Section 2.2.2).

3.2.1.2 Method

40 mg of H.R.P. was dissolved in 1 ml of F.O. Solution (3.2.1.1.a) and 0.15 ml of this solution was injected into the peritoneal cavity of six 2+ year-old rainbow trout. Six other females of the same age group were injected with 0.15 ml of the F.O. solution and they were used as controls. One H.R.P. injected fish and a control fish were sacrificed at 2 hour intervals for 12 hours. Their ovaries removed and the differentiated follicles placed in modified Karnovsky's fixative. This primary fixation lasted for 1 hour and then the follicles were washed for 2-4 hours in a 0.1 M phosphate buffer containing 4% sucrose. Both the control and experimental follicles were rinsed with 0.05 M phosphate buffer (pH 7.6) and incubated in 0.05% D.A.B. containing 0.01% hydrogen peroxide in 0.05 M phosphate buffer (pH 7.6) for 20-40 minutes. Follicles from the F.O. solution-injected fish were also incubated in a D.A.B. reaction mixture which did not contain hydrogen peroxide. Follicles larger than 0.40 mm in diameter were bisected prior to D.A.B. treatment. After several washes in 0.1 M phosphate buffer (pH 7.4) containing 4% sucrose, all the follicles were post fixed in 1% osmium tetroxide (O_sO_4). Following rapid dehydration in ethanol, they were embedded in Poly/Bed 812 (Section 2.2.2.2.b). Sections were stained with uranyl acetate followed by lead citrate and examined under the transmission electron microscope. For light microscopy, whole ovaries were prepared as outlined above and embedded in JB-4 plastic. Sections of 10-20 μ were stained and examined under a light micrscope.

3.3 Results

3.3.1 Morphology of the ovary.

The ovary of the rainbow trout develops from a white thin thread-like microscopic structure, weighing less than 0.0001 g, at 60 days, to a large 15-20 cm long bright orange-coloured structure, weighing 75-85 g, at 2 years of age. Although, in under-yearling fish the different stages of oocyte development could not be identified on superficial examination. In older fish, oocytes above stage 4 were easily distinguished.

The ovaries are suspended in the body cavity by the peritoneum which is a thin tissue attached to the dorsolateral wall of the peritoneal cavity. This was already evident in the sections of 60 day-old rainbow trout (Plate 3.1). The ovary was partially covered by the peritoneum and through the uncovered dorsolateral area of the ovary ovigerous lamellae could be seen. Ovigerous lamellae were arranged transversely along the longitudinal axis of the ovary. These were clearly separated in post-ovulated ovaries. A branch of the dorsal aorta and the ovarian vein run parallel to the ovarian axis, along the margin where the peritoneal membrane meets the ovary. Both the artery and the vein send capillary branches to each developing oocyte. The outer wall of the peritoneum was lined with a ciliated columnar epithelium. These ciliated cells were especially well-developed on the peritoneal attachment of the post-ovulated ovary (Plate 3.2). The peritoneum, which covers a major part of the ovary, was thicker than the epithelial covering of the ovigerous lamellae; the latter consisting of flattened more or less squamous cells (Plate 3.3).

3.3.2 Histology of the ovary.

Primary and secondary oogonia, previtellogenic and vitellogenic oocytes, mature oocytes,pre- and post-ovulatory follicles and the different stages of atresia were present within the ovaries in variable proportions depending on the time of sampling in relation to the annual cycle (Chapter 4). All the oocytes and follicles were embedded in a loosely bound connective tissue consisting of trabeculae and blood capillaries (plate 3.3). These trabeculae were clearly seen in post-ovulated ovaries. Oocyte size (expressed as diameter) increased many-fold during oocyte development (Plate 3.4 and Fig. 3.3). The structure of the oocytes at each of the different developmental stages and the follicular and extra follicular tissues are described in more detail below.

3.3.2.1 Oogonia

Oogonia were present in almost all the ovaries studied. Two types of oogonia were observed, ie. primary and secondary oogonia (plates 3.3, 3.6).

3.3.2.1.1 Primary oogonia

Primary oogonia are undifferentiated cells with a large nucleus containing one or more small nucleoli and an indistinct band of cytoplasm. These oogonia have a irregular shape (Plate 3.5). The ovary of 60 day old fish consisted solely of primary oogonia (Plate 3.1). These primary oogonia became larger and more spherical to form secondary oogonia.

3.3.2.1.2 Secondary oogonia

Secondary oogonia were found singly or more often in clusters or nests (Plate 3.6). The diameter of these cells was less than 17.5 μ and these oogonia had a large nucleus containing a single large nucleolus. The cytoplasm composed of an indistinct narrow band around the nucleus. Prefollicle cells were also observed in the vicinity of the secondary oogonia. Mitotic figures were frequently seen within these oogonial cells (Plate 3.7). Subsequently, secondary oogonia acquired increased amounts of cytoplasm to become primary stage 1 oocytes; this change also involved the beginning of the first meiotic prophase.

3.3.2.2 Prefollicle cells.

Prefollicle cells are smaller than oogonia and have a flattened shape. They were found adjacent to the clusters of oogonia. During development they become increasingly flattened and migrate to form a primary follicle cell layer around the growing oocytes (Plates 3.6, 3.7).

3.3.2.3 Stage 1 oocytes.

Very few of the ovaries sampled contained oocytes at this stage of development. They were slightly larger than secondary oogonia with diameters ranging from 24.5 μ to 46.5 μ. The nuclei of stage 1 oocytes consisted of several strands of basophilic chromatin just inside the nuclear membrane. The nucleus occupied a major portion of the oocyte with only a narrow encircling band of slightly basophilic cytoplasm. At this stage the follicle consisted of a single layer composed of 3-4 flattened cells (Plate 3.8). Oocytes of this stage

were first observed with stage 2 oocytes and were not found only amongst oogonia.

3.3.2.4 Stage 2 oocytes.

Three types of stage 2 oocytes were identified, ie. 2a, 2b and 2c, (Fig. 3.3 - 2a, 2b, 2c; Plates 3.9, 3.10, 3.11). Their diameters ranged from 80 μ to 241 μ. Oocytes of stage 2a were slightly larger than stage 1 oocytes and had strongly basophilic cytoplasm which stained readily with haematoxylin, methylene-blue and acid fuchsin (Plate 3.9). The nucleus contained one large spherical nucleolus and several smaller nucleoli. The basophilic nature of the cytoplasm became gradually reduced as the oocyte developed from stage 2b to 2c. In stage 2b the basophilia was restricted to the yolk nuclei or "Balbiani bodies" which had appeared in the juxta-nuclear region (Plate 3.10). The "Balbiani bodies" moved peripherally when the oocytes entered stage 2c, and their basophilia was reduced (Plate 3.11). The nuclei of both stage 2b and 2c included several smaller nucleoli each lying close to the nuclear envelope (Plates 3.10 and 3.11). At the end of stage 2c, the oocytes were surrounded by a follicle which was composed of a single squamous granulosa cell layer, and one or two squamous thecal cell layers (Plate 3.11). Stage 2 oocytes were present in all the ovaries examined with an especially large number in the underyearling fish.

3.3.2.5 Stage 3 oocytes.

These oocytes were larger than those of stage 2c and with diameters ranging from 313 μ to 430 μ. Like stage 2c their nuclei contained several small nucleoli each lying close to the nuclear

envelope which had become slightly undulated (Plate 3,12). "Balbiani bodies" had disappeared by this stage and the cytoplasm had become slightly acidophilic. The follicle of the stage 3 oocytes like those of stage 2c consisted of a single layer of flattened granulosa cells and double layer of squamous thecal cells.

3.3.2.6 Stage 4 oocytes.

These oocytes were clearly identified by the presence of yolk vesicles. The vesicles contained faintly basophilic material which stained pale mauve with haematoxylin (Plates 3.13 and 3.14); blue with Mallory's triple stain and magenta with periodic-acid Schiff's (Plate 3.15). Oocytes at this stage were divided into two substages, ie. stages 4a and 4b, according to the number and distribution of cytoplasmic vesicles (Fig. 3.3, 4a and 4b; Plates 3.13 and 3.14). In early stage 4 (4a) the cytoplasmic vesicles had just appeared, at the periphery of the ooplasm. Gradually, they moved towards the centre of the oocyte, increasing in size during this movement. By late stage 4 (ie. 4b), the whole ooplasm was full of vesicles giving a net-like or reticulate appearance. The vesicles in the juxta-nuclear region were larger than the rest and some of them had no vesicular inclusions (Plate 3.14). Electron micrographs of stage 4 oocytes revealed close contact between the Golgi complex, the endoplasmic reticulum and the ooplasmic vesicles (Plate 3.16). In some vesicles the cristae of the endoplasmic reticulum enclosed the growing vesicles. During the development of stage 4 oocytes a zona radiata was found between the granulosa and the oocyte membrane. In the zona radiata of the stage 4b oocytes radial striations were observed. These are microvilli from the oocyte membrane (Plate 3.17). The

granulosa cells were cuboidal in shape whilst the thecal cells still have a flattened appearance (Plate 3.17).

Yolk vesicles were also present within the oocytes at later stages of development, ie. in oocytes where yolk deposition have also begun.

3.3.2.7 Stage 5 oocytes.

The oocytes at this stage were easily distinguished from stage 4 by the presence of small exogenous yolk granules in the ooplasm adjacent to the microvilli of the oocyte membrane (Plates 3.18, 3.19). The yolk granules stained deep orange with Mallory's triple stain and pink with haematoxylin and eosin. As development progressed these yolk granules coalesced to form larger yolk globules. At the same time they became pushed towards the centre of the oocyte. The path taken by the exogenous yolk during its incorporation into the vitellogenic or stage 5 oocytes was also followed in fish which had been injected with H.R.P. Six hours after the treatment an electron dense material was seen within the zona radiata, the ooplasm and also in the yolk globules (Plates 3.20a, 3.20b). The follicular layers were mainly unchanged from stage 4 except for an increased thickness of the zona radiata (Plate 3.19). Electron micrographs of these oocytes showed an enhanced development of rough endoplasmic reticulum within the thecal and granulosa cells (Plate 3.21). The lamellae of the thecal endoplasmic reticulum lay parallel to the oocyte surface. The nuclei of the oocytes at this stage situated in the centre of the oocyte with the nucleoli remaining close to the oocyte membrane.

3.3.2.8 Stage 6 oocytes.

These oocytes were characterised by a migrating nucleus or germinal vesicle (Plate 3.22, Fig 3.3) and the presence of many aggregations of yolk within the ooplasm; this yolk had pushed the yolk vesicles towards the periphery. Empty vacuoles or vesicles were found close to the peripherally migrating germinal vesicle. There were differences in size of the yolk globules with the largest being found in those parts of the oocyte which were away from the direction of the germinal vesicle migration. At this stage the yolk globules occupied greater volume of the oocyte than the yolk vesicles; the sperical yolk globules also began to develop into polygonal yolk platelets (Plate 3.23). The nuclei of the stage 6 oocytes were smaller and had more highly folded nuclear envelopes. The nucleoli were small and indistinct by this stage. The yolk vesicles were now restricted to portions immediately adjacent to the oocyte membrane (Plate 3.23). The follicle was wider than in any of the previous stages mainly as a result of the growth of the zona radiata (Plate 3.24). The granulosa cells had also become more flattened by the end of stage 6. At this time the germinal vesicle was close to the oocyte membrane and smaller amounts of yolk granules were being incorporated. The yolk globules which were close to the nucleus were smaller than those at the opposite pole of the oocyte (Plate 3.23).

3.3.2.9 Stage 7 oocytes.

These are the largest oocytes in a mature ovary. They are yolk-filled structures with peripherally-located and ruptured nuclear envelopes (germinal vesicle breakdown) (Plate 3.25, Fig 3.3). Unlike the previous stages the nucleoli were found in the middle of the

nuclear mass. No small yolk granule formation occurred during this stage and the yolk vesicles still remained adjacent to the nuclear membrane. The follicle had reached its maximum thickness at this stage and the zona radiata contained prominent radial striations. Within the zona radiata just above the germinal vesicle the micropyle could be observed (Plate 3.25). The entrance to this opening was covered by one of the enlarged granulosa cells. The thecal layers were squamous and difficult to differentiate from extra-follicular tissues.

3.3.2.10 Twin-oocytes.

A few twin-oocytes were found amongst stage 2 and 4 oocytes (Plates 3.26, 3.27 respectively). These consisted of two oocytes enclosed within a single follicle. Between the stage 2 twin-oocytes no cells were seen (Plate 3.26) whilst between stage 4 twin-oocytes a single layer of flattened, possibly granulosa cells was found (Plate 3.27).

3.3.2.11 Post-ovulatory follicles.

These are hollow collapsed structures with a hypertrophied granulosa in the space previously occupied by the oocyte. Some of the post-ovulatory follicles had an opening to the peritoneal cavity through which oocyte had been released (Plates 3.28a,b, Fig 3.3). The theca of post-ovulatory follicles were continuous with the epithelial cell layer lining the ovigerous lamellae (Plate 3.28b). The basement membrane of the hypertrophied granulosa was of variable thickness and in some places the granulosa was separated from the theca at the basement membrane (Plate 3.29). Occasionally oogonial

nests were found amongst the connective tissue surrounding the post-ovulatory follicles (Plate 3.30). Trabeculae or connective tissue strands were folded and clearly seen among post-ovulated ovaries (Plates 3.28b, 3.32).

3.3.2.12 Atresia.

Atresia occurred at a number of different stages of the oocyte developmental cycle. Although previtellogenic stage 1, 2 and 3 oocytes decreased in large numbers during the ovarian development (Chapter 4), no atresia was identified in these oocytes. Only atretic oocytes of stages 4,5,6 and 7 were observed, with the majority occurring at the beginning of vitellogenesis and in the post-ovulated ovary. Atretic oocytes could be identified superficially by their dark-orange colour, the occasional presence of dark pigment spots (Plate 3.31a) and also by their irregular shape (Plate 3.31b). The dark spots were most prominent at terminal stages of the atretic process.

3.3.2.12.1 Stage 4 atretic oocytes.

These oocytes were found to undergo two types of atresia (Plates 3.32, 3.35). In one type the oocytes were shrunken and separated from the theca of the follicle by a hypertrophied granulosa (Plate 3.32). In some of these atretic oocytes which were found close to the epithelium of the ovigerous lamellae the oocyte membrane was ruptured having released its contents into the extra-ovarian cavity (Plate 3.33). These atretic stage 4 oocytes were most commonly found in post-ovulated ovaries (Plate 3.32) or in mature ovaries with stage 7 oocytes (Plate 3.34). The other type of stage 4 atretic oocyte was

seen in the ovaries of fish subjected to food deprivation. In these the hypertrophied granulosa penetrated the zona radiata and the oocyte membrane and the oocytes were filled with connective tissue (Plate 3.35).

3.3.2.12.2 Stage 5 atretic oocytes.

These oocytes contained a hypertrophied granulosa whose basement membrane was irregular in thickness in places. These atretic structures were filled with a mass of loosely-bound cells derived from the hypertrophied granulosa and acidophilic fragments of the zona radiata and yolk granules (Plate 3.36).

3.3.2.12.3 Stage 6 atretic oocytes.

Two types of atretic stage 6 oocytes were found. One type was similar to those of stage 5 although there were larger amounts of fragmented zona radiata and yolky material (Plate 3.37). Subsequently, these structures became shrunken and the acidophilic inclusions were clustered toegther with a few dark-brown or black pigmented bodies within the irregular cell mass in the atretic oocytes (Plate 3.38). The second type of atresia was of the large, yolky, late stage 6 oocytes. These oocytes were separated from the follicular tissue by a hypertrophied granulosa but, unlike stage 5 the zona radiata remained intact. The yolk mass within the oocyte was separated from the zona radiata and had lost its globular or granular appearance forming a uniform acidophilic mass in the middle of the oocyte. The gap between the yolk mass and the zona radiata was filled with a faintly-acidophilic fine granulated material (Plate 3.39). Subsequently, the whole yolk mass disappeared and the

follicular cavity enclosed by zona radiata became filled with this fine granular material (Plate 3.40). Finally, this material disappeared leaving a hollow collapsed structure margined only by the zona radiata (Plate 3.41).

3.3.2.12.4 Stage 7 atretic oocytes.

Atresia was only found in the stage 7 oocytes which had failed to ovulate. They were separated from a hypertrophied granulosa. The radial striations of the zona radiata were still visible. The platelet structure of the yolk mass was not seen and within the oocyte was a uniform mass of acidophilic material (Plate 3.42). The later stages of atresia development in these oocytes was similar to that seen in the large stage 6 atretic oocytes with the zona radiata remaining intact until the whole of the internal structure had been resorbed.

3.3.2.12.5 Brown bodies.

These are the terminal stages of atretic oocytes. These were prominently represented in both mature and post-ovulated ovaries (Plate 3.43) and also in those containing oocytes only up to stage 4 (Plate 3.44). They consisted of a loosely-bound cell mass in which dark-brown or black pigmented bodies were embedded.



Plate 3.1

Plate 3.1 - Transverse section of the trunk region of a 60 day old fish showing the situation of the ovary within the peritoneal cavity. Stained with haematoxylin and Eosin. Magnification x 500

Og - oogonia, Ov - ovary, P - peritoneum or mesovarium, Pc peritoneal cavity, Tm - trunk muscles.

Plate 3.2 - Section of the peritoneum (mesovarium) which suspend a post-ovulated ovary within the peritoneal cavity. Stained with haematoxylin and Eosin.
Magnification x 312.5

Ge - germinal epithelium, Oc - oocytes at different stages, Og - oogonia, p - peritoneum, S - stroma, Ta - tunica albugenia, 1 - stage 1 oocytes, 2a - stage 2a oocytes.

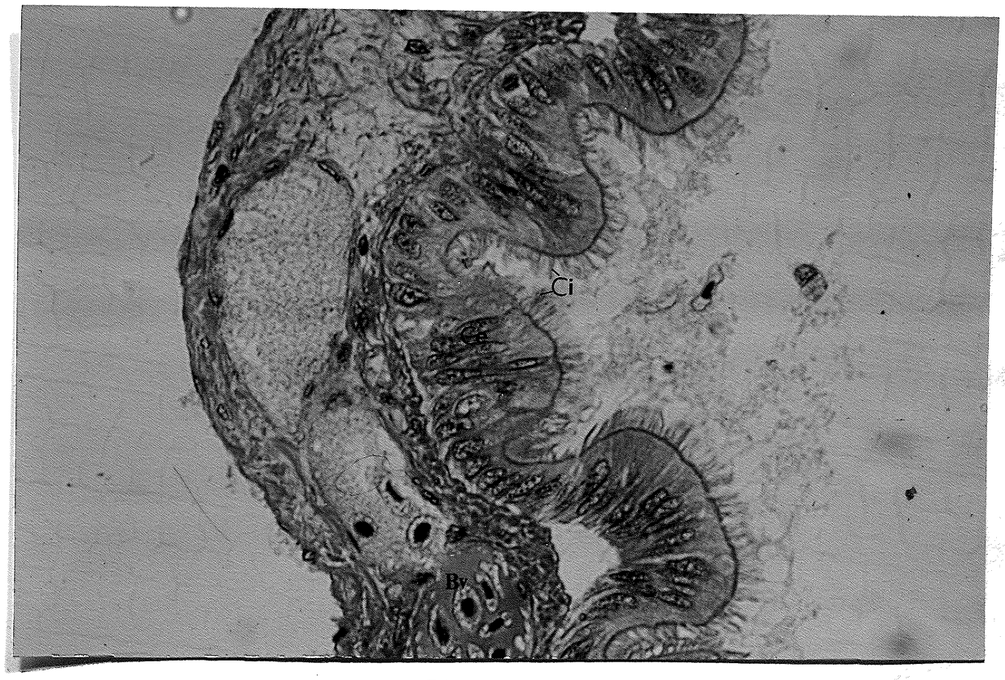


Plate 3.2

Plate 3.3 - Section of an ovary of a 5 month-old fish. Stained with haematoxylin and Eosin.
Magnification x 312.5

Ge - germinal epithelium, Oc - oocytes at different stages, Og - oogonia, P - peritoneum, S - stroma, Ta - tunica albugenia, 1 - stage 1 oocytes, 2a - stage 2a oocytes.

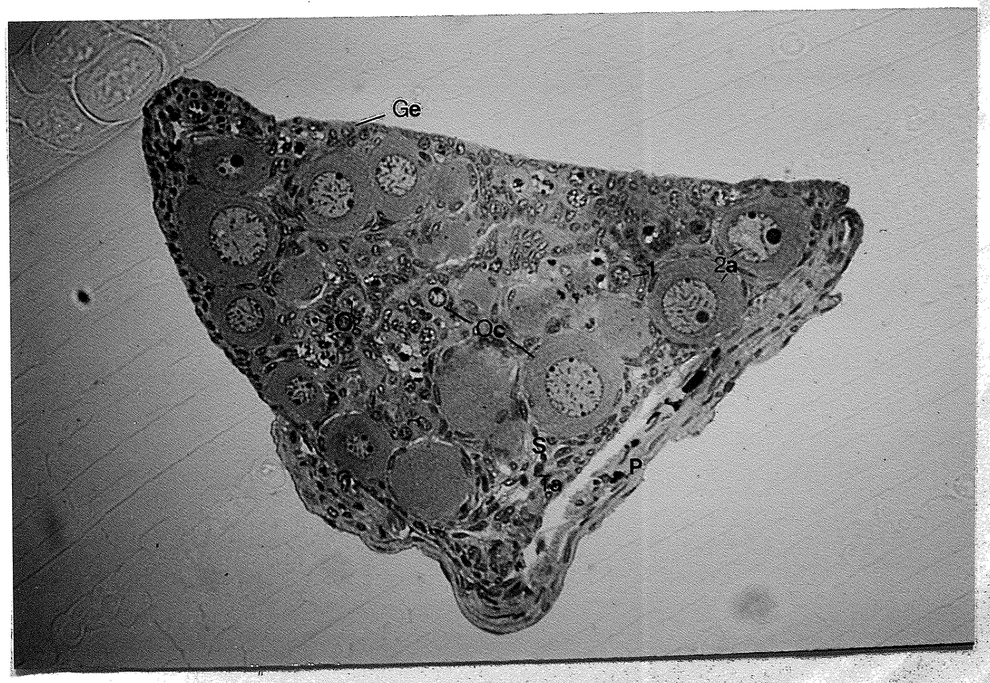


Plate 3.3

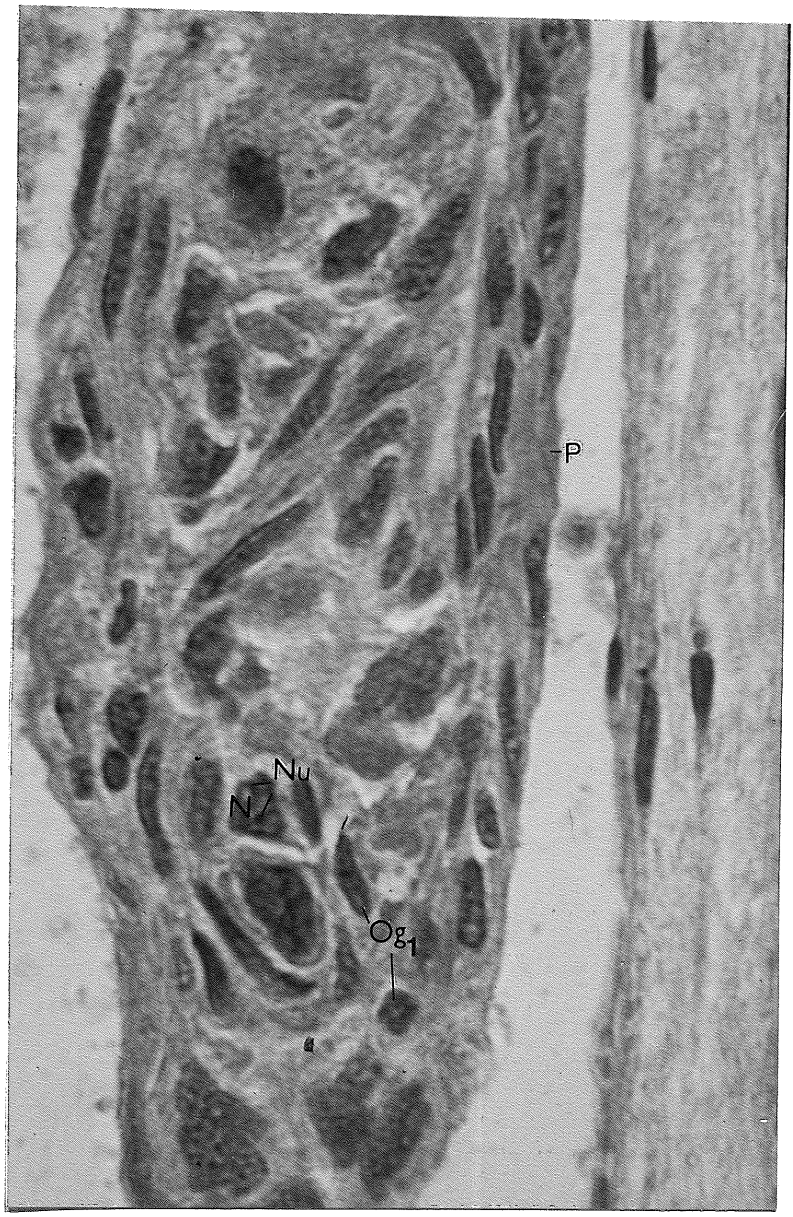


Plate 3.5

Plate 3.5 - Section of an ovary of a 60 day old fish containing primary oogonia. Stained with haematoxylin and Eosin. Magnification x 1250

N - nucleus, Nu - nucleoli, Og_1 - primary oogonia, P - peritoneum

Plate 3.6 - Section of an ovary showing the secondary oogonia. Stained with haematoxylin and eosin.
Magnification x 1250

Cp - cytoplasm of oogonia, N - nucleus of oogonia, Ne - nuclear envelope, Nu - nucleoli, Og_1 - primary oogonia, Og_2 - secondary oogonia, PFC - pre-follicle cells.

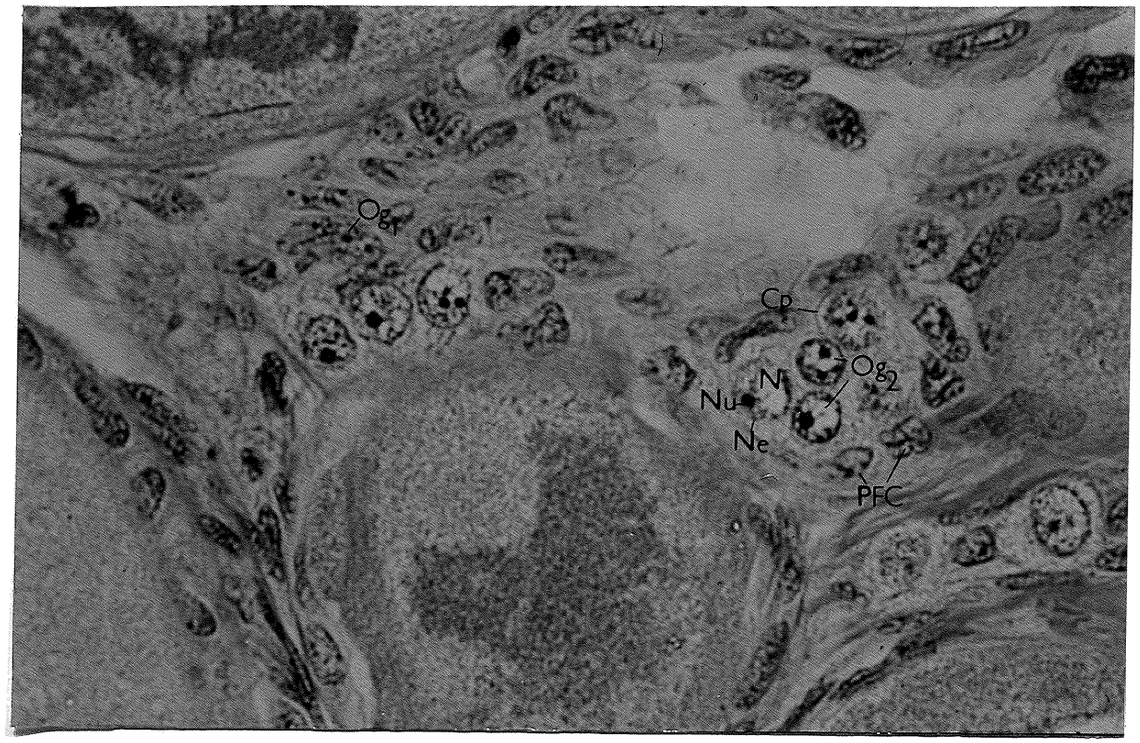


Plate 3.6

Plate 3.7 - Section of an ovary of a 5 month-old fish showing secondary oogonia undergoing mitosis. Stained with haematoxylin and eosin.
Magnification x 2000

G - granulosa, ..
Og_1 - primary oogonia, Og_2 - secondary oogonia, PFC - pre-follicle cells, St_{2a} - stage 2a oocyte.

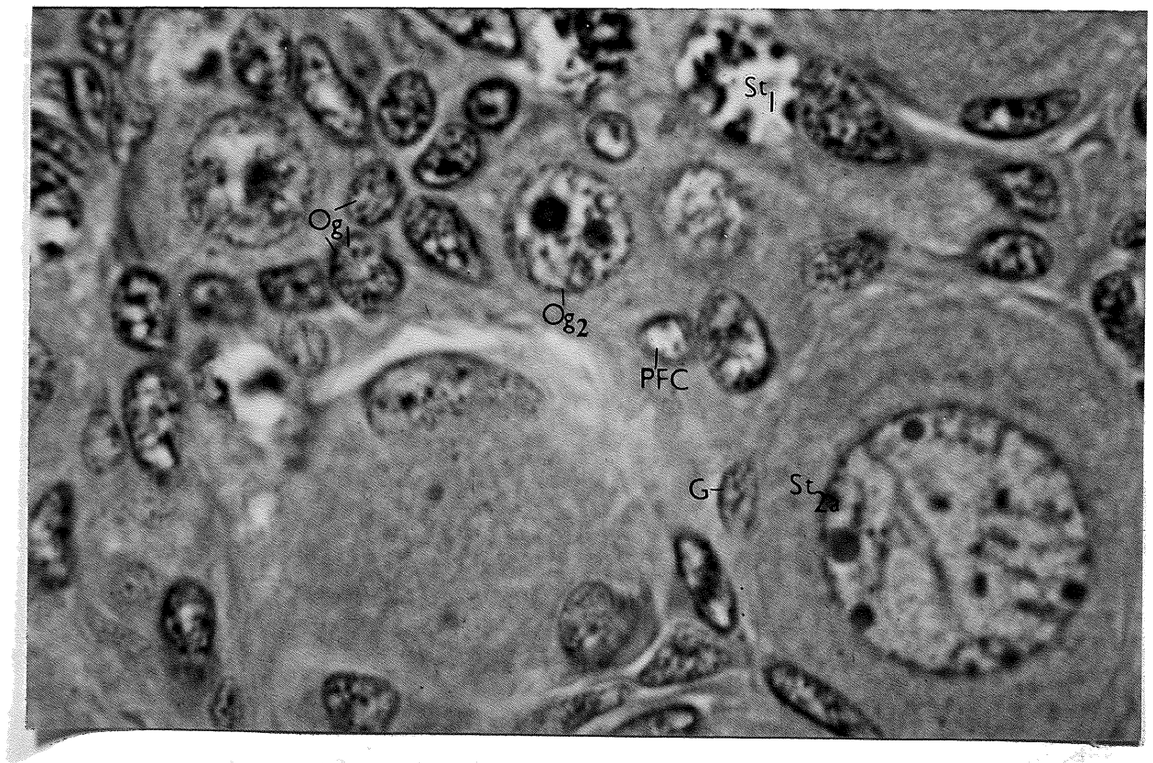


Plate 3.7

Plate 3.8 - Section of an ovary showing stage 1 oocytes. Stained with haematoxylin and eosin.
Magnification x 1250

Ch - chromatin strands, N - nucleus, Ne - nuclear envelope, Op - ooplasm, PFC - pre-follicle cell, St_1 - stage 1 oocytes.

Plate 3.9 - Section of an ovary showing a stage 2a oocyte. Stained with haematoxylin and eosin.
Magnification x 800

C - connective tissue, G - granulosa, N - nucleus, Ne - nuclear envelope, Nu - nucleoli, Op- basophilic ooplasm, St_{2a} - stage 2a oocytes.

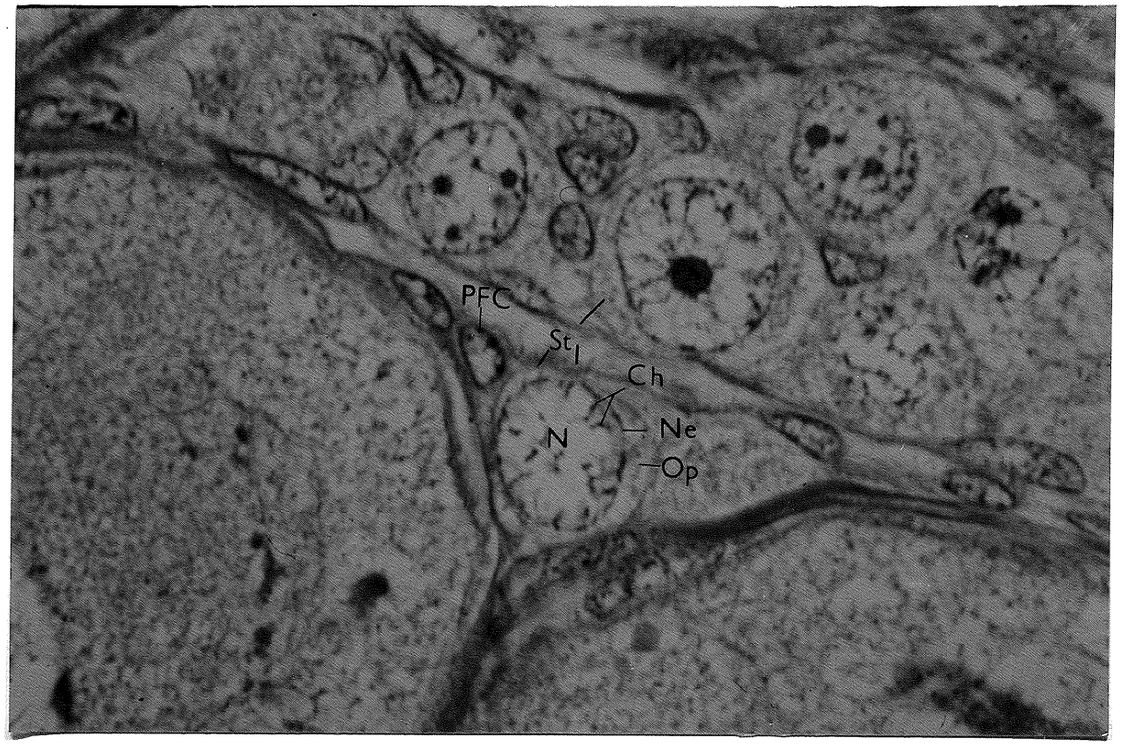


Plate 3.8

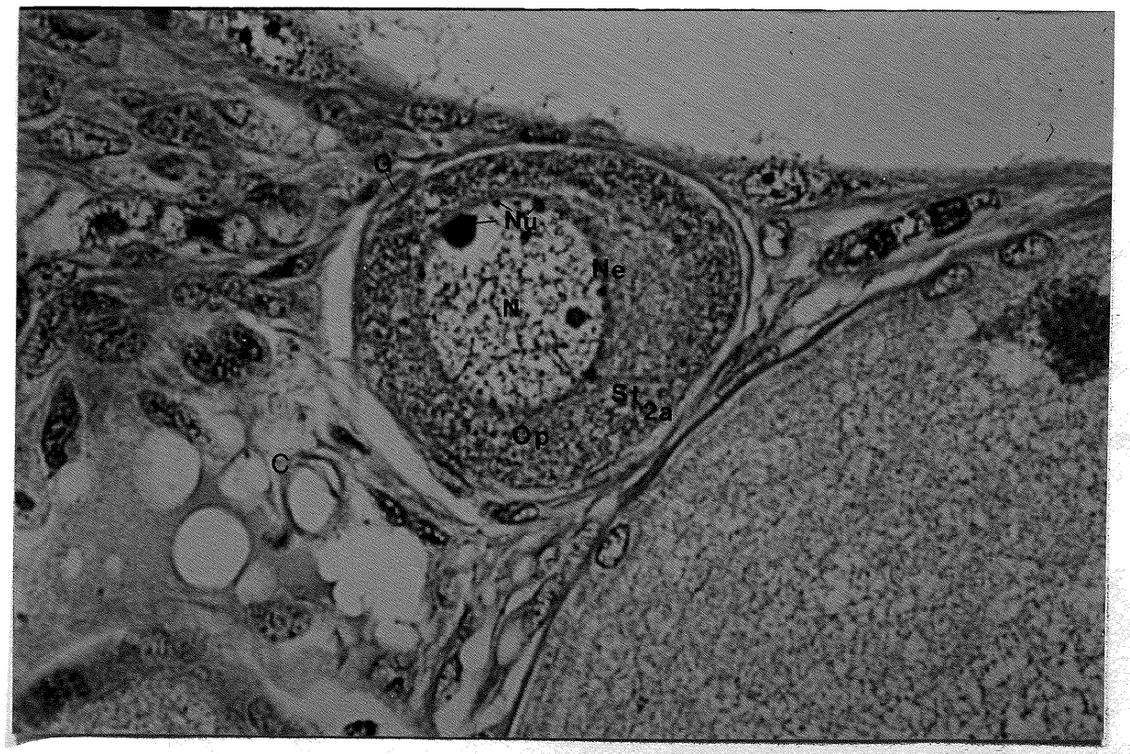


Plate 3.9

Plate 3.10 - Section of an ovary showing a stage 2b oocyte. Stained with Lee's methylene-blue basic-fuschin.
Magnification x 625

Bb "Balbiani bodies" or yolk nuclei, C - connective tissue, F - follicle, N - nucleus, Ne - nuclear velope, Nu - nucleoli, Op - ooplasm, St_{2b} - stage 2b oocyte, St_{4a} - stage 4a oocyte.



Plate 3.10

Plate 3.11 - Section of an ovary showing stage 2c oocytes. Stained with haematoxylin and eosin.
Magnification x 500

C - connective tissue, G - granulosa, N - nucleus, Ne - nuclear envelope, Nu - nucleoli, Op - ooplasm, St_{2c} - Stage 2c oocyte, Th - theca, Yn - yolk nuclei or "Balbiani bodies".

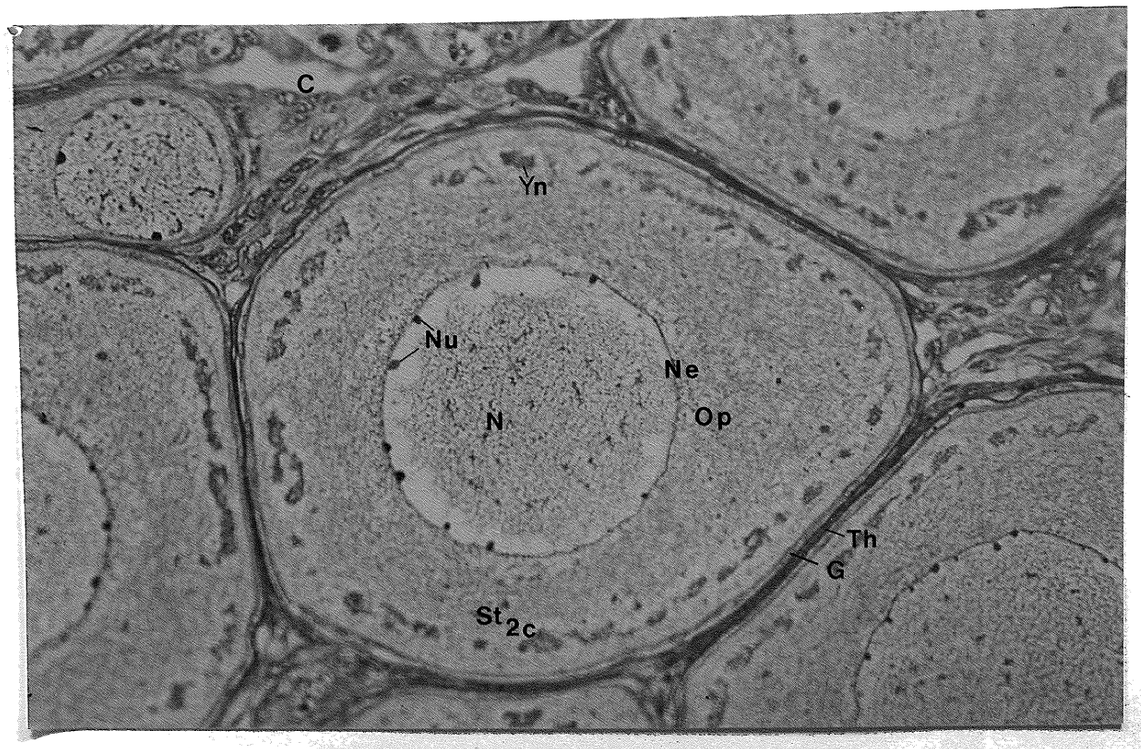


Plate 3.11

Plate 3.12 - Section of an ovary showing a stage 3 oocyte. Stained with haematoxylin and eosin.
Magnification x 250

C - connective tissue, G - granulosa, N - nucleus, Ne - nuclear envelope, Nu - nucleoli, Op - ooplasm, St_3 - stage 3 oocyte, Th - theca, Tr - trabeculae.

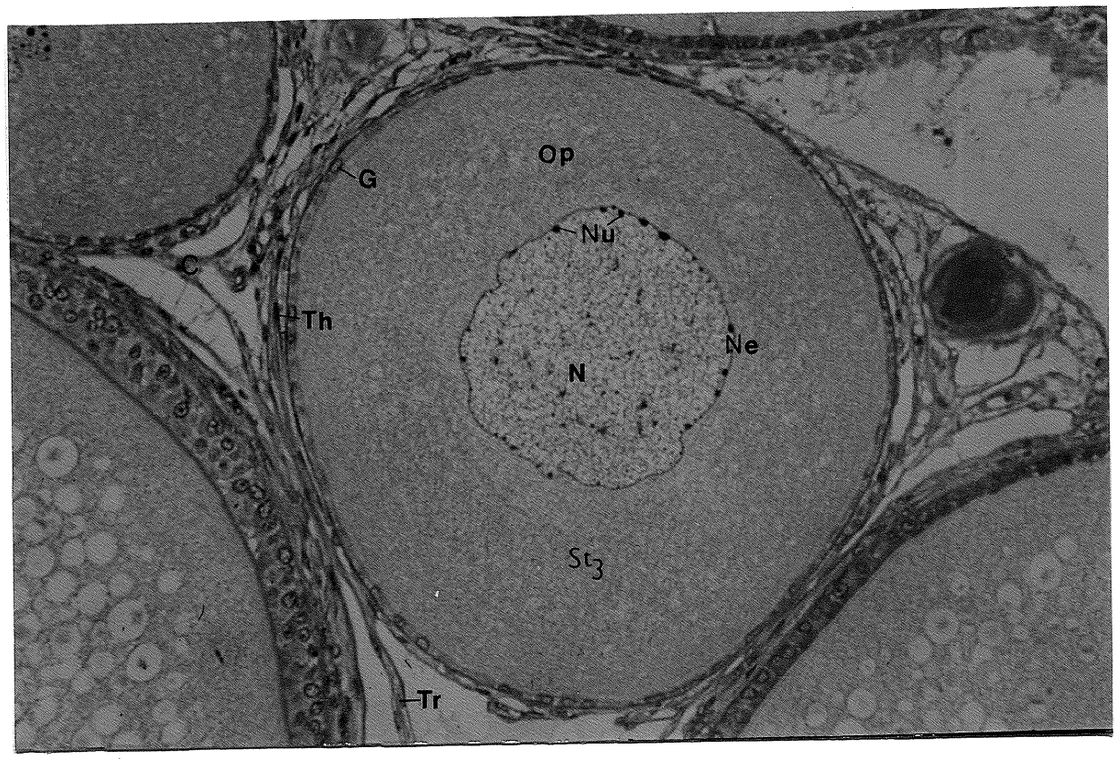


Plate 3.12

Plate 3.13 - Section of an ovary showing a stage 4a oocyte. Stained with haematoxylin and eosin.
Magnification x 160

G - granulosa, N - nucleus, Op - ooplasm, St_{4a} - stage 4a oocyte, Th - theca, Yv - ooplasmic vesicles or yolk vesicles, Zr - zona radiata.

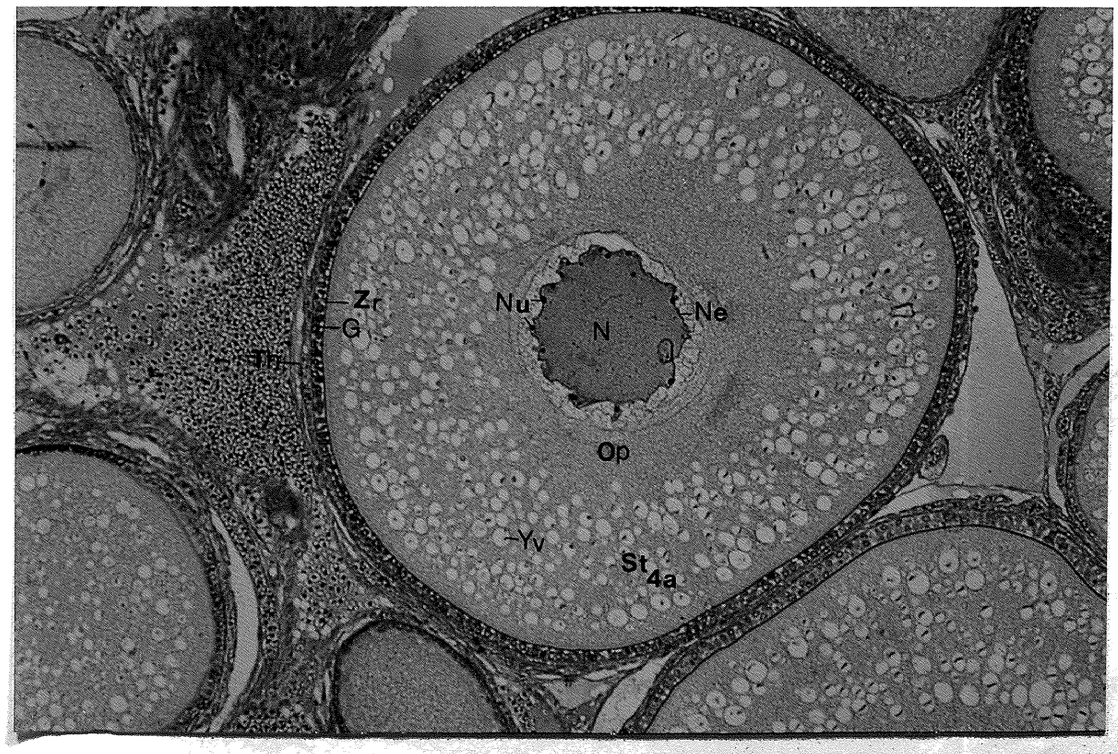


Plate 3.13

Plate 3.14 - Section of an ovary showing a stage 4b oocyte. Stained with haematoxylin and eosin.
Magnification x 160

G - granulosa, N - nucleus, Ne - nuclear envelope, Nu - nucleoli, Op - ooplasm, St_{4b} - stage 4b oocyte, Th - theca, Yv - ooplasmic vesicles or yolk vesicles, Zr - zona radiata.

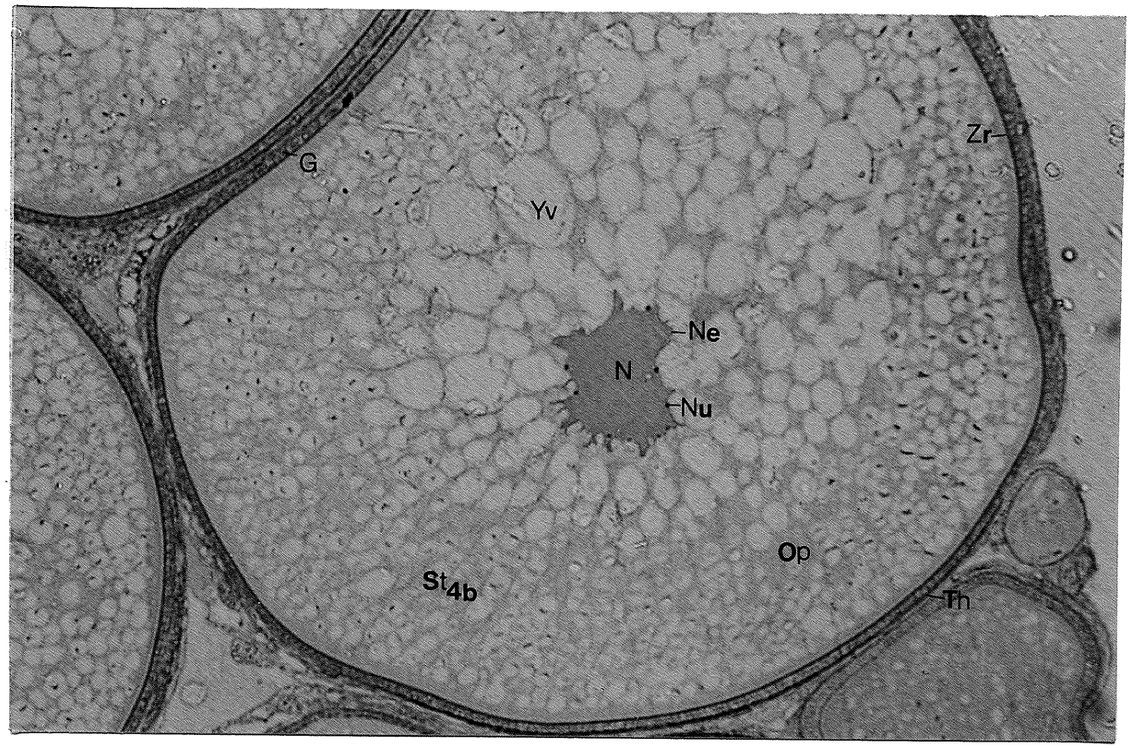


Plate 3.14

Plate 3.15 - Section of a stage 4a oocyte showing PAS + granules within the ooplasmic vesicles. Stained with periodic acid Schiff's (PAS).
Magnification x 1250

C - connective tissue, G - granulosa, Op - ooplasm, PAS+ - PAS positive granules, St_{4a} - stage 4a oocyte, Th - theca, Yv - ooplasmic vesicles or yolk vesicles, Zr - Zona radiata.

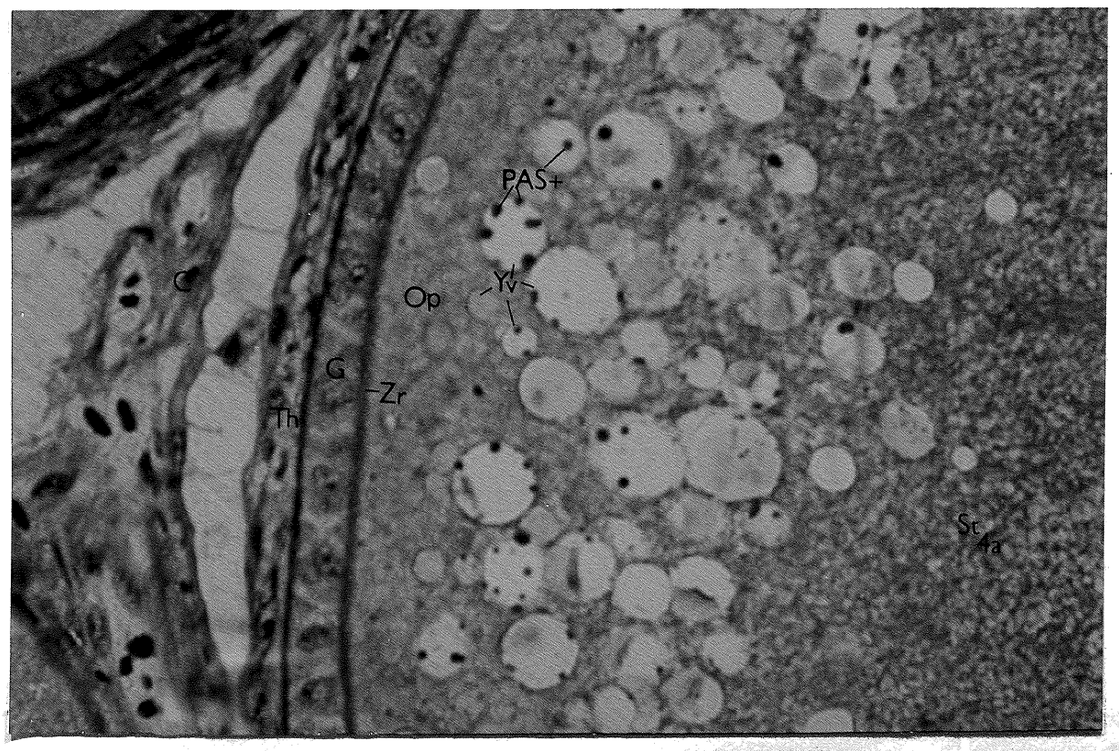


Plate 3.15

Plate 3.16 - Electron micrograph of a section of a stage 4a oocyte showing the association of endoplasmic reticulum and Golgi complex with the ooplasmic vesicles. Stained with uranyl acetate and lead citrate.
Magnification x 70,000

ER - endoplasmic reticulum, Gc - Golgi complex, Op - ooplasm, Yv - ooplasmic vesicles or yolk vesicles.

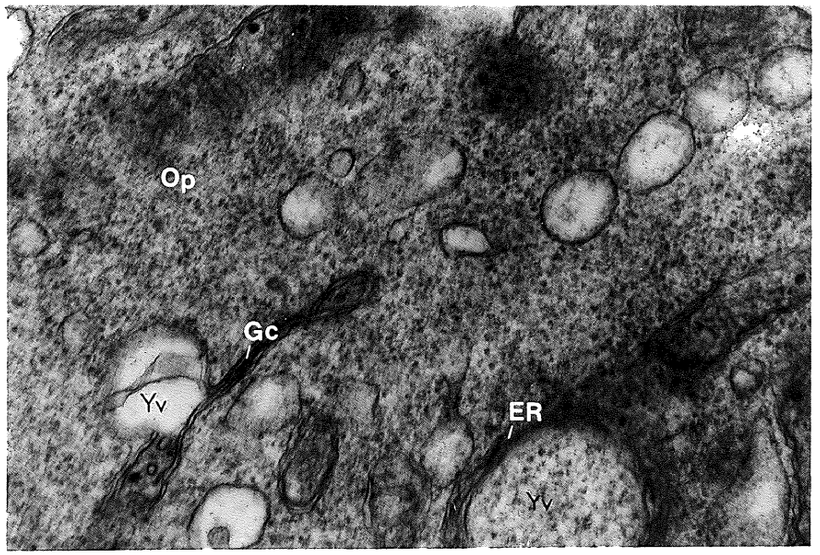


Plate 3.16

Plate 3.17 - Section of a stage 4b oocyte showing the follicle consisted of granulosa, theca and zona radiata. Stained with haematoxylin and eosin.
Magnification x 1250

C - connective tissue, G - granulosa, Mv - microvilli of oocyte membrane penetrating the zona radiata, Op - ooplasm, St_{4b} - stage 4b oocyte, Th - theca, Yv - ooplasmic vesciles, Zr - zona radiata.

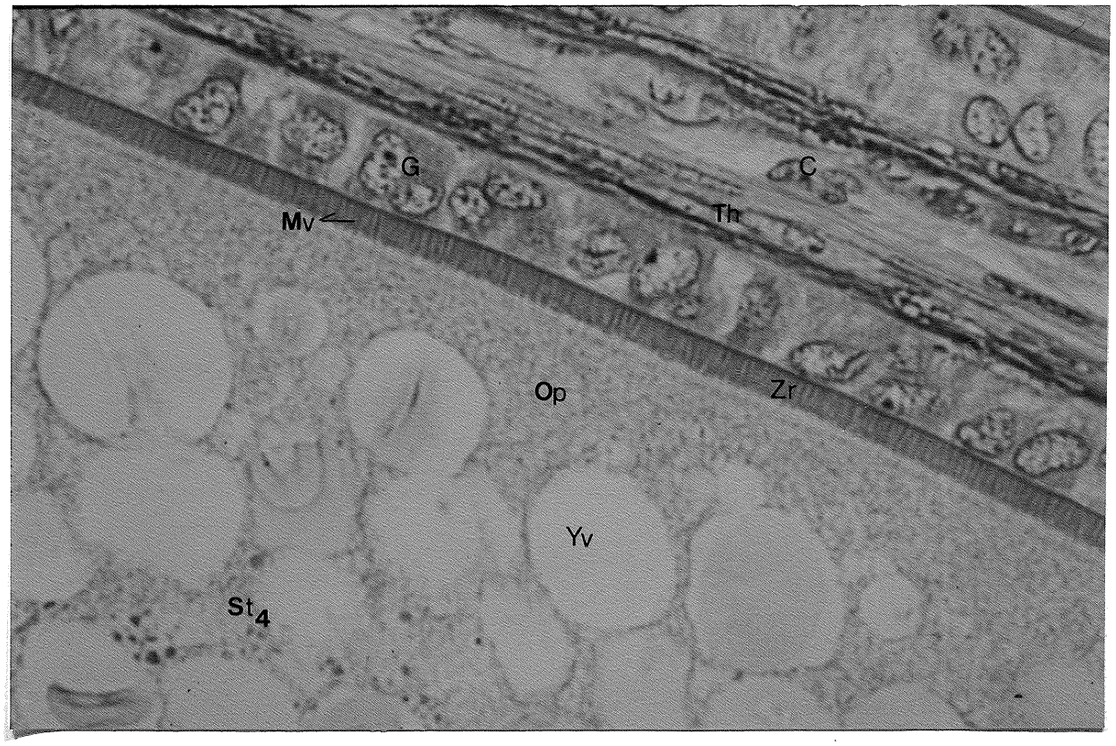


Plate 3.17

Plate 3.18 - Section of an ovary showing stage 5 oocytes. Stained with Mallory's triple stain.
Magnification x 80

C - connective tissue, G - granulosa, N - nucleus, Ne - nuclear envelope, Nu - nucleoli, Op - ooplasm, St_5 - stage 5 oocyte, Th - theca, Yg - yolk granules and globules, Yv - ooplasmic vesicles, Zr - zona radiata.

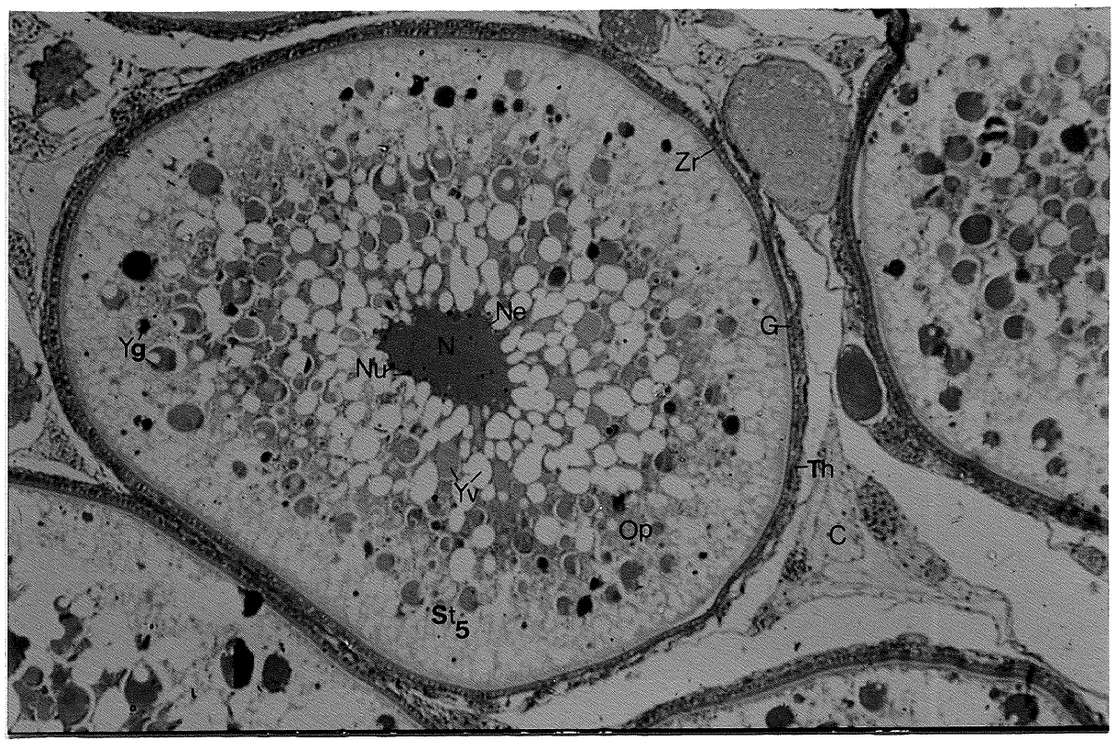


Plate 3.18

Plate 3.19 - Section of a stage 5 oocyte showing the follicle. Stained with haematoxylin and Eosin.
Magnification x 800

C - connective tissue, G - granulosa, Op - ooplasm, St_5 - stage 5 oocyte, Th - theca, Ygl - Yolk globules, Ygr - yolk granules, Yv - ooplasmic vesicles, Zr - zona radiata.

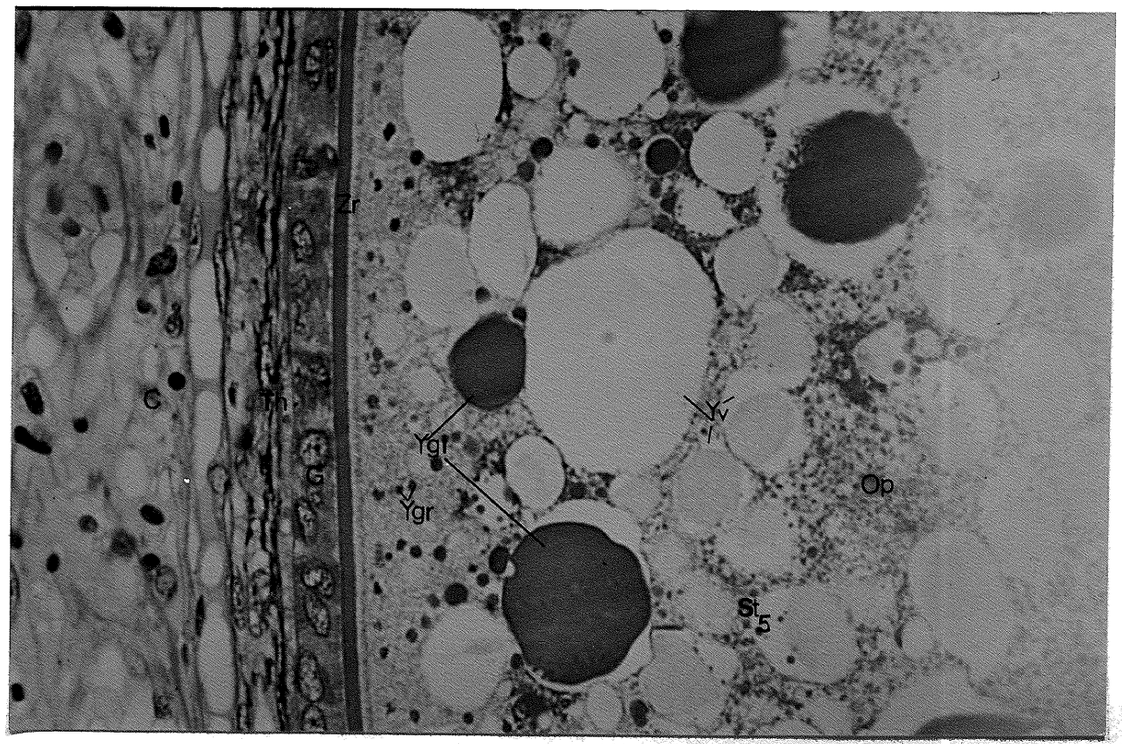


Plate 3.19

Plate 3.20a - Electron micrograph of a follicle of a stage 5 oocyte from a horseradish peroxidase (H.R.P.) injected fish, showing electron dense material on the zona radiata. Stained with uranyl acetate and lead citrate.
Magnification x 28000.

HRP - electron dense material deposited on places with high HRP concentrations, Mv - microvilli, Op - ooplasm, Zr - zona radiata.

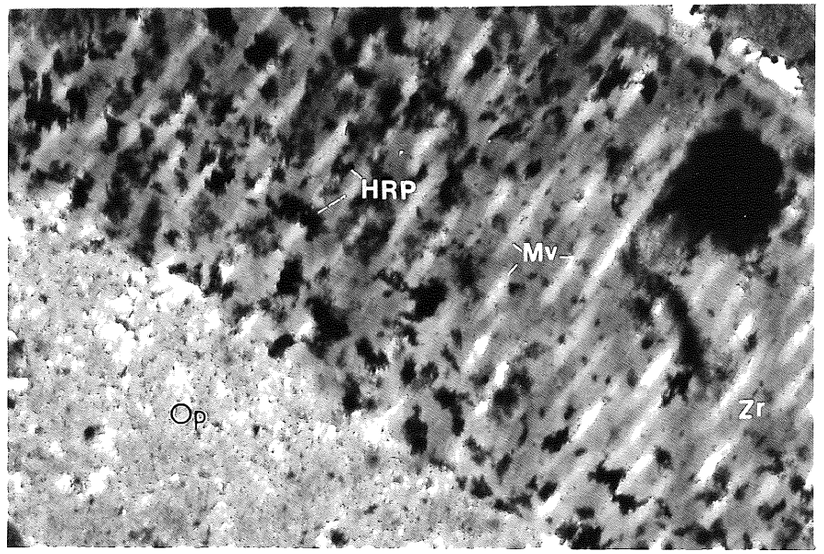


Plate 3.20a

Plate 3,20 b - Electron micrograph of a section of a stage 5 oocyte from a horseradish peroxidase (HRP) injected fish showing the deposition of electron dense material within the growing yolk globules. Stained with uranyl acetate and lead citrate.
Magnification x 48000

HRP - electron dense material on places where HRP is found. Op - ooplasm, Yg - yolk globule.

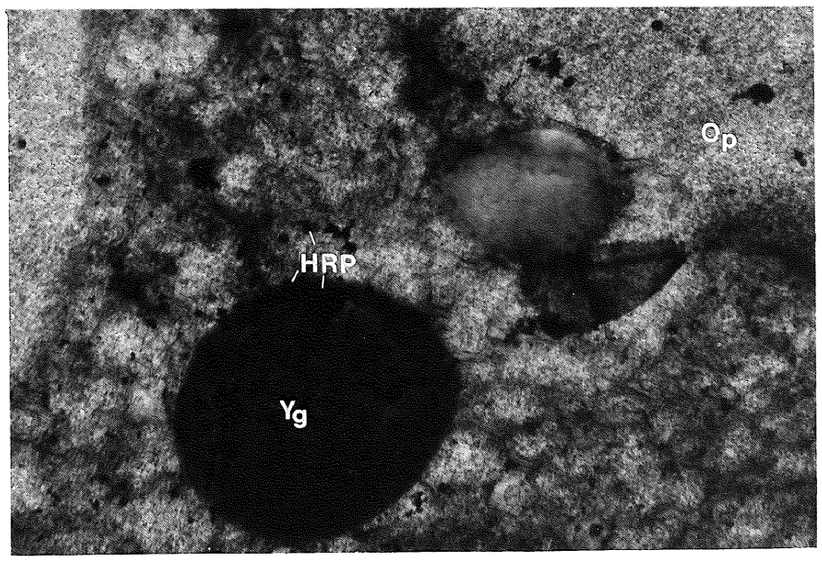


Plate 3.20b

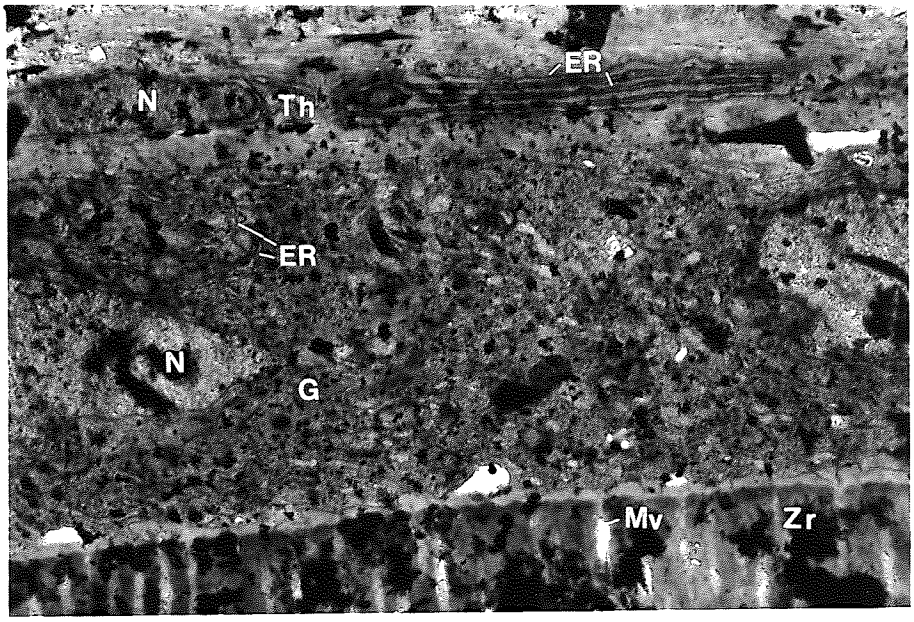


Plate 3.21

Plate 3.21 - Electron micrograph of a follicle of a stage 5 oocyte. Stained with a uranyl acetate and lead citrate. Magnification x 100,000

ER - rough endoplamic reticulum, G - granulosa, Mv - microvilli, N - nucleus, Th - theca, Zr - zona radiata.

Plate 3.22 - Section of an ovary showing early stage 6 (germinal vesicle migration stage) oocytes. Stained with haematoxylin and eosin.
Magnification x 50

G - granulosa, N - nucleus, Ne - nuclear envelope, Nu - nucleoli, St_6 - early stage 6 oocyte, Th - theca, Yg - yolk granules and globules, Yp - yolk platelets, Yv - ooplasmic vesicles, Yv- - empty vesicles, Zr - zona radiata.

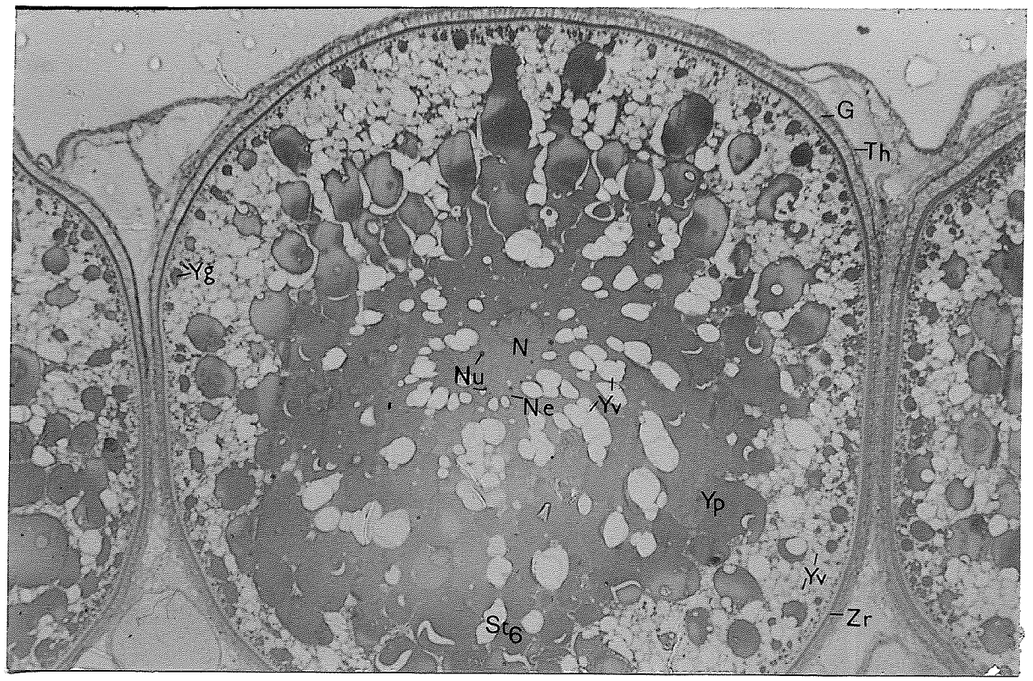


Plate 3.22

Plate 3,23 - Section of an ovary showing part of a late stage 6 (germinal vesicle migration stage) oocyte. Stained with haematoxylin and eosin.
Magnification x 50

Ca - cortical alveoli or peripheral ooplasmic vesicles, G - granulosa, N - nucleus, Ne - nuclear envelope, St_6 - late stage 6 oocyte, Th - theca, Yg - yolk granules and globules, Yp - yolk platelets, Yv - ooplasmic vesicles, Zr - zona radiata.

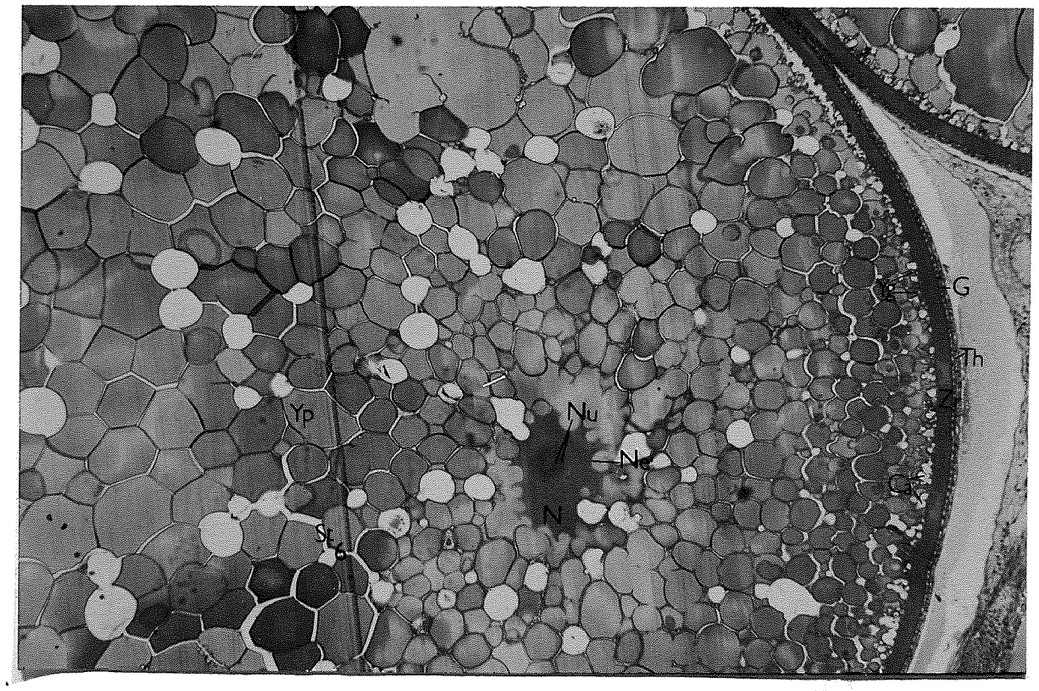


Plate 3.23

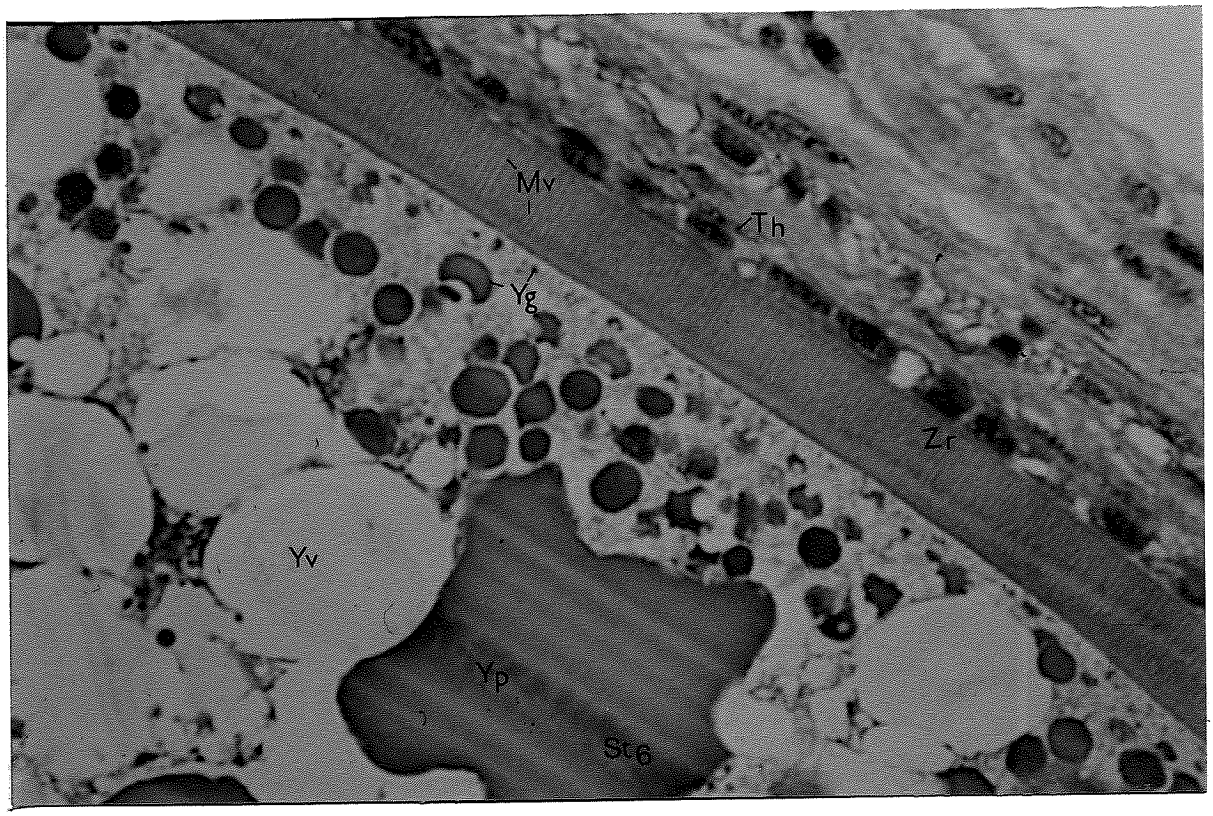


Plate 3.24

Plate 3.24 - Section of a late stage 6 oocyte showing its follicle. Stained with haematoxylin and eosin. Magnification x 800

G - granulosa, Mv - microvilli, St_6 - late stage 6 oocyte, Th - theca, Yg - yolk granules and globules, Yp - yolk-platelets, Yv - ooplasmic vesicles.

Plate 3.25a - Section of a stage 7 (germinal vesicle breakdown stage) oocyte showing the animal pole of the oocyte. Stained with Mallory's triple stain. Magnification x 500

C - connective tissue, Ca - cortical alveoli, G - granulosa, Gv - germinal vesicle or nucleoplasmic mass, M - micropoyle, Mc - micropylar cell, Nu - nucleoli, St_7 - stage 7 oocyte, Th - theca, Yg - yolk globules, Yp - yolk platelets, Zr - zona radiata.



Plate 3.25a

Plate 3.25b - Section of a stage 7 oocyte showing its follicle. Stained with Mallory's triple stain. Magnification x 800

Ca - cortical alveoli, G - granulosa, Mv - microvilli, St_7 - stage 7 oocyte, Th - theca, Yg - yolk globules, Yp - yolk platelets, Zr - zona radiata.

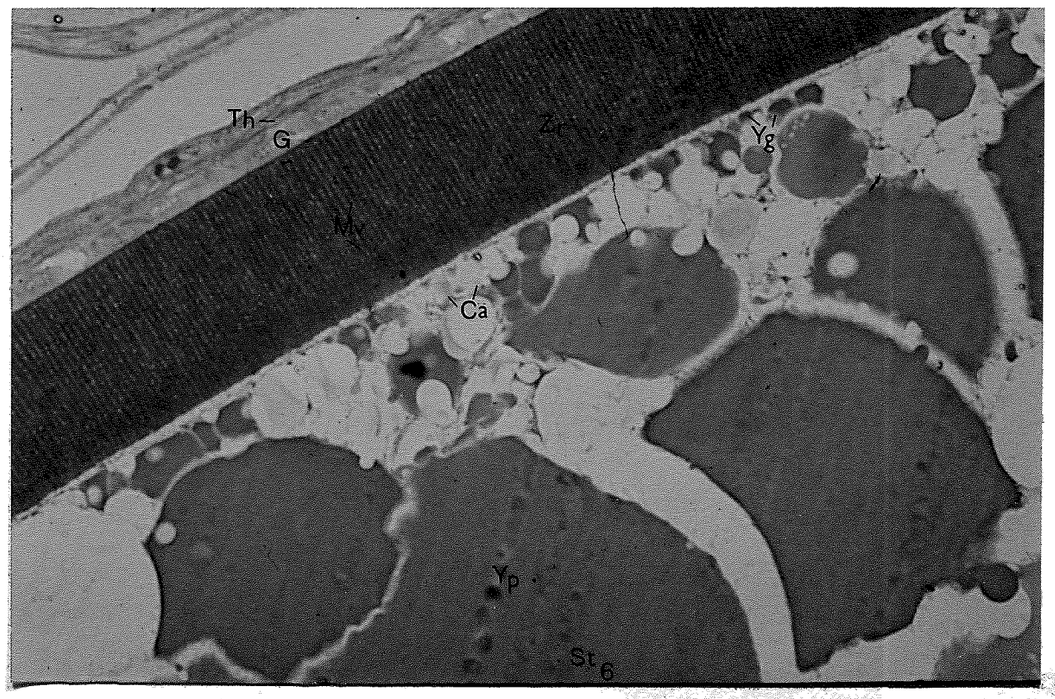


Plate 3.25b

Plate 3.26 - Section of an ovary showing stage 2b twin-oocytes. Stained with haematoxylin and eosin. Magnification x 625

F - follicle, N - nucleus, Nu - nucleoli, T - twin oocytes of stage 2a, Yn - Balbiani bodies.

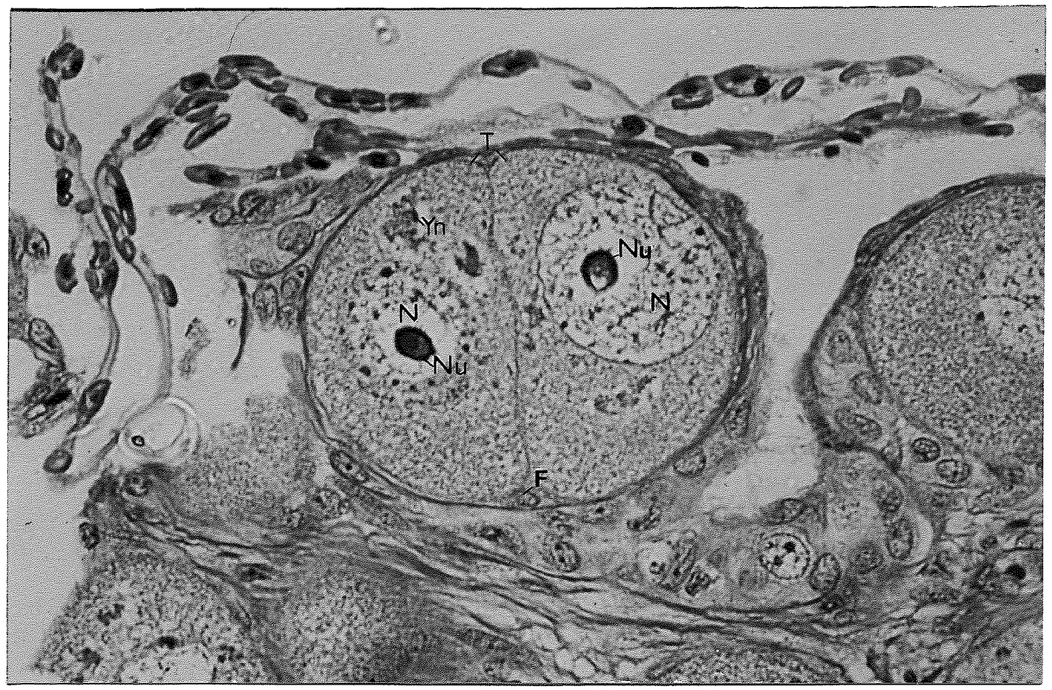


Plate 3.26

Plate 3.27 - Section of an ovary showing stage 4a twin-oocytes. Stained with haematoxylin and eosin. Magnification x 160

G - granulosa, N - nucleus, Th - theca.

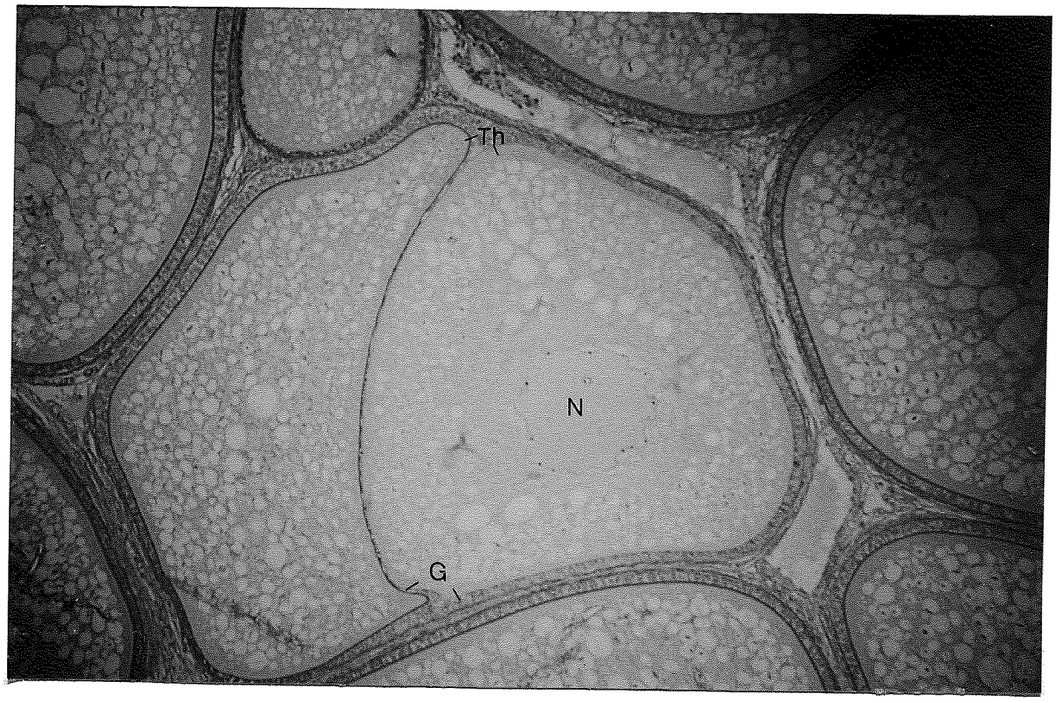


Plate 3.27

Plate 3.28a - Superficial view of a post-ovulatory follicle.
Magnification x 125

O - opening of the follicle through which oocyte was released, POF - post-ovulatory follicle.

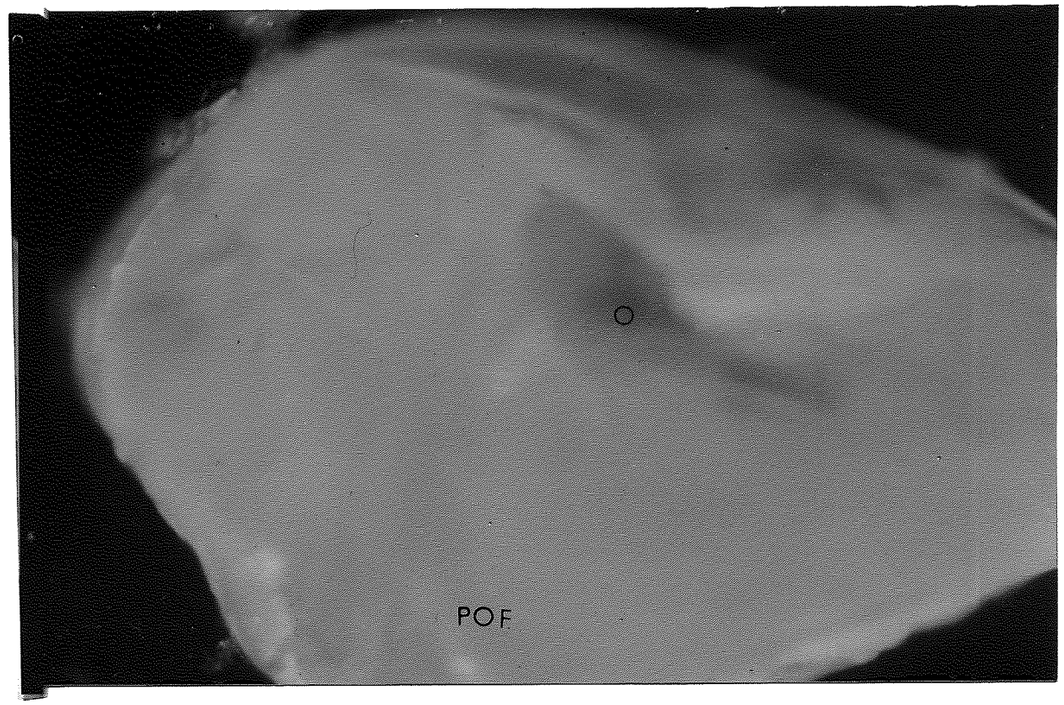


Plate 3.28a

Plate 3.28b - Section of an ovary showing a post-ovulatory follicle.
Stained with Mallory's triple stain.
Magnification x 80

G - hypertrophied granulosa, Ge - germinal epithelium, O - opening of the follicle, POF - post-ovulatory follicle St_3 - stage 3 oocyte, St_4 - stage 4a oocyte, Th - theca.



Plate 3.28b

Plate 3.29 - Section of a post-ovulatory follicle showing the separation of hypertrophied granulosa from the theca at the basal lamina (or basement membrane). Stained with haematoxylin and eosin.
Magnification x 400

Bl - basal lamina, C - connective tissue, G - granulosa, Th - theca.

Plate 3.30 - Section of a post-ovulatory follicle showing oogonial nests in the connective tissue adjacent to the theca. Stained with haematoxylin and eosin.
Magnification x 640

C - connective tissue, G -granulosa, Og - oogonial nests, Th - theca, Tr - trabeculae.

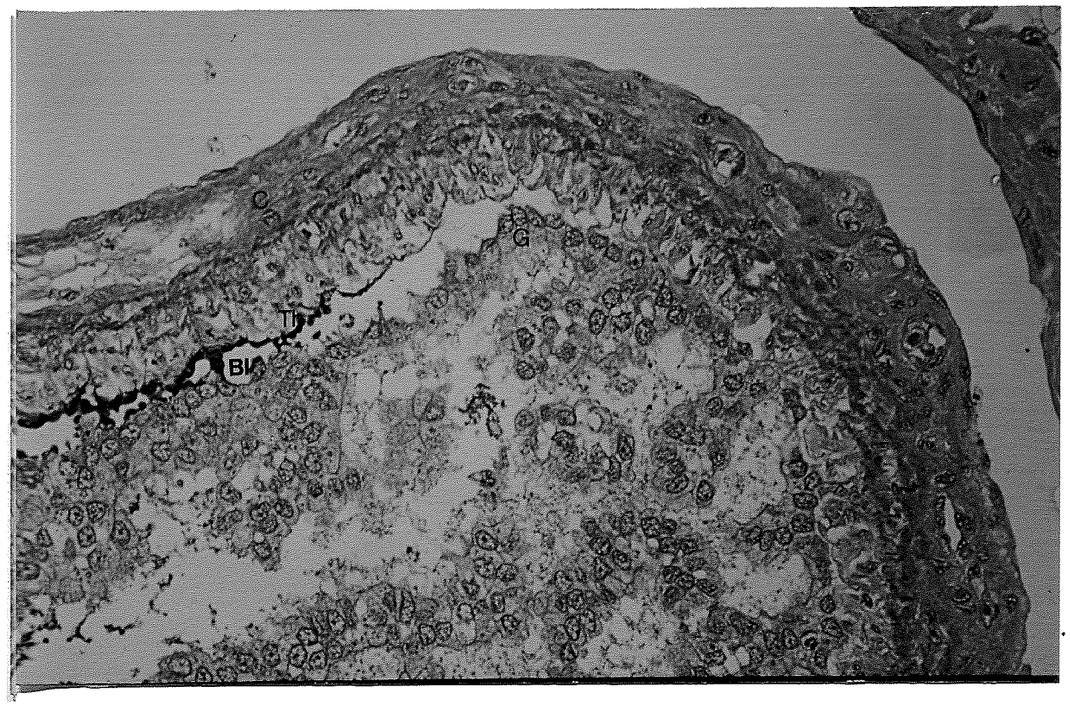


Plate 3.29

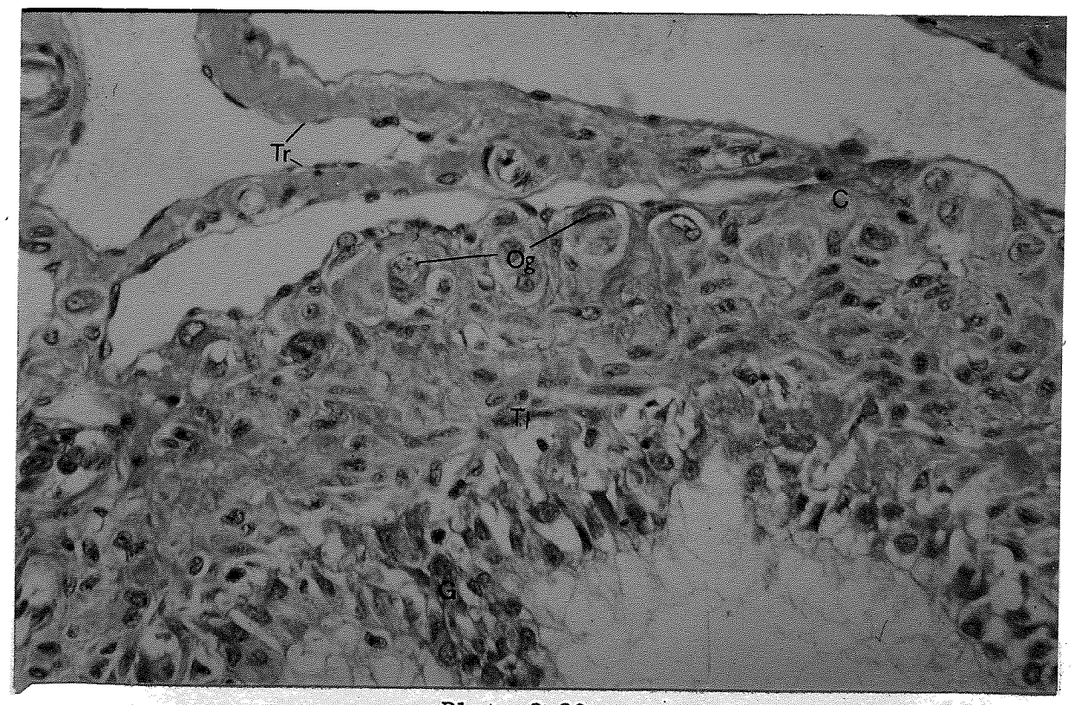


Plate 3.30

Plate 3.31a - Superficial view of an atretic oocyte
Magnification x 200

At - atretic oocyte.

Plate 3.31b - Superficial view of atretic oocytes, intact oocytes and post-ovulatory follicles.
Magnification x 80

At - atretic oocytes, St_2, St_3, St_4 and St_5 - intact oocytes, POF - post-ovulatory follicles.

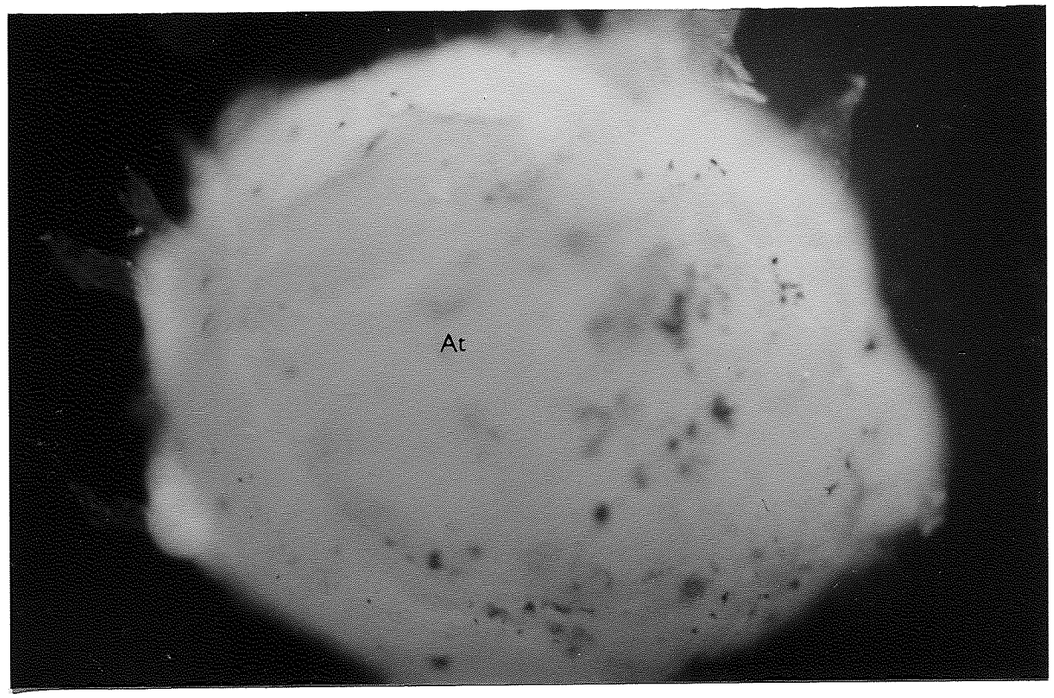


Plate 3.31a

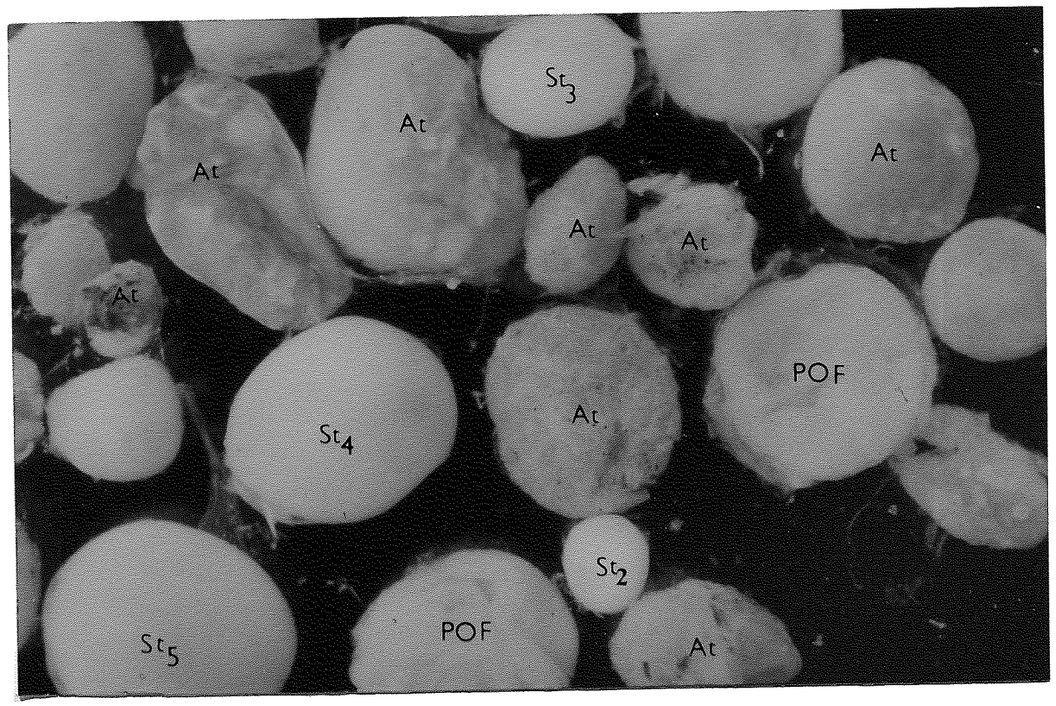


Plate 3.31b

Plate 3.32 - Section of a post-ovulated ovary showing stage 4 non-hypertrophic type atretic oocytes. Stained with Mallory's triple stain.
Magnification x 64

At_4 - stage 4 atretic oocyte, POF - post-ovulatory follicle.

Plate 3.33 - Section of an ovary showing a ruptured non-hypertrophic type stage 4 atretic oocyte.
Stained with haematoxylin and eosin.
Magnification x 160

G - granulosa, At_4 - stage 4 atretic oocyte.

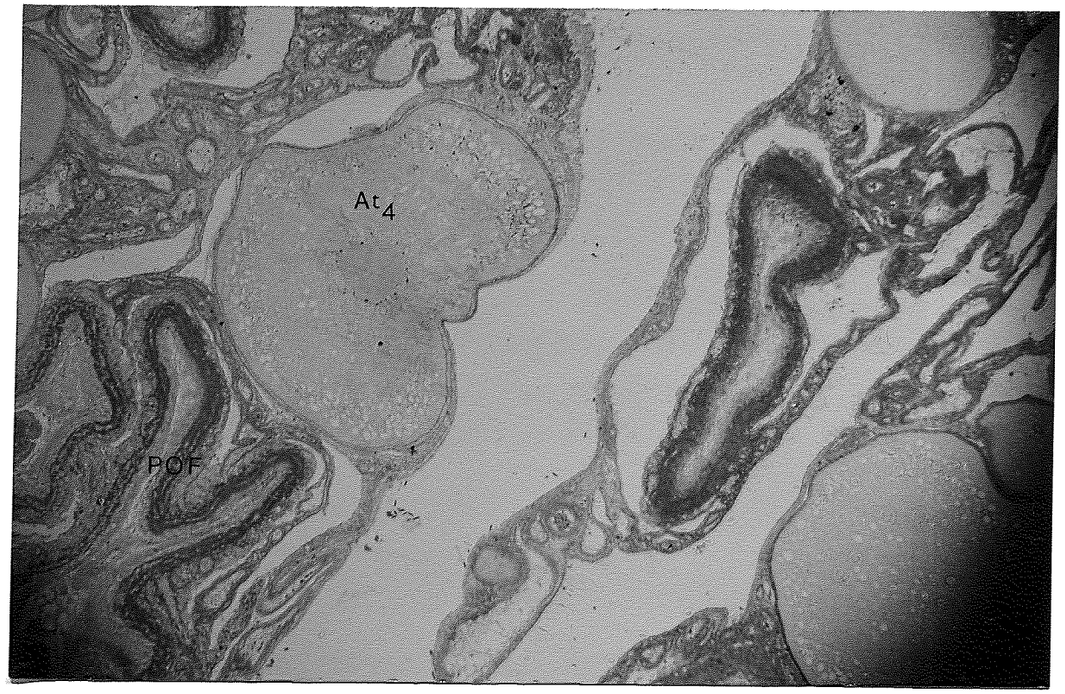


Plate 3.32

Plate 3.32

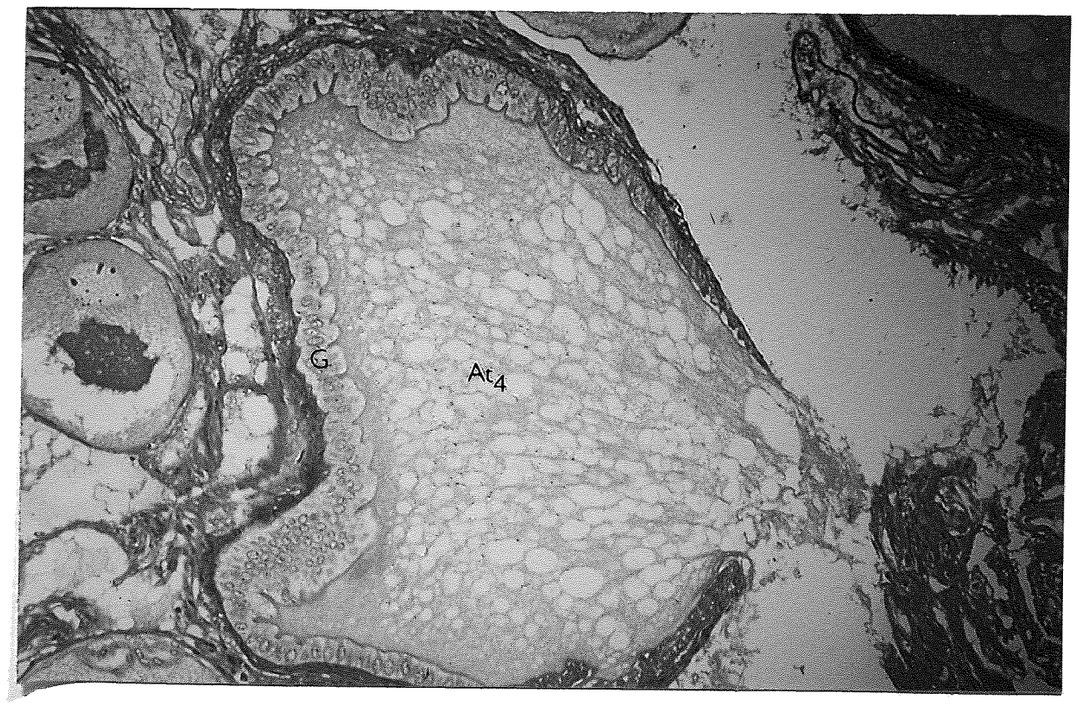


Plate 3.33

Plate 3.34 - Section of a mature ovary showing non-hypertrophic type stage 4 atretic oocytes among stage 7 mature oocytes. Stained with haematoxylin and eosin.
Magnification x 50

At_4 - stage 4 atretic oocyte, C - connective tissue, Gv - germinal vesicle, 7 - stage 7 mature oocytes.

Plate 3.35 - Section of an ovary of a starved fish showing hypertrophic type stage 4 atretic oocytes. Stained with haematoxylin and eosin.
Magnification x 125

At_4 - atretic stage 4 oocyte.

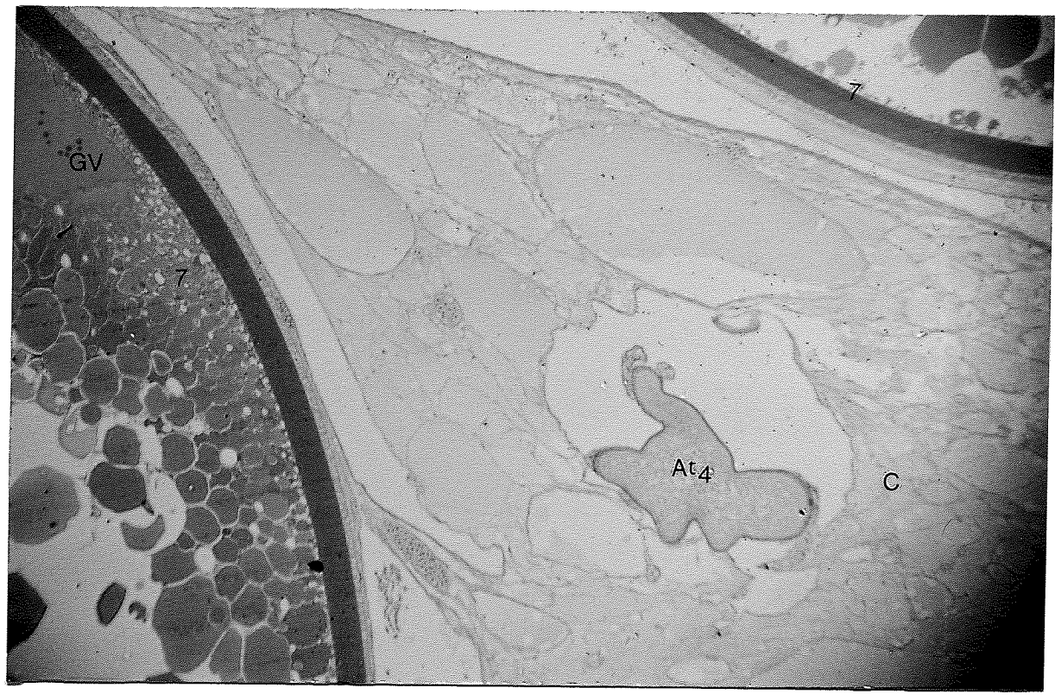


Plate 3.34

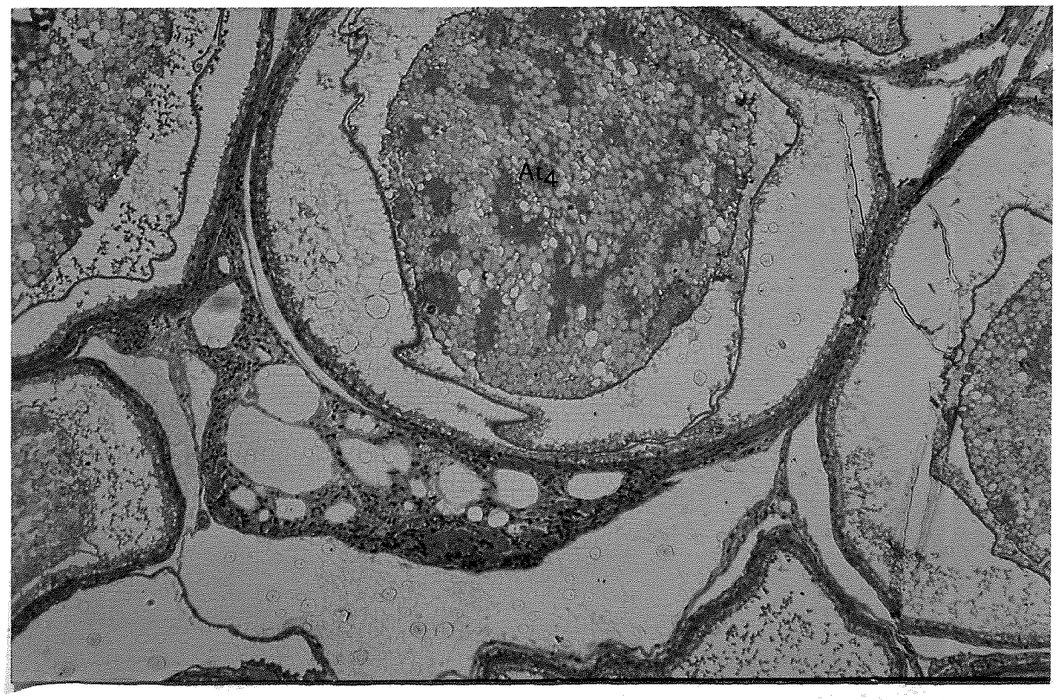


Plate 3.35



Plate 3.36

Plate 3.36 - Section of an ovary showing hypertrophic type stage 5 oocyte among intact stage 6 oocytes. Stained with haematoxylin and eosin.
Magnification x 64

At_5 - stage 5 atretic oocyte, Dy - degenerating yolk globules, Dz - degenerating zona radiata, G - hypertrophied granulosa, St_2 and St_6 - stage 2 and 6 intact oocytes.

Plate 3.37 - Section of a post-ovulated ovary showing an early stage 6 hypertrophic type atretic oocyte at the beginning of atresia. Stained with Mallory's triple stain.
Magnification x 64

At_6 - early stage 6 atretic oocyte, Dy - degenerating yolk, Dz - degenerating zona radiata, G - granulosa, Th - theca, Tr - trabeculae, POF - post-ovulatory follicle.

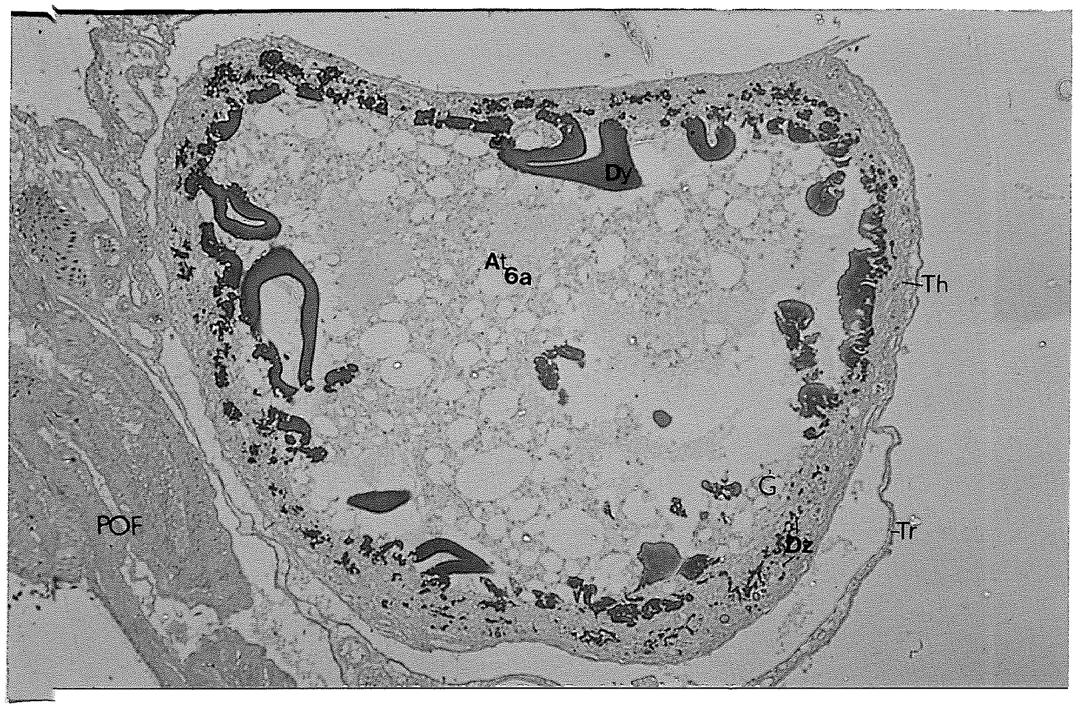


Plate 3.37

Plate 3.38 - Section of a post-ovulated ovary showing an early stage 6 hypertrophic type atretic oocyte at a later stage of atresia. Stained with haematoxylin and eosin.
Magnification x 64

At_6 - atretic stage 6 oocyte, Dy - degenerating yolk, F - follicle, G - granulosa, Pb - pigmented bodies, Tr - trabeculae.

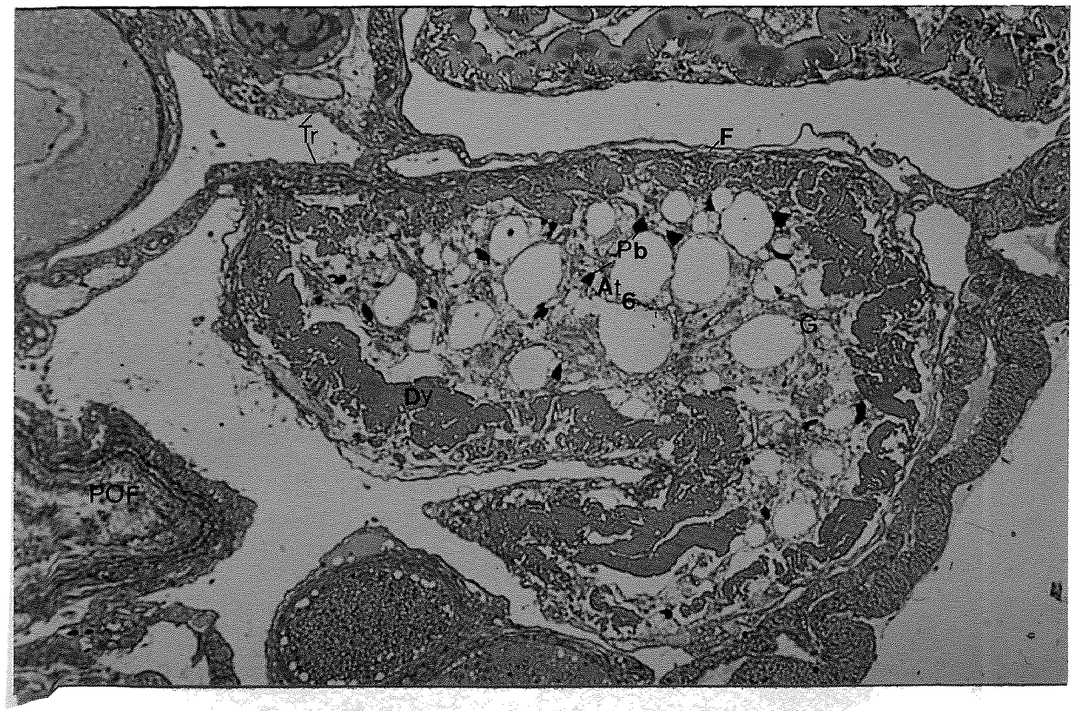


Plate 3.38

Plate 3.39 - Section of a post-ovulated ovary showing part of a late stage 6 non-hypertrophic type atretic oocyte at early stages of atresia. Stained with Mallor's triple stain.
Magnification x 50

At_6 - stage 6 atretic oocyte, Dy - degenerating yolk mass, POF - post-ovulated follicle, St_4 - stage 4 intact oocyte, Zr - zona radiata.

Plate 3.40 - Section of an ovary showing a stage 6 non-hypertrophic atretic oocyte at later stages of atresia. Stained with Mallory's triple stain.
Magnification x 50

At_6 - stage 6 atretic oocyte.

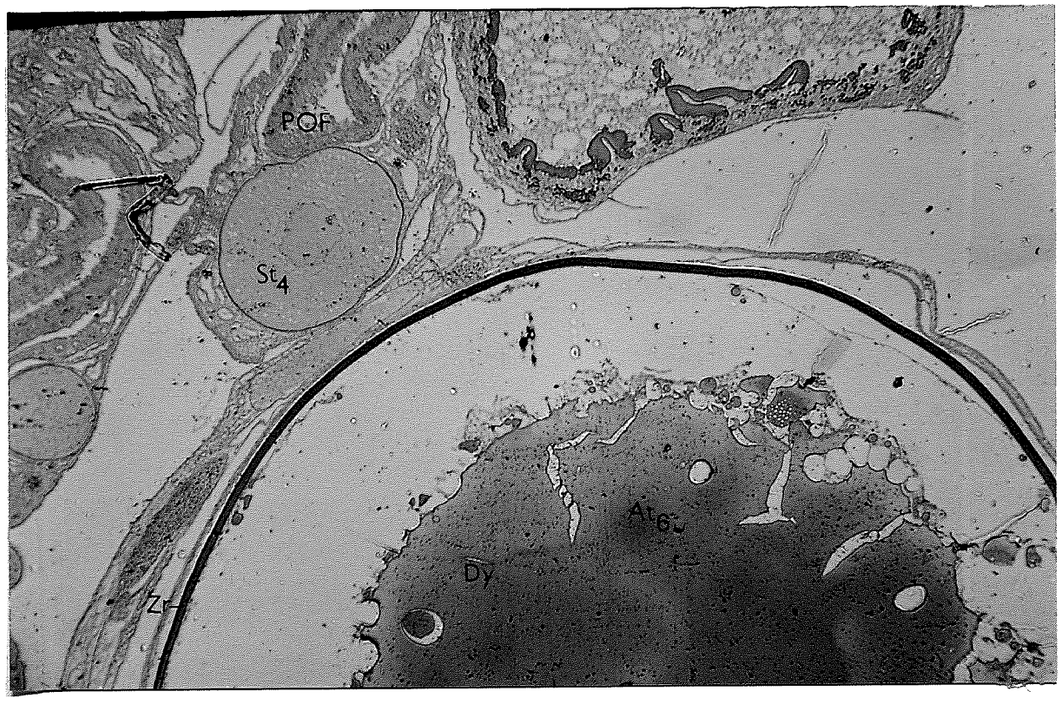


Plate 3.39



Plate 3.40

Plate 3.41 - Section of a starved fish ovary showing a stage 6 atretic oocyte at final stages of atresia with a collapsed zona radiata. Stained with haematoxylin and eosin.
Magnification x 64

At_6 - stage 6 atretic oocyte, St_4 - stage 4 atretic oocyte.

Plate 3.42 - Section of a stage 7 non-hypertrophic type atretic oocyte at early stages of atresia. Stained with haematoxylin and eosin.
Magnification x 640

At_7 - stage 7 atretic oocyte, Bc - blood capillaries, C - connective tissue, G - granulosa, Mv - microvilli, Th - theca, Y - yolk mass, Zr - zona radiata.



Plate 3.41

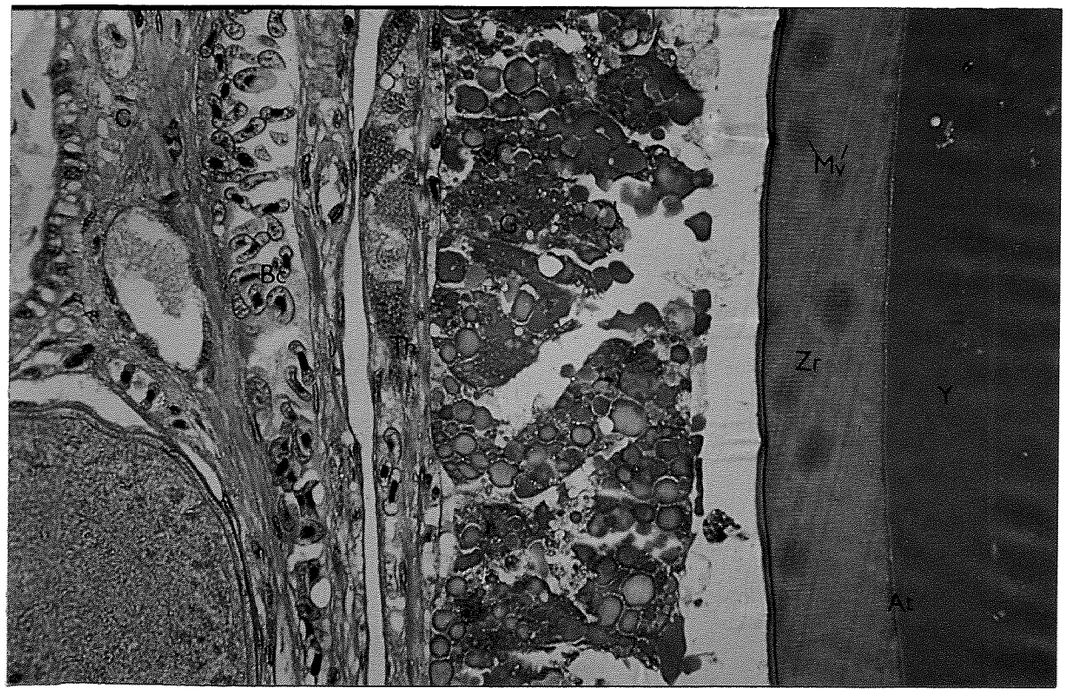


Plate 3.42

Plate 3.43 - Section of a post-ovulated ovary showing brown bodies, which are the terminal stages of atresia, among post-ovulatory follicles and early stage 4 oocytes. Stained with haematoxylin and eosin. Magnification x 160

Br - brown bodies, POF - post-ovulatory follicle, St_{2a} and St_{4a} - stage 2a and 4a intact oocytes.

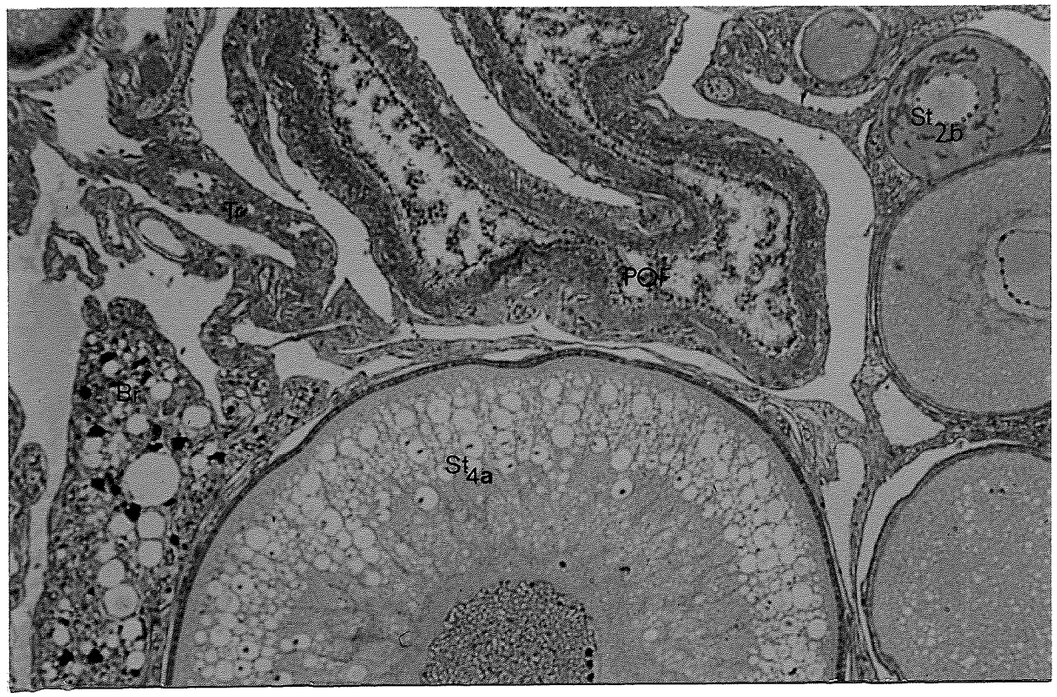


Plate 3.43

Plate 3.44 - Section of an ovary of a 1+ year-old fish which was not destined to spawn, showing brown bodies when spawning fish had vitellogenic stage 5 oocytes. Stained with haematoxylin and eosin. Magnification x 312.5

Br - brown body, St_{4a} - stage 4a oocyte.

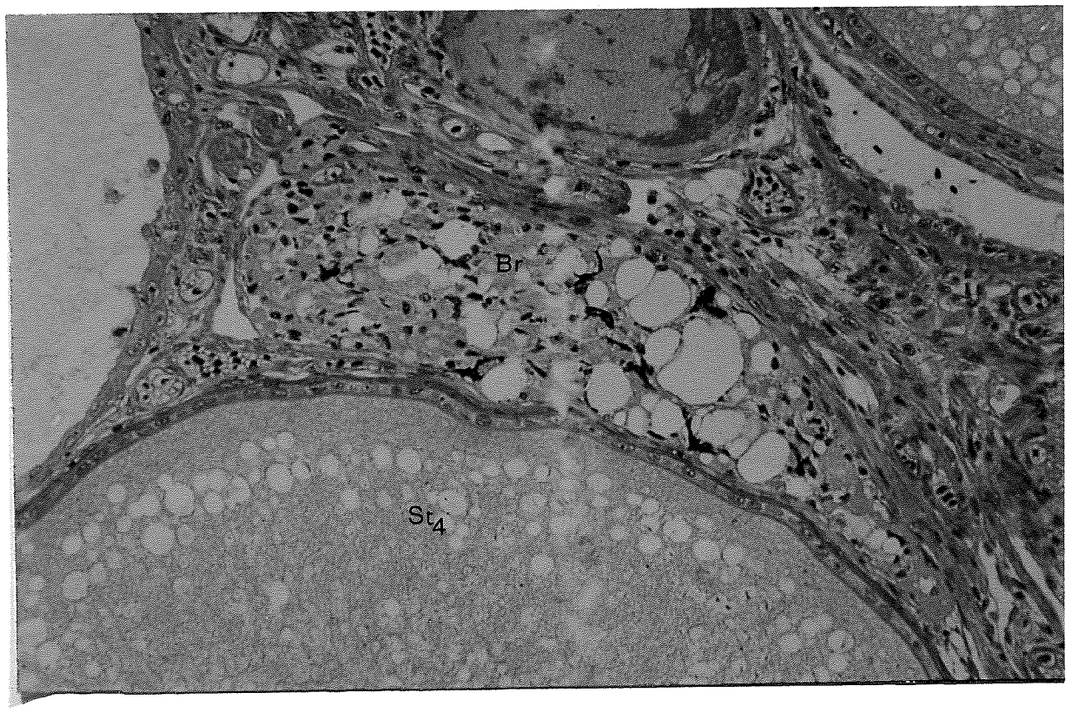
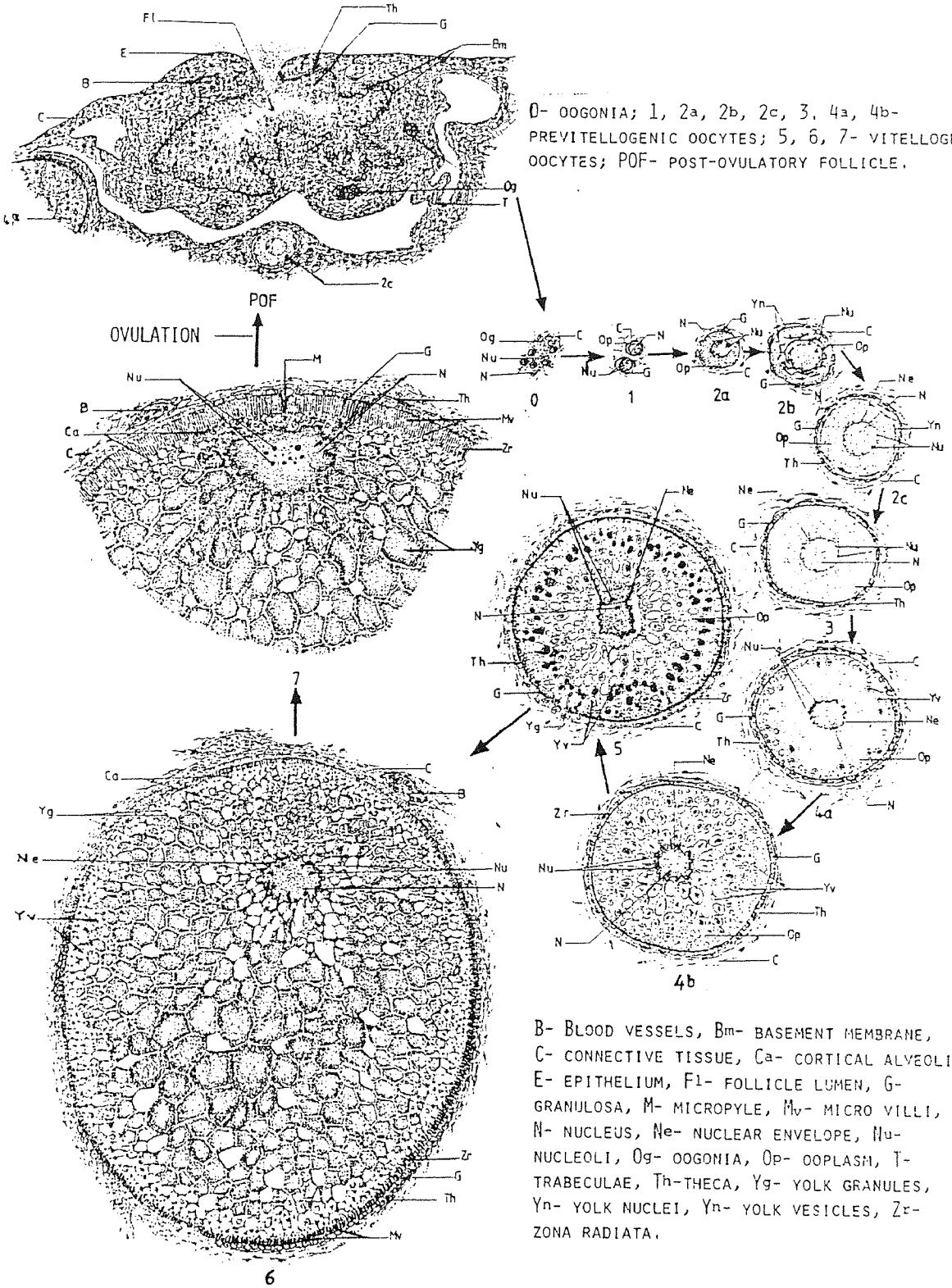


Plate 3.44

OOCYTE DEVELOPMENT CYCLE OF RAINBOW TROUT
(SALMO GAIRDNERI)



O- OOGONIA; 1, 2a, 2b, 2c, 3, 4a, 4b- PREVITELLOGENIC OOCYTES; 5, 6, 7- VITELLOGENIC OOCYTES; POF- POST-OVULATORY FOLLICLE.

B- BLOOD VESSELS, Bm- BASEMENT MEMBRANE, C- CONNECTIVE TISSUE, Ca- CORTICAL ALVEOLI, E- EPITHELIUM, Fl- FOLLICLE LUMEN, G- GRANULOSA, M- MICROPYLE, Mv- MICRO VILLI, N- NUCLEUS, Ne- NUCLEAR ENVELOPE, Nu- NUCLEOLI, Og- OOGONIA, Op- OOPLASM, T- TRABECULAE, Th-THECA, Yg- YOLK GRANULES, Yn- YOLK NUCLEI, Yn- YOLK VESICLES, Zr- ZONA RADIATA.

Fig 3.3 - Oocyte developmental cycle of rainbow trout (Salmo gairdneri) showing the cytological changes which occur within an oocyte.

3.4 Discussion

The ovarian histology of the three strains of rainbow trout considered in this work was similar to the descriptions given by Hurk and Peute (1979) for the same species and by Matthews (1938), Braekevelt and McMillan (1967), Hoar (1967) and Brummett et al (1982) for other telosts. Seven different stages of oocyte development could be identified thus confirming the earlier findings of Yamamoto et al (1965) and Hurk and Peute (1979). In addition several different stages of atresia were also described. Similar stages have also been reported for other teleost species (Vakaet, 1955; Gokhale, 1957; Polder, 1961; Takano, 1964; Abraham et al, 1966; Braekevelt and McMillan, 1967; Shakley and King, 1977; Tokaz, 1978; Khoo, 1979; Kagawa et al, 1981; Wallace and Selman, 1981; Forberg, 1982 and Matsuyama and Matsuura, 1982) although few have used the terminology of Hurk and Peute (1979) which was adopted in this study. In general the maturational process in the oocytes have previously only been poorly described.

Yamamoto et al (1965) obtained histological sections of different stages of oocyte development in rainbow trout, although descriptions of the cytological changes which occur within the oocyte during its development to the mature oocyte were not included. Hurk and Peute (1979) identified seven stages of oocyte development in rainbow trout, ie. chromatin nucleolar stage, early perinucleolar stage, late perinucleolar stage, yolk vesicle stage, peripheral yolk granule stage and maturational stage, but included few details of the nuclear and cytoplasmic changes of the last three stages of development of the oocyte. Although seven stages of oocyte development can be recognised histologically, it is important to

explain these in terms of the developmental processes which are occurring within the ovary. Differences in the staining affinity of the ooplasm and in the positioning of the "Balbiani bodes" (Wallace and Selman, 1981) also allowed further subdivision of early perinucleolar stage into three; ie. 2a, 2b and 2c. The stage 4 oocytes, ie. those with the vesicles, could also be subdivided on the basis of the distribution of ooplasmic vesicles. Although seven stages of oocyte development can be recognised in the teleost ovary these stages may be functionally grouped into four phases of development, ie. oogenesis and folliculogenesis, endogenous vitellogenesis, exogenous vitellogenesis and maturation, with oogenesis being preceeded by oogonial proliferation (see review by Tokaz, 1978).

During the early stages of sex differentiation the ovaries of all the strains of rainbow trout contained only oogonia. Similar observations were also made by Hurk and Slof (1981). This condition prevailed for two to three months after sex differentiation. During this period the ovary grew only as a result of oogonial proliferation. Primary and secondary oogonia were identified. The primary oogonia were undifferentiated cells with the prominent nucleus containing several small nucleoli and enclosed by an indistinct narrow band of cytoplasm. The secondary oogonia were slightly larger than the primary oogonia and their nuclei contained one or more prominent nucleoli. Occasionally mitotic figures were observed. Generally most of the earlier studies failed to describe the structure of the oogonia and only mentioned the timing of their appearance. Oogonia were seen in almost all the ovaries of fish of all age groups studied, often in nests surrounded by other smaller

cells, ie. smaller than the primary oogonia. Similar cells have been identified for several other teleosts and they are known to be the pre-follicle cells which later form the follicle around the growing oocyte (see review by Tokaz, 1978; Brusle, 1980).

In the majority of studies of ovarian histology in teleosts it is often not clear whether the follicle consists of the oocyte together with the extra-oocyte tissue. However, according to the review by Tokaz (1978) "folliculogenesis" should include only the extra-oocyte tissue. Therefore, in the present study the word "follicle" is used to describe the extra-oocyte tissues.

Transformation of oogonia into primary oocytes, ie. oogenesis is marked by the growth of cytoplasm and also by changes within the nucleus. During the present study it was not possible to define the time at which oogenesis commenced although one could conclude the first stage of primary oocyte growth, ie. chromatin nucleolar stage, appear within the ovary four months after hatching or 2 months after the first appearance of primary oogonia. They appears for the first time together with both oogonia and early perinucleolar stage (stage 2a) oocytes, which suggests that the transformation of secondary oogonia into chromatin nucleolar stage oocytes and then their recruitment to perinucleolar stage occurs within a period less than a month. Oocytes of the chromatin nucleolar stage were slightly larger than oogonia with a narrow, slightly basophilic band of cytoplasm surrounding a large nucelus. Similar observations have been made for rainbow trout (Yamamoto et al, 1965; Hurk and Peute, 1979) and for other teleost species (Takano, 1964; Braekevelt and McMillan, 1967; Lambert, 1970a; Tokaz, 1978; Hurk and Peute, 1979; Khoo, 1979; Brusle, 1980; Kessel, 1981; Forberg, 1982). In a few studies the

nuclei of the chromatin nucleolar stage oocytes contained several peripherally-located, thread-like, basophilic nucleoli attached to the inside of their nuclear envelope, whilst in later stages of stage 1 oocytes one or more spherical nucleoli were observed in association with chromatin strands before the oocytes entered the second stage (early perinucleolar stage). After this stage of development the cytoplasm of oocytes increases in volume and undergoes a complex series of structural changes (Braekevelt and McMillan, 1967; Lambert, 1970a; Wallace and Selman, 1981; Forberg, 1982; Nagahama, 1983).

The second stage of oocyte development was divisible into three substages (2a, 2b and 2c) according to the basophilic nature and the distribution of "Balbiani bodies" described by Wallace and Selman (1981). Kessel (1981) reported that these Balbiani bodies were primarily made up of nucleolar fragments (nuage) which are released into the ooplasm through the pores in the nuclear envelope. The coincidental disappearance of the chromatin strands, which are attached to the nuclear envelope, and the appearance of one or more small spherical nucleoli supports Kessel's (1981) proposition. The nuage associates later with mitochondria, polyribosomes and annulate lamellae to form the "cement" (Kessel, 1981) or "Balbiani bodies" (Wallace and Selman, 1981). During the present work the ooplasm of the stage 2 oocytes was initially highly basophilic (stage 2a) but with further growth basophilic bodies were only seen close to the nucleus (stage 2b). Finally in the largest stage 2 oocytes the basophilia of these cytoplasmic bodies diminished and at the same time they migrated towards the periphery of the oocyte (stage 2c) and gradually disappeared. These basophilic bodies are comparable to "Balbiani bodies" or "cement" described by Wallace and Selman (1981)

and Kessel (1981). The disappearance of the "Balbiani bodies" defines the time of entrance of the oocytes into stage 3.

When the oocytes entered stage 3 the ooplasm was clear and slightly acidophilic. Up to this stage the increase in size of the oocyte was due to the growth of both the nucleus and the cytoplasm whereas in the later stages, growth was due solely to increases in cytoplasmic volume and in the numbers of cytoplasmic inclusions. At the end of stage 3, the follicle which surrounded the oocyte consisted of a single granulosa and two thecal layers (Hurk and Peute, 1979; Wallace and Selman, 1981). Braekevelt and McMillan (1967) and Hurk and Peute (1979) reported that the oocyte membrane formed microvilli towards the end of stage 3, with the granulosa producing proteins which are then incorporated into the zona radiata formed between the microvilli of oocyte membrane and granulosa itself (Wourms, 1976; Wourms and Sheldon, 1976; Hoar and Nagahama, 1978). However, in the present study microvilli were not observed light-microscopically, until late stage 4 although the zona radiata was first seen in early stage 4 oocytes.

The zona radiata is formed external to the oocyte membrane below the granulosa cell layer. Within the zona radiata microvilli could be seen as radial striations under both the light and electron microscopes. With the growth of the maturing oocytes the microvilli lengthened and the zona radiata became thicker. In mature oocytes the zona radiata forms the chorion which has a thickness of more than 30 μ.

When the stage 3 oocytes reached stage 4 the first structures to appear in the cytoplasm were the vesicles. They appeared in the ooplasm near the oocyte membrane. As this stage progressed the

vesicles became larger and moved towards the centre of the oocyte. Similar observations were also made by Braekevelt and McMillan (1967) and Khoo (1979). At the end of stage 4 the whole ooplasm was filled with vesicles, with those situated at the juxta-nuclear region being larger than the rest and without any inclusions. The remaining vesicles contained basophilic and PAS positive material. Although it is difficult to interpret these results it might be suggested that the vesicular material had been utilized during the growth of the oocyte leaving them empty.

Autoradiographic studies on zebra fish oocytes have shown that these inclusions are synthesised within the oocytes and are glycoprotein in nature (Korfsmeier, 1966). Supportive evidence for such an origin has also been provided in electrophoretic (Heesen and Engles, 1973) and immunological studies (Heesen, 1977) both of zebra fish oocytes. Earlier work had proposed that the vesicles contained an intravesicular yolk (Marza et al, 1937) and consequently they were named yolk vesicles or "gouttes claires" (Konaopacka, 1935), yolk vacuoles (Yamamoto, 1956b; Bisht and Joshi, 1975), primary yolk globules (Beach, 1959) lipid vesicles or granules (Krajhanzl et al, 1984; Nosek et al, 1984) or cortical alveoli (Anderson, 1968). Although most of the above terms indicate the presumed relation of those vesicles to the yolk formed during the secondary growth phase, their structure, formation and functional importance are poorly described. The electron microscopical studies reported here indicated that vesicles can arise in association with the Golgi complex and the endoplasmic reticulum, with lamellae of the endoplasmic reticulum arranged around the growing vesicles. The involvement of the Golgi complex and endoplasmic reticulum in the

formation of these vesicles has also been reported by Anderson (1968) whereas, Yamamoto (1971) suggested that only the Golgi complex is involved in vesicle formation. Irrespective of the organelles from which vesicles are derived all these observations confirm the endogenous origins of the ooplasmic vesicles.

Elliott (1982) observed protein synthetic organelles such as mitochondria with lamellar cristae close to the growing vesicles. Although vesicle formation is succeeded by true yolk deposition, which is probably the most significant phase of oocyte growth, it is not clear whether this yolk is derived from a precursor material manufactured during the ooplasmic vesicle stage. The presence of large empty vesicles close to the nucleus during the late vesicle stage and prior to the yolk deposition indicates that the vesicular contents are used in the growth of the oocyte which occurs before true yolk deposition begins. To confirm this proposition further biochemical investigations are necessary.

The vesicles are also said to give rise to cortical alveoli during the final stages of oocyte development (Yamamoto, 1956b; Malone and Hisoaka, 1963; Khoo, 1979). During the present study the incoporation of true yolk pushed the ooplasmic vesicles towards the periphery of the oocyte. This observation supports a role for the vesicles in the formation of cortical alveoli. Recent biochemical and immunofluorescent studies provide clearer evidence also for the vesicular origins of the cortical alveoli (Krajhanzl et al, 1984a, b; Nosek, 1984; Nosek et al, 1984).

When the oocytes had become filled with vesicles small acidophilic granules appeared in the vicinity of the microvilli. Subsequently these minute granules coalesed with each other and

migrated towards the centre of the oocyte later to form large yolk globules. These globules were arranged into polygonal-shaped yolk platelets. Similar reports have also been made for the development of yolk in Fundulus heteroclitus (Selmand and Wallace, 1983). This deposition of yolk constitutes by far the major portion of the growth of the oocyte. The yolk proteins are derived from the macromolecule vitellogenin which is produced within the liver (Thurston, 1967; Aida et al, 1973; Heesen and Engels, 1973; Wallace, 1978; Hurk and Peute, 1979; Campbell and Idler, 1980; Wallace and Selman, 1981). Vitellogenin is sequestered by the oocytes from the blood by a micropinocytotic process (Korfsmeier, 1966; Heesen and Engels, 1973; Campbell and Jalabert, 1979). Autoradiographic and horseradish peroxidase techniques have shown that in Fundulus heteroclitus, the vitellogenin is transferred into the oocyte via perivitelline capillaries and the follicular tissues (Selmen and Wallace, 1982, 1983). During the present work electron micrographs of the vitellogenic oocytes of rainbow trout injected with horseradish peroxidase showed after 6 hours aggregations of electron-dense material randomly distributed in the zona radiata and also in small clusters within the ooplasm and on the yolk globules. It was difficult to ascertain the path taken by the horseradish peroxidase, although the presence of electron-dense material within the oocyte suggests that large molecular weight materials like horseradish peroxidase and vitellogenin are transported through the zona radiata of the rainbow trout oocytes in a similar way to that seen in Fundulus heteroclitus (Selman and Wallace, 1982, 1983). Yolk precursor proteins are incorporated into the oocyte via micropinocytotic vesicles at the oocyte membrane (Selman and Wallace,

1982, 1983). The appearance of eosinophilic spherical granules near the microvilli at the beginning of stage 5, supports this proposition.

The incorporation of yolk into the oocytes and the subsequent formation of yolk platelets were seen light microscopically. Similar observations have been made in Fundulus. It has also been reported that formation of the crystalline platelets of yolk involves the proteolytic cleavage of vitellogenin to form the yolk proteins; lipovitellin and phosvitin (Selman and Wallace, 1983). Although the present investigation did not include any information regarding the cleavage of yolk materials, there were clear histological differences between the yolk granules or globules and the yolk platelets. The yolk globules were spherical and included an oily substance whilst the yolk platelets were larger and had a crystalline structure.

The incorporation of yolk is referred to as true yolk deposition or true vitellogenesis (Yamamoto, 1957; Hurk and Peute, 1979; Khoo, 1979; Wallace and Selman, 1981; Kagawa et al, 1981; Nagahama, 1983). In the rainbow trout three stages of oocyte development actively incorporated yolk; they are the peripheral yolk granular stage (stage 5), the centrally-migrating yolk granular stage (stage 6) and the stage of final maturation (stage 7). Similar stages were identified by Hurk and Peute (1979) on the basis of yolk incorporation by the oocyte and the movement of yolk globules within the oocyte. In addition the last two stages showed a polarization of the oocyte due to differences in size of the yolk platelets, and to the structure and the positioning of the germinal vesicle (nucleus). The germinal vesicle migrated towards the periphery of the oocyte during the stage 6 which not surprisingly is called the germinal vesicle

migration stage (Wallace and Selman, 1978). This migration continued until the germinal vesicle reached the periphery of the oocyte; this position designates the animal pole (Wassermann and Smith, 1978; Wallace and Selman, 1978, 1981). The yolk globules or platelets which were situated close to the animal pole were smaller than those of the opposite pole in stage 6 and 7 oocytes. In addition the empty vesicles or vacuoles which appeared around the germinal vesicle moved with the germinal vesicle during its migration to the animal pole. The significance of these empty vacuoles or vesicles remains unclear. All these stages were identified for the rainbow trout by Yamamoto et al (1965) although the detailed structural changes were not fully described. Hurk and Peute (1979) only described the migration and aggregation of the yolk granules and globules but no information was given on the germinal vesicle migration or breakdown.

Once the germinal vesicle had migrated to the periphery, the nuclear membrane ruptured, a process which is known as germinal vesicle breakdown (Wallace and Selman, 1978) or nuclear membrane breakdown (Wasserman and Smith, 1978). In the present study breakdown of germinal vesicle also involved a change in the position of the nucleoli. From stage 4 until stage 6, nucleoli were seen within the peripheral undulations of the nuclear membrane whereas during the stages of final maturation, ie. by late stage 6 and 7, the nucleoli were in the centre of the nucleoplasmic mass. In contrast, Wasserman and Smith (1978) reported that the breakdown of the nuclear membrane involves a protrusion of the nucleoplasm into the yolk mass which then releases the nucleoli into the ooplasm amongst the yolk globules. Resumption of the first meiotic division of oocyte is said to occur at this stage and it is at this time that the first polar

body is extruded (Huver, 1960). Unfortunately, this phenomenon is not well documented for teleosts. Furthermore, resumption of meiosis is known to be timed by the penetration of the sperm (Wasserman and Smith, 1978). The aggregation of the nucleoli at the centre of the nucleoplasmic mass during germinal vesicle breakdown could be representing the later stages of first meiotic division prior to the release of the first polar body. Germinal vesicle breakdown has been observed in rainbow trout both in vivo (Yamamoto et al, 1965) and in vitro (Jalabert and Fostier, 1984). It is also at this time that the micropyle is formed. The micropyle appeared , during the final stages of maturation, within the zona radiata just above the germinal vesicle of the oocytes. Similar observations were made by Yamamoto et al (1965) again with the rainbow trout. However, the present study also shows the presence of a micropyler cell covering the funnel-shaped micropyle; this appeared to be a large granulosa cell as was first proposed by Yaron (1971). All the granulosa cells near the micropyle were larger than similar cells elsewhere on the oocyte suggesting that the micropyle could have been derived from any one of a number of granulosa cells.

In addition to the structural and functional changes within the oocyte, many changes were observed in the extra-oocyte tissues of the ovary. The granulosa and thecal cells increased in number and changed their shape during development. Initially the granulosa cells were flattened in shape but after the previtellogenic phase of development they became more cuboidal. During stage 4 and 5 they again became flattened and remained this shape until after the final maturation of the oocytes. The thecal cells became more flattened as a result of the proportionately greater growth of the oocyte enclosed

within the follicle. Electron micrographs of the follicle of vitellogenic oocytes showed increased amounts of endoplasmic reticulum in both the thecal and granulosa cells. Rough endoplasmic reticulum was clearly evident within the thecal cells, with the lamellae lying parallel to each other and to the granulosa cell layer. Granulosa cells are said to be involved in the formation of proteins which are then incorporated into the zona radiata (Wourms, 1976; Wourms and Sheldon, 1976; Hoar and Nagahama, 1978; Kagawa et al, 1981). The full complement of organelles necessary for protein synthesis is present within the granulosa cells, ie. rough endoplasmic reticulum, mitochondria with lamellar cristae etc. (Hoar and Nagahma, 1978; Kagawa et al, 1981). The thecal cells have high enzyme activity and are said to be involved in the synthesis of steroids (Hoar and Nagahama, 1978; Hurk and Peute, 1979; Kagawa et al, 1981; Kagawa et al, 1982b; Nagahama et al, 1982; Young et al, 1982c). A "two cell type model" has been put forward to describe the synthesis of steroids within the follicle (Kagawa et al 1982b; Nagahama et al, 1982; Young et al, 1982c). According to this model the theca is involved in the production of testosterone which is aromatised to oestradiol-17β within the granulosa. This aromatisation is said to occur under the catalytic action of 17β hydroxy steroid dehydrogenase enzymes. Probably the highly developed rough endoplasmic reticulum in the theca and also those in granulosa are involved in the production of enzymes involved in steroidogenic functions of the follicular tissues. The most prominent change within the follicle layers was in the zona radiata, which increased in thickness to more than 30 μ. The radial striations represent microvilli from the oocyte membrane. These microvilli are thought to

play a role in the metabolic exchange between the oocyte and the follicle until the oocyte maturation (Guraya, 1978). According to Guraya (1978) the zona radiata can be mono, bi or tri-layered although no such stratification was seen in the present study.

After final maturation oocytes were released into the peritoneal cavity leaving behind the follicle consisting of a hypertrophied granulosa and the theca. Similar structures were described by Hurk and Peute (1979) in their study of the rainbow trout. Additionally, the thecal cell layer was found to form a continuous layer with the epithelium lining the ovigerous lamellae. The hypertrophied granulosa, which surrounded the hollow space earlier occupied by the oocyte, grew into the collapsed structure of the post-ovulatory follicle. The basement membrane of the granulosa of the post-ovulatory follicle varied in thickness and in places the granulosa was separated from the thecal cell layer at the basement membrane. These observations suggest that after ovulation, the granulosa becomes dissociated from the theca which itself is joined to the epithelium lining the ovigerous lamellae, ie. the germinal epithelium. This view is supported by the appearance of oogonial nests in the connective tissue in the vicinity of the thecal cell layer of the post-ovulatory follicles. One month after ovulation the ovarian tissues were highly contracted and contained no sign of the earlier existence of post-ovulatory follicles.

In addition to the normal development of oocytes, a rare phenomenon amongst stage 2 and 4 oocytes was the occurrence of twin oocytes. These consisted of two oocytes enclosed by the same one cell-layered follicle, but with no follicular cells between the stage 2 twin oocytes. In twin oocytes of stage 4 both cells were

surrounded by a common theca. Both oocytes had their own zona radiata but the only cell layer found between them was a single layer of squamous granulosa cells. It is possible that after the oogonial proliferation, either these cells had not moved away from the oogonial nests of cells or that mitosis had occurred after the completion of oogenesis. However, mitotic division were not seen after oogenesis, although they are of common occurrence during oogonial proliferation.

In addition to the complex changes which occur within the ovary during oocyte development, the peritoneum of a 60 day old trout consisted of undifferentiated cells but in a mature or post-ovulated ovary the outer epithelial cell layer consisted of ciliated columnar cells. Possibly these cilia move the body fluid and the ovulated ova towards the urogenital sinus during ovulation in the gymnovarian ovaries, ie. where oocytes are released directly into the peritoneal cavity. Another feature observed in post-ovulated ovaries were the trabeculae which are folded connective tissue strands found within the spaces among collapsed post-ovulated follicles. In immature ovaries which were compact and containing developing oocytes these trabeculae could not be distinguished from the follicular tissue.

Aside from the developing oocytes and follicular and extra-follicular tissue the other important structures in the ovary were the atretic oocytes and the terminal stages of this process which have been termed "brown bodies" (Chan et al, 1967). Atresia is said to occur at any stage of oocyte development (Rastogi, 1966) although its importance has not been fully appreciated in the majority of the studies of ovarian development in teleosts. Initially many oocytes begin development but in all species this number is considerably

reduced before oocytes undergo final maturation and are ovulated (Saidapur, 1978, Chapter 4). Ingram (1962) suggested that atresia was a significant cause of this reduction in most vertebrates. Bretschneider and Duyvene de Wit (1947) described four stages of atresia in fish namely α, β, γ and δ. Similar stages have been described for plaice (Barr, 1963), rice-field eel (Chan et al, 1967), goldfish (Khoo, 1975) and perch (Lang, 1981). The present study revealed stages of atretic oocytes which are comparable to those identified initially by Bretschneider and Duyvene de Wit (1947). In addition atretic oocytes of stages 4, 5, 6 and 7 were identified but atresia in stages 1, 2 and 3 oocytes did not appear. However, the disappearance of oocytes up to stage 3 suggests that resorption may also have affected these oocytes. All the stages of atretic oocytes included two forms of atresia as first described by Rastogi (1966), ie. phagocytotic or hypertrophic atresia and non-hypertrophic atresia or oolysis. Generally, other studies in teleosts have revealed only the former type (Bretschneider and Duyvene de Wit, 1947; Sathyanesan, 1963; Beach, 1959; Barr, 1963; Nair, 1963; Chan et al, 1967; Lambert, 1970; Khoo, 1975; Lang, 1981).

Although no atretic previtellogenic oocytes were identified atretic stage 4 oocytes were observed both macroscopically and after sectioning for light microscopy. They included both hypertrophic and oolytic types. in hypertrophic atretic stage 4 oocytes forming an irregular cell mass within the oocyte. These atretic oocytes were similar in shape to developing oocytes of the same stage whereas those of the non-hypertrophied or oolytic type were shrunken and the hypertrophied granulosa had separated the oocyte from the rest of the follicle. The stage 4 atretic oocytes of the oolytic form, which

were situated close to the epithelium covering the ovigerous lamellae, had ruptured and released their contents into the extra-ovarian space. Hypertrophic stage 4 atretic oocytes were only found in fish which had been subjected to starvation whilst the oolytic form was present amongst late stage 4 (ie. 4b) oocytes found in both mature and post-ovulated ovaries. Rastogi (1966) observed only hypertrophic type stage 4 atretic oocytes in Xenentodon cancila, and suggested that atresia occurs very quickly in oocytes of this stage. The resorption of stage 4 oocytes has also been evident in other studies on atresia (Bretschneider and Duyvene de Wit, 1947; Chan et al, 1967; Khoo, 1975; Lang, 1981).

The present study showed that stage 4, 5 and 6 oocytes can all be subject to hypertrophic atresia. Initially, the hyertrophied granulosa penetrates the zona radiata and subsequently the whole oocyte becomes filled with a connective tissue-like mass in which are embedded fragments of zona radiata and degenerating yolk. Similar reports were made by Bretschneider and Duyvene de Wit (1947) and Rastogi (1966), although neither of these studies described atresia in three stages of vitellogenic oocytes (stage 5, 6 and 7). The present work revealed differences both in the structure and the timing of occurrence of the stage 5, 6 and 7 atretic oocytes. Stage 5 atretic oocytes were mainly found during the beginning of exogenous vitellogenic phase amongst intact stage 5 and/or early stage 6 vitellogenic oocytes. In contrast stage 6 and 7 atretic oocytes were only encountered in fully mature or post-ovulated ovaries.

The stage 5 atretic oocytes contained a few fragments of zona radiata and a yolk substance. They appeared as a mass of cells margined by an irregular band composed of the basal lamina of the

hypertrophied granulosa and surrounded by the outer thecal and connective tissue layer. Despite their larger size and the presence of fragments of thick zona radiata and yolk material, the stage 5 atretic oocytes were similar in structure to the hypertrophic atretic oocytes of stage 4. Early stage 6 atretic oocytes were also similar in structure but in addition had larger acidophilic fragments of zona radiata and yolky material. Hypertrophic atresia was not evident among later stages of stage 6 or stage 7 oocytes.

In mature and post-ovulated ovaries the atretic oocytes of both hypertrophic and non-hypertrophic form were present. The hypertrophied atretic oocytes were more shrunken than earlier stages and the fragments of zona radiata and yolky material were clumped together. As this process continued these fragments became smaller, subsequently giving rise to connective tissue-like cell masses in which black or dark brown pigmented bodies were embedded. Similar pigmented bodies have earlier been described by Bretschneider and Duyvene de Wit (1947) and Khoo (1975) who named these stages α, β, γ and δ, although no clear histological description of these stages was provided. The observations of this study proved the presence of atretic oocytes both in healthy well-fed rainbow trout raised under farmed conditions and also in fish which had been subjected to starvation, low-rations of food and hormonal manipulations. In each case a cellular mass consisting of connective tissue-like cells with occasional dark-brown or black pigmented bodies was all that remained of hypertrophic atresia. It is not clear whether these bodies are identical to the "brown bodies" described by Chan et al (1967). however, for convenience this term has also been used in the present study. Brown bodies were more often found in post-ovulated ovaries

although ovaries of fish sampled later from the same group showed that they subsequently disappear from the ovary without leaving any trace. Khoo (1975) reported that the terminal stage of atresia which he named the ε stage contained oogonia. However, oogonia were not seen within atretic oocytes observed during the present investigation. In addition to hypertrophied and oolytic atresia, observed in stage, 4 5 and early stage 6 oocytes, late stage 6 and 7 oocytes also became atretic but their structure was different from the earlier forms. Unlike in earlier vitellogenic stages the zona radiata formed a thick band around the oocyte with no penetration by the hypertrophied granulosa. Subsequently, the zona radiata separated from the granulosa and because shrunken thus forming an irregular structure. At the same time the yolk mass within the oocyte had detached from the zona radiata and the space between the yolk mass and zona radiata had filled with a faintly acidophilic-staining fine granular material. Before this granulated mass appeared the yolk platelets or globules lost their structure to form a liquified acidophilic mass, which may have occurred due to autolysis of oocyte contents, which mainly composed of the thick yolk mass. This process appeared to affect mainly the mature oocytes which had failed to ovulate. The radial striations of the zona radiata were seen in the atretic stage 7 oocytes and the hypertrophied granulosa showed acidophilic spherical bodies. During the later stages of atresia in larger yolky oocytes, the yolk mass disappeared leaving a space within the collapsed structure which was margined by the thick zona radiata. It is not clear whether this is similar to the oolysis mentioned by Rastogi (1966) because of insufficient histological details in his study.

These results show that under normal conditions atresia occurs in all stages of the oocyte developmental cycle. In addition to the reduction of the total numbers of oocytes in the ovary, atresia also decreases the proportion of the total numbers of oocytes which are recruited into the later developmental stages. Atresia does not appear to be just a "mopping up" process for oocytes which have failed to ovulate as suggested by Wallace and Selman (1981) but represents a mechanism of controlling the numbers of oocytes which are recruited into the higher developmental stages. Clearly this process is of profound importance to the determination of the fecundity of fish.

Chapter 4

The annual developmental cycle of the ovary and its relationship to changes in the levels of serum oestradiol-17β, testosterone and calcium in three strains of rainbow trout.

4.1 Introduction

The rainbow trout is the principal farmed fish in Europe and, although its spawning is rountinely carried out in captivity, surprisingly very little is known about the dynamics of ovarian development and the timing of this process in relation to environmental and endocrinological changes. In the rainbow trout and many other annually-spawning teleosts ovarian development begins at least a year before spawning (Elliott et al, 1984; Sumpter et al, 1984) and over this period the oocytes progress through a series of four distinct growth phases, ie. oogenesis, folliculogenesis, vitellogenesis and maturation (see chapter 3).

Recent improvement in sensitivity of assay methods, particularly radioimmunoassay, have enabled the seasonal changes in circulating levels of oestradiol, testosterone, progesterone, gonadotropin and vitellogenin to be described for salmonids and other teleosts (Breton et al, 1972, 1975; Crim et al, 1973, 1975; Billard et al, 1978; Crim and Idler, 1978; Fostier et al, 1978; Lambert et al, 1978; Whitehead et al, 1978a, b, 1983; Bromage et al, 1982a; Scott & Sumpter, 1983a; Elliott et al, 1984; Sumpter et al, 1984). With the exception of Sumpter et al (1984) none of these investigations involved concurrent histological studies of gonadal development. Furthermore, the majority have merely related, the hormonal changes which occur during oocyte development to the period of ovulation or spawning ie. the release of ripe eggs. Although ovulation is the most easily defined stage of oocyte development it represents only a small portion of the year-long cycle. There have been few studies which have aimed to correlate or time the seasonal patterns of hormone secretion with the structural changes which occur within the ovary, because of the

terminal nature of histological sampling and the expense of sacrificing large mature fish.

A few studies on rainbow trout have described some of the seasonal histological changes in ovarian development (Yamamoto et al, 1965; Hurk and Peute, 1979; Schulz et al, 1981; Schulz, 1984; Sumpter et al, 1984). Yamamoto et al, (1965) described only the structure of the different stages of oocyte development in the rainbow trout but gave no indication of the timing of these changes or their relationships to the underlying mechanisms of control. Hurk and Peute (1979) attempted a limited description of the timing of each of the seven stages of oocyte development, ie. chromatin nucleolar stage, early perinucleolar stage, late perinucleolar stage, yolk vesicle stage, peripheral yolk granule stage, aggregation and central migration of yolk and oocyte maturation, but included no endocrine information . Schulz et al (1981) and Schulz (1984) correlated the seasonal alterations in blood protein and oestradiol levels respectively with oocyte development in the trout.

However, in both these studies the oocyte cycle was only divided into four stages, ie. small oocytes with a cytoplasm without any vacuolisation, oocytes with periplasmic vacuolisation, oocytes with cytoplasmic vacuolisation and light green-positive yolk platelets. This staging does not include the complex changes which occur within the ovary during the early or final stages of oocyte development and maturation. Sumpter et al (1984) correlated the number and size of oocytes with alterations in the levels of serum gonadotropic and steroidogenic hormones, vitellogenin and calcium, in the rainbow trout, although the work was mostly limited to early stages of oocyte development. Sumpter et al (1984) was primarily concerned with

oocytes of three types, ie. primary oocytes, yolk vesicle stage and yolk globule stage, and included no information of environmental and endocrine factors which govern oogonial proliferation, oogenesis, germinal vesicle migration, and germinal vesicle breakdown stages. No mention was made of atresia, ie. the degeneration of ovarian ova, which is considered to be important in the determination of the numbers of eggs which are produced, ie. fecundity. During the present study monthly samples of the ovary were taken from 1+ and 2+ year old fish in parallel with samples of blood for analyses of serum levels of oestradil-17β, testosterone and also calcium which provides an index of vitellogenin. This enabled the changes in levels of these constituents to be related to the development of the ovary. Histological samples were also taken from the ovaries of 0+ year old fish in order to describe the changes which occur during the earliest stages of oocyte development.

In teleosts as in other vertebrates ovarian development is known to occur under the stimulation of pituitary gonadotropin(s) (Upadhyay et al, 1978). These induce the ovary to produce female specific hormones which are then involved in controlling oocyte development. Pituitary gonadotropins are known to increase plasma oestradiol levels in salmonids (Billard et al, 1978; Crim and Idler, 1978) and in other teleosts (Fostier et al, 1979; Nagahama et al, 1982; Kagawa et al, 1982a). In salmonids and possibly all other annually-spawning teleosts, the levels of gonadotropin(s) show periodic fluctuations during the reproductive cycle. Most workers agree that the highest levels of gonadotropin occur just before and after ovulation (Crim et al, 1973, 1975; Billard et al, 1978; Fostier et al, 1978; Lambert et al, 1978; Jalabert and Breton, 1980; Bromage et al, 1982a; Breton

et al, 1975; Scott and Sumpter, 1983; Whitehead et al, 1983; Lou et al, 1984; Sumpter et al, 1984). Gonadotropin levels are also at their highest around ovulation in rainbow trout which spawn twice a year under artificially-long photoperiods (Bromage et al, 1984; Scott et al, 1984). There is, however, less agreement regarding the levels of gonadotropin during the earlier stages of ovarian development, ie. stages including the growth and recruitment of previtellogenic and vitellogenic oocytes. Bromage et al (1982a) and Whitehead et al (1983) found that the levels of gonadotropin were first increased during gonadal recrudesence, at a time when the ambient daylength is increasing. Raised levels of gonadotropin are known to be followed by increases in the levels of oestradiol-17β, phosphoprotein phosphorous and calcium (Whitehead et al, 1983) and also of vitellogenin (Sumpter et al 1984). In addition to its effect on vitellogenesis and also directly on oocyte development (see chapter 5) it is also suggested that oestradiol-17β exerts a negative feedback control on the secretion of gonadotropin by the pituitary (Egami, 1955; Egami and Ishii, 1962; Sundararaj and Goswami, 1968; Whitehead et al, 1983). In the rainbow trout a highly significant positive correlation in gonadotropin and oestradiol levels also been observed during the early stages of ovarian development whereas during the later stages of maturation, ie. in the month before ovulation, these hormones showed a negative relationship (Whitehead et al, 1983). Somewhat similar observations have been made by other workers for trout and also other teleosts (Lambert et al, 1978; Fostier et al, 1978; Billard et al, 1978; Crim and Idler, 1978; Lou et al, 1984). Thus, the increases in oestradiol-17β levels during active vitellogenesis in rainbow trout are thought to be induced by

the raised levels of gonadotropin observed during the early stages of oocyte development. Unfortunately, none of these studies have related the above changes to structural developments in the ovary. Consequently, the relationship which exist between gonadotropic hormones and oestradiol-17β under the previtellogenic growth, vitellogenin uptake, maturation and atresia of the oocytes is obscure. Although gonadotropin levels are not measured in the present study the alterations in oestradiol-17β levels are related to the seasonal changes in ovarian histology and to the proportions of oocytes which have reached the different developmental stages and to the levels of atresia in rainbow trout.

In the rainbow trout, testosterone levels have also been found to increase in parallel with those of oestradiol and vitellogenin (Scott and Baynes, 1982; Elliott et al, 1984; Sumpter et al, 1984). Scott and Baynes (1982) proposed that the increase in testosterone was due to the alterations in the enzyme functions, although Elliott et al (1984) suggested that it may also have a role in the control of yolk resorption and/or atresia, a function which has already been suggested for mammals (Louvet et al, 1975). However, no histological evidence was provided to support this hypothesis. Yamazaki (1972) reported a resorption of yolk in salmonids as a result of testosterone treatment. In the "two cell type model" proposed to explain the oestrogen production by the ovarian follicle (Kagawa et al, 1982b; Nagahama et al, 1982; Young et al 1982c), testosterone is produced in the theca after which it is aromatised to oestrogens by the granulosa cells. Peak levels of testosterone have been observed in rainbow trout and other salmonids during the period of final oocyte maturation and ovulation (Kagawa et al, 1983; Young et al,

1983; Elliott et al, 1984; Scott et al, 1984; Sumpter et al, 1984), at a time when levels of gonadotropic hormone are at their highest (Crim et al, 1973, 1975; Billard et al, 1978; Fostier et al, 1978; Lambert et al, 1978; Jalabert and Breton, 1980; Bromage et al, 1982a; Breton et al, 1975; Scott and Sumpter, 1983; Whitehead et al, 1983; Lou et al, 1984; Sumpter et al, 1984).

Similar observations have been made for other teleosts (Schmidt and Idler, 1962; Katz and Eckstein, 1974; Schreck and Hopwood, 1974; Campbell and Idler, 1976, 1980; Wingfield and Grimm, 1977; Lance and Callard, 1978). The aromatisation of testosterone is catalysed by the enzyme 17β hydroxysteroid dehydrogenase (Sire and Dèpêche, 1981) and the activity of this enzyme appears to be controlled by pituitary gonadotropins (Young et al, 1983; Sower et al, 1984). Although the role of testosterone on oocyte development is controversial, Kagawa et al (1983) suggested that it may affect oocyte maturation. Most of the above studies indicate that testosterone is involved directly or indirectly in the ovarian development, although no supportive histological evidence has been provided. Thus, the present study will compare the testosterone levels of three strains of rainbow trout with their seasonal ovarian development and proportions of oocytes at different developmental stages and also of the oocytes which have become atretic.

In seasonally-breeding teleosts including salmonids many environmental factors have been implicated in the control of reproduction and of these photoperiod, temperature and nutritional status appear to be the most important (see review by Lam, 1983). Each of these environmental cues is thought to control reproduction by the induction and entrainment of hormonal change (de Vlaming, 1972). In

salmonids daylength is known to be of prime importance (Henderson, 1963a; McQuarrie et al, 1979; Bromage et al, 1984). The effect of altered photperiod on ovarian development of rainbow trout has been the subject of many investigations (Allison, 1951; Whitehead et al, 1978b; Baggerman, 1980; Whitehead and Bromage, 1980; Bromage et al, 1984; Lou et al, 1984). Most of these studies, however, like those concerned with the natural reproductive cycles, have used ovulation and/or spawning as the reference point for correlations with ovarian development. Bromage et al (1982b) and Scott et al (1984) suggested that trout are long-day animals, and that long days are necessary for early growth and vitellogenesis of the oocytes. In addition, short days or possibly the absence of long days are thought to provide a further cue for the synchronisation of later events (Whitehead and Bromage, 1980; Bromage et al, 1982b, 1984; Scott and Sumpter, 1983b). However, in none of these studies was any correlation made between structural changes in the ovary and the seasonally-changing photoperiod. It is thus not clear which phases of oocyte development coincide with the period(s) of increasing (long) or decreasing (short) daylength or whether daylength per se is important in the recruitment to higher developmental stages including exogenous vitellogenesis or in the development of atresia. Although the present work did not consider the effects of artificially-altered photoperiods on ovarian development, a study was made of the timing of the different stages of oocyte development, recruitment and the proportions of oocytes at each stage and, those undergoing atresia, in relation to the natural changes in daylength.

The primary aim of this study was to describe the structural changes which occur in the ovary of the rainbow trout from the time

of sexual differentiation until the end of the first and second reproductive cycles. This will indicate the changes in proportion of oocytes during maturation and the timing of their recruitment to each of their successive developmental stages, especially that relating to vitellogenesis. Furthermore, changes within the ovaries were related to seasonal changes in serum oestradiol-17β, testosterone and calcium levels and also to the changing daylength. Extending the above studies to three different strains of rainbow trout (Caribou, Grampian and Whitebrook) which spawn in October/November, November/December and December/January respectively, may help to explain the differences between strains, as far as the timing of important events in ovarian development, ie. oogonial proliferation, oogenesis, vitellogenesis, ovulation, atresia etc., and also the egg size and fecundity. Such findings may also help to improve photoperiod manipulations and other important modifications adopted by the fish farming industry to increase egg and hence fry production.

4.2 Materials and Methods.

All the rainbow trout used in this investigation were obtained from stocks maintained at Avon Spring Hatcheries. Broodstocks of three major strains viz. Caribou, Grampian and Whitebrook, are maintained on this farm for the commercial production of eggs and fry. Each month blood and ovarian samples were taken from three 1+ and 2+ year-old fish from each of the three strains. Samples were also taken from 0+ year-old fish, but only from the ovary because of difficulties in obtaining sufficient blood for the various hormonal and vitellogenin assays.

Each of the fish was weighed and measured and after sacrifice, the right and left ovaries removed and separately weighed. The right ovary was fixed in Bouin's fixative and then processed for microscopical examination (section 2.2), whilst the left one was preserved in Gilson's Fluid for macroscopic examination, measurements of oocyte diameter and counts of the numbers of oocyte stages (section 2.3). Serum was separated from the blood samples and stored at -20°C for later analyses of oestradiol-17β and testosterone (section 2.4) and for calcium by fluorimetric method (section 2.5).

4.3 Results

Ovarian development followed similar patterns in the different age groups and strains although two types of ovarian development were observed in 1+ year-old fish (Fig. 4.1; Tables 4.1.1; 4.1.2; 4.1.3). In one group of the 1+ year-old fish development was less advanced than the other and members of this group did not spawn at the end of their second year. The presence of stage 5 vitellogenic oocytes distinguished the 1+ year-old fish which were to spawn. This difference was clearly seen after August in Caribou and Grampian strains and after July in Whitebrook strain (Figs. 4.1 A,C,E). In contrast the ovaries of the groups which were destined not to spawn showed no signs of vitellogenesis at these times (Figs. 4.1 B,D,F).

In all the 2+ year-old fish spawning occurred at the end of their third year (Fig. 4.2). However, two types of development were observed in the Whitebrook fish; one spawning 2 to 3 months earlier than the other (Fig. 4.2 C,D).

In all three strains, the 2+ year-old fish spawned at least one month in advance of the time of spawning of the corresponding group of 1+ year-old fish (Compare Figs. 4.1 and 4.2).

4.3.1 Body weight.

The body weights of the three strains of trout showed similar patterns of increase during the three years of sampling (Fig. 4.3).

4.3.2 Gonadosomatic index (G.S.I).

In all spawning 1+ and 2+ year-old fish gonadosomatic indices were at their highest value just before spawning (Figs. 4.3 to 4.6;

Tables 4.3.1 to 4.4.3.2). Slightly raised G.S.I. values were also evident during the previtellogenic phase of development and they decreased before the commencement of active exogenous vitellogenesis. Amongst the 1+ year-old fish, which were destined not to spawn at the end of their second year of life, a slight increase in G.S.I. was observed when the ovaries of the corresponding spawning groupswere showing active vitellogenesis (Fig. 4.4). There were no significant differences in G.S.I.s of fish belonging to 1+ and 2+ age groups during corresponding stages of ovarian development.

4.3.3 Oocyte numbers.

The highest total numbers of oocytes were present in ovaries with previtellogenic oocytes and stage 4 oocytes (Tables 4.1.1 to 4.2.3.2). A large proportion of the oocytes in these ovaries consisted of stage 1 and 2 (Figs. 4.1 and 4.2). With the appearance of vitellogenic oocytes the total numbers of oocytes gradually decreased in large numbers a process which could be seen until spawning. Subsequently, there was a further increase in the total number of oocytes in the post-ovulated ovary (Tables 4.1.1 to 4.2.3.2). The ovaries of 1+ year-old fish which were not showing any signs of vitellogenesis contained oocytes up to stage 4 at a time when the spawning group of that strain showed stage 5 oocytes (Fig. 4.1). The numbers of stage 4 oocytes of the non-spawning group also gradually decreased over the year and in a few ovaries they were completely absent. In contrast 1+ year-old fish with stage 5 oocytes, progressed through the higher stages until maturation.

In all the 1+ year-old fish, destined to spawn at the end of their second year of life vitellogenesis was delayed by 3 to 4 months

in comparison with the ovaries of the 2+ year-old fish of corresponding strains (compare Figs. 4.1 and 4.2). Active exogenous vitellogenesis in the 1+ year-old fish of all three strains commenced in July/August (Fig. 4.1) whilst in 2+ year-old Caribou, Grampians and early spawning Whitebrooks it was first observed in March/April (Fig. 4.2). In late spawning 2+ year-old Whitebrooks the unavailability of ovarian samples during July, August and September makes it difficult to indicate the correct time of the commencement of active vitellogenesis but the information obtained from ovaries collected in June and October shows clearly that their vitellogenesis may have occurred in July/August (Fig. 4.2).

A few vitellogenic (stage 5) oocytes were observed in 1+ year-old Caribous and 1+ year-old and 2+ year-old late-spawning Whitebrooks in March and May respectively, although they disappeared over the succeeding months leaving oocytes up to stage 4 before the commencement of active vitellogenesis. Similarly stage 5 and 6 vitellogenic oocytes were observed within the ovaries of 2+ year-old Grampians in February but stage 6 oocytes disappeared during the two succeeding months. The disappearance of these oocytes coincided with the appearance of atretic oocytes. Atretic oocytes were also found in the ovaries of 1+ year-old fish which were not to spawn at the end of their second year of life; they appeared at the same time as the appearance of exogenous vitellogenic oocytes within the ovaries of spawning fish of the same age (Fig. 4.1). Atretic oocytes were seen in almost all vitellogenic ovaries, and also in the post-ovulated ovaries of 1+ year-old fish and 2+ year-old early-spawning Whitebrooks (Figs. 4.1 and 4.2 D).

During development there were considerable reductions in total

numbers of oocytes present in the ovaries of all age groups and strains of fish (Tables 4.1.1 to 4.2.3.2). Reductions in total numbers of oocytes from each of the developmental stages were seen before their recruitment to next higher developmental stage. In vitellogenic ovaries these reductions coincided with the appearance of atresia in the vitellogenic oocytes although the reductions in total numbers of oocytes were far higher than the numbers of vitellogenic atretic oocytes which were observed. There were also marked reductions in oocyte numbers in previtellogenic ovaries especially those contain[illegible] stage 1, 2 and 3 oocytes, despite the absence of well defined atresia among these oocyte stages. These reductions in previtellogenic oocytes affected the total number of oocytes at different stages as a percentage over total number of oocytes appeared misleading. Especially, the same number of atretic oocytes may result in different percentages at different phases of ovarian development. Therefore, it was clear that it is much more appropriate to indicate atresia as the proportion of atretic oocytes $100g^{-1}$ body weight rather than the[illegible] representing aspercentages(Fig. 4.7). This made it possible to compare [illegible] proportions of oocytes of different stages and those becoming atretic in fish belonging to different age groups and strains, at corresponding phases of ovarian development, regardless of their differences in body weight.

4.3.4 Mean highest oocyte diameter.

The mean highest oocyte diameter was observed in vitellogenic oocytes prior to spawning (Fig. 4.4 to 4.6; Tables 4.3.1 to 4.4.3.2). Mature 2+ year-old fish had larger stage 7 oocytes than those of spawning 1+ year-old fish of the same strain. Similar

differences were found in the Whitebrook strain of fish with later-spawning variety having larger stage 7 oocytes (Fig. 4.5 C & D). An increase in oocyte diameter was observed in the previtellogenic or early vitellogenic ovaries of all fish although it decreased again before the commencement of active vitellogenesis (Figs. 4.4 to 4.6). This increase in ova diameter during the early months of the year was due to the presence of exogenous vitellogenic oocytes in 1+ year-old Caribous, 1+ and 2+ year-old Whitebrooks and 2+ year-old Grampians as described above (Figs. 4.1 A,E; 4.2 D,B). In the remaining fish this increase was due to the appearance of late stage 4 endogenous vitellogenic oocytes (Figs. 4.1 & 4.2). Subsequently, these oocytes disappeared from the ovaries, resulting in a decrease in the mean highest oocyte diameter. Further increases subsequently occurred with commencement of active exogenous vitellogenesis in the spawning groups. In 1+ year-old fish which were not undergoing exogenous vitellogenesis the mean highest oocyte diameter increased gradually, although this increase only involved the growth of the stage 4 previtellogenic oocytes. These changes in diameter coincided with an increase in G.S.I. in both spawning and non-spawning groups (Tables 4.3.1 to 4.4.3.2; Figs. 4.4 to 4.6).

4.3.5. Ovarian histology.

The ovaries of 0+, 1+ and 2+ year-old fish showed a number of significant histological changes. In 0+ fish 60 days after hatching the whole ovary consisted of primary oogonia (Plate 3.5, Chapter 3). Eggs of Caribou, Grampian and Whitebrook strains hatched in January, February and March respectively and sixty days after hatching the ovary consisted solely of primary oogonia. Four to five months after

hatching the ovaries contained secondary oogonia and stage 1 and early stage 2 (2a) oocytes (Plate 3.6, Chapter 3). Eight months after hatching the ovary consisted solely of stage 2 oocytes of various diameters (stages 2a, 2b & 2c). Stage 3 oocytes appeared 9 to 10 months after hatching and by the end of the first year of life the majority of fish sampled contained ovaries with oocytes up to early stage 4 (4a). These stage 4a oocytes developed to stage 4b just prior to their recruitment to exogenous vitellogenesis. With the commencement of vitellogenesis atretic oocytes also appeared and were present in the ovaries until the remainder of the developing oocytes had reached stage 6 (Plate 4.1). The atresia occurring at this time was only of stage 5. In vitellogenic ovaries with stage 7 mature oocytes, stage 4 atretic oocytes were observed (Plate 3.34). Atretic stage 4 oocytes were also observed in post-ovulated ovaries (Plate 3.43, Chapter 3). In a few post-ovulated ovaries stage 5 and 6 atretic oocytes were also observed (Plates 3.38 and 3.39, Chapter 3). In the majority of post-ovulated ovaries early stage 4 oocytes (4a) and atretic brown bodies, which are the terminal stages of hypertrophic atretic oocytes, were also seen. Atretic oocytes and brown bodies were also present amongst the oocytes below stage 4b in the ovaries of 1+ year-old fish which were not destined to spawn at the end of their second year of life (Plate 3.44). Atresia was consistently found within the ovaries of fish of all age groups and strains.

4.3.6 Oestradiol-17β changes.

Serum oestradiol-17β levels were raised in all groups and strains during the previtellogenic phases of development (Figs. 4.4

to 4.7) with the maximum levels being reached just before final oocyte maturation. The raised levels observed during the previtellogenic phase of development were slightly higher than basal values. These returned to basal levels prior to the commencement of active exogenous vitellogenesis, during which period they were at their highest levels. Oestradiol levels were also increased during September, October and November in the non-spawning 1+ year-old fish, although the peaks were much lower than those observed in the spawning fish of the same age group (Compare 4.4 A,C,E with 4.4 B,D,F). In all cases peak levels of oestradiol-17β coincided with the appearance of oocytes with the highest mean diameters (Figs. 4.6; Tables 4.3.1 to 4.3.2).

4.3.7 Testosterone changes.

Changes in serum testosterone levels were similar in all three strains and age groups with the initial changes paralleling those of oestradiol-17β although testosterone levels were almost always much higher than those of oestradiol-17β (Figs. 4.4 to 4.6; Tables 4.3.1 to 4.4.3.2). Testosterone levels continued to rise until after spawning unlike the oestradiol levels which were at basal values just prior to spawning. In 1+ year-old fish which were destined not to spawn, slightly raised levels of testosterone were seen at the same time when very high levels appeared in spawning fish of the same group in parallel with the growth of vitellogenic oocytes. These testosterone levels of the non-spawning groups returned to basal or below the limits of the assay whilst in the non-spawning groups they continued to increase until oocyte maturation.

4.3.8 Calcium changes.

Levels of serum calcium, measured as an index of vitellogenin, showed similar patterns of change during the year in fish of all age groups and strains. These changes also paralleled those of oestradiol-17β and testosterone (Figs. 4.4 to 4.6). In all fish groups the maximum calcium levels occurred just before spawning (Figs. 4.4 to 4.7; Tables 4.3.1 to 4.4.3.2).

Amongst the 1+ year-old fish which were destined not to spawn at the end of their second year of life, calcium levels were raised from basal levels during the period in which the spawning fish of the same age had very high levels of calcium. Subsequently, the calcium levels in the non-spawning group returned to basal during November and December in a similar manner to the spawning groups. The peak levels of calcium in the non-spawning group were very much lower than those observed for spawning groups. In spawning groups the peak levels of calcium coincided with the appearance of stage 7 oocytes within the ovaries (Figs. 4.4 to 4.6; Tables 4.3.1 to 4.4.3.2).

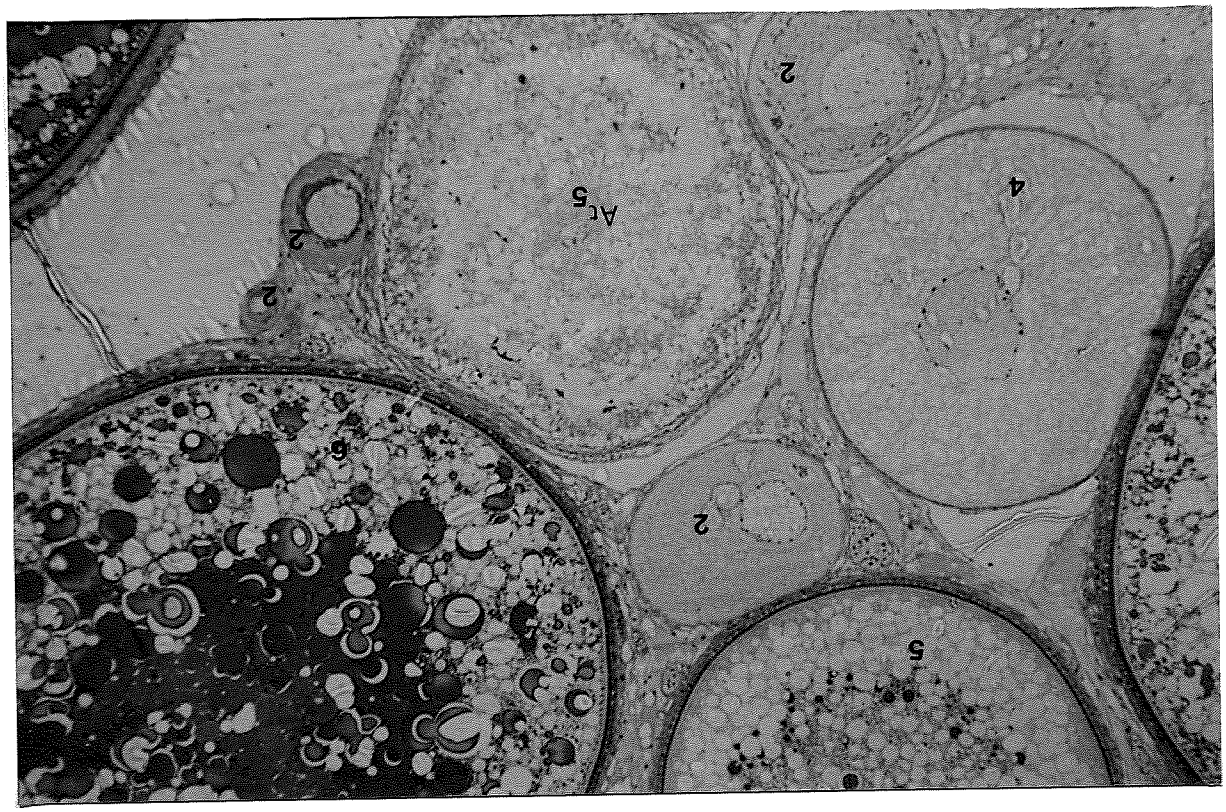


Plate 4.1

Plate 4.1 - Section of an ovary of a 2+ year-old Grampian sampled in July during active vitellogenesis, showing stage 5 atresia among stage 2, 4 , 5 and 6 intact oocytes. Stained with haematoxylin and eosin.

Magnification x 50

At_5 - stage 5 atretic oocyte, 2, 4, 5, 6 - 2, 4, 5 and 6 intact oocytes.

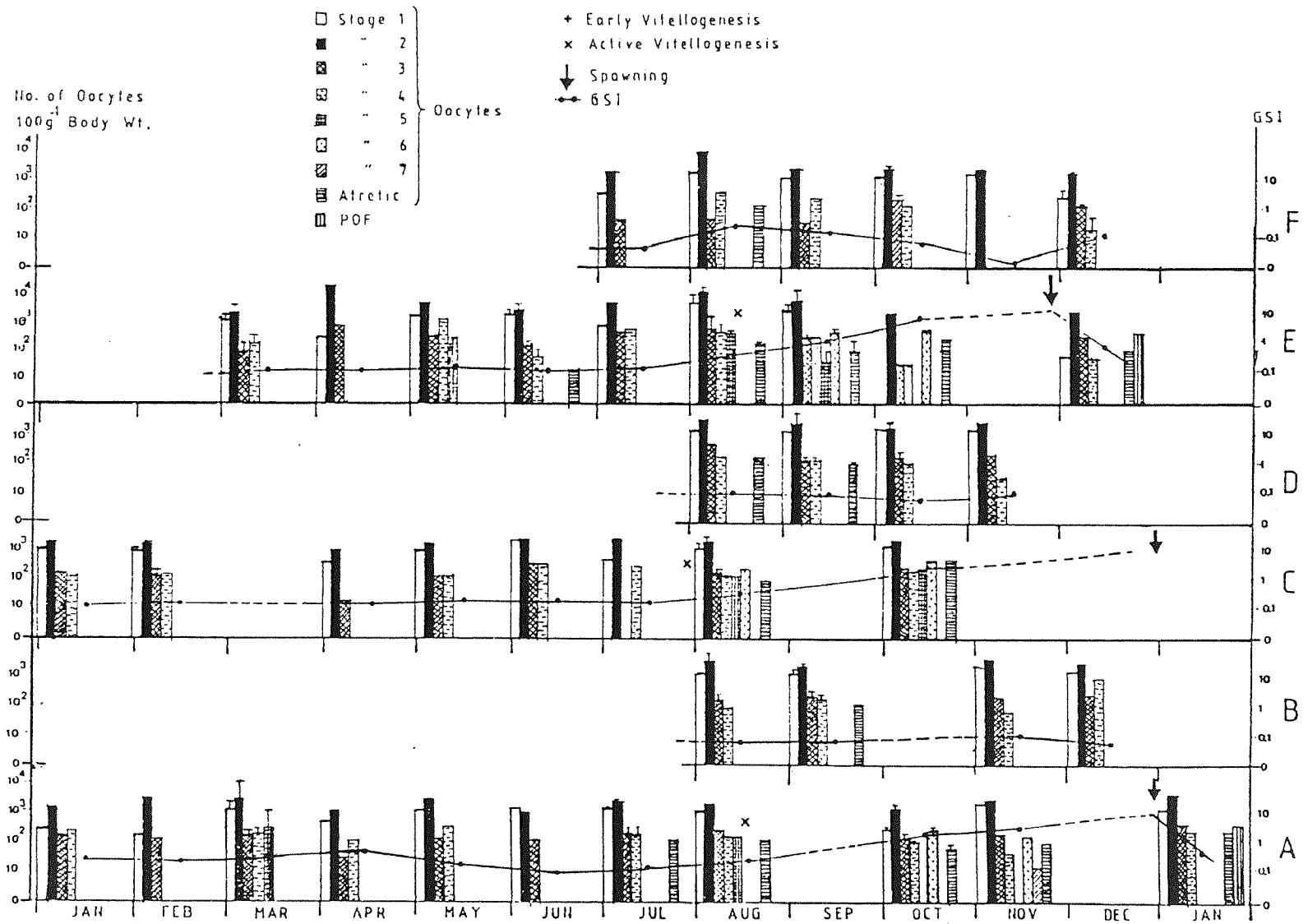


Fig 4.1 - Seasonal changes in the numbers of oocytes at different developmental stages (1-7 and atretic) and post-ovulatory follicles in 1+ year-old spawning and non-spawning Caribous (A,B), Grampians (C,D) and Whitebrooks (E,F) in relation to changes in gonadosomatic index (G.S.I.). Each value indicates the mean number of oocytes $100g^{-1}$ body weight ± standard deviation (S.D.) where appropriate.

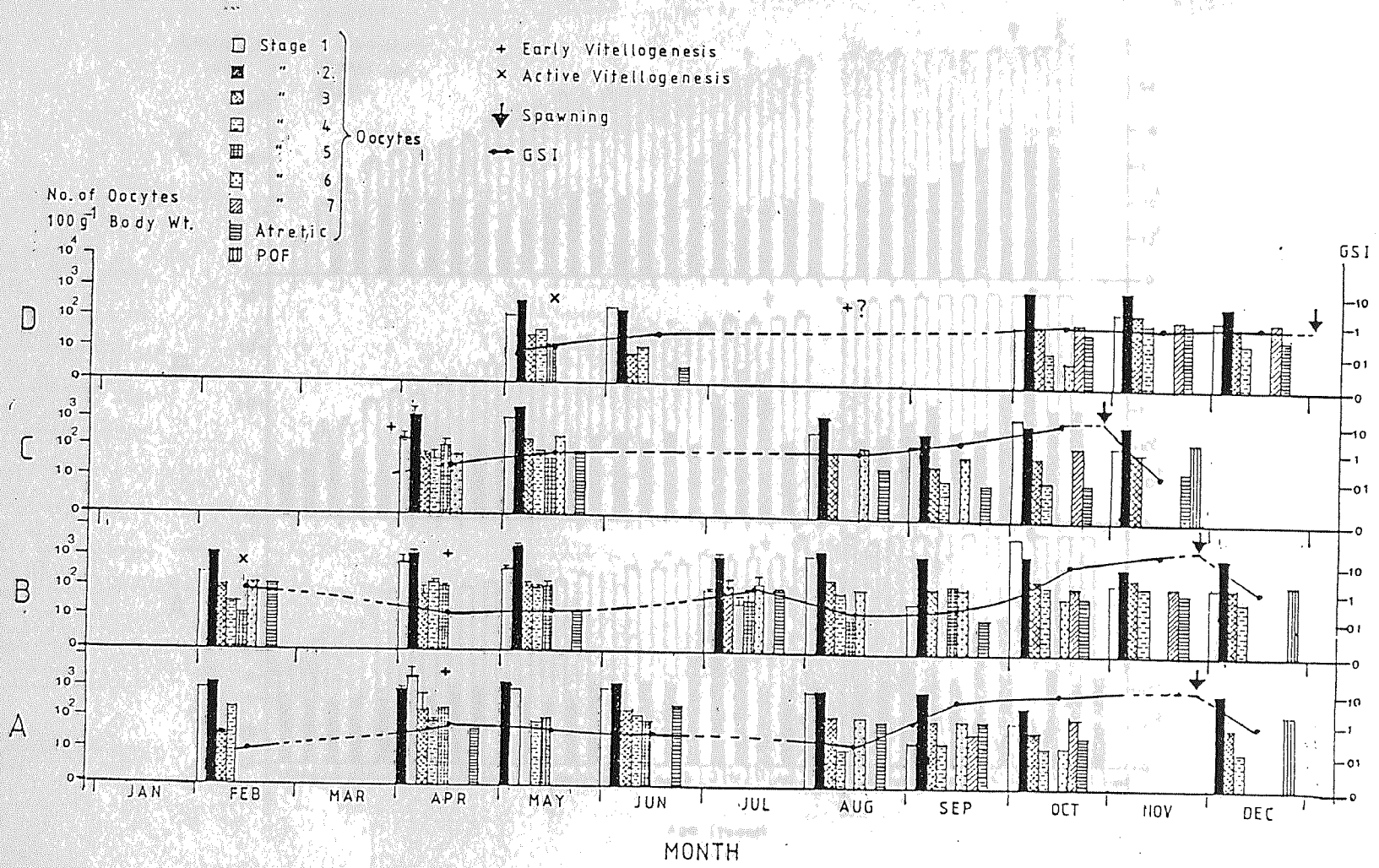


Fig 4.2 - Seasonal changes in the numbers of oocytes at different developmental stages (1-7 and atretic) and post-ovulatory follicles in 2+ year-old Caribous (A), Grampians (B) and early and late spawning Whitebrooks, in relation to changes in gonadosomatic index (G.S.I.). Each value indicate the mean number of oocytes $100g^{-1}$ body weight ± standard deviation (S.D.) where appropriate.

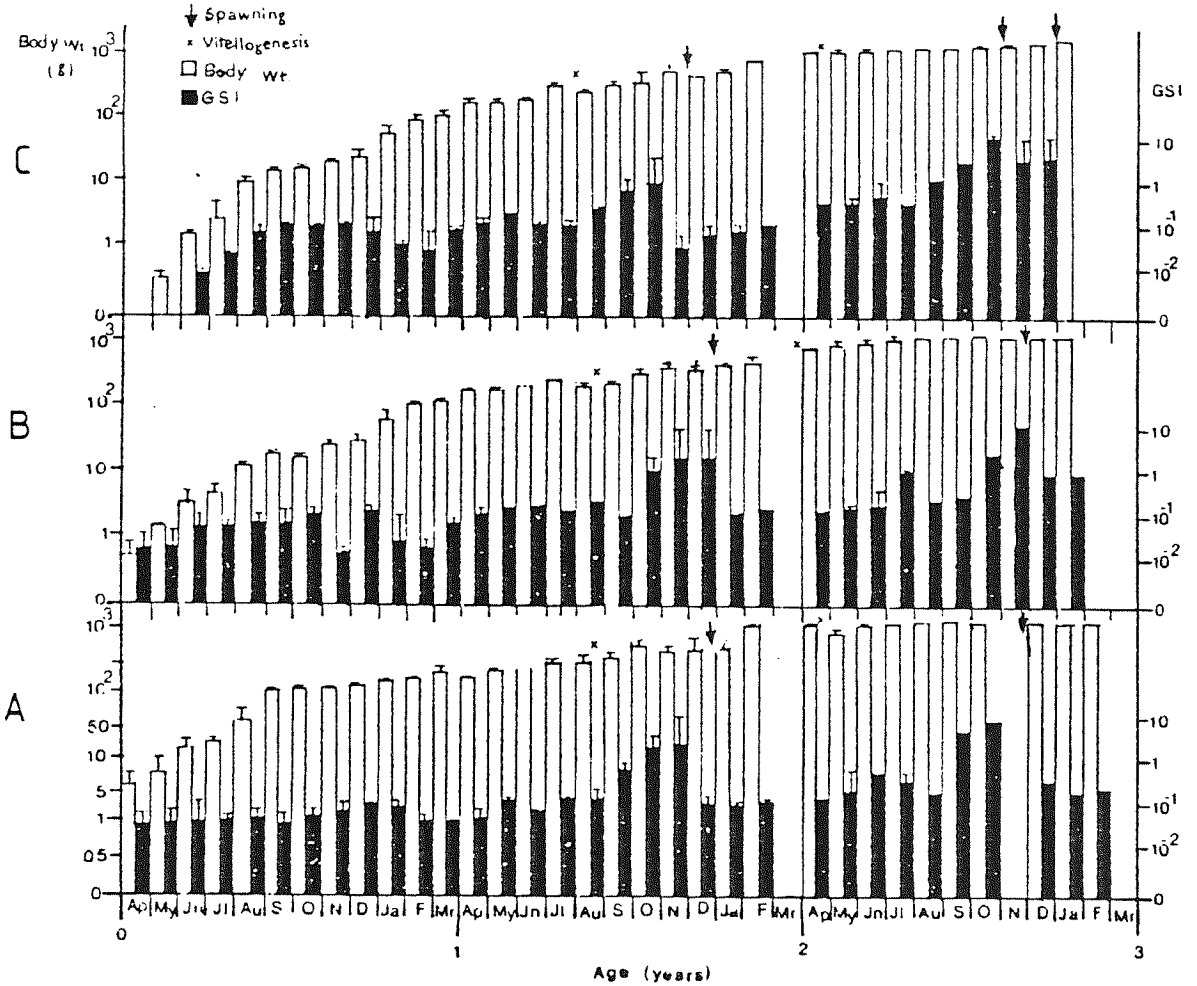


Fig 4.3 - Seasonal changes in the Body weight (B.Wt) and gonadosomatic index (G.S.I.) of fish up to 3 years of age in Caribou (A), Grampian (B) and Whitebrooks (C).

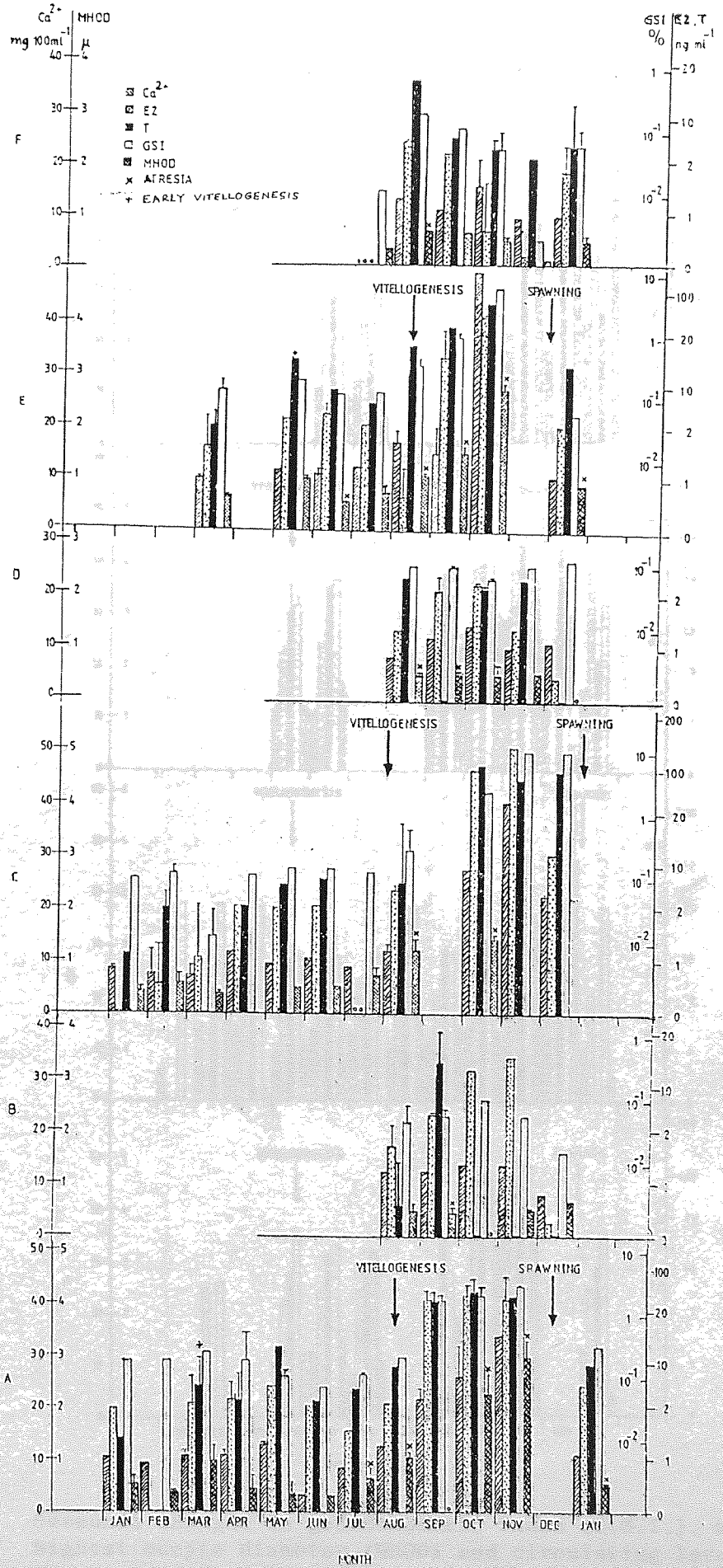


Fig 4.4 - Seasonal changes in gonadosomatic index (G.S.I.), mean highest oocyte diameter (MHOD) and circulating levels of oestradiol-17β (E2), testosterone (T) and calcium (Ca^{2+}), in 1+ year-old spawning and non-spawning Caribous (A,B), Grampians (C.D) and Whitebrooks (E,F).

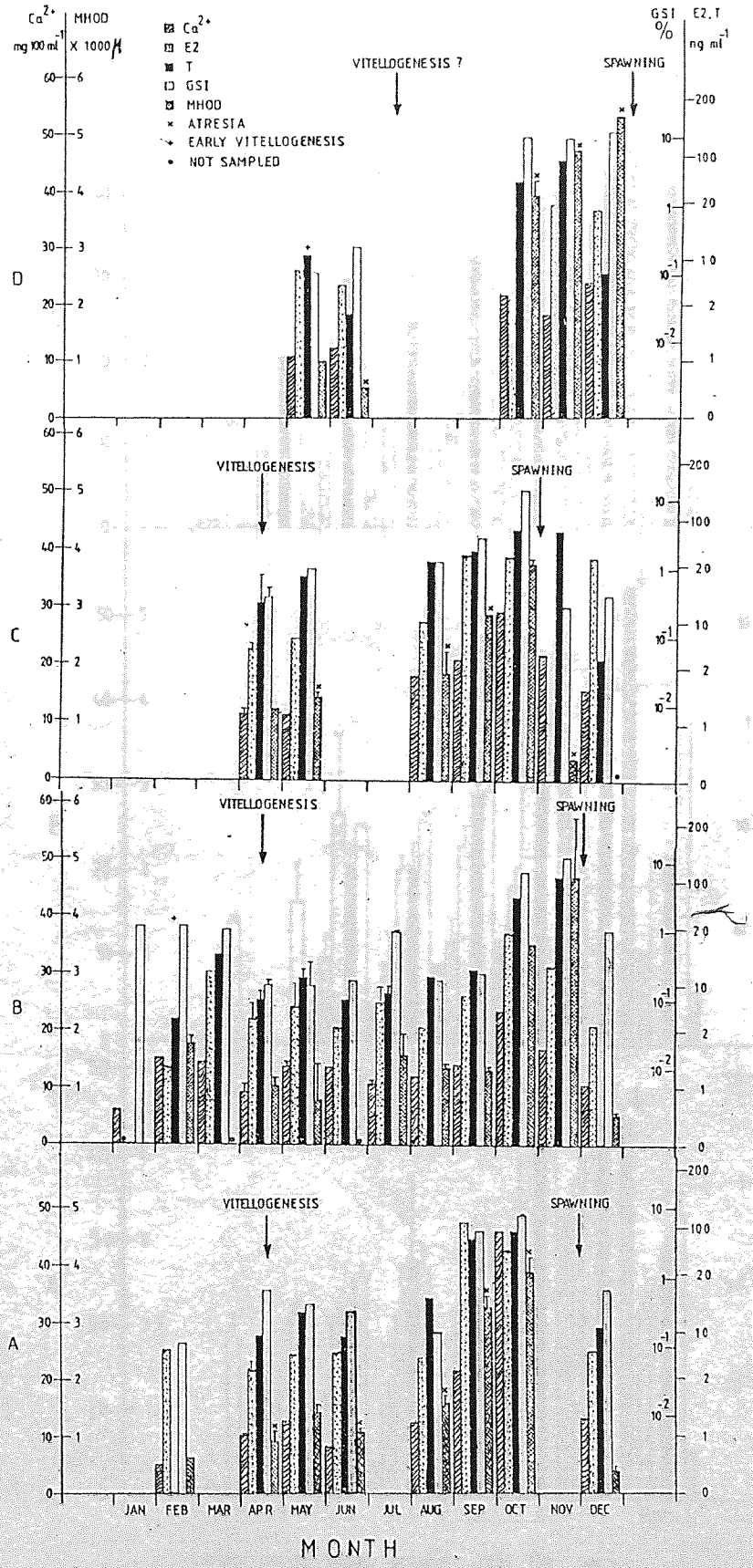


Fig 4.5 - Seasonal changes in gonadosomatic index (G.S.I.), mean highest oocyte diameter (MHOD) and circulating levels of oestradiol-17β (E2), testosterone (T) and calcium (Ca), in 2+ year-old Caribous (A), Grampians (B) and early and late-spawning Whitebrooks (C,D).

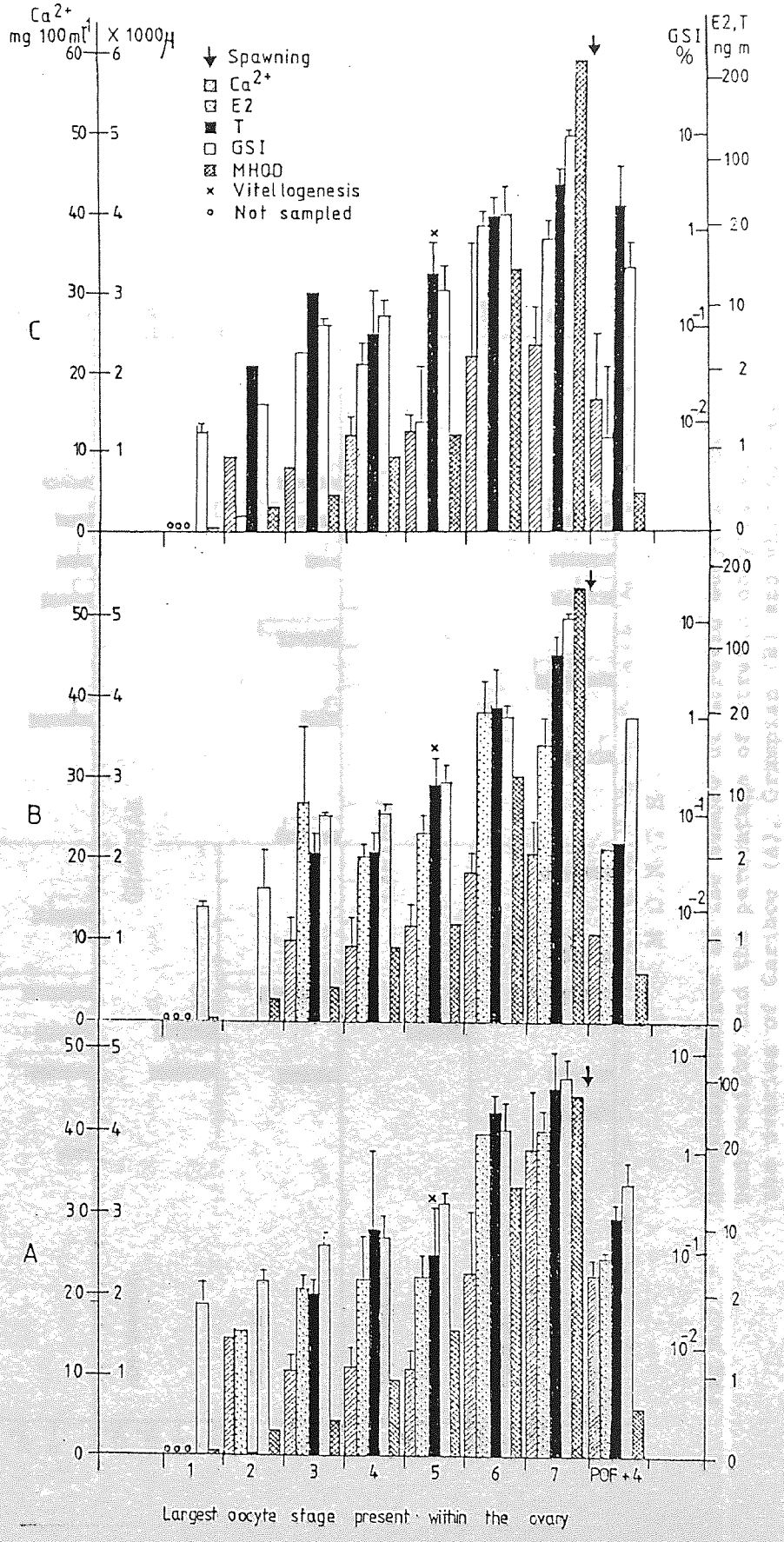


Fig 4.6 - Changes in gonadosomatic index (G.S.I.), mean highest oocyte diameter (MHOD) and circulating levels of estradiol-17β (E2), testosterone (T) and calcium (Ca) during the development of oocytes from stage 1 to 7 and after ovulation in Caribou (A), Grampian (B) and Whitebrooks (C).

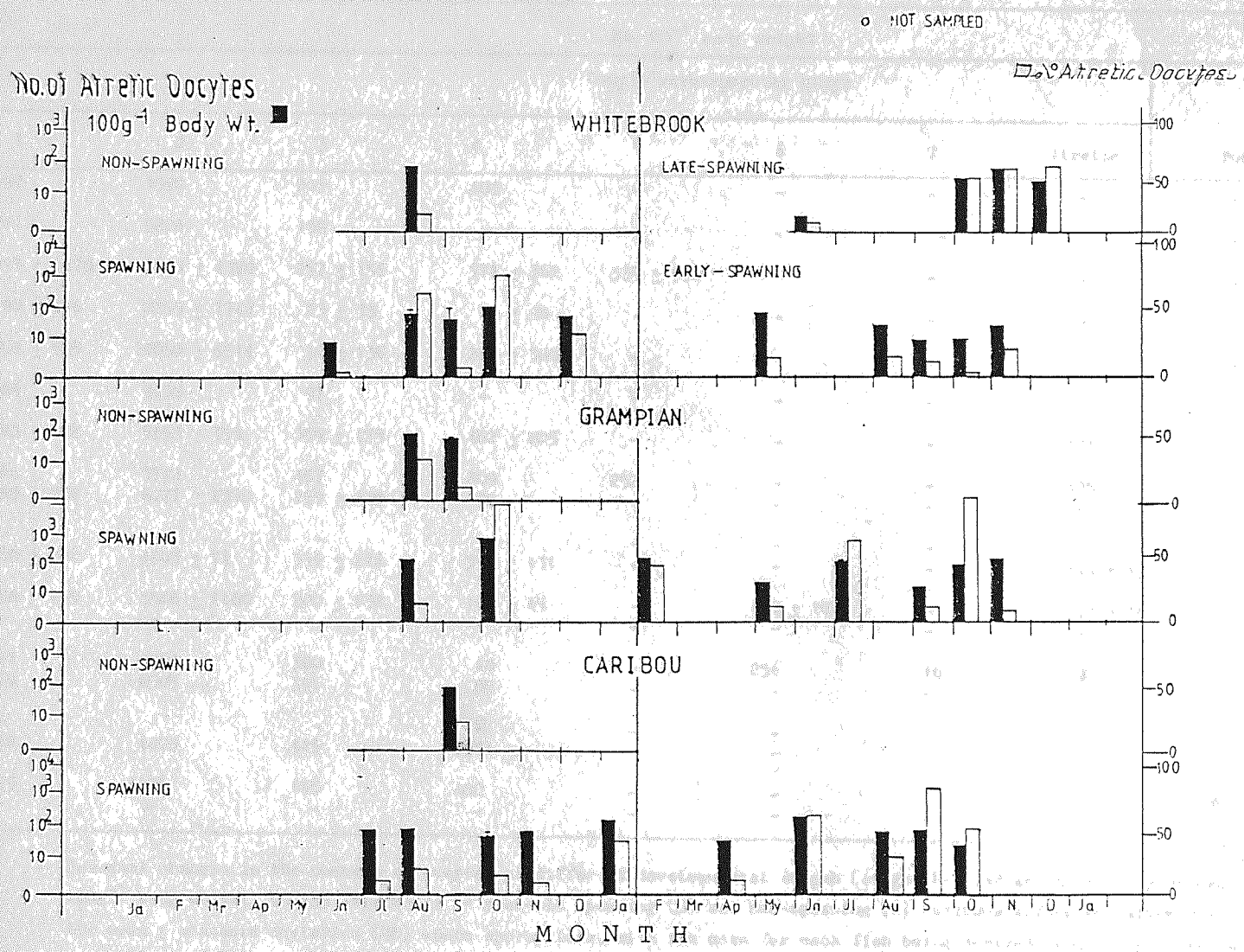


Fig 4.7 - Seasonal changes in the number of atretic oocytes $100g^{-1}$ body weight and the percentage of atretic oocytes within the ovaries of Caribou (A), Grampian (B) and Whitebrooks (C).

Month		No. 100^{-1} body weight										
		Oocyte developmental stage										
		1	2	3	4	5	6	7	Atretic	POF	Total	n
JAN		497	1990	205	409	-	-	-	-	-	3101	1
FEB		211	5224	159	-	-	-	-	-	-	5593	1
MAR		2097 ± 1678	5120 ± 4370	257 ± 155	374 ± 362	539 ± 530	-	-	-	-	9041 ± 6542	3
APR		1190 ± 716	2259 ± 1747	23 ± 33	69 ± 98	-	-	-	-	-	3540 ± 2334	2
MAY		2958 ± 595	10050 ± 5514	96 ± 136	269 ± 380	-	-	-	-	-	13334 ± 5647	2
JUN		2084	9259	97	-	-	-	-	-	-	11440	1
JUL		2083 ± 122	5159 ± 359	359 ± 175	482 ± 205	-	-	-	121	-	7984 ± 1072	2
AUG	S	1664	3742	457	209	252	-	-	125	-	5984	1
	N	1589 ± 204	6077 ± 2370	259 ± 178	96	-	-	-	-	-	7976 ± 2417	2
SEP	S	-	-	-	-	-	-	-	-	-	-	-
	N	2369 ± 701	4203 ± 71	359 ± 216	318 ± 131	-	-	-	145 ± 13	-	7239 ± 1488	3
OCT	S	458 ± 159	3508 ± 1180	200 ± 132	102 ± 45	-	410 ± 18	-	77 ± 16	-	4602 ± 1615	3
	N	-	-	-	-	-	-	-	-	-	-	-
NOV	S	3611	4563	300	60	-	251	16	90	-	8894	1
	N	3660	6505	395	85	-	-	-	-	-	10644	1
DEC	S	-	-	-	-	-	-	-	-	-	-	-
	N	278	4000	445	1004	-	-	-	-	-	5607	1
*JAN	S	2479	6536	675	480	-	-	-	450	676	11296	1
	N	-	-	-	-	-	-	-	-	-	-	-

Table 4.1.1 - Seasonal changes in the numbers of oocytes at different developmental stages (stages 1-7 and atretic) and post-ovulatory follicles (POF), $100g^{-1}$ body weight, in the ovaries of 1+ year-old spawning (S) and non-spawning (N) Caribous before and after deviation. Numbers given are the mean ± standard deviation (SD) where appropriate, with the mean for each fish being derived from 5 replicate samples (* - spawning time, n - number of fish).

Month	No. 100^{-1} body weight										
	Oocyte developmental stage										
	1	2	3	4	5	6	7	Atretic	POF	Total	n
JAN	1333 ± 136	3336 ± 15	189 ± 4	143	-	-	-	-	-	4886 ± 121	2
FEB	1642 ± 33	3279 ± 285	265 ± 85	258 ± 6	-	-	-	-	-	5443 ± 650	3
MAR	608	1201	33	-	-	-	-	-	-	1842	1
APR	-	-	-	-	-	-	-	-	-	-	-
MAY	2499	5087	268	267	-	-	-	-	-	8123	1
JUN	2019	6315	390	390	-	-	-	-	-	9116	1
JUL	4036	3437	-	330	-	-	-	-	-	8205	1
AUG S	1657 ± 1405	4055 ± 1759	359 ± 104	173 ± 19	142 ± 81	470	-	95	-	7161 ± 4187	2
N	2836	6078	718	336	-	-	-	335	-	10293	1
SEP S	-	-	-	-	-	-	-	-	-	-	-
N	1544 ± 573	4976 ± 2999	202 ± 93	259 ± 62	-	-	-	110 ± 78	-	7264 ± 3331	3
OCT S	1403	4168	479	64	413	732	-	718	-	8020	1
N	1829 ± 237	3673 ± 960	291 ± 222	134	-	-	-	-	-	6123 ± 475	2
NOV S	-	-	-	-	-	-	-	-	-	-	-
N	2614	5357	446	64	-	-	-	-	-	8479	1
*DEC	-	-	-	-	-	-	-	-	-	-	-

Table 4.1.2 - Seasonal changes in the numbers of oocytes at different developmental stages (stages 1-7 and atretic) and post-ovulatory follicles (POF), $100g^{-1}$ body weight, in the ovaries of 1+ year-old spawning (S) and non-spawning (N) Grampians before and after deviation. Numbers given are the mean ± standard deviation (SD) where appropriate, with the mean for each fish being derived from 5 replicate samples (* - spawning time, n - number of fish).

Month		No. 100g body weight Oocyte developmental stages										
		1	2	3	4	5	6	7	Atretic	POF	Total	n
MAR		1469 ± 947	2729 ± 1163	150 ± 146	258 ± 197	-	-	-	-	-	4605 ± 2405	2
APR		3329	11500	784	-	-	-	-	-	-	15771	1
MAY		1118	5595	407	916	306	-	-	-	-	8337	1
JUN		2470 ± 720	2952 ± 1933	96 ± 74	59 ± 21	-	-	-	22	-	5590 ± 2733	2
JUL	S	723	4589	882	541	-	-	-	-	-	6504	1
	N	563	3439	59	-	-	-	-	-	-	4061	1
AUG	S	5031 ± 2483	8507 ± 1811	572 ± 441	423 ± 157	457 ± 11	-	-	97 ± 11	-	1507 ± 5382	2
	N	3402	9251	60	599	-	-	-	179	-	13489	1
SEP	S	2386 ± 1944	5182 ± 3849	256 ± 89	274	40 ± 30	424 ± 89	-	75	-	9543 ± 4680	2
	N	1276	4515	49	392	-	-	-	-	-	6233	1
OCT	S	-	1155	35	35	-	501	424 ± 84	140	-	1862	1
	N	1008 ± 23	4286 ± 119	315 ± 164	114 ± 8	-	-	-	-	-	5222 ± 1605	2
*NOV	S	-	-	-	-	-	-	-	-	-	-	-
	N	1888	2591	-	-	-	-	-	-	-	4478	1
DEC	S	49	1252	219	49	-	-	-	73	389	2239	1
	N	415 ± 262	2293 ± 925	13 ± 78	42 ± 30	-	-	-	-	-	2854 ± 136	3

Table 4.1.3 - Seasonal changes in the numbers of oocytes at different developmental stages (stages 1-7 and atretic) and post-ovulatory follicles (POF), $100g^{-1}$ body weight, in the ovaries of 1+ year-old spawning (S) and non-spawning (N) Whitebrooks before and after deviation. Numbers given are the mean ± standard deviation (SD) where appropriate, with the mean for each fish being derived from 5 replicate samples (* - spawning time, n - number of fish).

Month	No. 100^{-1} body weight										
	Oocyte developmental stage										
	1	2	3	4	5	6	7	Atretic	POF	Total	n
JAN	-	-	-	-	-	-	-	-	-	-	-
FEB	2045	3613	68	1362	-	-	-	-	-	6954	1
MAR	-	-	-	-	-	-	-	-	-	-	-
APR	1040 ± 434	4765 ± 3245	479 ± 359	130 ± 77	503	-	-	75	-	6702 ± 3550	2
MAY	2477	1926	-	118	220	-	-	-	-	8123	1
JUN	1885	3732	490	301	152	-	-	415	-	6978	1
JUL	-	-	-	-	-	-	-	-	-	-	-
AUG	1178	1436	256	20	-	272	-	102	-	3673	1
SEP	43	1315	173	43	-	168	70	159	-	1972	1
OCT	128	587	71	28	-	32	250	57	-	1153	1
*NOV	-	-	-	-	-	-	-	-	-	-	-
DEC	-	1123	86	17	-	-	-	-	381	1609	1
JAN	-	-	-	-	-	-	-	-	-	-	-

Table 4.2.1 - Seasonal changes in the numbers of oocytes at different developmental stages (stages 1-7 and atretic) and post-ovulatory follicles (POF), $100g^{-1}$ body weight, in the ovaries of 2+ year-old Caribous. Numbers are the mean ± standard deviation (SD) where appropriate, with the mean for each fish being derived from 5 replicate samples (* - spawning time, n - number of fish)

Month	No. 100^{-1} body weight										
	Oocyte developmental stage										
	1	2	3	4	5	6	7	Atretic	POF	Total	n
JAN	-	-	-	-	-	-	-	-	-	-	-
FEB	461	1473	11	50	19	290	-	110	-	2514	1
MAR	-	-	-	-	-	-	-	-	-	-	-
APR	691 ± 221	1924 ± 600	83 ± 21	224 ± 24	115 ± 33	-	-	-	-	3090 ± 332	2
MAY	608 ±117	1314 ± 91	160 ± 83	119 ± 59	1295 ± 110	-	-	27	-	2357 ± 203	2
JUN	-	-	-	-	-	-	-	-	-	-	-
JUL	154 ± 7	1340 ± 863	189 ± 199	55 ± 35	66 ± 45	213 ± 111	-	132 ± 16	-	2152 ± 1229	2
AUG	2083	2764	381	88	10	140	-	59	-	5518	1
SEP	61	2142	153	-	225	196	-	275	-	2965	1
OCT	7551	2478	410	248	-	80	174	82	-	10687	1
NOV	82	771	488	246	-	-	258	130	-	2735	1
*DEC	188	1727	236	72	-	-	-	-	364	2586	1

Table 4.2.2 - Seasonal changes in the numbers of oocytes at different developmental stages (stages 1-7 and atretic) and post-ovulatory follicles (POF), $100g^{-1}$ body weight, in the ovaries of 2+ year-old Grampians. Numbers are the mean ± standard deviation (SD) where appropriate, with the mean for each fish being derived from 5 replicate samples (* - spawning time, n - number of fish)

Month	No. 100^{-1} body weight										
	Oocyte developmental stage										
	1	2	3	4	5	6	7	Atretic	POF	Total	n
APR	400 ± 224	2102 ± 2444	96 ± 22	76 ± 30	270 ± 219	89	-	-	-	2981 ± 2870	2
MAY	1708	4998	432	108	80	505	-	108	-	7939	1
JUN	-	-	-	-	-	-	-	-	-	-	-
JUL	-	-	-	-	-	-	-	-	-	-	-
AUG	740	3208	139	-	-	344	-	63	-	4495	1
SEP	370	780	69	28	-	125	-	14	-	1385	1
OCT	3224	1369	149	19	-		199	11	-	5049	1
*NOV	416	1722	246	-	-	-	-	59	551	2994	1

Table 4.2.3.1 - Seasonal changes in the numbers of oocytes at different developmental stages (stages 1-7 and atretic) and post-ovulatory follicles (POF), $100g^{-1}$ body weight, in the ovaries of 2+ year-old Whitebrooks. Numbers are the mean ± standard deviation (SD) where appropriate, with the mean for each fish being derived from 5 replicate samples (* - spawning time, n - number of fish)

Month	No. 100^{-1} body weight										
	Oocyte developmental stage										
	1	2	3	4	5	6	7	Atretic	POF	Total	n
APR	-	-	-	-	-	-	-	-	-	-	-
MAY	140	855	48	61	17	-	-	-	-	818	1
JUN	388	313	8	12	-	-	-	4	-	727	1
JUL	-	-	-	-	-	-	-	-	-	-	-
AUG	-	-	-	-	-	-	-	-	-	-	-
SEP	-	-	-	-	-	-	-	-	-	-	-
OCT	87	1111	130	-	-	7	342	72	-	1789	1
NOV	391	1511	391	131	-	-	179	130	-	2755	1
DEC	871	96	48	-	-	-	206	64	-	1331	1
*JAN	-	-	-	-	-	-	-	-	-	-	-

Table 4.2.3.2 - Seasonal changes in the numbers of oocytes at different developmental stages (stages 1-7 and atretic) and post-ovulatory follicles (POF), $100g^{-1}$ body weight, in the ovaries of late spawning 2+ year-old Whitebrooks. Numbers are the mean ± standard deviation (SD) where appropriate, with the mean for each fish being derived from 5 replicate samples. (spawning time, n - number of fish).

Month		Ca mg 100ml^{-1}	E_2 ng ml^{-1}	T ng ml^{-1}	GSI	MHOD μ	n
JAN		10.51	1.98	1.40	0.3682	557 ± 92	1
FEB		9.18	0.0	0.0	0.3605	371 ± 56	1
MAR		10.73 ± 1.61	2.97 ± 5.14	6.36 ± 5.20	0.4754 ± 0.009	998 ± 281	3
APR		10.85 ± 0.88	3.68 ± 3.00	3.50 ± 4.95	0.3613 ± 0.3989	445.5 ± 256.7	2
MAY		12.965 ± 0.431	6.2	13.4	0.1328 ± 0.0931	314 ± 272	2
JUN		3.38	2.4	3.32	0.0893	330	1
JUL		8.54	1.56	5.72	0.1744 ± 0.023	659 ± 185	2
AUG	S	12.54	2.72	9.6	0.4018	1056 ± 86	1
	N	12.11 ± 0.48	1.71 ± 1.37	0.58 ± 0.82	0.0706 ± 0.0251	471 ± 152	2
SEP	S	21.5 ± 2.03	44.34	40.2	3.2728 ± 0.75	-	3
	N	12.24 ± 0.24	5.01	14.77 ± 12.39	0.0797 ± 0.0094	451 ± 83	
OCT	S	28.74 ± 5.86	52.6 ± 26.5	58.8 ± 24.1	3.8382	2260 ± 368	3
	N	13.43	13.2	0.0	0.1259	-	1
NOV	S	33.39	45.54	48.8	5.2830	2939 ± 309	1
	N	13.34	15.6	0.0	0.0789	480 ± 31	1
DEC	S	-	-	-	-	-	-
	N	7.7	0.24	0.0	0.0278	643 ± 122	
*JAN	S	11.01	6.2	10.0	0.5635	517 ± 38	1
	N						

Table 4.3.1 - Seasonal changes in circulating levels of calcium (Ca), oestradiol-17β (E_2) and testosterone (T), gonadosomatic index (G.S.I.) and mean highest oocyte diameter (MHOD) of 1+ year-old spawning (S) and non-spawning (N) Caribous before and after deviation. Values given are the mean ± standard deviation (S.D.) where appropriate. (* - spawning time, n - number of fish).

Month		Ca mg 100ml^{-1}	E_2 ng ml^{-1}	T ng ml^{-1}	GSI	MHOD μ	n
JAN		8.35	0.0	1.13	0.1236 ± 0.0011	412.5 ± 101.1	2
FEB		7.525 ± 4.48	0.54	1.05 ± 1.48	0.18453 ± 0.1020	597.7 ± 76.5	3
MAR		7.225 ± 2.06	1.1 ± 1.56	0.0	0.0444 ± 0.038	346.5 ± 19.1	3
APR		11.53	2.39	2.16	0.1671	-	1
MAY		9.57	4.1	6.4	0.2529	512	1
JUN		8.89	-	-	0.2058	627 ± 112	1
JUL		10.5	4.34	7.42	0.2592	528 ± 77	1
AUG	S	11.965 ± 1.365	5.28 ± 1.02	7.5 ± 10.61	0.5308 ± 0.3041	1212 ± 205	2
	N	8.48	1.34	5.34	0.1276	490 ± 7	1
SEP	S	-	-	-	-	-	-
	N	12.12	2.9 ± 2.52	0.0	0.105 ± 0.0223	527.7 ± 47.4	3
OCT	S	27.22	102	110.8	4.4816	1421 ± 80	1
	N	14.245	4.15 ± 0.35	3.24 ± 0.51	0.08335	504.5 ± 197.3	2
NOV	S	40.38	124	84.8	11.4871	-	1
	N	10.65	1.36	4.92	0.1169	528.0	1
*DEC	S	21.33	12.4	98	10.8607	-	1
	N	11.0	0.44	0.0	0.1764	-	1

Table 4.3.2 – Seasonal changes in circulating levels of calcium (Ca), oestradiol-17β (E_2) and testosterone (T), gonadosomatic index (G.S.I.) and mean highest oocyte diameter (MHOD) of 1+ year-old spawning (S) and non-spawning (N) Grampians before and after deviation. Values given are the mean ± standard deviation (S.D.) where appropriate. (* - spawning time, n - number of fish).

Month		Ca mg 100ml^{-1}	E_2 ng ml^{-1}	T ng ml^{-1}	GSI	MHOD μ	n
MAR		9.88 ± 0.44	1.59 ± 0.45	1.98 ± 2.52	0.2294 ± 0.1312	653 ± 11.3	2
APR		7.86	4.56	12.0	0.2069	355 ± 38	1
MAY		11.56	3.23	15	0.3643	1001 ± 50	1
JUN		10.9 ± 1.07	4.23 ± 3.21	9.2 ± 2.26	0.1762 ± 0.003	526 ± 2.12	2
JUL	S	12.25	4.3	6.6	0.2058	744 ± 130	1
	N	-	-	-	0.0544	330	1
AUG	S	13.83 ± 2.23	-	17.7 ± 97	0.5931	1037 ± 20.5	2
	N	13.25	6.0	17.4	0.3929	683 ± 181	1
SEP	S	15.13 ± 5.45	15.5 ± 11.74	34.4 ± 13.56	1.2266 ± 0.6556	1514 ± 138.59	2
	N	10.95	3.5	6.8	0.1677	639 ± 93	1
OCT	S	50.12	55	80.8	8.2152	2751 ± 117	1
	N	15.225	0.66 ± 0.93	4.4 ± 6.22	0.07645 ± 0.038	500.5 ± 54.4	2
*NOV	S	-	-	-	-	-	1
	N	9.24	0.18	2.76	0.0037	101 ± 39	1
DEC	S	10.64	2.3	14.2	0.8479	402	1
	N	9.58 ± 0.609	1.8 ± 3.1	5.07 ± 8.08	0.1521 ± 0.0801	465.33 ± 107.7	3

Table 4.3.3 - Seasonal changes in circulating levels of calcium (Ca), oestradiol-17β (E_2) and testosterone (T), gonadosomatic index (G.S.I.) and mean highest oocyte diameter (MHOD) of 1+ year-old spawning (S) and non-spawning (N) Whitebrooks before and after deviation. Values given are the mean ± standard deviation (S.D.) where appropriate. (* - spawning time, n - number of fish).

Month	Ca mg $100ml^{-1}$	E_2 ng ml^{-1}	T ng ml^{-1}	GSI	MHOD μ	n
FEB	4.87	7.02	0.0	0.1648	614 ± 100	1
MAR	-	-	-	-	-	-
APR	10.03 ± 0.226	3.60 ± 1.41	10.1 ± 2.69	0.8553 ± 0.3319	894 ± 190.9	2
MAY	12.66	6.2	13.6	0.6744	1416 ± 118	1
JUN	8.23	6.4	9.2	0.5844	1048 ± 83	1
JUL	-	-	-	-	-	-
AUG	12.20	5.4	16.0	0.3093	1563 ± 138	1
SEP	21.32	110	81.2	7.0701	3225 ± 200	1
OCT	45.33	60	92	9.3899	3852 ± 238	1
*NOV	-	-	-	-	-	-
DEC	13.57	6.62	6.2	0.8458	386 ± 77	1

Table 4.4.1 - Seasonal changes in circulating levels of calcium (Ca), oestradiol-17β (E_2) and testosterone (T), gonadosomatic index (G.S.I.) and mean highest oocyte diameter (MHOD) of 2+ year-old spawning (S) and non-spawning (N) Caribous. Values given are the mean ± standard deviation (S.D.) where appropriate. (* - spawning time, n - number of fish).

Month	Ca mg $100ml^{-1}$	E_2 ng ml^{-1}	T ng ml^{-1}	GSI	MHOD μ	n
JAN	6.06	0.0	0.0	1.6039	-	1
FEB	18.28	1.35	14.12	1.6659	1761.2	1
MAR	14.47	12.4	15.2	1.2680	1365.2	1
APR	9.265 ± 1.54	4.28 ± 2.01	8.14 ± 1.5	0.2952 ± 0.694	1030.0 ± 94.75	2
MAY	13.93 ± 0.75	6.32 ± 4.07	11.3 ± 1.56	0.293 ± 0.213	798 ± 521.8	2
JUN	13.68	2.76	7.4	0.3642	-	1
JUL	10.765 ± 0.771	7.0 ± 2.82	8.70 ± 1.27	1.65485 ± 0.0728	1586 ± 388.91	2
AUG	12.14	2.72	11.6	0.3716	1374 ± 81	1
SEP	14.28	8.3	12.8	0.4641	1332 ± 67	1
OCT	23.51	18.6	75.2	8.8814	3524	1
NOV	17.05	13.4	110	17.4505	4692 ± 1225	1
*DEC	10.69	3.08	0.0	1.2057	545 ± 45	1

Table 4.4.2 - Seasonal changes in circulating levels of calcium (Ca), oestradiol-17β (E_2) and testosterone (T), gonadosomatic index (G.S.I.) and mean highest oocyte diameter (MHOD) of 2+ year-old Grampians. Values given are the mean ± standard deviation (S.D.) where appropriate. (* - spawning time, n - number of fish).

Month	Ca mg $100ml^{-1}$	E_2 ng ml^{-1}	T ng ml^{-1}	GSI	MHOD μ	n
MAR	-	-	-	-	-	-
APR	11.66 ± 0.947	4.85	12.9 ± 4.48	0.5976	1244	2
MAY	11.44	6.80	15.6	1.2902	1450 ± 98	1
JUN	-	-	-	-	-	-
JUL	-	-	-	-	-	-
AUG	18.40	9.9	23.8	1.7019	1904 ± 125	1
SEP	21.35	33.0	43.2	5.0163	2934	1
OCT	29.56	33.0	82.0	17.0404	3802 ± 158	1
*NOV	22.52	0.0	84.0	0.4776	357 ± 29	1
DEC	16.26	31.0	3.68	0.6485	-	1

Table 4.4.3.1- Seasonal changes in circulating levels of calcium (Ca), oestradiol-17β (E_2) and testosterone (T), gonadosomatic index (G.S.I.) and mean highest oocyte diameter (MHOD) of 2+ year-old early-spawning Whitebrooks. Values given are the mean ± standard deviation (S.D.) where appropriate. (* - spawning time, n - number of fish).

Month	Ca mg $100ml^{-1}$	E_2 ng ml^{-1}	T ng ml^{-1}	GSI	MHOD μ	n
MAY	10.67	8.3	10.8	0.1314	998 ± 62	1
JUN	12.58	5.60	1.84	0.516	528 ± 33	1
JUL	-	-	-	-	-	-
AUG	-	-	-	-	-	-
SEP	-	-	-	-	-	-
OCT	21.94	3.4	58.4	11.1298	3957 ± 244	1
NOV	18.35	19.8	102.0	10.7549	4720 ± 244	1
DEC	24.0	18.6	6.16	11.6654	5322	1
*JAN	-	-	-	-	-	-

Table 4.4.3.2- Seasonal changes in circulating levels of calcium (Ca), oestradiol-17β (E_2) and testosterone (T), gonadosomatic index (G.S.I.) and mean highest oocyte diameter (MHOD) of 2+ year-old late-spawning Whitebrooks. Values given are the mean ± standard deviation (S.D.) where appropriate. (* - spawning time, n - number of fish).

4.4 Discussion

There were no differences in the patterns of ovarian development or in the sequences of changes of the various serum constituents measured in the different strains and age groups of rainbow trout. However, there were differences in the timing of important events associated with oocyte development, ie. oogenesis, vitellogenesis, ovulation etc., and also in the circulating levels of vitellogenin, oestradiol-17β and testosterone. Similar changes in hormone and serum vitellogenin levels have been observed in an earlier study of the same three strains of rainbow trout from this laboratory (Elliott et al, 1984). These differences were evident not only between fish of the different strains but also in the different age groups of the same strain (eg. spawning 1+ and 2+ year-old fish of all three strains). There were also significant numbers of non-spawning fish in the stocks of the 1+ year-old group of all three strains. A feature which is found in some later spawning Scandinavian strains (Elliott et al, 1984; Bromage et al, 1984) but has not previously been reported for early spawning (October to December) varieties. Generally, the majority of female fish of Danish strains where natural spawning times are in January, February and March in the U.K., fail to mature as 2 year-old fish and have their first spawning at 3 years of age. In contrast, most female fish whose spawning periods are in the months from September through to December, and they are often of American stock, spawning as one or two year-olds.

Although the eggs of the three strains hatched at different times of the year the development of their ovaries followed essentially similar sequences during the first year of life. At the end of the first year fish of each of the strains contained oocytes

up to early stage 4. After the completion of the first year of life ovarian development followed a similar sequence of changes as 2+ year-old fish although the development of the younger age group of fish was delayed. In 1+ year-old fish active exogenous vitellogenesis commenced in July/August in all three strains whereas this process began in April in 2+ year-old Caribous and Grampians and in March/April in early-spawning Whitebrooks. Although it is not possible to exactly time exogenous vitellogenesis in the late-spawning group of 2+ year-old Whitebrooks, probably the process started in July/August. A few vitellogenic oocytes (stage 5) also appeared within the ovaries of 1+ year-old Caribous in March and of 1+ year-old and 2+ year-old late-spawning Whitebrooks in May, although these yolky oocytes disappeared during the succeeding months. The disappearance of stage 5 oocytes in these fish coincided with the first appearance of atretic oocytes. One month before the commencement of active exogenous vitellogenesis the ovaries contained oocytes only up to vesicle stage (stage 4). Similar changes were experienced by 2+ year-old Grampians with stage 5 and 6 exogenous vitellogenic oocytes which appeared in February, but with the stage 6 oocytes being lost by April leaving only stage 5 oocytes. Again the loss of these vitellogenic stage 6 oocytes coincided with the appearance of a large number of atretic oocytes. These observations show that vitellogenin uptake may commence during the late winter and early spring when ambient daylength is of short duration but increasing. However, before the commencement of the most active phase of exogenous vitellogenesis, the stage 5 oocytes which had been observed early in the cycle had become atretic. In contrast, in most of the oocytes which entered vitellogenesis in July/August, when

ambient daylength is long and beginning to decrease development was continuous until full maturation and ovulation. Thus, one might suggest that the early vitellogenic phases requires increasing daylengths, while the later phases of oocyte development, ie. the stages of germinal vesicle migration (stage 6) and final maturation (stage 7) require decreasing daylengths. In salmonids daylength is known to be of prime importance in the cuing of reproductive development (Allison, 1951; Henderson, 1963b; Whitehead et al, 1978b; McQuarrie et al, 1979; Baggerman, 1980; Whitehead and Bromage, 1980; Bromage et al, 1984; Elliott et al, 1984; Scott et al, 1984), although most of these studies have used only ovulation and/or spawning as the reference point for correlations with ovarian development. Thus it is not clear which of the phases of oocyte development coincide with the long and also the short days of a seasonally-changing natural photoperiod. Bromage et al (1982b) and Scott et al (1984) suggested that trout are long-day animals, and that long-days are necessary for early growth and vitellogenesis, although neither study offered histological evidence for these proposals. Similar reservations also apply to the suggestion that short days or possibly the absence of long days provide further cues for the synchronisation of later events (Whitehead and Bromage, 1980; Bromage et al, 1982b, 1984; Scott and Sumpter, 1983b). In the present study vitellogenic oocytes first appeared on long or increasing daylengths with the rest of the developmental cycle occurring under a decreasing photoperiod, thus confirming that trout are long-day animals. One might also suggest that if the appropriate cue is not provided during the correct phase of oocyte development, then the development may be arrested and the oocytes subject to

atresia. Possibly this was most clearly seen amongst the non-spawning groups of 1+ year-old fish where recruitment to each of early previtellogenic stages was delayed, when compared to the rates of development of the corresponding spawning groups. The ovaries of the non-spawning groups only developed late stage 4 (4b) oocytes when the fish of the spawning groups were undergoing active exogenous vitellogenesis (stage 5 and stage 6).

The above changes in oocyte development were paralleled by changes in oocyte diameter and G.S.I. Oocyte diameter and G.S.I. slightly increased in all 1+ and 2+ year-old fish during the early months of the year only to decrease in the months which preceded the phase of active exogenous vitellogenesis. Subsequently, the G.S.I.s and oocyte diameters increased during the exogenous vitellogenesis to reach maxima just before ovulation. Both the early and late increases in G.S.I. and oocyte diameter were paralleled by increases in the levels of serum oestradiol-17β, testosterone and calcium; these changes also coincided with the appearance of stage 4, stage 5 and sometimes stage 6 oocytes which belong to the vitellogenic phase of development. The slightly raised levels in hormones which occurred during the late winter or early spring months have not previously been reported for rainbow trout or any other seasonally-breeding salmonid. However, Sumpter et al (1984) also observed for the rainbow trout, early increases in vitellogenin levels which fell during the middle part of the cycle only to increase again at the time when exogenous vitellogenesis began. The significance of these early changes in vitellogenin levels is unexplained, although Bromage et al (1982a) and Whitehead et al (1983) both reported increases in gonadotropin in rainbow trout, during the early months of the year

and suggested that this increase was concerned with gonadal recrudescence. Although gonadotropin levels were not measured during the present investigations, the appearance of exogenous vitellogenic oocytes during the early months of the year may be related to the raised levels of gonadotropin and vitellogenin which were reported by Bromage et al (1982a), Whitehead et al (1983) and Sumpter et al (1984) in their studies.

In addition to these increases Elliott et al (1984) and Sumpter et al (1984) both reported increased levels of circulating testosterone during active vitellogenesis. Similar observations were also made during the present investigation. Elliott et al (1984) suggested that the increased levels of testosterone may be involved in the degeneration of ovarian ova. High levels of testosterone also coincided with higher incidence of atretic oocytes in the present work, although it is not possible to say that these changes are causatively linked. In Chapter 5 and 6 it is reported that injections of oestradiol-17β and starvation induced atresia, although in both experiments the treatment also produced significantly reduced testosterone levels when compared to those of controls. Hence, it is unlikely that testosterone is involved in the development of atresia. A more likely function for testosterone has been suggested by Kagawa et al (1982b); Nagahama et al (1982) and Young et al (1982c), in their "two cell type model". This model implicates the involvement of theca in the production of testosterone which, after transfer to granulosa is aromatised to oestradiol-17β which in turn is involved in the induction of exogenous vitellogenesis. Aside from its importance in the formation of oestrogen testosterone may affect oocyte maturation. The present work suggests that testosterone may

be involved with exogenous vitellogenesis as well as oocyte maturation. These propositions are supported by low but significantly raised levels of testosterone in 1+ year-old Caribou and Whitebrooks and in 2+ year-old Grampians and late spawning Whitebrooks at time when their oocytes first showed signs of exogenous vitellogenesis.

In all ages and strains of trout studied here oestradiol-17β and testosterone levels both showed parallel increases until just before oocyte maturation at which part oestradiol levels began to fall while testosterone values continued to increase until ovulation. Similar observations have also been made in other studies of rainbow trout (Elliot et al, 1984 and Sumpter et al, 1984), and other salmonids (Kagawa et al 1983 and Young et al, 1983). In the present study testosterone levels were almost always above the levels of oestradiol-17β except, during early previtellogenic and post-ovulatory periods, when the ovaries only contained oocytes up to early stage 4 (4a). Therefore, it is possible that the thecal layers of oocytes below stage 4a are not equipped with the necessary organelles to produce testosterone. The rough endoplasmic reticulum was particularly well-developed in the thecal cells of follicles surrounding oocytes which were undergoing vitellogenesis. However, no such development was present in the follicular layer of stage 4a oocytes. Kagawa et al (1983) suggested that 17α-hydroxy progesterone is converted to testosterone within the thecal layer of the preovulatory follicle and that two enzymes, 17β hydroxysteroid dehydrogenase and C_{17}-C_{20} lyase, are responsible for this conversion. Furthermore, these enzymes are said to become inactive in post-ovulatory follicles which produce 17α-hydroxy progesterone

instead of testosterone (Kagawa et al, 1983). However, the reasons for the change in patterns of hormone synthesis by the follicle during development is still unexplained, although their possible involvements in the control of various events associated with oocyte development have been suggested in various studies. Oestradiol-17β is known to be involved in the direct control of exogenous vitellogenesis in the trout (Lambert et al, 1978; Whitehead et al, 1978a,b, 1983; Elliott et al 1979, 1980; Scott et al, 1980; Van Bohemen and Lambert, 1981; Bromage et al, 1982a; Scott and Sumpter, 1983a; Schulz, 1984; Sumpter et al, 1984) and other teleosts (Campbell and Idler, 1976, 1977; Yaron et al, 1977; Crim and Idler, 1978; Khoo, 1979; Kagawa et al, 1981, 1983; Sundararaj and Nath, 1981). However, the role of testosterone in ovarian development is still controversial. Progesterones, and specifically 17α hydroxy 20β dihydro progesterone (17α 20β-OHP) is said to be involved in the final stages of oocyte development, ie. germinal vesicle migration and breakdown (Jalabert, 1976; Jalabert and Fostier, 1984). However, 17α hydroxy progesterone (17α OHP) and 17α 20β-OHP reach very high levels during the maturational phase of oocyte development in rainbow trout (Scott et al, 1983; Springate et al, 1984). The changes in aromatisation which occur within the follicle to produce oestradiol-17β, testosterone or progesterone have been attributed to subtle differences in gonadotropin concentrations, which in turn act on the activity of 17β hydroxysteroid dehydrogenase (Sire and Dépêche, 1981). At low gonadotropin levels aromatisation produces oestradiol-17β a pathway which normally occurs during exogenous vitellogenesis. During subsequent maturation the increased levels of gonadotropin produce testosterone (Sire and Dépêche, 1981). Whereas, during final

maturation this reaction is replaced and 17α hydroxy 20β dihydro progesterone is formed (Fostier et al, 1981). Although 17α hydroxy 20β dihydro progesterone (17α 20β OHP) was not measured during the present study high levels of testosterone were observed during germinal vesicle migration and breakdown and ovulation but, in post-ovulated fish these levels had dropped to basal levels. These observations indicate that testosterone may have a direct or an indirect role in final oocyte maturation. However, further histochemical and biochemical studies are necessary to elucidate the functions of the high testosterone levels which prevail during this stage of oocyte development.

There were also changes in oocyte development, G.S.I. and in levels of oestradiol-17β, testosterone and calcium in non-spawning 1+ year-old fish of all three strains. Generally, the patterns of change of oestradiol, testosterone and calcium in the non-spawning groups of fish were similar in profile to those exhibited by the spawning groups. In all cases, however, the peak heights of these materials were much lower in the non-spawning groups. Exogenous vitellogenesis also did not occur in these fish. Elliott et al (1984) reported similar changes in immature members of a later spawning strain of rainbow trout, ie. two year-old Whitebrooks which were not to spawn until they were 3 years of age. Elliott et al (1984) proposed that immature fish undergo "dummy" or "practice runs" at times when spawning fish of the same strain undergo maturation. Similar developments have been noted in other fish species in the years before their first spawnings (Davis, 1977; Eliassen and Vahl, 1982). It has been suggested that the changes in steroid hormones in these immature fish are related to feedback mechanisms which prepare

the hypothalmic-hypophysial axis for the first spawning (Elliott et al, 1984). Positive feedback induction of pituitary gonadotropin synthesis has already been demonstrated in trout (Crim and Evans, 197?). Despite the absence of any signs of vitellogenin incorporation in the non-spawning groups of 1+ year-old fish large numbers of atretic oocytes and brown bodies were present within their ovaries, at a time when the ovaries of the spawning fish of the same strain and age were undergoing vitellogenesis and maturation. These non-spawning fish also showed slight increases in G.S.I. and oocyte diameter and changes in hormone and calcium levels when late stage 4 (4b) oocytes appeared. Subsequently, the G.S.I. and oocyte diameter decreased in these fish whilst in the spawning groups they continued to increase until spawning. These slight changes in gonadal development and serum constituents could be the "dummy run" or "practice run" referred to by Elliott et al (1984). The observations of the present study indicate that the ovaries of the non-spawning 1+ year-old fish may have initially started development slightly later than the others, and the oocytes would have developed up to the late vesicle (4b) or early yolk granule stage with the increasing day-length of summer. These oocytes may have failed to reach a sufficiently advanced stage of development before July/August when the daylength was decreasing. In contrast, in the spawning 1+ year-old fish probably development started earlier and the oocytes were able to reach early stage 6 before any reduction in daylength began. In the non-spawning group the oocytes which had failed to reach stage 5 or early stage 6 before the daylength had begun to decrease were unable to undergo further development under prevailing conditions of photoperiod and consequently may have become subject to atresia.

The most important observation made during the present investigation was the occurrence of atretic oocytes during all stages of vitellogenesis and also after ovulation. Similar observations were also made by Macer (1974) on the horse mackerel. However, Macer (1974) failed to identify atretic oocytes in ovaries preserved in Gilson's fluid and concluded that they had been dissolved during preservation. In contrast, during the present study different stages of atretic oocytes were clearly identifiable in the ovaries before and after processing for histology and also after preservation in Gilson's fluid. This enabled actual counts of the numbers of atretic oocytes to be made. Macer (1974) estimated the numbers of atretic oocytes from sections of ovary but did not take into account any oocytes which were less than 0.2 mm in diamter. Consequently there may have been errors in his estimates of atresia. Similar problems have complicated interpretation in other studies (Vladykov, 1956; Scott, 1962; Bagenal, 1969; de Vlaming, 1971; Townshend and Wootton, 1984); these are more fully reviewed in Chapter 6. However, atresia has been the subject for several other studies including those of Bretschneider and Duyvene de Wit (1947), Sathyanesan (1963), Scott (1962), Henderson (1963b), Rastogi (1966), Chan et al (1967), Guraya (1973, 1976), Khoo (1975), Byskov (1978), Saidapur (1978) and Lang (1981), although their numbers have not been taken into account and structures of atretic oocytes are not clearly explained.

In the present investigation in addition to the proportion of atretic oocytes, proportions of all oocyte stages were taken into account and it was found that the total number of oocytes were significantly reduced during development with the major portion of this reduction occurring during previtellogenesis. Despite the

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In the present investigation in addition to the proportion of atretic oocytes, proportions of all oocyte stages were taken into account and it was found that the total number of oocytes were significantly reduced during development with the major portion of this reduction occurring during previtellogenesis. Despite the

overall losses of previtellogenic oocytes atretic structures were not observed during these stages possibly because they are rapidly resorbed by the ovary. Support for this suggestion may also be found in the work of Byskov (1978) who reported that previtellogenic oocytes disappear without leaving any trace. These observations do not agree with the study of Henderson (1963b) who concluded that atresia could not occur in healthy well-fed populations of trout. However, in the present work atresia was commonly found in vitellogenic, mature and post-ovulated ovaries of Caribou, Grampian and Whitebrook strains of rainbow trout grown on a nutritionally balance diet and at rates recommended by the feed manufacturers.

In addition to the above observations, it was found that the atretic oocytes or developing oocytes when represented as a percentage over total number of oocytes present within the ovary may produce misleading results, as the total number of oocytes fluctuate within a broad range during the annual ovarian developmental cycle. This may result in different percentages for the same number of atretic oocytes or oocytes at different stages, during different phases of ovarian development. Therefore, it was found that it is more appropriate to indicate the oocyte proportions as the number of oocytes 100 g^{-1} body weight. This allowed one to compare not only the differences among fish of the same strain and age at different phases of ovarian development but also the differences between strains and age groups within a strain, at corresponding phases of ovarian development, regardless of the differences in body weight of individual fish. A similar method has been adopted to compare the fecundities of fish belonging to different strains and age groups and those subjected to different feeding regimes, and fecundity has been

represented as relative fecundity, ie. number of eggs produced Kg^{-1} of fish (Springate and Bromage, 1984a,b). Furthermore, despite the differences observed in absolute numbers of oocytes within the fish belonging to different groups, relative numbers (numbers 100 g^{-1} body weight) did not show differences between fish of different age groups at corresponding phases of ovarian development. Similar observations on relative fecundity have been made for rainbow trout up to 5 years of age (Springate and Bromage, 1984a).

In summary, the present study shows that different strains of trout follow essentially similar patterns of ovarian development albeit with some differences in the timing of development which are related to their spawning times. In all strains it was found that oocytes undergo exogenous vitellogenesis under increasing daylength whilst later events occurred under short or decreasing daylength. Delays of development particularly those up to stage 5 may lead to atresia in the developing oocytes and an inability of the fish to spawn during that cycle. These changes in ovarian development were closely correlated with alterations in serum oestradiol, testosterone and calcium levels, thus confirming the involvement of the two hormones in the control of ovarian development and also the importance of calcium in vitellogenesis. Atresia was found to be of common occurrence within vitellogenic, mature and post-ovulated ovaries, and it is found to be the main reason for reductions in the proportions of vitellogenic and maturing oocytes. Although previtellogenic oocytes were found to disappear in large numbers atresia was not observed among oocytes at those stages.

CHAPTER 5

Effects of oestradiol-17β on vitellogenic oocytes with special reference to atresia.

5.1 Introduction

Oestrogenic hormones are known to be intimately involved in stimulating the formation of yolk in oviparous vertebrates (Follett and Redshaw, 1974). Of all the oestrogens, oestradiol-17β is thought to be the most potent steroid in this process, although much of the evidence in support of this proposition is restricted to a few species of birds, reptiles and amphibians (Reviewed by Chester Jones et al, 1972). Few studies have been carried out on fish. Investigations on rainbow trout conducted in this laboratory strongly indicate a role for oestradiol-17β in the direct control of vitellogenesis (Whitehead et al, 1978a, b 1983; Elliott et al, 1979, 1980; Bromage et al, 1982a). Similar studies have been carried out by other workers on rainbow trout (Lambert et al, 1978; Scott et al, 1980; Van Boheman and Lambert 1981; Scott and Sumpter, 1983a; Schulz, 1984; Sumpter et al, 1984) as well as on other teleosts (Campbell and Idler, 1976; Emmersen and Petersen, 1976; Wingfield and Grimm, 1977; Yaron et al, 1977; Crim and Idler, 1978; Khoo, 1979; Kagawa et al, 1981, 1983; Sundararaj and Nath, 1981). Although a role for oestradiol-17β in the synthesis of vitellogenic proteins within the liver is now established, the possible involvement of this hormone in the control of oogonial proliferation, oogenesis, vitellogenin incorporation during the secondary growth phase of the oocyte, recruitment of oocytes to higher developmental stages and atresia of developing oocytes remain obscure. Therefore, the present study will investigate the effects of intra-peritoneal injections of oestradiol-17β on the oocytes in a vitellogenic ovary.

High concentrations of oestrogens are observed in the blood serum of rainbow trout just before and during vitellogenesis (Lambert et al, 1978; Whitehead et al, 1978a, b, 1983; Elliott et al, 1979, 1984; Idler and Campbell, 1980; Scott et al, 1980, 1984; Van Boheman and Lambert, 1981; Bromage et al, 1982a; Scott and Sumpter, 1983; Sumpter et al, 1984; Aida et al, 1984). Somewhat similar observations have also been made for other teleosts (Schreck and Hopwood, 1974; Emmersen and Petersen, 1976; Wingfield and Grimm, 1976, 1977; Yaron et al, 1977; Billard et al, 1978; Crim and Idler, 1978; Sundararaj et al, 1982; Ueda et al, 1984). Furthermore oestradiol-17β levels are found to return to basal values prior to oocyte maturation and ovulation (Whitehead et al, 1978a, b, 1983; Fostier et al, 1978; Elliott et al, 1979, 1984). Oestradiol is known to be produced by the ovarian follicular tissue as a result of aromatisation of androgens (Nagahama et al, 1982; Young et al, 1982c). Testosterone is said to be the major aromatisable androgen produced by the follicle (Kagawa et al, 1982b). In rainbow trout seasonal changes in plasma oestradiol are found to be followed by seasonal changes in testosterone levels (Elliott et al, 1984; Scott et al, 1984; Sumpter et al, 1984). Peak levels of testosterone are found to occur at the time of oocyte maturation and ovulation (Kagawa et al, 1983; Young et al, 1983a; Elliott et al, 1984; Scott et al, 1984; Sumpter et al, 1984). Similar results were also seen during the present work of three strains of rainbow trout (Chapter 4). Thus, it seemed appropriate to study the changes of both testosterone and oestradiol-17β in the present experiment.

Following the high levels of oestradiol-17β, values for phosphoprotein phosphorus and calcium have been found to increase, and a highly significant correlation has been observed between the levels of oestradiol-17β and those two components. Both phosphoprotein phosphorus and calcium are known to be natural constituents of the vitellogenin molecule (Whitehead et al, 1983; Elliott et al, 1979, 1984). In the rainbow trout, peaksof serum vitellogenin are preceded by raised levels of oestrogens (Elliott, 1984; Sumpter et al, 1984). Furthermore, oestradiol-17β injections are able to induce viteloogenesis, irrespective of the sex of the treated fish (Plack and Fraser, 1971; Campbell and Idler, 1976, 1980; Emmersen and Petersen, 1976; Craik, 1978; Emmersen et al, 1979; Nath and Sunderaraj, 1981; Korsgaard et al, 1983). Conversely, the absence of vitellogenin in male serum has been attribted to the lack of oestrogens (Idler and Campbell, 1980). Collectively, these observations demonstrate that oestrogens are involved in vitellogenin synthesis (Whitehead, et al, 1983; Elliott et al, 1984; Sumpter et al, 1984). In addition, it has been suggested that oestradiol-17β has a role in the regulation of carbohydrate and lipid metabolism (Plack and Pritchard, 1968; de Vlaming et al, 1977a, b; Emmersen et al, 1979) and also in the mobilization of calcium from the scales (Mugiya and Watabe, 1977; Mugiya and Ichii, 1981) and of lipids (de Vlaming et al, 1977a), both these changes possibly serving to prepare fish for the demands of vitellogenesis.

Although vitellogenin synthesis is believed to occur within the liver, evidence for this is sparse. Generally, studies of oestrogen-treated trout, have failed to detect vitellogenin in the liver (Van Bohemen et al, 1982a,b) despite enormous increases in circulating

levels of this complex (Van Boheman et al, 1982a,b; Elliott et al, 1979). Oestrogens also induce hepatic hypertrophy in the rainbow trout (Campbell and Idler, 1980) and other teleosts (Kobayashi, 1953; Egami et al, 1955; Nath and Sundararaj, 1981). Furthermore, hepatic hypertrophy (see review by Ng and Idler, 1983), increases in lipid content of the liver and decreases in its glycogen content (de Vlaming et al, 1977a, b) have been observed in response to oestrogen treatment. Ultrastructural studies have also shown that there are clear signs of synthetic acitivity in the hepatocytes of mature females and also oestrogen-treated fish, whereas the corresponding cells of males and immature females appear quiescent (Peute et al, 1978; Olivereau amd Olivereau, 1979; Van Boheman et al, 1981; Selman and Wallace, 1983; Ng et al, 1984). The hepatocytes of the oestrogen-treated fish also have a well-adapted rough endoplasmic reticulum (Ishii and Yamamoto, 1970; Aida et al, 1973; Peute et al, 1978) and elevated amounts of RNA (Emmersen and Emmersen, 1976; Emmersen et al, 1979; Medda et al, 1980; Emmersen and Korsgaard, 1982; Korsgaard et al, 1983). Although the above studies suggest that vitellogenin synthesis occurs within the liver, it is unclear whether the incorporation of calcium into the vitellogenin molecule is completed before it is released into the blood. Van Boheman et al (1982a), suggested that fish vitellogenin is not stored in large quantities in the liver and that once synthesised it is rapidly released into the circulation. Therefore, together with the changes in serum calcium levels, this study will investigate the changes in liver calcium levels in oestradiol injected mature rainbow trout with vitellogenic ovaries.

In addition to its effects on vitellogenein synthesis by the liver, oestradiol administration is also known to influence gonadal development and function (Review by Ng and Idler, 1983). However, the physiological significance of oestrogens during previtellogenesis remains unclear. Khoo (1975) suggested that oestradiol may induce oogonial recrudescence. An oestradiol induced oogonial proliferation has also been reported in the minnow (Bullough, 1942), medaka (Egami, 1954) and goldfish (Khoo, 1979). However, Bullough (1942) observed an inhibition of primary oocyte development and their disintegration following oestrogen administration. However, the oestrogen synthetic capacity of the ovary of rainbow trout is known to increase from May during endogenous vitellogenesis to a maximum in October during exogenous vitellogenesis (Elliott et al, 1979). Similar observations were made during the present studies of three strains of rainbow trout (Chapter 4). In hypophysectomised goldfish oestrogens have induced ... endogenous vitellogenesis (yolk vesicle formation) (Khoo, 1979). Furthermore, in immature pink salmon treated with a combination of oestrogen and salmon gonadotropin, yolky oocytes have been found to develop more quickly than in those treated with salmon gonadotropin alone (Funk et al, 1972). In oestradiol treated eel an increase in gonado-somatic-index and oocyte diameters have been observed (Olivereau and Olivereau, 1979). In immature carp oogonial proliferation, oogenesis, the formation of ooplasmic vesicles and yolk granule formation are all said to be induced by oestrogen treatment and it has been suggested that oestradiol treatment may have different effects during different phases of ovarian development (Pantic and Lovren, 1978). This proposition is supported by the observation of a significant increase in gonado-somatic-index as a result of oestrogen treatment during the middle and late phases of

gonadal cycle of Notemigonus (de Vlaming et al, 1977a). However it has been suggested for a number of teleosts that oestrogens have no direct effects on the ovary (Ho and Vanstone, 1961; Plack et al, 1971; Campbell and Idler, 1976; Medda et al, 1980). This controversial evidence regarding the effects of oestrogens on teleost ovarian development makes it difficult to understand the role of oestrogens in the development of ovarian ova.

Dodd (1960) suggested that oestrogens have an inhibitory action on the ovarian development of fish. This proposition is supported by the reports that oestradiol administration causes ovarian degeneration (atresia) in Phoxinus laevis (Bullough, 1942), Platypoecilus maculatus (Tavolga, 1949), Lebistes (Berkowitz, 1941), Oryzias latipes (Egami, 1954, 1955); Onchorhynchus gorbuscha (Funk et al, 1972), Lopomis (Simon and Reinboth, 1974) and Carassius auratus (Khoo, 1955). The administration of oestradiol to mature gravid goldfish has induced atresia in oocytes at second growth phase (vitellogenesis) Khoo (1975). Similarly Funk et al (1972) observed that a large number of vitellogenic oocytes became atretic and only a fewer number of oocytes reached maturation in pink salmon treated with oestradiol alone or together with salmon gonadotropin, when compared to those which were untreated. However, the endocrine mechanism behind the degeneration of ovarian ova (atresia) is still obscure, although it is said to be a naturally occurring phenomenon during the oocyte development cycle (Rastogi, 1966). Possibly this contradictory evidence regarding the effects of oestradiol on oocyte development, relate to the complexity of its relationships with gonadotropin(s).

Yamazaki (1965) suggested that the pituitary may be involved

in the oogonial mitoses as a result of the action of gonadotropins on the ovarian hormones. Olivereau and Olivereau (1979), as a result of their observation, that the pituitary gonadotropic cells hypertrophied and contained increased amounts of glycoprotein granules after oestradiol administration in freshwater eel, suggested that oestradiol may act on the pituitary and/or hypothalmus via a positive feedback mechanism to induce gonadotropin synthesis. However, in contrast most other studies favour a concept of negative feedback control of oestrogen in pituitary gonadotropin secretion (Egami, 1955; Egami and Ishii, 1962; Sundararaj and Goswami, 1968). Furthermore, treatment with pituitary extracts and gonadotropic hormone preparations are known to stimulate oestradiol secretion in salmonids (Billard et al, 1978; Crim and Idler, 1978) and also in other teleosts (Fostier et al, 1979, Nagahama et al, 1982; Kagawa et al, 1982a). Thus, gonadotropin(s) are involved indirectly in vitellogenin synthesis as a result of their influence on oestradiol secretion. Although the majority of studies suggest the existence of a single gonadotropic hormone, some authors suggest that there may be two gonadotropins: a maturational gonadotropin and a vitellogenic gonadotropin (Campbell and Idler, 1976; Ng and Idler, 1978a, b, 1979). However, this proposition needs further investigation.

In a number of studies on teleosts vitellogenesis has been arrested by the removal of the pituitary (Vivien, 1941; Barr, 1963; Yamazaki, 1965; Sundararaj and Goswami, 1968), whereas hypophysectomy has decreased the gonadosomatic index and diminished the incorporation of vitellogenin into the gonad (Campbell and Idler, 1976). In addition, hypophysectomy induced atresia in the

yolky oocytes of goldfish (Khoo, 1975). Furthermore, administration of salmon gonadotropin had induced ovarian development, ie. vitellogenesis ovulation, etc., in hypophysectomised Carassius auratus (Yamazaki and Donaldson, 1968), Poecilia reticulata (Liley and Donaldson, 1969) and Heteropneustes fossilis (Sundararaj et al, 1972). Similarly salmon gonadotropin extractions injected into immature pink salmon has accelerated the ovarian maturation in immature pink salmon (Funk et al, 1972). However, this evidence would suggest a direct or indirect influence of pituitary gonadotropin on the synthesis of vitellogenin and/or in the vitellogenin incorporation by the oocytes although there is other evidence which suggests the involvement of steroids (Crim et al, 1973, 1975; Billard et al, 1978; Whitehead et al, 1978a, b, 1983; Fostier et al, 1979; Bromage et al, 1982a; Kagawa et al, 1982a; Nagahama et al, 1982; Scott and Sumpter, 1983). Whitehead et al (1983) observed a highly significant correlation between serum gonadotropin and oestradiol levels. Somewhat similar observations for rainbow trout and other teleosts have been made in other studies (Lambert et al, 1978; Fostier et al, 1978; Billard et al, 1978; Crim and Idler, 1978; Lou et al, 1984). Thus, the increases in oestradiol-17β levels which occur during active exogenous vitellogenesis in the rainbow trout, and possibly all other teleosts, may themselves be induced by the higher levels of gonadotropin observed during the early stages of oocyte development.

In the trout ovariectomy has been shown to induce a rise in gonadotropin (Bommelaer et al, 1981) whilst in Tilapia it decreased the plasma calcium and protein levels (Yaron et al, 1977). In

ovariectomised fish the increases in gonadotropin are thought to occur due to the absence of ovarian feedback on pituitary (Bommelaer et al, 1981). This concept is supported by the observation of raised levels of serum calcium and protein, and hence vitellogenin levels in oestrogen-injected fish (Yaron et al, 1977; Nath and Sundararaj, 1981) whilst the administration of salmon gonadotropin, SG-G100 (Donaldson et al, 1972) did not show any influence on the vitellogenin levels of ovariectomised catfish (Nath and Sundararaj, 1981). Although the majority of studies indicate the involvement of oestrogens in the synthesis of vitellogenins, the control of the uptake of vitellogenin is far less clear. Thus, further investigations are necessary to understand this phenomenon, as the major part of the oocyte growth is dependent on the vitellogenin uptake.

The primary aim of this study is to investigate the effects of oestradiol-17β administration on vitellogenic oocytes. Attention will be focused mainly on the vitellogenin incorporation, atresia and proportions of previtellogenic and vitellogenic oocytes within the ovaries after oestradiol-17β treatment. In addition to its effects on the ovary, effects on the hepatosomatic index, liver and serum calcium levels, and serum oestradiol and testosterone will be studied. This may help to get a better understanding on the importance of oestradiol-17β in the process of vitellogenesis in the rainbow trout.

5.2 Materials and Methods

The primary aim of this study was to investigate the effect of oestradiol treatment on the vitellogenic ovary of therainbow trout, in particular the effects of this hormone on oogenesis, vitellogenin incorporation, oocyte recruitment and on resorption of ovarian ova.

All fish used in this study were obtained from 2+ year old stocks held at Aston Fish Culture Unit. This age group was selected because the ovary of these fish would expect to be undergoing maturation during their third year of life. The investigation was conducted during the summer and autumn months, a time when the ovaries of maturing fish are known to comprise of stage 5 and 6 vitellogenic oocytes and also have high levels of circulating oestradiol.

Fifteen female rainbow trout were transferred from the main stock into a recirculating system (Appendix 1) and allowed to acclimatise for two weeks. Following this period the fish were weighed, blood sampled and tagged. Six fish were selected at random from the above group and injected with 5 $\mu g\ g^{-1}$ body weight of oestradiol-17β suspended in an arachis/ethanol (4:1 vv) emulsion. A further 6 fish, which were to act as controls, were injected with a similar volume of the arachis-ethanol emulsion (vehicle). Three fish from the initial stock were sacrificed at the start of the experiment and their ovaries removed for histological examination (section 2.2), oocyte counts (section 2.3) and determination of G.S.I. (section 2.1.6.1). Serum samples from all fish were stored at -20°C until radioimmunoassay of oestradiol-17β and testosterone (section 2.4) and analysis of calcium (section2.5).

Oestradiol-injected fish and the vehicle-injected controls were reinjected 4 weeks later with the same doses of oestradiol-17β and vehicle respectively. Six weeks after reinjection the fish were sacrificed and ovarian and liver samples removed for histological analysis and oocyte counting. At the same time blood samples were collected for serum oestradiol, testosterone and calcium analyses. Liver weight was used to calculate the Hepato-Somatic-Index (HSI) according to the method outlined below (section 5.2.1). Hepatic calcium content was determined according to the method indicated in section 5.2.2.

5.2.1 Calculation of Hepato-Somatic-Index (HSI).

Liver weight = L

Body weight = Bg

Hepato-Somatic-Index (HSI) = Liver weight $100g^{-1}$ body weight

$$\therefore \text{H.S.I.} = \frac{L}{B} \times 100$$

5.2.2 Evaluation of liver calcium.

Calcium in the liver was extracted (Tew et al, 1981) using the modification of the method of Gochman and Givelber (1970). The materials and method were as follows.

5.2.2.1 Extraction medium.

La_2O_3 (Lanthanum oxide)	=	5.85 g
Concentrated hydrochloric acid	=	25 ml
n-butanol	=	40 ml
Deionised water		

Above components were mixed and made up to 1000 ml with deionised water.

5.2.2.2 Method of calcium extraction.

Each liver was weighed and for each gram of liver weight 2 ml of the above extraction medium was added. The liver was cut into small pieces in order to facilitate the extraction. The mixture was allowed to stand for a week in a tightly-closed container. Following this period the extract was centrifuged at 2500 rpm and the supernatant solution analysed for calcium using a Corning Calcium Analyser - Model 940. The amount of calcium in 100grams of liverweight was calculated according to the method given in section 5.2.2.3.

5.2.2.3 Calculation of liver calcium.

Liver weight = Lg

Volume of extraction medium added per 1g of liver = 2 ml

Volume of extraction medium added per Lg of liver = 2 x L ml

Amount of calcium in 100 ml of the extract)

) = C mg

(Read from the calcium analyser)

∴ Amount of calcium in 2 L ml of extract = $\frac{C}{100}$ x 2 L mg

∴ Amount of calcium in Lg of Liver = $\frac{2LC}{100}$ mg

Amount of calcium in 100 g of liver = $\frac{2LC}{100} \times \frac{100}{L}$

= 2C mg

All the data were subjected to variance ratio test (F-test) followed by a student's t-test.

5.3 Results

Initially, no significant differences in serum calcium, oestradiol and testosterone levels and also body weight were seen between the two fish groups used for oestradiol and vehicle injections. However, 6 weeks after the reinjection with oestradiol-17β, significant differences were observed between the oestradiol-injected fish and vehicle-injected controls. These differences are described below.

5.3.1 Body weight.

No significant change in body weight was observed as a result of oestradiol-17β treatment.

5.3.2 Gonado Somatic Index.

In the oestradiol-injected fish, the mean G.S.I. at 0.936 ± 0.724 was significantly lower than the initial value, 2.46 ± 0.98, and also than the G.S.I. of the vehicle-injected fish (4.54 ± 2.82) (Fig. 5.1). The difference in size of the ovaries of the oestrogen and vehicle-injected fish could easily be seen on gross examination (Plate 5.1).

5.3.3 Mean highest oocyte diameter.

The mean highest diameter of the vehicle-injected control fish, 3117 ± 776 μ was significantly higher than that of the oestradiol-injected fish (1557 ± 1212 μ) and also that of the initially sampled fish (2003 ± 153 μ) ($P<0.025$ and $P<0.01$ respectively) (Fig. 5.1).

5.3.4 Numbers of oocytes at different developmental stages.

5.3.4.1 Previtellogenic oocytes.

Comparison of the oocyte numbers in the different developmental stages of previtellogenesis showed that the oestradiol treatment had produced a number of changes. Stage 1 oocytes were found in very large numbers in the ovaries of oestradiol-injected fish (11538 ± 7398 oocytes $100g^{-1}$ body weight). This number was significantly higher ($P<0.01$) than in the vehicle-injected controls (766 ± 542 oocytes $100g^{-1}$ body weight) and also higher than ($P<0.025$) in the ovaries of the initial samples (2752 ± 1112 $100g^{-1}$ body weight). Furthermore, the number of stage 1 oocytes of the vehicle-injected controls was significantly lower than that of the initial samples ($p<0.01$). In both treated and control groups numbers of stage 2 oocytes (2976 ± 1604 and 3228 ± 905 oocytes $100g^{-1}$ body weight) were significantly lower than those of the initially sampled fish (7118 ± 3271 oocytes $100g^{-1}$ body weight, $P<0.05$). However, no significant difference was observed between the number of stage 2 oocytes in oestradiol-injected and vehicle-injected fish. There were no differences in the numbers of stage 3 and 4 oocytes either between the oestrogen and vehicle-injected groups or the initially-sampled fish and the injected groups (Fig. 5.2, Table 5.2).

5.3.4.2 Vitellogenic oocytes.

In the ovaries sampled initially, stage 5 vitellogenic oocytes were not observed but in the oestradiol-injected fish their numbers increased significantly up to 99 ± 62 oocytes $100g^{-1}$ body weight

($P<0.01$). However the stage 5 oocytes were not found in the vehicle-injected controls, this was a significant difference ($P<0.005$) from the oestradiol-injected group. In the initial ovarian samples 746 ± 249 stage 6 oocytes $100g^{-1}$ body weight were found and this number was significantly reduced in both the vehicle-injected controls ($P<0.025$) and the oestradiol-injected fish ($P<0.005$) although the number of stage 6 oocytes in the controls (327 ± 57 oocytes $100g^{-1}$ body weight) was significantly higher ($P<0.0005$) than that of the oestradiol-injected fish (15 ± 33 oocytes $100g^{-1}$ body weight). Stage 7 oocytes were found in one of the fish from each of the treated groups but their numbers were not significantly different (Fig. 5.2, Table 5.2).

5.3.4.3 Atretic oocytes.

The number of atretic oocytes present in the oestradiol-injected ovaries was 271 ± 62 oocytes $100g^{-1}$ body weight, which was significantly higher ($P<0.0005$) than the 10 ± 12 atretic oocytes $100g^{-1}$ body weight present in the ovaries of the vehicle-injected control fish. No atretic oocytes were found within the ovaries of the initial samples (Fig. 5.2, Table 5.2).

5.3.5 Histology of the ovary.

Histological studies of the ovaries of the initial samples showed that they contained stage 6 vitellogenic oocytes and previtellogenic oocytes up to stage 4 (Plate 5.2). Sections of the oestradiol-injected fish showed a large number of stage 5 and 6 atretic oocytes (Plate 5.3). In one of the oestradiol-injected fish, in addition to large numbers of atretic oocytes, small

numbers of developing stage 5 and 6 oocytes were observed (Plate 5.4). In the ovaries of the vehicle-injected control fish stage 6 and/or 7 oocytes and previtellogenic oocytes up to stage 4 were found (Plates 5.5 and 5.6).

5.3.6 Hepato-Somatic-Index (HSI).

The liver weight was not measured in the fish which were sampled initially. The hepato-somatic-index of the oestradiol-injected fish did not show any significant difference from that of the vehicle-injected controls (Fig. 5.1, Table 5.1). However in all the oestradiol-17β treated group, the liver was much paler in colour.

5.3.7 Liver calcium.

The mean calcium level in the liver of oestradiol-17β-treated fish was 4.648 ± 0.8963 mg $100g^{-1}$ liver weight and this was significantly higher ($P<0.025$) than that of the vehicle-injected controls where levels were only 3.46±0.39 mg $100g^{-1}$ liver weight (Fig. 5.1, Table 5.1).

5.3.8 Serum calcium.

In both the oestradiol-17β and vehicle-treated fish, the serum calcium levels were significantly higher than that of the initially sampled fish ($P < 0.0005$). However, no significant difference in serum calcium level was observed between the oestradiol and vehicle-treated fish (Fig. 5.1, Table 5.1).

5.3.9 Serum oestradiol-17β.

In the initial samples serum oestradiol-17β levels were 1.81 ± 0.77 ng ml^{-1} and subsequently in both oestradiol and vehicle-injected controls they showed significant increases ($P<0.0005$ for both groups). The serum oestradiol-17β level of the oestradiol-injected fish was not significantly different from that of the controls (Fig. 5.1, Table 5.1).

5.3.10 Serum testosterone.

The mean serum testosterone level of the oestradiol-injected fish was significantly lower than that of the vehicle-injected controls and also those of the initial samples ($P<0.05$ and $P<0.0005$ respectively). In the oestradiol-treated group the mean serum testosterone level was 1.86 ± 1.76 mg ml^{-1} whilst in the vehicle-injected controls and initial it was 75.76 ± 83.81 mg ml^{-1} and 25.46 ± 0.98 mg ml^{-1} (Fig. 5.1, Table 5.1).

Plate 5.1 - Superficial view of the ovaries of oestradiol-17β-injected fish and vehicle injected controls showing the difference in their size.

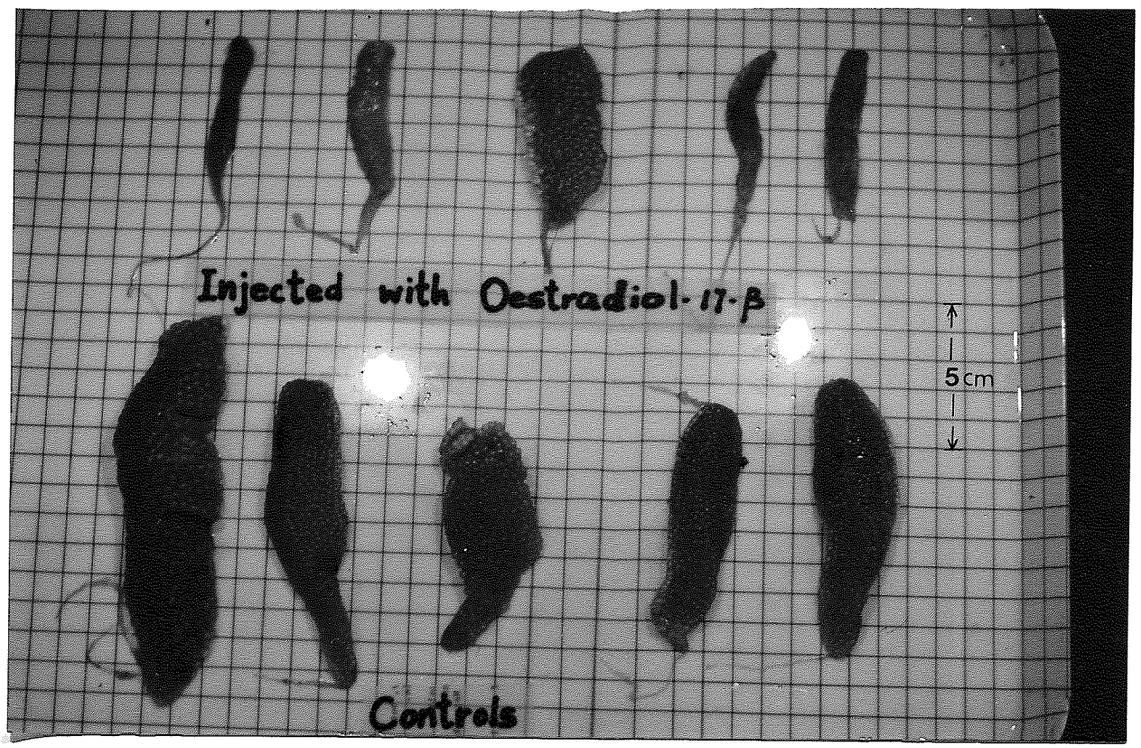

Plate 5.1

plate 5.2 - Section of an ovary of a 2+ year-old rainbow trout prior to oestradiol-17β injection showing oocytes at early stage 6. Stained with haematoxylin and eosin. Magnification x 50

2, 6 - stage 2 and 6 oocytes.

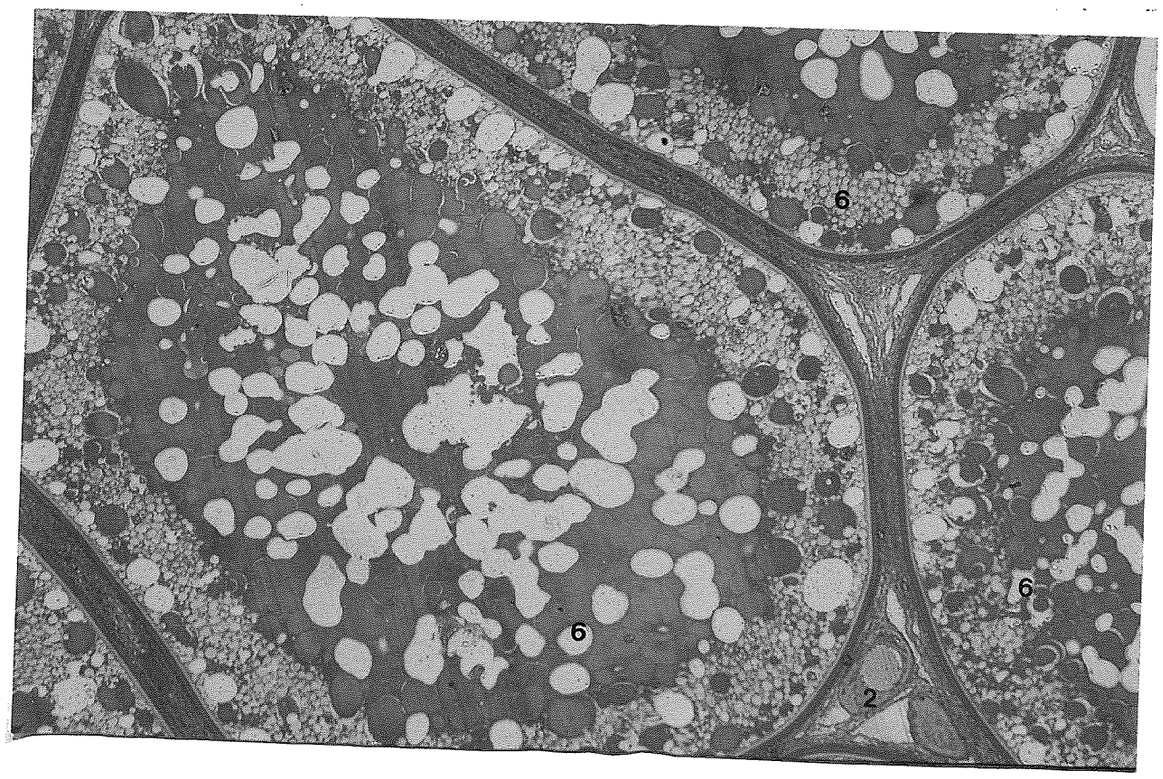

Plate 5.2

Plate 5.3 - Section of an ovary of a 2+ year-old fish injected with oestradiol-17β during active vitellogenesis showing stage 4, 5 and 6 atretic oocytes.
Magnification x 80

4, 5, 6 - atretic stage; 5 and 6 oocytes, St_{2a} - stage 2a intact oocytes.

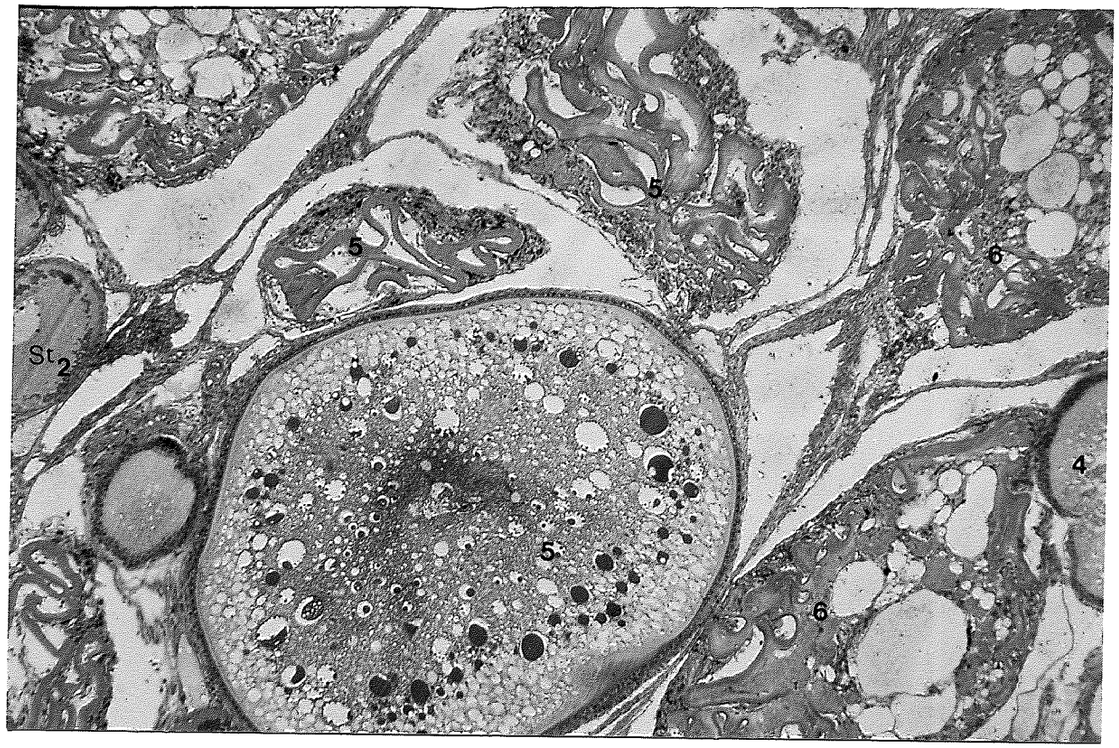

Plate 5.3

Plate 5.4 - Section of an ovary of a 2+ year-old fish injected with oestradiol-17β during active vitellogenesis showing stage 4 and 6 atretic oocytes among stage 5 and 6 intact oocytes. Stained with haematoxylin and eosin.
Magnification x 50

St_2, St_5, St_6 - stage 5 and 6 intact oocytes, 4, 6 - atretic stage 4 and 6 oocytes.



Plate 5.4

Plate 5.5 - Superficial view of an ovary of a 2+ year-old fish injected with oestradiol-17β during active vitellogenesi showing atresia among intact oocytes.
Magnification x 20

At - atretic oocytes, 2, 3, 4, 5 - stage 2, 3, 4 and 5 intact oocytes.

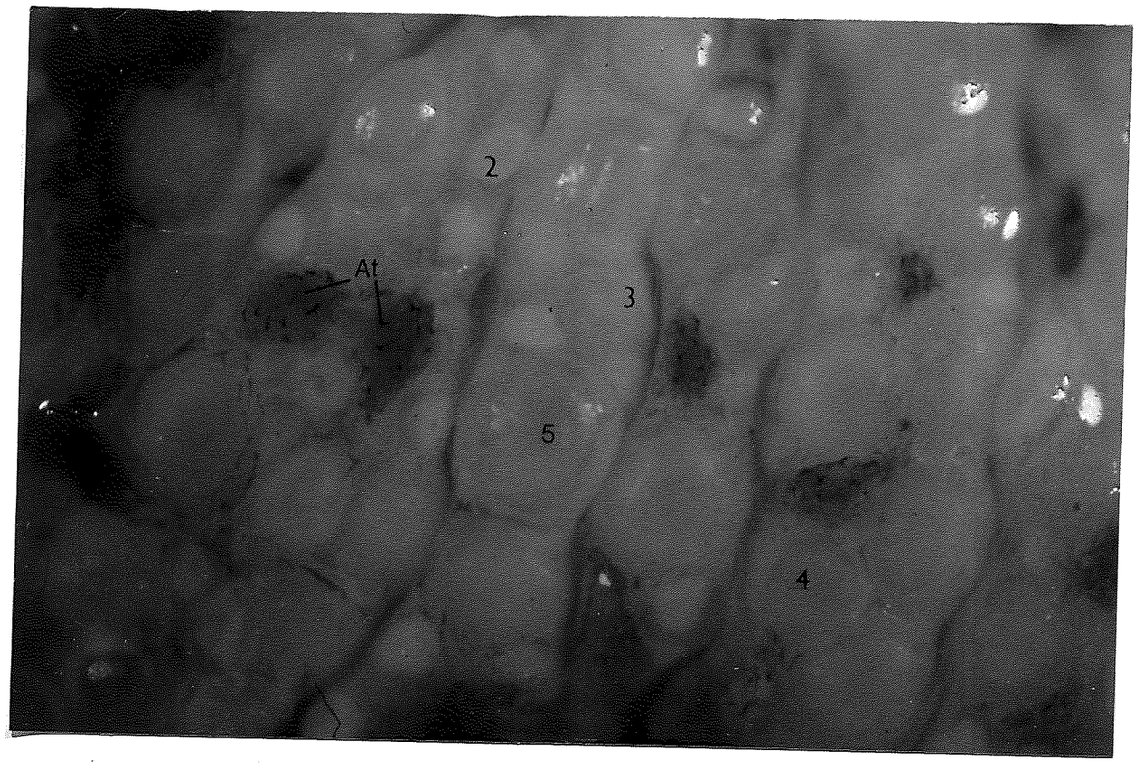

Plate 5.5

Plate 5.6 - Section of an ovary of a 2+ year-old fish injected with Vehicle (at the same time when others were treated with oestradiol-17β) showing stage 6 oocytes.

Magnification x 800

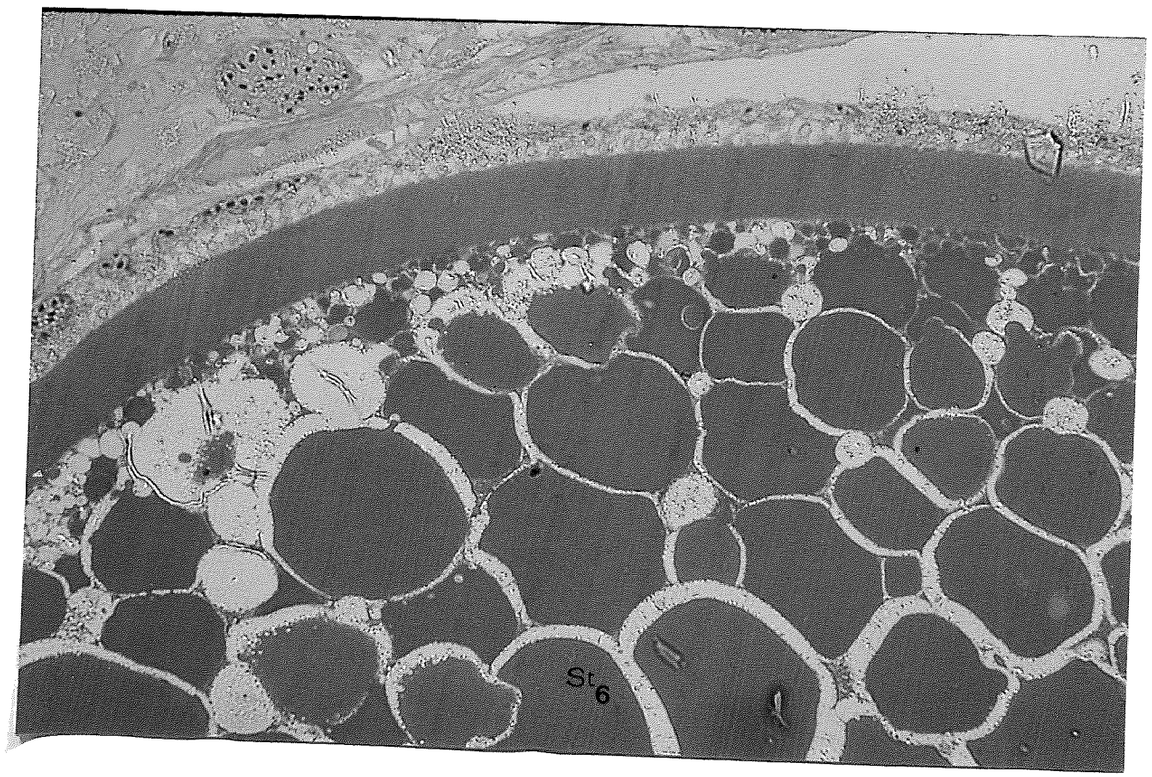

Plate 5.6

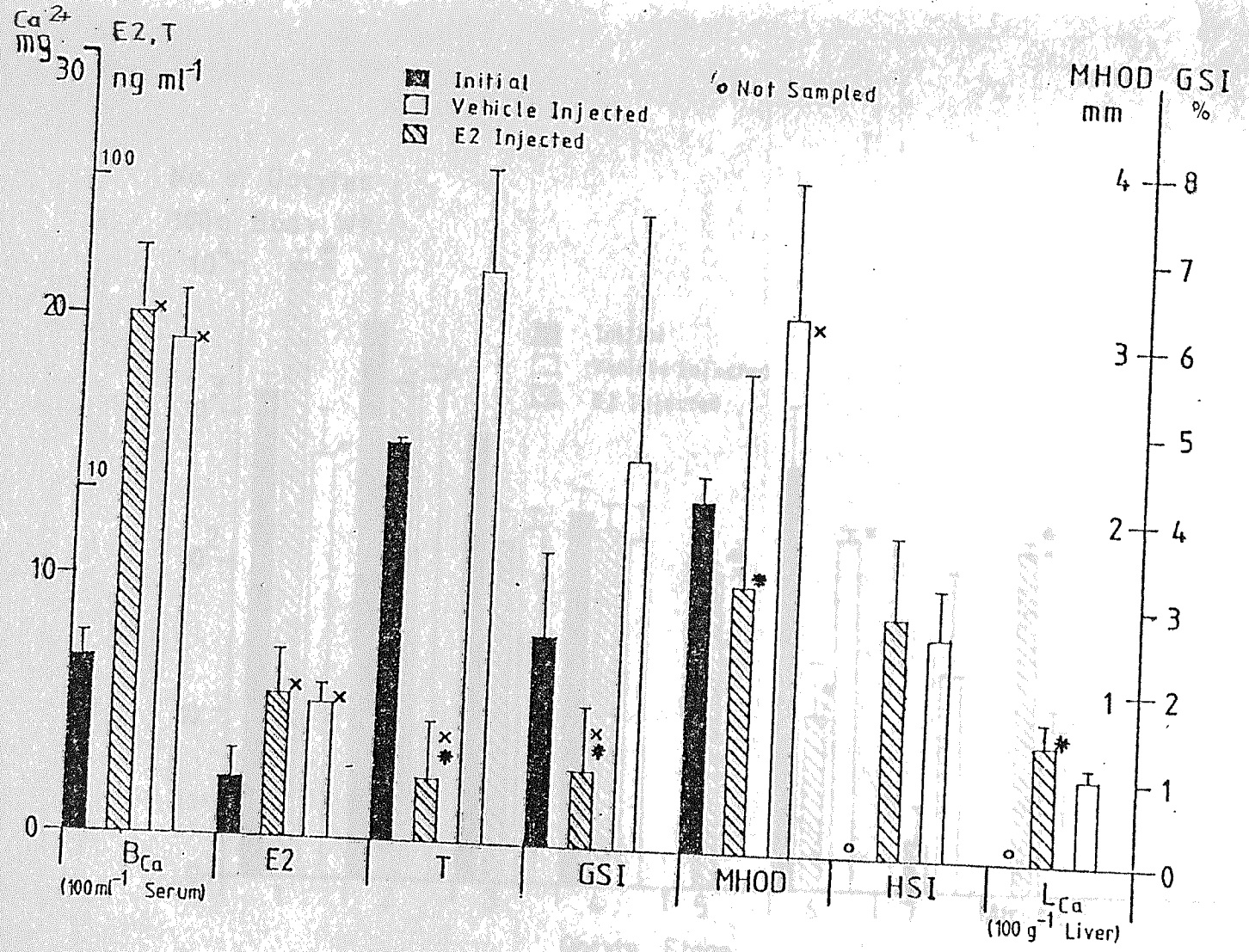

Fig 5.1 - Effects of oestradiol-17β injection on the gonadosomatic index (G.S.I.), mean highest oocte diameter (MHOD), hepatosomatic index (H.S.I.), Liver calcium (L_{Ca}) and circulating levels of oestradiol-17β (E2), testosterone (T) and calcium (B_{Ca}) of 2+ year-old fish with vitellogenic ovaries. Each value indicates the mean ± standard deviation (S.D.). (* - significant difference from the control, x - significant difference from the initial).

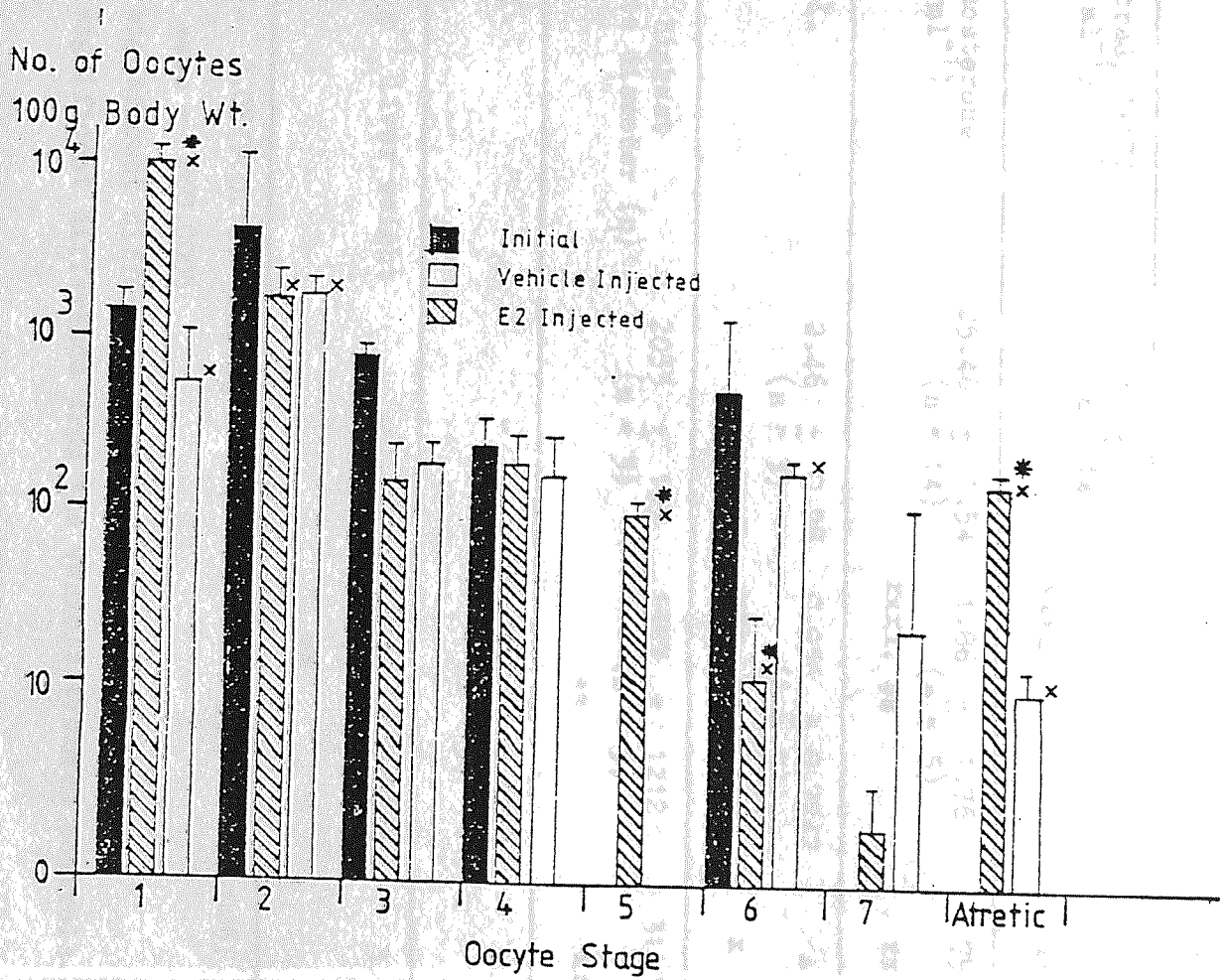

Fig 5.2 - Effects of oestradiol-17β injection on the numbers of oocytes at different developmental stages (1-7 and atretic) in the ovaries of 2+ year-old fish with vitellogenic ocaries. Each value indicate the mean number of oocytes $100g^{-1}$ body weight ± standard deviation (S.D.). (* - significant difference from the control, x - significant difference from the initial).

	Initial	Oestradiol-17β treatment	Vehicle injected control
Serum Ca^{2+} ($mg\ 100ml^{-1}$)	6.90 ± 0.90 (n = 14)	21.23 ± 1.6514 (n = 5) xxxx, *	19.158 ± 2.1739 (n = 5) xxxx
Oestradiol-17β ($ng\ ml^{-1}$)	1.81 ± 0.77 (n = 14)	4.27 ± 1.25 (n = 5) xxxx	4.06 ± 0.484 (n = 5) xxxx
Testosterone ($ng\ ml^{-1}$)	25.46 ± 1.54 (n = 14)	1.86 ± 1.76 (n = 5) xxxx, **	75.76 ± 83.81 (n = 5) xx
G.S.I.	2.46 ± 0.98 (n = 3)	0.936 ± 0.7242 (n = 5) xxx, **	4.54 ± 2.82 (n = 6) x
Mean highest oocyte diameter (μ)	2033 ± 153 (n = 3)	1557 ± 1212 (n = 5) **	3117 ± 776 (n = 6) xx
H.S.I.	-	2.828 ± 0.9215 (n = 5)	2.5992 ± 0.5650 (n = 6)
$Ca^{2+}\ g^{-1}$ liver weight	-	4.648 ± 0.8963 (n = 5) **	3.46 ± 0.3905

x, * - $0.05<P<0.10$; xx, ** - $P<0.05$; xxx, *** - $P<0.005$; xxxx, **** - $P<0.0005$

x - significant difference from the initial fish

* - significant difference from the vehicle treated controls.

Table 5.1 - Effects of oestradiol-17β treatment on the gonad, liver and serum calcium, oestradiol-17β and testosterone, in the rainbow trout. Each value indicates the mean ± standard deviation (S.D.). G.S.I. - gonadosomatic index, H.S.I. - hepatosomatic index, n - number of fish.

No. of oocytes $100g^{-1}$ body weight

Oocyte Stage	Initial	Oestradiol treatment	Control
1	2752 ± 1112	11538 ± 7398 xx **	766 ± 542 xx
2	7118 ± 3271	2976 ± 1604 xx	3228 ± 905 xx
3	971 ± 683	274 ± 179 x	344 ± 118 x
4	449 ± 144	356 ± 150	287 ± 205
5	00	99 ± 62 xx ***	00
6	746 ± 249	15 ± 33 xxx ****	327 ± 57 xx
7	00	3 ± 7	43 ± 104
Atretic	00	271 ± 62 xxx ****	10 ± 12 xx
	n = 3	n = 5	n = 6

x,* - $0.05<P<0.10$; xx,** - $P<0.05$; xxx,*** - $P<0.005$;
xxxx,**** - $P<0.0005$.

x - significant difference from the inital fish

* - significant difference from the vehicle-injected controls.

Table 5.2 - Effects of oestradiol-17β treatment on the proportions of oocytes at different developmental stages in rainbow trout. Each value indicates the mean ± standard deviation (S.D.) n - number of fish

5.4 Discussion

Ovarian regression or atresia was clearly evident in mature rainbow trout treated with oestradiol-17β during the phase of active vitellogenesis. The primary purpose of injecting oestradiol-17β at a time when circulating levels are naturally high and when maximum vitellogenin incorporation is occuring, was to ascertain whether the rate of vitellogenin uptake could be further enhanced to favour the growth and development of oocytes. The presence of large numbers of atretic oocytes within the ovaries of oestradiol-injected fish, when compared with the vehicle-injected controls, showed that oestradiol has inhibitory effects on the growth of vitellogenic oocytes if administered during active vitellogenesis. This supports the suggestion made by Dodd (1960) that oestrogens exert inhibitory influences on the ovarian development of fish.

It has been suggested that oestradiol administration has different effects during different phases of ovarian development (Bullough, 1942; Egami, 1954; Pantic and Lovren, 1978; Khoo, 1979). Oogonial proliferation was said to be stimulated by oestrogen treatment in the minnow (Bullough, 1942), medaka (Egami, 1954), carp (Pantic and Lovren, 1978), goldfish (Khoo, 1979). It was not possible to confirm these findings in the present work because no counts were made on the numbers of oogonia. However oogonia were present in both oestradiol and vehicle injected fish. There were also significantly increased numbers of stage 1 primary oocytes after oestradiol injection. Similar results were also reported by Pantic and Lovren (1978). In contrast Bullough (1942) observed an inhibition in the formation of primary oocytes

in oestrogen-treated minnow. During the present investigation there were no changes in the proportions or growth of stage 2, 3 and 4 oocytes. Therefore, it would appear that oestradiol does not inhibit oogonial proliferation or oogenesis. On the contrary, the presence of significantly increased proportions of stage 1 oocytes after oestradiol treatment, suggest that oogenesis, ie. the recruitment of oogonia into primary oocyte stage 1 may have been stimulated.

The most significant effect of the injection of oestradiol was on the growth and development of vitellogenic oocytes. In the ovaries of oestradiol-17β-injected fish, the numbers of stage 5 oocytes, ie. the initial stage of vitellogenin uptake, were significantly increased, compared with the vehicle-injected fish or with the ovaries sampled before oestradiol-treatment, where no stage 5 oocytes were present. This suggested that the initiation of vitellogenin incorporation may have been enhanced by the oestradiol-treatment. This conflicts with the results of Khoo (1979), who reported that oestradiol had no effects on vitellogenin incorporation and the numbers of vitellogenic oocytes in the goldfish. Khoo (1979) showed that the treatment with oestradiol stimulated vesicle formation (endogenous vitellogenesis). In the present experiment the proportion of stage 4 oocytes was not changed by oestradiol, indicating that the recruitment of stage 3 previtellogenic oocytes into stage 4 and the stage 4 oocytes into stage 5 exogenous vitellogenic oocytes, were not affected by the treatment. Pantic and Lovren (1978) observed that the adminsitration of oestrogen to carp, induced both ooplasmic vesicle formation (endogenous vitellogenesis) and yolk granule formation

(exogenous vitellogenesis), although no indication was given of the effects of this hormone on the subsequent maturation of the oocyte. In the present study oestradiol-17β exerted significant inhibitory effects on oocyte maturation and in the ovaries of these fish there were fewer stage 6 oocytes and significant increases in the numbers of vitellogenic oocytes undergoing atresia.

There were also reductions in the mean diameter of the highest stage of oocytes present within the ovary of the oestradiol-injected fish. This suggests that oestrogen administration during the exogenous vitellogenic phase of the oocytes may inhibit the development of stage 6 (germinal vesicle migration stage) oocytes and most probably the recruitment of stage 5 oocytes into stage 6. This suggestion is supported by the presence of significantly larger proportions of atretic vitellogenic oocytes in the ovaries of oestradiol-treated fish. Similar observations were reported by Funk et al (1972) after a series of injections with oestradiol and salmon gonadotropin in the pink salmon. In contrast, Ho and Vanstone (1961), Plack et al (1971), Campbell and Idler (1976) and Medda et al (1980), were unable to show any effects of oestrogens on the ovaries. Similar negative effects with oestrogen were also reported by Olivereau and Olivereau (1979) on vitellogenin incorporation in the eel. The present results suggest that oestradiol-treatment may inhibit the recruitment of stage 5 (peripheral yolk granule stage) to stage 6 (germinal vesicle migration stage) and the growth of the oocytes over the later stages of maturation.

The inhibitory effects of oestradiol on the vitellogenic ovary is further supported by the significant reduction in gonadosomatic

index of the oestradiol-treated fish. This reduction contrasts with the observations of Olivereau and Olivereau (1979) who reported an increase in gonadosomatic index in oestradiol treated eels suggesting that this was due to a reduction of body weight as a result of the treatment. However, in the present study there were no differences in body weight between the oestradiol and vehicle-injected fish, and it is concluded that the reductions in gonadosomatic index was due to the significant reductions in oocyte size. However, gonadosomatic index is not a sensitive index of gonadal activity because differences in gross weight of the gonad may conceal a variety of developmental changes (de Vlaming et al, 1984). Consequently it is important that measurements of gonadosomatic index are carried out together with the measurements and counts of the proportions of the different oocyte stages.

Besides its possible effects on oocyte maturation oestradiol-17β has an important role in the synthesis of hepatically-produced vitellogenic proteins (vitellogenins) (see review by Ng and Idler, 1983). However, 6 weeks after reinjection with oestradiol-17β there was no significant change in the hepatosomatic-index of mature rainbow trout. The absence of any gross change in the liver is surprising for there have been reports of hepatic hypertrophy, increases in lipid content and decreases in liver glycogen in other fish treated with oestrogen (see review by Ng and Idler, 1983). Ultrastructural studies have also revealed enhanced activity of the protein synthetic organelles, ie. endoplasmic reticulum, Golgi complex and mitochondria and also increases in RNA (Emmersen and Emmersen, 1976; Emmersen et al, 1979; Medda et al, 1980; Emmersen and Korsgaard, 1982; Korsgaard et al, 1983) within the hepatic

cells after oestrogen-treatment. Although these studies show that the liver synthesises vitellogenin and its precursor proteins as a result of oestradiol stimulation, no information is available regarding the time at which the calcium is incorporated into the vitellogenin molecule. The present study revealed the significantly higher levels of liver calcium in oestradiol-17β treated fish when compared with the controls. This suggests that the calcium is incorporated before vitellogenin is released into the circulation. The increase in calcium levels in the liver are of interest because previous studies have failed to detect increases in vitellogenin in the liver despite many-fold increases in its serum levels following oestradiol administration (Van Boheman et al, 1982a, Elliott et al, 1979 and 1980).

In the normal reproductive cycle of the rainbow trout peak levels of vitellogenin are known to be preceded by raised levels of oestrogens (Whitehead et al, 1978a; Scott and Sumpter, 1983; Sumpter et al, 1984). The levels of vitellogenin and also calcium and phosphoprotein phosphorus have also been shown to be highly correlated with the levels of circulating oestradiol-17β (Whitehead et al, 1983; Elliott et al, 1984). Similar conclusions were reached as a result of monthly sampling of Caribou, Grampian and Whitebrook strains of rainbow trout (Chapter 4). In the present experiment normal oestradiol levels would have been masked by the injections of this hormone and hence the measurements of this hormone may have been misleading. However, there were significant reductions in the levels of serum testosterone in the oestradiol-17β-treated fish. Under normal conditions most of the circulating oestradiol is derived from testosterone by aromatisation within the

theca (see review by Fostier et al, 1983). The production of oestradiol within the follicle is explained by a "two cell type model" (Nagahama et al, 1982; Kagawa et al, 1982b; Young et al, 1982), in which testosterone is said to be produced within the thecal cells and transferred into the granulosa, where it is aromatised to oestradiol-17β.

During the natural reproductive cycle of salmonids and possibly other teleosts testosterone levels generally occur parallel to those of vitellogenin with the highest levels occurring at the time of oocyte maturation and ovulation (Kagawa et al, 1983; Elliott et al, 1984; Scott et al, 1984; Sumpter et al, 1984; see Chapter 4). In addition to its utilization in the synthesis of oestrogen (Kagawa et al, 1982b) several other functions have been attributed to the high concentrations of testosterone found in mature females. These include effects on oocyte maturation (Kagawa et al, 1983), positive feed-back effects on pituitary gonadotropin secretion (Crim et al, 1981; Young et al, 1982a; Kagawa et al, 1983) and in the degeneration of ovarian ova (Yamazaki, 1972; Elliott et al, 1984). However, neither Yamazaki (1972) nor Elliott et al (1984) provided any histological evidence to confirm a role for testosterone in the development of atresia. In contrast, during the present study low testosterone levels were paralleled by high proportions of atresia. It has been suggested that the increased capacity of the mature follicle to produce testosterone and not oestradiol may be related to decreased aromatase activity (Young et al, 1982b). In isolated thecal cells testosterone production has been increased by salmon gonadotropin (SG-G100)

(Kagawa et al, 1983), although the aromatisation of testosterone to oestradiol-17β has not been induced by this hormone. Thus it has been suggested that the substrate required for this aromatisation is only provided within the granulosa (Kagawa et al, 1982b). The high plasma testosterone levels experienced during the periods of final maturation and ovulation in the amago salmon are also thought to be caused by the rapid elevations in plasma levels of gonadotropin which occur at this time (Young et al, 1982b), The current uncertainty regarding the number of existing gonadotropins makes it difficult to fully understand roles for gonadotropin both in the production of testosterone within the theca and the aromatisation of testosterone to oestradiol-17β. However, the presence of low testosterone levels in oestradiol-17β treated rainbow trout suggest an inhibitory effect of oestradiol on the production of testosterone possibly through a negative feedback on the secretion of pituitary gonadotropin(s). During the final stages of ovarian development there is an inverse correlation between the levels of gonadotropin and oestradiol and the rapid increase in gonadotropin at this time is thought to be due to diminished oestradiol feed-back (Fostier et al, 1978; Whitehead et al, 1978a, 1983). Unfortunately, the present work did not include measurement of the levels of serum gonadotropin and consequently it is not possible to confirm this relationship.

The presence of large numbers of atretic stage 6 vitellogenic oocytes in the oestradiol-17β-treated fish, in parallel with the decreased levels of serum testosterone provide some support for a role for testosterone in the development of stage 6 vitellogenic oocytes. An oocyte-maturational function for testosterone has been suggested by Kagawa et al, 1983. Moreover, recent studies on the

steroid metabolism in the brain have revealed that aromatisable androgens, particularly testosterone, may elevate pituitary gonadotropin levels in sexually immature salmon (Crim and Peter, 1978; Crim and Evans, 1979; Crim et al, 1981) and juvenile rainbow trout (Hurk et al, 1984). Whether these effects are also important in sexually-mature fish remains to be clarified. Kagawa et al (1983) reported that, in preovulatory follicles, gonadotropin stimulates those enzymes in the thecal cells which catalyses the conversion of 17α hydroxy-progesterone to testosterone whereas, in the post-ovulatory follicles, this activity is decreased resulting in high levels of 17α hydroxy-progesterone. Progestagens added to aquarium water have been shown to exert a stimulatory effect on the levels of gonadotropin in the piuitary in juvenile rainbow trout (Hurk et al, 1984). Progestagens are also said to induce germinal vesicle breakdown (see review by Goetz, 1983) with the most effective progestagen being 17α 20β dihydroxy-4-pregnen-3-one (Fostier et al, 1973; Jalabert et al, 1976; Goetz and Theofan, 1979; Duffey and Goetz, 1980; Nagahama, 1983; Jalabert and Fostier, 1984).

The output of 17α 20β dihydroprogesterone (17α 20β OH-P) by the follicle is said to be partially inhibited by a reduction in the follicular sensitivity to gonadotropin (Jalabert and Fostier, 1984), and this in turn affects the rate of maturation of the oocyte (Jalabert, 1976). Furthermore, the sensitivity of the follicle to gonadotropin is said to be negatively correlated with plasma oestradiol-17β levels (Jalabert and Fostier, 1984). The migration of the germinal vesicle is associated with an increase in oocyte sensitivity (Jalabert, 1976; Jalabert and Fostier, 1984).

Furthermore, during maturation the sensitivity of the oocyte to 17α 20β OH-P is significantly correlated with the sensitivity of the follicle to salmon gonadotropin. In contrast there is no correlation between the follicular sensitivity to 17α 20β OH-P and to oestradiol-17β (Jalabert and Fostier, 1984). Oestradiol-17β is said to have no effect on 17α 20β OH-P-induced maturation in trout (Jalabert, 1976), although it has been suggested that oestradiol-17β may inhibit gonadotropin-induced maturation through inhibition of 17α 20β OH-P synthetic ability (Jalabert and Fostier, 1984). This could be the reason for the presence of significantly increased proportions of atretic stage 5, 6 and 7 oocytes within the ovaries of oestradiol-17β-treated rainbow trout in the present study.

17α 20β OH-P has only been found in the granulosa layer of amago salmon (Nagahama, 1983). However, Fostier et al (1981) induced the production of 17α 20β OH-P from clusters of rainbow trout follicles in an in vitro study. Pregnenolone is said to be the precursor of progesterone in amphibians (Spiegel et al, 1978), although in vitro studies of rainbow trout follicles suggest that pregnenolone may act as a precursor for oestrogen synthesis during early vitellogenesis (Sire and Dépêche, 1981). Later in development aromatisation of pregnenolone may result in the formation of 17α 20β OH-P. The mechanism behind this sequence of changes is however unclear. Khoo (1979) observed that pregnenolone but not oestradiol induced exogenous vitellogenesis in the goldfish. It has been suggested that, in amphibians the conversion of pregnenolone to progesterone is inhibited by oestrogens (Spiegel et al, 1978). This could be the reason for the inhibition of maturation in the oestradiol-treated fish in the present study.

Also the low levels of testosterone present in these fish could be attributed to this inhibitory effect of oestrogen on the conversion of pregnenolone to progesterone as the latter is said to be responsible for the formation of testosterone within the theca of developing pre-ovulatory follicles (Kagawa et al, 1983). Although the role of testosterone on oocyte development is controversial Kagawa et al (1983) suggested that it may affect oocyte maturation. The increased proportion of stage 6 atretic oocytes, along with the reduction in serum testosterone levels observed in oestradiol-17β-treated rainbow trout during the present investigation supports Kagawa et al's (1983) proposition. However, to understand the mechanisms behind the steroidogenic control of gonadal development and the involvement of pituitary gonadotropin in these events clearly requires further work.

Pituitary gonadotropins are known to stimulate vitellogenin uptake by stimulation of pinocytotic activity in the oolemma (Abraham et al, 1982). The majority of authors suggest that there is only one gonadotropic hormone and that this is able to induce both vitellogenic uptake and oocyte maturation (see review by Idler and Ng, 1983). However, some workers suggest that there are two separate gonadotropins: one a carbohydrate-rich "maturational" gonadotropin and the other a carbohydrate-poor "vitellogenic" hormone (Campbell and Idler, 1976; Ng and Idler, 1978a,b, 1979). Ng and Idler (1979) proposed that vitellogenic gonadotropin induces vitellogenin synthesis and its uptake by the oocytes, whereas the maturational form involves primarily in the control of final maturation and ovulation and also in vitellogenesis. Possibly, the apparent vitellogenic and maturational functions of gonadotropic principles are due to the presence of multiple forms of the hormone

which under normal conditions are not physiologically active. Stuert-Kreger et al (1981) has described such a phenomenon in salmon parr. During the present study, although stage 5, 6 and 7 maturing oocytes were subject to atresia, there was a significant increase in the numbers of stage 5 oocytes in the ovaries of the oestradiol-17β injected fish. This shows clearly that oestradiol is not affecting gonadotropin-induced uptake of vitellogenin and oestradiol-17β acts differently on the growth of the oocyte during the different phases of exogenous vitellogenesis. Oestrogens may induce the recruitment of oocytes from endogenous (stage 4) into exogenous vitellogenesis (stage 5). Alternatively, it may inhibit either the recruitment of stage 5 oocytes into germinal vesicle migration stage (stage 6) and/or the growth of the stage 6 oocytes. These effects of oestradiol-17β on oocyte development suggests that, it may have an inhibitory action on the physiological conditions required for the growth of oocytes at germinal vesicle migration stage. This may have occurred through feedback control of gonadotropin secretion which in turn would have affected the secretion of the steroids involved in the promotion of oocyte development and maturation.

In summary the present results show that oestradiol-treatment administered during active vitellogenesis produced significant increases in the levels of atresia and reduced the numbers of oocytes undergoing the final stages of maturation, ie. germinal vesicle migration and breakdown. These effects may have occurred as results of the inhibition of aromatase activity and/or the production of testosterone by the oocyte follicle, which in turn would have reduced the levels of circulating testosterone.

CHAPTER 6

Effects of two different ration levels and starvation on oocyte development in the rainbow trout.

6.1 Introduction

Food availability is known to be of prime importance in the determination of the reproductive potential of fish (Woodhead, 1960; Nicholskii, 1969; Bagenal, 1973). Supportive evidence for this hypothesis is provided by investigations carried out on Salmonids and also on other teleosts both in the wild (Fry, 1949; Vladykov, 1956; McFadden et al., 1965; Martin, 1970; de Vlaming, 1971; Kuznetsov and Khalitov, 1978) and in captivity (Scott, 1962; Bagenal, 1969; Wootton, 1973, 1977; Mirnova, 1977; Baiz, 1978; Hislop et al, 1978; Townshend and Wootton, 1984; Springate et al, 1985). However, much of the evidence , from the earlier studies is circumstantial, mainly because it is dervied from records of wild stocks of fish where dietary influences are difficult to quantify and to separate from the other environmental variables. Therefore, to avoid such inherent difficulties of associating cause and effect in the determination of fecundity and egg composition amongst wild stocks of fish, and also to establish a proper relationship between the nutrition and reproduction, it is necessary to carry out carefully controlled laboratory investigations. Despite the enormous literature in fecundity, surprisingly few laboratory studies have been conducted (Scott, 1962; Bagenal, 1969; Kato, 1975; Baiz, 1978; Hislop, 1978; Ridelman, 1984; Towshend and Wootoon, 1984) and further more they include equivocal results.

Reduced supplies of food are said to delay spawning (Scott, 1962; McFadden et al, 1965; Mackay and Mann, 1969; Mirnova, 1977) decrease fecundity (Scott, 1962; McFadden et al, 1965, Mackay and Mann, 1969; Baiz, 1978; Kuznetsov and Khalitov, 1978; Townshend and

Wootton, 1984) and reduce egg diameters (Townshend and Wootton, 1984; Springate et al, 1985). McFadden et al, (1965) reported that brown trout from oligotrophic streams reached sexual maturity later and also their fecundities were lower than similar sized fish from more fertile streams. Better feeding conditions were also said to advance the age of first spawning and increase fecundity in brook trout (Vladykov, 1956) although this finding was not substantiated in a similar study of the same species and area by Gibson et al, (1976). Scott (1962) in a three year investigation of the rainbow trout showed that the proportion of fish which became mature was lower in fish fed on approximately half ration; these fish also produced fewer eggs although the eggs were unchanged in size. The findings of this paper are difficult to interpret because only 5% of all the experimental fish attained maturity at the end of the experiment. Studies on prespawned brown trout has shown that diet restrictions delayed maturation, reduced total fecundity and altered the wet and dry weight of the eggs (Bagenal, 1969), although again interpretation is difficult because the measurements were made on the pre-ovulated oocytes and at this part in the cycle the ovary at different stages of development (See results in Chapter 4). In several other studies no change in egg size has been reported as a result of reductions in food supplies (Scott, 1962; Baiz, 1978; Kuznetsov and Khalitov, 1978). However, as in the study of Bagenal (1969) some of these findings (Baiz, 1978; Kuznetsov and Khalitov, 1978) are compromised because measurement of ova diameter were made on unovulated ova which may have belonged to different stages of oocyte development. Although Scott (1962) measured ovulated ova, closer examination of the final weights of fish fed on highest rations of food and the

number of fish spawned at the end of the experiment would suggest that all the experimental and control groups were being subjected to restricted diets. Hislop (1978) suggested that the egg production and feed levels are positively correlated in laboratory maintained haddock. More recent studies on convict cichlid, which is a multiple spawner, suggested that ration size is positively correlated with fecundity and egg size but negatively correlated with the length of time between spawnings. However, Katoh (1975) and Ridelman et al (1984) working with rainbow trout, reported that starvation for 4 months and 40 days before spawning respectively produced no reductions in either fecundity or egg size. Although the majority of studies in this area indicate that there is a significant relationship between ration size and the fecundity, time of spawning and egg size of the broodstocks, the mechanism(s) by which these effects are achieved are largely unexplained.

In a few studies the reductions in oocyte numbers, which are said to have been produced by reduced food supplies, are suggested to have occurred as a result of the atresia of ovarian ova (Vladykov, 1956) and/or to a modification in the recruitment of oocytes into vitellogenesis (Robb, 1982), although in none of these studies was sufficient histological evidence provided to support this contention. Townshend and Wootton (1984) also suggested that the low ration levels affect the rate of recruitment of vitellogenic oocytes and the rate of atresia in convict cichlid, although the relative importance of these two factors in determining the fecundity and number of spawnings at reduced rations was not evaluated.

According to the observations made in chapter 4 on ovarian development of Caribou, Grampian and Whitebrook strains of rainbow

trout, it is clear that atresia is of regular occurrence during the normal oocyte developmental cycle. Counts of the oocytes in the monthly ovarian sampling of the above strains of rainbow trout, revealed that the total number of oocytes present in a previtellogenic ovary could reach a maximum of approximately 15,000 100 g^{-1} body weight but that this number is reduced to 1000-3000 oocytes 100 g^{-1} body weight in the vitellogenic ovary during the final stage of maturation. This may have been due to the atresia which occurred in the vitellogenic oocytes and also possibly in the previtellogenic oocytes. Previtellogenic oocytes are thought to disappear without any trace unlike the larger vitellogenic oocytes (Byskov, 1978). It is likely therefore, that reduction in oocyte number can occur even if fish are fed to satiation, and furthermore it is possible that such reductions can involve oocytes of a wide range of size and stage. This proposition does not agree with the findings of Henderson (1963b) who suggested that the levels of atresia are too low to significantly affect the number of eggs in healthy well-fed populations. In contrast Vladykov (1956) reported reductions of 80% and 45% in the number of oocytes reaching 1mm and 3mm in diameter respectively, in the brook trout and suggested that this was due to atresia (Vladykov, 1956), although evidence for this proposition was not provided. Similar findings have also been provided by de Vlaming (1971) working with gobiid fish.

In review of the possible importance of atresia in determining oocyte numbers, the present chapter will focus on the numbers of atretic follicles which are observed after starvation of both previtellogenic and vitellogenic fish. A further investigation will also be made of the effects of reduced rations rather than starvation

in this process. A few studies have reported a reduction in the recruitment of oocytes to higher developmental stages as a result of feeding on low rations (Hester, 1964; Tyler and Dunn, 1976; Townshend and Wootton, 1984). In contrast de Vlaming (1971) showed that starvation did not reduce recruitment although it appeared to increase the levels of atresia. According to the observation made in chapter 4, it is clear that there are reductions in oocyte numbers, possibly during recruitment at each development stage, during the normal ovarian development of well-fed trout populations. Clearly, the question of recruitment needs further investigations and thus in the present chapter a study will be made of the effect of feeding regimes on oocyte recruitment rates at both previtellogenic and vitellogenic levels.

In the majority of studies which have been so far conducted on the effects of feeding different rations and diet restrictions on oocyte development, measurements have been made of the underlying changes in hormonal and vitellogenin levels which may influence or be influenced by modifications in oocyte development. Springate et al, (1985) observed significantly lower serum levels of vitellogenin and testosterone in the rainbow trout and it was suggested that the alterations might be related to the reductions in size and number of eggs produced, although no evidence was provided for a causative link.

From the review of early studies on the effect of altered ration on oocyte development it is clear that there are many unresolved questions relating to the mechanisms by which these dietary alterations are connected to ovarian development. Therefore the primary aims of the present study are to investigate the effects of altered rations and starvation on the numbers of oocytes, their

recruitment to the higher developmental stages and the proportion of these stages, atresia and also on the hormonal changes which accompany their development. This will be achieved by histological examination, counting oocytes at different developmental stages and parallel serum sampling for analysis of testosterone, oestradiol-17β and calcium as an index for vitellogenin.

This work is also of considerable commercial significance because fish farmers currently use a bewildering range of feeding regimes with little understanding or consideration of their effects on reproduction. This means that there are possible losses in production from hatcheries as a result of the use of inappropriate feeding regimes for broodstocks during important phases of gonadal development. Feeding strategies which enable the optimal numbers and sizes of eggs to be obtained from the available broodstock and water supplies may offer considerable opportunities for significant improvements to be made in the efficiency of commercial hatcheries.

6.2 Materials and methods.

6.2.1 Effects of starvation on the previtellogenic ovaries of immature rainbow trout.

The aim of this study was to investigate the effects of starvation on the growth and development of previtellogenic oocytes and also of the levels of atresia.

Thirty-eight female rainbow trout were selected at random from 1+ year old stock held in the Fish Culture Unit and allowed to acclimate for one week in two circular tanks of a recirculating system (Appendix 1). Following acclimation, all the fish were marked with numbered tags, weighed and blood samples taken for later analyses of serum oestradiol-17β, testosterone (Section 2.4) and calcium (Section 2.5). At the beginning of the experiment 6 fish were sacrificed to obtain ovarian samples for histological examination (Section 2.2). Unlike the other studies described herein, the ovaries were not preserved in Gilson's fluid and all the oocyte measurements and counting were made on histological sections obtained from JB-4 plastic embedded ovarian samples.

The remaining fish were randomly separated into two groups of 16 and returned into the two experimental tanks. Fish in one of the tanks were fed daily with a full ration of food as recommended in the feed manufacturer's Tables (0.7% of the body weight) and those in the other tank were starved. The experiment lasted for a period of six weeks. Every 2 weeks at least 5 fish from the starved group were blood sampled and then sacrificed in order to provide ovarian samples. At the same time all the fish fed on full ration (controls) and the remaining starved fish were weighed. At the end of the sixth week the remaining starved fish and 7 of the control fish were blood

sampled for analyses of serum oestradiol-17β and testosterone and calcium as an index of vitellogenin (Sections 2.4 and 2.5). Fish were then sacrificed and samples of ovary taken for histological analysis (section 2.2). Results are given in section 6.3.1.

6.2.2 Effects of starvation on the vitellogenic oocytes of mature rainbow trout.

This study was carried out to investigate the effects of starvation on the growth and development of vitellogenic oocytes and also to determine the incidence of atresia.

Fifteen 2+ year old female rainbow trout belonging to the Whitebrook strain of the broodstock from Avon Springs Hatcheries were used for this experiment. Initially three fish were blood sampled and sacrificed, to provide ovarian samples for histological studies (section 2.2) and oocyte counting (section 2.3). A further 9 fish were maintained in a 2m circular tank and starved for a period of 21 weeks. Groups of three fish were sacrificed in order to provide ovarian samples for histology and oocyte counting (section 2.3). Each time starved fish were sacrificed blood and ovarian samples were taken from one fish of the same stock in an adjacent tank which was receiving daily a full ration of food (0.7% of body weight). It was not possible to sacrifice more control fish because of the high cost of each of the 2+ old broodstock fish. All the serum samples taken were later analysed for measurements of testosterone, oestradiol-17β and calcium as an index for vitellogenin (section 2.4 and 2.5).

6.2.3 Effects of half and full rations of food on ovarian development of mature rainbow trout.

This work was carried out at the Avon Spring Hatcheries in collaboration with J.R.C. Springate of the Fish Culture Unit of the University of Aston.

Groups of 40 two year-old female rainbow trout were maintained in 2m circular tanks and fed daily in replicate at either half ration (0.35% of body weight) or full ration (0.7% of body weight). Two fish from each group was sacrificed before the time of spawning at the end of their third year. Immediately after spawning 4 more fish were sacrificed. Ovarian samples were removed from all eight fish for histological study (section 2.2) and oocyte counting (section 2.3). After spawning the experiment was continued during the next reproductive cycle and a further 3 fish from both the half and full ration groups were sacrificed during the initial stages of the next vitellogenesis. Ovarian samples from these fish were used for histological analysis and oocyte counting.

Concurrently, blood samples were taken at monthly intervals for analyses of serum testosterone and vitellogenin and fecundity and egg size determinations were also made at spawning.

6.2.3.1 Measurement of Egg Diameter

Egg size was determined by aligning the water-hardened eggs along a measuring groove of fixed length (either 120mm or 300mm). The number of eggs aligned along the groove were counted to the nearest quarter of an egg. Mean egg diameter was then calculated as below:

$$\text{Ova diameter O.D.} = \frac{\text{length of groove}}{\text{number of eggs along groove}}$$

6.2.3.2 Egg number (fecundity)

After measuring the egg diameter (O.D.), the volume of eggs produced female^{-1} was determined. The number of eggs produced can then be calculated using the formula shown below: this was derived from the work of Von Bayer (1950).

$$Y = -0.283X + 5.41$$

$Y = \log_{10}$ No. eggs length^{-1}; X = O.D. (mm)

total fecundity T.F. = Antilog Y^{*} ($^{z}/1000$)

z = volume of eggs produced (ml)

$$\text{Relative fecundity} = \frac{\text{Total fecundity}}{\text{somatic weight kg}}$$

Measurement of post-stripped weight enabled determination of relative fecundity to be made (No. egg kg^{-1} fish).

6.3 Results

6.3.1 Effects of starvation on the previtellogenic ovaries of the rainbow trout.

6.3.1.1 Body weight.

In the starved fish body weight decreased significantly by the end of the 6th week from an initial weight of 203.0 ± 75.75 g to 140.8 ± 63.5 g ($P<0.05$). In the fish fed at full ration for 6 weeks, body weight significantly increased from an initial value of 245.00 ± 60.78 g to 395.57 ± 100.06 g ($P<0.001$). By the end of the sixth week the body weight of the starved fish was significantly lower than that of those on full ration ($P<0.0005$) (Fig. 6.1).

6.3.1.2 Gonado-Somatic Index (G.S.I.)

In fish fed on full ration the G.S.I. did not show a significant change over a period of 6 weeks but in the starved fish it increased significantly from an initial value of 0.0937 ± 0.0165 to 0.1405 ± 0.0354 ($P<0.02$) by the end of the sixth week of starvation (Fig. 6.2). However, gonadal weight did not significantly change during the above period, ie. the significant increase in G.S.I. of the starved fish was due to the significant decrease in body weight (Fig. 6.1).

6.3.1.3 Histology of the ovary.

Initially the ovary consisted of previtellogenic oocytes up to early stage 4, with only small number of stage 4 oocytes when compared with the numbers of stage 2 and 3. The inter-oocyte spaces

were filled with connective tissue strands (plate 6.1). After 6 weeks of starvation oocytes up to stage 4 were present (plate 6.2) but the stage 4 oocytes were at a less advanced stage of development when compared to those of the fish fed on full ration (plate 6.3). Furthermore, in the ovaries of the starved fish, empty inter-oocyte spaces were also seen (plate 6.2) whereas in the ovaries of the fully-fed fish these spaces were filled with connective tissue.

6.3.1.4 Oocyte counts

6.3.1.4.1 Stage 1 oocytes

Six weeks of starvation or feeding with full ration did not change the number of stage 1 oocytes from the numbers initially found.

6.3.1.4.2 Stage 2 oocytes.

In the starved fish number of stage 2 oocytes did not show any significant difference from the number in the initial sample. In contrast, the fish fed with full ration for 6 weeks had 61.67 ± 18.96% stage 2 oocytes, a figure which was significantly lower than that of the initial control sample (80.75 ± 5.20%) and of the fish which had been starved for 6 weeks (82.61 ± 0.84%) ($P<0.025$ and $P<0.01$ respectively) (Fig. 6.3).

6.3.1.4.3 Stage 3 oocytes.

Initially the number of stage 3 oocytes was 18.37 ± 5.50%. Six weeks of starvation or feeding with full ration did not significantly change the numbers of these oocytes. However, the number of stage 3 oocytes in the starved fish, which amounted to 16.40 ± 0.67%, was

significantly lower than that of the fully-fed fish (22.99 ± 8.59%). ($P<0.025$) (Fig. 6.3).

6.3.1.4.4 Stage 4 oocytes.

Initially the number of stage 4 oocytes was 0.12 ± 0.29% and this increased to 15.10 ± 16.3% after six weeks of feeding ($P<0.001$). In the fish starved for 6 weeks stage 4 oocytes constituted 0.10 ± 0.22% of the total, a figure which was not significantly lower than that of the fully-fed fish ($P<0.005$) (Fig. 6.3).

6.3.1.5 Oocyte diameter.

6.3.1.5.1 Stage 1 oocytes.

Initially the diameter of the stage 1 oocytes was 31.08 ± 0.40 μ. This was significantly increased to 34.89 ± 1.22 μ after 6 weeks of starvation and significantly decreased to 28.60 ± 0.14 μ following 6 weeks of feeding with full ration ($P<0.0005$). The diameter of stage 1 oocytes in starved fish was significantly greater than that of the fish fed for 6 weeks ($P<0.0005$) (Fig.6.4).

6.3.1.5.2 Stage 2 oocytes.

The diameter of the stage 2 oocytes from both the starved and fed fish, showed no difference from the initial values (Fig. 6.4).

6.3.1.5.3 Stage 3 oocytes.

The diameter of the stage 3 oocytes was initially 398.10 ± 41.62 μ and this decreased significantly after 6 weeks in both the starved

and fed groups ($P<0.0005$ for both). The diameter of the stage 3 oocytes was 341.30 ± 7.17 μ in fish starved for 6 weeks and this was significantly lower ($P<0.0005$) than the diameter observed in fish fed with full ration (354.69 ± 29.6μ) (Fig. 6.4).

6.3.1.5.4 Stage 4 oocytes.

The diameter of stage 4 oocytes in both the starved and fed fish showed no significant differences from the initial values. In starved fish the stage 4 oocytes had a diameter of 564.40 ± 6.19 μ which was significantly lower ($P<0.01$) than that of the fish fed on full ration (609.0 ± 118.52 μ) (Fig. 6.4).

6.3.1.6 Serum Calcium.

Initially the mean serum calcium level was 10.368 ± 2.026 mg $100ml^{-1}$ in the fish maintained on full ration for six weeks. In the fish which had been starved for 6 weeks the calcium level was 8.832 ± 1.749 mg $100ml^{-1}$ and this was significantly lower ($P<0.05$) than that of the fully-fed fish although not significantly different from the initial values (Fig. 6.5).

6.3.1.7 Serum Oestradiol-17β.

Initially, the mean serum oestradiol-17β level of the fish fed on full-ration was 3.52 ± 2.23 ng ml^{-1} and this showed a significant decrease to 0.602 ± 0.641 ng ml^{-1} ($P<0.01$) in the starved fish. Similarly in fish fed for 6 weeks serum oestradiol-17β level was significantly lowered to 1.302 ± 1.00 ng ml^{-1} ($P<0.025$) (Fig. 6.6).

3.1.8 Serum testosterone.

Following 6 weeks of starvation the mean serum testosterone levels were decreased from 6.688 ± 9.6 ng ml^{-1} to 0.708 ± 0.23 ng ml^{-1}. This was significantly lower than the mean testosterone level found in fish maintained on full ration for 6 weeks, where the value was 8.489 ± 10.5 ng ml^{-1} ($P<0.05$) (Fig. 6.7).

6.3.2 Effects of feeding and starvation on vitellogenic ovaries of rainbow trout.

Initially all fish sampled had ovaries at early stages of true vitellogenesis (stage 5 oocyte). After 21 weeks of feeding the control fish spawned whilst the fish which had been starved during this period failed to spawn. Superficial examination of the ovaries of fish starved for 12 and 21 weeks showed clearly that one of the three fish sampled each time had ovaries more advanced in development than the other two, and by the end of the 21st week of starvation this fish had oocytes at final stages of vitellogenesis while the other two did not have any vitellogenic oocytes. However, the body weights of all three fish were significantly decreased due to starvation.

6.3.2.1 Body weight.

Initially body weight was 1236.67 $\pm$ 275.6 g, this decreased to 936.67 $\pm$ 57.52 g; 808.33 $\pm$ 153.08 g and 908.33 $\pm$ 38.18 g as a result of starvation for 7, 12 and 21 weeks respectively and each of these values was significantly lower than the body weights of the controls fed on full ration for the same periods (respectively:- $P<0.005$, $P<0.01$ and $P<0.0005$). The body weight of the fish fed for 21 weeks was also significantly higher than that of the fish sampled initially ($P<0.05$). (Fig. 6.8, Table 6.1.1).

6.3.2.2 Gonado Somatic Index.

Initially the G.S.I. was 0.7972 $\pm$ 0.1186 and following feeding for 12 weeks this increased significantly to 5.0163 ($P<0.0005$), which

was also significantly higher than that of the fish starved for a similar period ($P<0.025$). Although fish starved for 12 weeks had a mean G.S.I. of 1.1500 $\pm$ 1.6514, one of the 3 fish sampled had a G.S.I. value significantly higher than the other two (Table 6.1.1). Feeding for 21 weeks resulted in the spawning of the controls. Following this the G.S.I. was reduced to 0.4976 . In fish which were starved over a similar period the mean G.S.I. was 4.2616 $\pm$ 7.0168. However, one of the starved fish had a G.S.I. of 12.3634 while the other two had G.S.I.s of 0.1289 and 0.2926 (Table 6.1.1, Fig. 6.9).

6.3.2.3 Histology of the ovary.

Initially all fish had oocytes up to stage 6 and atresia was not seen in any of the samples taken for histological examination. Vitellogenic oocytes were at the point of transformation from stage 5 to stage 6 (*germinal vesicle migration* stage) (Plate 6.4). Seven weeks later the only vitellogenic oocytes seen amongst the previtellogenic oocytes in the ovaries of the fed group were stage 6 (Plate 6.5). However, the stage 6 oocytes of fed fish were more advanced in development than the stage 6 oocytes observed at the initial sampling. In starved fish, although oocytes up to stage 6 were observed these stage 6 oocytes were at a less advanced stage of development than those of the fed controls. At the same time a large number of atretic oocytes was observed in the ovaries of starved fish (Plate 6.6). After 12 weeks of starvation atretic oocytes were found in larger numbers (Plate 6.7), although one of these starved fish sampled had stage 6 vitellogenic oocytes (Plate 6.8), in contrast the other two had only previtellogenic oocytes and stage 5 atretic oocytes (Plate 6.7). In the control fish which had been fed on full-

nation for 12 weeks stage 6 oocytes, which were undergoing their final stages of vitellogenesis, were observed and they were larger and more advanced in development than the stage 6 oocytes observed in starved fish (Plate 6.9). By the end of the 21st week spawning had occurred in the control fish and post-ovulatory follicles and previtellogenic oocytes up to stage 4 were seen in the ovaries (Plate 6.10). In the fish which had been starved over this period ovulation had not occurred. However, in one of the fish stage 6 oocytes undergoing the final stages of vitellogenesis and also stage 7 oocytes were observed (Plate 6.11). Whereas in the other two fish sampled, oocytes only up to stage 4 were present. In all the starved fish there were large numbers of stage 5 and 6 vitellogenic oocytes undergoing atresia (Plate 6.12).

6.3.2.4 Oocyte counts.

6.3.2.4.1 Stage 1 oocytes.

Initially, the number of stage 1 oocytes present was 1556 ± 366 100 g^{-1} body weight. As a result of normal feeding or starvation over a period of 21 weeks these numbers were reduced to 416 and 265 ± 216 100 g^{-1} body weight respectively. However no significant difference was seen between starved and fed groups (Table 6.1.2).

6.3.2.4.2 Stage 2 oocytes.

The numbers of stage 2 oocytes did not show any significant change after starvation for 21 weeks. However in the starved fish, after 12 and 21 weeks of starvation, one of the three fish sampled each time had a lower number of stage 2 oocytes within the ovary (Table 6.1.2).

6.3.2.4.3 Stage 3 oocytes.

Initially 137 ± 51 $100g^{-1}$ body weight stage 3 oocytes were present within the ovaries and in the fish fed or starved over a period of 21 weeks this number was significantly increased to 246 $100g^{-1}$ body weight and 240 ± 280 $100g^{-1}$ body weight, respectively ($P < 0.05$ for both). However, no significant difference was seen in the number of stage 3 oocytes between the starved and fed groups (Fig. 6.10). Again one of the fish starved for 21 weeks had 558 stage 3 oocytes $100g^{-1}$ body weight which was higher than that of the other two fish of the same group (Table 6.1.2).

6.3.2.4.4 Stage 4 oocytes.

Initially 137 ± 51 $100g^{-1}$ body weight stage 4 oocytes were present in the ovaries. After 21 weeks no stage 4 oocytes were present in the fed fish whereas in the starved ones 75 ± 107 $100g^{-1}$ body weight stage 4 oocytes were observed. However, one of the starved fish from the above group had 198 $100g^{-1}$ body weight stage 4 oocytes which was higher than that found in the other two. The former fish had a vitellogenic ovary while the latter two had previtellogenic ovaries (Fig. 6.10, Table 6.1.2).

6.3.2.4.5 Stage 5 oocytes.

Initially, stage 5 oocytes were found in very small numbers (7 ± 8 $100g^{-1}$ body weight). This number increased to 42 ± 42 $100g^{-1}$ body weight in the fish starved for 7 weeks. However, one of these fish did not contain any stage 5 oocytes. No stage 5 oocytes were present in the ovaries of fish fed for 7, 12 and 21 weeks and those of the fish starved for 12 and 21 weeks (Fig. 6.10, Table 6.1.2).

6.3.2.4.6 Stage 6 oocytes.

In the ovaries of the initially-sampled control fish 170 ± 25 100g^{-1} body weight stage 6 oocytes were seen. After 12 weeks of feeding this increased to 125 ($P<0.005$). In one of the fish starved over this period 370 100g^{-1} body wt. stage 6 oocytes were observed (Table 6.10). Somewhat similar observations were made after 21 weeks of feeding and starvation. However, in the ovaries of one of the starved fish 46 stage 6 oocytes 100g^{-1} body weight were present (Table 6.1.2), whereas in the fed group all the vitellogenic oocytes had been ovulated (Fig. 6.10).

6.3.2.4.7 Stage 7 oocytes.

Stage 7 oocytes were not present at the initial or after starvation or feeding for 12 weeks. After 21 weeks of starvation stage 7 oocytes were present in one of the starved fish whilst the other two had no vitellogenic oocytes (Fig. 6.10, Table 6.1.2).

6.3.2.4.8 Atretic oocytes.

Initially atretic oocytes were present in very small numbers (11 ± 10 100g^{-1} body weight). The levels of atresia significantly increased in both the starved and fed fish to 372 ± 278 100g^{-1} body weight and 69 100g^{-1} body weight respectively. However, the number of atretic oocytes found in the fish fed for 21 weeks was significantly lower than that of the fish starved over a similar period ($0.05 < P < 0.10$) (Fig. 6.10, Table 6.1.2).

6.3.2.4.9 Post-ovulatory follicles.

Post-ovulatory follicles were observed only in the fed fish. Their ovaries were more advanced than those of the fish starved for a similar period (Fig. 6.10 and Table 6.1.2).

6.3.2.5 Mean highest oocyte diameter.

The mean diameter of the highest oocyte stage present in the ovary was initially 1546 ± 37 μ. This significantly increased to 2934 ± 132 μ after 12 weeks of feeding ($P<0.005$). In one of the starved fish the oocyte diameter increased to 2365 ± 113 μ whilst in the other two it decreased to 490 ± 30 μ and 450 ± 26 μ. Similar changes were observed after 21 weeks of starvation. Although the oocyte diameter also decreased in the control group, this decrease was due to the ovulation of the largest vitellogenic oocytes. In contrast in the starved fish the reduction was due to the resorption of vitellogenic oocytes i.e. atresia (Figs. 6.10 and 6.11, Tables 6.1.1 and 6.1.2).

6.3.2.6 Serum Calcium.

Initially the mean serum calcium level was 11.55 ± 0.95 mg $100ml^{-1}$ and this is significantly increased to 21.34 mg $100ml^{-1}$ as a result of feeding for 12 weeks ($P<0.005$). In fish starved over a similar period serum calcium was 11.46 ± 2.83 mg $100ml^{-1}$, significantly lower than that of the fed controls ($p<0.025$). Further feeding for 21 weeks resulted in a post-ovulatory decrease in serum calcium to 10.12 ± 8.27 mg $100ml^{-1}$. In one of the fish starved for 21 weeks serum calcium level was 14.66 mg $100ml^{-1}$ which was significantly higher than the initial control values and this fish had vitellogenic ovaries. The other two fish of the same group had

pre-vitellogenic ovaries and also low serum calcium levels (Table 6.1.1 and Fig. 6.12)

6.3.2.7 Serum Oestradiol-17β.

Initially the mean serum oestradiol-17β level was 6.40 ± 1.85 ng ml^{-1} and this showed a significant increase to 33 ng ml^{-1} after 12 weeks. After 21 weeks the fed group ovulated and at the same time their serum oestradiol levels decreased significantly to 2.3 ng ml^{-1}. In the fish starved for 12 and 21 weeks differences were observed between the fish in the starved group. After 21 weeks one of the starved fish had a significantly higher serum oestradiol-17β level than the other two and also higher than the initial control sample (Table 6.1.1 and Fig. 6.13).

6.3.2.8 Serum testosterone.

Initially the mean serum testosterone level was 3.95 ± 6.12 ng ml^{-1} and this significantly increased to 21.6 ng ml^{-1} in the controls after 12 weeks. High testosterone levels were also observed in one of the starved fish whereas in the other two starved fish levels were not significantly different from the initial controls. Similar reduced levels were also seen after starvation for 21 weeks. However, in the controls feeding up to 21 weeks resulted in ovulation and the serum testosterone level did not show difference from the initial value (Fig. 6.14 and Table 6.1.1).

6.3.3 Effects of feeding on full and half rations on the ovaries of mature rainbow trout.

6.3.3.1 Effects of feeding on full and half-rations on mature rainbow trout before and after spawning.

6.3.3.1.1 Body weight.

The body weights of the fish fed on half-ration were 655 ± 134 g and 803 ± 103 g in the fish sampled just before and after spawning respectively and these were significantly lower than those of the fish fed on full-ration whose body weights were 1117 ± 168 g and 1333 ± 95 g before and after spawning respectively ($P < 0.05$ and $p < 0.025$) (Fig. 6.15).

6.3.3.1.2 Gondado Somatic Index.

There were no differences in the G.S.I.s of the fish fed on full and half-ration either before or after spawning (Fig. 6.15, Table 6.2).

6.3.3.1.3 Ovarian histology.

In the pre-spawned ovaries of fish fed on full-ration the only vitellogenic oocytes observed among the previtellogenic oocytes were stage 7 as might be expected as the fish were close to ovulation. In the half-ration fed group one of the fish had only stage 7 vitellogenic oocytes although they were smaller and less advanced in development than those of the fed group. In the other fish stage 6 vitellogenic oocytes predominated amongst the few stage 7 oocytes present. Atretic oocytes were not seen in the sections of pre-ovulatory ovaries of either group probably because the oocytes were

large and only a few are included in a histological sample. As will be seen below parallel sampling for determination of oocyte numbers revealed increased levels of atresia in the half-ration fed fish. However, atretic oocytes were present in the post-ovulated ovaries of both full and half-ration fed fish, with the fish fed on half-ration showing a wider distribution (Plates 3.39). These ovaries contained stage 4, 5 and 6 atretic oocytes.

6.3.3.1.4 Oocyte counts.

Previtellogenic oocytes.

No significant difference in the numbers of previtellogenic stage 1, 2, 3 and 4 oocytes were seen between the full and half-ration fed groups (Table 6.2, Fig. 6.15).

Vitellogenic oocytes.

No significant difference was seen in the total number of vitellogenic oocytes between the full and half-ration fed groups before spawning although in the full ration fed group all the vitellogenic oocytes were at stage 7 and late stages of maturity whilst in the half fed group stage 6 oocytes predominated and/or the stage 7 oocytes were less advanced in development than those of the fully fed group. There were no significant differences in the numbers of retained stage 7 oocytes in the post-ovulatory ovaries of the half and full-ration fed groups (Table 6.2, Fig. 6.15).

Atretic oocytes.

In the pre-ovulated ovaries of half-ration fish 86 $\pm$ 49 atretic oocytes 100 g^{-1} body weight were found and this number was higher

than that found in the fed group where the atretic oocytes amounted to 25 ± 2 100 g^{-1} body weight ($0.05 < P < 0.10$). Significant differences were seen in the levels of atresia in the post-ovulated ovaries (Fig. 6.15, Table 6.2).

Post-ovulated follicles.

No significant difference was observed between the numbers of post-ovulatory follicles in the two groups. All ovarian samples taken for this comparison were obtained from fish of which had been stripped on the same day (Fig. 6.15, Table 6.2)

6.3.3.1.5 Mean highest oocyte diameter.

The mean diameter of stage 7 oocytes from fish fed on full-ration was 4600 ± 66 μ, significantly higher than the 3555 ± 676 μ of half-ration fed fish ($0.05 < P < 0.10$). In the post-ovulated ovaries highest number of previtellogenic oocytes found were at stage 4 and their mean diameter had not been changed by the different feeding regimes (Fig. 6.15, Table 6.12).

6.3.3.1.6 Ovulation, fecundity and egg size and quality.

Ovulation of the full-ration fed fish commenced 2-3 weeks earlier than those on half-ration (Fig. 6.16) from which group 11% failed to mature. Total fecundity of full ration fish was significantly higher than that of the half ration fed fish ($P < 0.0001$) but the relative fecundity was higher in the fish fed with half ration ($P < 0.001$) (Fig. 6.17). The eggs of the full ration group were larger and weighed more than those of the half ration group (Fig. 6.17). Egg quality, expressed as the survival of eggs at fertilization, eying and hatching was not affected by the rates of feeding.

6.3.3.1.7 Serum calcium and testosterone.

Measurements of serum calcium and testosterone revealed similar profiles to those reported elsewhere (Elliott et al, 1984) (Fig. 6.18). However, the peak levels of both calcium and testosterone were reduced in the half-ration group when compared to those of full-ration fed group.

6.3.2 Effects of the long-term feeding on full and half rations on the early stages of vitellogenesis of rainbow trout.

6.3.2.1 Body weight.

The mean body weights of the fish which were fed on full and half rations, for the year preceding spawning and the subsequent 5 month period of the next cycle were 1370 ± 381 g and 687 ± 170 g respectively ie. significantly different ($P<0.025$) (Fig. 6.19, Table 6.3).

6.3.2.2 Gonado-Somatic-Index.

There was no difference in G.S.I. between the groups (Fig. 6.21, Table 6.3).

3.2.3 Oocyte counts.

Previtellogenic oocytes.

With the exception of stage 1 oocytes there were no other differences in numbers of previtellogenic oocytes in two experimental groups. The number of stage 1 oocytes was 174 ± 35 100 g^{-1} body weight in the fish fed on full-ration whereas in the half-ration groups the number at 103 ± 32 100 g^{-1} body weight was significantly lower ($P<0.05$) (Fig. 6.19, Table 6.3).

Vitellogenic oocytes.

Two of the full ration fed fish had both stage 5 and 6 oocytes whilst the remaining fish had no vitellogenic oocytes (Table 6.16). However, no difference in the total numbers of vitellogenic oocytes was seen (Fig. 6.19, Table 6.3).

Atretic oocytes.

In the half ration fed fish 180 ± 38 atretic oocytes 100 g^{-1} body weight were observed, a significantly higher level than the 15 ± 6 100 g^{-1} body weight ($P<0.0005$) (Fig. 6.19) found in the full ration group (Table 6.3).

6.3.3.2.4 Histology of the ovaries.

Late stage 5 oocytes predominated in the sections of the ovaries of fully-fed fish (Plate 6.13) whilst in the half-ration fed fish mainly stage 5 vitellogenic oocytes were found. Additionally, in the ovaries of the half-ration fish large numbers of atretic stage 4 oocytes and some atretic stage 5 previtellogenic oocytes were observed (Plate 6.14). Atretic remains of stage 6 and/or 7 vitellogenic oocytes retained from the previous developmental cycle were also seen (Plate 6.15).

6.3.3.2.5 Mean highest oocyte diameter.

The mean highest oocyte diameter of the full and half ration fed fish were respectively 1242.45 ± 140.91 μ and 926.97 ± 313.7 μ. The vitellogenic oocytes of full-ration fed fish were larger than those of the half-ration group ($0.05<P<0.10$) and some of the half-ration fish had only previtellogenic oocytes (Fig. 6.19, Table 6.3).

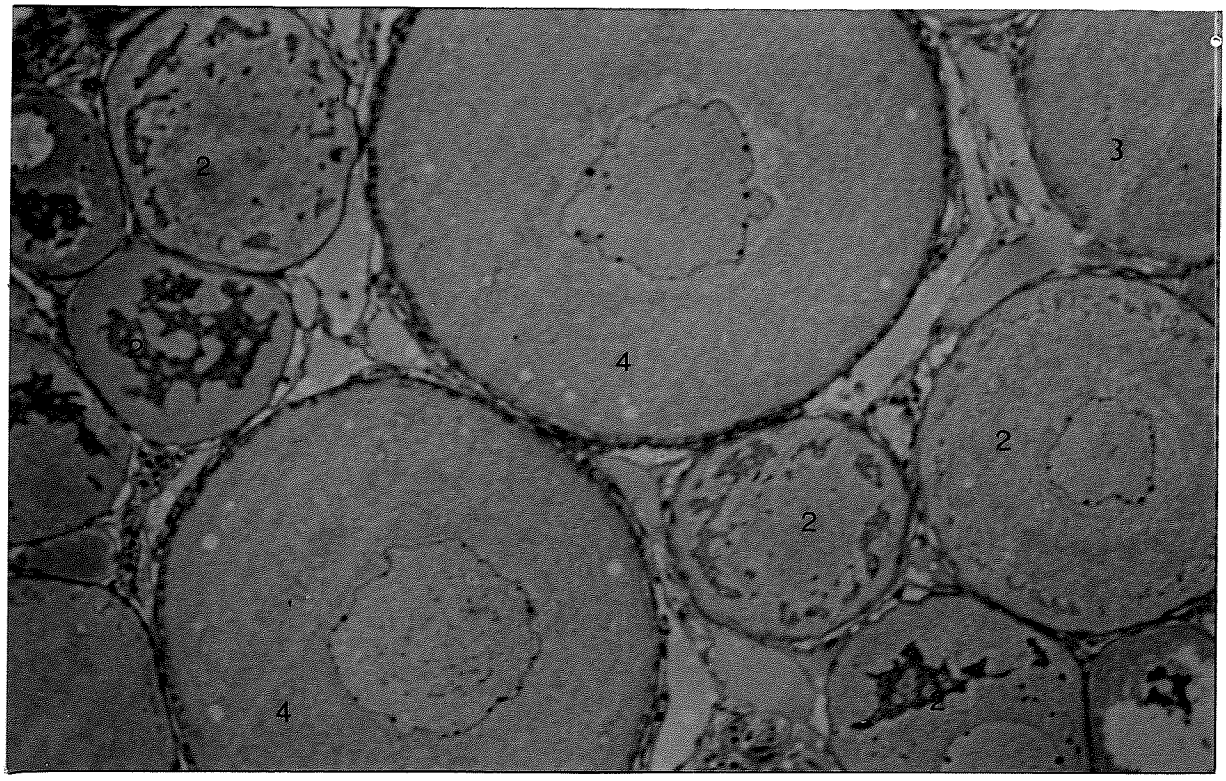

Plate 6.1

Plate 6.1 - Section of an ovary of a 1+ year-old immature fish prior to starvation, showing oocytes up to stage 4. Stained with haematoxylin and eosin. Magnification x 125

2, 3, 4 - previtellogenic oocytes at stage 2 and 3, and early vesicle stage oocytes.

Plate 6.2 - Section of an ovary of a 1+ year-old immature fish after 6 weeks of starvation showing oocytes up to early stage 4 with large inter-oocyte spaces. Stained with haematoxylin and eosin.
Magnification x 125

2, 3 4 - stage 2, 3, 4 previtellogenic oocytes,
x - inter-oocyte spaces.

Plate 6.3 - Section of an ovary of a 1+ year-old immature fish after feeding on full-ration for 6 weeks, showing oocytes up to large stage 4 (4a) . Stained with haematoxylin and eosin.
Magnification x 125

2, 3, 4 - stage 2, 3, and 4 oocytes

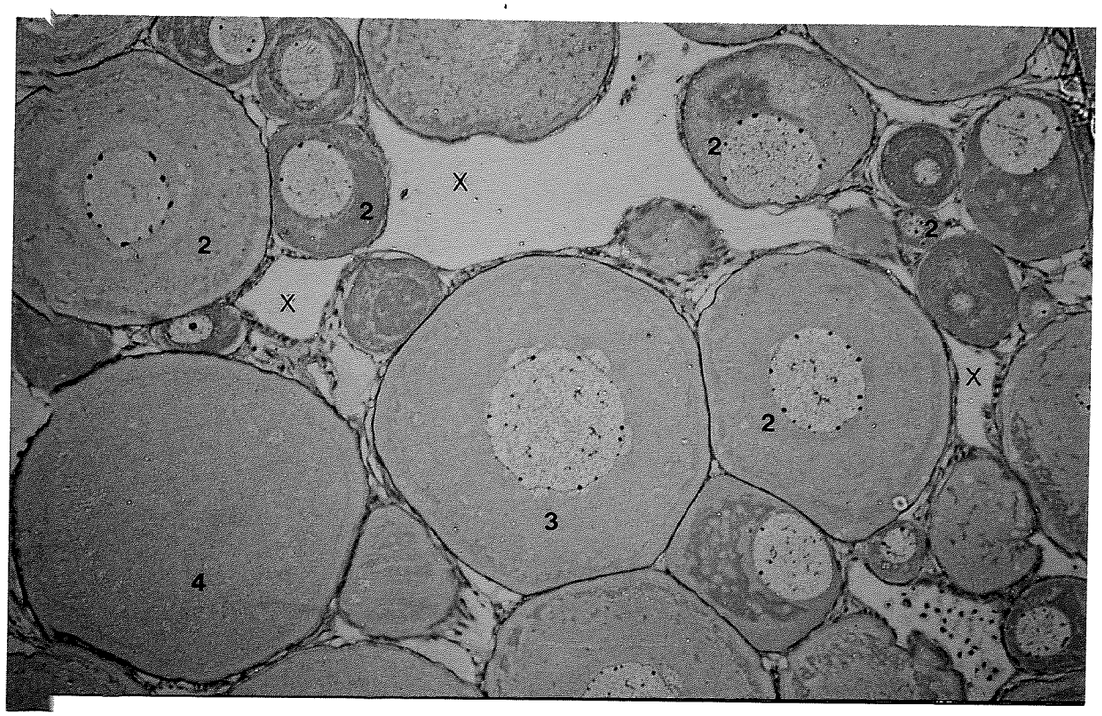

Plate 6.2



Plate 6.3

Plate 6.4 - Section of an ovary of a 2+ year-old vitellogenic fish prior to starvation showing early stage 6 oocytes. Stained with haematoxylin and eosin. Magnification x 50

4, 6 - stage 4 and 6 oocytes.

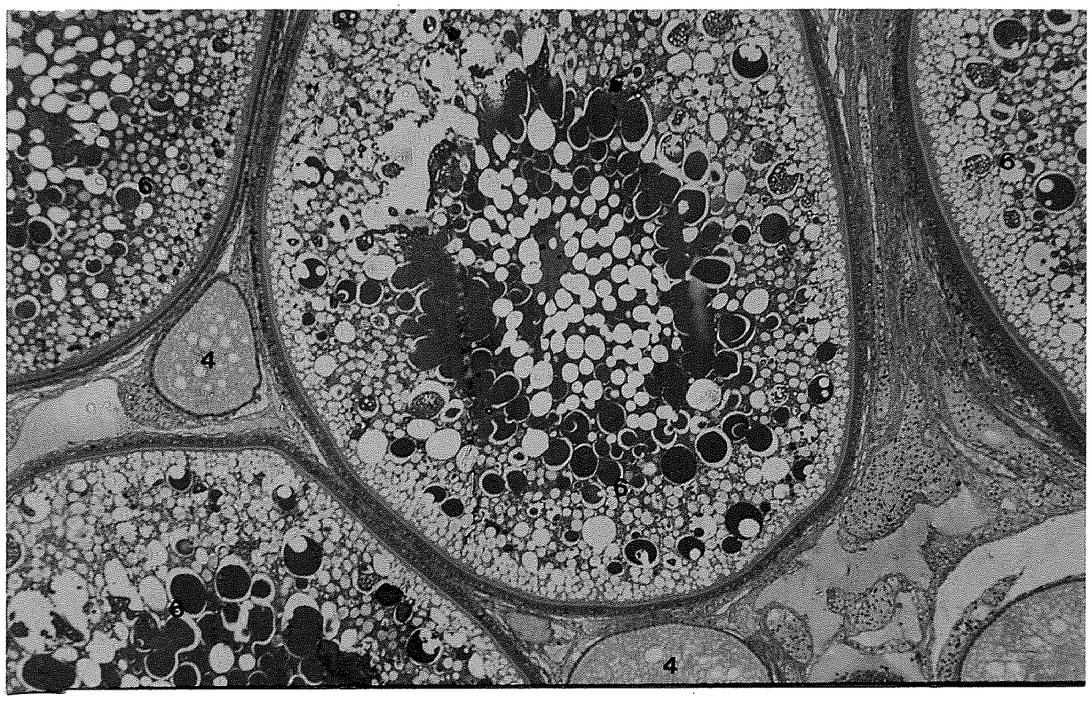

Plate 6.4

Plate 6.5 - Section of an ovary of a 2+ year-old vitellogenic fish fed with full-ration for 7 weeks showing mid stage 6 oocytes. Stained with haematoxylin and eosin. Magnification x 80



Plate 6.5

Plate 6.6 - Section of an ovary of a 2+ year-old vitellogenic fish starved for 7 weeks showing stage 6 non-hypertrophic atretic oocytes among early-stage 6 intact oocytes. Magnification x 80

At_6 - stage 6 atretic oocyte, 6 - early stage 6 intact oocytes.

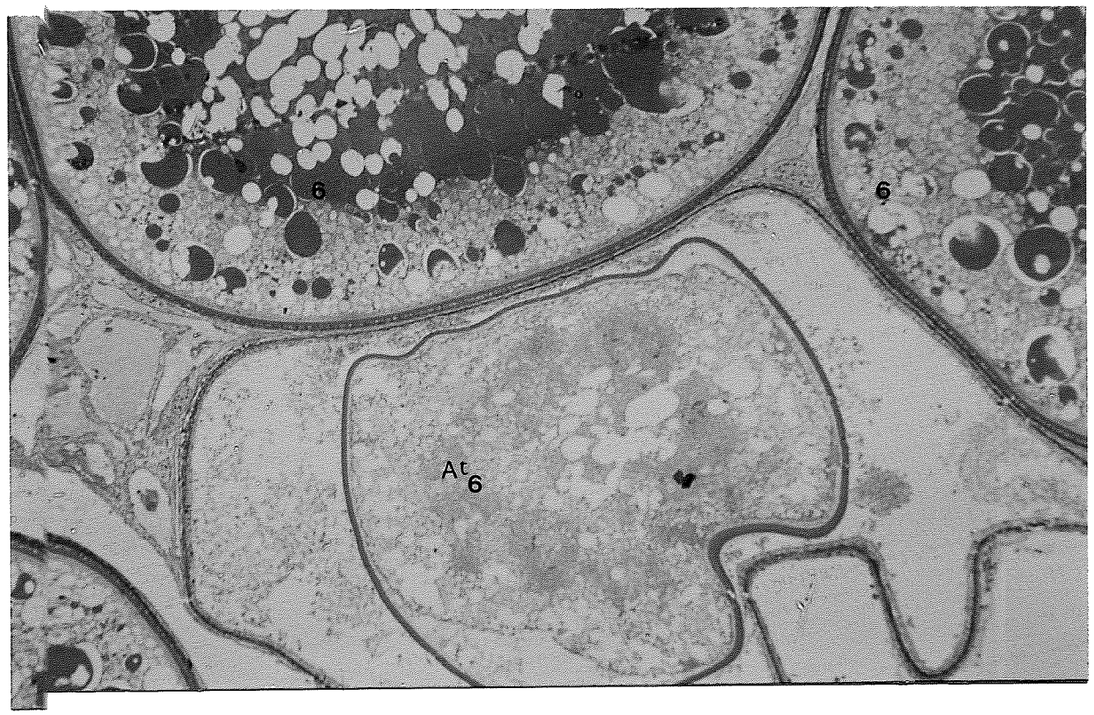

Plate 6.6

Plate 6.7 - Section of an ovary of a 2+ year-old vitellogenic fish starved for 12 weeks showing stage 5 atretic oocytes. Stained with haematoxylin and eosin. Magnification x 50

5 - stage 5 atretic oocytes, St_2, St_3 - stage 2 and 3 intact oocytes.

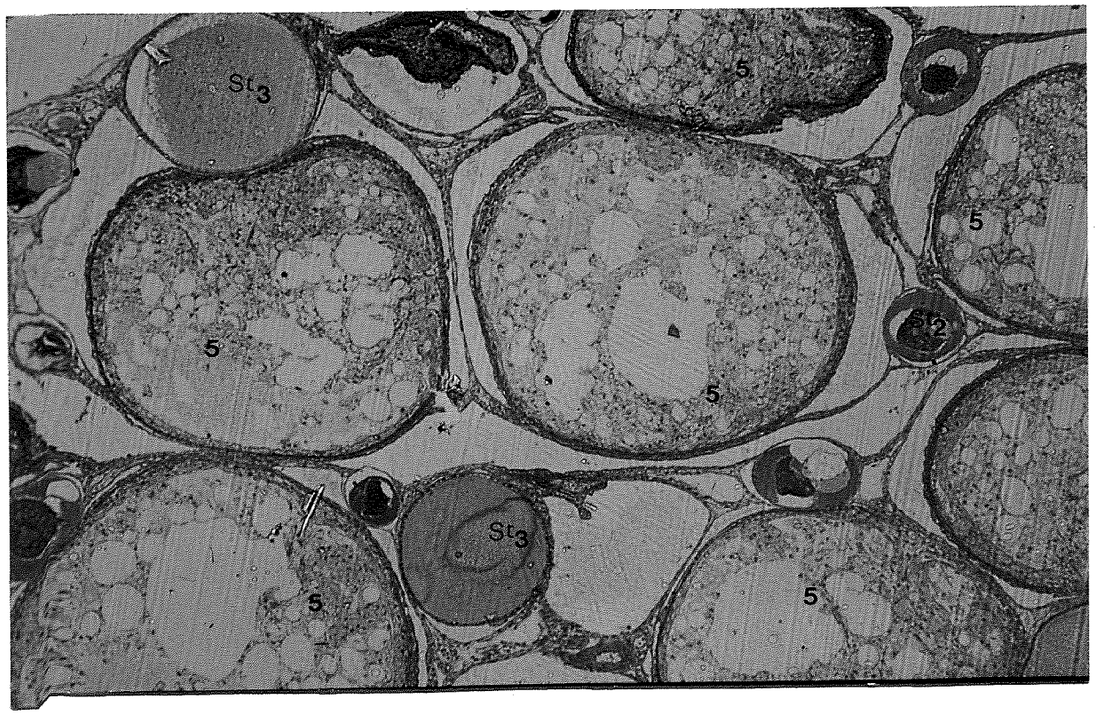

Plate 6.7

Plate 6.8 - Section of an ovary of a 2+ year-old fish starved for 12 weeks showing stage 6 atretic oocytes among stage 6 intact oocytes. Stained with haematoxylin and eosin.
Magnification x 125

At_6 - stage 6 atretic oocyte, 6 - stage 6 intact oocytes.

Plate 6.9 - Section of an ovary of a 2+ year-old fish fed for 12 weeks with full ration showing late stage 6 oocytes. Stained with haematoxylin and eosin.
Magnification x 50

St_6 - late stage 6 oocyte, N - migrating germinal vesicle.

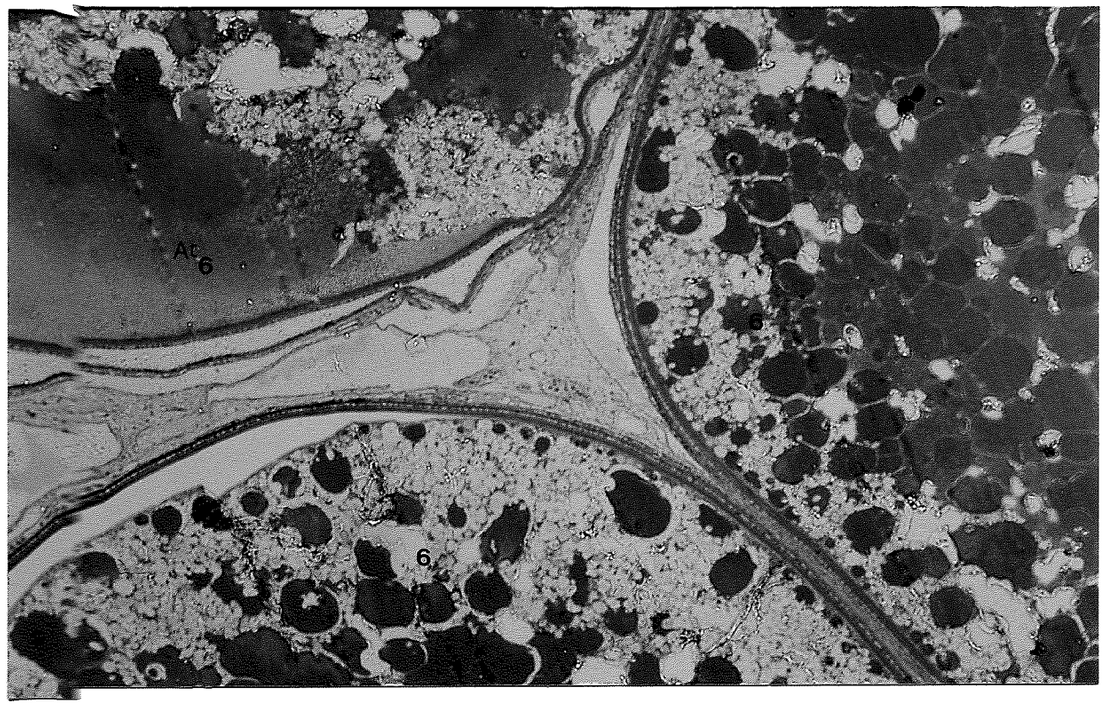

Plate 6.8

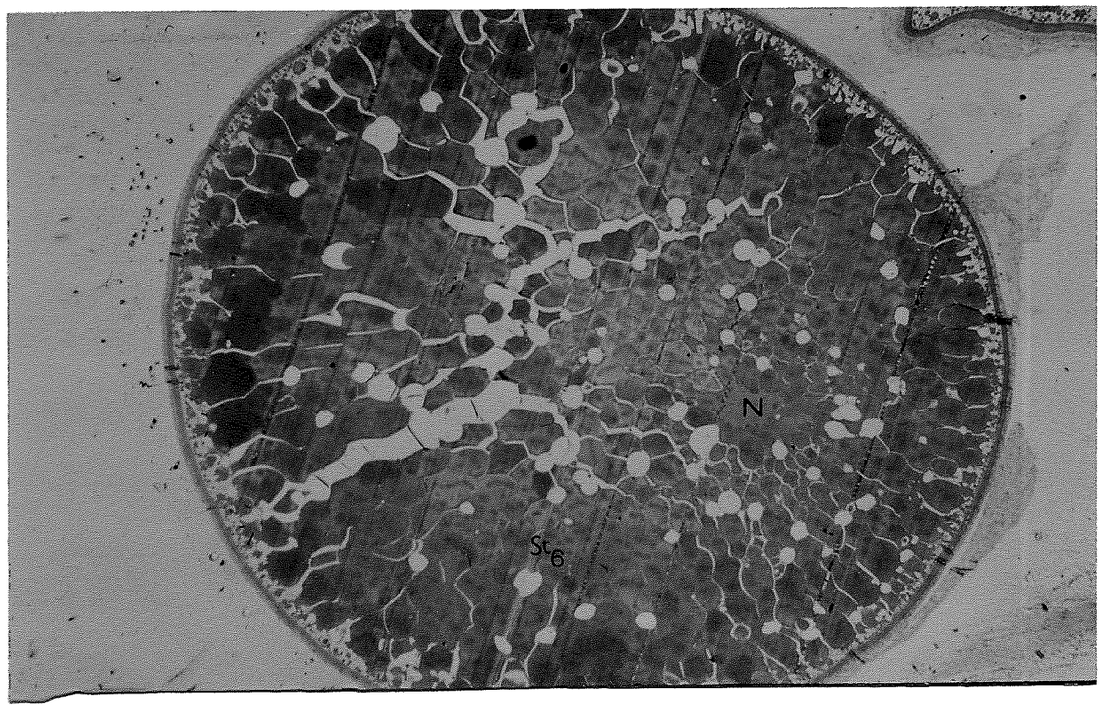

Plate 6.9

Plate 6.10 - Section of an ovary of a 2+ year-old mature fish fed with full ration for 21 weeks showing post-ovulatory follicles. Stained with haematoxylin and eosin.
Magnification x 125

St_4 - stage 4 oocytes, POF - post-ovulatory follicle.

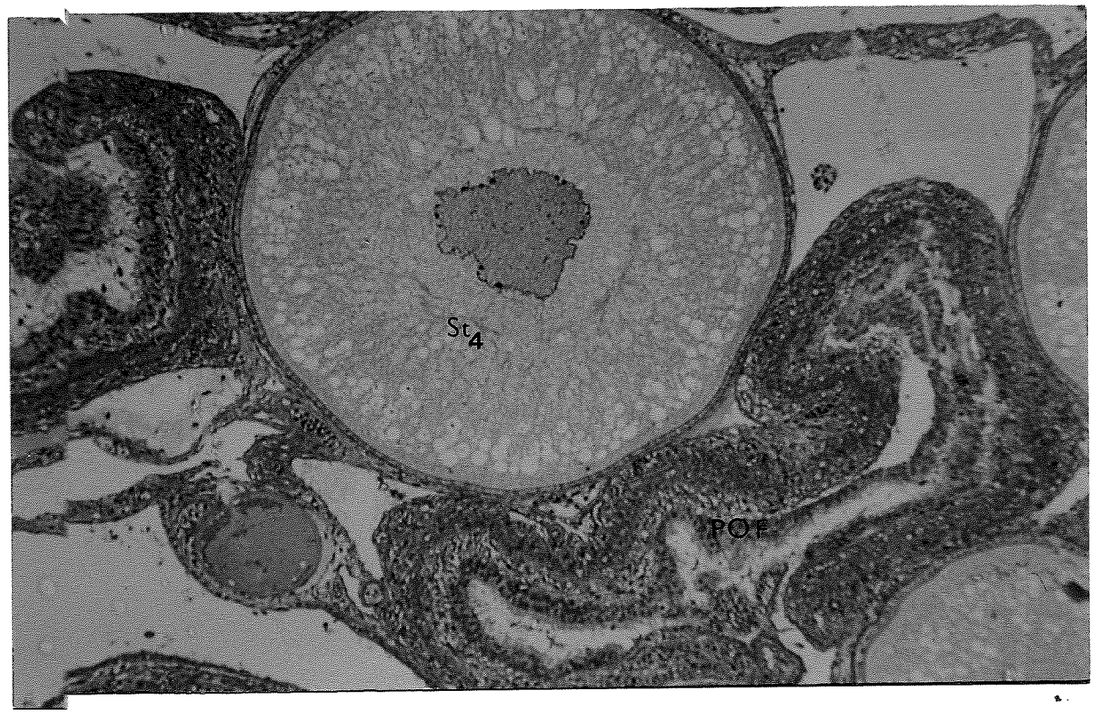

Plate 6.10

Plate 6.11 - Section of an ovary of a 2+ year-old mature fish starved for 21 weeks showing stage 6 oocytes. Stained with haematoxylin and eosin.
Magnification x 125

6 - stage 6 oocyte.

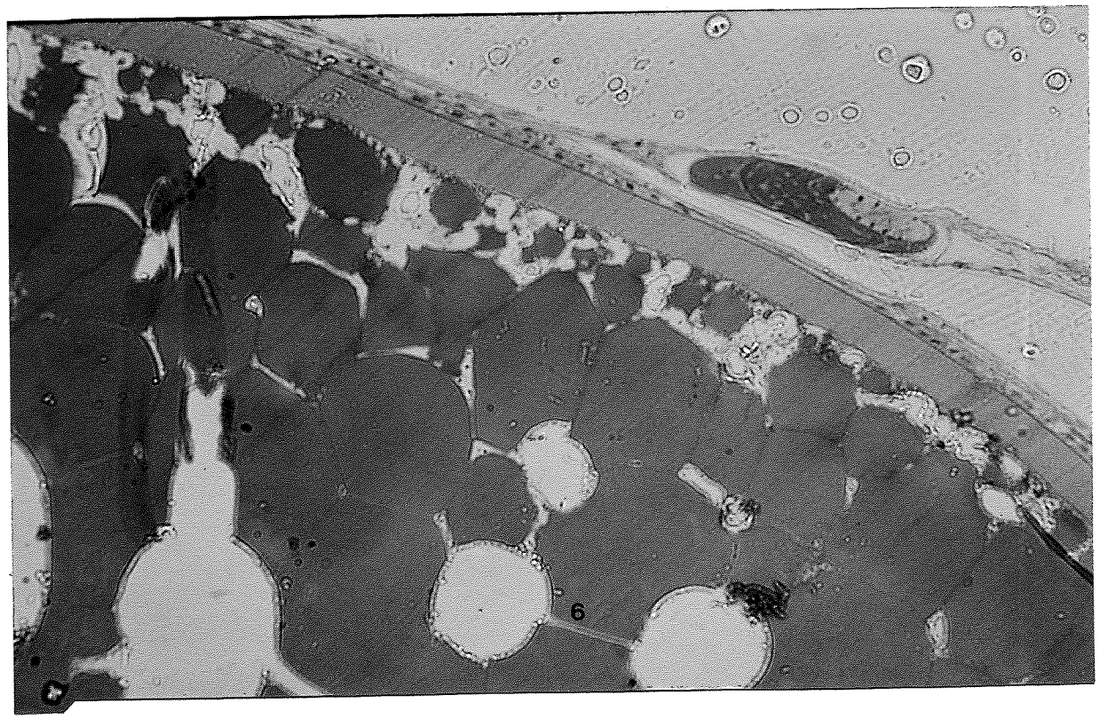

Plate 6.11

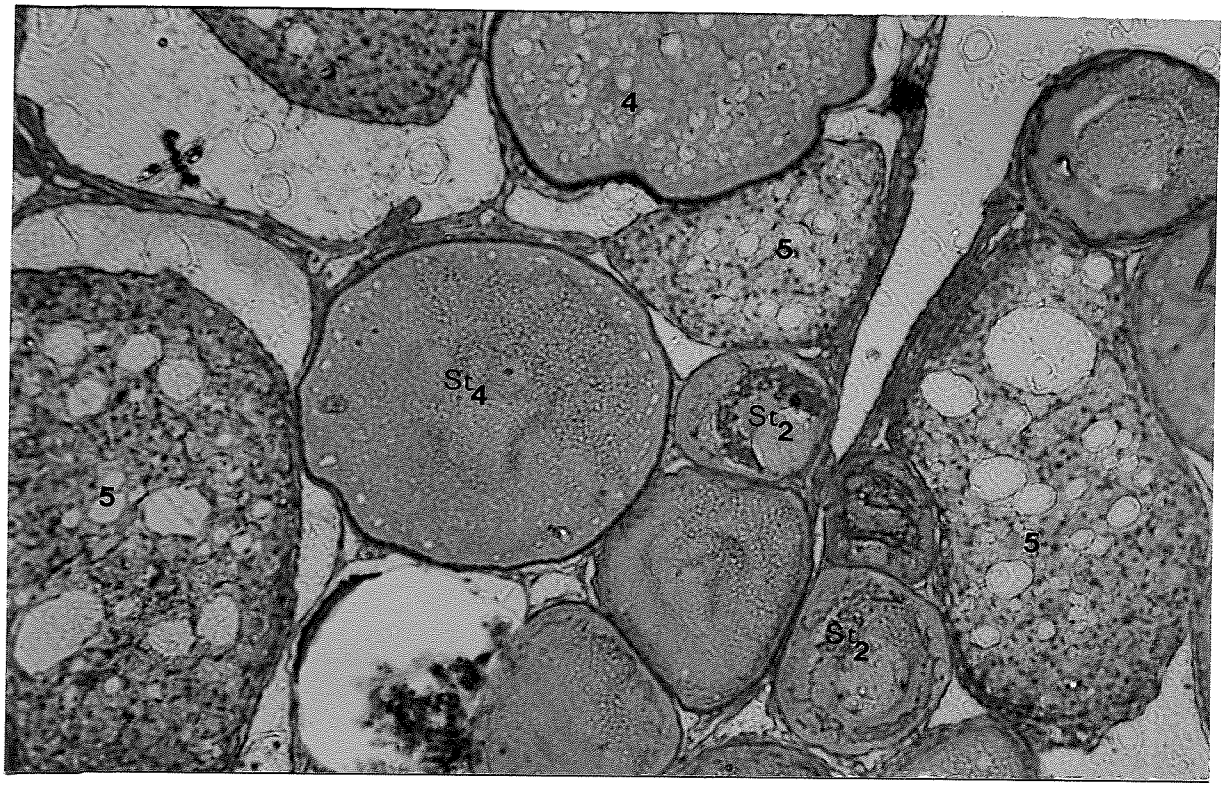

Plate 6.12

Plate 6.12 - Section of an ovary of a 2+ year-old fish starved for 21 weeks showing stage 4 and 5 atretic oocytes. Stained with haematoxylin and eosin.
Magnification x 80

4, 5 - stage 4 and 5 atretic oocytes, St_2, St_4 - stage 2 and 4 intact oocytes.

Plate 6.13 - Section of an ovary of a 3+ year-old fish fed with full ration for 18 months, showing stage 5 oocytes. Stained with haematoxylin and eosin. Magnification x 64

5 - stage 5 oocytes.



Plate 6.13

Plate 6.14 - Section of an ovary of a 3+ year-old fish fed on half-ration for 18 months showing stage 4 atretic oocytes. Stained with haematoxylin and eosin. Magnification x 80

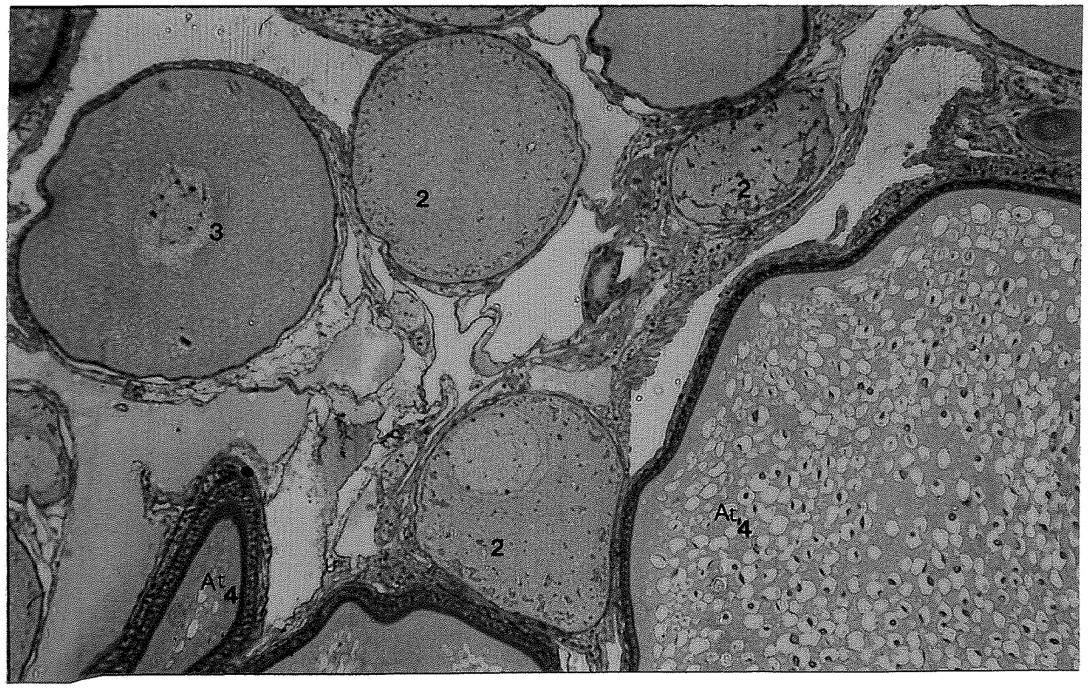

Plate 6.14

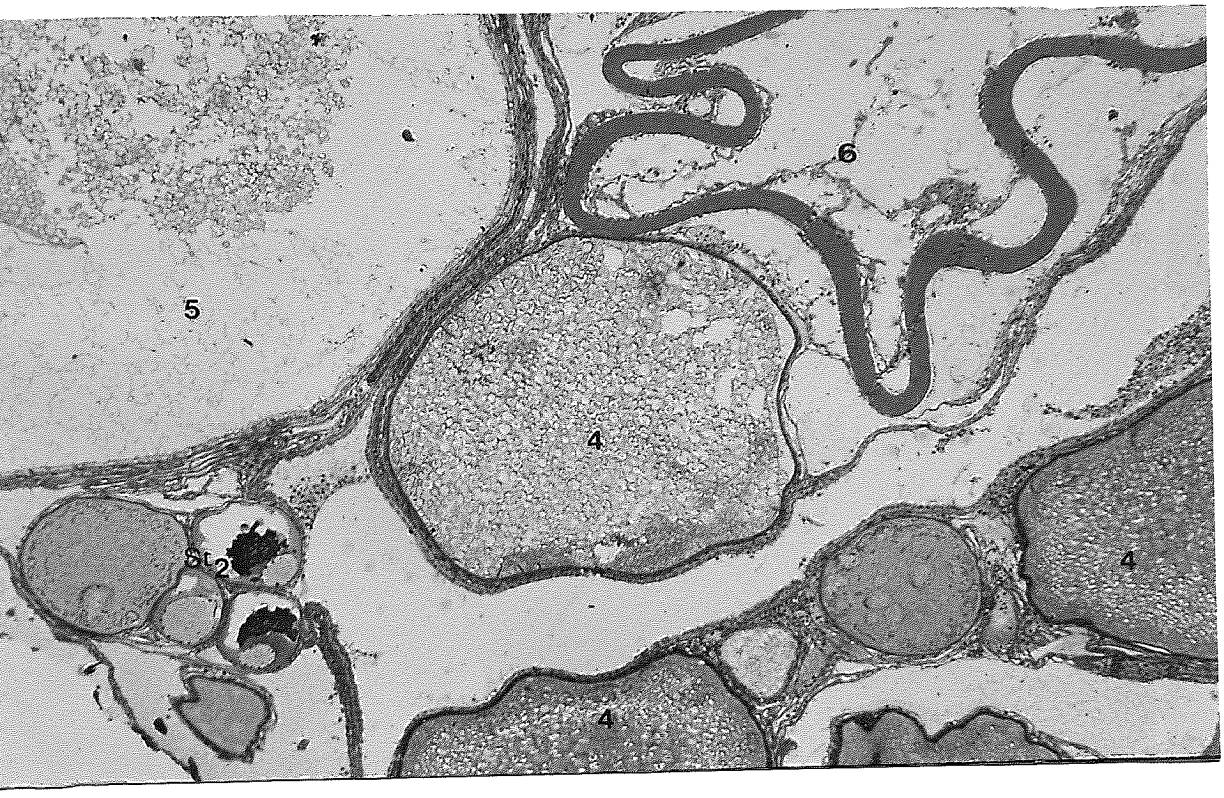

Plate 6.15

Plate 6.15 - Section of an ovary of a fish fed on half-ration for 18 months showing stage 4, 5 and 6 atretic oocytes. Stained with haematoxylin and eosin. Magnification x 80

4, 5, 6 - stage 4, 5, and 6 atretic oocytes, St_2 - stage 2 intact oocytes.

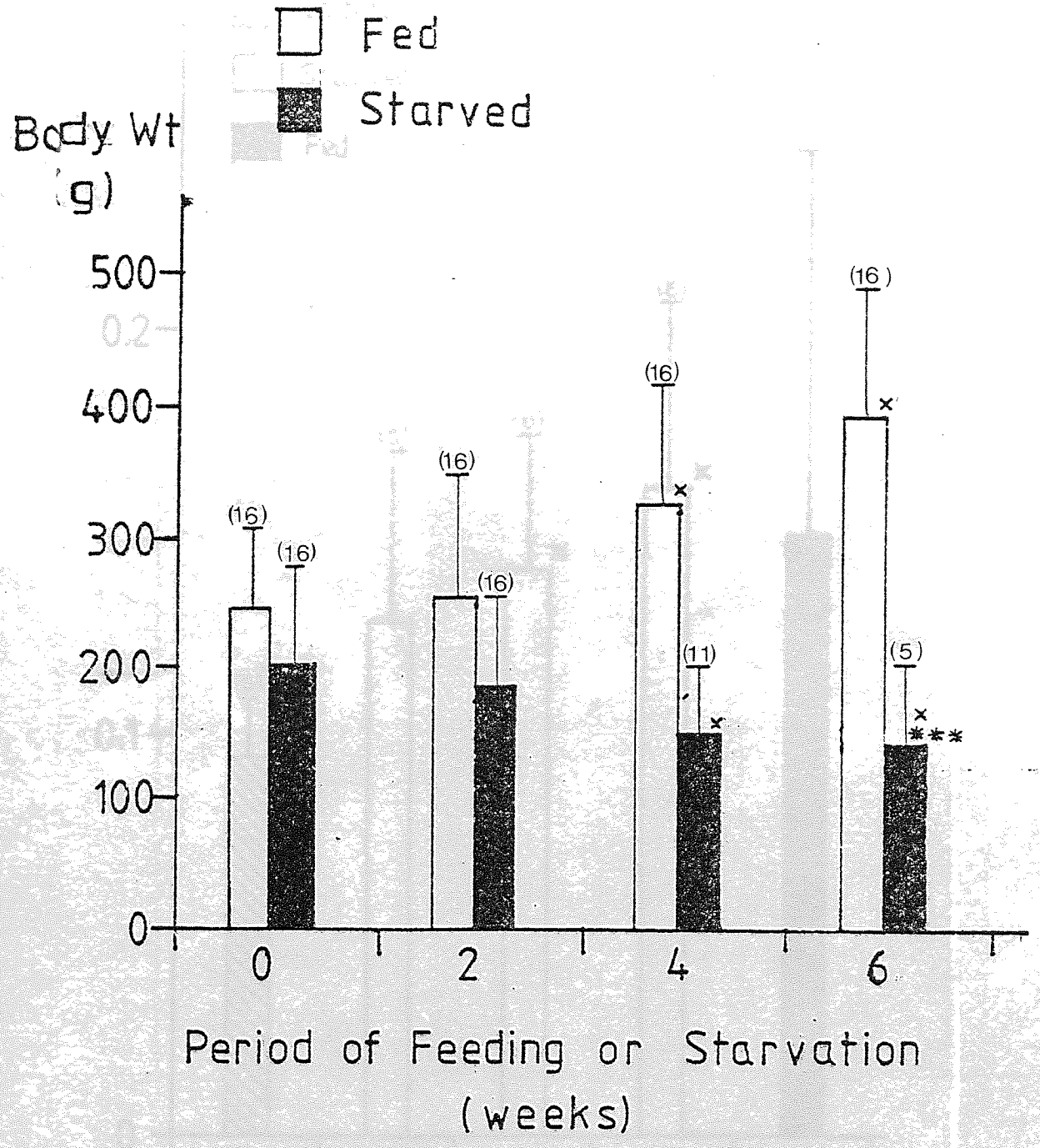

Fig 6.1 - Effects of starvation up to 6 weeks on the Body weight (B.Wt.) of immature 1+ year-old fish. Each value indicates the mean ± S.D. for number of fish (n). (* significant difference from the fed controls; x - significant difference from the initial). x $P<0.005$, *** - $P<0.0005$

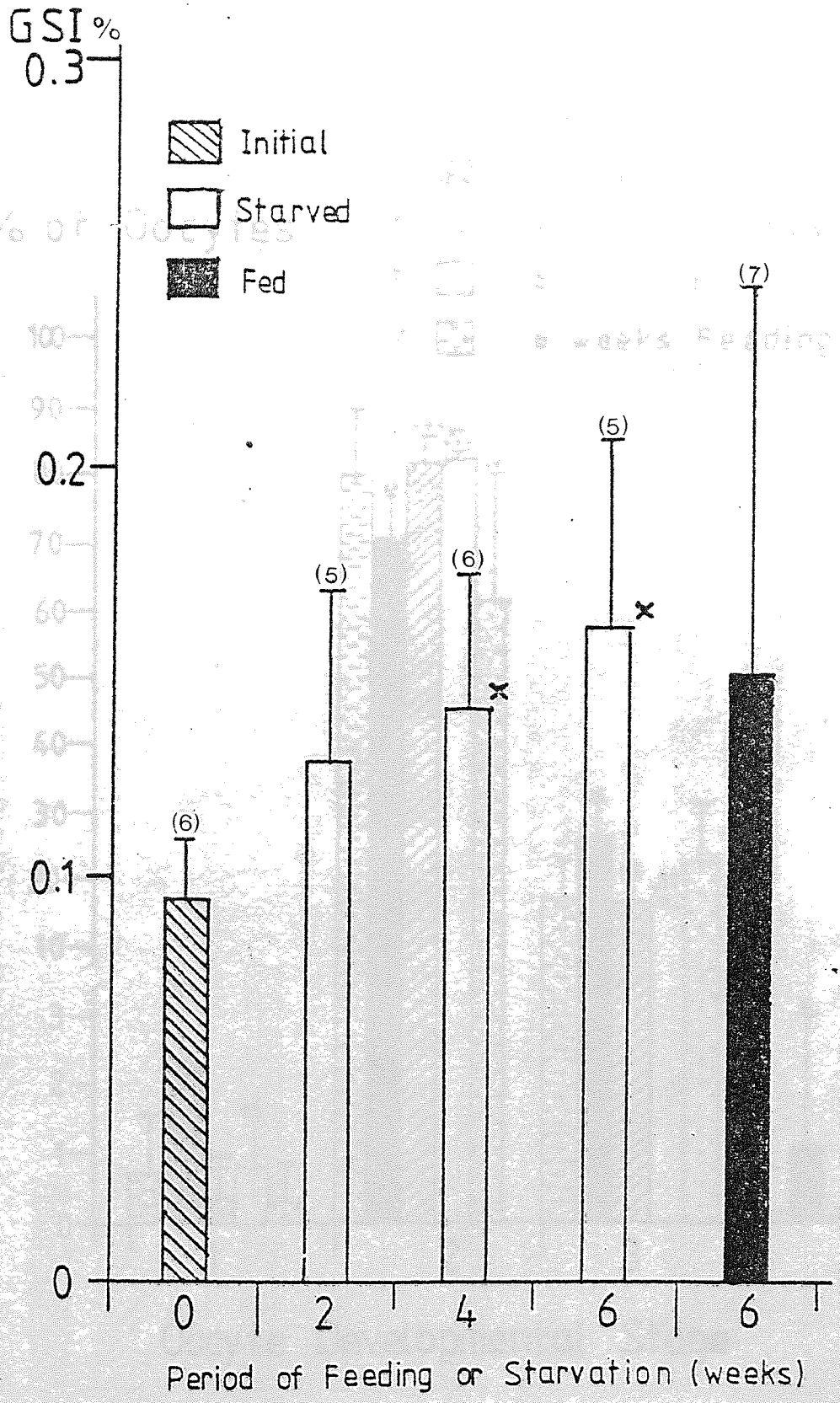

Fig 6.2 - Effects of starvation up to 6 weeks on the gonadosomatic index (G.S.I.) of immature 1+ year-old fish. Each value indicates the mean ± S.D. for number of fish (n). (x - significant difference from the initial). $P<0.05$

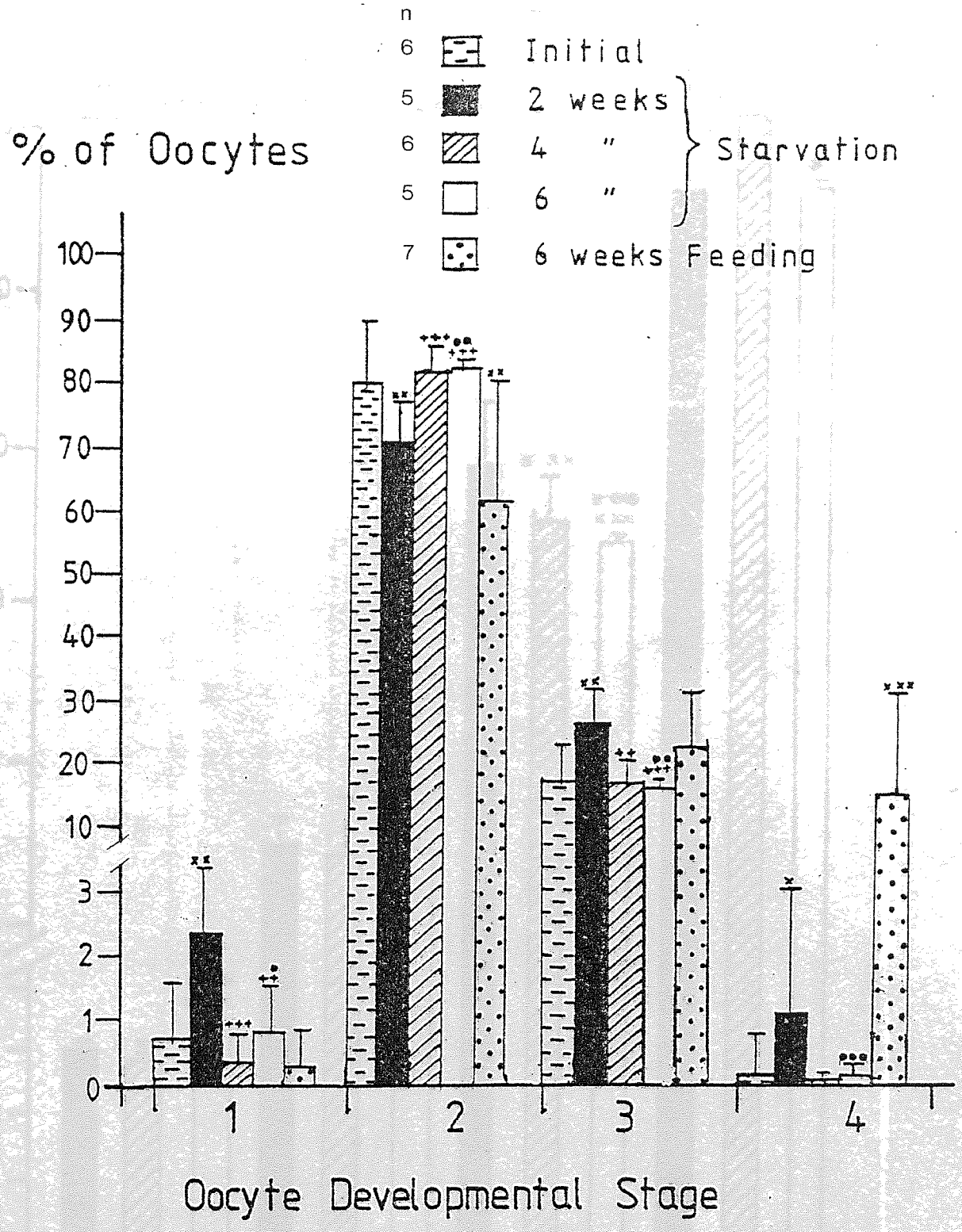

Fig 6.3 - Effects of starvation up to 6 weeks on the percentage of oocytes at different developmental stages (1-4) in 1+ year-old fish. Each value indicates the mean ± S.D. for number of fish (n). (× - significant difference from the initial, + - significant difference from fish starved for 2 weeks , ● -significant difference from fed-fish. *, x, ● - $0.10 < P < 0.50$; **, xx, ●● - $P < 0.05$; ***, xxx ●●● - $P < 0.005$.

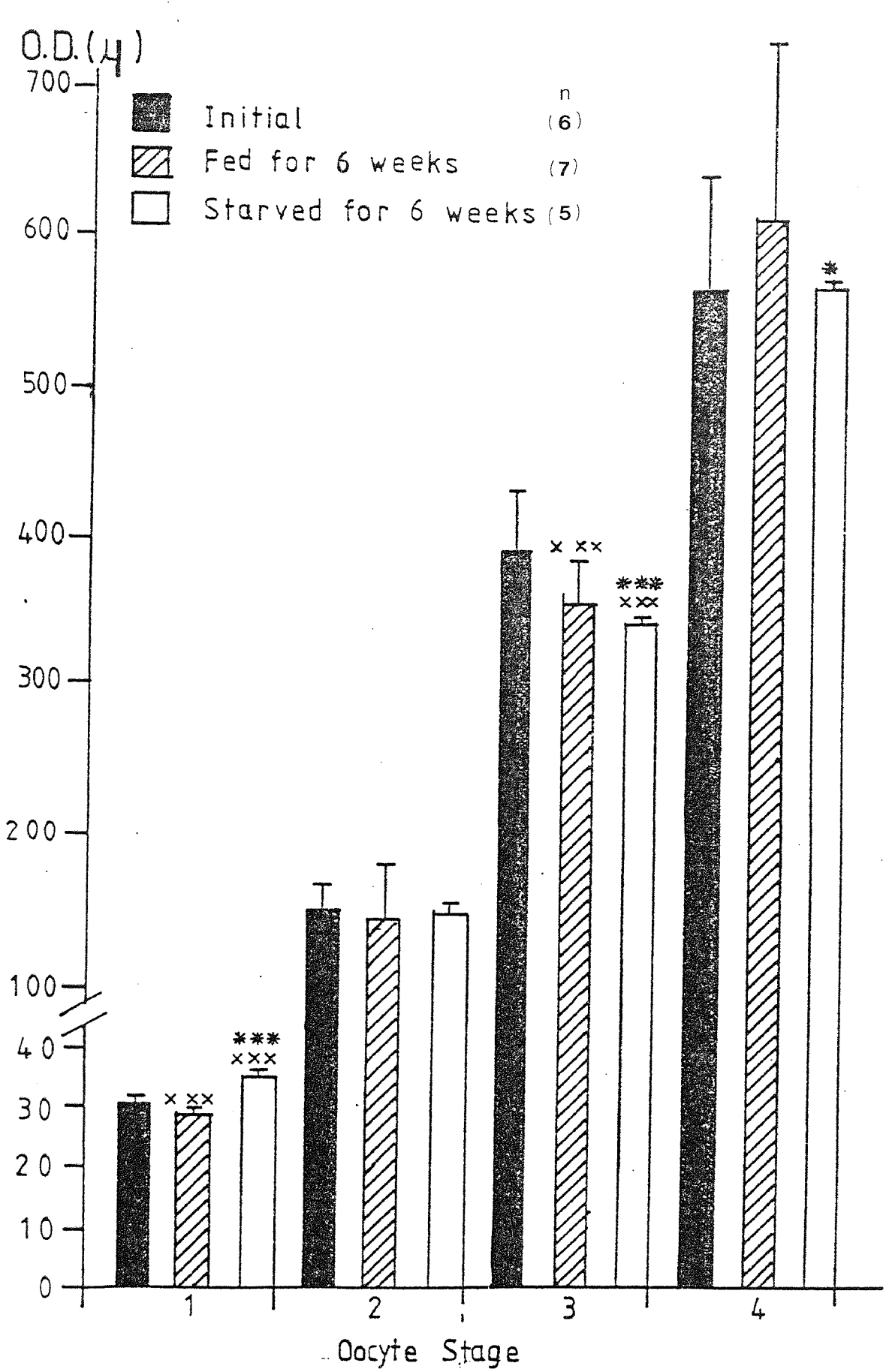

Fig 6.4 - Effects of starvation up to 6 weeks on the size (diamter - OD) of previtellogenic oocyte at different stages (1-4) in the ovaries of 1+ year-old immature fish. Each value indicates the mean ± S.D. for number of fish (n). (* - significant difference from the fed controls, x - significant difference from the initial) * - $P<0.05$, ***, xxx - $P<0.0005$.

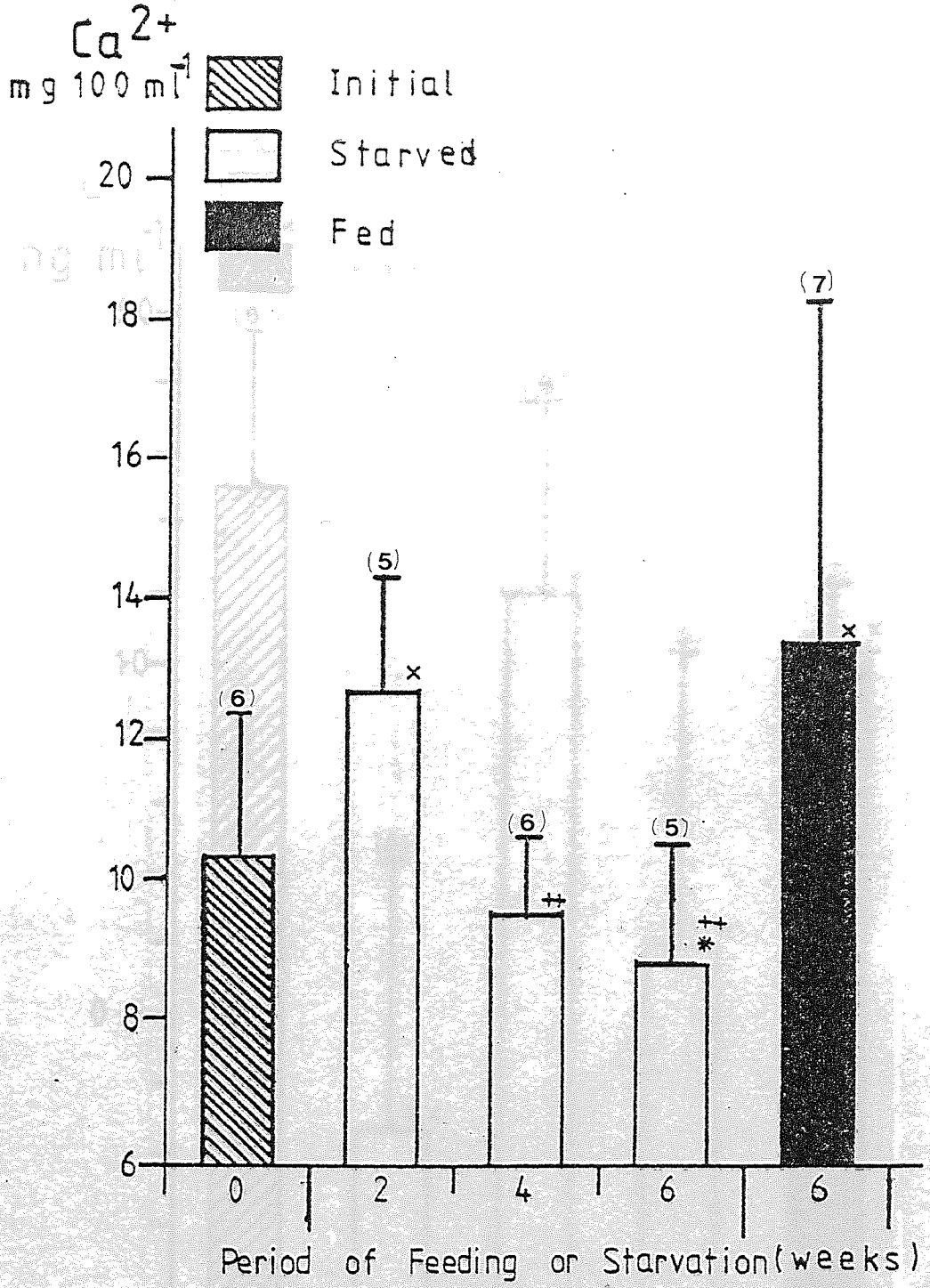

Fig 6.5 - Effects of starvation up to 6 weeks on the serum calcium (Ca) levels of 1+ year-old immature fish. Each value indicates the mean ± S.D. for number of fish (n).
(* - significant difference from fed controls, x - significant difference from the initial, + - significant difference from the fish starved for 2 weeks).
*, x, + - $P<0.05$; ++ - $P<0.005$.

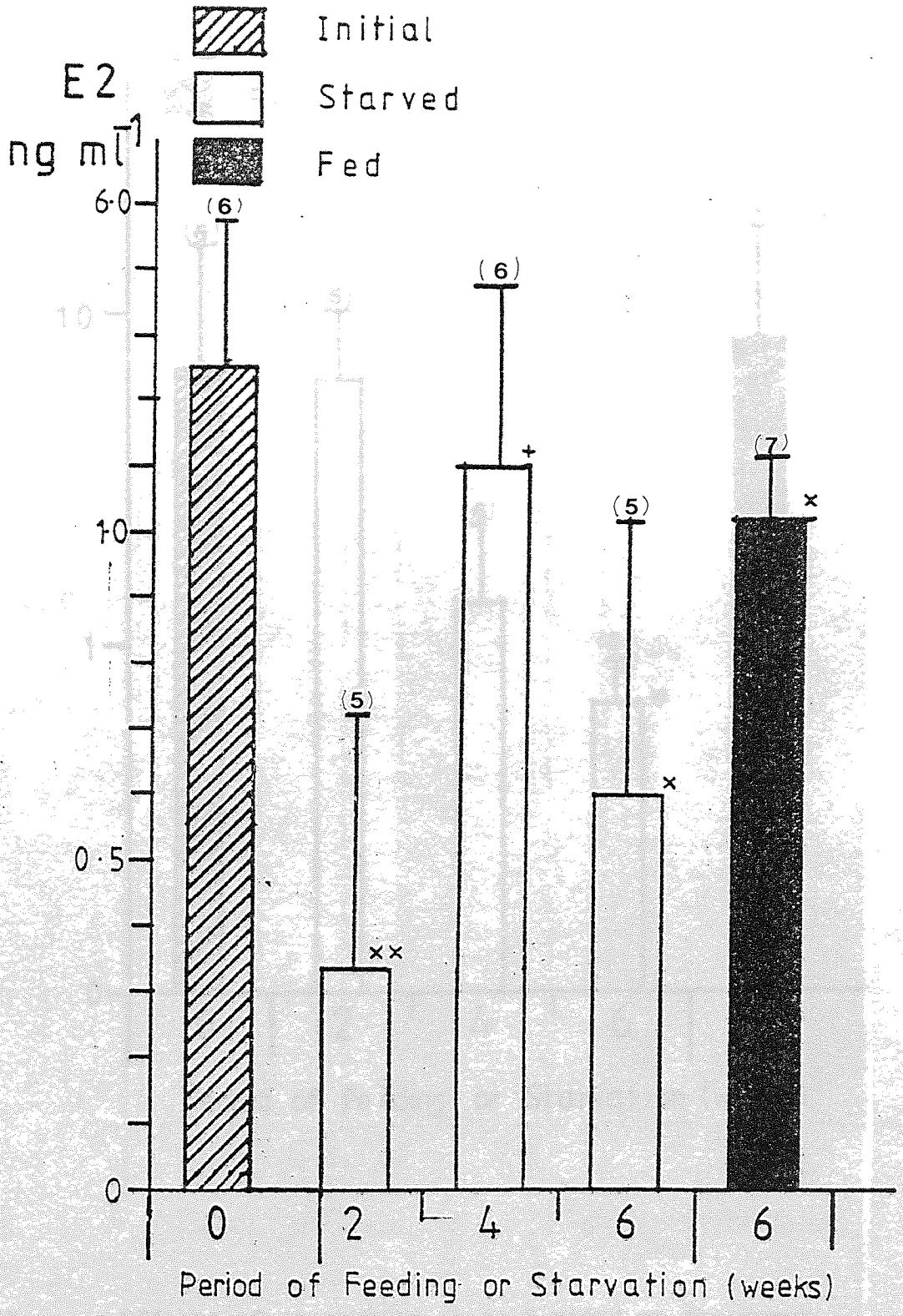

Fig 6.6 - Effects of starvation up to 6 weeks on the serum oestradiol (E2) levels in 1+ year-old immature fish. Each value indicates the mean ± S.D. for number of fish (n). (x - significant differencefrom the initial, + - significant difference from the fish starved for 2 weeks). x, + - $P<0.05$; xx - $P<0.005$.

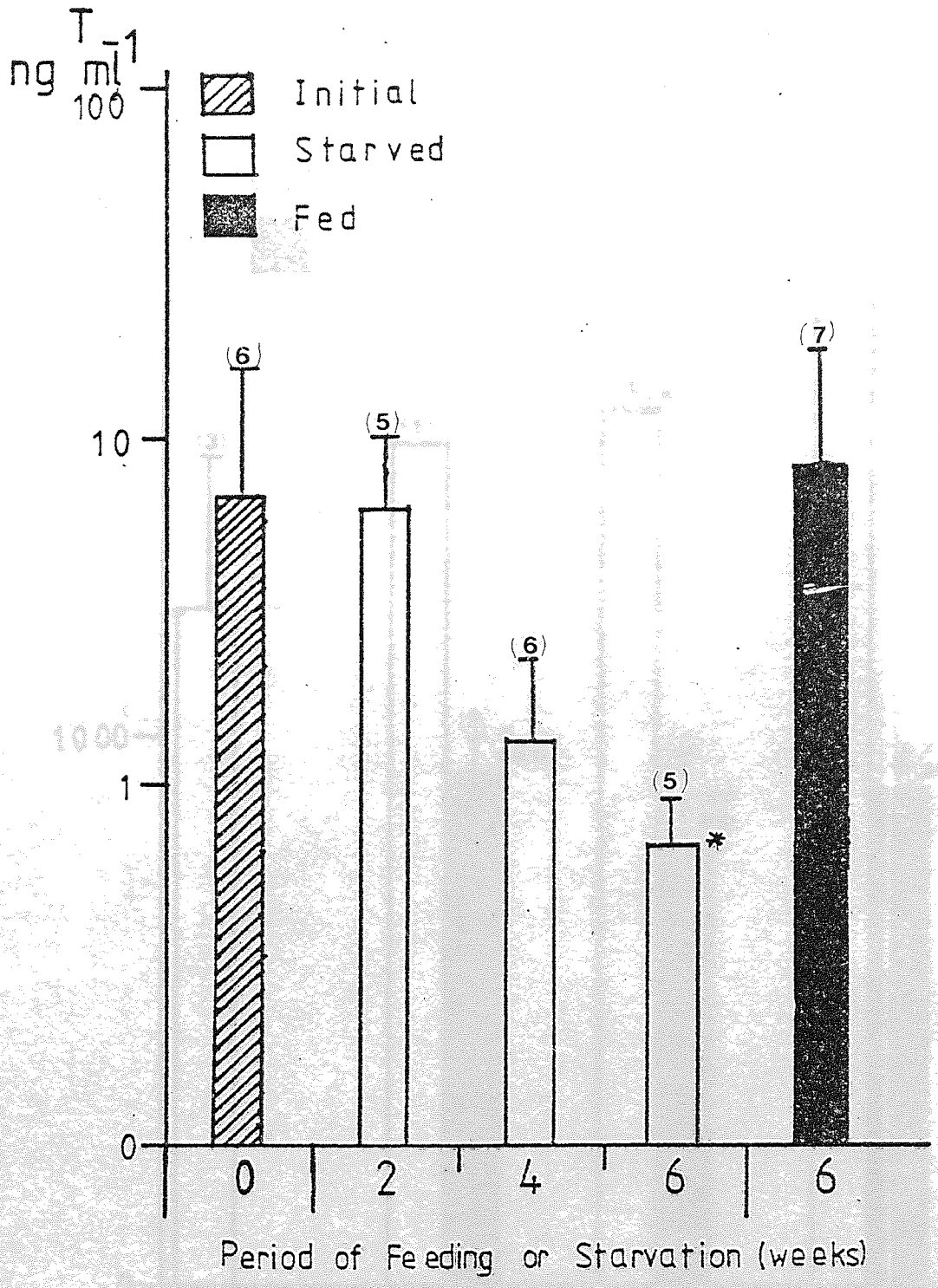

Fig 6.7 - Effects of starvation up to 6 weeks on the serum testosterone (T) levels in 1+ year-old immature fish. Each value indicates the mean ± standard S.D. for number of fish (n). (* - significant difference from the fed controls $P<0.05$).

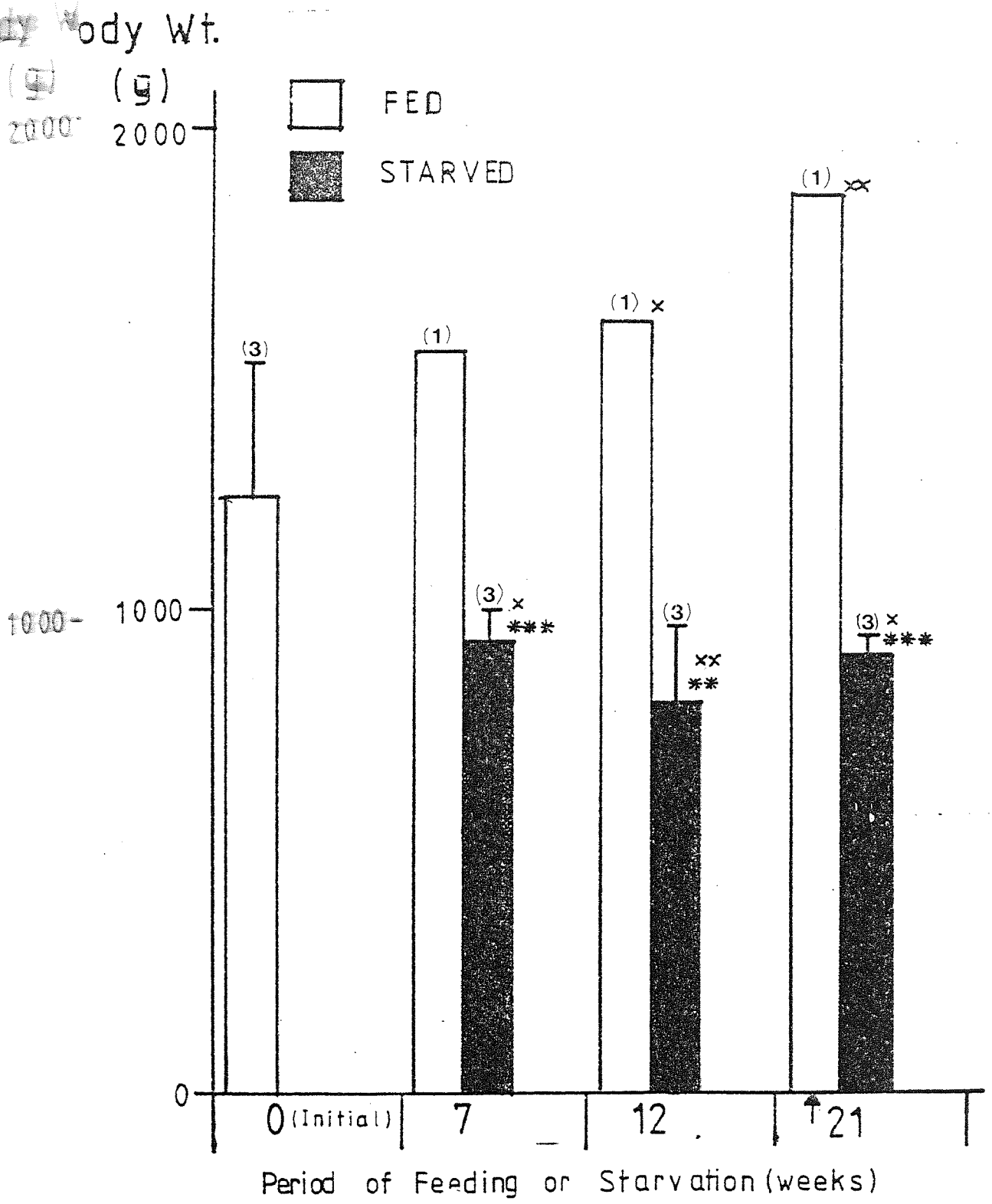

Fig 6.8 - Effects of starvation up to 21 weeks on the Body weight (B.Wt.) of 2+ year-old fish with vitellogenic ovaries. Each value indicates the mean ± S.D. for number of fish (n). (* - significant difference from the controls, x - significant difference from the initial, ↑ - spawning of controls.
x - 0.10 P<0.5; **, xx - P<0.05; *** - P<0.005; **** - P<0.0005.

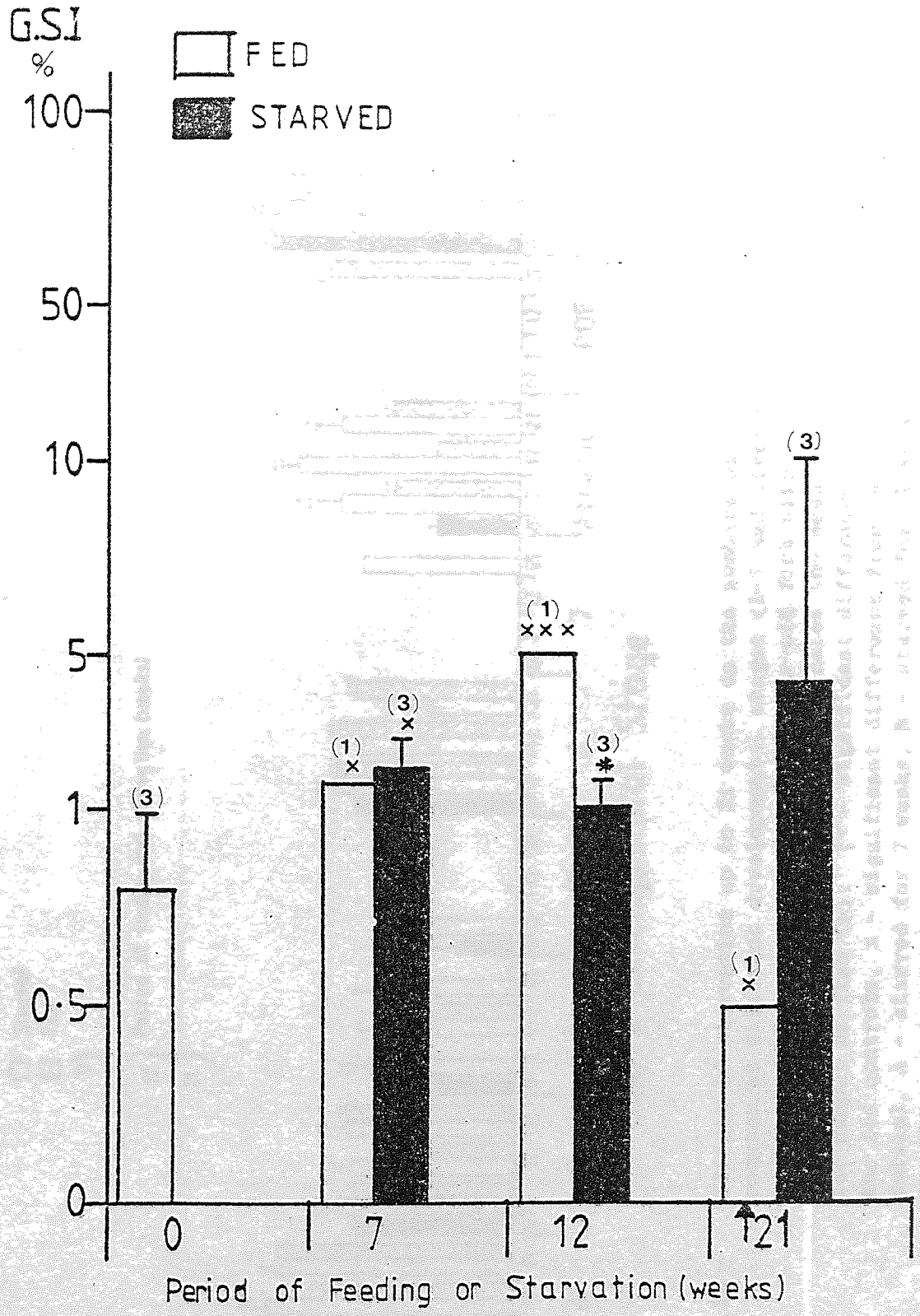

Fig 6.9 - Effects of starvation up to 21 weeks on the gonadosomatic index (G.S.I.) of 2+ year-old fish with vitellogenic ovaries. Each value indicates the mean ± S.D. for number of fish (n). (* - significant difference from the controls, x - significant difference from the initial, - spawning of controls).
*, x - $P<0.05$; xxx - $P<0.0005$.

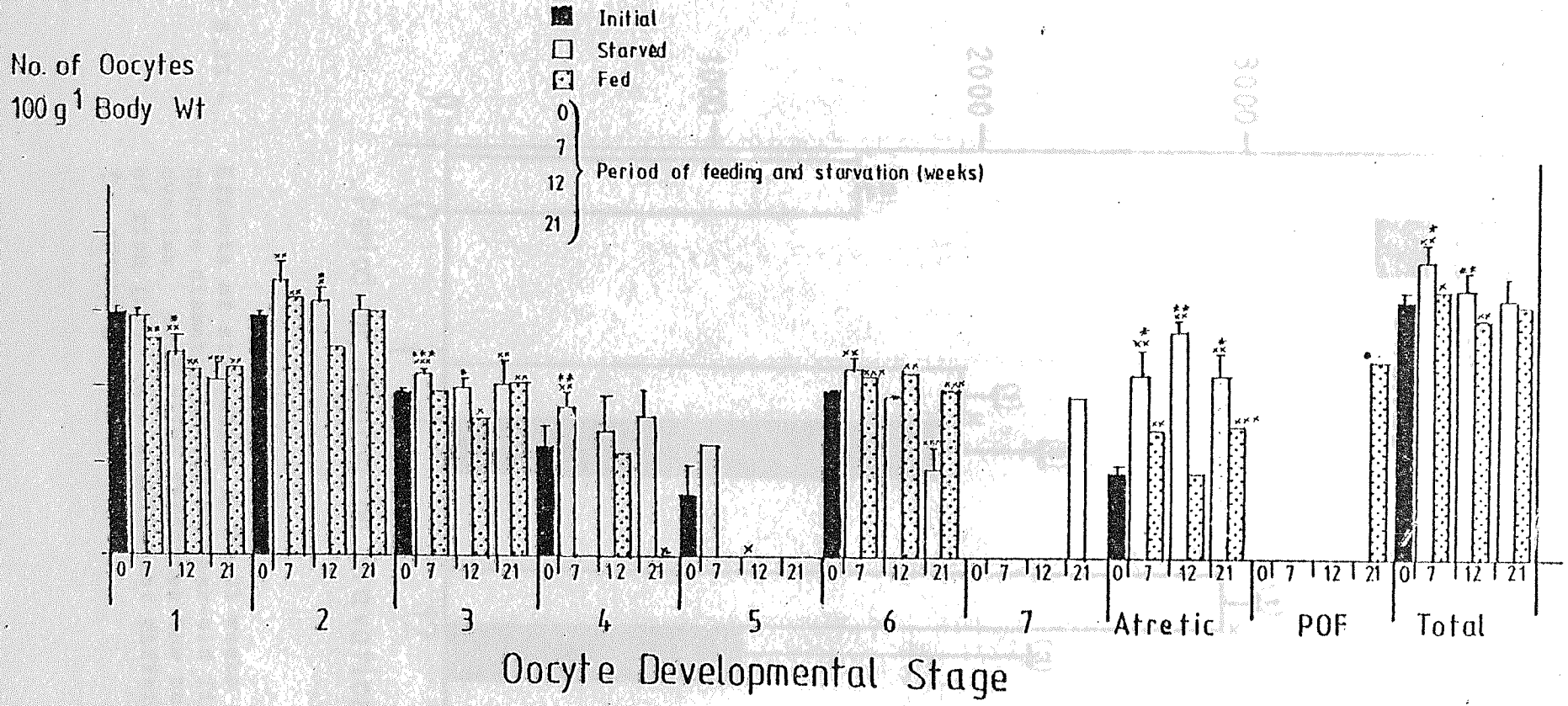

Fig 6.10 - Effects of starvation up to 21 weeks on the numbers of oocytes at different developmental stages (1-7 and atretic) and post-ovulatory follicles in 2+ year-old fish with vitellogenic ovaries. Each value indicates the mean ± S.D. for number of fish (n). (* - significant difference from the fed controls, x - significant difference from the initial, A - starved for 7 weeks, B - starved for 12 weeks, C - starved for 21 weeks, • - spawning of controls.

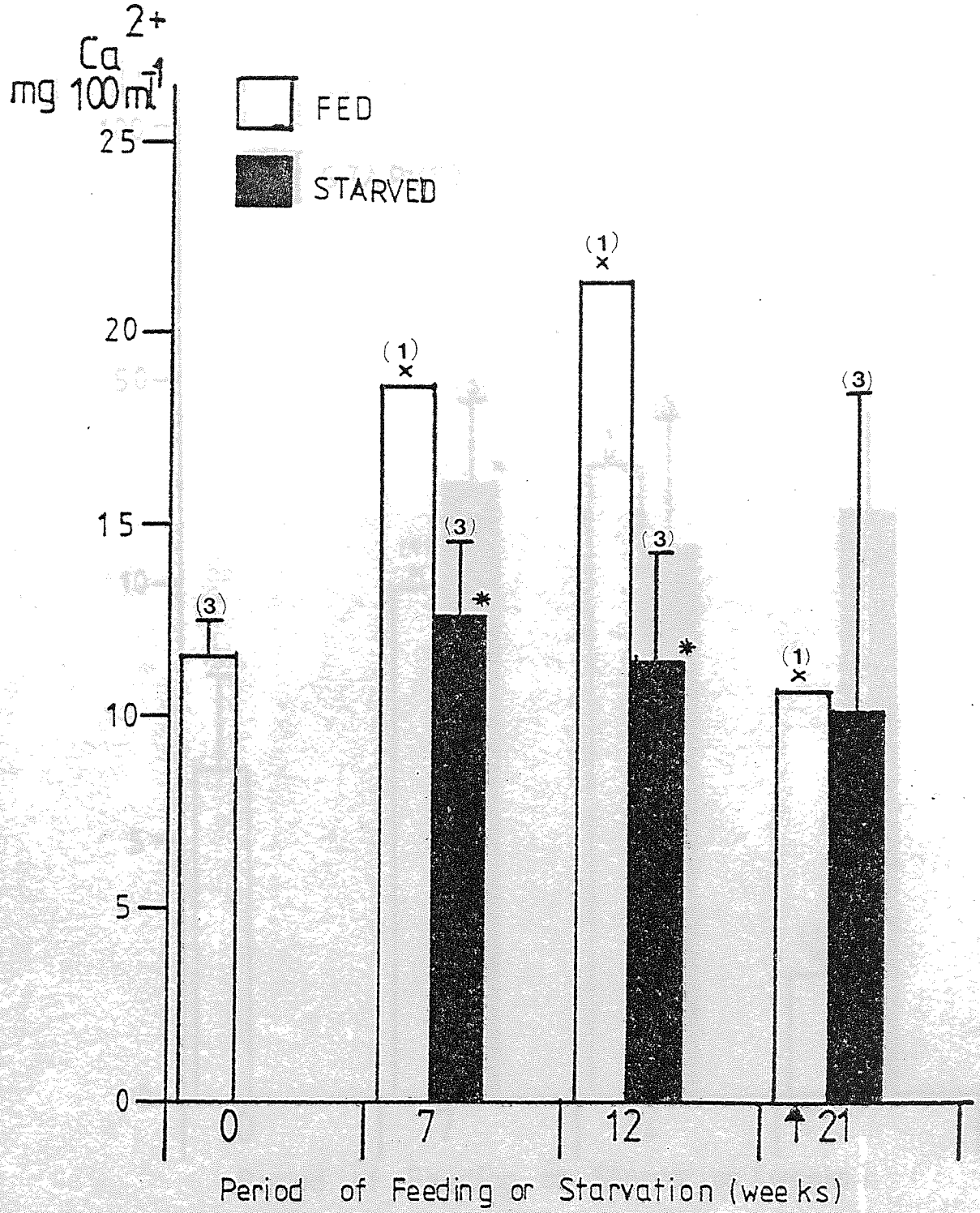

Fig 6.12 - Effects of starvation up to 21 weeks on the serum calcium levels of 2+ year-old fish with vitellogenic ovaries. Each value indicates the mean ± S.D. for number of fish (n). (* - significant difference from controls, x - significant difference from the initial, ↑ - spawning of controls).

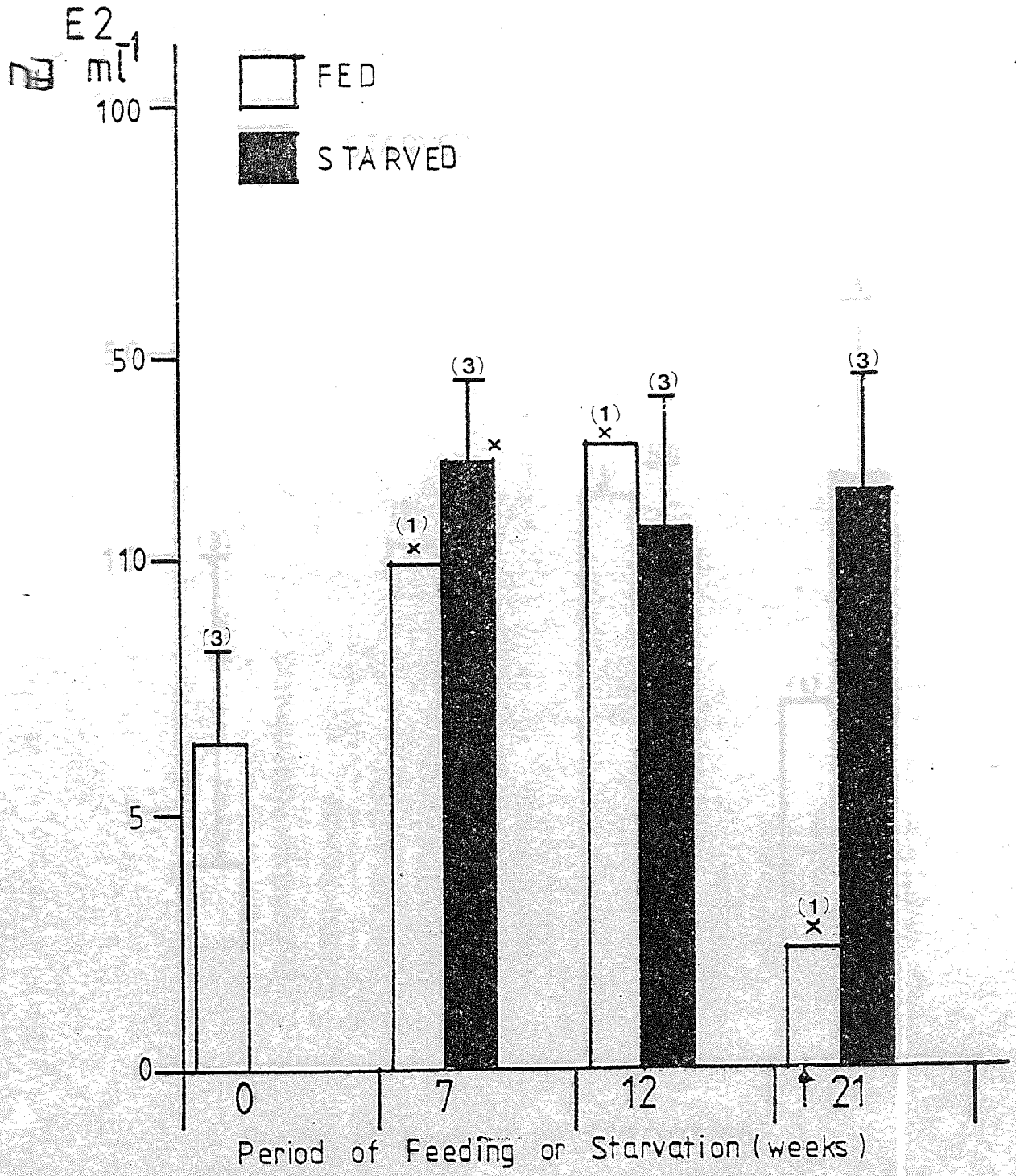

Fig 6.13 - Effects of starvation up to 21 weeks on the serum oestradiol-17β (E2) levels of 2+ year-old fish with vitellogenic ovaries. Each value indicates the mean ± S.D. for number of fish (n). (x - significant difference from the initial, ↑ - spawning in controls).

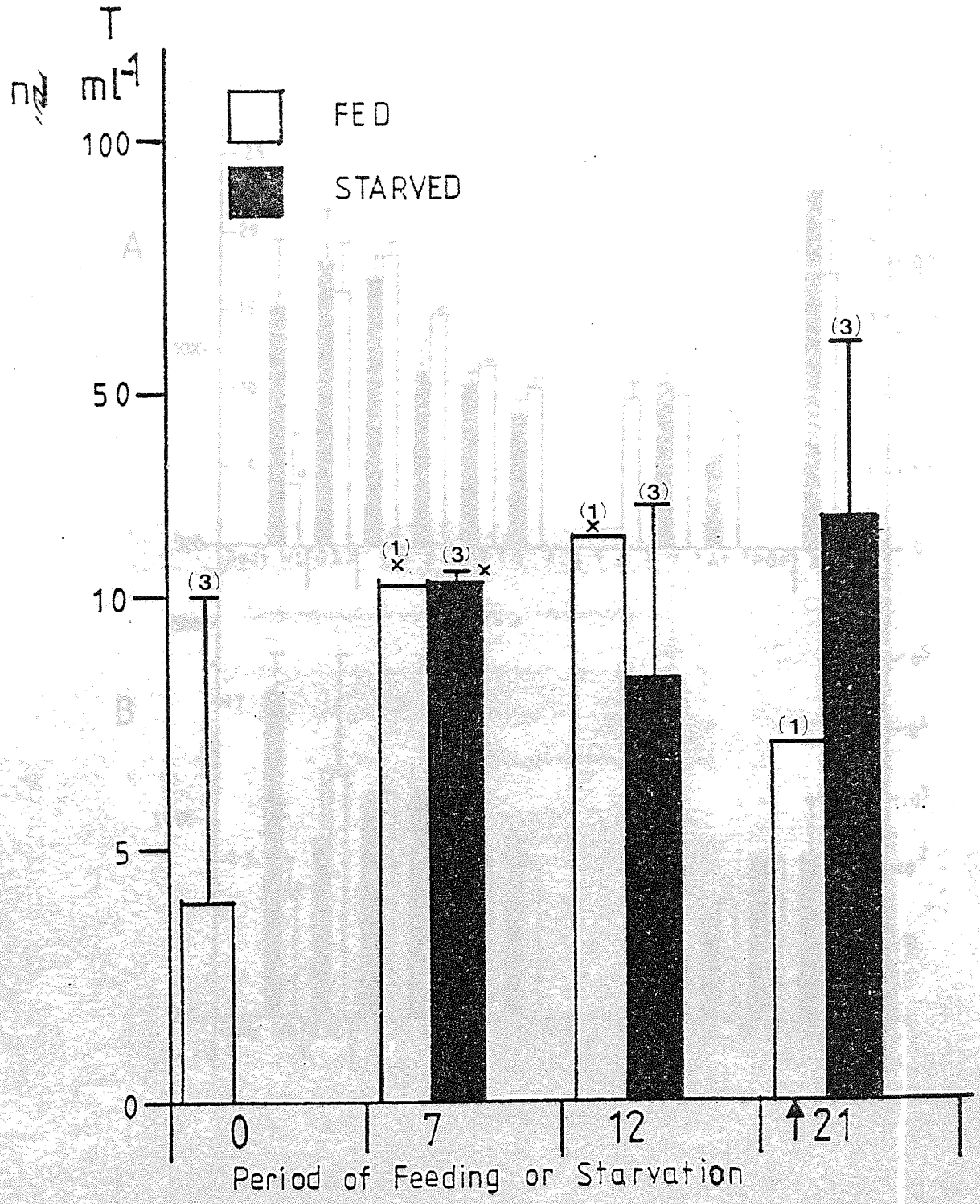

Fig 6.14 - Effects of starvation up to 21 weeks on the serum testosterone (T) levels of 2+ year-old fish with vitellogenic ovaries. Each value indicates the mean ± S.D. for number of fish (n). (x - significant difference from the intial, spawning in controls).

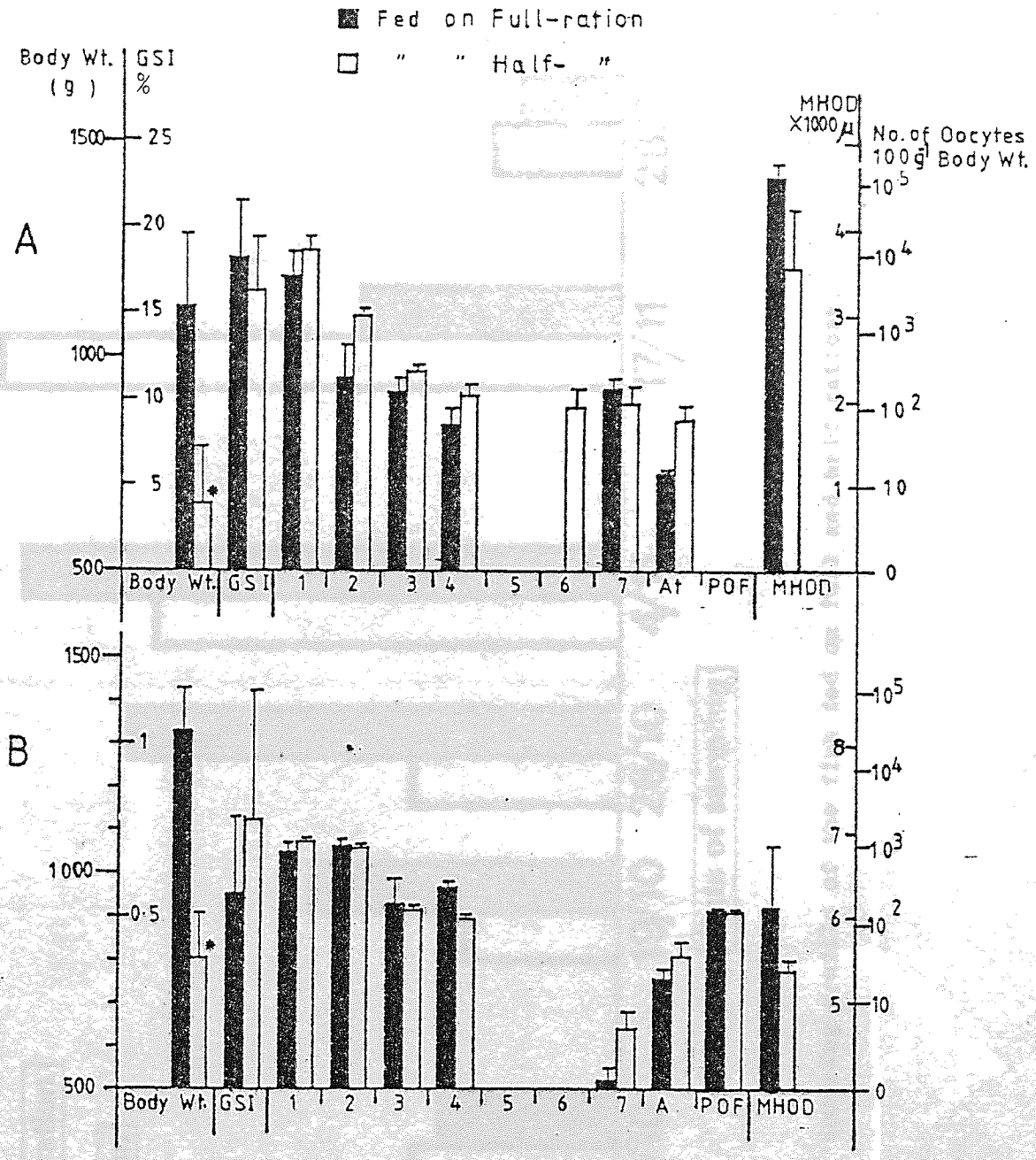

Fig 6.15 - Effects of feeding on full and Half-rations for 12 months on the Body weight (B.Wt.); gonadosomatic index (G.S.I.), mean highest oocyte diameter (MHOD) and number of oocytes at different developmental stages (1-7 and atretic) and post-ovulatory follicles in 3+ year-old fish before (A) and after (B) spawning. Each value indicates the mean ± S.D. (* - significant difference from the full-ration fed fish).

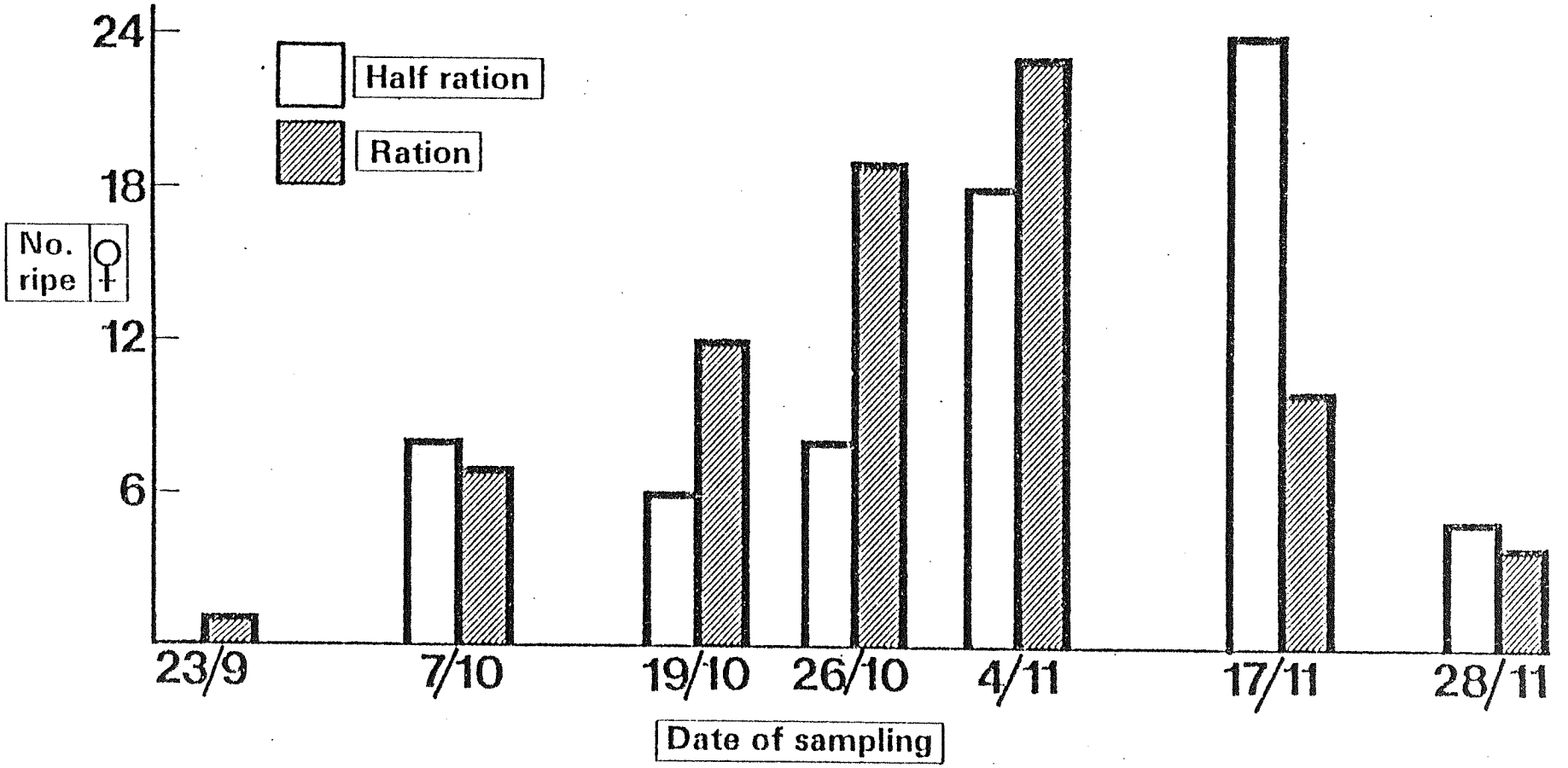

Fig 6.16 - Spawning profile of the fish fed on full and half rations.

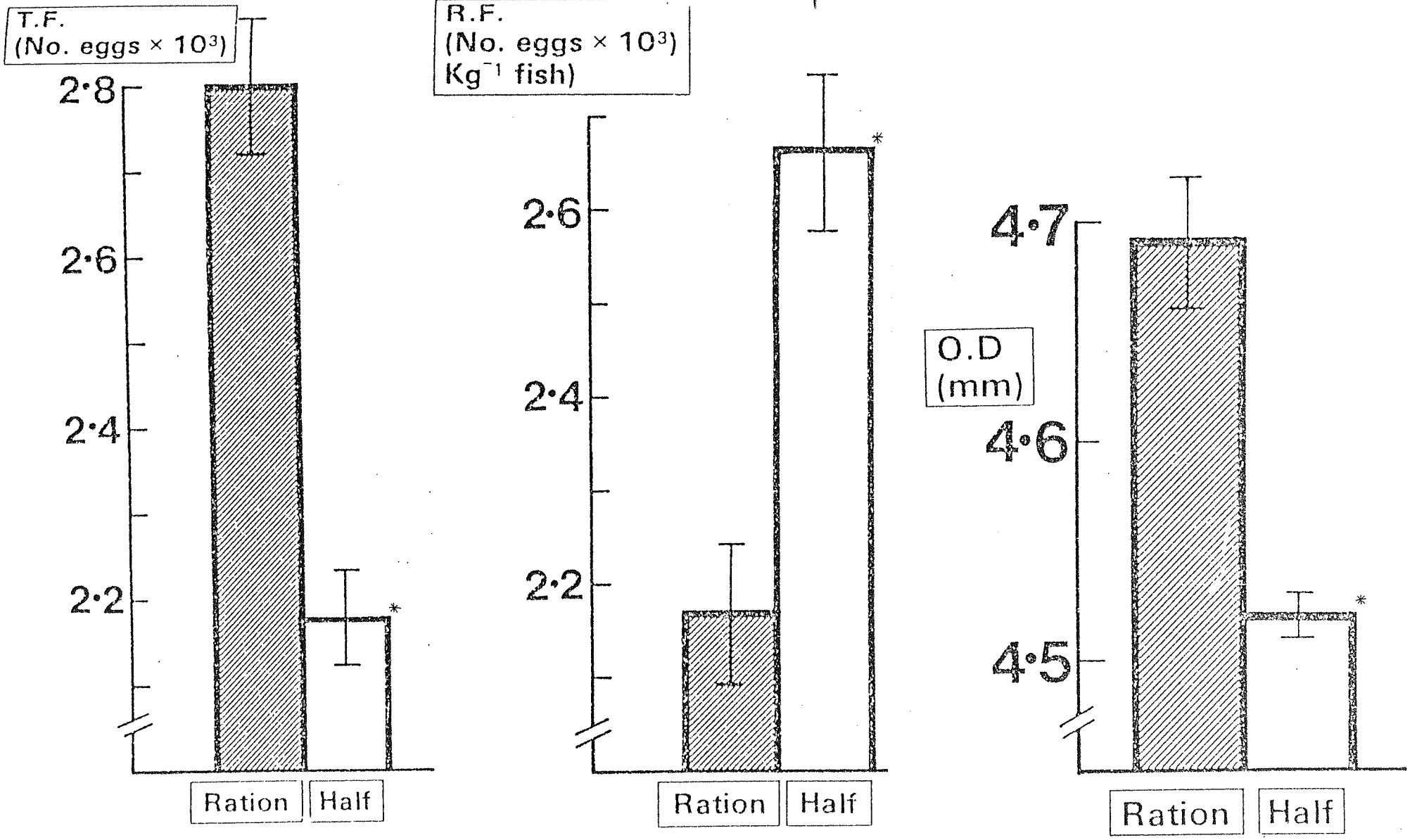

Fig 6.17 - Total fecunidty (T.F.), relative fecundity (R.F.) and ovar diameter of the fish fed on full and half rations. (* - significant difference from the full-ration fed-fish).

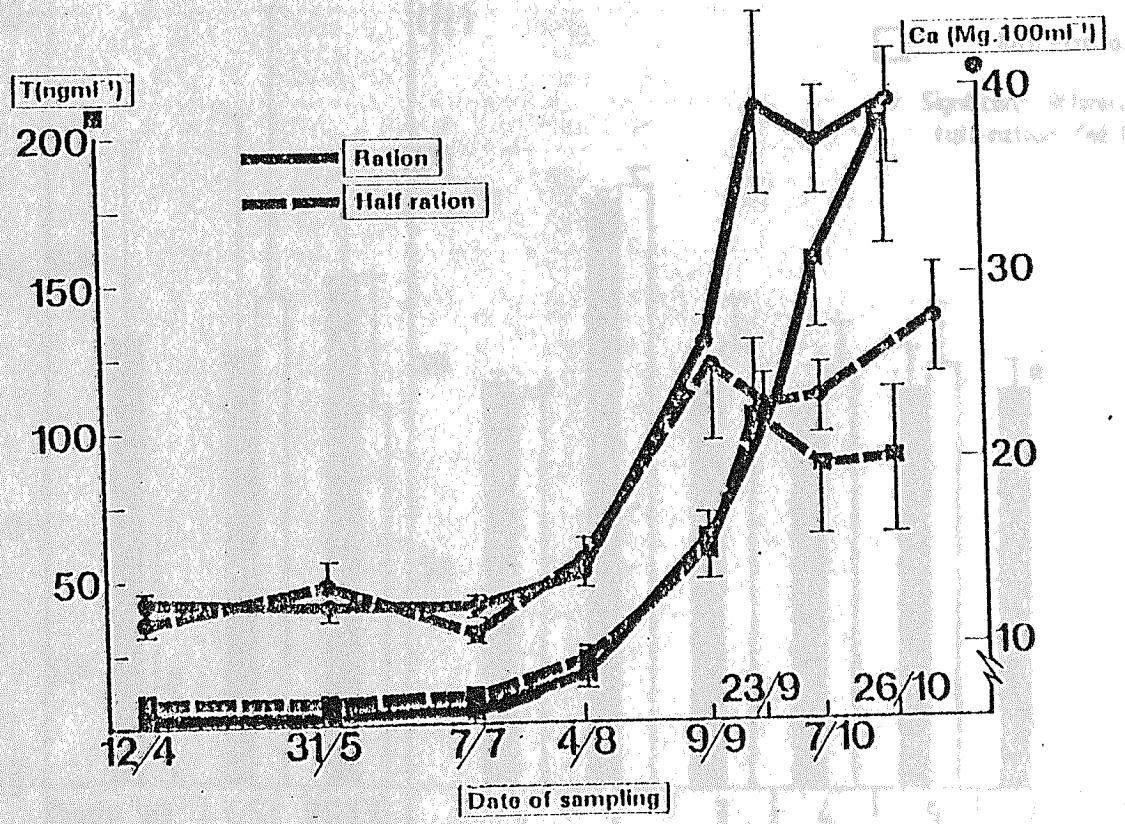

Fig 6.18 - Changes in circulating levels of testosterone and calcium in fish fed on full and half rations.

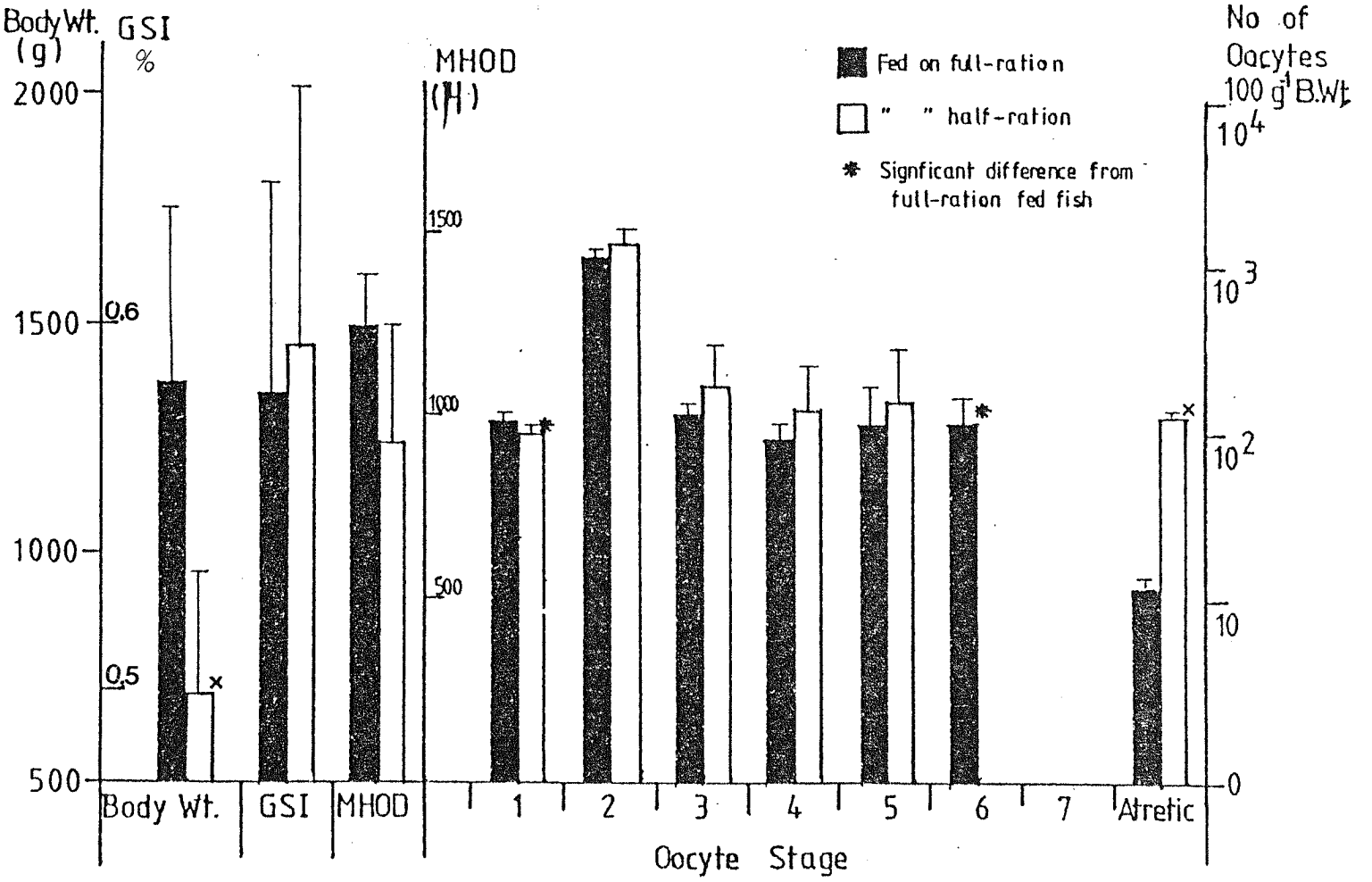

Fig 6.19 - Effects of feeding on full and half rations for 18 months on the body weight (B.Wt.) gonadosomatic index (G.S.I.), mean highest oocyte diamter (MHOD) and the numbers of oocytes at different developmental stages (1-7 and atretic) in 3+ year-old fish. Each value indicates the mean ± standard deviation (S.D.). (* - significant difference from the full-ration fed-fish).

		3		11.44		6.8	0.0	

Period weeks	Treatment	n	Body weight g	GSI	MHOD μ	Ca mg 100ml^{-1}	E_2 ng ml^{-1}	T ng ml^{-1}
0	Initial		1125	0.7164	1558	11.44	6.8	0.0
			1035	0.9333	1575	10.67	8.3	11.0
			1550	0.7419	1505	12.55	4.6	1.85
		3	1236.67 ± 275.06	0.7972 ± 0.1186	1546 ± 37	11.55 ± 0.95	6.4 ± 1.85	3.95 ± 6.12
7	Starved		935	1.4342	1703	12.27	43.0	10.6
			880	1.6341	1517	11.16	11.0	10.08
			995	2.9477	2290	14.42	34.0	15.2
		3	x *** 936.67 ± 57.52	xx 2.0053 ± 0.8222	xxx 1837 ± 403	** 21.34 ± 2.83	xx * 29.33 ± 16.50	x 11.96 ± 2.82
	Fed	1	1530	1.7019 xxx	1940 xxx	18.40 xxx	9.9 xx	11.9 x
12	Starved		725	0.1945	490	9.92	10.06	2.32
			985	3.0569	2365	14.72	31.8	22.6
			715	0.1986	473	9.73	7.50	0.0
		3	xx * 808.33 ± 153.08	** 1.1500 ± 1.6514	* 1103 ± 132	** 11.46 ± 2.83	* 16.45 ± 13.35	8.31 ± 12.43
	Fed	1	1600 x	5.0163 xxxx	2934 xxx	21.34 xxx	33 xxx	21.6 xx
21	Starved		875	12.3634	3603	14.66	4.9	65.2
			900	0.1289	495	9.64	5.3	5.8
			950	0.2926	495	11.48	17.0	5.0
		3	x **** 908.33 ± 38.18	4.2616 ± 7.6168	xxx 1531 ± 1794	11.93 ± 2.54	23.77 ± 22.62	x 25.33 ± 34.53
	Fed	1	1865 x	0.4976 xx	357 •	10.64	2.3 xx	7.1

Table 6.1.1 - Effects of long-term starvation (for 21 weeks) on the body weight (B.Wt.), gonadosomatic index (G.S.I.), mean highest oocyte diameter (MHOD) and circulating levels of calcium (Ca), oestradiol-17β (E_2) and testosterone (T) in 2+ year-old vitellogenic rainbow trout. Values given are the mean ± standard deviation (SD) where appropriate. (n - number of fish, * significant difference from the fed-controls, x significant difference from the initial, • - spawning in fed-controls).
*,x - $0.10 < P < 0.05$; **,xx - $P < 0.05$; ***,xxx - $P < 0.005$; ****,xxxx - $P < 0.0005$).

N.B. Values for individual fish are also given to show the differences within a group.

Period weeks	Treatment	n	No 100^{-1} Body weight Oocyte developmental stage 1	2	3	4	5	6	7	Atretic	POF	Total
0	Initial		1276 1970 1423	1388 2029 774	100 195 117	68 26 12	00 16 6	162 198 151	00 00 00	00 16 18	00 00 00	2991 4438 2501
		3	1556 ± 366	1397 ± 628	137 ± 51	35 ± 29	7 ± 8	170 ± 25	00	11 ± 10	00	3252 ± 1080
7	Starved		1137 1925 628	4856 7973 3828	371 329 383	24 88 146	84 42 00	393 599 340	00 00 00	139 561 421	00 00 00	7006 11515 5746
		3	1229 ± 655	5552 ± 2158 xx	361 ± 28 xxx ***	86 ± 61 xx **	42 ± 42	444 ± 137 xx	00	374 ± 215 xx *	00	8089 ± 3033 xx *
	Fed	1	740 xx	3208 xx	139	00	00	344 xxx	00	63 xx	00	4495 x
12	Starved		499 453 814	1488 3989 4428	148 155 293	74 00 98	00 00 00	00 370 00	00 00 00	797 602 1248	00 00 00	3007 5366 6881
		3	589 ± 196 xx *	3302 ± 1586 x *	199 ± 82 *	57 ± 51	00	123 ± 214	00	882 ± 331 xx **	00	5151 ± 1970 **
	Fed	1	370 xx	780	69 x	28	00	125 xx	00	14	00	1385 xx
21	Starved		427 348 19	3696 2659 204	558 129 33	198 12 15	00 00 00	46 00 00	351 00 00	600 372 145	00 00 00	5676 3579 415
		3	265 ± 216 xxx	2186 ± 1793	240 ± 280 xx	75 ± 107	00	15 ± 27 xxx	117 ± 203	372 ±278 xx *	00	3669 ± 2737
	Fed	1	416 xx	1722	246 xx	00 x	00	00 xxx	00	69 xxx	551 •	2994

Table 6.1.2 - Effects of long-term starvation (for 21 weeks) on the numbers of oocytes at different developmental stages (stages 1-7 and atretic) and post-ovulatory follicles (POF) in 2+ year-old, vitellogenic rainbow trout. Numbers are the mean ± standard deviation (SD) where appropriate; (n - number of fish, * significant difference from the fed-controls, x significant difference from the initial, • - spawning in fed-controls). (*,x - $0.10 < P < 0.05$; **,xx - $P < 0.05$; ***,xxx - $P < 0.005$).

N.B. Values for individual fish are also given to show the differences within a group.

State of fish	Ration $100g^{-1}$ B.wt.	n	B. wt. g	G.S.I.	No 10^{-1} Body weight Oocyte stage 1	2	3	4	5	6	7	Atretic	POF	Total	MHOD μ
Pre-spawned	0.7		998	15.1784	11046	7305	417	104	00	00	264	26	00	19162	4733 ± 223
			1235	20.9530	3336	1957	162	61	00	00	425	23	00	5967	4479 ± 214
		2	11165	18.0657	7193	4631	290	83	00	00	345	25	00	12565	4606
			± 168	± 4.0833	± 3782	± 3782	± 180	± 30	00	00	± 114	± 2	00	± 9330	± 180
	0.35		750	18.4960	23493	3428	291	156	00	00	317	51	00	27736	4033 ± 224
			560	14.0393	569	2478	806	348	00	367	41	120	00	4738	3077 ± 208
		2	655 *	16.2677	12031	2953	549	252	00	125	179	86 *	00	16237	3555 *
			± 134	± 3.1514	± 16210	± 672	± 364	± 136	00	± 217	± 195	± 49	00	± 16262	± 676
Post-spawned	0.7		1265	0.5202	1291	724	145	84	00	00	2	30	277	2553	544 ± 87
			1400	0.7386	605	1660	554	518	00	00	00	45	272	3654	675 ± 149
		2	1333	0.6294	948	1192	350	301	00	00	1	38	275	3104	610
			± 95	± 0.1544	± 485	± 662	± 289	± 307	00	00	± 1	± 11	± 4	± 779	± 93
	0.35		730	1.0438	1873	1129	303	148	00	00	5	42	273	3773	527 ± 78
			875	0.504	1743	971	220	197	00	00	8	65	220	3424	544 ± 86
		2	803 **	0.7739	1808 *	1050	262	173	00	00	7	54	247	3599	536
			± 103	± 0.3817	± 92	± 112	± 59	± 35	00	00	± 2	± 16	± 37	± 247	± 12

Table 6.2 - Effects of feeding on full and half-rations for 12 months on the body weight (B.wt.), gonadosomatic index (GSI), mean highest oocyte diameter (MHOD) and number of oocytes at different developmental stages (stages 1-7 and atretic) and post-ovulatory follicles (POF) in 3 year-old rainbow trout before and after spawning. Values given are the mean ± standard deviation (SD) where appropriate. (* significant difference from the full-ration fed-fish). (* - $0.10 < P < 0.05$; ** - $P < 0.05$).

N.B. Values for individual fish are also given to show the differences within a group.

Ration $100g^{-1}$ B.wt.	n	B. wt. g	C.S.I.	No of oocytes at different stages. $100g^{-1}$ body weight. 1	2	3	4	5	6	7	Atretic	Total	MHOD μ
0.7		1165	0.5931	154	1764	258	37	36	163	00	12	2424	1385.34 ± 116.16
		1135	0.6308	215	1953	187	142	22	288	00	11	2818	1238.49 ± 51.48
		1810	0.5188	154	1323	157	110	384	00	00	22	1967	1103.85 ± 73.9
	3	1370 ± 381	0.5809 ± 0.0570	174 ± 35	1680 ± 323	201 ± 52	96 ± 54	147 ± 205	150 ± 144	00	15 ± 6	2403 ± 426	1242.45 ± 140.91
0.35		690	0.5899	66	3225	608	497	569	00	00	155	5120	1080.42 ± 69.96
		855	0.6655	117	2120	265	142	248	00	00	160	3052	1133.88 ± 71.94
		515	0.5262	125	1378	188	54	00	00	00	224	1969	566.61 ± 105.27
	3	687 ** ± 170	0.5939 ± 0.0697	103 ** ± 32	2241 ± 929	354 ± 224	231 ± 235	272 ± 285	00 * 00	00	180 ** ± 38	3380 ± 1601	926.97 * ± 313.17

Table 6.3 – Effects of feeding on full and half-rations for 18 months on the body weight (B.wt.), gonadosomatic index (GSI), mean highest oocyte diameter (MHOD) and number of oocytes at different developmental stages (stages 1-7 and atretic) and post-ovulatory follicles (POF) in 3+ year-old rainbow trout before and after spawning. Values given are the mean ± standard deviation (SD). (* significant difference from the full-ration fed-fish). (* - $0.10 < P < 0.05$; ** - $P < 0.05$).

N.B. Values for individual fish are also given to show the differences within a group.

6.4 Discussion.

The most significant finding in the present investigation was the larger number of atretic oocytes present within the ovaries of rainbow trout subjected to either starvation or feeding on low rations. Previous studies of the effects of feeding on the reproduction of fish have focussed mainly on the reductions in the number of mature ova, ie. the fecundity, produced by fish fed on low rations (McFadden et al, 1965; Mackay and Mann, 1969; Bagenal, 1969; Wootton, 1973; Baiz, 1978; Kuznetsov and Khalitov, 1978), with little consideration of the mechanisms by which this reduction was achieved. Reductions in total fecundity were also observed in the present work on rainbow trout maintained on half the ration specified by the Feed Manufacturer's tables. Both Scott (1962) and Townshend and Wootton (1984) suggested that the reductions in fecundity which followed reduced feeding were due to atresia. In contrast Bagenal (1969) reported that only low levels of atresia were present in mature brown trout fed on low rations and concluded that the differences in fecundity could not be accounted for by this process. Possibly, Bagenal (1969) reached these conclusions because he was not aware that atresia can occur at any time in the oocyte developmental cycle; and not just in the mature oocytes. Furthermore, it is often not realised that the "brown bodies" which are widely found in mature ovaries, are the terminal stages of atretic process.

Scott (1962) reported reductions of 27% of oocytes in rainbow trout fed on low rations and attributed these reductions to atresia. However, in an earlier chapter (Chapter 4) it was revealed that levels of atresia of this magnitude can occur during the natural

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Scott (1962) reported reductions of 27% of oocytes in rainbow trout fed on low rations and attributed these reductions to atresia. However, in an earlier chapter (Chapter 4) it was revealed that levels of atresia of this magnitude can occur during the natural

cycle even in apparently well-fed fish. In contrast Henderson (1963b) recorded levels of 6% atresia in brook trout and suggested that atresia may not occur in natural populations although no mention was made of feeding rate, Vladykov (1956) also working with the brook trout suggested that the 80% and 45% reductions which occurred in oocytes' numbers up to 1 mm and 3.5 mm in diameter respectively were due to atresia althouogh no counts of atretic oocytes were made. Levels of 27% atresia were also recorded for natural populations of chub sucker with figures for individual fish ranging from 4-72% (Wagner and Cooper, 1963). In the present experiments the total number of oocytes present within previtellogenic ovaries was more than 15,000 oocytes $100g^{-1}$ body weight whereas in mature ovaries this number was reduced to between 2000 and 3000 oocytes $100g^{-1}$ body weight. Of these only 200-500 oocytes $100g^{-1}$ body weight would be expected to be ovulated at the end of the maturation cycle. Therefore the 27% reduction in number of oocytes reported by Scott (1962) may be a naturally occurring phenomenon and not due to the feeding of low rations. Furthermore, the maximum number of oocytes reported by Scott (1962) was 12.655 oocytes g^{-1} body weight, well below the total number of oocytes observed for the fish fed on half-ration during the present study. Thus, it would appear that all the fish used in Scott's (1962) study, even those with the highest fecundities, had been subjected to reduced rations. This is supported by the low final weights of fish (100-200g) which served as "fully-fed" controls. Under normal farmed conditions in the U.K. it would be expected that 3 year-old fish would weigh upwards of $1\frac{1}{2}$ kg. Townshend and Wootton (1984) in a controlled experiment with convict cichlid observed a reduction in the percentage of vitellogenic

oocytes as a result of feeding low-rations and attributed this reduction to atresia, although again no counts of the numbers of ateretic oocytes were included. During the present work, reductions in vitellogenic oocytes were observed in both the fish fed on half-ration and those starved over a prolonged period and, furthermore, these reductions were paralleled by corresponding increases in the proportions of atretic oocytes.

In the experiments on pre-spawned fish it was difficult to estimate the number of oocytes which might be ovulated at the end of the oocyte developmental cycle, because in the fish subjected to restricted diets much higher rates of atresia and delays in recruitment to higher developmental stages were observed. However, in the fish which had been starved or fed on half ration of food, vitellogenic oocytes were reduced in number or totally absent. This shows that diet restriction either reduces fecundity or inhibits the production or maturity of ripe eggs. During the present study 11% of the fish on half-ration failed to spawn whereas the remainder of the group produced eggs.

Without exception all the studies mentioned above estimated levels of atresia indirectly by calculating either the reductions in percentage of vitellogenic oocytes (Townshend and Wootton, 1984), or in the total number of oocytes (Vladykov, 1956; Scott, 1962; Wagner and Cooper, 1963).

Only Scott (1962) and Towshend and Wootton (1984) identified atretic oocytes in the sections of the ovary. No atretic oocytes were reported in the other investigations although, the reductions in oocyte numbers were attributed to atresia. This may have been due to the difficulties of identifying atretic oocytes macroscopically or

under the microscope; the random distribution of the atretic oocytes within the ovary may also have led to their exclusion from the small portions of ovary which are generally used for histological studies. Atretic oocytes and especially the "brown bodies" which represent the final stages of resorptive process, are much smaller and less obvious than the generally larger vitellogenic or mature oocytes. Thus, the number of atretic oocytes within a mature ovary may not be fully appreciated on superficial examination. Atretic oocytes whether of stage 4, 5 or 6 or of the brown body stage constituted important proportions of the total number of oocytes in the vitellogenic ovary and were widely seen both macroscopically and microscopically in the present study.

The highest number of atretic oocytes were seen in fish starved for 12 weeks; here atresia amounted to 882 ± 331 oocytes $100g^{-1}$ body weight, whereas, only 14 atretic oocytes $100g^{-1}$ body weight were observed in the control fish. Coincident with the increases in proportion of atretic oocytes in starved fish there were also reductions in the total number of vitellogenic oocytes. Most of the starved fish had no vitellogenic oocytes of stages 5, 6 or 7, in their ovaries after 12 weeks of starvation. Comparatively low but significant increases in the proportion of atretic oocytes were also observed in fish maintained on reduced rations for 18 months, and again these increases were matched by corresponding reductions in the numbers of vitellogenic oocytes. Similar reductions in the numbers of vitellogenic oocytes have also been observed in convict cichlids fed on low-rations (Townshend and Wootton, 1984) although in this work proportion of vitellogenic oocytes was expressed as a percentage of the total number of oocytes. This mode of expression may give

rise to some errors because the total number of previtellogenic and vitellogenic oocytes show considerable seasonal variation even in fish maintained under apparently constant conditions (Chapter 4). In order to limit over or under-estimations of the levels of atresia or of the proportions of the different stages of oocytes, the relative numbers of oocytes at different stages were calculated on the basis of body weight. Body weight was used because G.S.I. and fecundity measurements are usually made on the basis of fish size. Only where the ovaries contained no vitellogenic oocytes, were the proportions of oocytes [illegible] expressed as a percentage of total number.

In the majority of studies which have considered the [illegible] relationships between food and reproduction, only the effects of [illegible] ration on the numbers of vitellogenic oocytes and the final stages of maturation have been considered. No information is provided of the effects on fish at earlier stages of development when only [illegible] previtellogenic oocytes are present. The starvation of immature fish produced significant increase in the numbers of stage 1 and 2 oocytes and reductions in numbers of stage 3 and 4 oocytes when compared to fish fed on full-ration. The decreases in number of stage 3 and 4 oocytes in the starved fish were associated with the appearance of large empty spaces between the oocytes. It is proposed that these spaces are possibly the only remaining evidence for the regression or atresia of small previtellogenic oocytes. Byskov (1978) suggested that previtellogenic oocytes may disappear in large numbers from the ovary without leaving any trace. During the present investigations, although atretic structures of stage 1, 2 and 3 oocytes were not identified, atretic structures of late stage 4 oocytes were observed both in maturing rainbow trout fed on half-ration for 12 or 18 months

and also in mature fish which had been starved for 12 weeks. The widespread atresia of stage 4 oocytes in these groups is probably the reason for the reductions in number or complete absence of vitellogenic stage 5 oocytes in the ovaries of these fish. Clearly, the regression of stage 4 oocytes would prevent the recruitment of previtellogenic oocytes into vitellogenesis. Both Robb (1982) and Townshend and Wootton (1984) suggested that reduced food supplies could cause modifications in the recruitment of oocytes into vitellogenic phases, although no histological evidence was provided in either of the studies.

Reduced ration or starvation have also been said to affect the recruitment of oocytes to higher developmental stages in non-salmonid fish (Hester, 1964; Tyler and Dunn, 1976; Townshend and Wootton, 1984). Conversely de Vlaming (1972) reported that starvation had no effect on the recruitment of oocytes into both the previtellogenic and vitellogenic phases of development. Reductions in numbers of vitellogenic oocytes may occur as a result of either atresia and/or a delay in recruitment of previtellogenic oocytes into vitellogenesis. In all mature fish subjected to starvation or low rations of food, vitellogenic oocytes were found to be at least one stage behind in development when compared to fish on full ration. This slowing in development was also confirmed by the 3 to 4 week delay in spawning of the fish fed on half-ration when compared with the fish on full ration. Histological examination of the ovaries of the starved or half-ration fish revealed large numbers of atretic stage 4 oocytes. This suggests that the delay in onset of vitellogenesis was due to the absence of sufficient oocytes in the late vesicle stage (stage 4 oocytes), a stage which links the

previtellogenic and exogenous vitellogenic phases of oocyte development. Consequently one might conclude that in addition to its effect on atresia of vitellogenic and possibly previtellogenic oocytes, food deprivation also reduces the proportions of oocytes which are recruited into the vitellogenesis. Townshend and Wootton (1984) also observed reductions in vitellogenic oocytes in convict cichlid subjected to low rations, although they failed to evaluate actual number of vitellogenic oocytes subjected to atresia. In addition, Scott (1962) observed a reduction in the number of maturing trout. However, interpretation of Scott's data are difficult because only 5% of his experimental and control fish attained maturity at the end of the experiment. A delay in maturation was also reported by Bagenal (1969) in brown trout subjected to diet restrictions. Vladykov (1956) found that the age of first spawning was advanced in brook trout fed on better diets. This conclusion was not however supported by a later study of the same species and area by Gibson et al, (1976). Spawning time was also delayed by reduced rations in the multiple-spawning convict cichlid (Townshend and Wootton (1984).

In addition to the possible effects on atresia and the final maturation of vitellogenic oocytes, reductions in ration may also modify the development of previtellogenic oocytes. The starvation of immature fish produced a significant increase in numbers of stage 1 and 2 previtellogenic oocytes. Observations of the oocyte distribution of these fish showed that oogenesis had occurred in both the starved and fed groups with oocytes having been recruited into stage 2. The numbers of stage 2 oocytes were far higher in starved fish whereas stage 3 oocytes were reduced. This suggests that the

recruitment of oocytes to stage 3 may have been delayed. In mature fish fed on half-ration for 18 months a significant reduction in stage 1 oocytes was seen. This could have been due to a resorption of the stage 1 oocytes or to a delay in the recruitment of secondary oogonia into stage 1 primary oocytes. Unfortunately, the proportions of oogonia were not taken into account during oocyte counting as they are far too small to be observed under binocular examination. However, oogonia were present in both the fed and starved fish.

It has also been suggested that, the availability of food may affect the size of eggs produced (Townshend and Wootton, 1984) although Scott (1962), Baiz (1978), Kuznetsov and Khalitov (1978) and Ridelman et al, (1984) found no evidence of such effects in their studies of fish on restricted diets. These results conflict with those of the present work where the size of the oocytes was reduced in both the pre and post-ovulatory ovary. The mean diameters of the highest developmental stage of oocyte present in the starved fish and in the fish fed on half-ration were also significantly lower than those of the controls fed on full-ration. This could have been due to a delay in oocyte recruitment in starved and half-ration fed fish. A corresponding size reduction was also seen at spawning time where the fish fed on half-ration produced significantly smaller ripe eggs than those from the fully-fed group.

In the majority of studies of food availability and fecundity, no information is provided of the effects of diet on the endocrine mechanisms which control ovarian development. At spawning, fish maintained on half-ration in the present study had significantly lower vitellogenin and testosterone levels than the fully-fed fish. Both changes may be related to the reductions in size and number of

eggs produced at spawning by these fish. Starved fish, whether mature or immature, also had significant reductions in vitellogenin, oestradiol-17β and testosterone. Collectively these results suggest a causative relationship between the reductions in levels of steroid hormones and vitellogenin, the number and sizes of the vitellogenic oocytes and the delay and/or reduction in the recruitment of previtellogenic oocytes into vitellogenesis. This relationship is supported by the range of levels of these serum constituents seen in the groups of fish which had been starved. Clearly, starvation had affected different fish in different ways. Initially all fish used for this experiment had stage 5 and 6 (germinal vesicle migration stage) oocytes but after 12 weeks of starvation in the majority of fish there were decreases in G.S.I., and proportions and diameters of vitellogenic oocytes together with decreases in serum calcium, oestradiol-17β and testosterone levels. In contrast, oocyte development continued in the remaining fish of the starved group and the levels of calcium, oestradiol-17β and testosterone were similar to those of the fully-fed controls. Possibly, the differences within the starved group may be due to the presence of a critical phase within stage 6 (germinal vesicle migration stage) at which further growth and vitellogenic development of the oocyte requires adequate supplies of food. After this stage, further oocyte growth and maturation occurs irrespective of the availability of food. This proposition, however, needs further attention. Kato (1975) and Ridelman et al, (1984) both reported that diet restrictions either for 4 months or 40 days before spawning respectively produced no changes in either fecundity or egg size in the rainbow trout although in neither case was any indication given of the percentage of fish which attained maturity.

In summary the present results show that the reduction in fecundity which follow diet restriction occur as a result of increases in the levels of atresia and delays in the recruitment of oocytes into vitellogenesis.

Decreased levels of circulating oestradiol-17β which in turn would have caused a decrease in vitellogenin may have affected the growth of vitellogenic oocytes resulting in smaller eggs in the fish subjected to restricted diets. Finally, there was evidence for the existence of a critical phase during the development of the stage 6 oocytes after which restrictions in diet produce no further effects on maturation.

CHAPTER 7

General Conclusions

General Conclusions

1. Ovarian development was studied in three strains of rainbow trout using histological techniques and also estimates of the total numbers of oocytes at the different developmental stages. Seven stages of oocyte development, primary and secondary oogonia and two types of atresia were identified. The seven oocyte stages were as follows: Stage 1 - chromatin nucleolar stage, stage 2 - early-perinucleolar stage, stage 3 - late-perinucleolar stage, stage 4 - ooplasmic vesicle stage, stage 5 - yolk incorporation stage, stage 6 - germinal vesicle migration stage and stage 7 - germinal vesicle breakdown stage. The twotypes of atresia were hypertrophic and non-hypertrophic (ie. oolytic) atresia.

2. Differences in the distribution of "Balbiani bodies" and staining affinities of the ooplasm allowed further subdivision of Balbiani body stage (stage 2) into three, ie. 2a, 2b and 2c. Subdivision of stage 4, ie. 4a and 4b, was also carried out on the basis of differences in distribution of the ooplasmic vesicles.

3. Sex differentiation occurred in the female fish at 50-60 days after hatching and was marked by the appearance of primary oogonia. After this time oogonia were present throughout the cyclclical development of the ovary, with largest numbers occurring during post-ovulatory period. As a result of oogonial proliferation oogonia increased in number and also produced secondary oogonia.

4. Oogenesis which was marked by the appearance of primary oocytes at chromatin nucleolar stage occurred when the fish were 4 to 5 months old. These oocytes reached stage 4 (ooplasmic vesicle stage) by the end of the first year of life in the fish from all three strains which were destined to spawn at the end of their second year of life. After the first spawning, stage 4 oocytes were found in all the ovaries sampled with the maximum numbers being found just before exogenous vitellogenesis. Thus, it is clear that ooplasmic vesicle formation does not occupy distinct phases of ovarian development unlike the exogenous vitellogenin incorporation which is restricted to the summer months.

5. The close association of both the endoplasmic reticulum and Golgi complex, with the ooplasmic vesicle support the endoplasmic origins of the vesicles. No link could be established between the formation of the ooplasmic vesicles and the yolk granules, globules or platelets. However, the vesicles close to the nucleus lost their contents during the early stages of vitellogenin incorporation which suggest that vesicular materials are utilized by the oocyte during development.

6. Use of the horseradish peroxidase (HRP) showed that large molecules (and probably vitellogenin) are taken up through the microvilli of the oocyte membrane of vitellogenic oocytes.

7. The development of the micropyle prior to ovulation and the positioning of the micropylar cell suggests that it is formed from one of the large granulosa cells near the animal pole of the maturing oocyte.

8. The theca of the post-ovulatory follicle reorganised its structure, shortly after ovulatiion to form a germinal epithelium. It is suggested that oogonia are formed from this epithelium.

9. Atretic oocytes were commonly encountered in both the microscopical sections of the ovary and during the macroscopic examination and counting of the numbers of oocytes at the different developmental stages. In some circumstances almost all the vitellogenic oocytes were atretic, eg. after starvation. In well-fed fish levels of atresia ranged between 150-170 atretic oocytes $100g^{-1}$ of body weight which amount to 5-10% of the total numbers of oocytes and up to 50% of the total numbers of vitellogenic oocytes in the ovary or numbers of the ovulated eggs. Two forms of atresia were seen among stage 4, 5, 6 and 7 oocytes, ie. hypertrophic and non-hypertrophic (oolytic) atresia. They were mostly found in vitellogenic or post-ovulated ovaries.

10. No atresia was seen in stages 1-4 although it is probable that this process is responsible for the 70-90% reduction in oocyte numbers which occurs during the period of maturation.

11. Similar patterns of ovarian development and profiles of changing serum oestradiol-17β, testosterone and calcium levels were seen in all three strains and age groups of rainbow trout. In each case there were differences in the timing of onset and duration of these changes, which were associated with the differences in

rates of maturation and spawning times of the different strains and age groups of fish.

12. Two forms of ovarian development were seen in each of the three 1+ year-old groups of fish. In the first type vitellogenesis began in August and the fish proceeded to spawn in the Autumn. In the second type which comprised approximately 33% of the total numbers of fish. No oocytes beyond early stage 4 (4a) were seen in the fish failed to mature fully at the end of their second year of life. However, atretic oocytes were present in the ovaries of non-spawning fish at the same time when fish of the spawning group started yolk incorporation.

13. Vitellogenin uptake began under increasing or long daylengths. There were differences in the timing of this change in the two age groups of spawning fish with yolk incorporation occurring during July/August in the 1+ year-old fish and March/April in the 2+ year-olds.

14. Levels of testosterone revealed very high values during oocyte maturation and ovulation suggesting a possible role for this hormone in final maturation.

15. Oestradiol-17β administration during the vitellogenic phase of ovarian development did not inhibit oogenesis or vitellogenin incorporation, although it produced significant increases in the levels of atresia. There were also significant reductions in the levels of testosterone after oestradiol-17β administration,

although it is not clear whether this change is linked to the increase incidence of atresia. It is more probable that this effect is due to the inhibition by oestradiol-17β of the secretion of vitellogenic and/or maturational gonadotropin.

16. Food deprivation caused an increase in the number of atretic oocytes, a delay or a reduction in the recruitment of oocytes into vitellogenesis and later stages of development, and reductions in fecundity and the sizes of eggs produced. Fish fed on half-ration showed significant reductions in body weight and total fecundity when compared to fish fed on full-ration, although their relative fecundities were significantly higher. In the majority of starved fish all the vitellogenic oocytes became atretic. There were significant reductions in the numbers of previtellogenic oocytes although there were no signs of atresia. This suggests that diet restriction affects the numbers of both previtellogenic and vitellogenic oocytes.

17. In both starved and low ration fed fish there was evidence for tht existence of a critical phase during germinal vesicle migration (ie. two month prior to maturation) after which restrictions in diet produce no further effects on oocyte development. Furthermore, diet restrictions produced reductions in serum oestradiol, testosterone and calcium levels (all changes which may have affected vitellogenic growth and maturation of oocytes.

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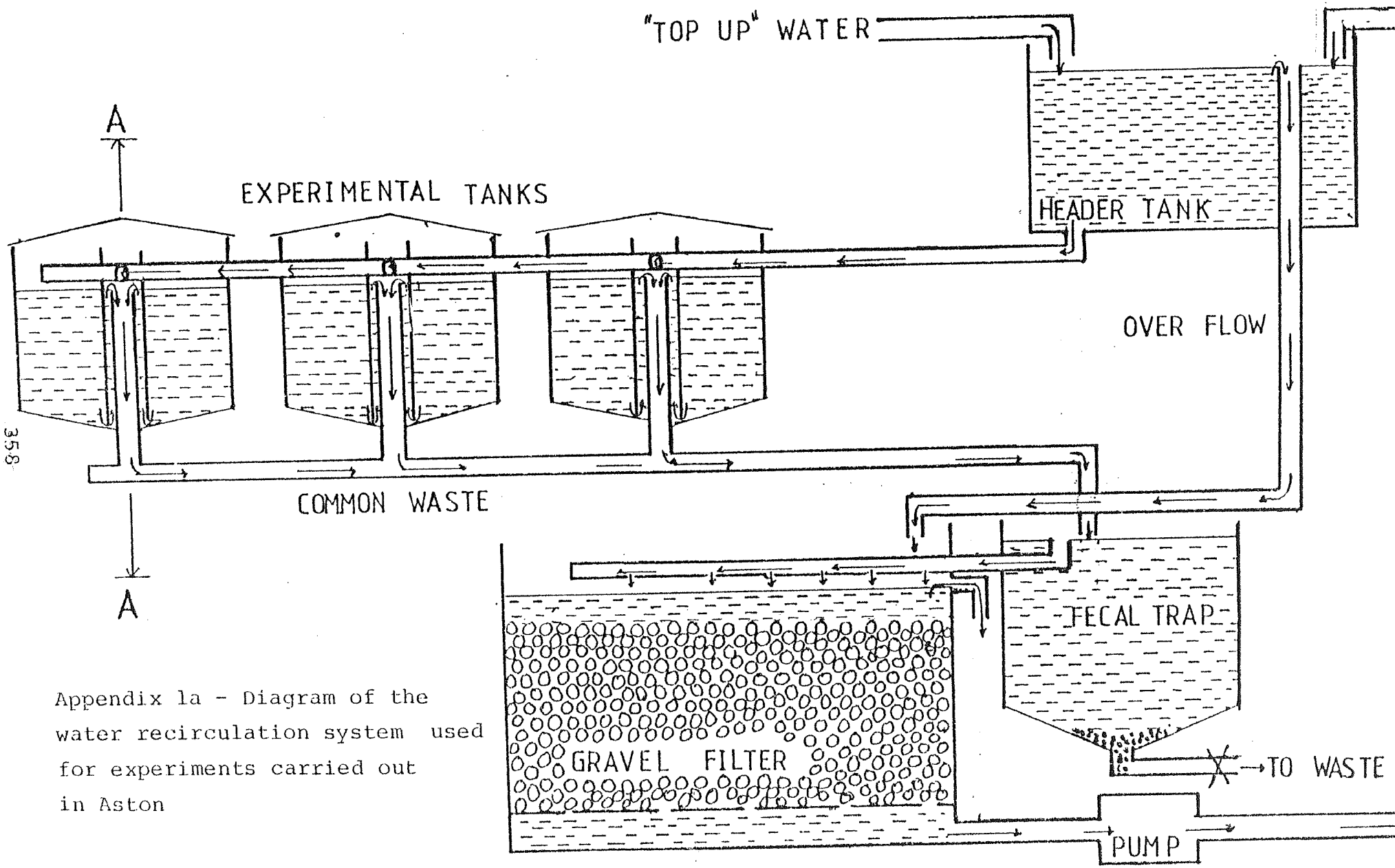
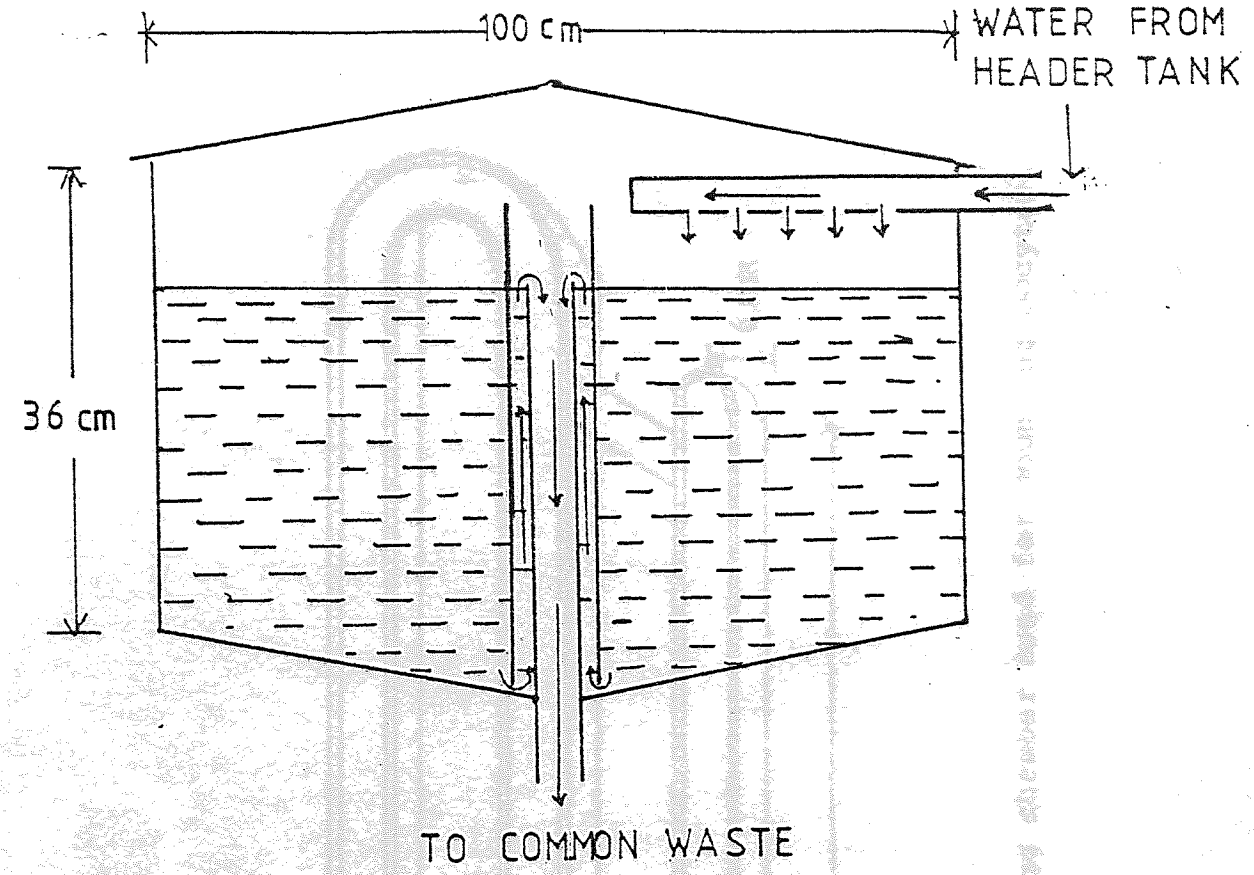
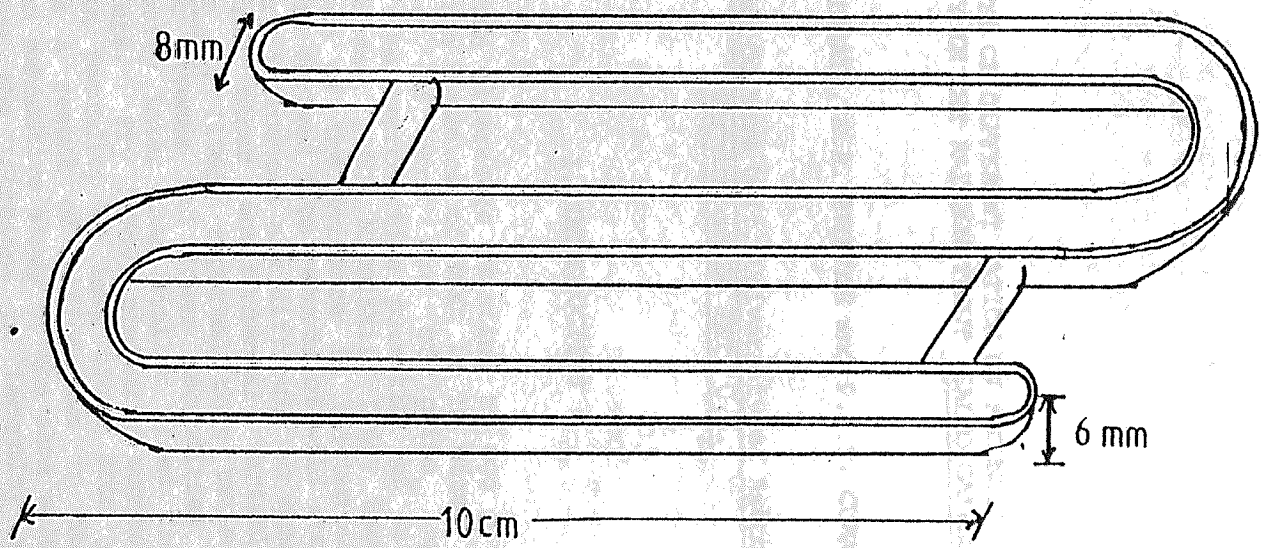


Appendix 1a - Diagram of the water recirculation system used for experiments carried out in Aston



Appendix 1b - A sectional diagram of a tank of the water recirculation system through A--A of Appendix 1a.



Appendix 2 - Diagram of the glass counting chamber used for counting oocytes.

Appendix 3

THE EFFECTS OF DIFFERENT RATION ON FECUNDITY AND EGG QUALITY IN THE RAINBOW TROUT (SALMO GAIRDNERI)

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I. INTRODUCTION

In the wide literature relating to egg production and the population dynamics of fish it is often implied that the supplies of food are of prime importance in determining the reproductive potential of different fish stocks (Woodhead, 1960; Nicolskii, 1969; Bagenal, 1973). However, much of the evidence is circumstantial mainly because it is derived from records of wild stocks of fish, where dietary influences are difficult to quantify and to separate from other environmental variables. Thus, McFadden et al. (1965) reported that brown trout from oligotrophic streams had lower fecundities and reached sexual maturity later than similar sized fish from more fertile waters. Fry (1949) similarly suggested that the variation in fecundity of lake trout from year to year occurred as a result of differences in diet. Martin (1970) in an exhaustive study of the same salmonid species proposed that the increases in fecundity and egg size shown in the later years of a 21 year series were due to improved food supplies. Better feeding conditions were also said to advance the age of first spawning and increase fecundity in brook trout (Vladykov, 1956), although this finding was not substantiated in a similar study of the same species and area by Gibson et al (1976).

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Working on non-salmonid species Hodder (1965), Bagenal (1966) and Raitt (1968) related variations in the fecundities of different stocks of haddock, plaice and Norway pout, respectively, to alterations in population density and/or food supplies. Various studies of the roach have indicted that diminished food supplies reduced fecundity (MacKay and Mann, 1969; Kuznetzov and Khalitov, 1978), delayed maturation (Mackay and Mann, 1969) but did not affect egg size (Kuznetzov and Khalitov, 1978).

Primarily, because of the inherrent difficulties of associating cause and effect in the determination of fecundity and egg composition amongst wild stocks of fish, the above data, at best, offers only supportive evidence for a direct relationship between nutrition and reproduction. Laboratory investigations of the effects of modified ration on egg size, number and quality (i.e. survival) would improve our understanding of this relationship but surprisingly, few such studies have been conducted. Scott (1962), in a three year investigation of the effects of varying ration on the rainbow trout, showed that the proportion of fish which became mature was lower in fish fed on approximately half ration; these fish also produced fewer eggs although the eggs were unchanged in size. Some questions, however, remain regarding these findings as less than 5% of all the fish matured by the end of the experiment. Working with the brown trout, Bagenal (1969a,b) showed that diet restriction reduced total fecundity and delayed maturation; there were also alterations in the mean wet and dry weights of the eggs, although these changes were probably due to the measurements being made on eggs from prespawned fish, and these would have included oocytes at different stages of growth and final maturation. Baiz (1978) in a study of the fecundity of the rainbow trout also reported that a period of starvation reduced egg numbers, but like Scott (1962), could find no effect on egg size. Hislop *et al.* (1978) showed that egg production and feed levels were positively correlated in laboratory maintained haddock. There was also a suggestion that a lower proportion of the fish under the lowest ration matured, and that their eggs were lighter on a dry weight basis. In contrast, Kato (1975) and Ridelman *et al.* (1984) reported that diet restriction for either 4 months or 40 days before spawning, respectively, produced no changes in either fecundity or egg size in the rainbow trout. In an extensive series of papers on the stickleback, Wootton and co-workers (see review Wootton,

1982) have shown that high food levels increase both the percentage of fish maturing and their spawning frequency and, that by improving the growth of fish, fecundity was increased. Food level however had no effect on egg size. More recently Townshend and Wootton (1984) showed for the convict cichlid that both fecundity and egg size were posively correlated with ration and that fish size per se had negligible effects on these parameters. Clearly, the weight of evidence points to direct effects of nutrition on varying aspects of reproduction, but the details of such changes remain obscure.

It is also not clear how the possible alterations in fecundity and egg size are achieved. It has been suggested that food deprivation reduces oocyte numbers by inducing atresia (Vladykov, 1956) and/or by modifying the recruitment of oocytes into vitellogenesis (Robb, 1982). However, Henderson (1963) reported that the levels of atresia in the brook trout were too low to account for these changes, and Townshend and Wootton (1984), although finding a reduction in ooocyte recruitment in convict cichlids on reduced ration, concluded that the relative importance of this process and atresia in determining fecundity could not be ascertained.

In view of the wide range of reported influences of diet on fish reproduction and the unresolved questions relating to atresia and recruitment, the present study investigates the effects of altered ration on the timing of reproduction, and the number, size and quality of the eggs produced in the rainbow trout under carefully controlled experimental conditions; and also by histological examination the ovarian changes which could mediate these changes.

II. MATERIALS AND METHODS

Groups of 40 female rainbow trout (Salmo gairdneri) were maintained in 2m circular tanks and fed in replicate at either 0.35% (half-ration) or 0.7% (full-ration) body weight day^{-1} on a dry commercial trout pellet (Ewos-Baker: Pellets 7 and 8. Proximate analysis: total protein 47%, carbohydrate 22%, ash 13%, moisture 9%, fat 7%, fibre 2%). Water inflow to each tank was 40L. min^{-1} at a constant temperature of 10°C. The fish, which were of North American origin, but with an

October/November spawning time under local conditions, were 2 years old at the beginning of the experiment on 13th October and weighed 0.581 ± 0.005 kg. Only fish which had spawned for the first time during the week before the experiment was begun were used.

The fish were individually marked with numbered tags attached by plastic ties through the dorsal fin. At approximately monthly intervals fish were individually weighed under phenoxyethanol anaesthesia (1 in 15,000), and also examined for signs of sexual maturation and the presence of ripe eggs. At the same time, blood samples were taken from the Cuverian Sinus and, after separation, the serum was deep frozen at -20°C for later analyses of calcium (as an index of vitellogenin) and testosterone using methods already described (Elliott et al., 1984).

As maturity approached the fish were examined each week for the presence of ripe or ovulated eggs. The eggs were removed from each mature fish by gentle pressure on the abdominal wall (i.e. stripping) and separately dry-fertilized in a plastic bowl with an aliquot of sperm from a pool of milt derived from several ripe male fish. Post-stripped fish weights and length were also recorded. 1 minute after fertilization the eggs were washed in running water and after water-hardening for one hour in the same bowls, the volume of eggs derived from each female was determined and the mean diameter of the eggs calculated by counting the number of eggs which could be aligned along a 30cm long groove in a measuring board. The number of eggs from each fish (i.e. the fecundity) was then calculated from the volume of eggs and their mean diameter using the method of von Bayer (1950). This method provides a consistent measure of fecundity which is not significantly different from fecundity determinations based on total egg counts (Springate and Bromage, unpublished observations).

After measurement, the eggs were transferred to hatchery trays and the fertilization rates and subsequent mortalities at eying and as feeding fry recorded using methods already described (Springate et al., 1984). Parallel groups of water-hardened, fertilized eggs from each female were taken for estimation of wet and dry weights (by weighing to constant weight after drying in a hot air oven for 24 hours at 70°C) and for analyses of their proximate composition. Total protein was measured by microkjeldahl analysis, total fats using a Soxhlet apparatus and extracting for 4 hours with petroleum ether (BP.40°-60°C), free amino acids using a Locarte

Analyser, mineral ions with a Perkin-Elmer 373 atomic absorption spectrophotometer and free fatty acids using a Pye-Unicam 304 gas liquid chromatograph. Ash content was determined by weighing to constant weight after heating samples in a muffle furnace for 24hr at 500°C.

After swim-up the fry were fed ad libitum on commercial fry diets (Ewos-Baker Ltd) and when they had reached a mean weight of approximately 1g (in March) they were transferred to 1.5m diameter fry tanks. Throughout this period, mortalities were recorded and, at approximately 3-week intervals from the time of first feed, individual fry weights were taken.

Ovarian samples were taken from the fish from each group approximately 14 days pre- and post-ovulation for histological examination after fixation in Bouin's fixative, alcohol dehydration and JB4 plastic (Polysciences) embedding. Sections were cut at approximately 1μm thickness. Parallel ovarian samples were also fixed and separated in Gilson's fluid for determination of the total numbers of each of the seven oocyte stages (van den Hurk and Peute, 1979). The levels of atresia in prespawning fish were expressed as percentages of the total number of vitellogenic and atretic oocytes whereas in the spawned fish the numbers of ovulated follicles were also included in the total count.

Differences between means were compared with a student's 't' test and in the case of percentages after 'arc sine' transformation. Regression analysis was carried out using a 'Minitab' statistical package on a Harris main frame computer. The estimated statistical parameters are given ± 1 standard error (SE) of the mean.

III. RESULTS

By the time of the first sampling in February, the differences in dietary input were clearly shown by the significant difference in growth between the groups (Fig. 1, $P < 0.05$). The growth divergence became more pronounced as the trial progressed and from May onwards the differences between means was highly significant ($P < 0.001$). At all times there was an overlap in the range of fish sizes in the two groups and at spawning the half-ration fish were 0.835 ± 0.020 kg in weight (range 0.580-1.225), whereas the full-ration fish were 1.330 ± 0.040 kg (range 0.500-2.000).

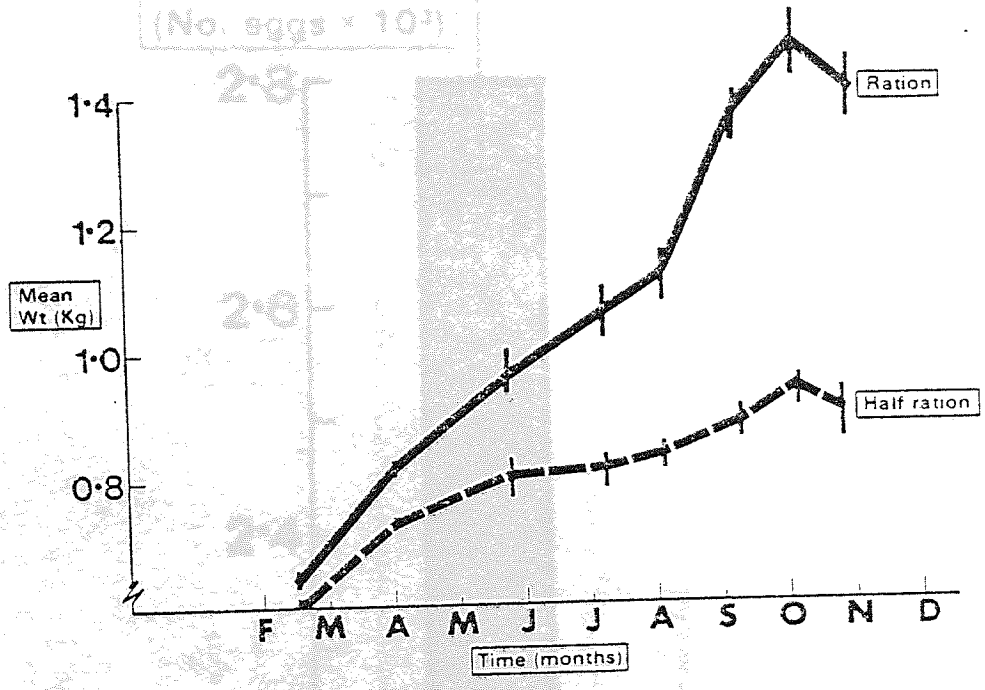

Fig. 1. Growth of rainbow trout fed on 0.35% (half-ration) and 0.7% (full-ration) body weight day^{-1} (Mean ± SE).

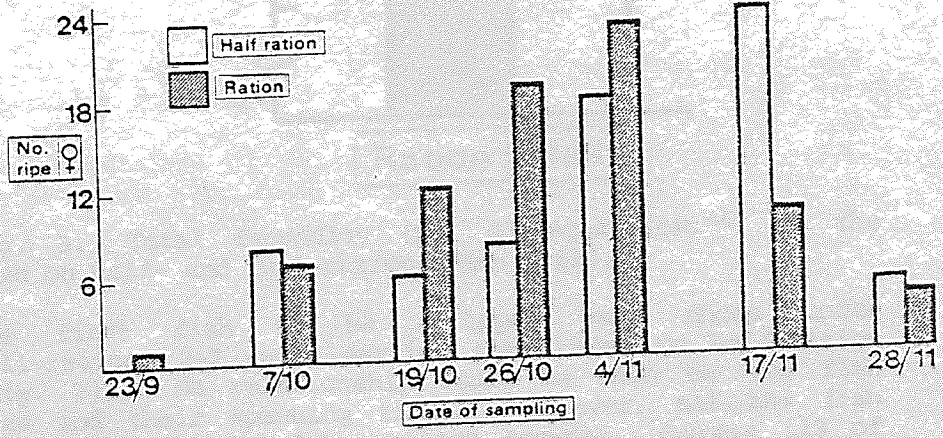

Fig. 2. Spawning profile of the fish on half- and full-ration.

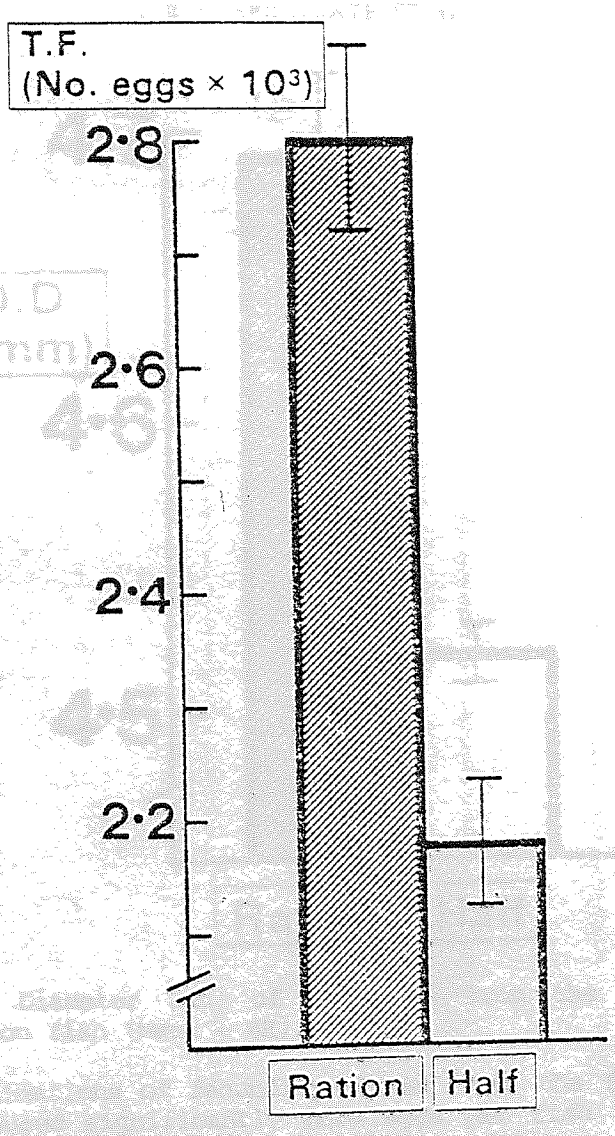

Fig. 3. Total fecundity (No. of eggs fish^{-1}) of the fish on half- and full-ration (Mean ± SE).

The first fish to be stripped were those under full-ration, 2-3 weeks earlier than those on half-ration (Fig. 2). No relationship could be seen between fish size and their spawning times. However, all the fish which had been fed full rations spawned, whereas 11% of the half-ration group failed to mature.

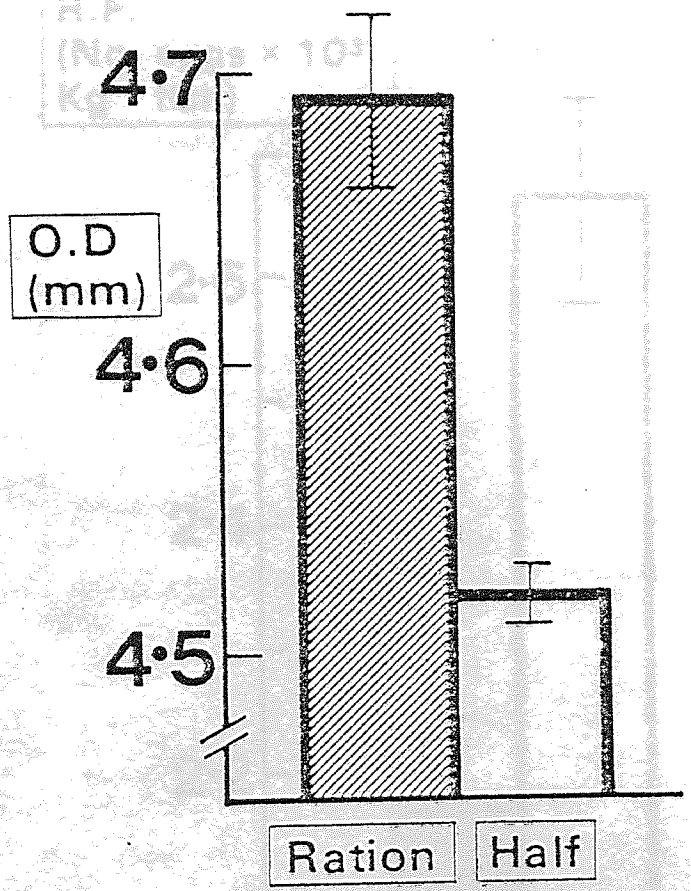

Fig. 4. Diameter (mm) of the eggs from the half- and full-ration fish (Mean ± SE).

Determinations of fecundity showed that the full-ration fish produced significantly more eggs per fish than those on half-ration (Fig. 3, $P < 0.001$). However, the eggs from the full-ration group were significantly larger (Fig. 4, $P < 0.001$). The concommitant changes in fecundity and egg size in the two groups were reflected in a significant increase in relative fecundity in the fish fed with half-rations (Fig. 5, $P < 0.001$) when compared with the full-ration group.

Measurements of serum calcium (as an index of vitellogenin) and testosterone revealed similar profiles to those reported elsewhere (Elliott et al., 1984) with peak values occurring around the time of ovulation. The

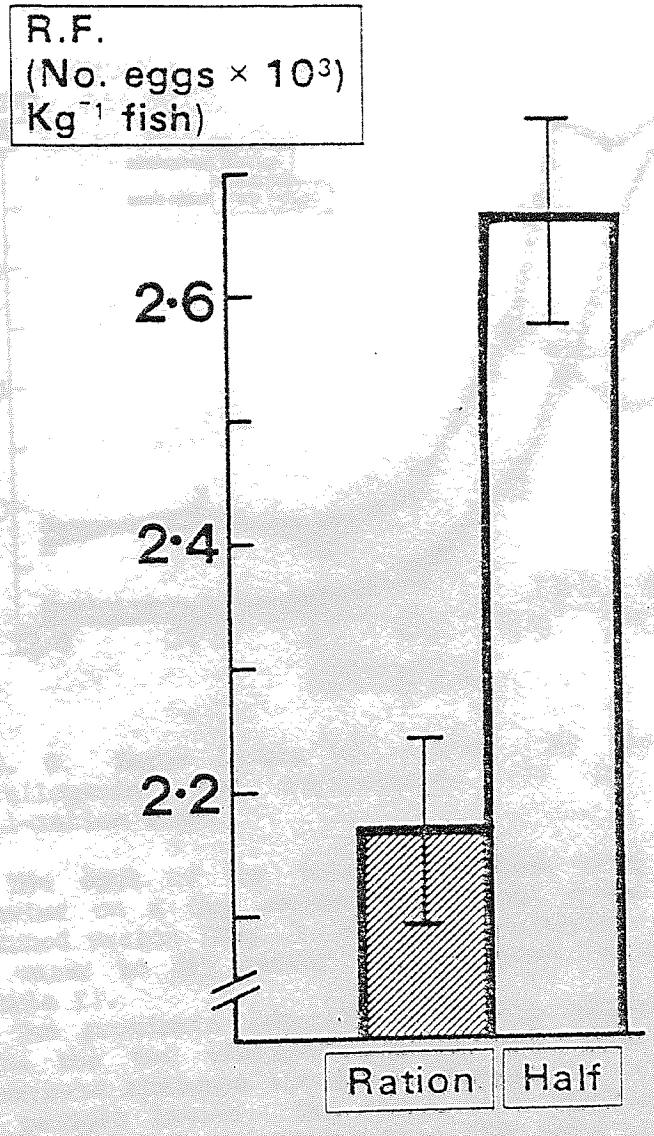

Fig. 5. Relative fecundity (No. of eggs kg^{-1} post-spawned fish weight) of the fish on half and full-ration (Mean ± SE).

peak heights for both calcium and testosterone were reduced in the half-ration fish (Fig. 6).

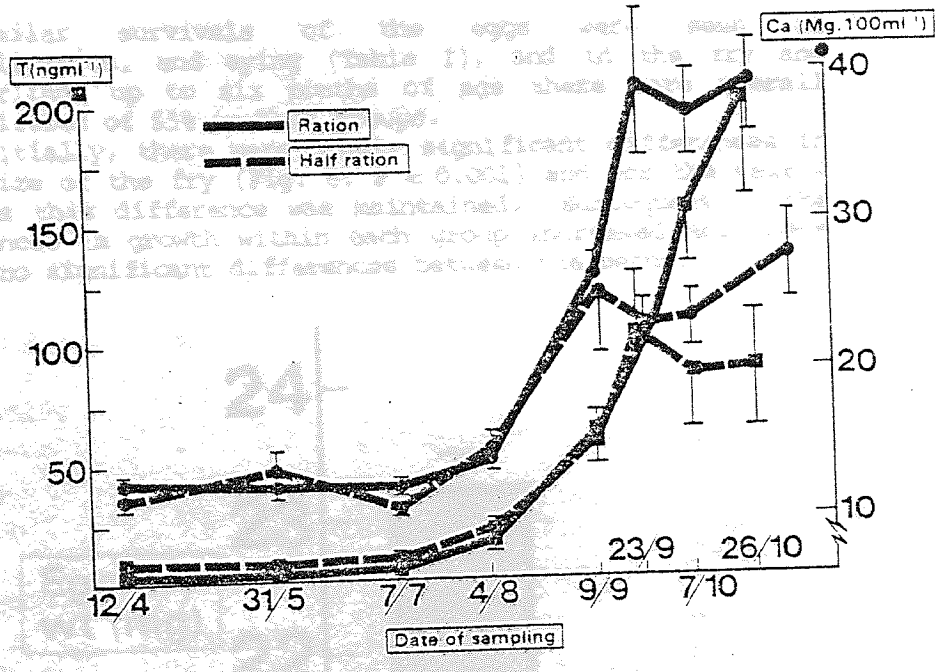

Fig. 6. Serum levels of calcium (Ca) (as an index of vitellogenin) and testosterone (T) in the half-and full-ration fish.

The eggs of the full-ration fish were significantly heavier on a dry weight basis than those from fish on reduced ration (Fig. 7, $P < 0.001$) although the proportion of water to dry matter was the same in the two groups (Table I).

The proximate composition of the water-hardened eggs from the two treatment groups are shown in Table I. Proximate analyses showed no changes in either total fat or protein levels. The amino acid profiles of the eggs from the full- and half-ration fish were similar, as were the concentrations of mineral ions, both expressed per unit weight of egg (Table II and III). There were few changes in free fatty acid composition although a moiety approximately described as 20:0 was consistently present in small amounts in the eggs from fish on half-ration but absent from the eggs of fish on full-ration (Table IV).

Similar survivals of the eggs were seen at fertilization, and eying (Table I), and in the fry and fingerlings up to six months of age there were overall mortalities of 55% in both groups.

Initially, there were highly significant differences in the size of the fry (Fig. 8, $P < 0.001$) and for the next 4 months this difference was maintained. Subsequently, the variances in growth within each group increased and there were no significant differences between the means.

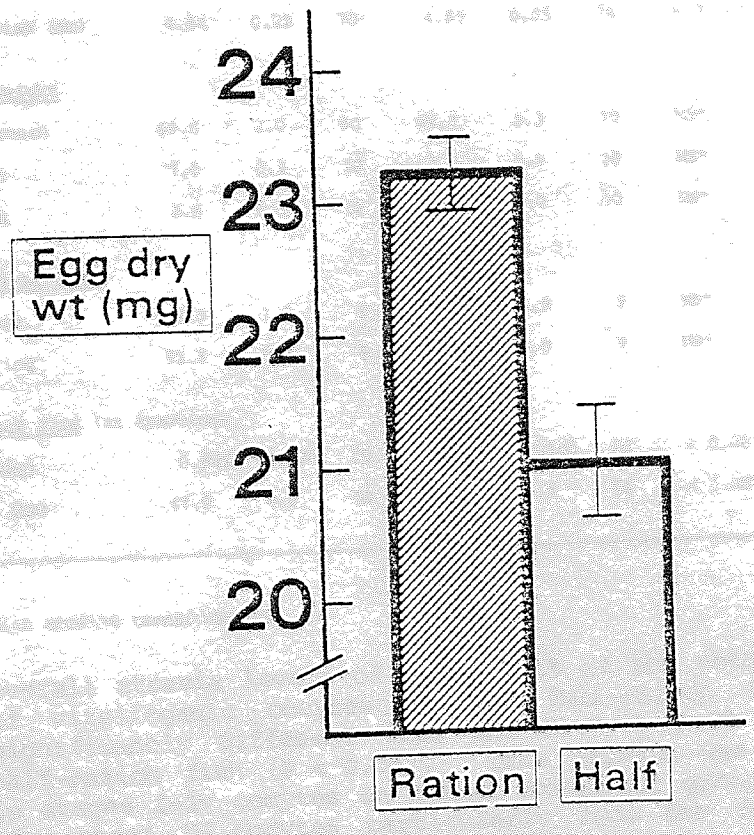

Fig. 7. Dry weight (mg) of the eggs from the half- and full-ration fish (Mean ± SE).

Histological examination of the preovulatory samples revealed that atresia was present in vitellogenic oocytes, stages V-VII (van den Hurk and Peute, 1979), of the ovaries of both full- and half-ration fish. However, the

Table I. Proximate analyses of the eggs.

	Half Ration			Full Ration			
	mean	± SE	n	mean	± SE	n	Significance of difference: P value
Wet Wt. (mg)	47.4	0.7	50	51.0	0.8	50	< 0.01
Dry Wt. (mg)	21.0	0.4	44	23.2	0.4	49	< 0.001
% water	55.7	0.4	44	54.5	0.3	49	NS*
Ova Diam (mm)	4.54	0.02	70	4.69	0.03	76	< 0.001
Dry weight							
% Protein	69.8	2.0	10	69.1	3.3	10	NS*
% Fat	7.5	0.2	10	7.3	0.1	10	NS*
% Ash	3.6	0.1	10	3.7	0.2	10	NS*
Survival Rates							
% Fert.	92.0	1.9	9	86.3	5.9	9	NS*
% Eying	82.5	5.8	9	74.8	9.0	9	NS*
Parent Size (at spawning)							
Wt (kg)	0.835	0.020	70	1.330	0.040	76	< 0.001
Lth (cm)	41.6	0.3	70	46.0	0.5	76	< 0.001

* With arcsine transformation

overall atresia levels of 6.9 ± 2.1% of the total numbers of vitellogenic oocytes in the full-ration fish were significantly different from the 21.9 ± 1.2% found in half-ration fish ($P < 0.05$). True atresia was not seen in stages I-IV oocytes in either treatment group although some stage IV oocytes particularly from the full-ration group were showing early signs of this process.

Total counts of the oocytes in the preovulatory ovaries of both half- and full-ration fish revealed similar numbers of vitellogenic oocytes to the numbers of fully-ripe eggs which would have been stripped at full maturity. The total numbers of oocytes of all stages were higher in the fish on full-ration as might have been

predicted from the mean total fecundity of this group at spawning. There was also a delay in the development of vitellogenesis in the ovaries of the half-ration fish. At the time of sampling of the preovulatory fish, most of the vitellogenic oocytes (i.e. stage V-VII) had reached late stage VI or early stage VII, whereas in the half-ration fish the majority of the vitellogenic oocytes were only in the early portion of stage VI. No stage V oocytes were present in either ration group. Examination

Table II. Levels of free amino acids (nmol g^{-1} dry wt egg x 10^3).

	Half-Ration		Full-Ration	
	mean	± SE	mean	± SE
Asp	17.3	0.2	15.6	1.1
Thr	12.4	0.4	12.8	0.4
Ser	15.8	1.1	15.7	0.8
Glu	21.2	0.4	21.3	0.5
Pro	17.3	0.5	17.2	0.7
Gly	10.4	0.5	11.1	0.3
Ala	20.1	0.2	20.0	0.3
Val	15.9	0.4	15.4	0.1
Met	6.5	0.1	6.5	0.1
Ile	11.8	0.2	11.5	0.2
Leu	14.9	0.2	16.2	0.4
Tyr	7.2	0.1	7.2	0.1
Phe	8.9	0.1	9.1	0.1
His	5.5	0.1	5.6	0.1
Lys	13.8	0.2	13.8	0.1
NH_3	16.1	0.2	14.9	0.5
Arg	11.7	0.3	8.9	0.2

of the post-ovulatory ovaries showed that in the full-ration fish only a small number of vitellogenic eggs remained unovulated. In contrast, up to 7% of the same stages remained in the ovaries of half-ration fish, although the significance of this change just failed to reach the $P < 0.05$ probability level. Atresia levels were 11.9% and 17.5% respectively in the full and half-ration groups, although this difference was not significant.

IV. DISCUSSION

The most significant effect of feeding a low ration to female rainbow trout was a 22% reduction in total fecundity, expressed as the number of ripe eggs produced per fish at stripping. Similar reductions in fecundity following experimental deprivation of food have also been reported for salmonids by other workers (Scott, 1962;

Table III. Mineral concentration (mg/g g^{-1} egg dry weight; n = 6).

Element	Half ration mean ± SE	Full ration mean ± SE
Na ($mg.g^{-1}$)	1.7 ± 0.7	2.1 ± 0.7
K ($mg.g^{-1}$)	4.7 ± 0.5	4.5 ± 0.2
Ca ($mg.g^{-1}$)	2.0 ± 0.1	2.5 ± 0.2
Mg ($mg.g^{-1}$)	1.9 ± 0.2	2.0 ± 0.1
Zn ($g.g^{-1}$)	86.7 ± 7.1	108.0 ± 7.1
Fe ($g.g^{-1}$)	60.4 ± 2.7	66.0 ± 4.4
Cu ($g.g^{-1}$)	8.8 ± 3.6	11.3 ± 0.9

Bagenal, 1969a; Baiz, 1978). However, only Scott (1962) and Baiz (1978) determined fecundity from measurements of ripe fish. Furthermore, in Scott's study overall

maturity levels in the experimental fish were very low probably because the fish on the highest ration only reached 150g in weight at three years of age. Non-salmonid species also experience reductions in fecundity following decreases in ration size (Wootton, 1973; Townshend and Wootton, 1984). Full interpretation of the results of the majority of these studies is,

Table IV. Percentage composition of fatty acid in the total lipid extract (n = 6).

Free Fatty Acid	Half ration mean ± SE	Full ration mean ± SE
14 : 0	3.3 ± 0.2	3.5 ± 0.1
16 : 0	18.6 ± 0.9	17.4 ± 0.4
16 : 1	7.4 ± 0.4	7.7 ± 0.2
18 : 0	3.1 ± 0.2	2.5 ± 0.2
18 : 1	16.9 ± 2.5	16.4 ± 1.4
18 : 2	6.5 ± 0.4	4.9 ± 0.2
18 : 3	0.9 ± 0.04	0.9 ± 0.04
20 : 1	4.0 ± 0.2	4.2 ± 0.4
20 : 2	0.7 ± 0.04	0.7 ± 0.08
20 : 3	1.4 ± 0.1	1.5 ± 0.1
20 : 4	2.2 ± 0.3	2.6 ± 0.6
20 : 5	7.2 ± 1.0	7.2 ± 0.4
22 : 5	2.5 ± 0.1	2.5 ± 0.1
22 : 6	22.1 ± 1.8	27.5 ± 0.7

however, complicated by the effects of diet on somatic growth because fecundity is primarily determined by fish size (Bagenal, 1973; Wootton, 1982). Consequently, if one is aiming to determine the direct influence of ration on fecundity then the primary effects of diet on fish size must be first removed by appropriate experimental

techniques (Wootton, 1982). Few studies are able to offer such differentiation and, even where such analyses have been possible, they have shown that there is considerable interspecific variation. Thus, in the stickleback, egg production is primarily a function of fish size, whereas in the convict cichlid the reductions in fecundity produced by lower rations were greater than would be expected from the reductions in fish weight (Wootton, 1982; Townshend and Wootton, 1984). A direct correlation of fecundity with the amount of food received, even after the size differences were taken into account, was also shown for the brown trout (Bagenal, 1969a). However, there is little evidence for a direct effect of ration on fecundity in the rainbow trout. The present work shows a significant correlation of size with fecundity in the full-ration fish ($r = 0.51$, $P < 0.001$), but not for those on reduced ration, suggesting that diet may have had a direct influence in this group. However, fish of equivalent sizes in the two ration groups had similar fecundities. Further partitioning of the multiple effects of diet in the rainbow trout must await factorial experimentation with a variety of ration levels.

In parallel with the change in fecundity, both the diameter of the eggs and their mean dry weight were significantly reduced in the fish on half-ration. Hislop *et al.* (1978) have also reported similar changes in laboratory-maintained haddock. In contrast, Bagenal (1969a) reported that diet restriction in brown trout led to the production of larger eggs by dry weight. However Bagenal's measurements were conducted on preovulatory fish and, as the feeding regime also delayed maturity, the sampling procedure would have included eggs of varying stages of final ripeness and hydration. Generally, other investigations on salmonids have shown that egg size is not affected by reduced ration (Scott, 1962; Ridelman *et al.*, 1984) or, as in the study of Baiz (1978), by a period of starvation enforced by disease problems in the experimental fish. Reductions in ration also failed to change egg size expressed as either wet or dry weight in the stickleback (Wootton, 1973) and the convict cichlid (Townshend and Wootton, 1984). However, both these species are multiple spawners and the length of the interspawning interval, which is extended by decreased ration, may also have effects on egg size. Increases in egg size have also been reported to occur in the rainbow trout after a delay in spawning time (Bromage *et al.*, 1984).

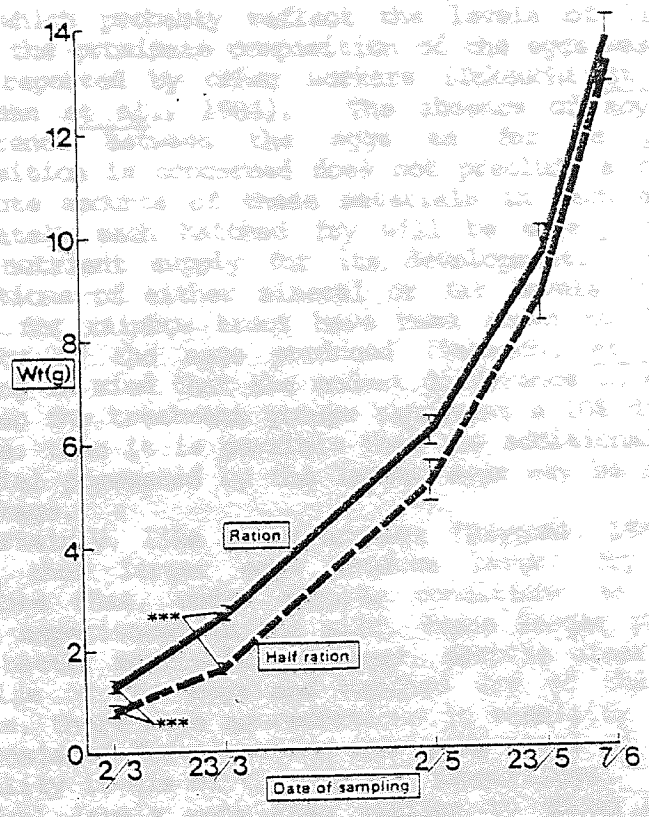

Fig. 8. Weight of fry (Mean ± SE) derived from the eggs of half- and full-ration fish.

Although there were no differences in percentage wet and dry weights of the eggs, the absolute dry weights of the eggs was reduced in the fish on half-ration and closely paralleled the reduction of egg diameter in this group. Possibly, some of the varied effects of diet on egg size described above relate more to the changes in water relations experienced by the eggs during their final maturation rather than to ration per se. Such difficulties would be minimised by only comparing the sizes of water-hardened, ovulated eggs.

Proximate analyses of the gross protein, fat and ash constituents of the eggs revealed no differences between the ration groups nor were there any alterations in mineral or amino acid composition, if expressed in terms of concentration. Apart from relatively low levels of

fat, which probably reflect the levels of lipid in the feed, the proximate composition of the eggs was similar to that reported by other workers (Takeuchi et al., 1981; Ridelman et al., 1984). The absence of any measurable difference between the eggs as far as proportional composition is concerned does not preclude a role for the absolute amounts of these materials in each egg, because ultimately each hatched fry will be solely dependent on this nutrient supply for its development. Experimental reductions of either mineral or fat levels in broodstock feeds for rainbow trout have been shown to decrease the quality of the eggs produced (Takeuchi et al., 1981). Bearing in mind that the modest difference in egg diameter between the treatment groups represent a 10% difference in volume, then it is possible that the additional amounts of material possessed by the larger eggs may be of selective advantage.

Certainly, like other workers (Bagenal, 1969b) we have shown that larger eggs produce larger fry and it is possible that, under adverse conditions as for example those experienced in the wild, these larger progeny might show better survivals. However, despite clear differences in size of the eggs and hatched fry of the two ration groups, there were no reductions in viability expressed on the basis of fertilization and eying rates of the eggs and mortality levels of the fry and fingerlings. The overall survival levels were also similar to those reported for other rainbow trout stocks (Ridelman et al., 1984). The absence of any reduction in viability of the developing eggs and hatched fry derived from small eggs supports the conclusion reached by Phillips and Dumas (1959) in their study of the brown trout that ample materials for viable embryo production exist even in the smallest egg.

In addition to the differences in egg number and size there was also a two week delay in spawning time in fish on half-ration. However, the failure of 11% of the low ration group to achieve maturity is probably of more significance. Delays of spawning and extensions of the interspawning interval after diet restriction have been reported for some multiple spawners (Wootton, 1982; Townshend and Wootton, 1984), whereas salmonids stocks appear to respond to lowered ration by reducing the overall percentage of maturing fish (Scott, 1962; Bagenal, 1969a). In the present work the fish on low ration which failed to spawn had a lower mean weight (0.68±0.05Kg) than the spawning members of this group. Low fish size may have also been the reason for the very low levels of

maturity described by Scott (1962) for fish on both high and low rations. Although, it is possible that a fish has to reach a certain weight before it can mature, from the present results, it would appear that size is not of importance in determining the timing of ovulation within the spawning spread of a stock. This is in contrast to the results of Kato (1975) who suggested that it was the larger fish amongst stocks of rainbow trout which spawned first.

Turning now to the possible mechanisms by which the alterations in fecundity and/or egg size are achieved, it is apparent even from the most cursory examination of the ovary of fish throughout the year that enormous reductions in oocyte number occur during development. In the present study up to 50,000 oocytes were present in the ovary a few weeks after ovulation but only 5-6000 of these would have been expected to reach full maturity at the following spawning. Three mechanisms might account for these changes: the first involves atresia or the resorption of oocytes particularly those undergoing yolk incorporation; the second the recruitment of stage I-IV oocytes into vitellogenesis; and lastly the extent of oogonial multiplications.

Although the relative importance of these processes in determining fecundity and egg size may vary with different fish, in the rainbow trout the most significant change following a reduction in food was an increase in the level of atresia up to 22% of the total numbers of vitellogenic oocytes present in pre-spawning fish. Much lower levels were found in the fish on full-ration. Levels up to 27% were also reported by Scott (1962) even for the group of fish which were fed on the highest rations of food. However, closer examination of the final weights of these fish would suggest that all the experimental and control groups were being subjected to restricted rations. Vladykov (1956) described 80% and 45% reductions in oocyte numbers up to 1mm and 3.5mm in diameter respectively in the brook trout and again suggested that this reduction was due to atresia. Levels of 27% atresia were also recorded for natural populations of the chubsucker although figures for individual fish ranged from 4-72% (Wagner and Cooper, 1963). In contrast Henderson (1963) found far fewer atretic oocytes in the brook trout although the levels, averaging 4.0%, were of the same order as those found in the fish on full-ration in the present experiment, probably because both groups were maintained under equivalent hatchery conditions and fed to

full-ration according to feed manufacturers' tables. It is clear that the levels of atresia reported here commonly occur in the rainbow trout in the months leading up to spawning. Furthermore, their alteration in response to reductions in ration suggests a physiological role for atresia in the determination of fecundity in the rainbow trout.

An effect of diet on the recruitment of oocytes is more difficult to assess from the present results primarily because the numbers of fish under experiment would not allow histological samples to be taken at more frequent intervals throughout the year and more specifically at the time of onset of vitellogenesis. Reductions in recruitment as a result of feeding low rations have been reported for the winter flounder (Tyler and Dunn, 1976), a cichlid (Townshend and Wootton, 1984) and Poecilia, an ovoviviparous fish (Hester, 1964). In contrast de Vlaming (1971) showed that starvation induced atresia in the yolky oocytes of the goby but did not affect recruitment. There was evidence from the present work that vitellogenesis was more advanced in the fish in full-ration. However, this apparent difference was probably due to an increase in the rate of maturation of these fish and the sampling of both groups on the same day. Vitellogenin and testosterone levels were significantly lower at spawning in the half-ration fish and although the reductions in size and number of eggs in these fish could be attributed to these serum changes, the relationship remains unclear. There were no changes in the proportion of eggs undergoing vitellogenesis in the full-ration fish despite the higher fecundities of this group and the presence in the pre-spawning ovaries of much higher numbers of yolky oocytes. One must conclude that effects on recruitment or the oogonial divisions, if present, must have occurred much earlier in the year-long cycle of ovarian development.

As a consequence of the parallel changes in total fecundity and egg size, there was an increase in relative fecundity i.e. the number of eggs per kilogram of body weight, in the fish on half-ration. The decrease in relative fecundity of the larger full-ration fish is partly explained by the increased size of their eggs. Other workers have also reported that larger fish often produce fewer eggs than would be expected from the regression of fecundity and fish size (Rounsefell, 1957; Nicholls, 1958). A probable explanation is that the rainbow trout and possibly other salmonids, produce a volume of eggs at stripping which bears a constant

relationship to body weight and that reductions in either fecundity or egg size, possibly caused by alterations in diet, will involve corresponding increases in the other parameter. A similar conclusion regarding a 'trade off' between egg size and number was also reached by Rounsefell (1957) in his extensive review of the fecundity of North American Salmonidae. Although the production by the trout of a weight-related volume of eggs would not preclude direct effects of ration or nutrient on fecundity and egg size it might provide a finite range of egg size and number on which subsequent alterations in atresia or recruitment induced by nutritional and other environmental changes might be superimposed.

The presence of a reciprocal relationship involving egg size and number which can be modified by dietary change, together with the decrease in relative fecundity and increase in egg size with increased body weight are potentially of considerable commercial importance. Currently, salmonid eggs are sold by number and not by size and the potential production of a hatchery is determined by the total weight of broodstock which can be maintained on the farm. Under such constraints, an increase in the total numbers of eggs from the same weight of broodstock might be achieved by varying the feeding regime or by using more broodfish but of smaller size. Providing the consequent decreases in size of the eggs do not affect their viability or their acceptance for general sale, such approaches may confer considerable economic advantage.

In summary, the present results provide evidence for multiple effects on the fecundity, egg size and rate of maturation of rainbow trout. Further work is necessary to investigate the effects of varying nutrient composition in the diet and of different feeding rates at different times of the year.

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