NEUROENDOCRINE MECHANISMS INVOLVED IN THE CONTROL OF OVARIAN DEVELOPMENT AND VITELLOGENESIS IN THE RAINBOW TROUT.

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Summary.

This thesis provides a detailed study of the neuroendocrine mechanisms that control vitellogenesis and ovarian development in the female rainbow trout. Fluorometric, electrophoretic and chromatographic techniques and radioimmunoassay were used to determine serum levels of oestrone, oestradiol-17β, testosterone, vitellogenin and calcium and these correlated with histological data both during the reproductive cycle and after treatment with hormones.

Single and double injections of oestradiol-17β respectively produced primary and much more rapid secondary increases in levels of vitellogenin, total calcium and phosphoprotein phosphorus. The dose of oestradiol-17β was correlated with the levels of vitellogenin and with hepatosomatic and gonadosomatic indices. Although the liver was at no time completely refractory to oestradiol-17β, there was a marked seasonal variation in sensitivity, being highest in October and lowest in January.

During the reproductive cycles of three strains of female trout, initial increases in oestrone and oestradiol-17β were followed by increases in vitellogenin, calcium and testosterone. Levels of both oestrogens were correlated with vitellogenin during vitellogenesis, which supports a role for both hormones in this process. All three strains showed similar sequences of changes in both endocrinological and histological development. Similar, although much reduced, changes were observed in immature fish of one strain at a time of year suggestive of a practice-run, one year before the first spawning, as a rehearsal for full reproductive development.

Exposing fish to long and then short days, or a condensed 6 month seasonal photoperiod, advanced spawning by 4-5 months. These results indicate that the different spawning times shown by the three strains may be the result of a requirement for a different number of stimuli by a specific length of daily photoperiod.

The application and value of techniques of hormonal and photoperiodic manipulation, for the improvement of egg quality and alteration of spawning time, in the farming of salmonid fish are discussed.

Vitellogenesis, Oestrogen, Egg, Photoperiod, Salmonid Culture.

TO MY MOTHER AND FATHER,

without whose support I would not have had

the opportunity to carry out this study.

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CHAPTER 1.

GENERAL INTRODUCTION.

Introduction.

In all seasonally breeding teleosts, the production of eggs is a complex process encompassing a number of individual stages which are all essential to the successful development of the ovary. Subsequently, these species have evolved a reproductive cycle such that oogonial mitosis, oogenesis, folliculogenesis, vitellogenesis, maturation and ovulation occur in the correct sequence and spawning occurs at the correct time of year. The control of the reproductive cycle is directly affected by changes in the hormonal milieu which are in turn modulated by cues from the external environment (de Vlaming, 1974; Bromage et al., 1982b; Dodd & Sumpter, 1982). During this period in trout, there is a dramatic reorganisation of the animals' energies and resources to meet the gross morphological and developmental changes that occur in the ovary.

At present, little data are available on the mechanisms controlling the first phase in reproduction, oogonial mitosis, which in trout probably occurs both at an early stage, and in the immediate post-spawning period (Tokarz, 1978). The cyclical nature of oogonial proliferation suggests that it is under some form of hormonal control, and an understanding of this mechanism may enable the number of mitoses and thus eggs produced by broodstock fish to be controlled. Whether the fecundity of fish is decided at this early stage, or later in development as a result of atretic processes, is not known, but the ability to control this process may be of commercial significance.

The next phase in development, oogenesis, closely follows oogonial mitosis and involves the meiotic transformation of oogonia

into primary oocytes (Tokarz, 1978; Dodd & Sumpter, 1982). Evidence suggests that this occurs independently of any hormonal regulation, since treatment with other hormones, including oestrogens and androgens, appears to inhibit this progression (Dodd, 1960). These primary oocytes are observed quite early in fish of only a few months old, and appear to remain at this stage until the onset of the second phase of gonadal development which leads to spawning (Vivien, 1939; Barr, 1968). Another process involving primary oocytes is folliculogenesis, where individual oocytes become closely associated with the different follicular cells that constitute individual follicles.

At the beginning of this second phase of ovarian growth, which ultimately results in the production of ripe gametes, the primary oocytes, probably under some form of pituitary trigger (Vivien, 1939) enter the phase which is commonly called 'vitellogenesis'. It is during this period that the large yolk-precursor given the name vitellogenin by Pan et al., (1969) is believed to be synthesised by the liver under the influence of oestrogenic control. Although the oestrogen principally involved in this mechanism in amphibians and birds has been positively shown to be oestradiol-17β, there is some doubt as to the role of other oestrogens and even androgens in this process in fish. Clearly, an investigation of the hormones responsible for this important phase of development must be carried out. The production and uptake of the yolk precursors are of considerable importance in the growth of the oocyte and to the growth of the embryo and fry derived from these eggs. An understanding of the mechanism controlling both the synthesis and uptake of this material is also of importance to the fish farming

industry, since hormonal manipulation of these processes may enable alteration to be made in the size and quality of trout eggs.

More recently it has been reported that amphibians and possibly fish (Wiley & Wallace, 1981; de Vlaming et al., 1980) may have more than one form of vitellogenin. That such a large and heterogenous molecule should be represented in more than one form suggests that different vitellogenins may have different physiological roles at different stages of gonad growth. This could be represented by differences in their nutritional value, or the relative speed of their uptake into the oocytes. Clearly, an understanding of the nature of vitellogenin(s) in trout may reveal what possible effect differences in this large complex may have on egg quality.

During the period of vitellogenin production by the liver, the incorporation of this large heterogenous molecule into the developing oocytes is indicated by large increases in gonad size and oocyte diameter. There is some evidence that the oocyte itself may produce some material which has subsequently been called endogenous yolk (Wallace, 1978). The significance of this material in ovarian development and its mechanism of control is still poorly understood. Indeed, chemically, it is unlike both the vitellogenin isolated from the serum and the granular material believed to be exogenous vitellogenin, which is laid-down after the appearance of the 'endogenous material' in the oocyte (Khoo, 1979). It is possible that it is another vitellogenin similarly synthesised exogenously, which undergoes drastic alteration when sequestered by the ovary. Clearly, this needs further investigation.

At the end of this period of gonadal growth, when the gonad may

represent 20% of the weight of the fish, the mature oocytes undergo a physicochemical changes which involves a rapid, though variable, uptake of water and a solubilisation of yolk (Fulton, 1898; Wallace, 1978). However, in all the species so far studied, it is not clear whether all developing oocytes reach maturity and are subsequently expelled as eggs at ovulation, or whether some are lost due to atresia. Thus some authors have shown that atresia may occur at all stages of gonadal development and after spawning, whilst others attribute virtually no loss of oocytes to this degenerative process (See Tokarz, 1978). The significance of atresia in trout may have a direct bearing on the number ie. fecundity, of eggs produced by broodstock and needs to be assessed. There is some evidence that certain environmental factors, including stress and nutrition can directly affect this process. Thus, the highly variable daily ration of food that some broodstock receive may, by modifying atresia, have effects on the fecundity of commercially farmed stocks. There is also a report that testosterone can cause atresia in vitellogenic oocytes (Yamazaki, 1972) and although high levels of testosterone have been observed in female teleosts, including trout, during the reproductive cycle (Campbell & Idler, 1976; Wingfield & Grimm, 1977; Scott et al., 1980b; Stuart-Kregor et al., 1981) it is not known whether this hormone has a physiological role in fish. It has been variously reported that androgens are inhibitory towards vitellogenesis in some cases (Dodd, 1960), but exert a vitellogenic action in others (Hori et al., 1979; Le Menn, 1979; Le Menn & Lamy, 1976). An investigation of the seasonal changes in this hormone, together with a study of its effect on immature fish is included to determine whether it is important in the control of vitellogenesis in the

female rainbow trout.

Although the changes in serum levels of these various hormones directly control the different phases of the reproductive cycle, the changing external environment ultimately controls the initiation and timing of these events. Of all the environmental factors that influence reproduction in fish including temperature, rainfall and nutrition, the daylength or photoperiod is the major factor in the overall control of the timing of reproduction in salmonids. Thus, in extensive experiments carried out previously in this laboratory using a number of strains of trout under different conditions, modifications of the photoperiod successfully altered the time of spawning such that eggs were available at all times of the year. A regular supply of eggs throughout the year would be of distinct commercial advantage, since the current limited seasonal availability of eggs imposes severe restrictions on the fish farming industry in the U.K. (See Bromage, 1982). The use of photoperiod manipulation to extend this availability would be even more flexible if used in conjunction with different strains which spawn at different times of the year.

Currently there has been no investigation of how different strains of trout respond to the same seasonal photoperiod to produce their different spawning times. If this occurs as a resultof changes in the rate, or nature, of the endocrine, or physiological, processes that make up their reproductive cycles, such differences may reflect the quality and/or fecundity of the eggs they produce. It is also possible that the different spawning times occur in response to different environmental cues, and thus it is important for these triggers to be determined if other strains are selected for use on

broodstock farms which already utilise light control to alter spawning times.

Throughout this introduction, reference has been made to 'good quality' eggs. At present there is no critical appraisal of what constitutes 'quality' in an egg apart from the mythical values of large size and colour. What limited data are available from a wide range of species held under various conditions provide little evidence to indicate that size and/or colour have any bearing on the future development of the young. For example, there is little published evidence regarding the differences in fecundity or quality of eggs produced by different strains of trout, or indeed what mechanisms might determine these factors in other teleosts. Clearly, any such differences may be related to differences in the neuro-endocrine mechanisms controlling the different phases of reproduction in these fish. Since hormone treatments may directly control or influence the distinct phases or processes in the reproductive cycle including fecundity and the production and assimilation of yolk, it is surprising that the application or use of hormones in aquaculture has not been widely investigated. Broodstock are very expensive fish to maintain, in terms of their water supply and food requirements and the production of good quality eggs from these fish more than once a year, or at an earlier age would give the fish farmer a greater return on his investment. It is possible that the endocrine changes, brought about by photoperiodic manipulation aimed at more frequent spawning, could be augmented by hormonal treatments to produce better eggs. Furthermore, female fish normally spawn at either 2 or 3 years of age, and at first stripping the eggs are often small and unsaleable. Thus, female broodstock

have to be maintained for almost 4 years before any real return on investment is realised. It may be possible for the hormonal changes observed in maturing adults to be artificially produced in younger fish by the addition of hormones, such that first spawning occurs at 1 or 2 years of age, and/or better quality eggs are produced at 3 years of age. An investigation of the neuroendocrine mechanisms that control the different phases of ovarian development in female rainbow trout, may provide a greater understanding of how these processes can best be manipulated to provide optimum commercial advantage.

Clearly, one of the determining factors that governs the quality of eggs is the nature of the yolk, which constitutes the future food supply for the independent development of the embryo and alevin. Thus, the production and assimilation of this material must hold a position of importance amongst the different phases of reproduction. Furthermore, a study of the mechanisms that control these processes are of equal importance. A common feature amongst oviparous vertebrates appears to be the role of oestrogens and in particular oestradiol-17β in the production of a large lipoglycophosphoprotein yolk-precursor, vitellogenin, by the liver which is carried in the blood to the developing ovaries. It is the investigation of the synthesis of this precursor, vitellogenesis, and the neuroendocrine mechanisms involved in its control, towards which this present study is directed.

CHAPTER 2.

GENERAL MATERIALS AND METHODS.

2.1. Fish Maintenance, Handling and Blood Sampling.

2.1.1. Fish Maintenance.

The experimental animal used throughout this study was the rainbow trout (Genus *Salmo gairdnerii*, Family *Salmonidae*); this is the major farmed species in Great Britain. Fish of different stocks and strains were used in this work, but care was taken to ensure that fish from the same source were used in related experiments. Although the majority of fish were purchased from commercial farms, some were reared from broodstock held at the Fish Culture Unit at Aston. Detailed information regarding size, age and stock used in each experiment is included in the materials and methods section for each chapter. Generally, experiments were carried out either at the University of Aston Fish Culture Unit or at a commercial trout Hatchery and farm at Pewsey in Wiltshire. Before starting each experiment, the fish were weighed, and then fed daily with the appropriate ration and pellet size of a commercially-produced dry trout diet as recommended by the manufacturers (Mainstream B.P. Nutrition U.K. Ltd., Witham, U.K. or Omega, E. Baker, Sudbury, U.K.). Fish were starved for 24-36 hours prior to injection, blood sampling, tagging or sacrifice for histological examination.

All the experiments conducted at Aston, were carried out in recirculation systems within the Fish Culture Unit. Two systems were used, both of a similar carrying capacity, but with marked differences in the methods of solid settlement. However, these provided water of parallel quality and chemical composition (See

Appendix 2). A more detailed description of the systems can be found in Appendix 1.

2.1.2. Anaesthesia.

All blood sampling and hormone injections were carried out under anaesthesia to avoid scale damage, and consequent fungal and bacterial infections. Two anaesthetics were used, benzocaine (ethyl -4-amino-benzoate, BDH Chemicals Ltd., Poole, U.K.) and 2-phenoxyethanol (BDH), both at concentrations of 1:20,000. 2-phenoxyethanol proved to be more effective when used at the commercial farm, firstly because the larger fish sampled here showed better recovery, and secondly because it is more readily miscible with water than benzocaine, which has to be initially dissolved in acetone before addition to water. Benzocaine, however, was used quite successfully with minimal mortality for all the experiments at Aston. Fish were starved overnight prior to anaesthesia and allowed to recover in separate tanks with increased water flow.

2.1.3. Blood sampling.

Blood was removed from the Cuverian vein with a 1,2 or 5ml syringe (depending on fish size) fitted with a $1\frac{1}{2}$ inch 21G needle. The volume withdrawn depended on the size of fish and the frequency of sampling eg. 0.5 - 1.0ml from a fish of 50g, 2.0 - 5.0ml from a fish in excess of 1kg, up to twice in one week. Post-sampling haemorrhage or mortality was only rarely seen. The blood obtained was allowed to stand for 15 minutes, the clot rimmed with a needle to improve serum separation, then centrifuged at 2,500 rpm for 20 minutes. The resultant serum was pipetted into clean plastic

tubes (LP3, Luckham Ltd., Sussex, U.K.), stoppered and either stored at -20°C for future analysis, or assayed immediately.

In the field experiments 5ml serum monovettes (Sarstedt, Leics., U.K.) were used for sampling. These are essentially combined syringes and centrifuge tubes containing glass beads which creates a greater surface area on which the clot can form. Using these, 5ml of whole blood were taken with a 2 inch (19G) needle and after clotting treated as above.

2.1.4. Identification of Fish.

Several methods of identifying different treatment groups were assessed in this study. Initially, fin clipping was used, but this was discontinued because of problems with fin regeneration and 'nipping' of wounds by other fish. Subsequently, fish were tagged with small plastic numbered tags (Charles Neal, Finchley Ltd., East Finchley, U.K.) of an appropriate size and colour. These were attached dorsally just anterior to the dorsal fin with silver or a nickel alloy wire, with due allowance for growth of the fish. Using this method, at Aston, very few tags were lost, even on fish as small as 30g, although occasionally repeated netting loosened them sufficiently to make re-tagging necessary. This method, however, proved unsatisfactory under commercial conditions, due to entanglement and subsequent extraction of the wire with the 'loose-mesh' nets used. A modification was therefore adopted in which the small numbered tags were attached at the same dorsal position by punching a plastic pin through the hole in the tag and then through the fish's back. The device used for punching

the pins, is a gun used by the clothing industry which accepts a cassette of plastic pins (Kimbal Systems Ltd., Leics., U.K.).

2.2. Assay Methods.

2.2.1. Radioimmunoassay for Serum Oestradiol-17β.

The initial method used for the radioimmunoassay of serum oestradiol-17B was that developed in this laboratory by Whitehead (1979). The sheep anti-17β-oestradiol serum was supplied by Searle Diagnostic, High Wycombe, Buckinghamshire, U.K.. Each vial contained freeze-dried anti-17β-oestradiol conjugated with bovine serum albumin at the 6 position because this gives improved specificity over conjugates linked in other positions (Leonard & Craig, 1974). When reconstituted and diluted with the appropriate buffer to the volume shown on the vial, a working dilution of the antiserum was obtained which bound approximately 50% of a trace of tritiated-17β-oestradiol. Each tube then received 200µl of this working dilution and an antibacterial agent, sodium azide, added to the buffer, gave the reconstituted antisera a shelf-life of 6 weeks at $4^{o}C$.

The standard curve on all occasions exhibited full parallelism with serial dilutions of extracted rainbow trout sera (Fig. 2.1.). Cross-reactivity with other steroids was tested by running parallel standards against oestradiol-17β antiserum (Fig. 2.2.). The sensitivity of the assay was approximately 50 pg ml^{-1} and the overall precision of the assay estimated by making 6 replicate determinations from a pool of female serum. The intra-assay coefficient of variation was 4.23% and the inter-assay coefficient of variation was 7.49%. The efficiency of extraction was 82.53 $\pm$ 2.38% over a total of 10 assays.

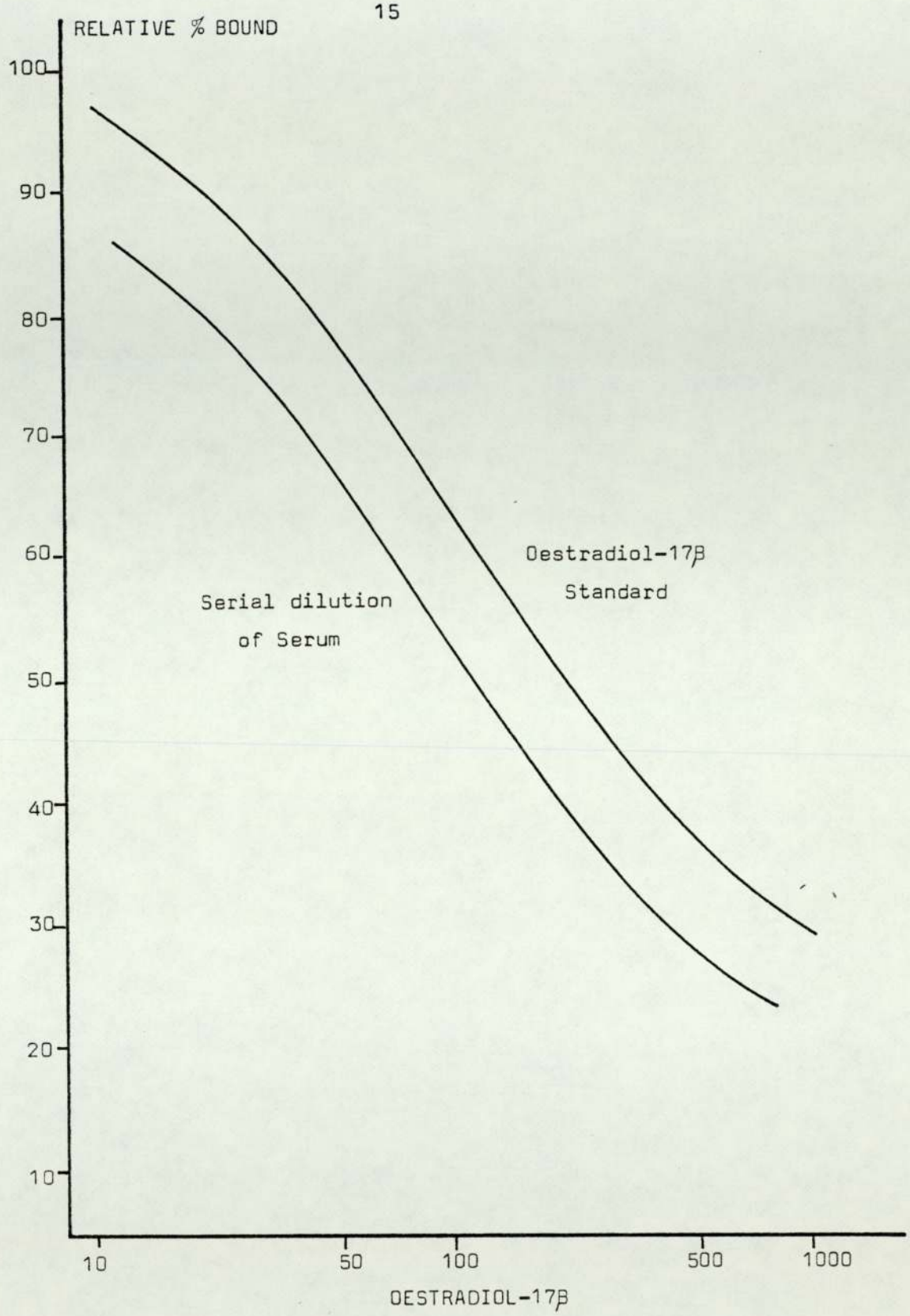


FIG. 2.1. PARALLELISM OF STANDARD CURVE FOR OESTRADIOL-17β WITH SERIAL DILUTION OF SERUM (AFTER C. WHITEHEAD, 1979).

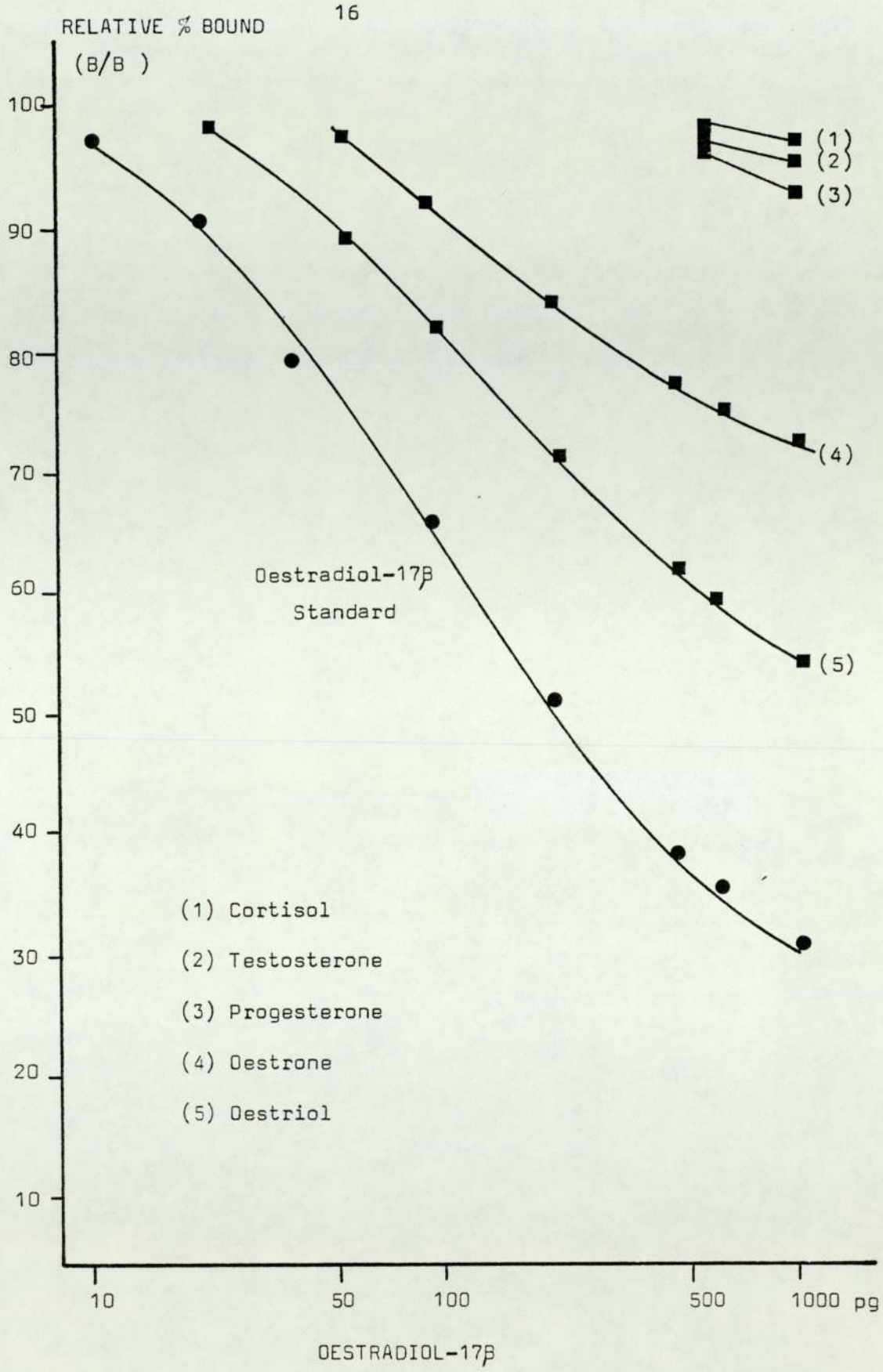


FIG. 2.2. CROSS REACTIVITY OF OESTRADIOL-17β ANTISERUM WITH SOME OTHER STEROIDS. (AFTER C. WHITEHEAD, 1979).

Materials :

(a) Dichloromethane with 1% methanol - frequently redistilled.

(b) Distilled water - double glass quality.

(c) Buffer A : (0.01M phosphate buffer pH 6.8 - 7.0)

Disodium hydrogen phosphate ($12H_2O$)	21.82g
Sodium dihydrogen phosphate ($2H_2O$)	6.08g
Sodium chloride	9.00g
Sodium azide	1.00g
Bovine serum albumen (BSA)	1.00g.

(d) Buffer B : As buffer A but without BSA.

(e) Charcoal suspension - 250mg powdered charcoal (Norito'l) in 50ml buffer B.

(f) Dextran solution - 25mg dextran (Pharmacia T40) in 50ml buffer B.

(g) (6,7,-^{3}H)-Oestradiol-17β-Radiochemical Centre Amersham

Specific activity - 44 Ci mM^{-1}

Stock solution - 20 uCi mM^{-1}

Working solution - 4 uCi in 100ml buffer B.

(h) Scintillator - 20g 2,5 Diphenyloxazole (PPO) in 2.5L toluene and 2.5L ethoxyethanol.

(i) Antiserum - from Searle Diagnostic (U.K.) - reconstituted as specified in buffer A.

(j) Standard oestradiol solutions - 0,5,10,20,50,100,200,500 and 1000 pg 100 ul^{-1} oestradiol in ethanol.

Assay Procedure :

(a) Shake vigorously 200µl of serum with 5ml dichloromethane (2 mins).

(b) Wash extracted phase with 2ml double distilled water and mix gently for 1.5 minutes.

(c) Centrifuge for 10 minutes at 2,500 rpm to separate emulsion.

(d) Set up water blanks by washing 5ml dichloromethane with 2ml double distilled water as in (c).

(e) Take 500µl aliquot of dichloromethane extract into a clean assay tube.

(f) Pipette 100µl of each standard into similar tubes.

(g) Take extracts and standards to dryness at 40°C under a stream of medical grade nitrogen.

(h) Add 200µl antiserum dilution.

(i) Add 200µl working label solution.

(j) Vortex for 30 seconds.

(k) Incubate at 37°C for 1 hour.

(l) Transfer to ice bath for minimum of 30 minutes.

(m) Add 200µl cooled dextran coated charcoal suspension, mix reagents (e) and (f) 1:1 v/v (not more than 30 minutes prior to use).

(n) Mix twice briefly using a vortex mixer - centrifuge for 10 minutes at 1,500 rpm (4°C).

(o) Transfer a 400µl aliquot to 10ml scintillation fluid in a counting vial.

(p) Count for 10 minutes or to 10,000 free counts.

(q) For total counts, count 200µl working label solution in 10ml scintillant.

Calculations :

(a) Correct free counts from 400µl aliquot to total free counts (ie multiply by 0.6/0.4).

(b) Calculate % free and hence % bound.

$$B = 100 (F/T \quad 1.5 \quad 100) \%.$$

(c) Plot standard curve of % bound against pg oestradiol.

(d) Calculate water blank correction (ie y - x).

(e) Correct % bound of assay samples.

$$B' = B + (y - x).$$

(f) Read off pg oestradiol from standard curve using B'.

(g) Correct for recovery.

(h) Correct results to pg/ml.

Explanation of symbols :-

B = % bound

B' = corrected % bound

F = % free

T = total counts

y = % bound in water blank

x = % bound in 0 standard

2.2.2. Second method for Oestradiol-17β.

Due to the cessation of production of the Searle Diagnostic antiserum, a new antisera was obtained from Steranti-Research, St. Albans, England. The antiserum preparation represents a pool of high titre, high specificity bleeds of successfully immunised New Zealand White rabbits. The antigen used was 17β-oestradiol-6-(CMO) BSA prepared by O-carboxy-methyl-oxime formation at the steroid '6' position and subsequent coupling to bovine serum albumen. Oestradiol-17β labelled in four positions instead of two (Radiochemical Centre, Amersham) had a higher specific activity. In addition further modifications were made to the method as follows :-

(1) Ethyl acetate was substituted for dichloromethane because it gave an improved efficiency of extraction and thus better recovery of the steroid.

(2) A recovery step was included.

(3) The incubation period of the assay was extended overnight at $4^{o}C$ to give a more consistent binding.

(4) A serial dilution of standard was used since this minimised possible sources of error.

The cross-reactivities were determined of a number of steroids having either structural similarities to oestradiol-17β, or which one might expect to find in teleost serum. The cross-reactivity of each steroid is expressed as the dimunition in the proportion of bound label produced by 100 pg of steroid relative to that produced by 100 pg of oestradiol-17β (Table 2.1.).

STEROID	% CROSS REACTIVITY
OESTRADIOL-17β	TAKEN AS 100%
OESTRONE	7.5%
OESTRIOL	12.2%
TESTOSTERONE	1.6%
11-KETOTESTOSTERONE	1.0%
ANDROSTENEDIONE	1.8%
17α HYDROXY-20β-DIHYDROPROGESTERONE	< 0.5%
CORTISOL	< 0.5%
PREGNENOLONE	< 0.5%
(NON SPECIFIC)	2.1

TABLE 2.1. CROSS REACTION DATA FOR OESTRADIOL-17β ANTI-SERUM WITH SOME OTHER STEROIDS.

The inter-assay coefficient of variation was found to be 6.05% and the intra-assay variation 3.97%. This meets the requirements for assay precision as laid down by Abraham, (1974) who indicated that within (intra) assay variance should be 10% or less. The accuracy of the assay, was determined by both the % recovery of oestradiol-17β and the assay of known amounts of the steroid added to charcoal-treated serum. The correlation between known amounts of oestradiol-17β added to the serum and those estimated by RIA was highly significant ($P < 0.001$; $r=0.999$). The sensitivity of the assay can be calculated by the following expression :-

$$S = \frac{s \times 100}{R \times F}$$

if

R = % recovery

F = fraction of sample used

s = sensitivity of standard curve (smallest amount of standard significantly different from 0 at 95% confidence limit - Abraham, 1974).

The sensitivity was calculated to be about 200pg ml^{-1} of serum.

Materials :-

(a) Ethyl acetate (Analar, BDH Chemicals).

(b) Analar water (Hopkin and Williams Ltd.).

(c) RIA Buffer (0.1M phosphate buffer pH 7.0; containing 0.9% NaCl; 0.1% Gelatine and 0.01% Sodium azide).

(i) 0.1M Disodium hydrogen phosphate (Na_2HPO_4 anhydrous) in Analar water.

(ii) 0.1M Sodium dihydrogen phosphate ($NaH_2PO_4 2H_2O$) in Analar water, (i) and (ii) mixed approximately 50/30 v/v and adjusted to pH 7.0.

(d) Dextran-coated charcoal - 'Separex' (Steranti Research) tablets. Each tablet when dissolved in 50ml RIA buffer produces a very fine and homogenous suspension of Dextran-coated charcoal. Made up freshly.

(e) (2,4,6,7-^{3}H) -Oestradiol-17β-Radiochemical Centre, Amersham.

As supplied - 80 Ci mM^{-1}

Stock solution - 10 uCi ml^{-1}

Working solution - 30,000 dpm 100 ul^{-1}.

(f) Recovery label - 5ml working solution up to 50ml with RIA buffer.

(g) Scintillant - 20g Diphenyloxazole (PPO) in 2.5L toluene and 2.5L ethoxyethanol.

(h) Antiserum (Steranti-Research, St. Albans, Herts., Code E002). Reconstituted as specified, in RIA buffer.

(i) Standard oestradiol solution - 1000 pg 100 ul^{-1} oestradiol in ethanol. Serial dilution 1000 - 4.0 pg 100 ul^{-1}.

Assay Procedure :

(a) Pipette 100µl of each serum sample into separate polypropylene tubes (Hughes and Hughes Ltd., Romford, U.K.).

(b) Add 100µl of recovery label.

(c) Mix well and incubate overnight at 4°C.

(d) Add 1ml Ethyl acetate.

(e) Mix on rotary mixer for 1 hour.

(f) Centrifuge briefly at 1500 rpm for 10 minutes.

(g) Transfer a 100µl aliquot of extract into 10ml scintillant in a counting vial and count against 100µl of recovery label for 10 minutes to obtain % recoveries.

(h) Take 100µl aliquot of extract into clean assay tubes (Radley and Co. Ltd., Sawbridge, U.K.).

(i) Prepare serial dilution of oestradiol standard in duplicate in similar assay tubes 1000 pg - 4.0 pg, and 0.

(j) Take extracts and standards to dryness in vacuum oven at less than 35°C.

(k) Cool to 4°C.

(l) Add 100µl antiserum - to bind 50% approximately.

(m) Add 100µl labelled oestradiol - to give 30,000 dpm.

(n) Vortex for 30 seconds and incubate overnight at 4°C.

(o) Reconstitute 'Separex' tablets with RIA buffer prepared not more than 30 minutes before use.

(p) Add 500µl of dextran-coated charcoal, prepared in (o) above, to each tube.

(q) Mix briefly and incubate for 10 minutes at 4°C.

(r) Centrifuge for 10 minutes at 1500 rpm (4°C).

(s) Transfer 400µl supernatant to 10ml scintillant in a counting vial.

(t) Count for 10 minutes.

(u) For total counts, count 100µl working label solution in 10ml scintillant.

Calculations :

(a) Correct % binding from 400µl aliquot to total % bound counts (multiply by 0.7/0.4).

(b) Calculate % bound.

(c) Plot standard curve of % bound against pg oestradiol.

(d) Read off pg oestradiol from standard curve.

(e) Correct for recovery.

(f) Correct results to ng ml^{-1} trout serum.

(g) No water-blank correction was used since in all assays it was found not to be significantly different from the 0 standard.

2.2.3. Radioimmunoassay for Oestrone.

The method used for measuring oestrone in trout serum was similar to the liquid phase RIA procedure for oestradiol-17β outlined above. The specific antiserum which was again obtained from Steranti Research Ltd., St. Albans, U.K. raised in New Zealand White rabbits against oestrone-6-O(Carboxymethyl) oxime-bovine serum albumen. 2,4,6,7-^{3}H oestrone was used as label (Radiochemical Centre, Amersham, U.K.) having a high specific activity (80 Ci mM^{-1}).

Cross-reactivity data for a number of steroids are shown in Table 2.2.. The intra-assay coefficient of variation was 4.07% and the inter-assay coefficient of variation 7.15%. The sensitivity of the assay was calculated by the following expression :-

$$S = \frac{s \times 100}{R \times F}$$

where R = % recovery

F = fraction of sample used

s = sensitivity of standard curve.

The sensitivity of this assay was calculated as approximately 160 pg ml^{-1}.

STEROID	% CROSS REACTIVITY
OESTRONE	TAKEN AS 100%
OESTRADIOL-17β	9.5%
OESTRIOL	10.2%
TESTOSTERONE	1.8%
11 KETOTESTOSTERONE	0.5%
ANDROSTENEDIONE	2.1%
17α HYDROXY-20β-DIHYDROPROGESTERONE	< 0.5%
CORTISOL	< 0.5%
PREGNENOLONE	0.9%
(NON SPECIFIC)	1.2%

TABLE 2.2. CROSS REACTION DATA FOR OESTRONE ANTI-SERUM WITH SOME OTHER STEROIDS.

Materials :

(a) Ethyl acetate (Analar).

(b) Water (Analar).

(c) RIA Buffer (0.1M phosphate buffer pH 7.0 containing 0.9% Sodium chloride; 0.1% gelatine and 0.01% Sodium methiolate)

(i)	Disodium hydrogen phosphate (Na_2HPO_4 anhyd.)	4.44g
(ii)	Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	2.91g
(iii)	Sodium chloride	2.25g
(iv)	Gelatine	0.25g
(v)	Sodium methiolate	0.03g.

(d) Dextran-coated charcoal - 'Separex' (Steranti Research, St. Albans, U.K.).

(e) 2,4,6,7-^{3}H oestrone - (Radiochemical Centre, Amersham)

Specific activity - 80 Ci mM^{-1}

Working solution - 30,000 dpm $100ul^{-1}$.

(f) Recovery label - 5ml working solution, made up to 50ml with buffer.

(g) Scintillant - 20g Diphenyloxazole (PPO) in 2.5L toluene and 2.5L ethoxyethanol (scintillator grade).

(h) Antiserum - (Steranti Research, St. Albans, U.K., Code E001). Reconstituted as specified in RIA buffer to bind 50%.

(i) Standard oestrone solution (Steranti Research, Code ST E001).

Stock A as supplied - 10 μg $100\mu l^{-1}$

Working solution - 1000 pg $100\mu l^{-1}$.

Assay procedure :

(a) Pipette 100µl of each serum sample into separate polypropylene tubes.

(b) Add 100µl of recovery label.

(c) Mix well and incubate overnight at 4°C.

(d) Add 1ml Ethyl acetate.

(e) Mix on rotary mixer for 45 minutes.

(f) Centrifuge briefly at 1500 rpm for 10 minutes.

(g) Transfer a 100µl aliquot of extract into 10ml scintillant in a counting vial and count against 100µl recovery label for 10 minutes to obtain % recoveries.

(h) Take 200µl aliquot of extract into clean assay tubes (Radley and Co. Ltd).

(i) Prepare serial dilution of oestrone standard in duplicate in similar assay tubes over range 1000pg - 4.0pg, and 0.

(j) Take extracts and standards to dryness in vacuum oven at less than 35°C.

(k) Cool to 4°C.

(l) Add 100µl working antiserum dilution.

(m) Add 100µl working oestrone label.

(n) Vortex for 30 seconds and incubate overnight at 4°C.

(o) Reconstitute 'Separex' tablet with RIA buffer. One tablet dissolved in 50ml is sufficient for 100 tubes. Prepared not more than 30 minutes before use.

(p) Add 0.5ml dextran-coated charcoal, as prepared above, to each tube.

(q) Mix briefly and incubate for 10 minutes at 4°C.

(r) Centrifuge for 10 minutes at 1500 rpm ($4^{o}C$).

(s) Transfer 0.4ml supernatant to 10ml scintillant in a counting vial.

(t) Count for 10 minutes.

(u) For total counts, count 100µl working label solution in 10ml scintillant.

2.2.4. Radioimmunoassay for Serum Testosterone.

A similar method was used for measuring testosterone in trout serum to that used to determine oestradiol-17B and oestrone (2.2.2. & 2.2.3.). The specific antiserum was also obtained from Steranti Research U.K. Ltd., and high specific activity tritiated testosterone supplied by the Radiochemical Centre (Amersham, U.K.). More ethyl acetate (2 or 4ml) was used in the extraction phase to account for the very high levels of testosterone (>200ng ml^{-1}) found in mature female trout. This was found preferable to reducing the aliquot volume taken from the extract to be assayed, since aliquots of less than 100ul caused considerable error and inaccuracy. Cross-reactivity data for a number of steroids are shown in Table 2.3. and Fig. 2.3. where the cross-reactivity is determined as the % depression of 50 pg of testosterone by 50 pg of the test steroid. The intra-assay coefficient of variation was 9.09% and the inter-assay coefficient of variation 11.5%. The sensitivity of the assay was determined as approximately 80 pg ml^{-1}. Whilst serial dilution of mature female serum (high testosterone) exhibited full parallelism with the standard curve, mature male trout serum did not always appear to do so (See Fig. 2.3.). This may be as a result of cross-reaction with 11-ketotestosterone (35%), often present at significant levels in male serum.

STEROID	% CROSS REACTIVITY
TESTOSTERONE	TAKEN AS 100%
11 KETOTESTOSTERONE	34.5%
DEHYDROEPIANDROSTERONE	20%
OESTRADIOL-17β	5.8%
OESTRONE	2.9%
CORTISOL	< 0.5%
17α HIDROXY-20β-DIHYDROPROGESTERONE	1.66%
17α HYDROXY PROGESTERONE	0.5%
PREGNENOLONE	3.3%
(NON SPECIFIC)	1.8

TABLE 2.3. CROSS REACTION DATA FOR TESTOSTERONE ANTI-SERUM WITH SOME OTHER STEROIDS.

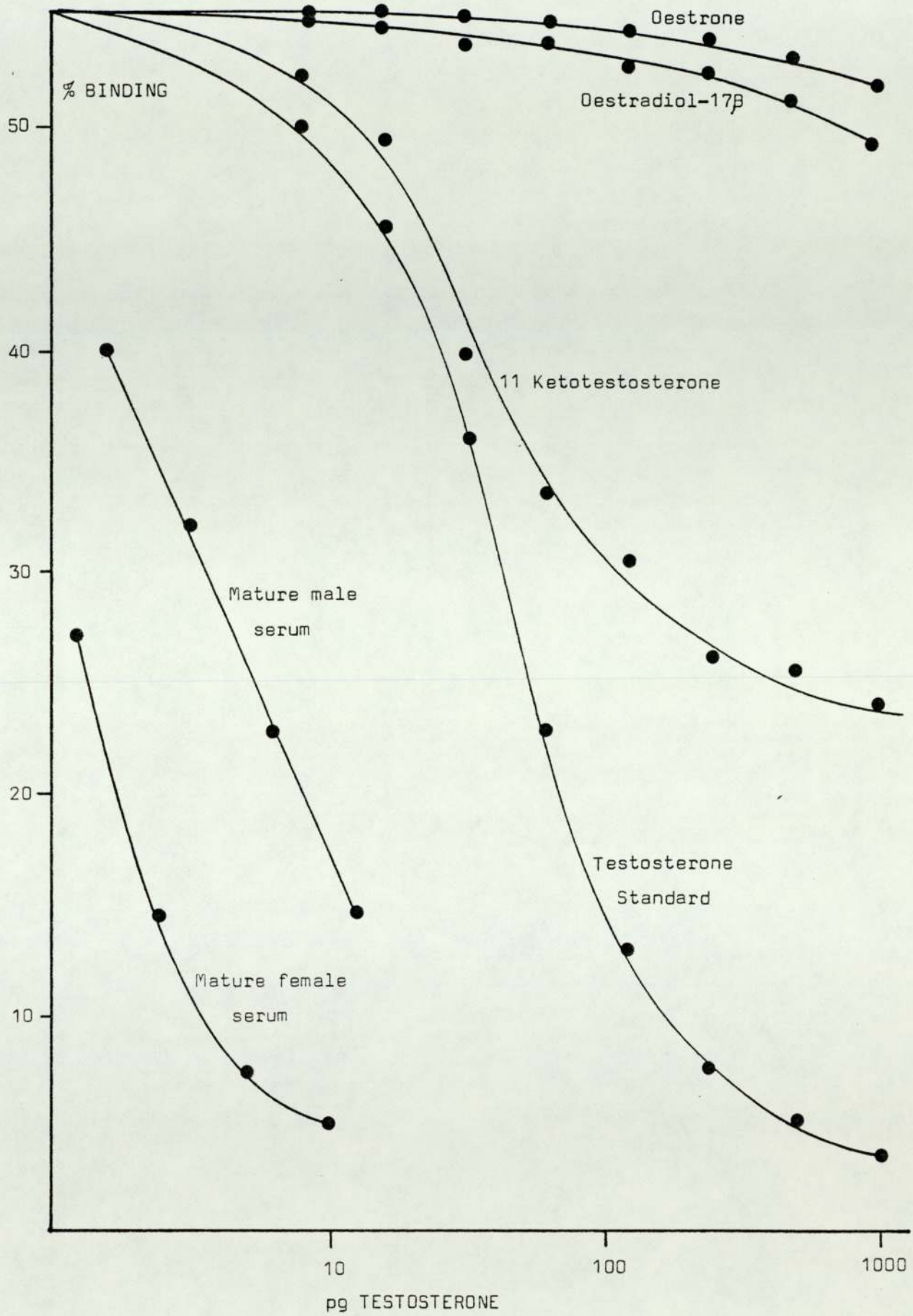


FIG. 2.3. SERIAL DILUTION OF SERUM FROM MATURE MALE AND FEMALE TROUT COMPARED WITH TESTOSTERONE STANDARD AND CROSS REACTION WITH SOME OTHER STEROIDS.

Materials :

(a) All buffer, scintillant and charcoal reagents were as used in the RIA methods for oestradiol-17β and oestrone.

(b) Tritiated testosterone - 1,2,6,7-^{3}H Testosterone prepared to working solution of 22,000 dpm 100μl^{-1}.

(c) Recovery label - 5ml working solution up to 50ml with RIA buffer.

(d) Antiserum (Steranti Research, St. Albans, Herts., U.K. Code A001) reconstituted as per manufacturers instructions.

(e) Standard testosterone solution - 1000 pg 100μl^{-1} testosterone in acetone. Serial dilution 1000 - 4.0 pg 100μl^{-1}.

Assay Procedure :

(a) Pipette 100µl of each serum sample into separate polypropylene tubes.

(b) Add 100µl of recovery label.

(c) Mix well and incubate overnight at 4°C.

(d) Add 2ml (or 4) of ethyl acetate.

(e) Mix on rotary mixer for 1 hour.

(f) Centrifuge briefly at 1500 rpm for 10 minutes.

(g) Transfer 100µl aliquot of extract into 10ml scintillant in a counting vial and count against 100µl of recovery label for 10 minutes to obtain % recoveries.

(h) Take 100µl aliquot of extract into clean glass assay tubes.

(i) Prepare serial dilution of testosterone standard in triplicate in similar assay tubes 1000 - 4.0 pg and zero.

(j) Take extracts and standards to dryness in vacuum oven at less than 35°C.

(k) Cool to 4°C.

(l) Add 100µl antiserum (to bind 50%)- leave on bench for 30 minutes.

(m) Add 100µl labelled testosterone - to give 22,000 dpm.

(n) Vortex briefly and incubate for 4 hours at room temp. or overnight at 4°C.

(o) Reconstitute 'Separex' tablets (dextran-coated charcoal) with RIA buffer just before use.

(p) Add 500µl of dextran-coated charcoal to each tube.

(q) Mix briefly and incubate for 10 minutes at $4^{o}C$.

(r) Centrifuge for 10 minutes at 1500 rpm ($4^{o}C$).

(s) Transfer 400µl supernatant to 10ml scintillant in a counting vial.

(t) Count for 10 minutes.

(u) For total counts, count 100µl working label solution in 10ml scintillant.

(v) For non-specific binding run assay tube with 100µl RIA buffer + 100µl label (no antisera).

Calculations :

(a) Correct % binding from 400µl aliquot to total % bound counts (multiply by 0.7/0.4).

(b) Calculate % bound.

(c) Plot standard curve of % bound against pg testosterone.

(d) Read off pg testosterone from standard curve.

(e) Correct for recovery.

(f) Correct results to ng ml^{-1} trout serum (ie account for Et Ac volume).

2.2.5. Assay of Serum Vitellogenin.

In this study vitellogenin levels were measured directly by a specific radioimmunoassay as well as indirectly by measurements of serum phosphoprotein phosphorus and total serum calcium. A further qualitative estimation was provided by polyacrylamide gel electrophoresis. Each is considered separately in the following sections :-

2.2.5.1. Radioimmunoassay of Trout Vitellogenin.

The double antibody technique used was that developed by Dr. J.P. Sumpter, School of Applied Biology, Brunel University, U.K. and the first assays were conducted under his direction at Brunel. Intact vitellogenin as standard was purified on sepharose 6B and antiserum raised in rabbit's showed little cross reactivity with the component parts of the trout vitellogenin molecule or salmon vitellogenin (J.P. Sumpter personal communication). The standard was iodonated using a recently developed method utilising an insoluble oxidising agent 'iodogen' (Salacinski et al., 1979). Approximately 38% of the standard incorporated I^{125} and pooled fractions were subsequently chromatographed on Concanavalin A-sepharose. Immunoprecipitation with anti-rabbit gamma globulin antibody raised in sheep, were used to separate free and bound fractions. The sensitivity of the assay was 10ng, the intra-assay coefficient of variation was approximately 7.02% and inter-assay coefficient of variation 8.83%.

Materials :

(a) RIA Buffer : Phosphate buffered saline (0.05M NaP, 0.15 NaCl) containing 1% egg albumin; 0.1% NaN_3 pH 7.05).

(b) Standard : Intact purified trout vitellogenin over Standard range 150ng ml^{-1} to 0.29ng ml^{-1}.

(c) 1st Antibody : Anti vitellogenin (R288) at 1:100,000 (made up in 1:400 Normal rabbit serum).

(d) Normal rabbit serum (NRS) : Normal rabbit serum at 1:400 with RIA Buffer.

(e) 2nd Antibody : anti-rabbit gamma globulin, diluted 1:20 with RIA Buffer.

(f) Label : I^{125} vitellogenin sufficient to give approximately 8000 cpm.

Method :

(a) Prepare total counts in triplicate :-

50µl RIA buffer, mixed with 50µl label (added at (i)) below.

(b) Prepare non-specific binding tubes (NSB) in triplicate :-

50µl RIA buffer mixed with 50µl NRS.

(c) Prepare maximum binding tubes in triplicate :-

50µl RIA buffer; 50µl of anti-vitellogenin (added at (g) below) mixed with 50µl of labelled vitellogenin (added at (i) below).

(d) Prepare standards in triplicate by serial dilution with buffer as follows.

(e) To all unknown assay tubes (LP2/2, Luckham, U.K.) add 50µl RIA buffer.

(f) Add 50µl of each serum sample (diluted with RIA buffer to fit within standard range) in triplicate.

(g) Add 50µl anti-vitellogenin to all assay tubes.

(h) Vortex briefly and incubate for 24 hours at $4^{o}C$.

(i) Add 50µl of labelled vitellogenin.

(j) Vortex and incubate for 24 hours at $4^{o}C$.

(k) Add 50µl anti-rabbit globulin.

(l) Vortex and incubate for 24 hours at $4^{o}C$.

(m) Centrifuge at 3000Xg for 30 minutes ($4^{o}C$) - not total counts tubes.

(n) Remove supernatant with aspirator - not total counts.

(o) Count all tubes - for 100 seconds to obtain cpm.

Calculations :

(a) Determine maximum binding - ie mean of maximum binding tubes less mean of non-specific binding tubes.

(b) Correct all standard and sample counts for non-specific binding - determine mean triplicate values.

(c) Plot log ng vitellogenin standard against % maximum binding.

(d) Read unknown from standard curve to give ng ml^{-1}, corrected to account for dilution to fit standard curve.

2.2.5.2. Assay of Phosphoprotein Phosphorus.

Prior to using this specific radioimmunoassay for vitellogenin, a method was used to measure serum phosphoprotein phosphorus, as the phosvitin component of the vitellogenin molecule is rich in inorganic phosphorus. The method used was the method outlined in the Boehringer Corporation Test Handbook (1969), as modified by Whitehead (1979). The levels of phosphoprotein phosphorus may then be converted to vitellogenin by multiplying by 71.43 assuming as indicated by Craik (personal communication to C. Whitehead) that vitellogenin contains 1.4% phosphoprotein phosphorus as in other vertebrates.

The sensitivity of the method was approximately 10μg ml^{-1}, equivalent to 0.71mg ml^{-1} vitellogenin. The inter-assay coefficient of variation was 11.74% and the intra-assay coefficient of variation 9.47%.

Materials :

(a) 20% Trichloroacetic acid.

(b) Ethanol.

(c) Chloroform : ether : ethanol (1:2:2).

(d) Acetone.

(e) Ether.

(f) 60% Perchloric acid (Analar).

(g) 100 vol. Hydrogen peroxide (Analar).

(h) Molybdate reagent : 40 mM ammonium molybdate, 2.5N H_2SO_4 (ie 49.44g $L^{-1}(NH_4)_6MO_7O_{24}4H_2O$; + 122.5g L^{-1} conc. H_2SO_4).

(i) Vanadate reagent : 21 mM ammonium vanadate, 0.28M HNO_3 (ie 2.46g L^{-1} NH_4VO_3; + 17.6g L^{-1} conc. HNO_3).

(j) Standard solutions prepared from Na_2HPO_4 hydrate crystals or $K_2H_2PO_4$ crystals to contain 0,25,50,75 and 100µg $100\mu l^{-1}$ phosphorus.

Method :

(a) Take 100µl serum sample.

(b) Precipitate proteins with 5ml 20% trichloroacetic acid.

(c) Centrifuge for 10 minutes at 2,500 rpm to isolate precipitate.

(d) Wash with organic solvent to remove lipid, ie wash successively with hot ethanol; chloroform: ether: ethanol (1:2:2); acetone and ether (Wallace, 1970).

(e) Dry protein pellet.

(f) To protein pellet add 0.5ml perchloric acid (60%) and 0.1ml hydrogen peroxide.

(g) Mix well and stand for 12 hours.

(h) Heat at 180-200^{o}C for 20 minutes. (If not completely colourless to eye and free of black solids, add a further 0.1 ml hydrogen peroxide and heat for a further 20 minutes).

(i) Add : 2ml H_2O
1ml Vanadate reagent
1ml Molybdate reagent.

(j) Stand for 10 minutes and measure optical density at 400 nm against a solution of :
2ml H_2O
1ml Vanadate reagent
1ml Molybdate reagent.

(k) Plot standard curve of ug phosphorus against optical density and read off samples.

2.2.5.3. Assay of Total Serum Calcium.

Total serum calcium was measured on a Corning calcium analyser, model 940. The complexiметric titration was first introduced by Schwarzenbach and Biedermann (1946). The method is based on measuring the fluorescence of a dye when associated with calcium. The dye used is calcein (first introduced by Diehl and Ellingboe, 1956) which is a fluorescein derivative and forms an intensely fluorescent non-dissociated complex with calcium ions in an alkaline medium (1M KOH). The analytical procedure used with model 940 is based upon the quenching of this fluorescence by chelating the calcium ions with the titrant Ethyleneglycol bis N,N' - tetraacetic acid (EGTA) - in preference to Ethylene diamine tetrachloroacetic acid (EDTA) especially in the presence of magnesium ions (Schmidt & Reilley, 1957).

The inter- and intra-assay coefficients of variation with fresh serum were 0.02% and 1.27% respectively. The inter-assay coefficients of variation for stored serum at 20°C; 4°C; -20°C and repeatedly thawed and -20°C (28 days) were 30.52%, 7.7%, 4.0% and 1.3% respectively. Although the majority of serum samples were assayed within a short period of bleeding, immediate freezing of samples at -20°C until assay without thawing, would appear to provide a reliable method of storage with no significant change in measured levels with time. This was proven by comparing calcium levels in replicate samples which had been stored at -20°C, 4°C, 20°C and also at -20°C but with repeated thawing (5 times in one month).

Method :

The method used was as described in the instruction manual as supplied with the Corning Calcium Analyser (940). The analyser consists essentially of a reaction cuvette with magnetic stirrer, EGTA reservoir and fluorescent light source, together with the appropriate electrical circuitry including digital read-out display.

(a) Switch machine on and allow to warm up for 15 minutes.

(b) Fill cuvette with 1M KOH to appropriate level, place in machine - check magnetic stirrer is working.

(c) Add 100µl 'Calcein' dye to cuvette.

(d) Add 100µl (10µg) calcium standard to cuvette.

(e) Partially close lid to check fluorescent light is working.

(f) Press 'TITRATE' button - which continues to flash.

(g) The machine is now calibrated by repeatedly adding 50µl aliquot (ie sample size) of calcium standard and pressing 'TITRATE' until the digital readout on successive samplings reads less than 0.2% error.

(h) Press 'CALIBRATE'.

(i) 50µl serum samples are added successively and titrated automatically.

(j) Digital readout gives calcium in mg% or mEq/L as determined by preselection at step (a).

2.2.5.4. Estimation of Vitellogenin in Trout Serum using Polyacrylamide-gel Disc Electrophoresis.

The method used was a modification of the technique developed by Yaron & Widzer (1978). Although this method can provide absolute levels of vitellogenin, the absence of a purified trout vitellogenin at the time of assay, together with our limited accessibility to a suitable densitometer and the heterogeneity of the vitellogenin molecule prevented such quantitative determinations in this work. However, this method did provide an important qualitative measure of the vitellogenin complex particularly when used in conjunction with the other three methods.

Materials :

(a) Tris HCl buffer (0.38M, pH 8.9) :

Tris 23g in 450ml distilled water adjusted to pH 8.9 with conc. HCl and made up to 500ml.

(b) Tris glycine buffer (0.005M Tris and 0.04M glycine) :

Glycine 2.8g and 0.6g tris made up to 1 litre and adjusted to pH 8.3 with tris.

(c) Cyanogum (Sigma) - 10% w/v in Tris-HCl buffer (store at $4^{o}C$).

(d) Saturated sucrose solution :

Prepare 5ml and add phenol to deep red colour.

Keep at room temperature.

(e) Sudan-black.

(f) Fast-green stain.

(g) Alizarin red(S) stain.

(h) 'Temed' catalyst (Sigma).

(i) Ammonium persulphate :

400mg in 2ml distilled water prepared freshly.

(j) Acetic acid :

7.5% v/v - prepare 2-3 litres for washing gels.

(k) 12 glass tubes in which to prepare the gels - approximately 75mm long by 5mm internal diameter.

Preparation of Gel columns :

Prepared not more than 24 hours before use, the main body of the gel (5.5%) acts as the separating gel, the top of which is a 3.5% starting gel to give a better demarcation of the protein bands.

(a) Mark glass tubes 65mm and 70mm from one end.

(b) Close the bottom ends tightly with parafilm.

(c) Place tubes upright as vertical as possible.

(d) Prepare 5.5% gel solution as follows :

10% cyanogum	-	12.3 ml
Tris-HCl buffer	-	10.2 ml
Temed	-	0.02ml
Ammonium persulphate	-	0.55ml.

(e) Fill glass tubes up to 65mm mark using pasteur pipette and avoiding air bubbles.

(f) Add a few drops of water very carefully to the top of the gel without stirring or mixing - this flattens the gel.

(g) When set - remove water from surface without damaging gel surface.

(h) Prepare 3.5% gel solution as follows :

10% cyanogum	-	5.3 ml
Tris-HCl buffer	-	9.7 ml
'Temed'	-	0.02ml
Ammonium persulphate	-	0.4 ml

(i) Fill tubes to 70mm mark and add water again to flatten gel.

Electrophoretic Separation :

(a) Fill lower tank with Tris-glycine buffer.

(b) Remove parafilm from bottom of tubes and insert into apparatus avoiding air bubbles.

(c) Fill upper tank with Tris-glycine buffer - cover tubes.

(d) Using a Hamilton 10 or 25µl precision syringe, take a 4µl sample of serum.

(e) Mix with an equal volume of saturated sucrose solution.

(f) Draw total volume into syringe and place on top of the gel column under the buffer.

(g) Put the complete apparatus in a cold chamber ($4^{o}C$).

(h) Connect positive of power supply (Shandon) to lower chamber and negative to upper chamber.

(i) Run gels at one Amp column^{-1} until the ion front reaches the separating gel.

(j) Increase current to 4 m Amp column^{-1}.

(k) Once ion front is about 5mm from end - switch off.

Fixation, Staining and Destaining :

(a) Remove gels quickly.

(b) Allow gels to slide into serological test-tubes.

(c) Fix and stain proteins simultaneously with one of the appropriate stains.

(d) Destain by washing in excess acetic acid (7.5% w/v).

(e) Store gels in 7.5% acetic acid.

Densitometry and Quantitative Method :

If known amounts of vitellogenin standard are run down the gels and densiometric readings taken with an appropriate densitometer, a standard curve of known vitellogenin versus densiometric reading can be plotted. Unknown samples can be read from the curve and the method can be applied quantitatively to estimate serum vitellogenin.

CHAPTER 3.

OESTRADIOL-17B AND VITELLOGENESIS.

3.1. Introduction.

An almost universal feature of oocyte development in animals is the accumulation within the cytoplasm of a "food store" or yolk, on which future embryonic development depends. Although collectively referred to as yolk, a rather loose term since in chemical structure there are species differences, Pan et al., (1969) proposed the generic name 'vitellogenin' to cover all protein precursors of yolk. There are further species differences in the relative amounts of yolk stored in the oocytes with respect to their degree of organisation. Thus, for example in species producing oligolecithal eggs such as the sea urchin (Arbacia) only about a quarter of the oocyte volume is taken up by yolk granules (Harvey, 1956). Whereas the telolecithal eggs of the teleosts, and birds contain so much yolk that the food reserves are segregated completely from the cytoplasm surrounding the oocyte nucleus. Vast amounts of vitellogenin must therefore be produced, most especially in oviparous vertebrates, for example in the birds where a chicken ovum increases from 1-16 g in the seven days preceding ovulation (Warren & Conrad, 1939). This rapid production of yolk is no less impressive in teleosts where the eggs, though smaller, may be much more numerous. In the trout (Salmo gairdneri) the oocyte grows from a previtellogenic size of 30 um to one of 3.5 mm over the 7 month period preceding spawning when the female gonad may be as much as 20% of the body weight, or 1000 g in a 5 Kg fish (Elliott, 1981 unpublished). Although almost 50% of this weight may be taken up with water, this 40,000 fold increase in oocyte volume is indicative of a vitellogenin uptake by the ovary

of approximately 1g a day.

It is apparent that vitellogenesis must be preceded and accompanied by a radical reorganisation of metabolic activity and resources in order to produce these yolk materials. Consideration must be given not only to the processes involved in vitellogenesis and where they take place, but also to the mechanisms effecting regulation.

In the oviparous vertebrates a consistent feature is the role of oestrogenic steroids in stimulating vitellogenesis, the evidence coming from studies using birds, reptiles, amphibians and fish (Bailey, 1957; Dessauer & Fox, 1959; Chung-Wai et al., 1961; Heald & Mclachlan, 1964; Hahn, 1967; Wallace & Dumont, 1968; Elliott et al., 1979). Thus, many workers have labelled vitellogenin as a sex-limited protein, being found in mature female animals but not at all in males unless after oestrogenic stimulation (Follett & Redshaw, 1974; Wallace, 1978; Knowland, 1980). Although the majority of this work is based on amphibians, further studies with fish have similarly indicated it's sex-limited nature in that it is found in mature female fish (Vanstone & Ho, 1961; Ridgeway et al., 1962; Drilhon & Fine, 1963; Thurston, 1967; Hara, 1975; Le Menn, 1979; Whitehead et al., 1978a) and in male and immature female fish after oestrogenic stimulation (Bailey, 1957; Ho & Vanstone, 1961; Plack et al., 1971; Aida et al., 1973; Hickey & Wallace, 1974; Emmersen & Petersen, 1976; Mugiya & Watabe, 1977; de Vlaming et al., 1977; Yaron et al., 1977; Craik, 1978a; Terkatin-Shimony & Yaron, 1978; Elliott et al., 1979; Hori et al., 1979; Korsgaard & Petersen, 1979; de Vlaming et al., 1980).

In almost all these studies oestradiol has been the oestrogen chosen to induce vitellogenesis. In only one study has the action of other oestrogens been studied, where oestriol was found to be much more potent in raising blood vitellogenin levels than either oestrone or oestradiol-17B (Terkatin-Shimony & Yaron, 1978). This is somewhat surprising since there is no evidence to suggest that oestriol is present in teleost blood and some recent work demonstrated the absence of oestriol in the rainbow trout (Diederik et al., 1981; Lambert & Van Oordt, 1981). One explanation for the potency of oestriol may be attributed to the oestrogen per se, rather than its conversion into oestradiol-17β. Furthermore, massive doses of androgens have also been reported to induce vitellogenin synthesis (Hori et al., 1979) which may be due to aromatisation of the androgen into oestrogen, although no evidence exists to support this.

In teleosts any evidence for oestradiol regulated vitellogenesis is circumstantial. It is only recently that measurements of the dynamic changes in levels of oestradiol-17β, phosphoprotein phosphorus and calcium in the serum during the reproductive cycle of the female rainbow trout have implicated a role for oestradiol-17B in the direct control of vitellogenesis (Whitehead, 1979; Scott et al., 1980b). Thus, our aim was to observe the changes in phosphoprotein phosphorus and calcium in the serum after injection of oestradiol-17B into immature male and female trout and compare these changes with the sequence of events which occurs during the normal reproductive cycle. An investigation was also made as to whether initial priming with oestradiol-17β was necessary, since

it has been suggested that this may be the case when using immature fish and indeed early tadpole (Xenopus) liver is unable to synthesise vitellogenin (Knowland, 1980).

By using different doses of oestradiol-17β it was aimed to observe whether the vitellogenic response was 'all or nothing' or correlated with the dose. Evidence in oviparous vertebrates demonstrates a dose response to oestradiol-17β (Follett & Redshaw, 1968; Emmersen et al., 1979; van Bohemen et al., 1981a). Furthermore, an investigation was made as to whether there was a level of oestradiol-17β below which vitellogenesis would not proceed. Such information would be valuable in interpreting the significance of serum changes in oestradiol-17β observed in the seasonal cycle of female rainbow trout.

In teleost fish as in other oviparous vertebrates (Follett & Redshaw, 1968; Tata, 1978; Knowland, 1980) there is some indication that the liver is the site of oestrogen-induced vitellogenesis (Plack & Fraser, 1971; Aida et al., 1973b; te Heesen & Engels, 1973; Campbell & Idler, 1976; Emmersen & Emmersen, 1976; de Vlaming et al., 1977). These studies have compared the histological changes in protein synthetic apparatus both after oestrogen treatment and during normal reproductive cycles. However, work with birds has shown that hepatectomy prevents the typical plasma changes associated with laying and that P^{32} is first incorporated into the liver as a protein before appearing in the blood (Ramney & Chaikoff, 1951; Flickinger & Rounds, 1956). More recent studies on amphibian vitellogenesis have provided irrefutable evidence that the liver is the

primary site of production of vitellogenin (Follett & Redshaw, 1974; Wangh et al., 1976; Tata, 1978a; Wallace, 1978; Ryffel, 1978; Wahli et al., 1979; Knowland, 1980; Wiley & Wallace, 1981).

The timing of specific phases of development to coincide with favourable external conditions is of considerable adaptive significance in seasonally-breeding fish. Since the liver, the most likely site for vitellogenesis, is the major storage organ, one might expect seasonal variation in the availability of essential basic materials necessary to synthesise vitellogenin. Recent work has demonstrated seasonal changes in the activity of certain enzyme systems in the liver of trout, which may affect clearance or metabolism of injected oestradiol-17β, or indeed vitellogenesis (Hansson & Gustafsson, 1981; Hansson, 1981). In many fish species the hepatosomatic index (HSI) has been observed to vary throughout the year (Zahnd, 1959; Larson, 1974; Wingfield & Grimm, 1977; Htun-Han, 1978; Wooton et al., 1978). Thus, one might also expect there to be some variation in vitellogenic response in fish injected at different times of the year and our aim was to investigate this phenomenon. Furthermore, workers have observed a post-spawning refractory period in fish, most especially with cyprinids (de Vlaming, 1972; Sundararaj & Vassal, 1976; Breton & Billard, 1977). During this post-spawning period, female carp brood fish for example, are unable to respond to a combination of raised water temperature and pituitary extract injection, a commercially used technique to induce spawning in carp. However, once this refractory-period is over, induced-spawning methods are effective. A similar refractoriness to oestrogen stimulation may also be

present in trout, either in the post-spawning period or after injection. Indeed, it may be suggested that sex or age differences in vitellogenic response are evident. Experiments aimed at investigating these phenomena were also carried out.

A further understanding of the processes involved in the induction of the vitellogenin gene only became available after development of an *in vitro* method using *Xenopus* liver. After early failures, *in vitro* induction of vitellogenin has now been successfully achieved (Wang & Knowland, 1975; Green & Tata, 1976). Furthermore, by using dissociated liver cells of a particular type, it was demonstrated that synthesis takes place in parenchymal cells. A scheme summarizing the major events triggered by oestradiol-17B leading to the secretion of vitellogenin into the blood of male or female *Xenopus* is shown in Diagram 1.

At present we must assume a receptor for oestrogen exists which is important in the induction process. Very few studies have had success in isolating oestrogen receptors in amphibians or birds because of its low level, lability and insolubility, although Westley & Knowland (1978) have partly characterised one in male *Xenopus* liver. Bergink & Wittliff (1975), have shown by polyacrylamide gel electrophoresis the presence in *Xenopus* liver of a low molecular weight protein which binds both oestradiol and testosterone. However, similar studies with chicken liver suggest that oestradiol interacts directly with the nucleus without first binding to a cytosol component. It is estimated that there are about 100 receptor sites each in both the nucleus and the cytoplasm of a single cell in *Xenopus* liver, suggesting

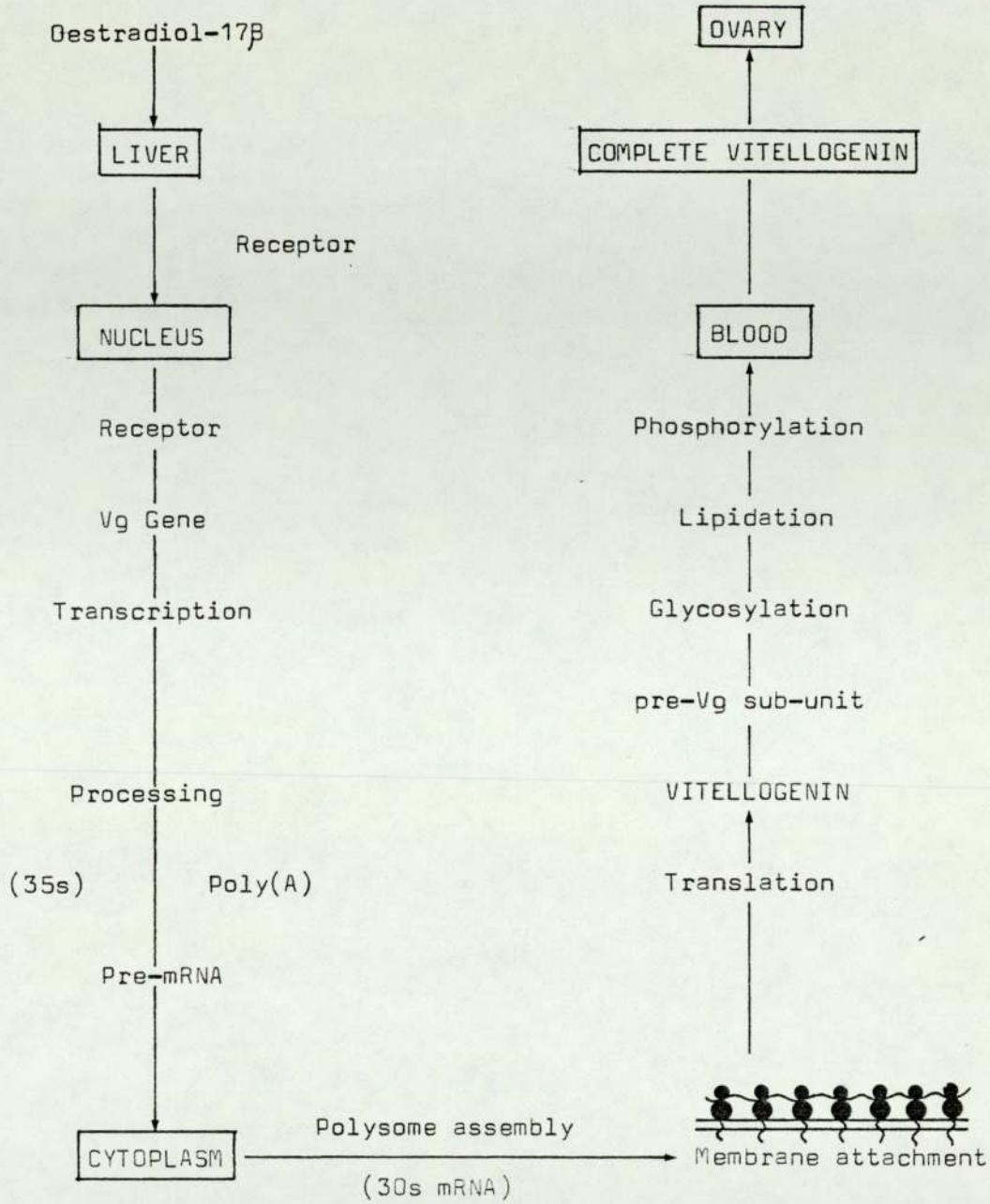


Diagram 1 Scheme summarizing the major events triggered by oestradiol-17B leading to the secretion of vitellogenin into the blood of male or female Xenopus (after Tata, 1978b).

that the receptor plays a significant part in vitellogenin induction (Westley & Knowland, 1978). It is surprising that there are as many receptors in the nucleus as in the cytosol in the absence of added oestrogen. This suggests that either its movement into the nucleus does not require oestrogen, or male Xenopus liver contains enough oestrogen to effect cytoplasm to nucleus receptor translocation without gene activation, or that translocation is not highly specific. Nuclear oestrogen receptors are increased 10-fold following oestrogen stimulation and since protein synthesis inhibitors block this increase, it cannot be due solely to translocation. However, this increased level is relatively stable (Knowland, 1980).

Following oestrogenization there is a large increase in vitellogenin mRNA (VgmRNA) (Baker & Shapiro, 1977) which is due primarily to the long half-life of VgmRNA of 40-48 hours in both Xenopus and chickens (Clemens et al., 1975; Beuving & Gruber, 1971; Greengard et al., 1964) which allows a large accumulation to occur. Synthesis of VgmRNA begins almost immediately (6-12 hours) after oestrogen treatment (Tata, 1978b) with 300 copies of mRNA per cell after 12 hours in Xenopus liver (Ryffel et al., 1977; Baker & Shapiro, 1977). Since no specific cytodifferentiation of cells making vitellogenin is required the reaction to oestrogens may be expected to be rapid. 12 days after a single injection of Xenopus with oestrogen there are some 35-36,000 copies, but after 40-60 days levels are almost undetectable.

In studies on vitellogenesis in birds and amphibians there is

a 'lag' period, the time between oestradiol-17β injection and appearance of vitellogenin in the serum (Tata, 1978b; Knowland, 1980). This 'lag' period is shorter when animals are treated with oestradiol -17β for a second time (Clemens, 1974; Tata, 1978b; Westley & Knowland, 1978; Knowland, 1980). It is not known whether the phenomenon of primary and secondary responses to oestradiol-17β occurs in fish.

What 'lag' phase there is between oestrogen administration and the appearance of vitellogenin appears to be due essentially to the time it takes for the polyribosomes involved in producing vitellogenin, to become functional. The presence of oestrogen during this phase seems essential to the maintenance of vitellogenin (Green & Tata, 1976). In Xenopus, vitellogenin is synthesised in membrane- bound ribosomes and undergoes post-translational modifications before the protein is packaged in the Golgi-apparatus prior to secretion. These modifications begin during translation on the rough endóplasmic reticulum and continue during packaging, since the enzymes for these processes are probably only available at the membrane site.

It is as a result of one of these post-translational modifications, phosphorylation, that has enabled many workers to estimate serum levels of vitellogenin indirectly in oviparous species, by measuring serum phosphoprotein levels, (Laskowski, 1936; Wallace, 1970; Elliott et al., 1979; Hori et al., 1979; Whitehead, 1979). This is hardly surprising since phosvitin, a highly phosphorylated protein, is a major constituent of many vertebrate eggs (Wallace et al., 1966) and of the vitellogenin complex (Fujii, 1960; Wallace, 1970; Wallace & Bergink, 1974;

Joubert & Cook, 1958a; 1958b). It has also been shown that both teleost vitellogenin and a trichloroacetic acid-extract of rainbow trout eggs are similarly high in phosphorus (Suzuki & Suyama, 1979; Campbell & Idler, 1980). The structure of trout vitellogenin is studied more closely in Chapter 4 where phosphoprotein phosphorus and calcium are correlated with vitellogenin in the normal reproductive cycle and after oestrogenization. Thus, after oestrogenization of immature male and female fish or during normal maturation in females, elevated levels of phosphoprotein phosphorus are observed (Elliott et al., 1979; Whitehead et al., 1978a). Concomittant with this increase in phosphoprotein phosphorus, levels of protein-bound calcium show similar changes (Elliott et al., 1979; Whitehead et al., 1978a ; 1978b). Such changes in total serum calcium thought to be due to the binding of calcium to the vitellogenin molecule in the serum, were detected in birds by Riddle & Reinhart in 1926 and sex differences in levels of blood calcium in the cod, by Hess et al., (1928). Many studies have since reported changes in total serum calcium in fish as a result of oestrogenization (Aida et al., 1973a; Elliott et al., 1979; 1980; Plack et al., 1971; Terkatin-Shimony & Yaron, 1978; Mugiya & Watabe, 1977) and like phosphoprotein phosphorus, elevated calcium levels have been used as an indicator of vitellogenesis. Although such indirect estimates are adequate (Chapter 4), the recent development of more sensitive methods of measuring vitellogenin by specific homologous radioimmunoassay, has enabled us to study the control of vitellogenesis much more closely.

Although much more of our knowledge of the physiology of

vitellogenesis comes from amphibian studies, there are so many similarities between the processes in Xenopus and other oviparous vertebrates, that the information from amphibian work can justifiably be applied to studies of the process in lower vertebrates. Although vitellogenesis is defined in this chapter as the synthesis of vitellogenin by the liver, Wallace (1978) has noted that teleosts may be unique in that some of their yolk components may be synthesised within the oocyte itself, rather than originating exclusively from the liver (Korfsmeier, 1966; Norrevang, 1968; Upadhyay et al., 1978). Furthermore, the yolk proteins lipovitellin and phosvitin isolated from teleosts are generally atypical, being heterogenous both in respect to their molecular and protein-bound phosphorus content and are generally soluble in solutions of low ionic strength (Mano & Lipman, 1966; Jared & Wallace, 1968; Markert & Vanstone, 1971; Hori et al., 1979).

More recently it has been suggested that in Xenopus, vitellogenin is coded for by a small family of genes and although the various polypeptides encoded by the different genes could be the same, it is highly unlikely (Wahli et al., 1979). It is now revealed that indeed there are multiple vitellogenins in Xenopus, and they give rise to multiple forms of yolk proteins (Wiley & Wallace, 1981).

The evidence available for teleosts so far suggests that the precise mechanism for vitellogenesis and its control, will undoubtedly be very similar to that already identified in amphibians and birds. However, certain aspects of that evidence also suggest, that in some respects, the teleosts are in themselves wholly unique.

Thus in our search for a fuller understanding of the physiology of vitellogenesis in teleosts, it is important to have an open mind and be prepared to accept whatever apparent anomalies may arise. Scientific interests apart, it is of economic viability that we have a closer understanding of vitellogenesis in trout in order to effect a more complete and efficient control of egg production in this commercially farmed species.

3.1.1. Primary aims of this series of Experiments.

Principally to investigate the physiology of hepatic vitellogenesis in rainbow trout, but also :-

(a) to Compare changes in total calcium and phosphoprotein phosphorus after injection of immature fish with oestradiol-17β, with changes observed in the normal cycle of maturing female trout.

(b) to Investigate whether virgin liver can respond to a single injection of oestradiol-17β.

(c) to Investigate whether a more rapid secondary response occurs on treatment of fish previously primed with oestradiol-17β.

(d) to Observe whether there is a dose response to injected oestradiol-17β.

(e) to Investigate whether there is a threshold level below which vitellogenesis cannot be induced.

(f) to Look for any seasonal variation in vitellogenic response or sensitivity to the hormone, and investigate whether other factors such as liver weight, clearance or metabolism of injected hormone may vary with season.

(g) to Investigate the phenomenon of refractory period, in the post-spawning period, or after vitellogenic response.

(h) to Investigate whether the sex, age or relative maturity of fish used may have any influence on vitellogenic response.

(i) to Develop methods of treating fish with oestradiol-17β that could be commercially acceptable in terms of ease and manageability.

3.2. Injection of Immature Male and Female Trout (Salmo gairdneri) with Oestradiol-17β (E2).

The aim of these initial experiments was to investigate the changes in egg protein or vitellogenin which occur following E2 injection in the rainbow trout. In all cases vitellogenin levels were assessed by measurement of phosphoprotein phosphorus (PP) and total serum calcium (TCa). A priming injection of E2 was used in these experiments since other workers had deemed it necessary when using immature fish. Two experiments were conducted.

3.2.1. Materials and Methods.

Short-term changes in E2, TCa and PP following E2 injection :
48 immature fish in the 150-200g range were selected from stock fish held at Aston Fish Culture Unit and allowed to acclimatise for 7 days in recirculation system B (See Appendix 1). Following this period, 24 fish were injected with E2 at a dose of 5 $\mu g\ g^{-1}$ body weight. The remaining 24 fish were injected with the same volume of arachis/ethanol suspension as controls. The two groups of fish were fin-clipped, mixed and randomly distributed between 2 tanks and allowed to acclimatise for another 4 weeks.

Following this period, treated fish received a further injection of the same dose of E2 and the control fish the same volume of vehicle. At the time of these injections, 8 fish (4 treated and 4 controls) were removed and blood sampled to obtain baseline values. A further 4 treated and 4 control fish were removed and bled at 3,6,9,10 and 11 hours after injection. After sampling, the fish were held in a separate tank to avoid the removal of

blood samples from the same fish. The protocol for this experiment is shown diagramatically in Diagram 2.

The serum samples obtained were stored at $-20^{o}C$ until assay for serum E2, TCa and PP as described in Chapter 2.

TIME

0 . . . . . 48 Immature mixed sex fish selected (150–200g)

7 days . . . . . 24 injected with E2 @ 5 $\mu g\ g^{-1}$

24 injected with vehicle

35 days . . . . . REINJECTED AS ABOVE

8 FISH BLED (4T + 4C) TO GIVE BASE VALUES

+3 hrs . . . . . 4T + 4C BLOOD SAMPLED

+6 hrs . . . . . 4T + 4C BLOOD SAMPLED

+9 hrs . . . . . 4T + 4C BLOOD SAMPLED

+10 hrs . . . . . 4T + 4C BLOOD SAMPLED

+11 hrs . . . . . 4T + 4C BLOOD SAMPLED

(T = Treated ; C = Control)

Diagram 2. Protocol of injection and blood sampling for experiment investigating the short-term changes in mean serum levels of E2, TCa and PP after injection with E2.

Long-term changes in E2, TCa and PP following E2 injection :

A further 50 immature rainbow trout with a mean weight of 200g were selected from the same stock and distributed between 3 experimental tanks. After an acclimatisation period of 7 days, 25 animals were selected at random and injected intraperitoneally with E2 at a dose of 5 $\mu g\ g^{-1}$ body weight. The remaining 25 fish were injected with an arachis/ethanol emulsion as controls.

5 treated fish and 5 control fish were bled to obtain baseline values. After fin-clipping, the 2 groups were randomly distributed between 2 experimental tanks and allowed to acclimatise for 28 days.

At the end of this period, treated fish were reinjected with the same dose of E2 and control fish with vehicle. The fish were divided into 5 groups of 10 fish (5 treated and 5 control), and bled sequentially at 0.5, 1, 2, 3, 4, 5, 6, 7, 9, 11, 14, 17, 21, 25, 46, 65, 85, 112 and 135 days after the 2nd injection. The protocol for this experiment is shown in Diagram 3. The serum obtained was stored at $-20^{o}C$ until assayed as above. The results are outlined in section 3.2.2..

TIME

0 . . . . . 50 Immature trout mean weight 200g

7 days . . . . . 25 animals injected ip with E2 @ 5 $\mu g\ g^{-1}$

25 animals vehicle only, AND BLED TO OBTAIN BASELINE VALUE

35 days . . . . . REINJECTED AS ABOVE

0.5 days	GROUP 1 BLOOD SAMPLED	14 days	GROUP 5 BLOOD SAMPLED
1 day	GROUP 2 " "	17 days	GROUP 3 " "
2 days	GROUP 3 " "	21 days	GROUP 4 " "
3 days	GROUP 4 " "	25 days	GROUP 5 " "
4 days	GROUP 5 " "	46 days	GROUP 3 " "
5 days	GROUP 3 " "	65 days	GROUP 4 " "
6 days	GROUP 4 " "	85 days	GROUP 5 " "
7 days	GROUP 5 " "	112 days	GROUP 3 " "
9 days	GROUP 3 " "	135 days	GROUPS 4 & 5 BLOOD SAMPLED
11 days	GROUP 4 " "		

Diagram 3. Protocol of injection and blood sampling for experiment investigating the long-term changes in mean serum levels of E2, TCa and PP after injection with E2.

3.2.2. Results.

In both experiments significant changes in E2 were observed in treated fish after the second injection. Significant increases in serum levels of PP and TCa were observed in treated fish in the long-term experiment when compared with controls. However, no such significant changes in PP and TCa were observed in treated or control fish in the short-term. Values are expressed as mean $\pm$ standard error of the mean. Student t-test used to test significance.

Short-term changes in mean serum levels of E2, TCa and PP :

There was a significant ($P < 0.05$) increase in the mean serum level of E2 in treated fish after 3 hours, rising from a basal value of 0.45 ± 0.2 ng ml^{-1} to 2.25 ± 0.7 ng ml^{-1}. A maximum mean value of 9.2 ± 4.5 ng ml^{-1} ($P < 0.05$) was observed in treated fish 10 hours after the second injection. There was no significant change in mean control values for serum E2 (See Table 3.1. & Fig. 3.1.).

The mean serum levels of PP and TCa showed no significant increases during the course of the experiment. However, mean serum levels of PP and TCa in treated fish rose from basal values of 16.4 ± 4.4 μg ml^{-1} and 9.6 ± 0.6 mg $100ml^{-1}$ to 59.5 ± 8.9 μg ml^{-1} and 15.3 ± 0.7 mg $100ml^{-1}$ respectively, at 11 hours. There were no significant changes in control fish (See Table 3.2. and Figs. 3.2. & 3.3.).

TIME AFTER 2ND. INJECTION.

	BASAL	3 HRS.	6 HRS.
CONTROL	0.45	0.3	0.5
± S.E.	0.2	0.04	0.4
TREATED	0.45	2.25	5.1*
± S.E.	0.2	0.7	0.8

	9 HRS.	10 HRS.	11 HRS.
CONTROL	0.7	0.37	0.4
± S.E.	0.1	0.1	0.1
TREATED	4.9	9.2*	5.3
± S.E.	2.6	4.5	2.1

* ($p < 0.05$)

n = 4

TABLE 3.1. SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) OVER THE 11 HOURS FOLLOWING A SECOND INJECTION OF E2 AT 5 μg g^{-1}.

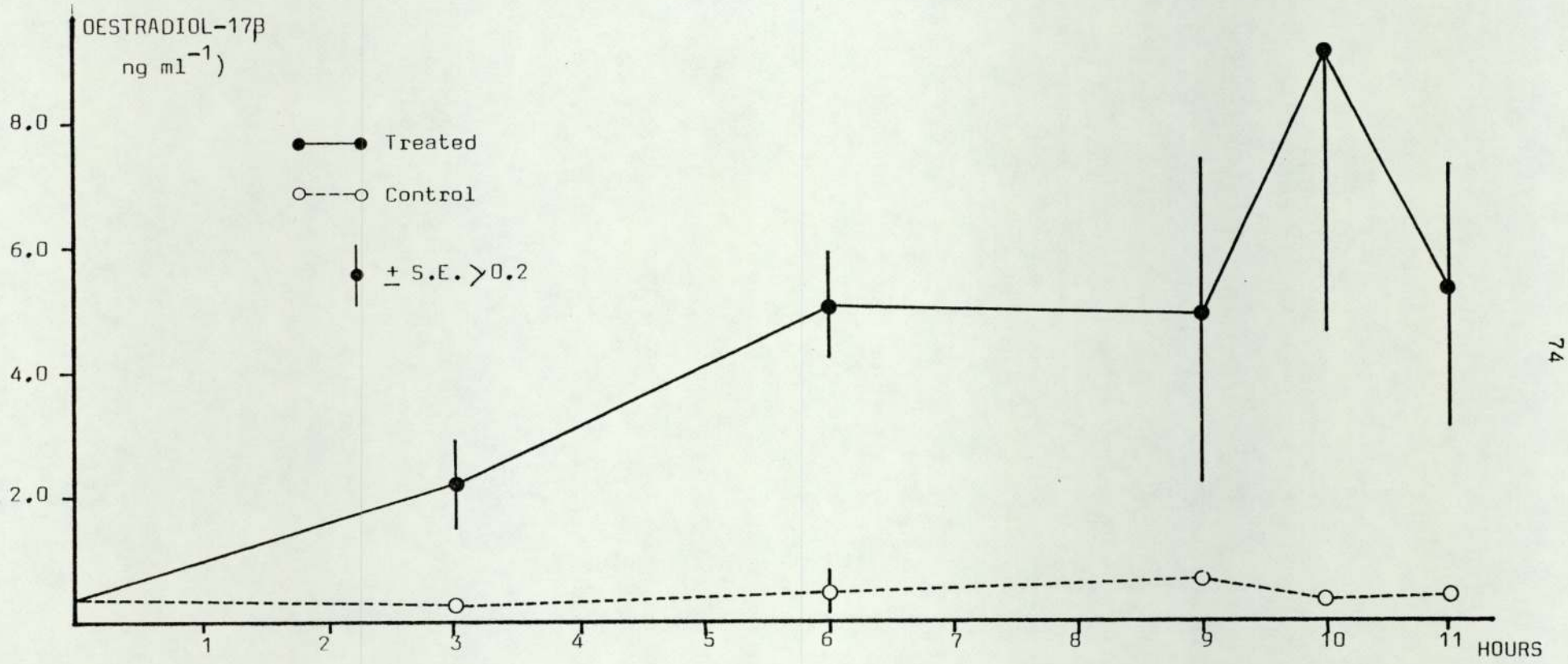


FIG. 3.1. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml $^{-1}$) OVER THE 11 HOURS FOLLOWING A SECOND INJECTION WITH OESTRADIOL-17β (E2) AT A DOSE OF 5 μg g $^{-1}$

TIME (HOURS)	BASAL	3	6	9	10	11
CONTROL	16.4	16.0	16.3	18.1	21.8	8.9
± S.E.	4.4	5.4	12.0	8.5	13.7	5.6
TREATED	16.4	26.0	20.0	17.1	20.5	59.5
± S.E.	4.4	19.3	12.0	28.7	19.8	8.9

TIME (HOURS)	BASAL	3	6	9	10	11
CONTROL	9.6	11.0	11.1	11.6	11.4	11.3
± S.E.	0.6	0.4	0.4	0.7	0.7	0.7
TREATED	9.6	11.4	11.4	12.3	12.8	15.3
± S.E.	0.6	0.5	0.4	1.2	1.9	0.7

TABLE 3.2. CHANGES IN MEAN SERUM LEVELS OF PHOSPHOPROTEIN PHOSPHORUS (PP $\mu g\ ml^{-1}$ TOP) AND TOTAL CALCIUM (TCa mg $100ml^{-1}$ BOTTOM) FOLLOWING A SECOND INJECTION OF OESTRADIOL-17β (E2 5 $\mu g\ g^{-1}$). n = 4.

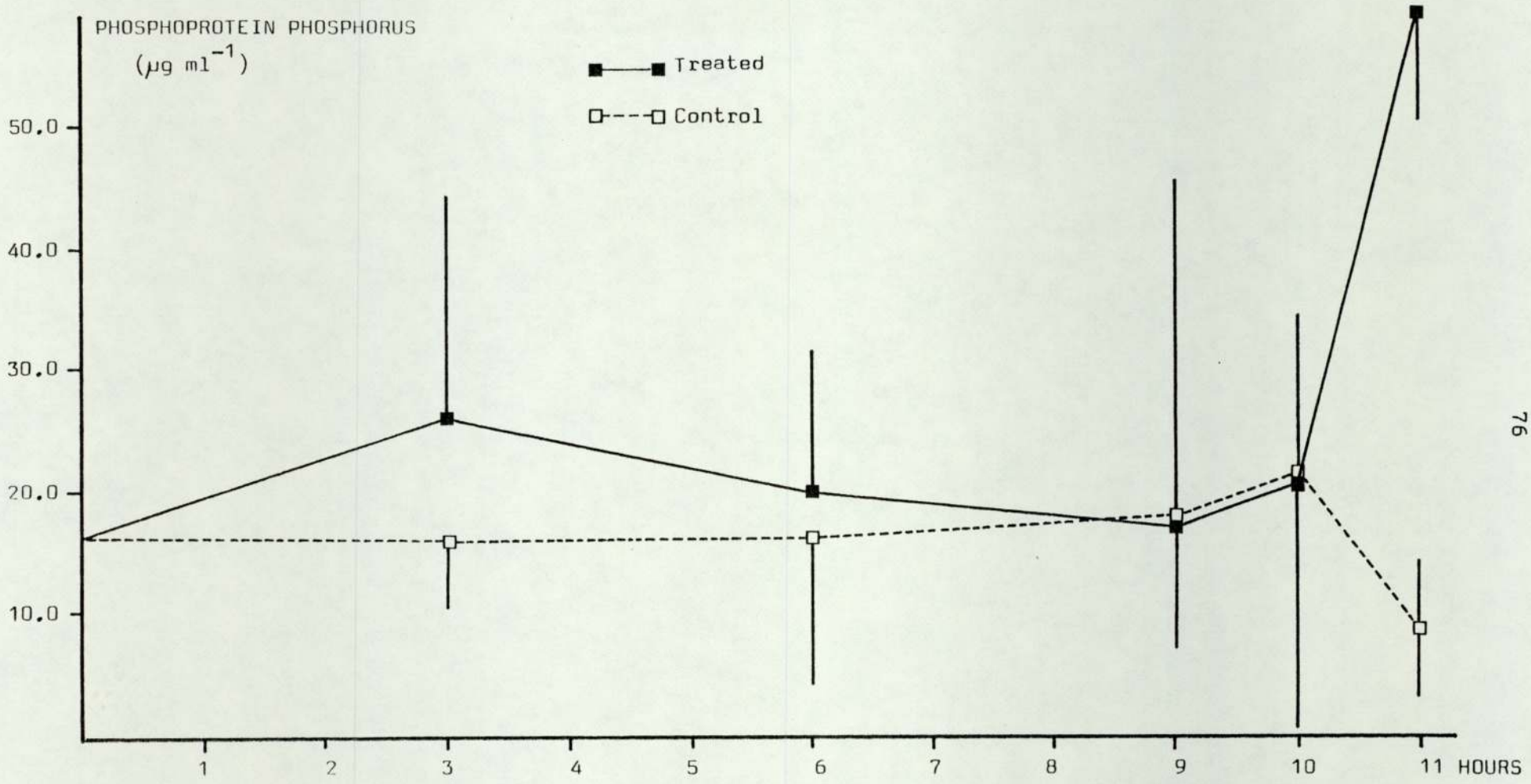


FIG. 3.2. CHANGES IN MEAN SERUM LEVELS OF PHOSPHOPROTEIN PHOSPHORUS (P P μg ml $^{-1}$) FOLLOWING A SECOND INJECTION OF OESTRADIOL-17β AT A DOSE OF 5 μg g $^{-1}$.

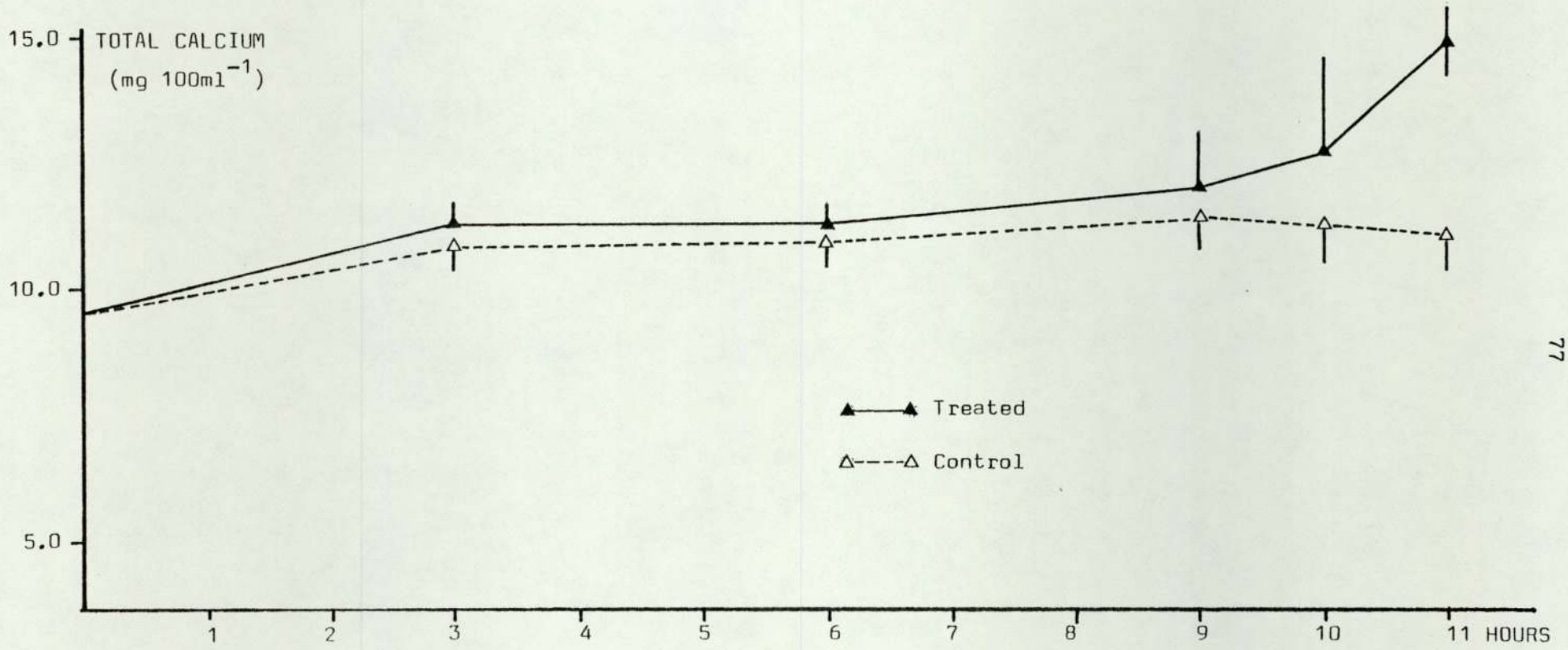


FIG. 3.3. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml $^{-1}$) FOLLOWING A SECOND INJECTION OF OESTRADIOL-17β AT A DOSE OF 5 μg g $^{-1}$.

Long-term changes in mean serum levels of E2, TCa and PP :

The serum hormone profile for injected E2 (Fig. 3.4.), differed markedly to those of PP (Fig. 3.5.) and TCa (Fig. 3.6.). In treated fish there was a significant ($P < 0.05$) increase in serum levels of E2 rising from basal levels of 0.5 ± 0.2 ng ml^{-1} to 10.9 ± 5.0 ng ml^{-1} 12 hours after the second injection. E2 reached a peak mean value of 38.0 ± 5.6 ng ml^{-1} 2 days after the second injection, falling to 1.6 ± 0.2 ng ml^{-1} after 9 days, and to control levels after 11 days. There were no such significant changes in levels of E2 in control fish during the course of the experiment (Table 3.3.).

In treated fish serum levels of PP increased from basal values of 21.0 ± 5.0 μg ml^{-1} to 307.0 ± 25.0 μg ml^{-1} ($P < 0.001$) 12 hours after the second injection. Serum levels of PP reached a peak mean value of 832.0 ± 95.2 μg ml^{-1} 21 days after the second injection, not returning to control values of 16.6 ± 2.1 μg ml^{-1} until 114 days later. There were no such similar changes observed in the control fish, a maximum value of 116.0 ± 22.0 μg ml^{-1} being observed after 25 days (See Table 3.4.).

Similarly in treated fish, TCa levels increased significantly ($P < 0.001$) from basal values of 11.0 ± 1.0 mg $100ml^{-1}$ to 41.1 ± 2.9 mg $100ml^{-1}$, 12 hours after the second injection. A peak mean value of 75.2 ± 6.0 mg $100ml^{-1}$ was observed after 21 days, not returning to control levels of 13.7 ± 0.6 mg $100ml^{-1}$ until 135 days after injection. Again no such similar changes were observed in control fish (Table 3.5.) where a maximum mean value of 16.3 ± 2.3 mg $100ml^{-1}$ was observed 46 days after the second injection.

TIME (DAYS)	BASAL	0.5	1	2	3
CONTROL	0.5	0.35	0.4	0.6	0.7
± S.E.	0.2	0.2	0.2	0.3	0.3
TREATED	0.5	10.9*	5.4**	38.0***	4.75**
± S.E.	0.2	5.0	0.1	5.6	1.4

TIME (DAYS)	4	9	11	14	17
CONTROL	0.9	0.4	0.8	0.6	0.5
± S.E.	0.3	0.2	0.3	0.1	0.1
TREATED	6.7*	1.6*	0.9	0.8	0.9
± S.E.	1.7	0.2	0.2	0.5	0.3

TIME (DAYS)	21	25	46	65	85
CONTROL	0.4	0.3	0.3	0.4	0.5
± S.E.	0.1	0.1	0.1	0.2	0.1
TREATED	0.6	0.5	0.3	0.3	0.4
± S.E.	0.2	0.2	0.1	0.05	0.3

* ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$)

n = 5

TABLE 3.3. LONG-TERM CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) FOLLOWING A SECOND INJECTION OF OESTRADIOL-17β AT A DOSE OF 5 μg g^{-1}.

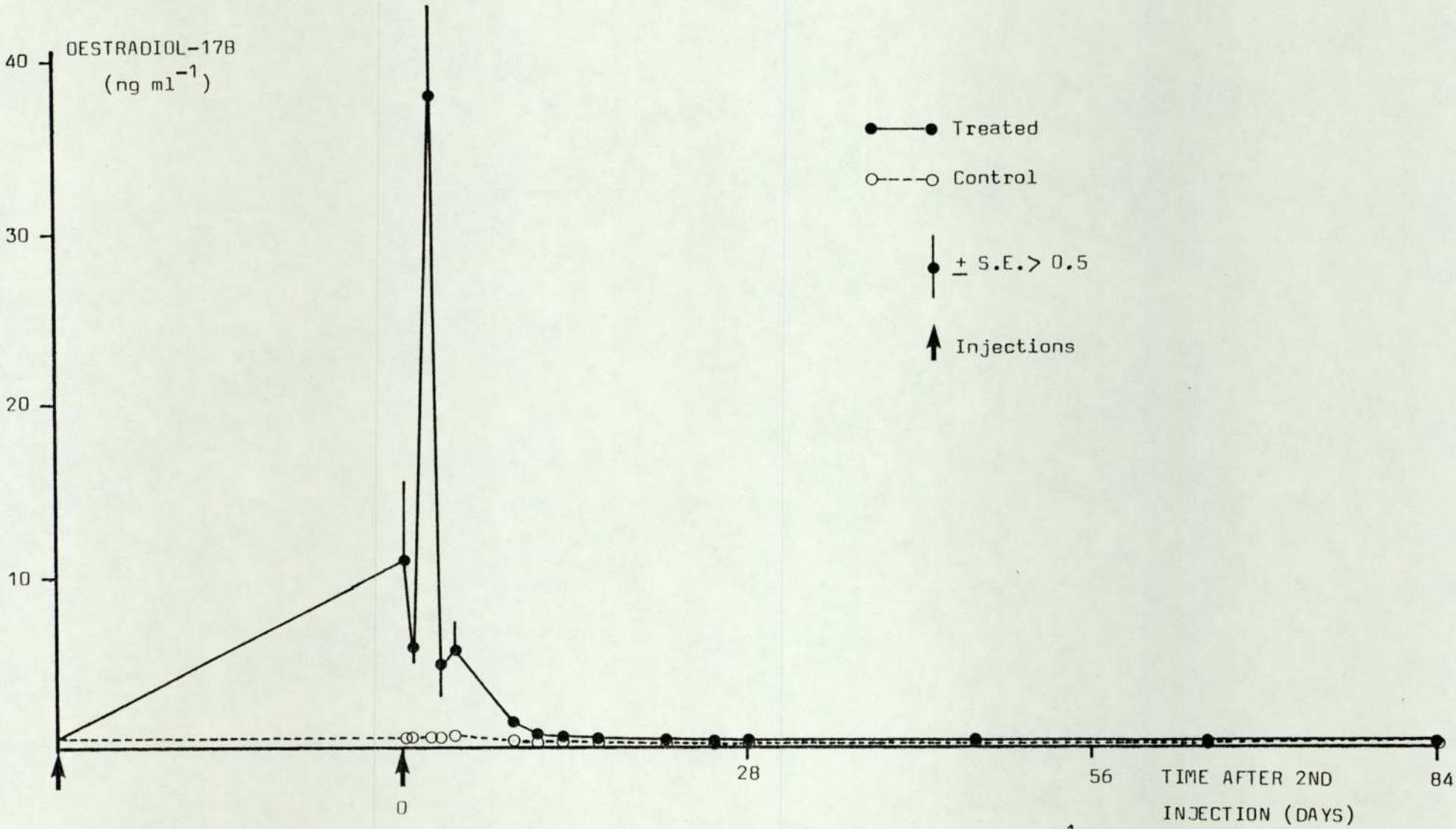


FIG. 3.4. LONG-TERM CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml $^{-1}$) FOLLOWING A SECOND INJECTION OF OESTRADIOL-17β (E2 5 μg g $^{-1}$).

TIME (DAYS)	BASAL	0.5	1	2	3	4
CONTROL	21.0	14.0	10.2	23.2	7.4	10.2
± S.E.	5.0	4.6	2.6	6.3	5.4	2.5
TREATED	21.0	307.0***	308.0***	351.0***	438.0***	292.0**
± S.E.	5.0	23.0	76.0	77.0	32.4	106.6

TIME (DAYS)	9	11	14	17	21
CONTROL	36.8	43.2	25.2	47.5	110.2*1
± S.E.	12.2	16.3	8.6	16.7	34.8
TREATED	316.0*	543.0***	449.0***	364.0*	832.0***
± S.E.	109.6	56.7	117.0	104.0	95.2

TIME (DAYS)	25	46	65	85	135
CONTROL	116.1*1	76.2*1	23.0	24.3	12.2
± S.E.	32.0	14.8	8.8	7.6	2.5
TREATED	745.0***	436.0***	385.0*	400.0*	16.6
± S.E.	54.4	77.1	132.8	85.7	2.1

* ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$) *1 versus basal control mean

n = 5

TABLE 3.4. LONG-TERM CHANGES IN MEAN SERUM LEVELS OF PHOSPHOPROTEIN PHOSPHORUS (P.P. $\mu g\ ml^{-1}$) FOLLOWING A SECOND INJECTION OF OESTRADIOL-17β (E2. 5 $\mu g\ g.^{-1}$).

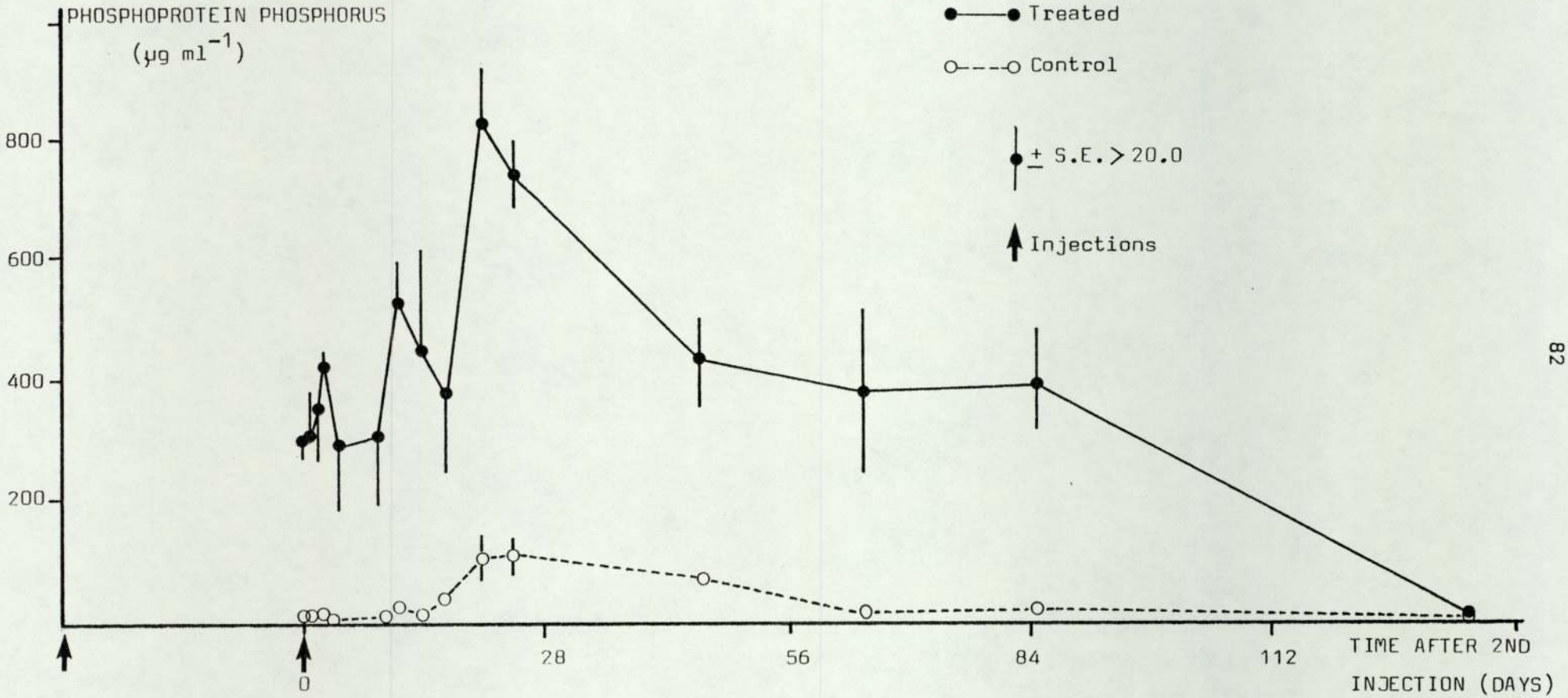


FIG. 3.5. LONG-TERM CHANGES IN MEAN SERUM LEVELS OF PHOSPHOPROTEIN PHOSPHORUS (P P µg ml $^{-1}$) FOLLOWING A SECOND INJECTION OF OESTRADIOL-17β (E2 5 µg g $^{-1}$).

TIME (DAYS)	BASAL	0.5	1	2	3	4
CONTROL	11.0	11.8	11.6	11.4	11.7	11.2
± S.E.	1.0	0.7	0.7	0.4	0.3	0.3
TREATED	11.0	41.2**	34.2**	38.1**	49.6***	41.9**
± S.E.	1.0	2.9	5.9	7.5	2.1	10.2

TIME (DAYS)	9	11	14	17	21
CONTROL	12.6	13.3	14.7**1	14.2**1	14.9
± S.E.	0.8	0.7	0.7	0.6	1.3
TREATED	44.5**	59.7***	52.3***	53.3**	75.2**
± S.E.	8.3	4.9	10.2	8.4	6.0

TIME (DAYS)	25	46	65	85	135
CONTROL	15.1*1	16.3	13.9*1	15.4***1	11.8
± S.E.	0.9	2.3	0.6	0.4	1.12
TREATED	72.7***	51.8**	54.1***	47.7**	13.7
± S.E.	4.9	8.6	6.7	15.1	0.6

* ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$) *1 versus mean basal control

n = 5

TABLE 3.5. LONG-TERM CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml $^{-1}$) FOLLOWING A SECOND INJECTION OF OESTRADIOL-17β (E2 5 μg g $^{-1}$).

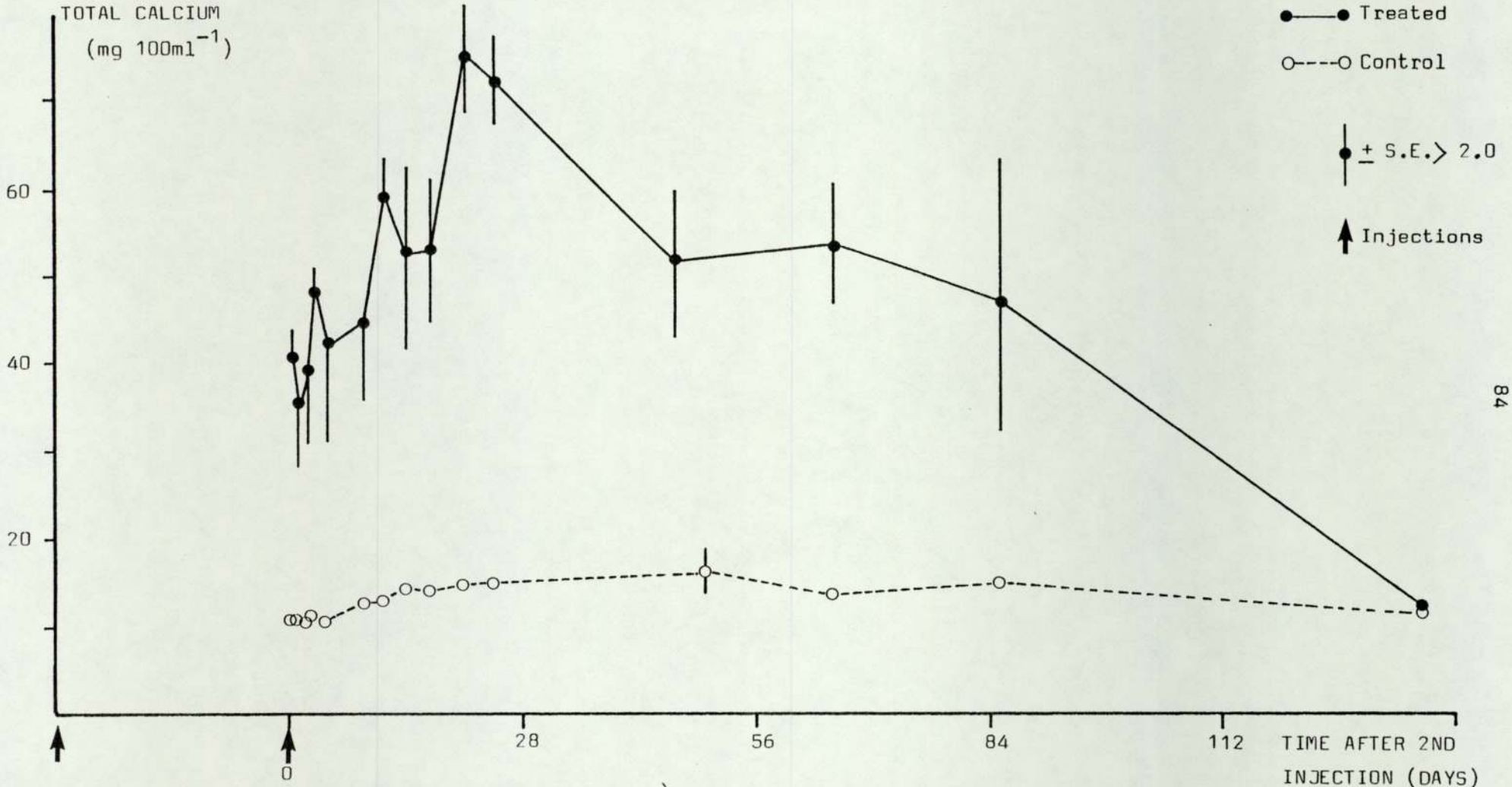


FIG. 3.6. LONG-TERM CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml $^{-1}$) FOLLOWING TWO INJECTIONS OF OESTRADIOL-17β (E2 5 μg g $^{-1}$).

3.2.3. Conclusions.

The results clearly demonstrate a role for E2 in the direct control of vitellogenesis in the rainbow trout as determined by measurement of PP and TCa. Measurement of serum E2 showed that uptake of the hormone to the serum occurred almost immediately after injection, reaching a peak 2 days later and not falling to control values until 9 days after injection.

In both experiments, concomitant increases in PP and TCa were observed and a 'lag-phase' of some 11 hours occurred after the injection of E2 before their appearance in the serum. However, following the 'lag-phase', gross changes in both parameters were observed which remained elevated for some 5 months. The results indicate that once initiated egg protein synthesis continues despite the return of E2 to control levels. The parallel increases in PP and TCa suggests a distinct relationship between the materials in the vitellogenin complex.

3.3. Primary Induction of Vitellogenesis and the Secondary Response after Reinjection with E2.

The aim of this experiment was to compare the vitellogenic response of a single injection of E2 into unprimed fish with that produced in fish which had previously been primed with a similar dose of this hormone.

3.3.1. Materials and Methods.

Primary induction with a single injection of E2 : 30 immature rainbow trout with a mean weight of 100g were selected and allowed to acclimatise to recirculation system A. 10 fish were picked at random and injected intraperitoneally with E2 at a dose of 5 $\mu g\ g^{-1}$ body weight in arachis oil/ethanol. A further 10 fish were injected with vehicle as controls. 10 fish were bled at the time of injection and at 1, 5, 7, 12, 14, 18, 22 and 60 days after this time. See Diagram 4 for protocol of injection and sampling. The serum samples were stored at $-20^{o}C$ until assay of E2 and TCa as described above.

DAYS

0 · · · · 30 Immature trout split into 3 groups of 10

	GROUP A	GROUP B	GROUP C
7	Injected ip with E2 @ 5 μg g^{-1}	Injected ip with vehicle	
1	S	S	
5	S	S	
7	S	S	
12	S	S	
14	S	S	
18	S	S	
22	S	S	
60	S	S	
62	E2 ip & S	VEH ip & S	E2 ip & S
2	S	S	S
4	S	S	S
6	S	S	S
9	S	S	S
13	S	S	S
21	S	S	S

S = FISH BLOOD SAMPLED

ip = Intraperitoneal injection of E2 at 5 μg g^{-1}

Diagram 4. Protocol of injection and blood sampling of E2 primed and unprimed fish with a single injection of E2 at 5 μg g^{-1}.

Secondary vitellogenic response in fish previously primed with E2 : Group A primary treated fish were reinjected with the same dose of E2 along with the remaining 10 fish, Group C, from above. Control fish, Group B, were injected with the same volume of arachis/ethanol emulsion. All fish were bled just before injection with E2, to give baseline values, and at 2, 4, 6, 9, 13 and 21 days after injection. The serum samples were assayed for serum E2 and TCa, the results for which are shown in section 3.3.2..

3.3.2. Results.

The results clearly demonstrate a vitellogenic response to a single injection of E2. Furthermore, on reinjection, a much more rapid secondary response occurred.

Primary response : In treated fish there were significant ($P < 0.001$) increases in serum E2, rising from basal values of 0.5 ± 0.25 to 35.0 ± 9.5 ng ml^{-1} after one day and falling to 2.5 ± 1.1 ng ml^{-1} 12 days after injection. Vehicle-injected control fish showed no such significant changes (Table 3.6. & Fig. 3.7.).

Mean TCa rose in treated fish from 10.2 ± 0.2 mg $100ml^{-1}$ to a peak mean value of 42.7 ± 4.0 mg $100ml^{-1}$ ($P < 0.001$), 22 days after injection. 60 days after injection the mean levels in treated fish had returned (14.2 ± 2.6 mg $100ml^{-1}$) to control levels of 11.8 ± 0.8 mg $100ml^{-1}$. No significant changes were observed in the vehicle-injected control fish over the same time period (Table 3.6. & Fig. 3.8.).

TIME (DAYS)	0	1	5	7	12
CONTROL	1.5	2.7	2.0	1.33	1.9
± S.E.	0.9	0.9	1.1	0.4	0.5
TREATED	0.5	35.0**	10.0	5.1***	2.5
± S.E.	0.3	9.5	4.0	0.8	1.1

TIME (DAYS)	0	1	5	7	12
CONTROL	10.2	9.8	10.61	10.9	11.7**1
± S.E.	0.2	0.3	0.3	0.4	0.3
TREATED	10.2	9.85	11.4	14.9	29.1***
± S.E.	0.2	0.2	0.2	0.5	1.9

TIME (DAYS)	14	18	22	60
CONTROL	11.11**1	12.9***1	12.9	11.8
± S.E.	0.2	0.2	0.8	0.8
TREATED	34.4***	41.3***	42.7***	14.2
± S.E.	2.9	3.0	4.0	2.6

* ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$) *1 versus mean basal control value

n = 10

TABLE 3.6. PRIMARY CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}, TOP) AND TOTAL SERUM CALCIUM (TCa mg $100ml^{-1}$, CENTRE & BOTTOM) FOLLOWING A SINGLE INJECTION OF OESTRADIOL-17β (E2) AT A DOSE OF 5 μg g^{-1}.

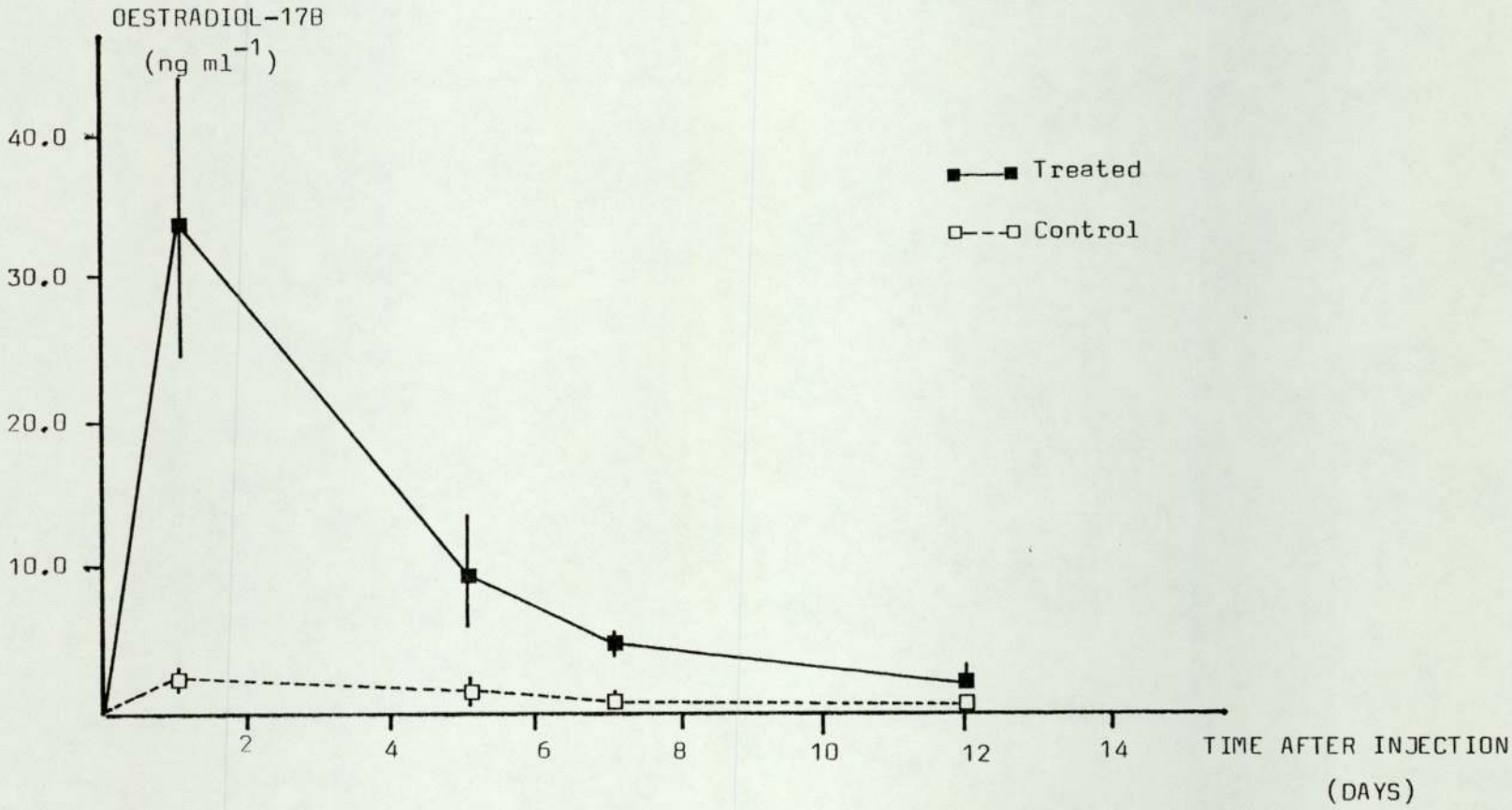


FIG. 3.7. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2) IN TREATED AND CONTROL FISH FOLLOWING A SINGLE INJECTION OF OESTRADIOL-17β (E2) AT A DOSE OF 5 μg g $^{-1}$.

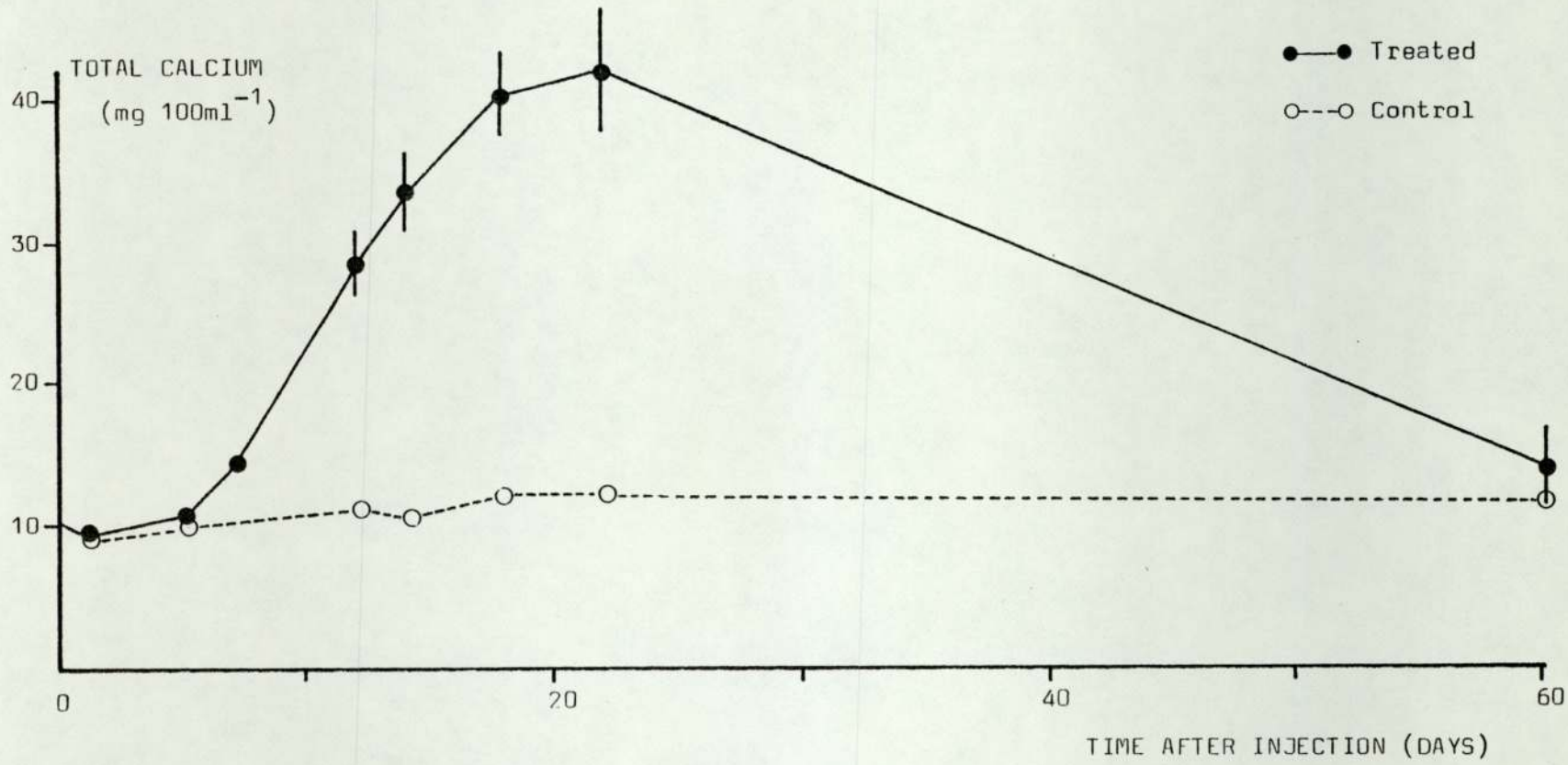


FIG. 3.8. CHANGES IN MEAN LEVELS OF TOTAL SERUM CALCIUM (TCa mg 100ml^{-1}) IN TREATED AND CONTROL FISH FOLLOWING A SINGLE INJECTION OF OESTRADIOL-17β (E2) AT A DOSE OF 5 μg g^{-1}.

Secondary vitellogenic response : Similarly, gross changes were seen in mean levels of TCa and E2 in both treated groups. Vehicle-injected controls did not show any significant changes in E2 or TCa.

In Group C fish, serum levels of E2 rose from a basal value of 0.75 ± 0.3 to 77.0 ± 18.25 ng ml^{-1} ($P < 0.01$) 2 days after injection and then fell to 3.5 ± 1.7 ng ml^{-1} after 9 days (Table 3.7.). Similarly, serum levels of E2 in fish treated for a second time, Group A, reached a peak mean value of 84.0 ± 14.0 ng ml^{-1} 2 days after injection and then fell to 2.0 ± 0.8 ng ml^{-1} after 9 days. (Table 3.7.). There were no significant differences in the injected serum hormone profile observed in the 2 groups (Fig. 3.9.).

In the treated group injected once, Group C, mean total calcium levels were significantly ($P < 0.01$) raised above basal levels of 10.13 ± 1.7 mg $100ml^{-1}$ after 9 days, at 20.0 ± 1.5 mg $100ml^{-1}$. Peak mean values of 41.7 ± 4.1 mg $100ml^{-1}$ were observed after 21 days ($P < 0.001$) (Table 3.8.). However, in Group A fish, mean TCa values were significantly ($P < 0.05$) raised at 18.8 ± 3.4 above basal levels of 11.12 ± 0.5 mg $100ml^{-1}$ after only 4 days. Only 13 days after injection TCa levels had risen to 49.95 mg $100ml^{-1}$ ($P < 0.001$) similar to the levels obtained by Group C some 8 days later. Control fish demonstrated no significant changes in TCa (Table 3.8. & Fig. 3.10.).

TIME (DAYS)	0	2	4	6	9
(B) CONTROL	0.5	1.0	2.3	0.5	0.5
± S.E.	0.1	0.25	0.4	0.2	0.3
(A) PRIMARY	0.75	77.0**	20.0*	10.25	3.5
± S.E.	0.3	18.25	6.8	4.4	1.7
(C) SECONDARY	2.0	84.0***	15.0	2.5**	2.0
± S.E.	0.8	14.0	7.6	0.5	0.8

* ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$)

n = 10

TABLE 3.7. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) FOLLOWING A SINGLE AND DOUBLE INJECTION OF THE HORMONE AT A DOSE OF 5 μg g^{-1}.

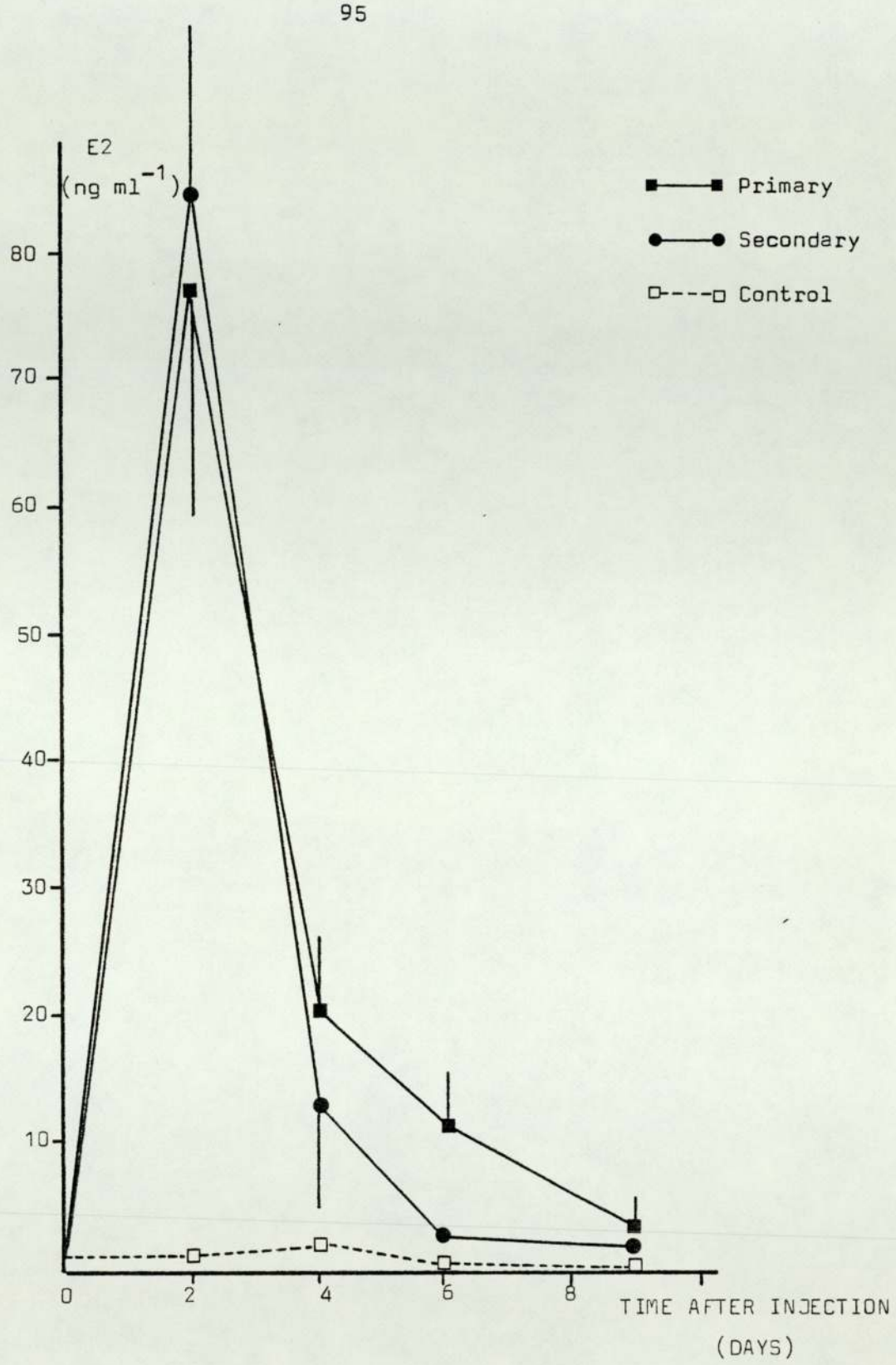


FIG. 3.9. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) FOLLOWING A SINGLE AND DOUBLE INJECTION OF OESTRADIOL-17β AT A DOSE OF 5 μg g^{-1}.

TIME (DAYS)	0	2	4	6
CONTROL	10.15	10.0	9.99	10.2
± S.E.	0.2	0.3	0.6	0.2
PRIMARY	10.12	10.0	10.7	12.5*
± S.E.	0.3	0.4	0.5	1.0
SECONDARY	11.13	13.9	18.8*1	23.7**1
± S.E.	1.7	3.6	3.4	3.9

TIME (DAYS)	9	13	21
CONTROL	10.7	11.7	11.3
± S.E.	0.45	0.9	0.7
PRIMARY	20.0***	30.8**	41.7***
± S.E.	1.5	4.9	4.1
SECONDARY	38.7***1	49.95***1	50.5***
± S.E.	2.8	4.3	7.5

* ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$) *1 significantly different from control & I^0

n = 10

TABLE 3.8. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) FOLLOWING A SINGLE & DOUBLE INJECTION OF OESTRADIOL-17β AT A DOSE OF 5 $\mu g\ g^{-1}$.

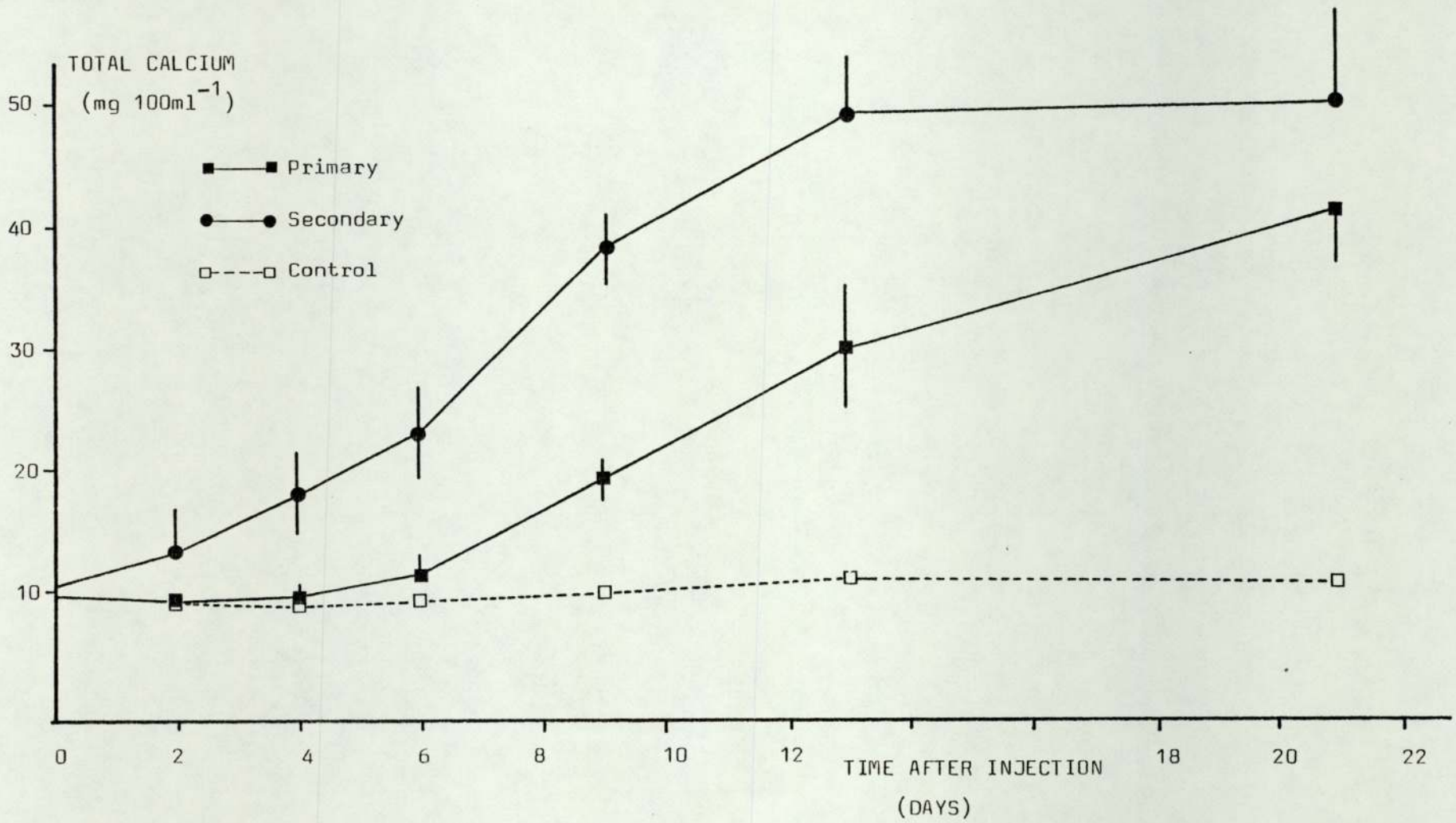


FIG. 3.10. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml^{-1}) FOLLOWING A SINGLE AND DOUBLE INJECTION OF OESTRADIOL-17β (E2 AT DOSES OF 5 μg g^{-1}).

3.3.3. Conclusions.

The results clearly demonstrate primary induction of vitellogenesis in response to a single injection of E2 in rainbow trout. Furthermore, when challenged for a second time with the same dose of E2, a much more rapid secondary response occurs. There would not appear to be any refractory period to subsequent E2. The 'lag-phase' during secondary stimulation is shortened, indicating that the difference in the 2 responses lies in the storage of information in the liver, most probably at the ribosomal level. Not only is the secondary response more rapid, but the amount of vitellogenin, as measured by TCa is also increased even though the dose of E2 administered was the same in each case.

3.4. Seasonal Variation in Vitellogenic Response to a Specific Dose of E2.

The aim of this series of experiments was to investigate whether there may be any seasonal variation in vitellogenic response to a specific dose of E2. Thus, different groups of 10 fish were injected successively at monthly intervals, each group receiving 2 injections 7 days apart.

3.4.1. Materials and Methods.

Monthly injections of groups of 10 fish, with the same dose of E2 : For each monthly injection, a group of 10 fish, of similar stock, were selected with a mean weight of 200 ± 45.8g, individually tagged and then bled to obtain base values for the serum parameters to be measured. After the fish had been acclimatised for 7 days in System A, 5 of the animals were injected intraperitoneally with E2. The dose selected was 5 $\mu g\ g^{-1}$ body weight with a final injection volume of 0.2ml. The remaining 5 animals were injected with the same volume of arachis/ethanol emulsion as controls.

2 days after the initial injection, blood samples were taken and 5 days later treated fish were reinjected with E2 at the same dose and control fish reinjected with vehicle. Following reinjection, each group was bled at 9, 21 and 28 days, and then at approximately monthly intervals from the time of the initial injection. Monthly sampling was continued until any measureable elevations in egg protein levels had returned to basal. The protocol for injection is shown in Diagram 5, and the protocol of sampling for each monthly injected group in Diagram 6.

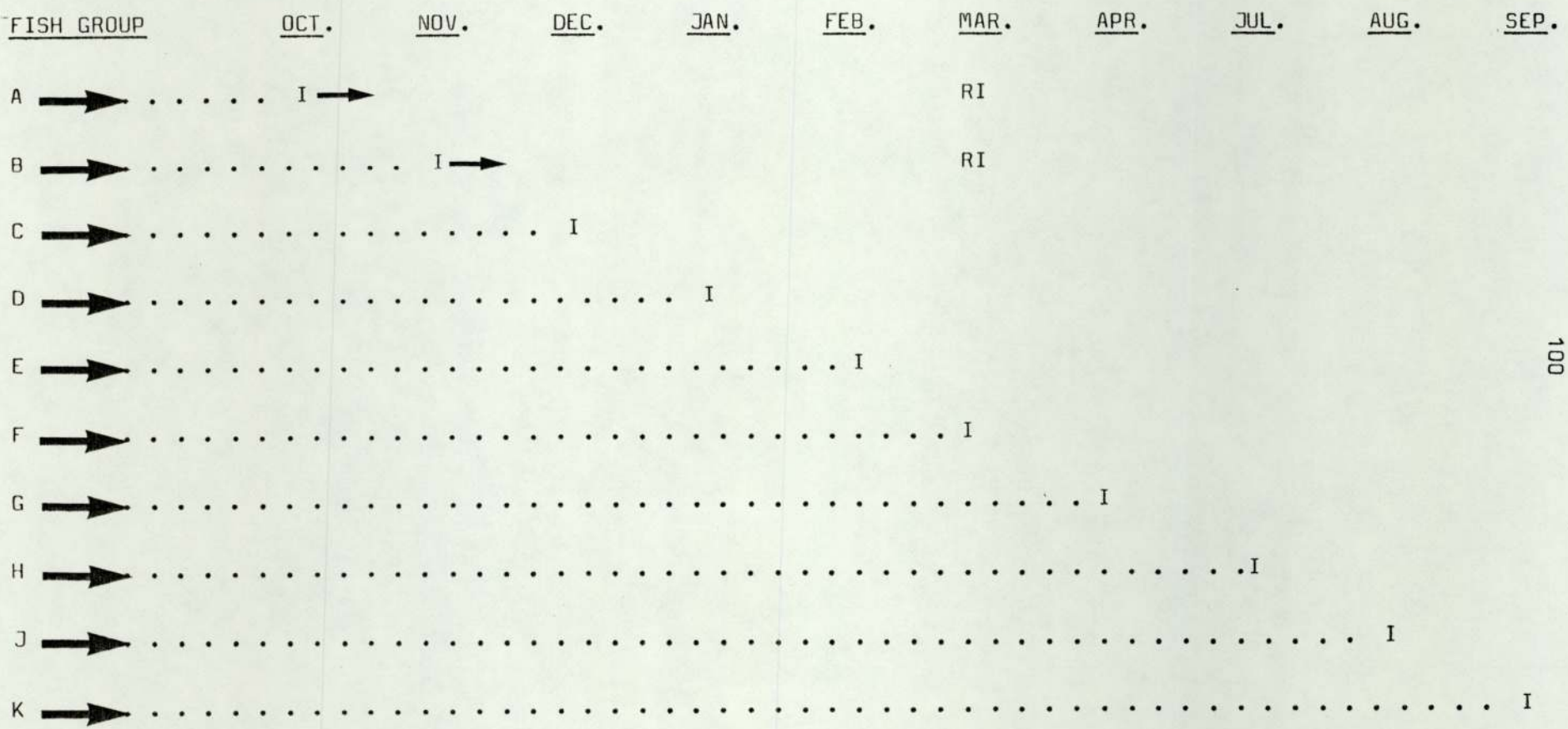


Diagram 5. Protocol for injection of groups of fish with E2 @ 5 μg g^{-1}, different groups being injected in successive months throughout the year.

I = Injection RI = Reinjection

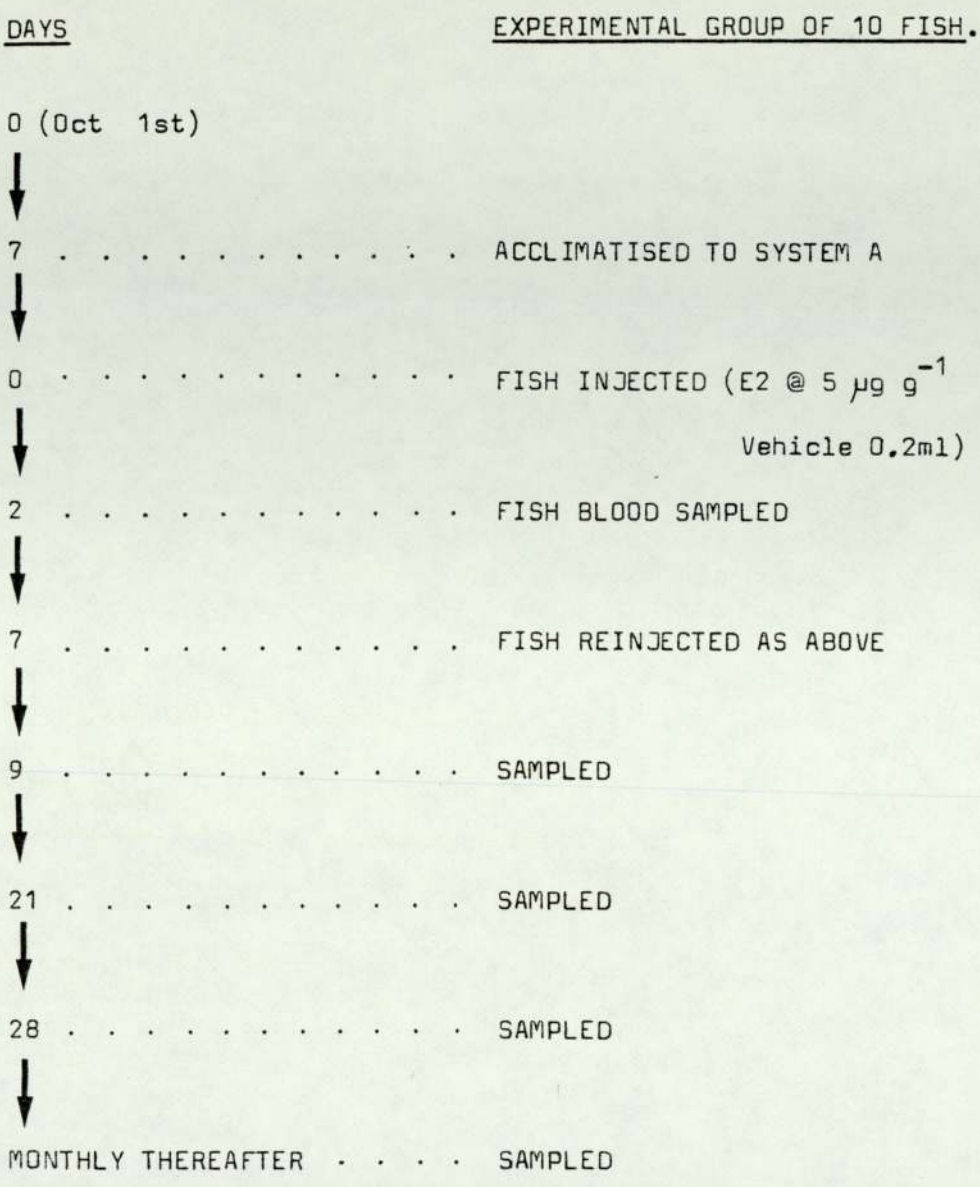


Diagram 6. Protocol of blood sampling for each experimental group, different groups being injected in successive months throughout the year.

Reinjection of Group A and B fish, with the same dose of E2, previously injected in Oct. and Nov. : Initial results from monthly injections revealed that the vitellogenic response of fish injected in winter months was reduced when compared with October. Thus the 2 groups injected in October and November were reinjected in March with the same dose as above. Similarly, 2 injections, one week apart were given, and blood samples treated in the same manner. The protocol for injection and blood sampling is given in more detail in Diagram 5.

3.4.2. Results.

Treated fish in all groups injected with the same dose of E2 at different times of the year demonstrated significant changes in TCa, as an indicator of vitellogenin. However, there were marked differences in the levels of TCa attained during the different months. Although assays for E2 were not conducted on all fish, measurements were made on those groups demonstrating maximal (October) and minimal (January) changes in levels of TCa in order to observe whether these changes were due to differences in uptake or clearance of the hormone.

Changes in mean serum levels of E2 & TCa after E2 injections at different times of the year : There were no differences between the serum hormone profile of E2-injected fish in October and January (Table 3.9.). In both groups peak mean E2 levels were recorded at 2 and 9 days and would appear to be similar in profile to those demonstrated in experiment 4 (Fig. 3.11.). Detailed comparison is difficult however, due to differences in sampling.

In Group A, injected with E2 in October (Table 3.10.) there was a significant ($P < 0.001$) increase in TCa from basal values of 11.02 $\pm$ 0.2 to 20.2 $\pm$ 3.2 mg $100ml^{-1}$ after only 9 days. Peak mean TCa levels of 49.8 $\pm$ 6.1 mg $100ml^{-1}$ were observed 21 days after the initial injection, falling to control values after 5 months (Fig. 3.12.). However, in fish injected with E2 in November, December and January, there was a marked reduction in peak mean TCa levels recorded, only reaching values of 30.1 $\pm$ 5.5 (Table 3.11.), 23.9 $\pm$ 2.8 (Table 3.12.) and 19.5 $\pm$ 3.8 mg $100ml^{-1}$ (Table 3.13.) respectively. Furthermore, the profile of the vitellogenic

response shown in October, November and January (Figs. 3.12. - 3.15.) changes significantly.

TIME (DAYS)	0	2	7	9	21
CONTROL	0.5	2.2	1.6	3.2	0.8
± S.E.	0.25	0.7	0.6	1.7	0.3
TREATED	0.7	47.5**	4.5	42.4***	0.6
± S.E.	0.3	9.1	1.2	7.3	0.25

TIME (DAYS)	0	2	7	9	21
CONTROL	0.5	1.5	1.2	2.0	1.0
± S.E.	0.3	0.4	0.3	0.3	0.4
TREATED	0.5	45.0***	9.95	54.3***	0.6
± S.E.	0.2	7.5	4.25	8.4	0.3

** ($P < 0.01$) *** ($P < 0.001$)

n = 5

TABLE 3.9. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) IN FISH INJECTED IN OCTOBER (TOP) AND JANUARY (BOTTOM).

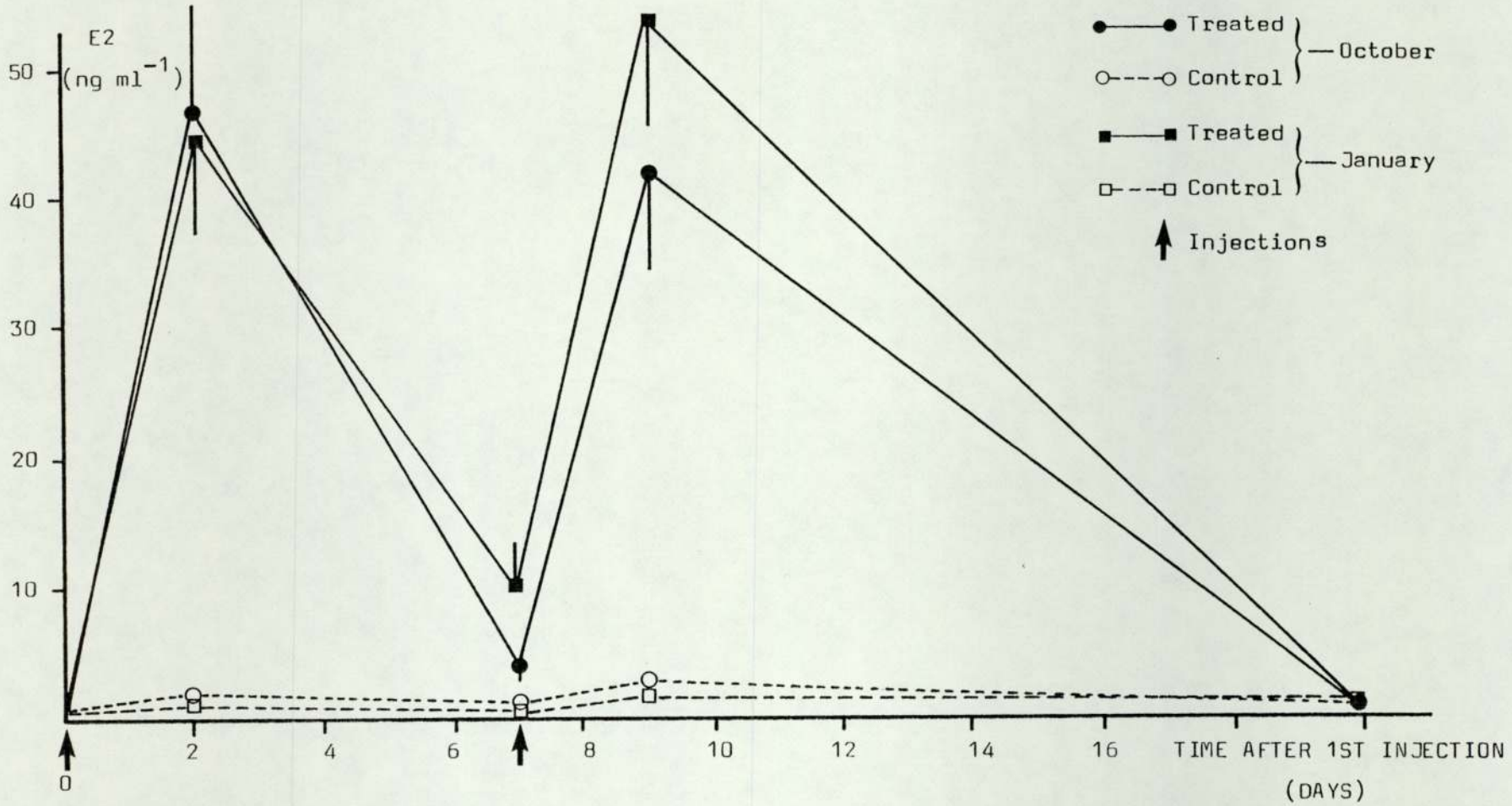


FIG. 3.11. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) IN FISH INJECTED WITH OESTRADIOL-17β (E2 5 μg g^{-1}) IN OCTOBER AND JANUARY.

TIME (DAYS)	BASAL	2	7	9	21
CONTROL	11.3	9.79	10.28	10.9	13.08
± S.E.	1.1	0.9	0.44	0.36	0.37
TREATED	11.07	10.4	14.94	20.19***	49.8***
± S.E.	0.2	0.8	2.05	3.2	6.1

TIME (DAYS)	28	56	84	112	140
CONTROL	10.24	11.24	10.62	11.01	12.47
± S.E.	0.34	1.03	2.07	0.79	0.58
TREATED	44.67**	41.9*	32.9*	18.06	13.15
± S.E.	10.1	9.3	7.5	3.72	2.19

TIME (DAYS)	168
CONTROL	9.97
± S.E.	0.36
TREATED	10.82
± S.E.	0.86

* ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$)

n = 5

TABLE 3.10. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml $^{-1}$) IN TREATED AND CONTROL FISH INJECTED IN OCTOBER WITH OESTRADIOL-17β (E2 5 μg g $^{-1}$).

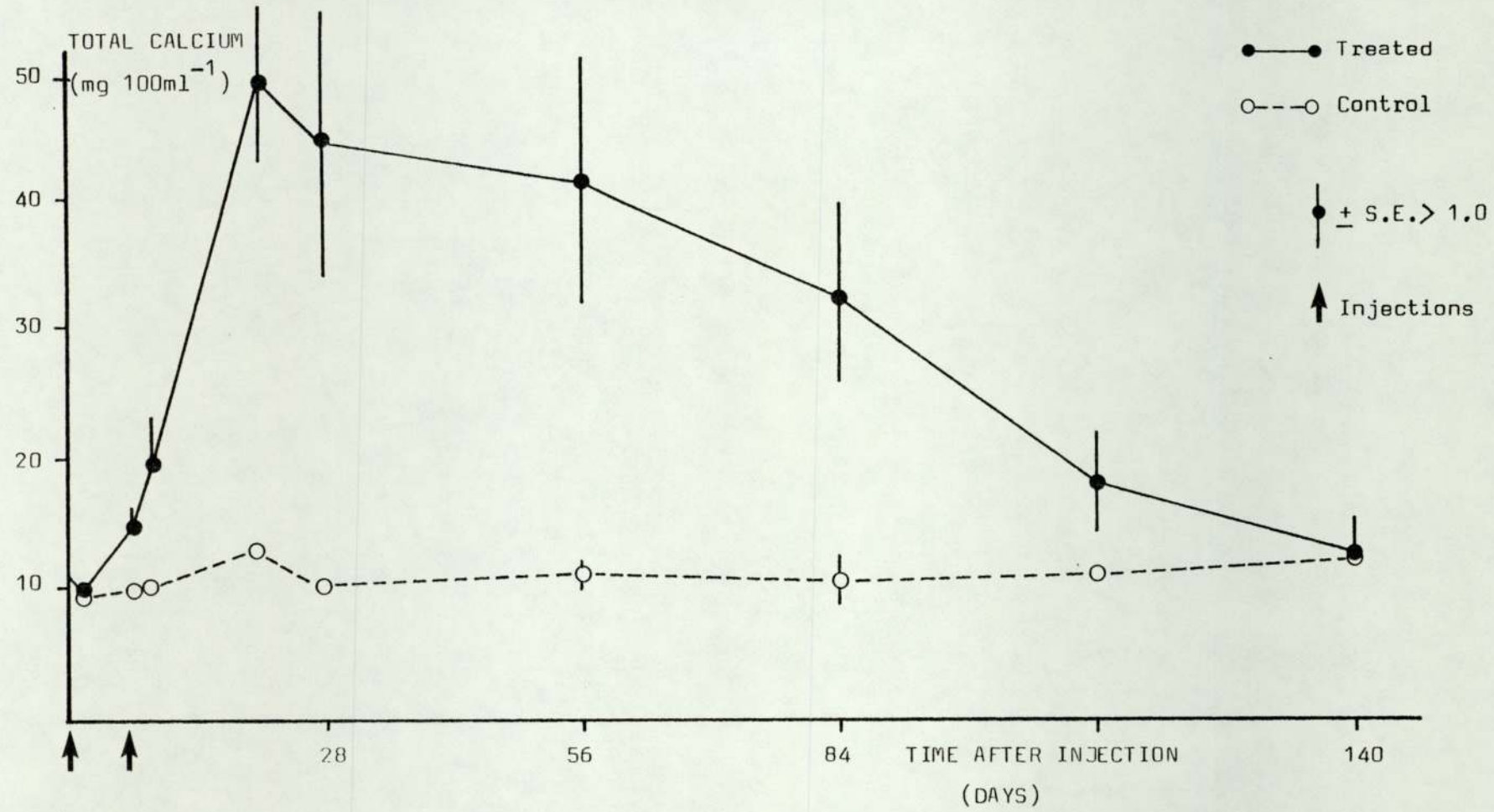


FIG. 3.12. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml^{-1}) IN TREATED AND CONTROL FISH INJECTED IN OCTOBER WITH OESTRADIOL-17β (E2 5 μg g^{-1}).

TIME (DAYS)	BASAL	2	7	9	21
CONTROL	11.45	10.54	9.83*1	9.91*1	10.81
± S.E.	0.46	0.26	0.30	0.40	0.32
TREATED	11.27	11.13	13.88**	15.48**	29.79**
± S.E.	0.38	0.29	0.85	1.19	3.9

TIME (DAYS)	28	56	84	112	140
CONTROL	10.18*1	11.04	8.54*1	10.56	10.85
± S.E.	0.33	0.14	0.9	0.7	0.3
TREATED	30.11**	17.4*	11.3	12.45	12.8
± S.E.	5.5	2.26	1.52	1.57	2.25

* ($P < 0.05$) ** ($P < 0.01$) *1 versus mean basal control value

n = 5

TABLE 3.11. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml $^{-1}$) IN TREATED AND CONTROL FISH INJECTED IN NOVEMBER WITH OESTRADIOL-17β (E2 5 μg g $^{-1}$).

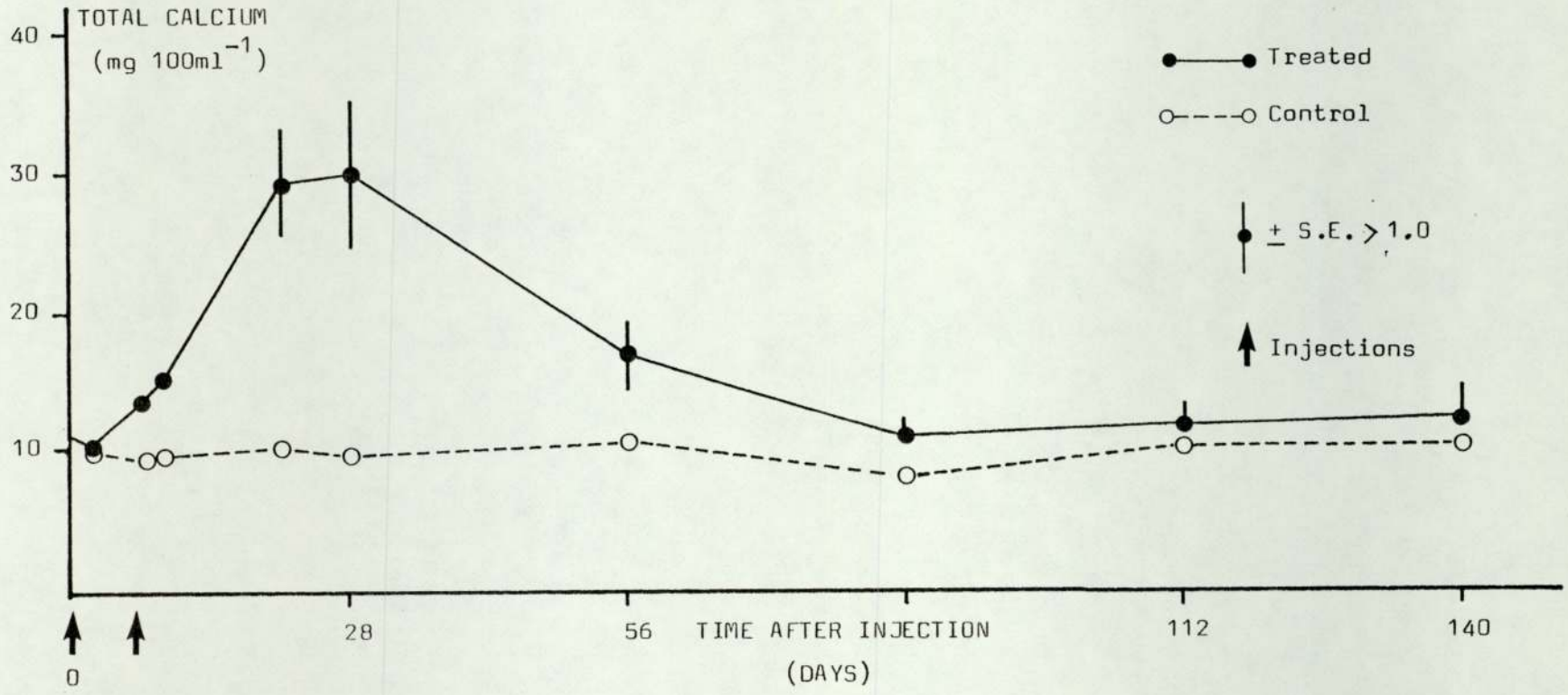


FIG. 3.13. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml^{-1}) IN TREATED AND CONTROL FISH INJECTED IN NOVEMBER WITH OESTRADIOL-17β (E2 5 μg g^{-1}).

TIME (DAYS)	BASAL	7	9	23	28
CONTROL	10.75	10.37	10.9	11.15	10.99
± S.E.	0.49	0.48	0.43	0.87	0.35
TREATED	10.1	12.81	13.10*	23.89*	23.15**
± S.E.	1.17	1.65	0.92	2.79	2.32

TIME (DAYS)	56	84	112
CONTROL	9.61	10.27	11.34
± S.E.	0.63	0.25	1.05
TREATED	19.78***	18.67	18.74
± S.E.	0.59	4.05	3.04

* ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$)

n = 5

TABLE 3.12. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa IN TREATED AND CONTROL FISH INJECTED IN DECEMBER WITH OESTRADIOL-17β (E2 5 μg g^{-1}).

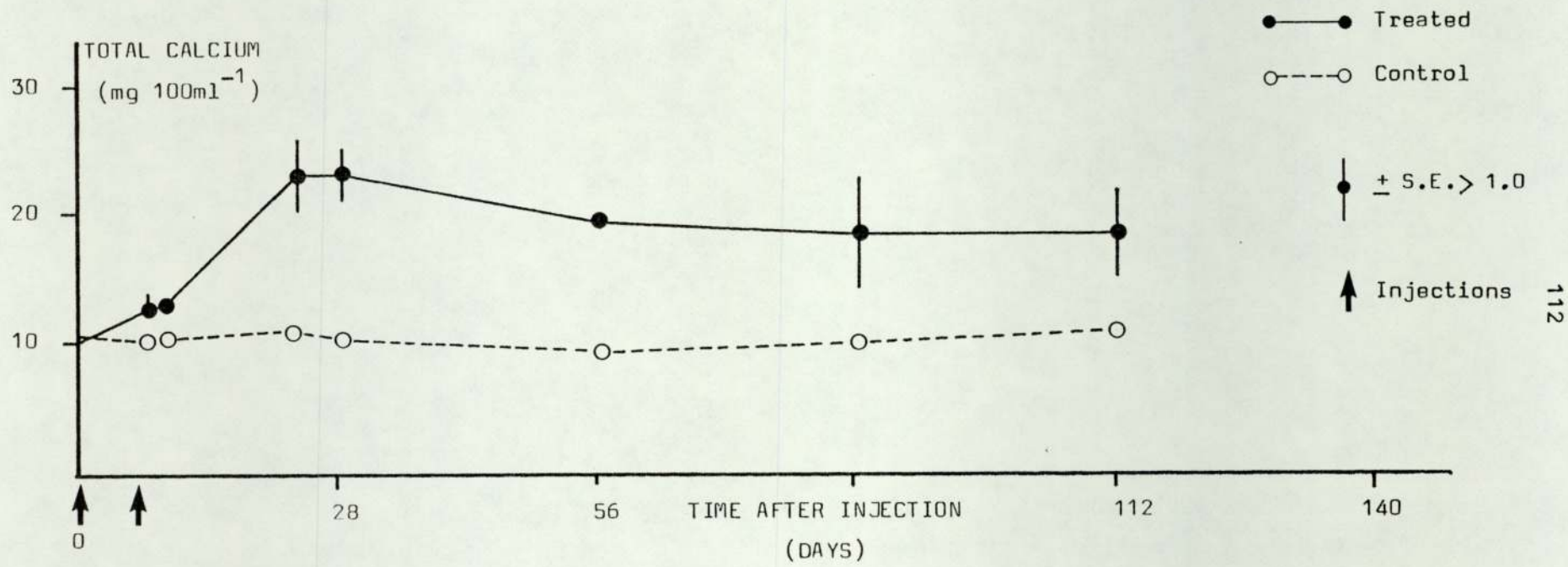


FIG. 3.14. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml^{-1}) IN TREATED AND CONTROL FISH INJECTED IN DECEMBER WITH OESTRADIOL-17β (E2 5 μg g^{-1}).

TIME (DAYS)	BASAL	2	7	9	23
CONTROL	8.69	9.24	8.54	8.14	10.0*1
± S.E.	0.4	0.27	0.33	0.13	0.13
TREATED	9.29	9.72	9.66	10.01**	18.5**
± S.E.	0.21	0.15	0.2	0.4	1.78

TIME (DAYS)	28	56	84	112	140
CONTROL	9.33	11.2**1	11.13*1	10.19**1	10.27*1
± S.E.	0.24	0.5	0.84	0.17	0.45
TREATED	18.21	19.46	14.22	9.94	10.3
± S.E.	2.53	3.8	1.72	0.89	0.54

* ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$) *1 versus mean basal control value

n = 5

TABLE 3.13. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) IN TREATED AND CONTROL FISH INJECTED IN JANUARY WITH OESTRADIOL-17β (E2 5 μg g^{-1}).

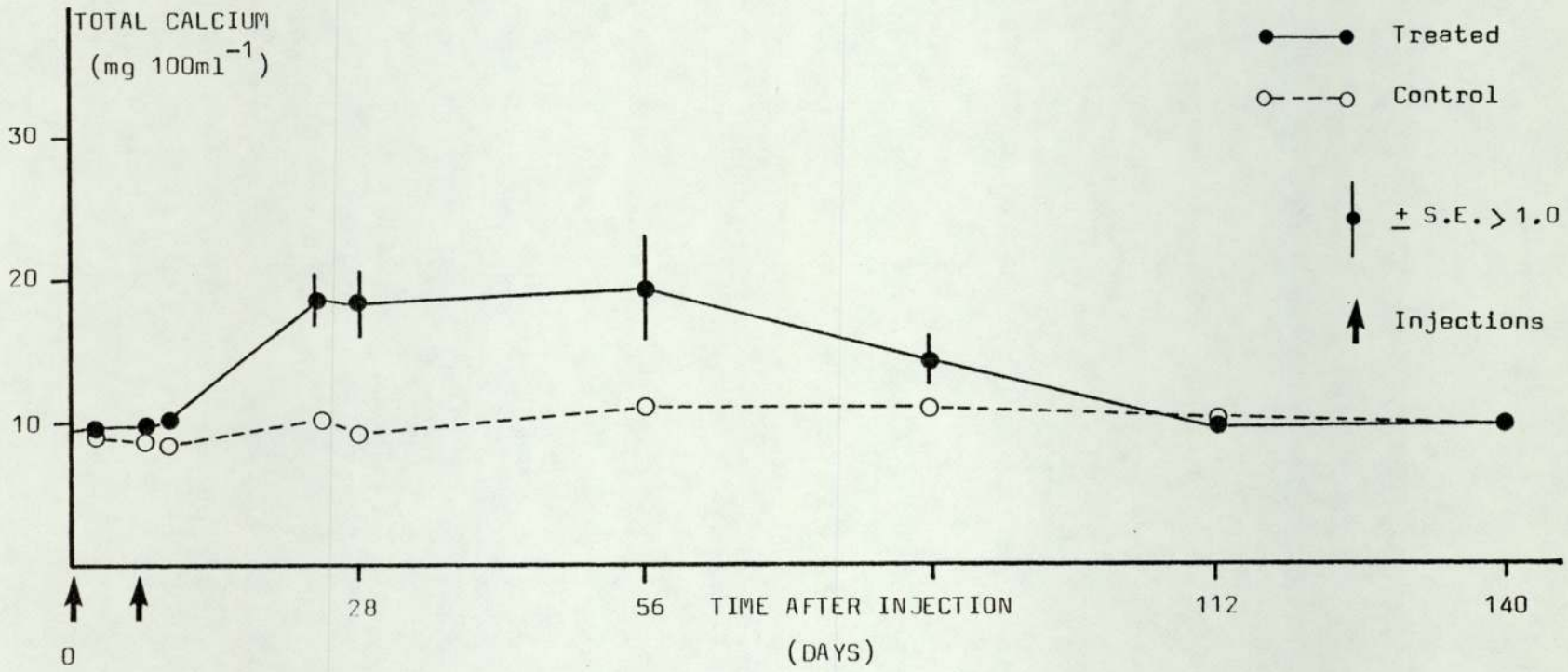


FIG. 3.15. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml^{-1}) IN TREATED AND CONTROL FISH INJECTED IN JANUARY WITH OESTRADIOL-17β (E2 5 μg g^{-1}).

In Group E fish, injected with E2 in February (Table 3.15.), TCa increased significantly from basal values of 9.94 ± 0.7 to 15.7 ± 1.03 mg $100ml^{-1}$ after 9 days. Peak mean values of 32.01 ± 6.4 mg $100ml^{-1}$ were observed 28 days after injection returning to control levels after 4 months. Unlike the response shown after injections in November - January, there was a subsequent increase in the peak mean TCa levels recorded in the groups treated in March, July, August and September, rising to 40.4 ± 6.7 (Table 3.16.), 43.0 ± 5.3 (Table 3.18), 44.1 ± 7.4 (Table 3.19.) and 55.0 ± 7.8 (Table 3.20.) mg $100ml^{-1}$ respectively. The response in Group G, treated in April (Table 3.17.) was similar to February-injected fish, with a peak mean TCa of 29.97 ± 0.9 mg $100ml^{-1}$. Furthermore, the profile of the vitellogenic response as determined by estimation of TCa in these groups injected with E2 in March, July, August and September, is similar to that originally observed in the group of fish treated in October (Figs. 3.16. - 3.21.).

TIME (DAYS)	BASAL	2	7	9
CONTROL	10.12	10.47	9.71	10.69
± S.E.	0.54	0.29	0.19	0.58
TREATED	9.94	10.92	12.19	15.65*
± S.E.	0.65	0.82	0.76	1.03

TIME (DAYS)	28	56	84	112
CONTROL	9.38	9.54	9.96	10.7
± S.E.	1.11	0.13	0.27	0.36
TREATED	32.02**	21.48**	12.3*	9.74
± S.E.	6.37	3.8	1.27	0.45

* ($P < 0.05$) ** ($P < 0.01$)

n = 5

TABLE 3.15. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) IN TREATED AND CONTROL FISH INJECTED IN FEBRUARY WITH OESTRADIOL-17β (E2 5 μg g^{-1}).

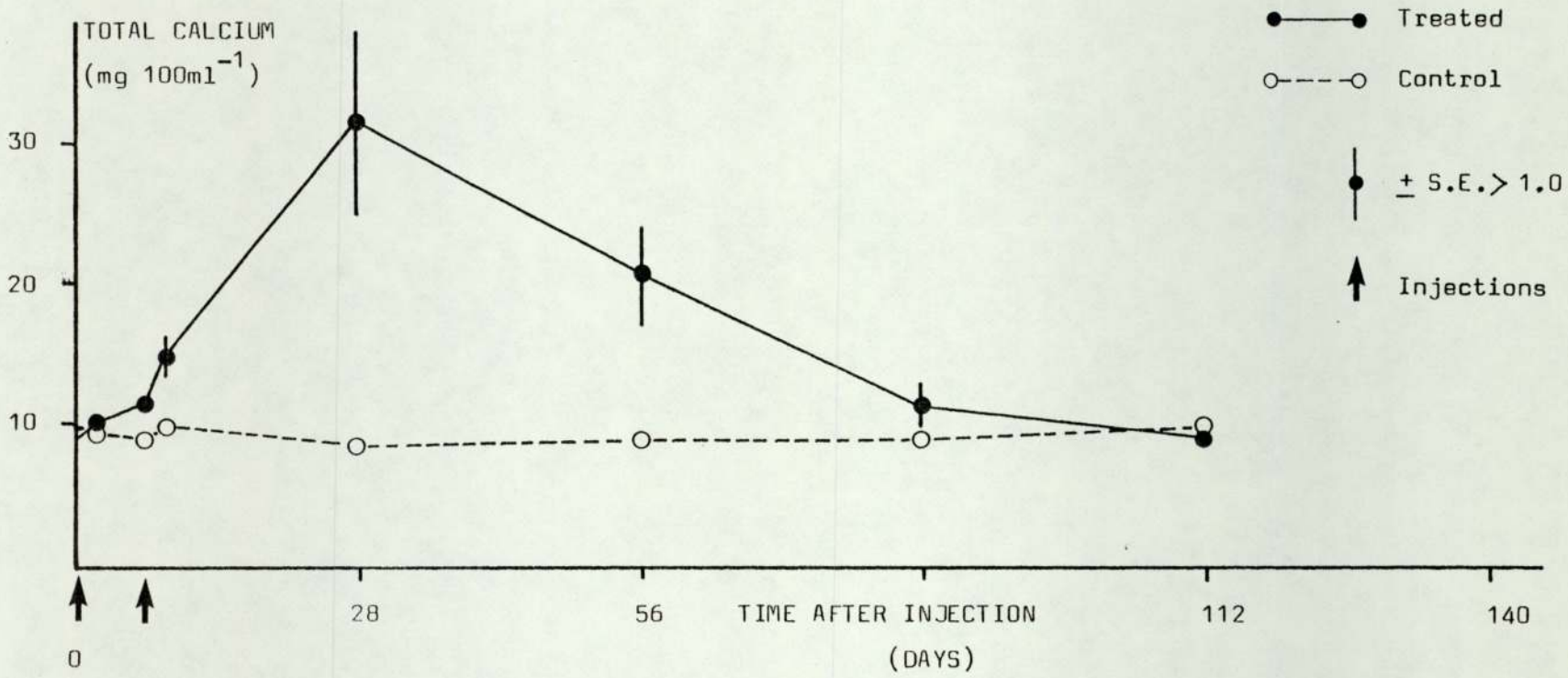


FIG. 3.16. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml^{-1}) IN TREATED AND CONTROL FISH INJECTED IN FEBRUARY WITH OESTRADIOL-17β (E2 5 μg g^{-1}).

TIME (DAYS)	BASAL	2	7	9
CONTROL	10.39	10.13	10.72	10.82
± S.E.	0.6	0.44	0.5	0.37
TREATED	10.74	9.77	12.11	12.77*
± S.E.	0.26	0.18	0.36	0.58

TIME (DAYS)	28	56	84	132
CONTROL	12.2	11.29	10.15	11.37
± S.E.	0.6	0.28	0.2	0.69
TREATED	40.42**	34.55**	16.05*	12.14
± S.E.	6.7	7.4	2.55	1.03

* ($P < 0.05$) ** ($P < 0.01$)

n = 5

TABLE 3.16. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) IN TREATED AND CONTROL FISH INJECTED IN MARCH WITH OESTRADIOL-17β (E2 5 $\mu g\ g^{-1}$).

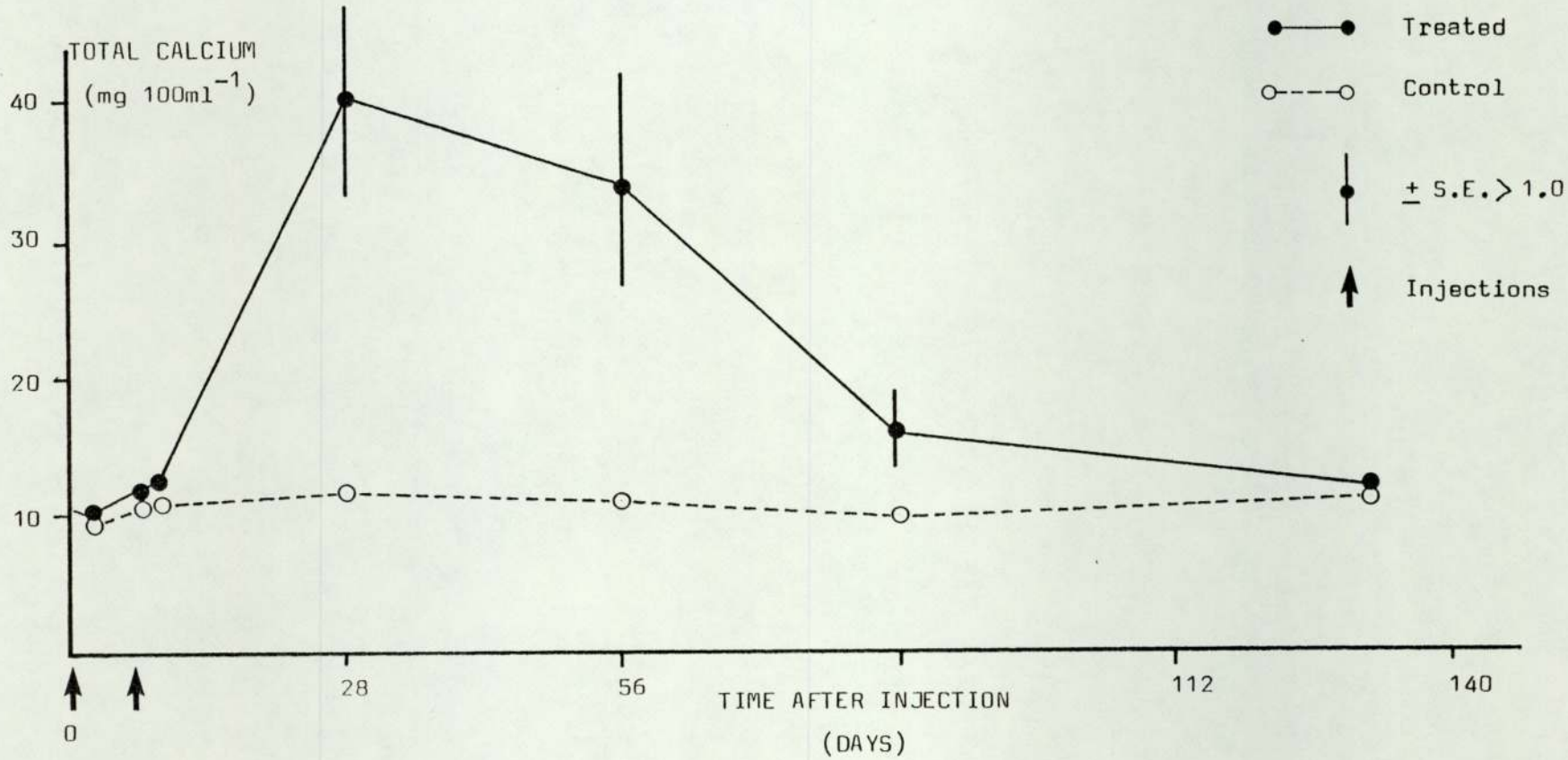


FIG. 3.17. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml^{-1}) IN TREATED AND CONTROL FISH INJECTED IN MARCH WITH OESTRADIOL-17β (E2 5 μg g^{-1}).

TIME (DAYS)	BASAL	2	7	9	21
CONTROL	10.87	9.39	9.596	9.19	10.2
± S.E.	0.17	0.24	0.19	0.2	0.5
TREATED	10.55	9.45	10.56*	10.59**	21.5***
± S.E.	0.16	0.16	0.24	0.3	0.2

TIME (DAYS)	28	56	84	130
CONTROL	10.14	10.33	10.73	10.55
± S.E.	0.16	0.59	1.03	0.66
TREATED	29.97***	13.56	12.14	11.47
± S.E.	0.92	2.9	2.1	1.98

* ($P < 0.05$) ** ($P < 0.01$) *** (P 0.001)

n = 5

TABLE 3.17. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml $^{-1}$) IN TREATED AND CONTROL FISH INJECTED IN APRIL WITH OESTRADIOL-17β (E2 5 μg g $^{-1}$).

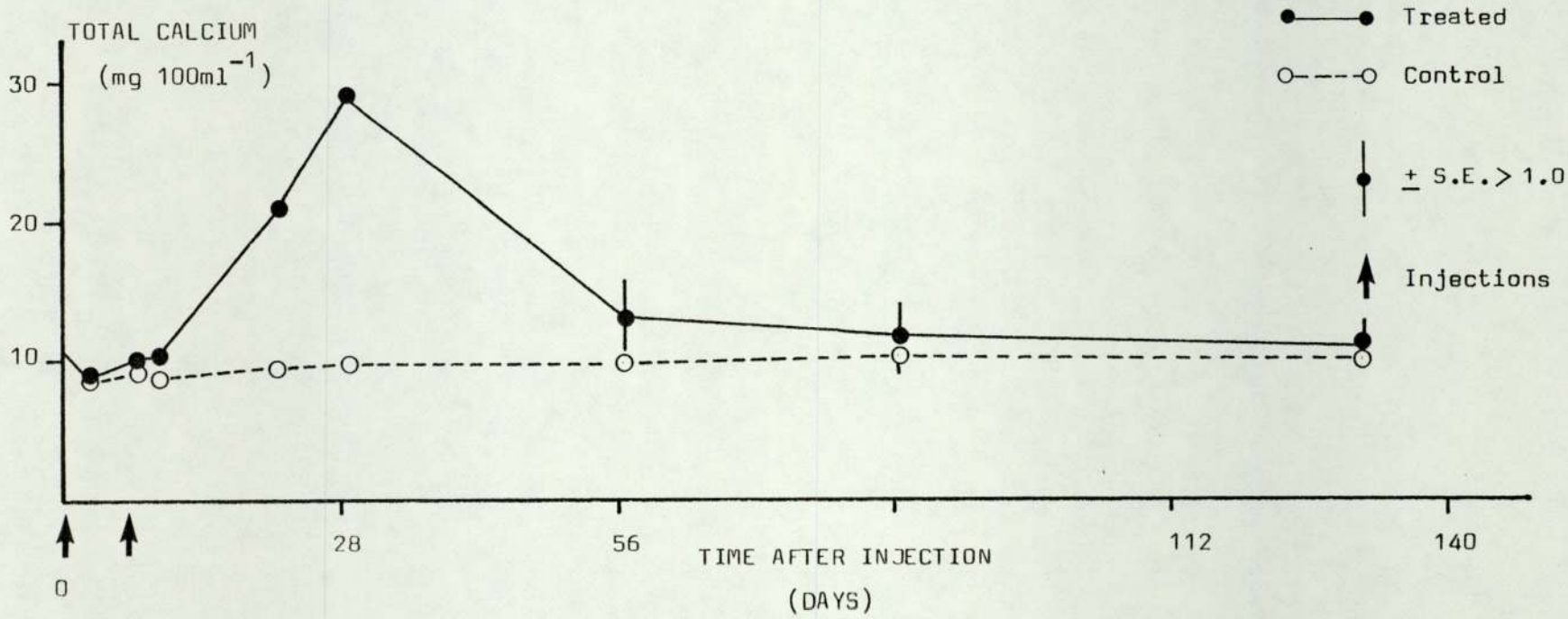


FIG. 3.18. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml^{-1}) IN TREATED AND CONTROL FISH INJECTED IN APRIL WITH OESTRADIOL-17β (E2 5 μg g^{-1}).

TIME (DAYS)	BASAL	2	7	9	21
CONTROL	10.98	9.08	10.28	11.15	11.01
± S.E.	0.2	1.0	0.67	0.4	1.35
TREATED	11.11	10.24	13.15	17.33*	43.0***
± S.E.	0.4	0.76	1.97	2.08	5.3

TIME (DAYS)	28	56	84	112	140
CONTROL	10.62	10.24	11.17	10.9	10.3
± S.E.	2.07	1.03	1.2	0.6	0.3
TREATED	38.64**	32.7**	22.5**	15.16**	11.78
± S.E.	5.71	7.3	2.1	0.9	1.7

* ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$)

n = 5

TABLE 3.18. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) IN TREATED AND CONTROL FISH INJECTED IN JULY WITH OESTRADIOL-17β (E2 5 $\mu g\ g^{-1}$).

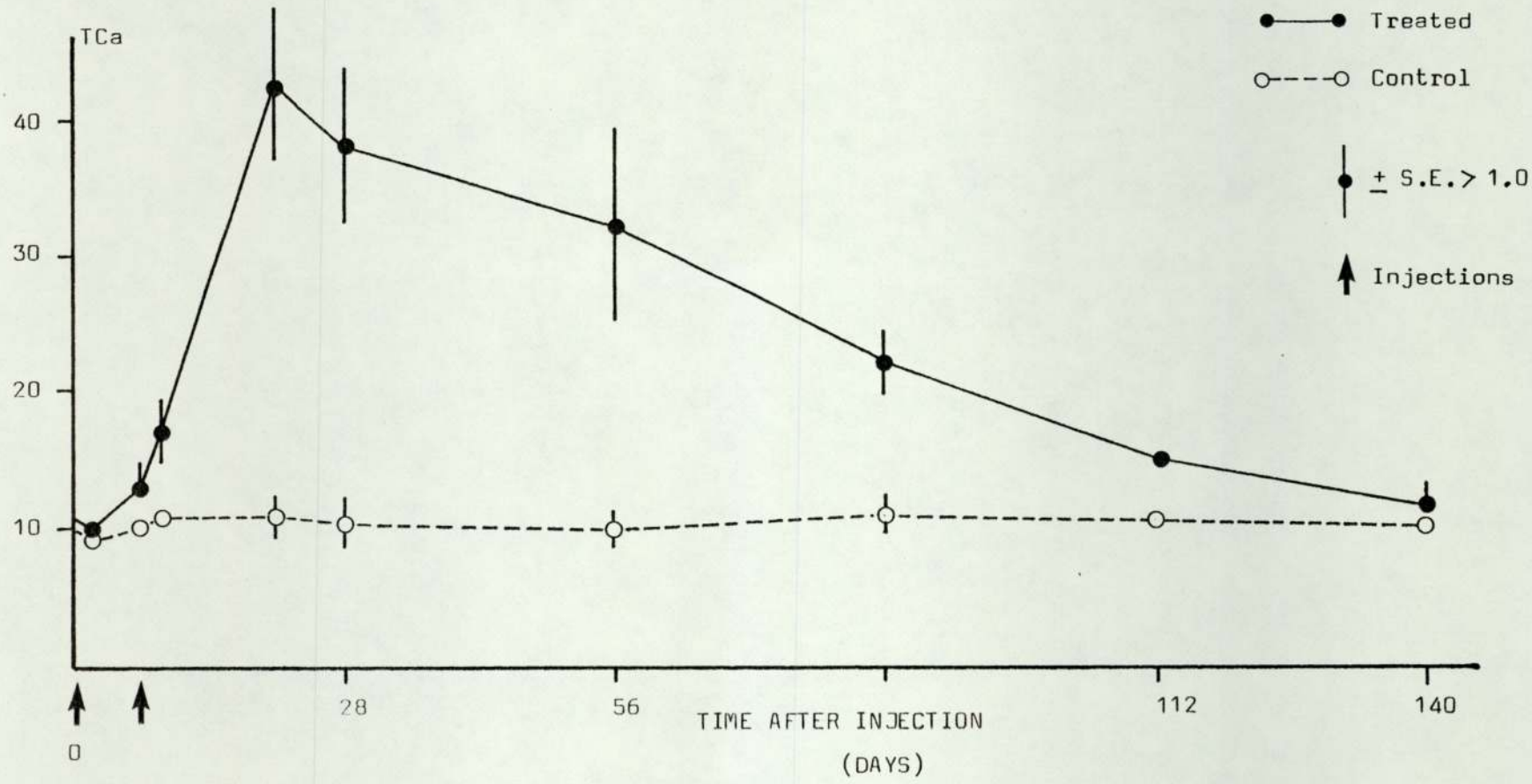


FIG. 3.19. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) IN TREATED AND CONTROL FISH INJECTED IN JULY WITH OESTRADIOL-17β (E2 5 $\mu g\ g^{-1}$).

TIME (DAYS)	BASAL	7	9	24
CONTROL	12.86	12.4	12.5	12.05
± S.E.	0.732	0.68	0.6	1.1
TREATED	12.68	16.25	20.03*	44.06**
± S.E.	0.88	2.7	2.95	7.44

TIME (DAYS)	28	56	84	112
CONTROL	10.54	11.01	11.07	10.84
± S.E.	0.46	0.2	0.7	0.5
TREATED	40.6**	37.5**	29.23*	21.71*
± S.E.	8.6	7.2	6.9	4.3

TIME (DAYS)	140
CONTROL	10.55
± S.E.	0.3
TREATED	11.04
± S.E.	0.8

* ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$)

n = 5

TABLE 3.19. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) IN TREATED AND CONTROL FISH INJECTED IN AUGUST WITH OESTRADIOL-17β (E2 5 $\mu g\ g^{-1}$).

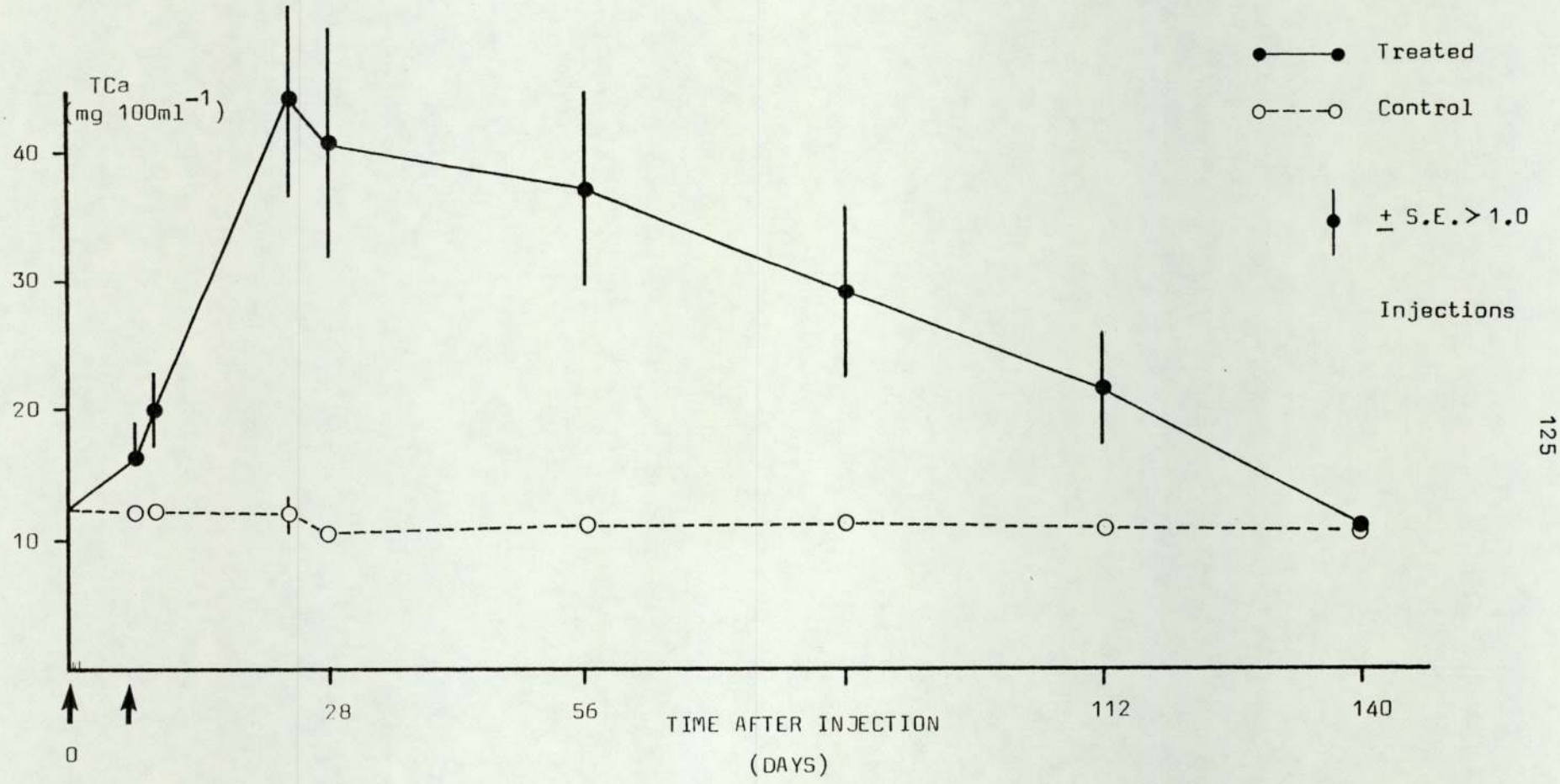


FIG. 3.20. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml^{-1}) IN TREATED AND CONTROL FISH INJECTED IN AUGUST WITH OESTRADIOL-17β (E2 5 μg g^{-1}).

TIME (DAYS)	BASAL	2	7	9	14
CONTROL	10.19	10.0	10.9	9.98	11.14
± S.E.	0.2	0.3	0.4	1.7	0.3
TREATED	10.72	9.95	13.78	19.21*	37.9***
± S.E.	0.4	0.6	1.2	2.3	4.9

TIME (DAYS)	21	28	56	84
CONTROL	11.1	11.55	12.1	11.0
± S.E.	0.5	1.03	1.0	0.7
TREATED	55.01***	50.26**	47.33***	36.48**
± S.E.	7.8	9.4	6.2	7.1

TIME (DAYS)	112	140
CONTROL	11.17	11.45
± S.E.	0.9	0.87
TREATED	20.71*	13.91
± S.E.	3.2	1.3

* ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$)

n = 5

TABLE 3.20. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) IN TREATED AND CONTROL FISH INJECTED IN SEPTEMBER WITH OESTRADIOL-17β (E2 5 μg g^{-1}).

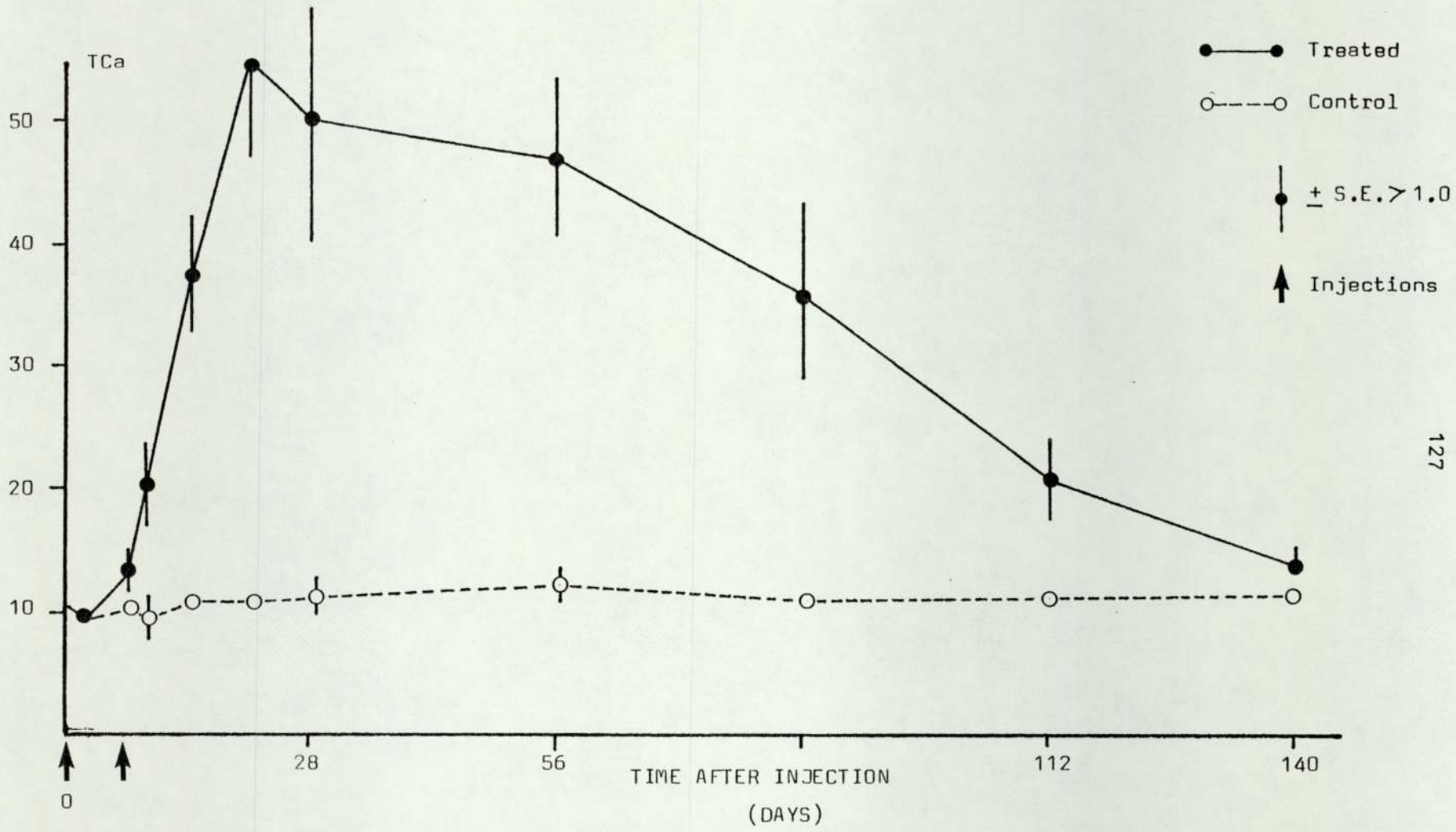


FIG. 3.21. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa. mg.100ml.$^{-1}$) IN FISH INJECTED WITH OESTRADIOL-17β (E2. 5 μg.g.$^{-1}$) IN SEPTEMBER.

Changes in mean serum levels of TCa on reinjection with the same dose of E2, after previous injection in October and November : On reinjection in March, Group A demonstrated a maximal vitellogenic response (Table 3.21. & Fig. 3.22.). Mean TCa levels rose from basal values, in treated fish, of 10.8 $\pm$ 0.9 mg $100ml^{-1}$, to peak mean levels of 41.7 $\pm$ 11.8 mg $100ml^{-1}$ after 28 days. TCa returned to control values 5 months after reinjection. Similarly, a maximal response was observed in Group B, on reinjection in March (Table 3.22. & Fig. 3.23.). TCa in treated fish rose significantly ($P < 0.001$) from basal levels of 12.8 $\pm$ 2.3 to 50.7 $\pm$ 6.2 mg $100ml^{-1}$ after 28 days. Control fish in both groups showed no such significant changes.

The peak mean TCa levels observed after the same dose of E2 given at different times of the year, is summarised in Fig. 3.24..

TIME (DAYS)	BASAL	2	7	9	28
CONTROL	9.97	10.13	10.72	10.82	12.2
± S.E.	0.30	0.44	0.5	0.37	0.6
TREATED	10.82	9.16	13.32*	14.76*	41.67*
± S.E.	0.86	0.95	1.03	1.5	11.79

TIME (DAYS)	56	84	132
CONTROL	11.29	10.15	11.37
± S.E.	0.28	0.2	0.69
TREATED	41.65*	24.35	11.92
± S.E.	13.5	10.35	1.5

* ($P < 0.05$)

n = 5

TABLE 3.21. MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml $^{-1}$) OF FISH INJECTED WITH OESTRADIOL-17β (E2) IN OCTOBER AND REINJECTED WITH THE SAME DOSE (5 μg g $^{-1}$) IN MARCH.

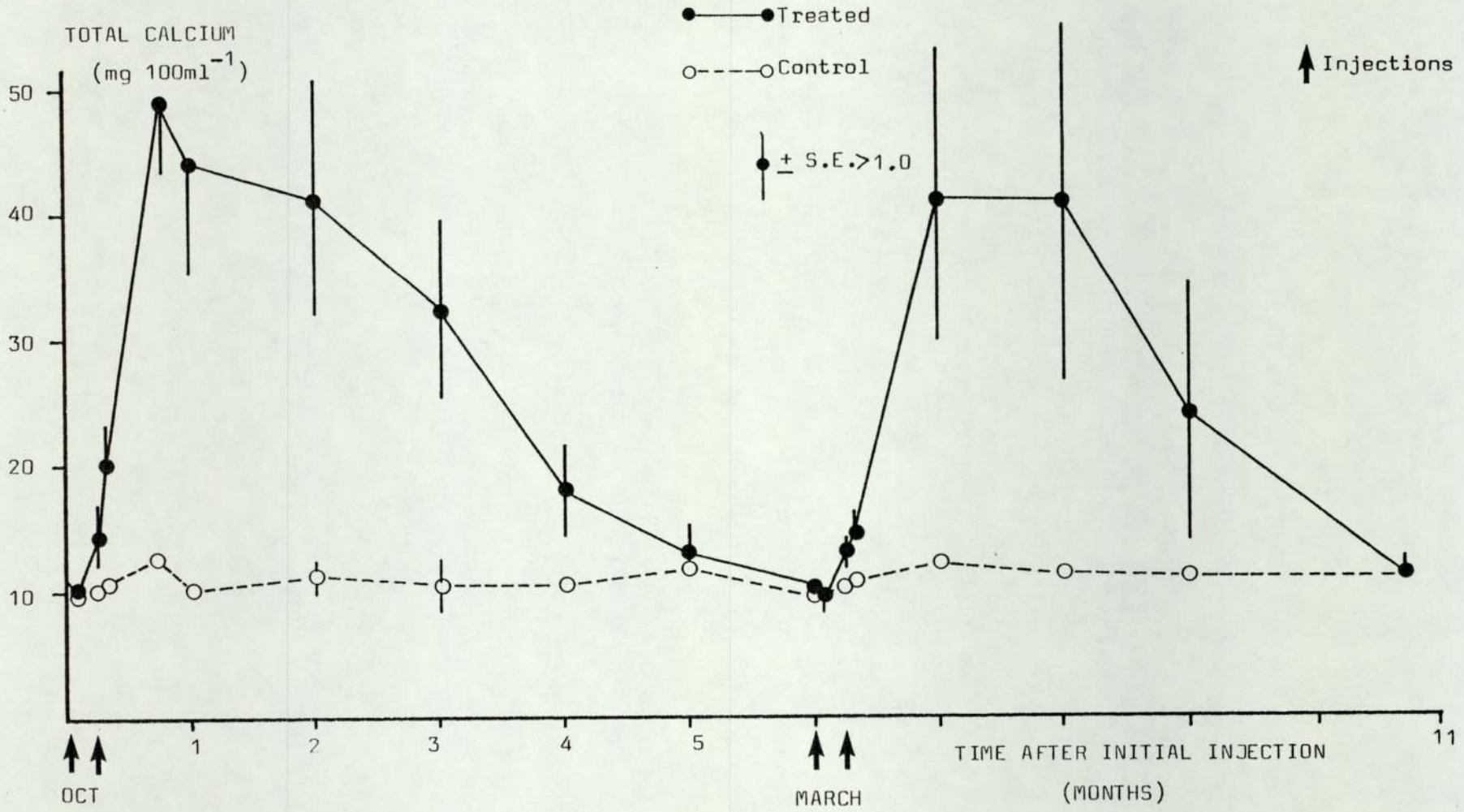


FIG. 3.22. MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml^{-1}) OF FISH INJECTED WITH OESTRADIOL-17β (E2) OCTOBER AND REINJECTED WITH THE SAME DOSE (5 μg g^{-1}) IN MARCH.

TIME (DAYS)	BASAL	2	7	9
CONTROL	10.85	10.13	10.72	10.82
± S.E.	0.3	0.44	0.5	0.37
TREATED	12.8	11.27	15.7	17.6^{*1}
± S.E.	2.25	2.52	3.9	4.07

TIME (DAYS)	28	56	84	132
CONTROL	12.2	11.29	10.15	11.37
± S.E.	0.6	0.28	0.20	0.69
TREATED	50.66^{***}	51.84^{***}_{**1}	31.98^{**}_{**1}	13.46^{*}
± S.E.	6.16^{**1}	6.9	5.8	0.77

* ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$) *1 versus mean treated levels of initial response in November.

n = 5

TABLE 3.22. MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) OF FISH INJECTED WITH OESTRADIOL-17β (E2) IN NOVEMBER AND REINJECTED WITH THE SAME DOSE (5 $\mu g\ g^{-1}$) IN MARCH.

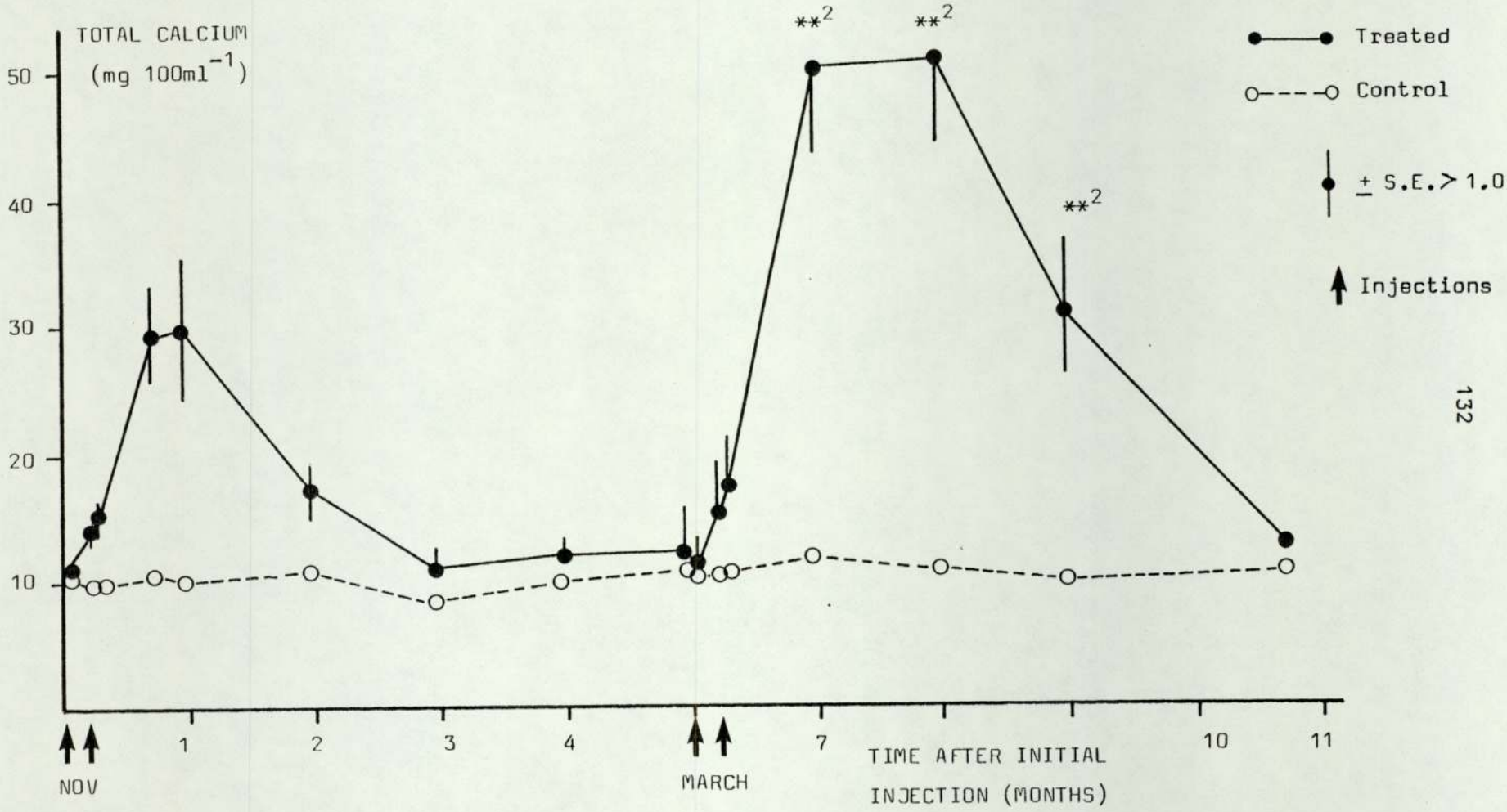


FIG. 3.23. MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml^{-1}) OF FISH INJECTED WITH OESTRADIOL-17β (E2) IN NOVEMBER AND REINJECTED WITH THE SAME DOSE (5 μg g^{-1}) IN MARCH. (*^{2} versus initial response).

Statistical Analysis of Results : The significance of differences between mean treated and mean control values for each monthly injected group was tested using the students t-test (1 tail). The peak mean TCa values for each month were also compared with the maximum value observed after treatment in October with the minimum value observed after treatment in January (Fig. 3.24.).

The t-test was used to test the significance of the differences in mean peak levels of TCa observed in Group B in response to E2 injection in November, compared to the response after reinjection with the same dose in March.

The different vitellogenin responses observed at different times of the year, after treatment with the same dose of E2 were analysed using a 10 $\times$ 2 factorial split-plot ANOVAR, where the data was of randomised design with 5 replications.

Results :

Significant differences between control and treatment groups where applicable are detailed on the relative tables of results above where :

*** ($P < 0.001$)

** ($P < 0.01$)

* ($P < 0.05$)

The Analysis of Variance was completed in 2 steps. In both instances "sub-treatment" was whether fish were treated with E2 or control. In the first analysis, the main treatment was "peak height" of calcium, and in the second analysis, "time to reach" peak height was the main treatment.

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	F-RATIO	(P)
TREATMENTS	9	3491.78	387.976	10.0401	P<0.001
LINEAR EFF.	1	805.21	805.21	20.8374	P<0.001
QUAD EFF.	1	1473.76	1473.76	38.1382	P<0.001
CUBIC EFF.	1	380.078	380.078	9.83573	P<0.001
RESIDUAL	6	832.733	138.789	3.5916	-
MAIN PLOT ERROR	40	1545.7	38.6426	-	-
SUB-TRTS	1	15314.1	15314.1	399.739	P<0.001
INTERACTION	9	3033.33	337.036	8.79757	P<0.001
SUB-PLOT ERROR	40	1532.41	38.3102	-	-
TOTAL	99				

1. Main Treatment - PEAK HEIGHT OF MEAN CALCIUM.
   sub treatment - treated or control.
   Conclusions:

(a) The time of year of injection significantly (P<0.001) affects the peak height of calcium (vitellogenin).

(b) This seasonal variation in response shows significant linear, quadratic and cubic changes(P<0.001).

(c) Treatment with oestradiol-17β significantly affects the peak height(P<0.001).

(d) A significant interaction(P<0.001), between main treatment and sub-treatment indicates that the relationship between the peak heights of treated and control groups changes with the time of year.

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	F-RATIO	(P)
TREATMENTS	9	17358.7	1928.75	2.06795	P<0.05
LINEAR EFF	1	2076.44	2076.44	2.22631	-
QUAD EFF	1	4803.41	4803.41	5.15009	P<0.05
CUBIC EFF	1	3204.48	3204.48	3.43576	-
RESIDUAL	6	7274.41	1212.4	1.2999	-
MAIN PLOT ERROR	40	37307.4	932.685	-	-
SUB-TRTS	1	11707.2	11707.2	14.9725	P<0.001
INTERACTION	9	9276.16	1030.68	1.31815	-
SUB-PLOT ERROR	40	31276.6	781.915	-	-
TOTAL	99				

2. Main Treatment - TIME TO REACH PEAK HEIGHT

Sub-treatment - treated or control.

Conclusions:

(a) The time to reach peak levels of calcium(rate) after treatment with oestradiol-17β is significantly affected by the time of year of injection($P<0.05$).

(b) This variation shows a significant quadratic effect($P<0.05$).

(c) Treatment with oestradiol-17β significantly affects the rate of increase in calcium($P<0.001$).

3.4.3. Conclusions.

The results indicate there is a marked seasonal variation in response to the same dose of injected E2 (Fig. 3.24.). A maximum response is observed in late-summer months at the time of year when vitellogenesis normally occurs in maturing female trout. A minimum response is observed in late winter/early spring at the time when spawning normally occurs.

The serum hormone profiles for injected E2 were not significantly different in October and January fish, the months where maximal and minimum responses were observed.

Since immature trout were used it appears that an inherent seasonal rhythm exists which either promotes the vitellogenic response in late summer or diminishes the response in late winter.

The responses observed in the 2 reinjected groups emphasise this seasonal variation in response. The significantly greater maximal response demonstrated by Group B on reinjection following an initial reduced response, indicates that this group was capable of a maximal response if injected at the correct time. Although the maximal response on reinjection in Group B could be interpreted as a secondary response, the 2 similar maximal responses in Group A suggest this is not the case.

The results also demonstrate that there is no refractory period to E2 stimulation in the production of vitellogenin from the liver.

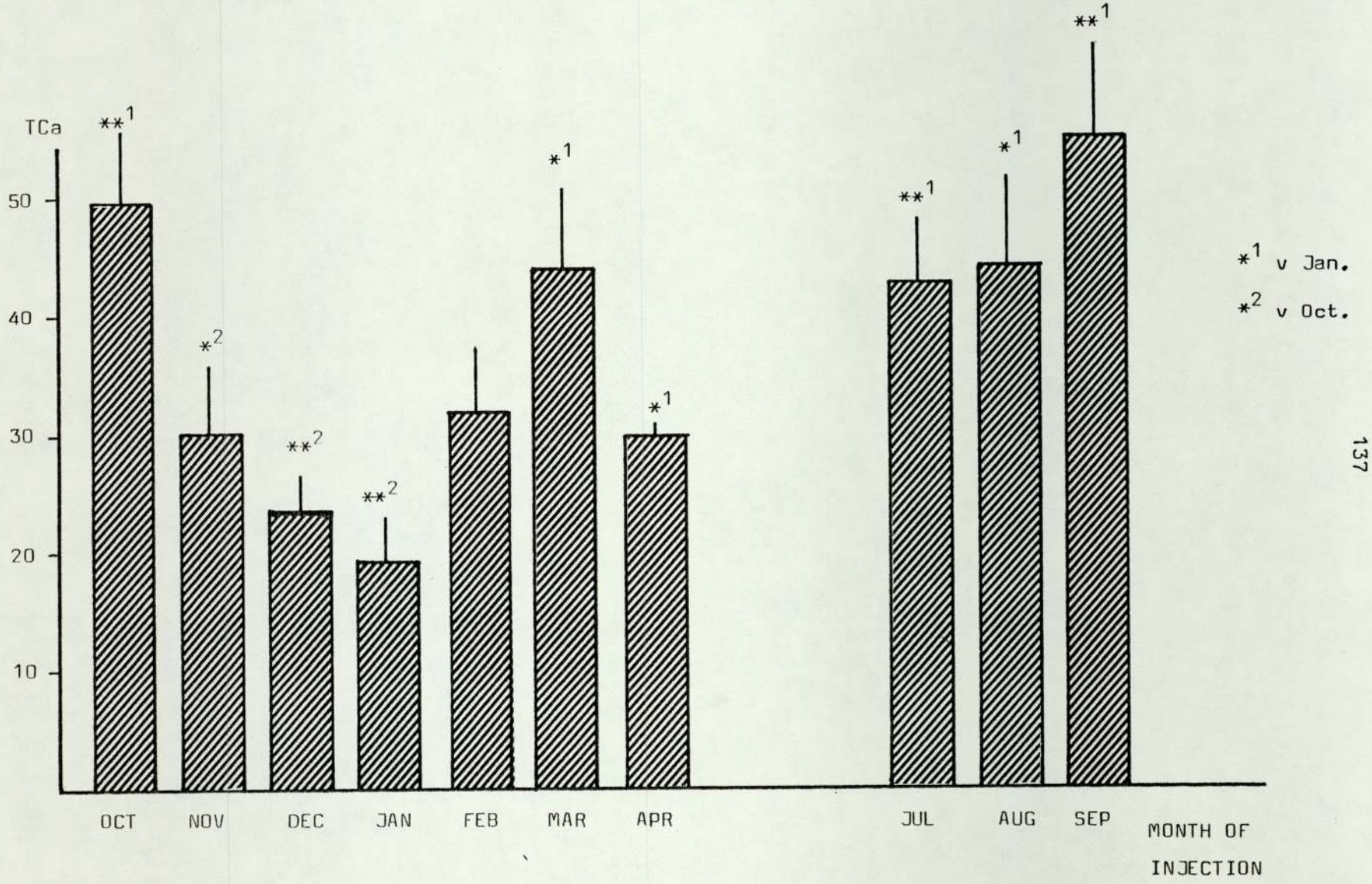


FIG. 3.24. SUMMARY OF SEASONAL RESPONSE TO A SPECIFIC DOSE OF OESTRADIOL-17β (E2 5 μg g^{-1}). PEAK MEAN SERUM CALCIUM ± S.E..

3.5. Vitellogenic Response in Trout Injected with Different Doses of E2.

The aim of this series of experiments was two-fold. Firstly, to establish if there was a dose response to injected E2. Secondly, by giving different doses at the time of the year when the response to a specific dose was minimal, an investigation could be made whether 'threshold' or refractory effects were evident. 2 months were chosen. October when the response was maximal, and January when that response was minimal.

3.5.1. Materials and Methods.

Injection of groups of fish with different doses of E2 in October: 45 immature 1-year+ fish were selected having an average weight of 150g. All fish were weighed, individually tagged and bled to obtain baseline values. The fish were allowed to acclimatise to system A for 6 days.

Groups of 5 fish were than injected once only with E2 suspended in arachis/ethanol at dose rates of 40, 20, 10, 5, 1.0, 0.5, 0.1 and 0.01 $\mu g\ g^{-1}$ body weight. The remaining 5 fish were injected with 0,3ml of vehicle only.

All fish were blood sampled at 0, 2, 5, 11, 14, 21, 39 and 56 days after injection and the serum thus obtained stored at $-20^{o}C$ until assay for TCa and E2. At the end of the experiment, the fish were weighed, sacrificed and hepatosomatic and gonadosomatic indices determined.

Injection of groups of fish with different doses of E2 in January: 72 immature trout were selected from the same stock with a mean weight of 200g. All fish were tagged, blood sampled and allowed to acclimatise for about 7 days.

Groups of 8 fish were injected once with E2 at the same dose rates outlined above. The remaining 8 fish acted as vehicle injected controls.

All fish were bled at 0, 2, 5, 8, 11, 25, 39 and 55 days after injection. At the end of the experiment, the fish were re-weighed, sacrificed and hepatosomatic and gonadosomatic indices determined.

3.5.2. Results.

Changes in mean serum levels of E2 & TCa after injection of different doses of E2 in October : Treated fish showed gross changes in serum levels of E2 and TCa following injection with different doses of this hormone in October. Peak mean levels of serum E2 were observed 2 days after injection (Table 3.23. & Fig. 3.25.). 11 days after injection, mean serum E2 levels in all treated groups, except 40 and 20 $\mu g\ g^{-1}$, were approaching control values. Mean serum E2 in control fish rose from basal values of 0.5 ± 0.3 ng ml^{-1} to a 2-day peak mean value of 1.2 ± 0.4 ng ml^{-1} before falling to 1.0 ± 0.2 ng ml^{-1} 11 days after injection.

All treated groups showed significant increases in TCa over the course of the experiment (Table 3.24. & Fig. 3.26.). The peak mean TCa levels were significantly correlated ($P < 0.001$) with the injected dose (Table 3.25. & Fig. 3.27.). There was no significant changes in the control fish. Peak mean TCa levels were observed 21 days after injection in all groups.

In treated groups the hepatosomatic index was significantly ($P < 0.001$) raised over basal values of $1.8 \pm 0.1\%$ to a maximum of $3.6 \pm 0.25\%$ at a dose of 20 $\mu g\ g^{-1}$ E2 (Table 3.25. & Fig. 3.27.). There was a significant correlation ($P < 0.01$) between the HSI and dose of E2 (Table 3.25.).

Although the GSI in treated groups was not significantly different from control values of $0.125 \pm 0.008\%$, there was a significant ($P < 0.05$) correlation between GSI and dose of E2 (Table 3.25. & Fig. 3.29.). A maximum mean GSI of $0.149 \pm 0.007\%$ was observed in

the group treated with 20 $\mu g\ g^{-1}$ and a minimum value of 0.116 $\pm$ 0.009, 0.116 $\pm$ 0.005% in the groups treated with 0.01 and 1.0 $\mu g\ g^{-1}$ respectively.

There was a marked reduction in growth in treated groups from control values of 25.23 $\pm$ 2.97 g 2 $month^{-1}$ to -6.4 $\pm$ 1.33 g 2 $month^{-1}$ ($P < 0.001$). See Table 3.25. & Fig. 3.30.. There was a significant ($P < 0.001$) negative correlation between dose of E2 and growth.

DOSE ($\mu g\ g^{-1}$)	DAYS AFTER INJECTION 0	2	5	11
CONTROL	0.5	1.2	0.9	1.0
± S.E.	0.3	0.4	0.5	0.2
0.01	0.5	4.1	2.5	1.2
± S.E.	0.1	1.5	1.25	0.5
0.1	0.5	6.3	3.0*	0.9
± S.E.	0.4	3.4	0.8	0.7
0.5	0.4	15.6*	3.5	1.7*
± S.E.	0.2	5.5	1.7	0.2
1.0	0.9	32.7**	5.0*	1.5
± S.E.	0.3	9.45	1.2	0.4
5.0	0.75	62.5***	12.5	2.1
± S.E.	0.4	10.5	5.5	0.5
10.0	0.5	84.7***	20.0***	3.5
± S.E.	0.1	8.0	4.0	2.0
20.0	0.5	100.0	40.0***	10.5***
± S.E.	0.2	-	7.5	1.5
40.0	0.65	100.0	35.0**	12.25*
± S.E.	0.3	-	9.5	3.4

* ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$) n = 5

TABLE 3.23. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 $ng\ ml^{-1}$) FOLLOWING THE INJECTION OF SIMILAR GROUPS OF FISH WITH DIFFERENT DOSES OF E2 IN OCTOBER.

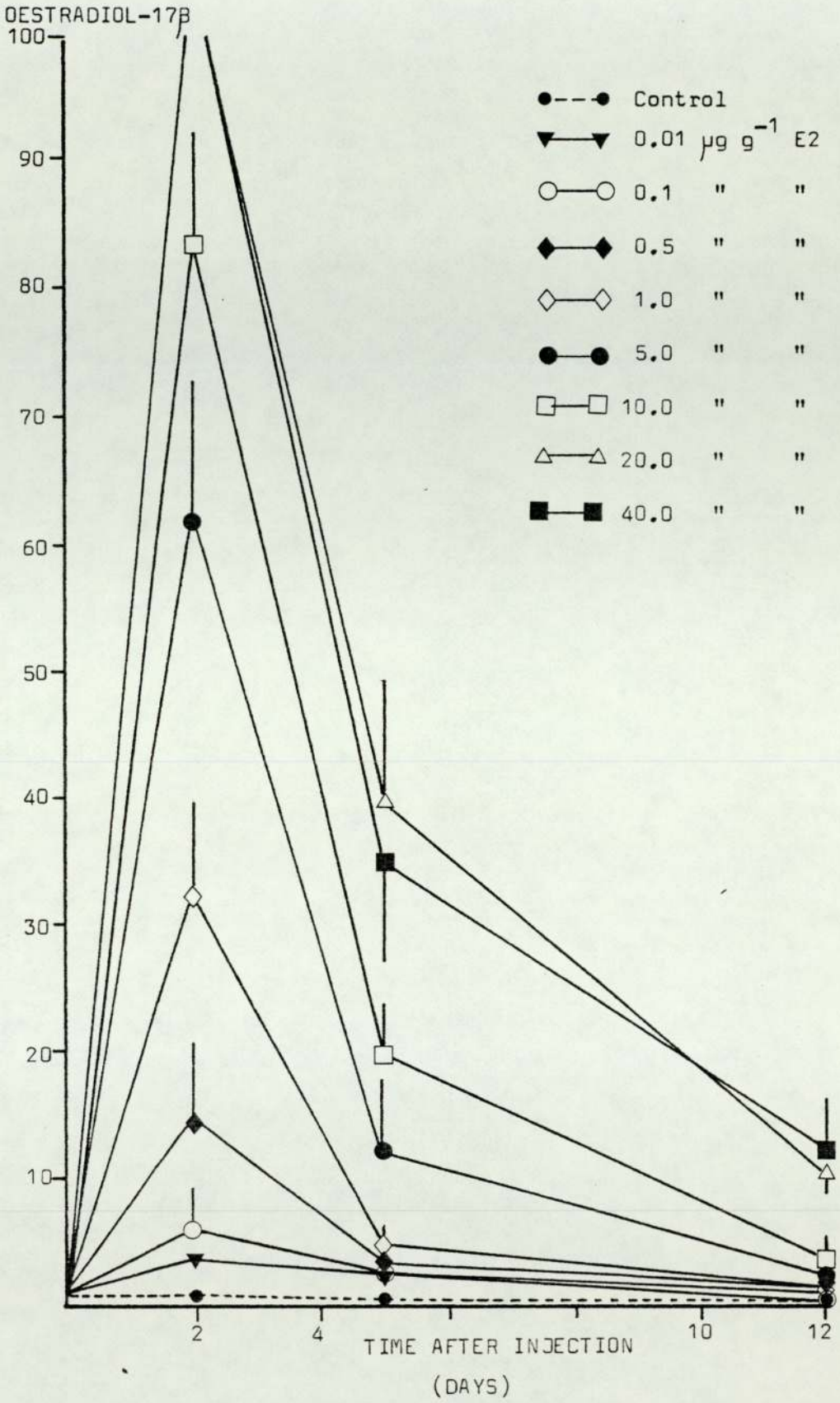


FIG. 3.25. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) FOLLOWING INJECTION OF SIMILAR GROUPS OF FISH WITH DIFFERENT DOSES OF OESTRADIOL-17β (E2 μg g^{-1}) IN OCTOBER.

DOSE ($\mu g\ g^{-1}$)	TIME (DAYS) 0	2	5	11	14	21	39	56
CONTROL	10.25	10.37	10.56	10.24	10.89	11.14	10.65	10.64
± S.E.	0.5	0.7	0.9	0.9	0.5	0.35	0.3	0.4
0.01	11.0	10.65	10.9	11.5	11.8	12.4*	12.0*	11.5
± S.E.	0.8	0.5	0.4	0.7	0.24	0.2	0.4	0.4
0.1	10.2	10.2	11.0	12.5*	13.0**	15.0***	14.01***	12.22
± S.E.	0.4	0.2	0.2	0.2	0.3	0.4	0.6	0.6
0.5	9.8	9.7	12.0	15.0**	16.5***	20.0***	18.11**	13.9
± S.E.	0.6	0.2	0.3	0.4	0.4	1.0	2.1	1.7
1.0	9.97	9.7	12.2	18.1***	21.1***	17.5***	24.98***	24.0***
± S.E.	0.7	0.2	0.4	0.8	1.3	1.8	1.5	2.1

TABLE 3.24. CHANGES IN MEAN SERUM LEVELS OF CALCIUM (TCa mg $100ml^{-1}$) IN FISH INJECTED WITH DIFFERENT DOSES OF OESTRADIOL-17β ($\mu g\ g^{-1}$) IN OCTOBER.

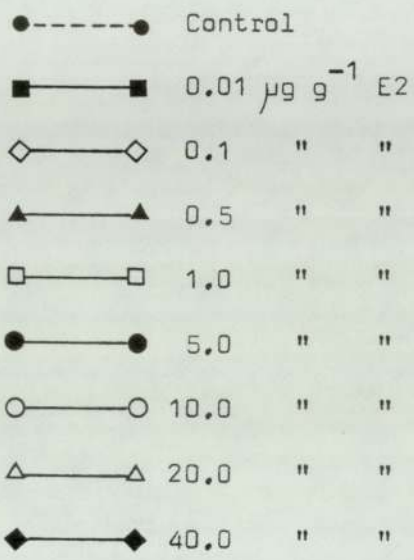
Continued .....

DOSE ($\mu g\ g^{-1}$)	0	2	5	11	14	21	39	56
5.0	10.24	9.97	13.03*	28.0*	35.6*	52.8*	48.1**	46.1**
± S.E.	0.4	0.4	0.9	4.7	9.7	10.4	8.4	11.3
10.0	10.01	9.98	14.0*	42.23***	57.5***	90.0***	82.7***	80.6***
± S.E.	0.3	0.6	0.6	5.0	2.5	3.7	4.5	7.0
20.0	10.8	10.2	14.4**	48.0***	64.1***	102.0***	97.0***	90.0***
± S.E.	0.8	0.8	0.7	3.0	5.5	14.5	9.0	4.5
40.0	10.2	10.0	14.0	46.0***	62.0***	99.0***	86.5***	82.5***
± S.E.	0.2	0.4	2.1	3.8	5.1	9.8	7.6	4.4

* ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.01$) n = 5

TABLE 3.24. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) IN FISH INJECTED WITH DIFFERENT DOSES OF OESTRADIOL-17β (E2 $\mu g\ g^{-1}$) IN OCTOBER.

LEGEND TO FIG. 3.26.



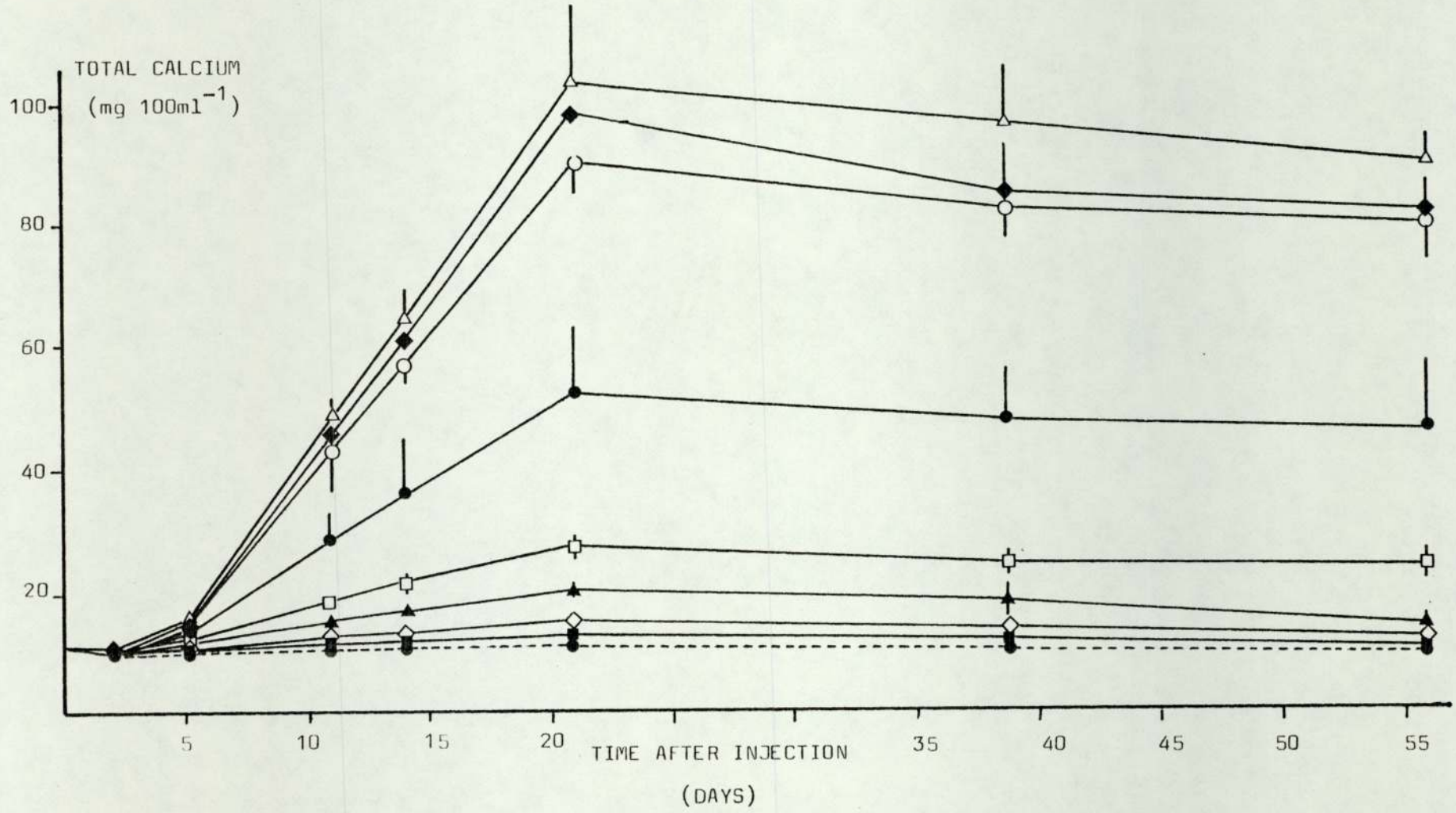


FIG. 3.26. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml^{-1}) IN FISH INJECTED WITH DIFFERENT DOSES OF OESTRADIOL-17β (E2 μg g^{-1}) IN OCTOBER.

TABLE 3.25. CORRELATION OF PEAK MEAN SERUM CALCIUM (TCa mg $100ml^{-1}$), HEPATOSOMATIC INDEX (HSI %), GONADOSOMATIC INDEX (GSI %) AND GROWTH (g $2month^{-1}$) WITH DOSE OF OESTRADIOL-17β (E2 $\mu g\ g^{-1}$) INJECTED IN OCTOBER.

DOSE	Peak Ca^{++}	HSI (n)	GSI (n)	Growth
CONTROL	11.14 ± 0.35	1.8 ± 0.1 (5)	0.125 ± 0.008 (3)	25.23 ± 2.97
0.01	12.4 ± 0.2*	1.85 ± 0.12(5)	0.116 ± 0.009 (1)	24.37 ± 3.72
0.1	15.0 ± 0.4***	2.45 ± 0.15(4)**	0.119 ± 0.006 (3)	18.4 ± 0.9
0.5	20.0 ± 1.0***	2.6 ± 0.08(5)***	0.13 ± 0.002 (2)	18.4 ± 0.9
1.0	27.5 ± 1.8***	2.85 ± 0.1 (5)***	0.116 ± 0.005 (2)	15.2 ± 1.4*
5.0	52.8 ±10.4**	2.95 ± 0.17(5)***	0.135 ± 0.008 (2)	15.0 ± 1.2***
10.0	90.0 ± 5.7***	3.1 ± 0.15(4)***	0.14 ± 0.009 (3)	2.47 ± 0.4***
20.0	102.0 ±14.5***	3.6 ± 0.25(5)	0.149 ± 0.007 (1)	-2.457± 1.28***
40.0	99.0 ± 9.8***	3.45 ± 0.3 (5)***	0.139 ± 0.01 (2)	-6.4 ± 1.33***
r.	0.95***	0.74**	0.68*	-0.85***

* ($p < 0.05$) ** ($p < 0.01$) *** ($p < 0.001$) r. = Correlation coefficient

TABLE. 3.25 LEGEND OPPOSITE.

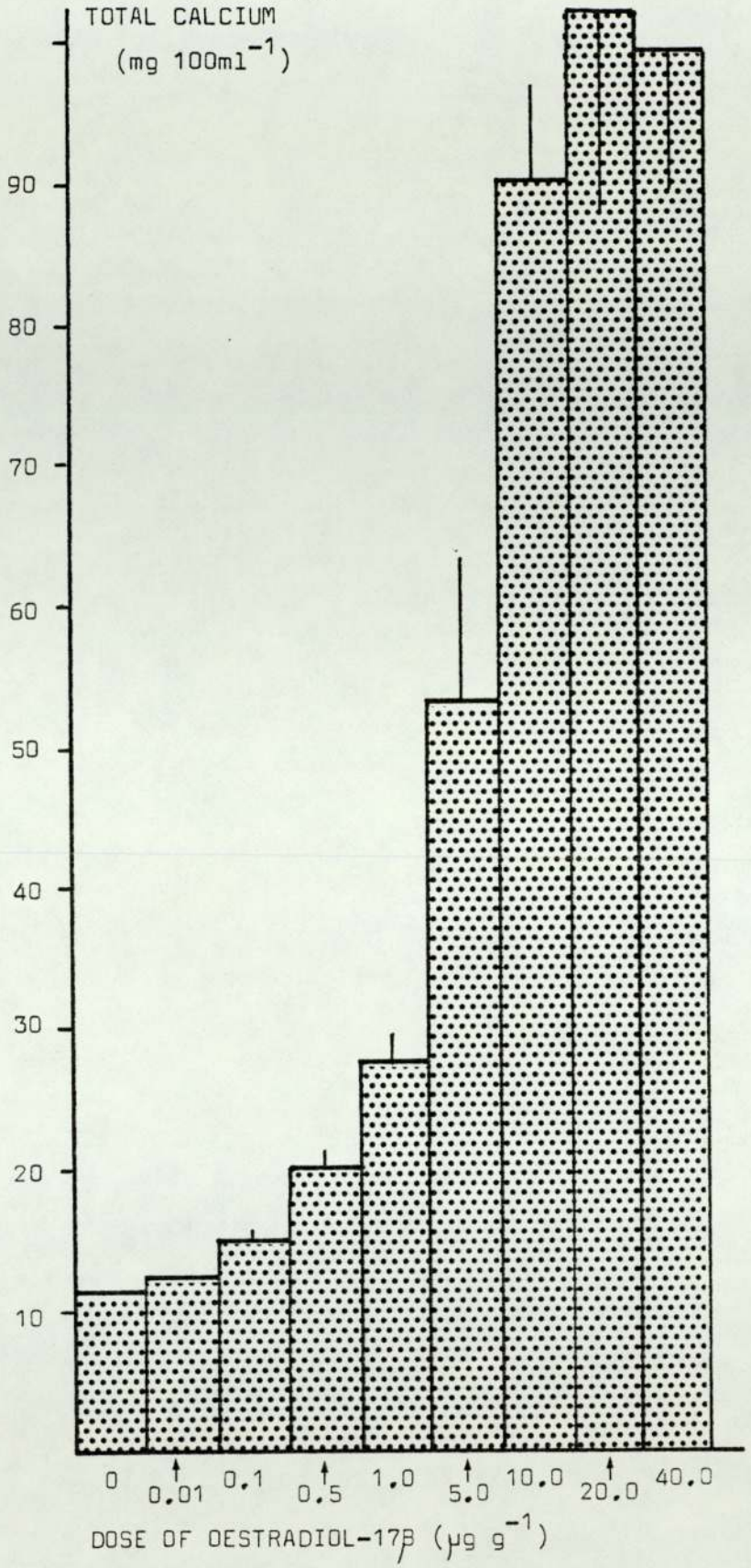


FIG. 3.27. PEAK MEAN SERUM CALCIUM LEVELS (TCa. mg.100ml.$^{-1}$) IN GROUPS OF FISH INJECTED WITH DIFFERENT DOSES OF OESTRADIOL-17β (E2. μg.g.$^{-1}$ body wt.) IN OCTOBER.

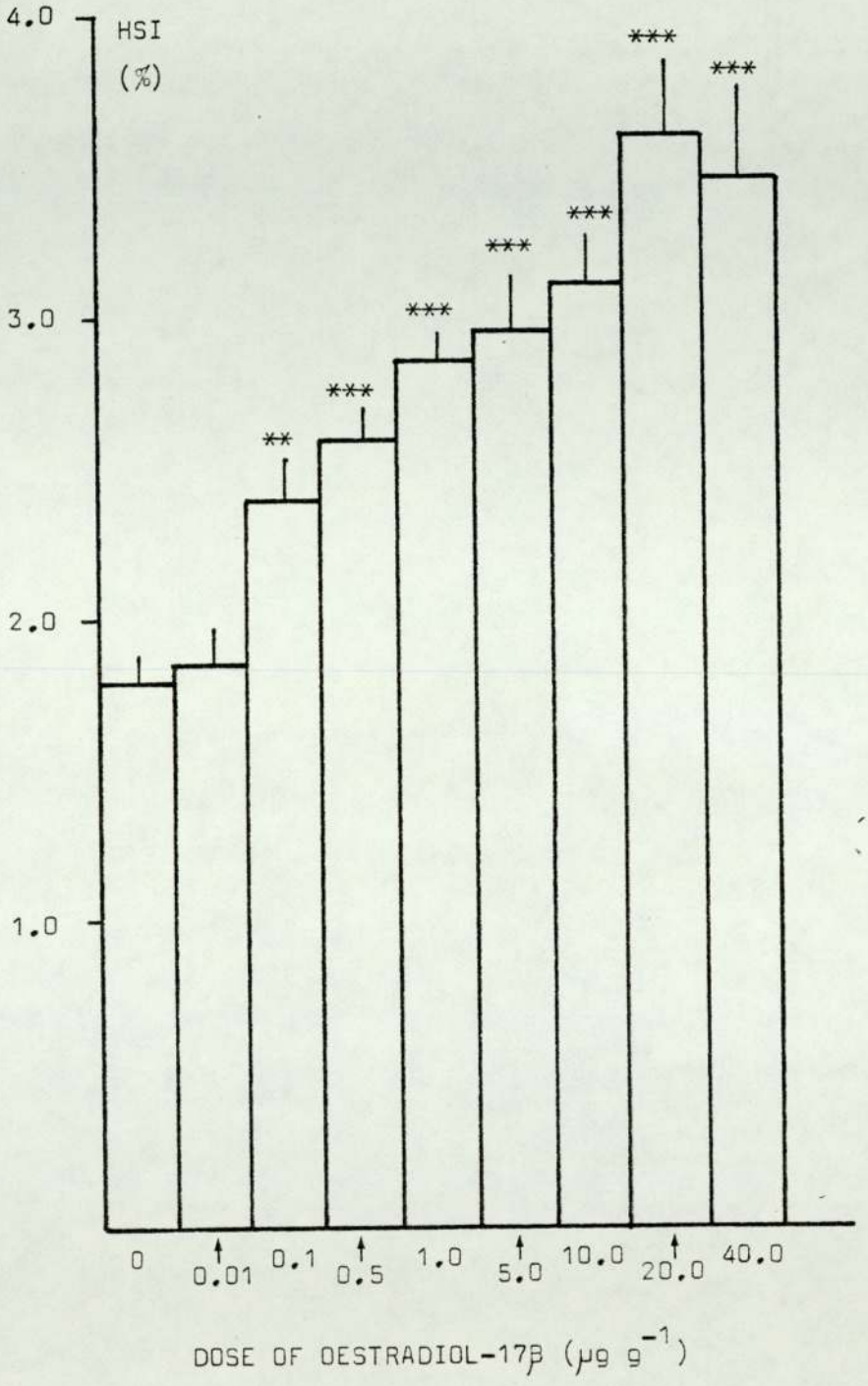


FIG. 3.28. MEAN HEPATOSOMATIC INDEX (HSI %) IN FISH 56 DAYS AFTER INJECTION WITH DIFFERENT DOSES OF OESTRADIOL-17β (E2 $\mu g\ g^{-1}$) IN OCTOBER.

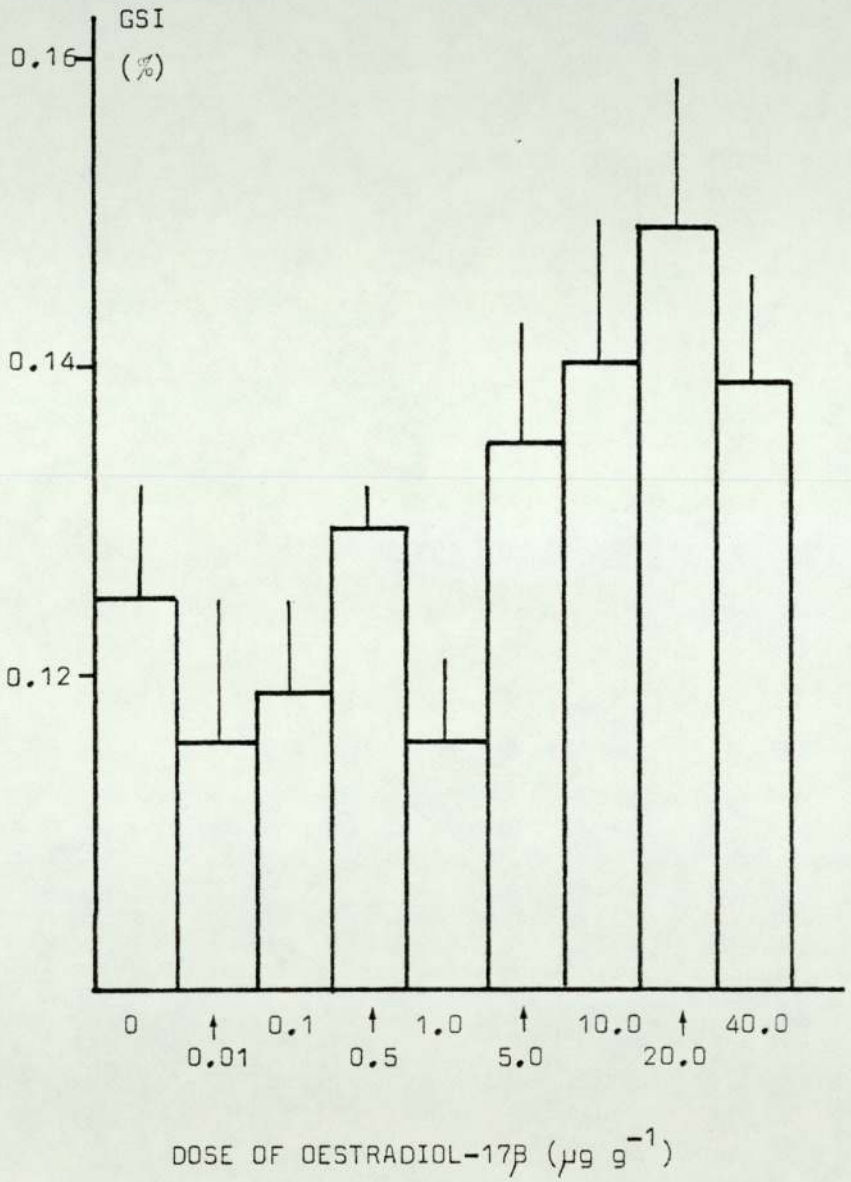


FIG. 3.29. MEAN GONADOSOMATIC INDEX (GSI %) IN FISH 56 DAYS AFTER INJECTION WITH DIFFERENT DOSES OF OESTRADIOL-17β (E2 μg g^{-1}) IN OCTOBER.

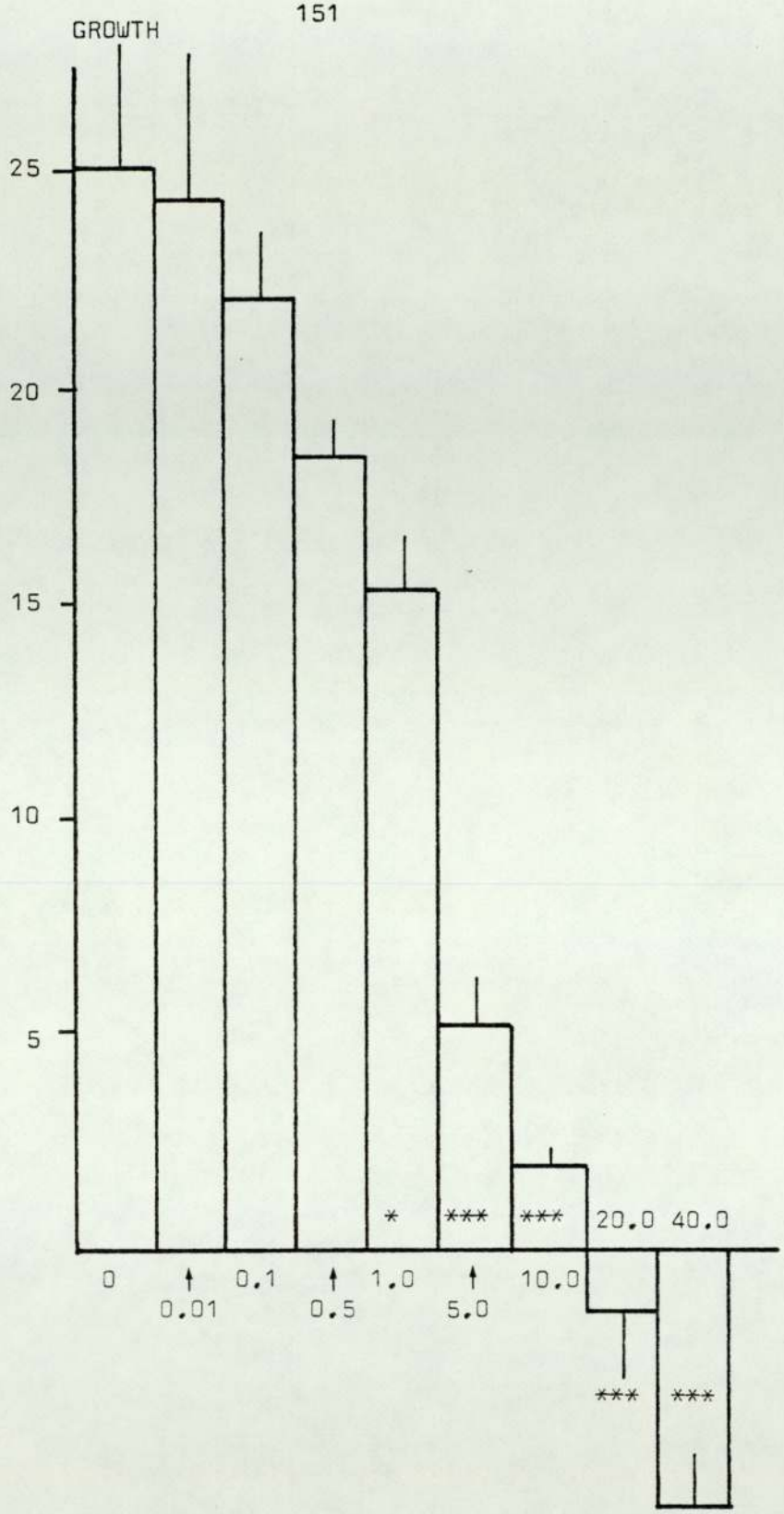


FIG. 3.30. MEAN GROWTH (g 2month^{-1}) IN FISH INJECTED WITH DIFFERENT DOSES OF OESTRADIOL-17β (E2 μg g^{-1}) IN OCTOBER.

Changes in mean serum levels of E2 and TCa after injection of different doses of E2 in January : Gross changes in serum E2 and TCa were observed in treated groups. Peak mean levels of serum E2 were observed 2 days after injection (Table 3.26. & Fig. 3.31.). In all but the 2 highest dose treatments, levels of serum E2 had returned to near control values 11 days after injection. Mean levels of E2 rose in control fish from basal values of 0.5 $\pm$ 0.25 ng ml^{-1} to a peak of 1.75 $\pm$ 0.5 ng ml^{-1} after 2 days, before falling to 0.8 $\pm$ 0.45 ng ml^{-1} after 11 days. Maximum mean E2 levels ($>$ 100.0 ng ml^{-1}) were observed in the group treated at 40 $\mu g\ g^{-1}$, and E2 levels in this group were still significantly ($P < 0.05$) raised above control levels of 0.8 $\pm$ 0.45 at 15.0 $\pm$ 6.4 ng ml^{-1} 11 days after injection.

Not all treated groups demonstrated significant increases in TCa during the course of the experiment (Table 3.27. & Fig. 3.32.). Mean serum levels of TCa were significantly raised above controls in those fish treated at 1.0, 5, 10, 20 and 40 $\mu g\ g^{-1}$. There was a significant correlation ($P < 0.001$) of peak mean TCa with injected dose (Table 3.28. & Fig. 3.33.). There was no significant changes in control fish. Peak mean serum TCa values were recorded 39 days after injection.

The hepatosomatic index in those groups treated with E2 at 1.0, 5, 10, 20 and 40 $\mu g\ g^{-1}$ was significantly ($P < 0.05$ & $P < 0.001$) above the control and index of 1.4 $\pm$ 0.12%, to a maximum of 3.1 $\pm$ 0.13% (20 $\mu g\ g^{-1}$) (Table 3.28. & Fig. 3.34.). The hepatosomatic index was significantly ($P < 0.05$) correlated with the injected dose of E2 (Table 3.28.).

In the 10 and 20 $\mu g\ g^{-1}$ treatment groups, the gonadosomatic index was significantly ($P < 0.05$ & $P < 0.001$) above the control index of $0.116 \pm 0.008\%$ at 0.14 ± 0.006 and $0.18 \pm 0.002\%$ respectively (Table 3.28. & Fig. 3.35.). There was a significant ($P < 0.001$) correlation between the dose of injected E2 and the GSI (Table 3.28.). A minimum GSI of $0.112 \pm 0.009\%$ was observed in the group treated with 1.0 $\mu g\ g^{-1}$ E2.

The mean growth of the fish over the course of the experiment was negatively correlated ($P < 0.05$) with the injected dose, with a mean control rate of 18.15 ± 3.16 g 2 $month^{-1}$ compared with the 40.0 $\mu g\ g^{-1}$ E2 treatment rate of -2.5 ± 5.3 g 2 $month^{-1}$ (Table 3.28. & Fig. 3.36.).

DOSE	0	2	5	8	11
CONTROL	0.5	1.75	1.0	0.9	0.8
± S.E.	0.25	0.5	0.4	0.3	0.45
0.01	0.4	5.8	3.1	1.5	1.5
± S.E.	0.2	2.4	1.2	0.5	1.4
0.1	0.4	7.75**	4.0	2.0	1.7
± S.E.	0.3	1.5	1.5	0.4	0.7
0.5	0.4	11.5**	3.0	2.7	2.25
± S.E.	0.15	2.9	1.7	0.5	1.5
1.0	0.35	25.0**	6.25*	3.5	2.4*
± S.E.	0.25	6.4	2.1	0.9	0.5
5.0	0.2	74.33***	15.0**	7.7*	3.5
± S.E.	0.1	10.0	3.5	2.35	1.7
10.0	0.7	82.0***	22.4**	11.25*	3.17*
± S.E.	0.4	7.5	6.5	4.0	1.0
20.0	0.5	99.8***	28.7***	14.26*	9.75*
± S.E.	0.3	20.0	4.5	5.25	3.6
40.0	0.5	100.0	33.3***	22.25***	15.0*
± S.E.	0.25	-	7.2	4.5	6.4

* ($p < 0.05$) ** ($p < 0.01$) *** ($p < 0.001$) n = 8

TABLE 3.26. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml $^{-1}$) FOLLOWING THE INJECTION OF SIMILAR GROUPS OF FISH WITH DIFFERENT DOSES OF E2 (μg g $^{-1}$) IN JANUARY.

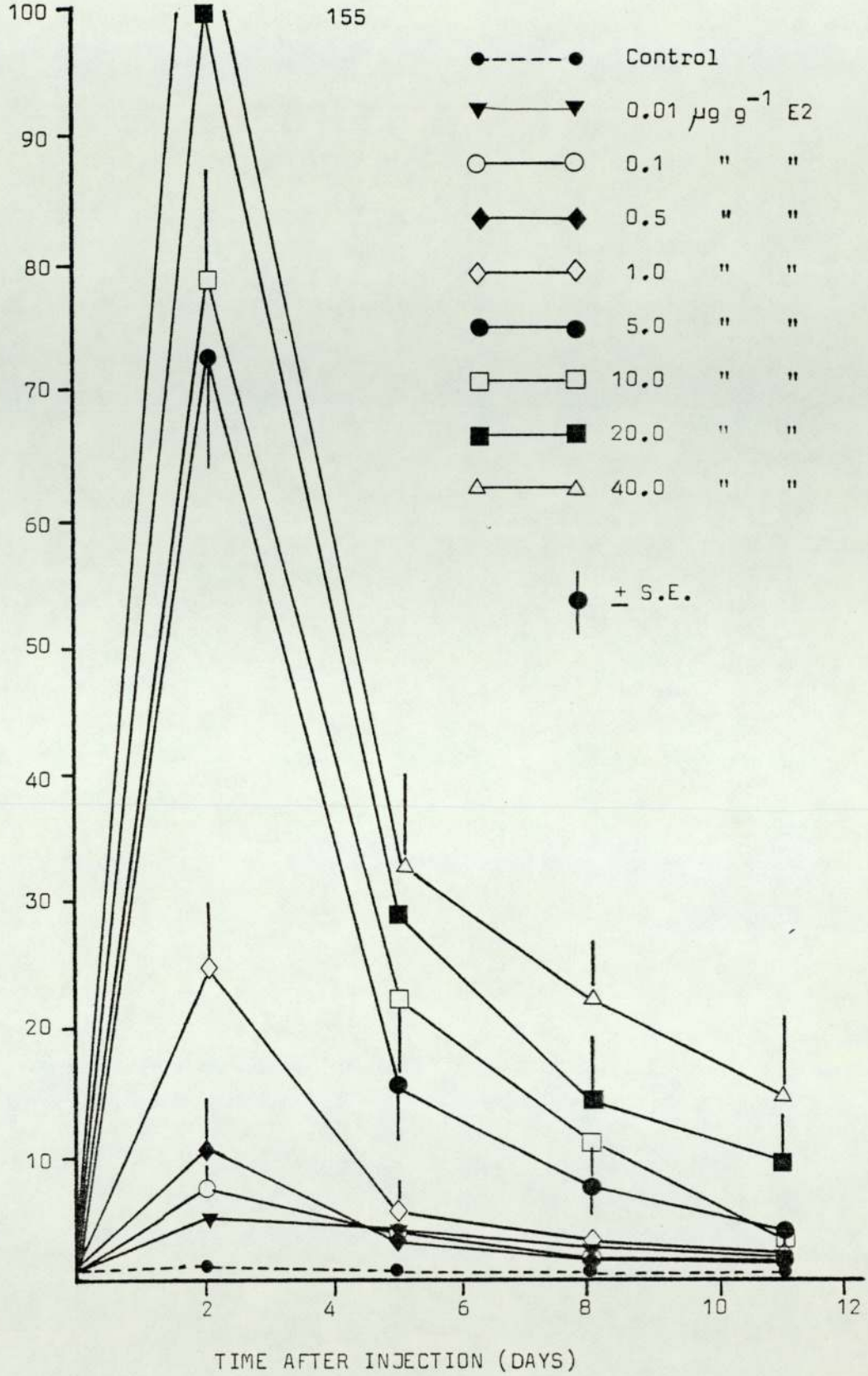


FIG. 3.31. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) FOLLOWING THE INJECTION OF SIMILAR GROUPS OF FISH WITH DIFFERENT DOSES OF E2 (µg g^{-1}) IN JANUARY.

DOSE ($\mu g\ g^{-1}$)	0	2	5	8	11	25	39	55
CONTROL	10.57	8.8	9.8	10.1	10.6	10.9	10.7	10.7
± S.E.	0.4	0.6	0.5	0.3	0.4	0.3	0.3	0.8
0.01	10.4	10.2	10.0	10.15	10.37	10.25	10.4	10.2
± S.E.	0.2	0.3	0.3	0.5	0.3	0.2	0.4	0.7
0.1	10.2	9.9	9.8	9.9	10.1	10.0	10.2	10.4
± S.E.	0.3	0.2	0.2	0.1	0.3	0.4	0.5	0.5
0.5	10.64	10.0	10.3	10.5	10.7	10.9	10.7	10.6
± S.E.	0.4	0.6	0.3	0.5	0.4	0.7	0.2	0.3
1.0	10.14	10.5	10.1	11.7**	12.1**	12.3**	12.2*	11.6
± S.E.	0.6	0.7	0.3	0.3	0.3	0.2	0.6	0.7

TABLE 3.27. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml $^{-1}$) FOLLOWING THE INJECTION OF SIMILAR GROUPS OF FISH WITH DIFFERENT DOSES OF OESTRADIOL-17β (E2 $\mu g\ g^{-1}$ body wt.) IN JANUARY.

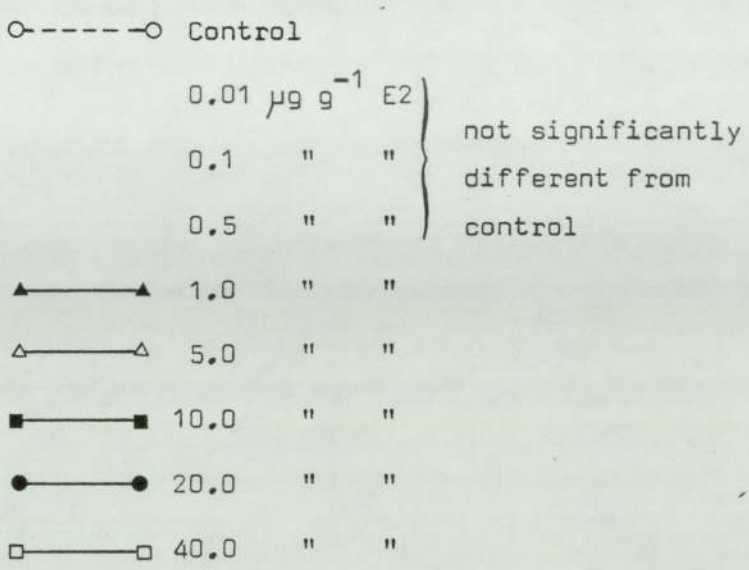
CONTINUED.....

DOSE ($\mu g\ g^{-1}$)	0	2	5	8	11	25	39	55
5.0	10.8	10.4	11.0	12.4***	14.2***	15.9***	13.8	11.0
± S.E.	0.8	0.6	0.2	0.3	0.4	0.9	1.5	0.4
10.0	10.1	10.0	10.4	12.6**	16.1***	28.9**	38.4***	21.7
± S.E.	0.1	0.6	0.3	0.6	0.6	5.5	5.8	5.5
20.0	10.3	10.2	10.6	13.1***	16.6***	51.8***	81.2***	69.9***
± S.E.	0.4	0.3	0.2	0.4	0.5	3.5	9.5	10.0
40.0	10.01	10.1	10.2	13.2**	16.6***	49.4***	74.4***	58.8***
± S.E.	0.3	0.3	0.2	0.8	0.9	4.6	2.9	7.5

* ($p < 0.05$) ** ($p < 0.01$) *** ($p < 0.001$) n = 8

TABLE 3.27. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml $^{-1}$) FOLLOWING INJECTION OF SIMILAR GROUPS OF FISH WITH DIFFERENT DOSES OF OESTRADIOL-17β (E2 $\mu g\ g^{-1}$ body wt.) IN JANUARY.

FIG. 3.32. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) FOLLOWING THE INJECTION OF SIMILAR GROUPS OF FISH WITH DIFFERENT DOSES OF OESTRADIOL-17β (E2 $\mu g\ g^{-1}$) IN JANUARY.



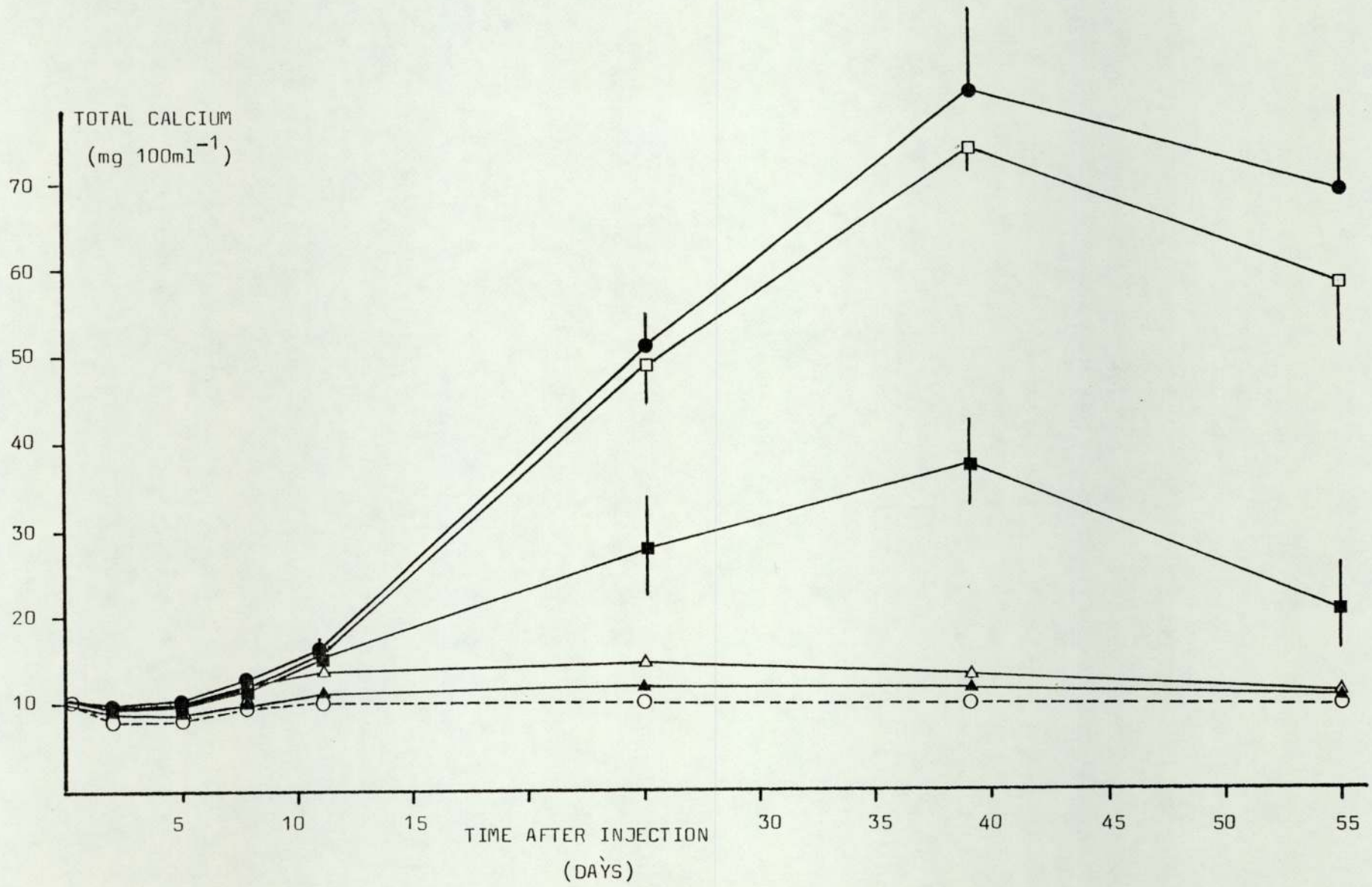


FIG. 3.32. LEGEND OPPOSITE.

TABLE 3.28. CORRELATION OF PEAK MEAN SERUM CALCIUM (TCa mg $100ml^{-1}$), HEPATOSOMATIC INDEX (HSI %), GONADOSOMATIC INDEX (GSI %) AND GROWTH (g $2month^{-1}$) WITH DOSE OF OESTRADIOL-17β (E2 $\mu g\ g^{-1}$) INJECTED INJANUARY .

DOSE	Peak Calcium	HSI (n)	GSI (n)	GROWTH
CONTROL	10.9 ± 0.3	1.4 ± 0.12 (8)^{*1}	0.116 ± 0.008 (3)	18.15 ± 3.16
0.01	10.4 ± 0.4^{**1}	-	-	18.75 ± 1.99
0.1	10.4 ± 0.5^{***1}	-	-	16.0 ± 1.7^{*1}
0.5	10.9 ± 0.7^{***1}	1.6 ± 0.09 (6)^{***1}	0.114 ± 0.007 (4)	17.02 ± 0.5
1.0	12.3 ± 0.2$^{**}_{***1}$	1.8 ± 0.1 (7)$^{*}_{***1}$	0.112 ± 0.009 (4)	10.09 ± 1.28^{*1}
5.0	15.9 ± 0.9$^{***}_{**1}$	2.05 ± 0.09 (7)$^{***}_{***1}$	0.13 ± 0.006 (2)	4.5 ± 2.4**
10.0	38.4 ± 5.8$^{***}_{***1}$	2.6 ± 0.06 (6)$^{***}_{*1}$	0.14 ± 0.006 (4)*	5.0 ± 1.88**
20.0	81.16 ± 9.5***	3.1 ± 0.13 (8)***	0.18 ± 0.001 (3)$^{***}_{***1}$	-1.5 ± 1.27***
40.0	74.4 ± 2.9$^{***}_{*1}$	2.9 ± 0.17 (6)***	all ♂	-2.5 ± 5.3***
r.	0.9***	0.8*	0.99***	-0.69*

* ($p < 0.05$) ** ($p < 0.01$) *** ($p < 0.001$) *1 versus corresponding October value

TABLE 3.28. LEGEND OPPOSITE.

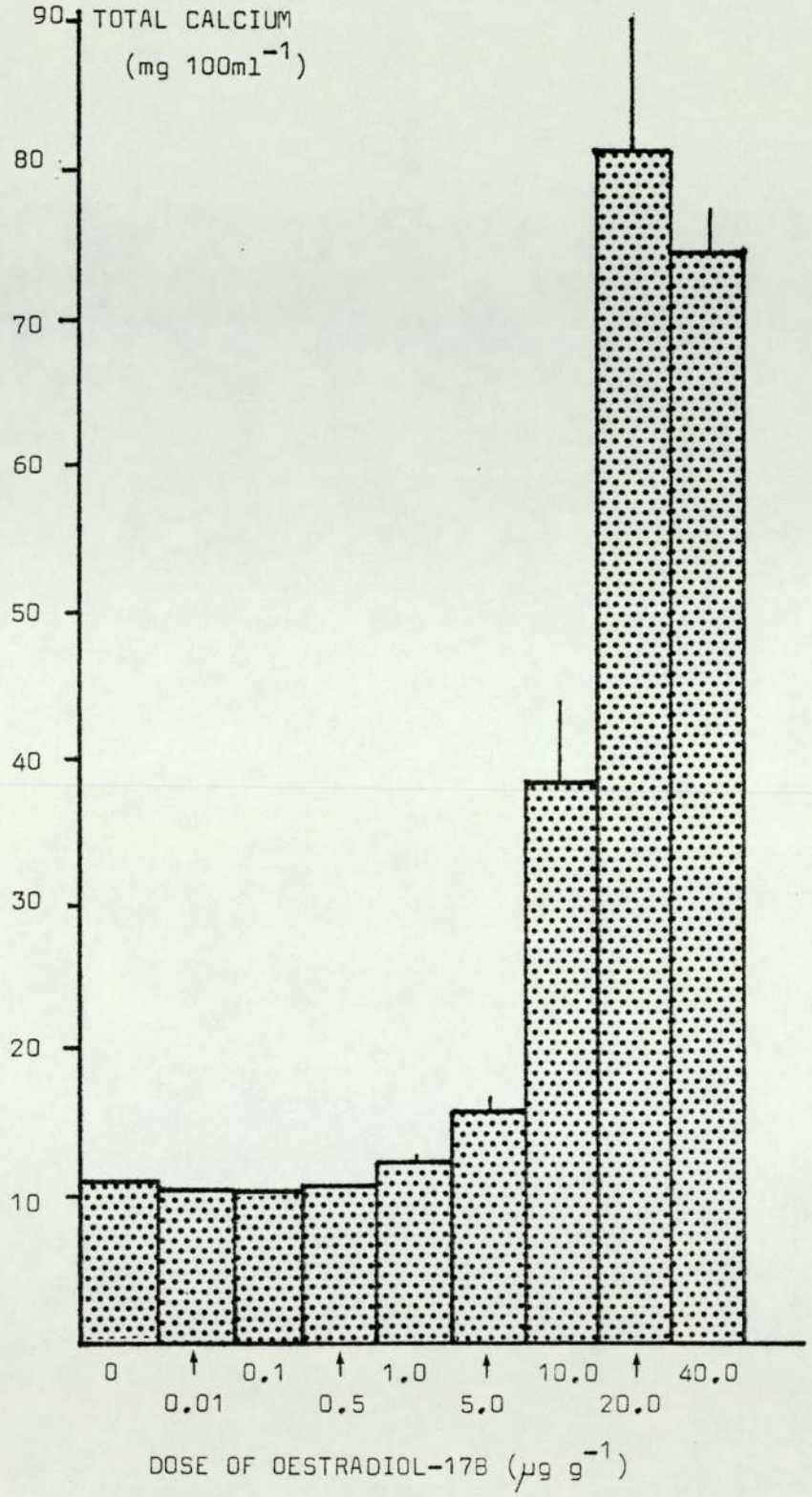


FIG. 3.33. PEAK MEAN SERUM CALCIUM LEVELS (TCa. mg.100ml.$^{-1}$) IN GROUPS OF FISH INJECTED WITH DIFFERENT DOSES OF OESTRADIOL-17β (E2. µg.g.$^{-1}$ body wt.) IN JANUARY.

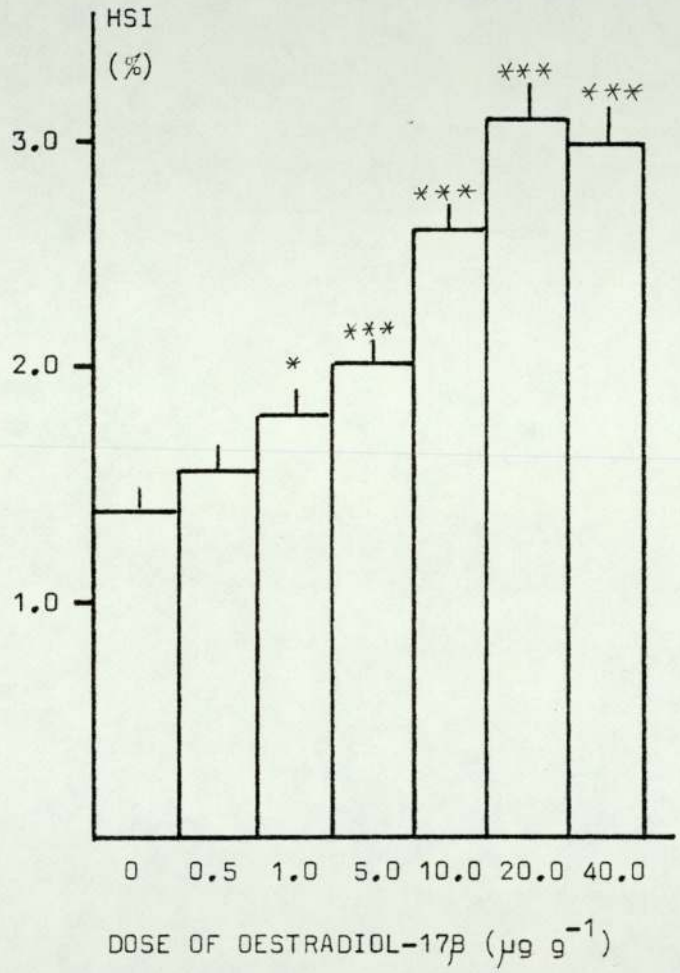


FIG. 3.34. THE MEAN HEPATOSOMATIC INDEX (HSI %) IN FISH 55 DAYS AFTER INJECTION WITH DIFFERENT DOSES OF OESTRADIOL-17β (E2 μg g^{-1}) IN JANUARY.

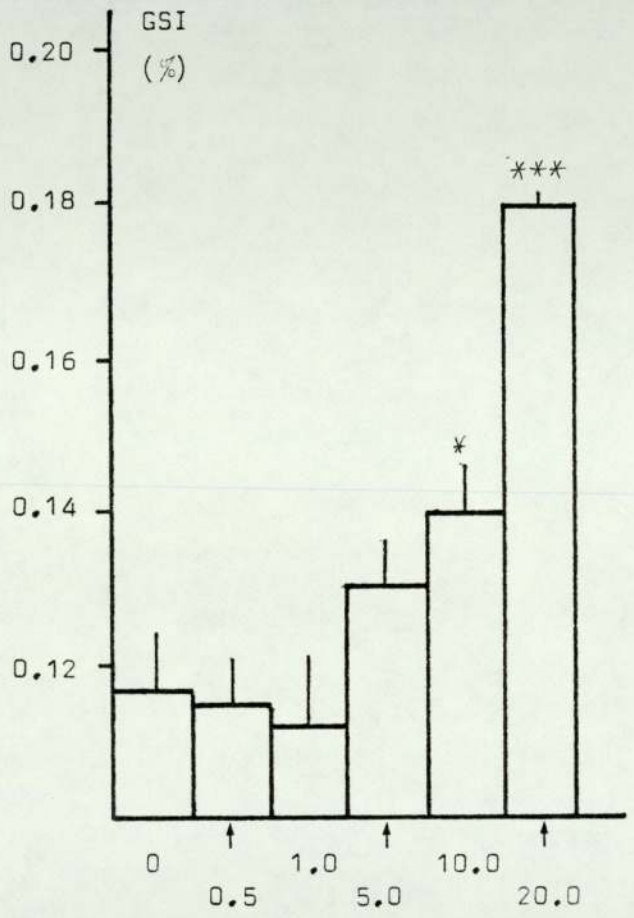


FIG. 3.35. THE MEAN GONADOSOMATIC INDEX (GSI %) IN FISH 55 DAYS AFTER INJECTION WITH DIFFERENT DOSES OF OESTRADIOL-17β (E2 μg g^{-1}) IN JANUARY.

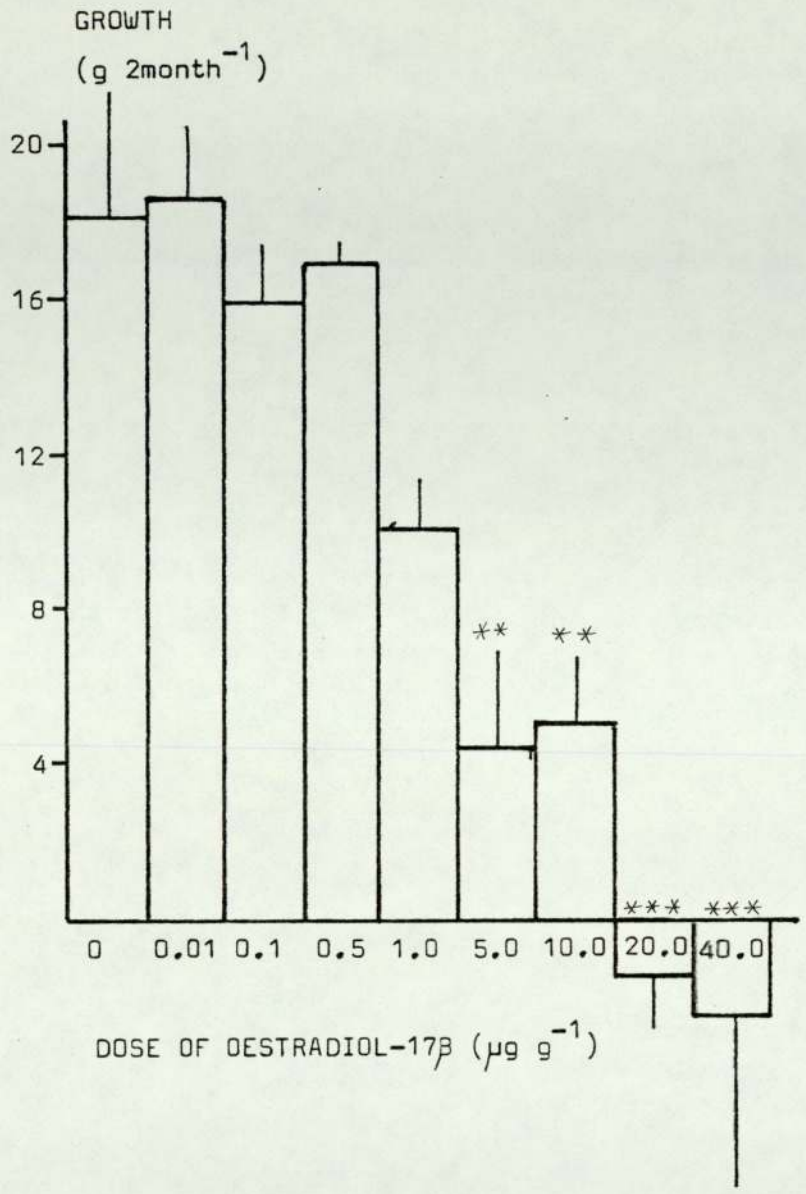


FIG. 3.36. MEAN GROWTH RATE (g 2month^{-1}) IN FISH INJECTED WITH DIFFERENT DOSES OF OESTRADIOL-17β (E2 μg g^{-1}) IN JANUARY.

Statistical Analysis.

The student t-test was used to test the significance of the differences in serum levels of E2, TCa, HSI, GSI and growth in treated and control fish at the different doses used, in both October and January experiments. The correlation coefficient r was calculated by the method of least squares to test the relationship of the dose of E2 with peak mean TCa, HSI, GSI and growth.

Comparison of Dose Responses observed in October and January.

There was no significant difference between the control levels of TCa in the October and January injected fish. Similarly, there was no significant difference between peak mean TCa observed in October and January at the dose of 20.0 $\mu g\ g^{-1}$, with a maximum response in both cases (Fig. 3.37.). However, the peak mean TCa levels observed in January were significantly lower for doses of 0.01 ($P < 0.01$), 0.1 ($P < 0.001$), 0.5 ($P < 0.001$), 1.0 ($P < 0.001$), 5.0 ($P < 0.01$), 10.0 ($P < 0.001$) and 40.0 ($P < 0.05$) $\mu g\ g^{-1}$ than the respective October groups.

The HSI in control fish was significantly ($P < 0.05$) lower in the January experiment than in the control fish at the end of October's experiment (Fig. 3.38.). The HSI in those groups treated at 0.5, 1.0, 5.0 and 10.0 were also significantly ($P < 0.05$ & $P < 0.001$) lower in January than the corresponding treated groups in October (Fig. 3.38.).

There was no significant difference in the GSI between like-treated groups in the 2 experiments except in the 20.0 $\mu g\ g^{-1}$ dose group. The GSI for the group injected in January at 20.0 $\mu g\ g^{-1}$ was significantly ($P < 0.001$) higher than the corresponding October value (Fig. 3.39.).

Mean growth of all groups (except 20.0 and 40.0) was higher in October's treatments, and significantly higher at doses of 0.1 ($P < 0.05$) and 1.0 $\mu g\ g^{-1}$ ($P < 0.05$) (Fig. 3.40.). The mean growth of 20.0 and 40.0 $\mu g\ g^{-1}$ treated groups was in real terms less in October than January, since negative values were recorded.

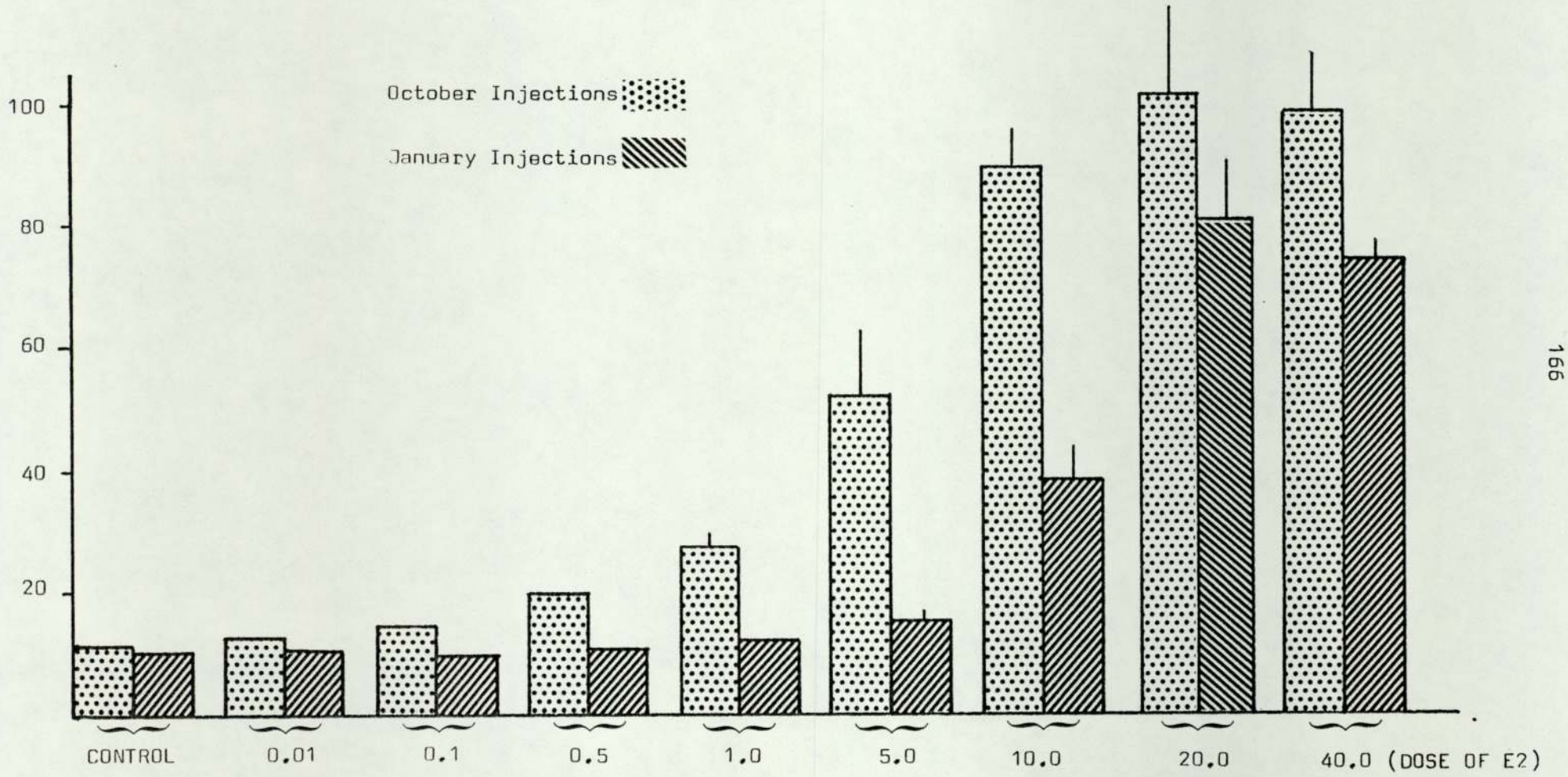


FIG. 3.37. A COMPARISON OF PEAK MEAN SERUM CALCIUM LEVELS (TCa mg $100ml^{-1}$) OBSERVED IN GROUPS OF FISH INJECTED WITH DIFFERENT DOSES OF OESTRADIOL-17β (E2 $\mu g\ g^{-1}$) IN EITHER JANUARY OR OCTOBER.

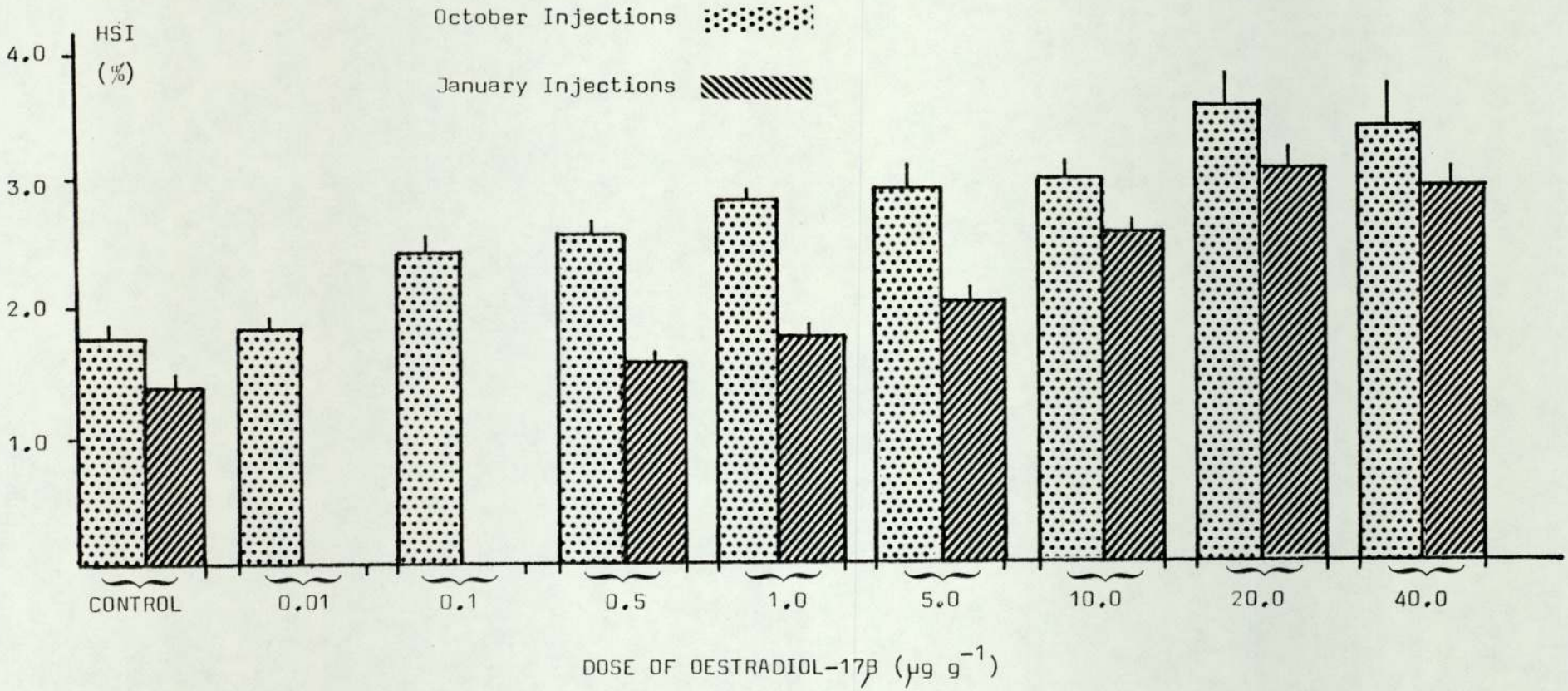


FIG. 3.38. A COMPARISON OF THE EFFECTS OF DIFFERENT DOSES OF OESTRADIOL-17β (E2 μg g^{-1}) ON THE MEAN HEPATOSOMATIC INDEX (HSI %) IN GROUPS OF FISH INJECTED IN EITHER JANUARY OR OCTOBER.

FIG. 3.39. A COMPARISON OF THE EFFECTS OF DIFFERENT DOSES OF OESTRADIOL-17β (E2 μg g^{-1}) ON THE MEAN GONADOSOMATIC INDEX (GSI %) IN GROUPS OF FISH INJECTED IN EITHER OCTOBER OR JANUARY.

FIG. 3.40. A COMPARISON OF THE EFFECTS OF DIFFERENT DOSES OF OESTRADIOL-17β (E2 $\mu g\ g^{-1}$) ON THE MEAN GROWTH RATES ($g\ 2month^{-1}$) IN GROUPS OF FISH INJECTED IN EITHER OCTOBER OR JANUARY.

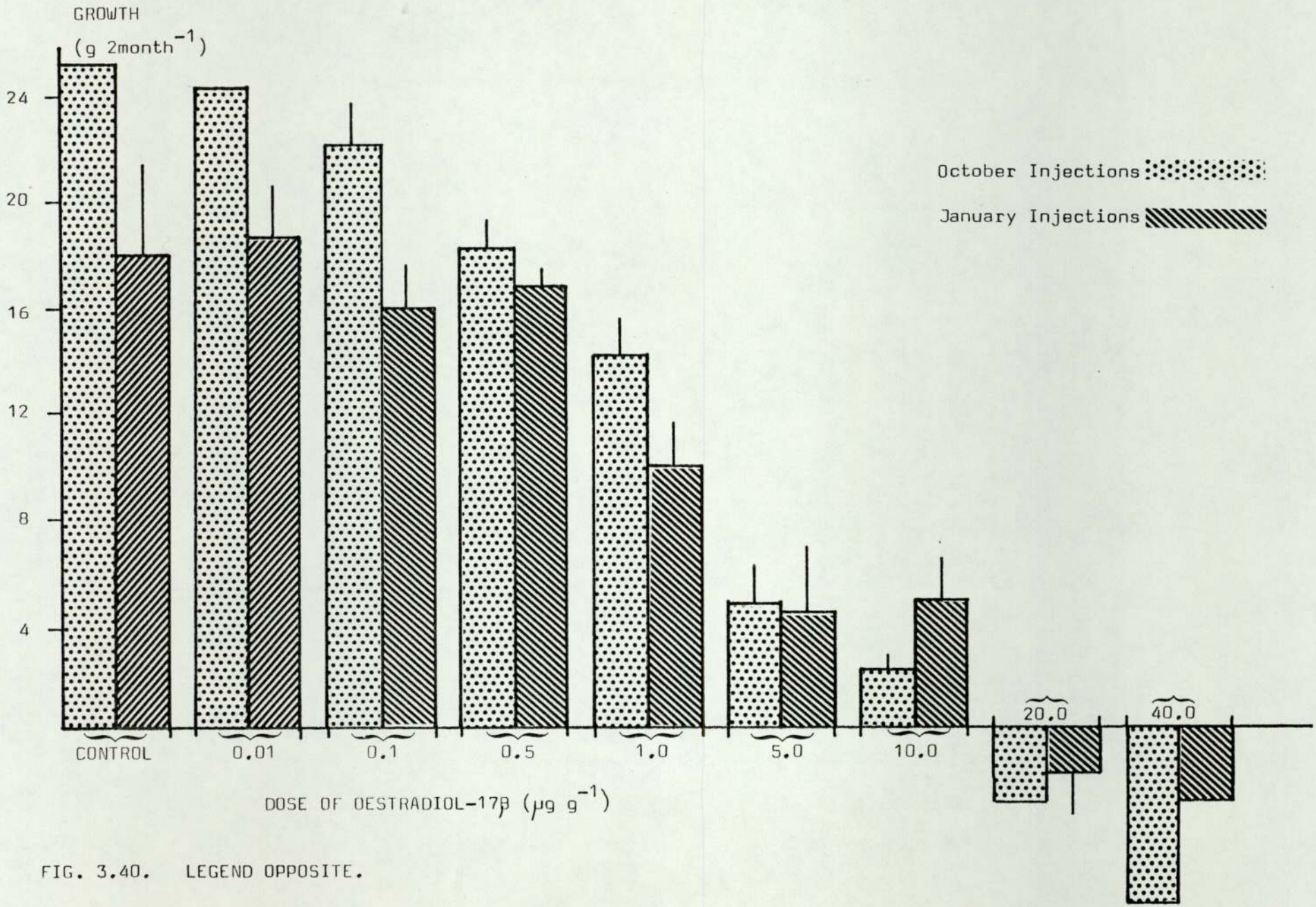


FIG. 3.40. LEGEND OPPOSITE.

3.5.3. Conclusions.

In both months the dose of injected E2 was significantly correlated with peak mean TCa, HSI, GSI and inversely correlated with growth. There was however, a clearly marked difference in sensitivity to the hormone. Whereas all treated groups in October showed a significant increase in TCa levels, only those treated at 1.0 $\mu g\ g^{-1}$ or above in January showed any significant increase. The response in January was both smaller and slower, with a peak in TCa observed after 39 days when compared to peak responses around 21 days in October treated groups.

The HSI was significantly higher in October control fish which suggests that liver weight relative to body weight may vary throughout the year. Correlation of dose with HSI supports the theory that the liver is the site of vitellogenin production.

Growth was faster in October-treated fish than in relative January-treated groups, and greater growth observed in the lowest dose groups in each case.

The serum-hormone profiles of injected E2 were very similar in both instances and were not significantly different.

3.6. Vitellogenic Responses of Different Size Fish to the same dose of E2.

The aim of this experiment was to determine whether the size or age of fish used may have any bearing on the vitellogenic response to a specific dose of E2 (5 $\mu g\ g^{-1}$).

3.6.1. Materials and Methods.

Groups of 5 fish with a mean weight of 50, 100, 200, 500, 900 and 2000g were selected. All fish had not previously spawned and ranged in age from less than 1 year to 2+ year olds. The fish were all individually tagged, bled to obtain base values and then injected with E2 at a dose of 5 $\mu g\ g^{-1}$ body weight. Adjustments were made so that final injection volume of 0.3ml was used.

All fish were sampled 2, 5, 9, 14, 21 and 28 days after injection, and the serum treated as above.

3.6.2. Results.

All groups demonstrated gross changes in TCa after injection with E2 (Table 3.29. & Fig. 3.41.). The peak mean TCa value in treated fish of 85.0 $\pm$ 8.3 mg $100ml^{-1}$ was observed 28 days after injection in the group with a mean weight of 2000g. The minimum peak mean TCa level in treated fish of 33.6 $\pm$ 3.5 mg $100ml^{-1}$ was observed in the smallest weight group, 21 days after injection.

The peak mean TCa for each weight group was positively correlated ($P < 0.001$) with the mean weight of the treated fish (Fig. 3.42.). Furthermore, the gradient of Linear increase in TCa (9-12 days, Fig. 3.41.) for each weight fish is positively correlated with the weight of fish (Fig. 3.42.).

TABLE 3.29. CHANGES IN TOTAL SERUM CALCIUM (TCa mg $100ml^{-1}$) IN DIFFERENT WEIGHT GROUPS INJECTED WITH A SIMILAR DOSE OF OESTRADIOL-17β (E2 5 $\mu g\ g^{-1}$)

MEAN WEIGHT (g)	DAYS AFTER INJECTION 0	2	5	9	14	21	28	GRADIENT OF LINEAR INCREASE 9-21 DAYS
50	9.95	9.5	10.0	12.5	21.2	33.6	33.1	1.76
± S.E.	0.2	0.2	0.5	0.5	2.5	3.5	4.1	
100	11.0	10.0	10.5	14.0	23.2	35.6	42.5	1.8
± S.E.	0.4	0.3	0.3	0.3	1.9	3.9	3.4	
200	10.9	11.5	11.0	12.5	22.01	35.0	46.6*	1.88
± S.E.	0.3	1.1	0.7	0.5	3.2	5.3	2.7	
500	10.95	11.0	11.0	14.7	25.5	41.0	49.8*	2.19
± S.E.	0.4	0.3	0.2	1.1	2.0	3.4	4.1	
900	11.3	10.0	11.5	18.5	31.2	49.2	57.5**	2.56
± S.E.	0.4	0.2	0.6	1.2	2.4	3.7	5.8	
2000	12.0	11.25	12.5	22.5	40.1	64.5	85.0***	3.5
± S.E.	0.4	0.6	0.7	2.5	5.4	7.1	8.3	

* ($p < 0.05$) ** ($p < 0.01$) *** ($p < 0.001$) n = 5

TABLE 3.29. LEGEND OPPOSITE.

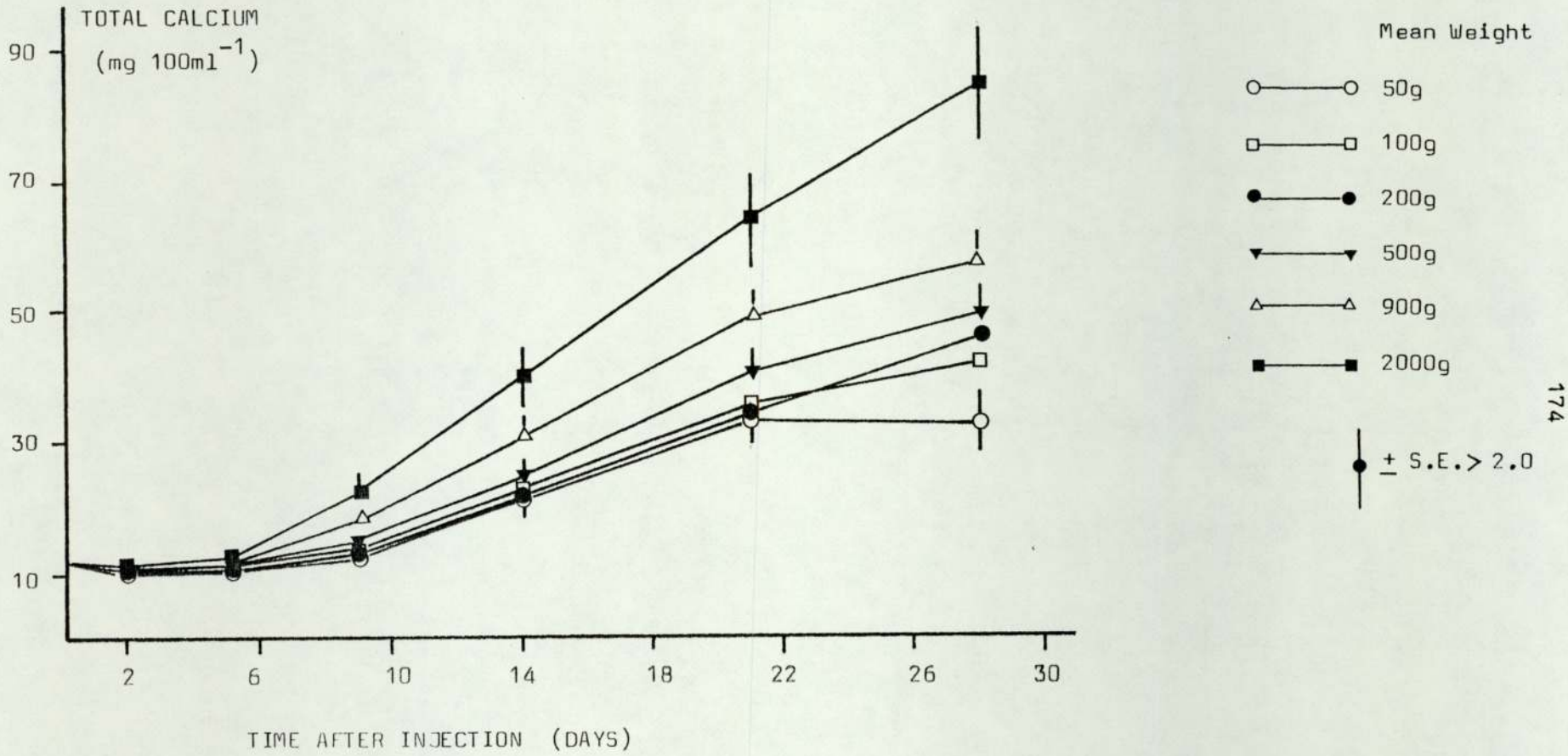


FIG. 3.41. CHANGES IN TOTAL SERUM CALCIUM (TCa mg 100ml^{-1}) IN DIFFERENT WEIGHT GROUPS INJECTED WITH A SIMILAR DOSE OF OESTRADIOL-17β (E2 5 μg g^{-1}).

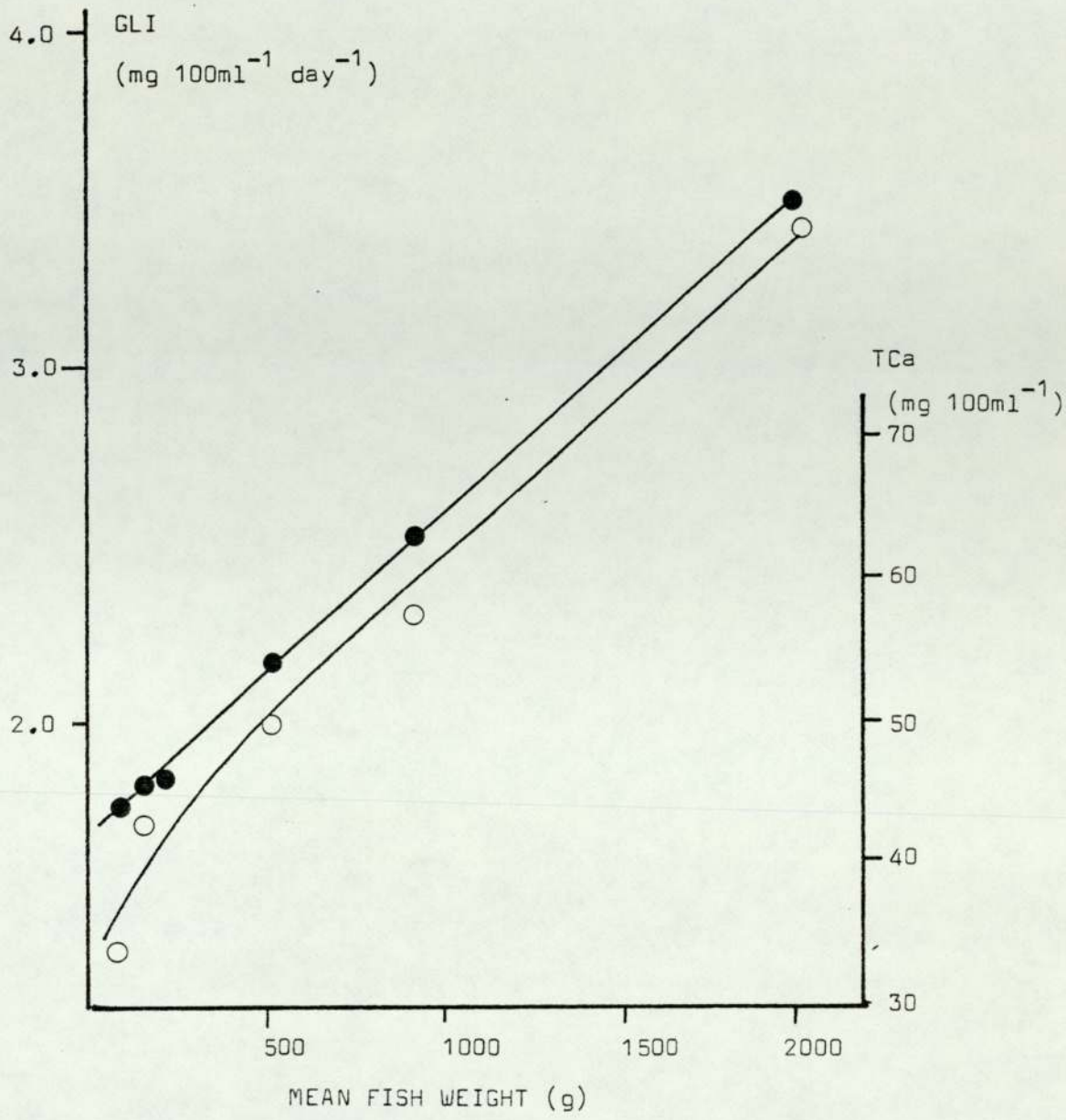


FIG. 3.42. COMPARISON OF CORRELATIONS BETWEEN :

○——○ (1) MEAN FISH WEIGHT (g) AND PEAK MEAN CALCIUM (TCa mg 100ml^{-1}).

●——● (2) MEAN FISH WEIGHT (g) AND GRADIENT OF LINEAR INCREASE (GLI mg 100ml^{-1} day^{-1}) IN TOTAL CALCIUM. (See TABLE 3.29.).

3.6.3. Conclusions.

Immature fish over a wide weight distribution are capable of vitellogenic response to a specific dose of E2. However, there is a distinct positive correlation between the weight of fish used and the vitellogenic response to a specific dose of E2. Whether the correlation with weight is also related to age or maturation is discussed in section 3.7..

3.7. Effect of Age, Maturity and Season on HSI.

The aim of this experiment was to investigate whether firstly, the HSI at a particular time varies with the age or weight of fish and secondly, whether HSI in a particular weight of fish varies with the time of year.

3.7.1. Materials and Methods.

Groups of 5 fish over the same weight range as in 3.6.1. and additionally down to 10.0 g were selected. All fish were then fed strictly to ration for 14 days. All fish were weighed and then sacrificed in order to calculate HSI and state of sexual development if any. This experiment was repeated in December, March, June and September. The HSI was determined in maturing female fish at 6 times during the year, and compared with HSI as determined for further groups of mature and immature male and female fish sampled in December.

3.7.2. Results.

At any time of the year the HSI between different weights of fish is not significantly different (apart from the 10.0 g group). See Table 3.30. & Fig. 3.43.. The HSI in all groups is significantly ($P < 0.001$) greater in December than in March.

Mature male (2.624 $\pm$ 0.39) and mature female (3.2 $\pm$ 0.25) rainbow trout have significantly ($P < 0.05$ & 0.01 respectively) greater HSI's than their respective immature siblings at 1.896 $\pm$ 0.04% and 2.12 $\pm$ 0.19% (Table 3.31. & Fig. 3.44.).

A seasonal variation in HSI (Table 3.31.) is observed in maturing female trout with a minimum of 1.3 $\pm$ 0.06% in February. HSI is significantly raised above February's index in December (1.79 $\pm$ 0.12%., $P < 0.05$); June (2.6 $\pm$ 0.15%., $P < 0.001$); August (3.5 $\pm$ 0.17%., $P < 0.001$) and October (3.65 $\pm$ 0.17%., $P < 0.001$). See Fig. 3.44..

WEIGHT (g)	2000	900	500	200	100	50	10
DECEMBER	1.8	2.1	1.9	1.85	1.7	1.9	1.4
± S.E.	0.1	0.15	0.05	0.06	0.12	0.17	0.13
MARCH	1.3	1.5	1.45	1.48	1.625	1.6	1.3
± S.E.	0.1	0.05	0.17	0.13	0.1	0.06	0.09
JUNE	2.6	2.9	2.7	2.6	2.7	2.6	1.6
± S.E.	0.09	0.06	0.16	0.17	0.08	0.2	0.09
SEPTEMBER	2.9	3.2	3.1	3.0	2.9	2.8	-
± S.E.	0.03	0.17	0.07	0.1	0.09	0.07	-

n = 5

TABLE 3.30. MEAN HEPATOSOMATIC INDEX (HSI %) IN GROUPS OF FISH WITH DIFFERENT MEAN WEIGHT (g) TAKEN AT FOUR TIMES DURING THE YEAR.

(a) Taken at December sampling.

	Imm. Males	Imm Females	Mature Males	Mature Females
Mean	1.896	2.12	2.624	3.2
± S.E.	0.04	0.19	0.39	0.25

(b) Seasonal variation for maturing females.

	Dec	Feb	Apr	Jun	Aug	Oct
Mean	1.79	1.3	1.5	2.6	3.5	1.65
± S.E.	0.12	0.06	0.09	0.15	0.17	0.17

n = 3

TABLE 3.31. (a) COMPARISON OF MEAN HEPATOSOMATIC INDEX (HSI %) IN MATURE AND IMMATURE MALE AND FEMALE FISH SAMPLED IN DECEMBER AT ASTON FISH CULTURE UNIT, AND

(b) IN MATURING FEMALE FISH SAMPLED AT DIFFERENT TIMES OF THE YEAR ON A COMMERCIAL FARM.

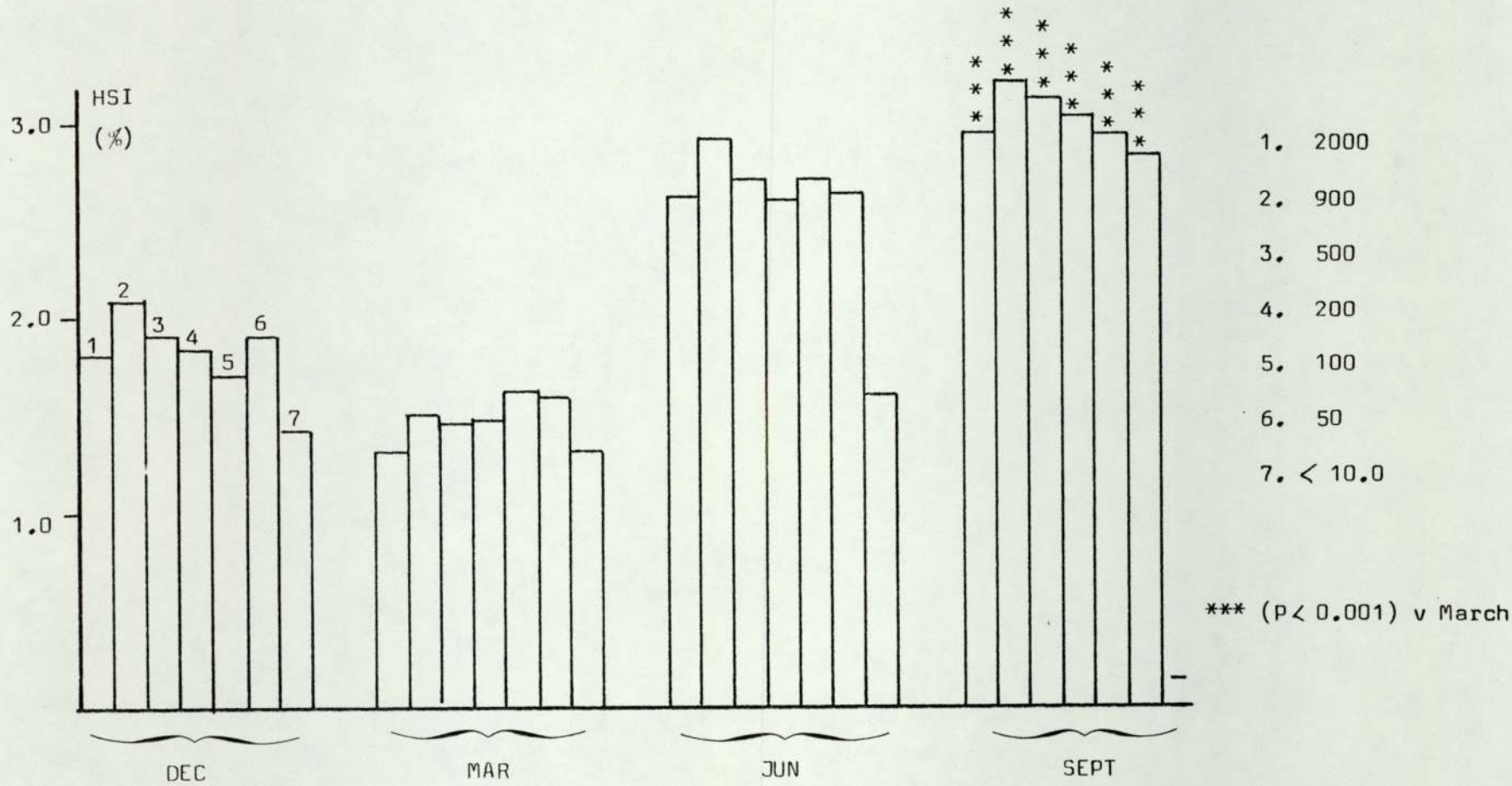


FIG. 3.43. MEAN HEPATOSOMATIC INDEX (HSI %) IN GROUPS OF FISH WITH DIFFERENT MEAN WEIGHT (g) TAKEN AT FOUR TIMES DURING THE YEAR.

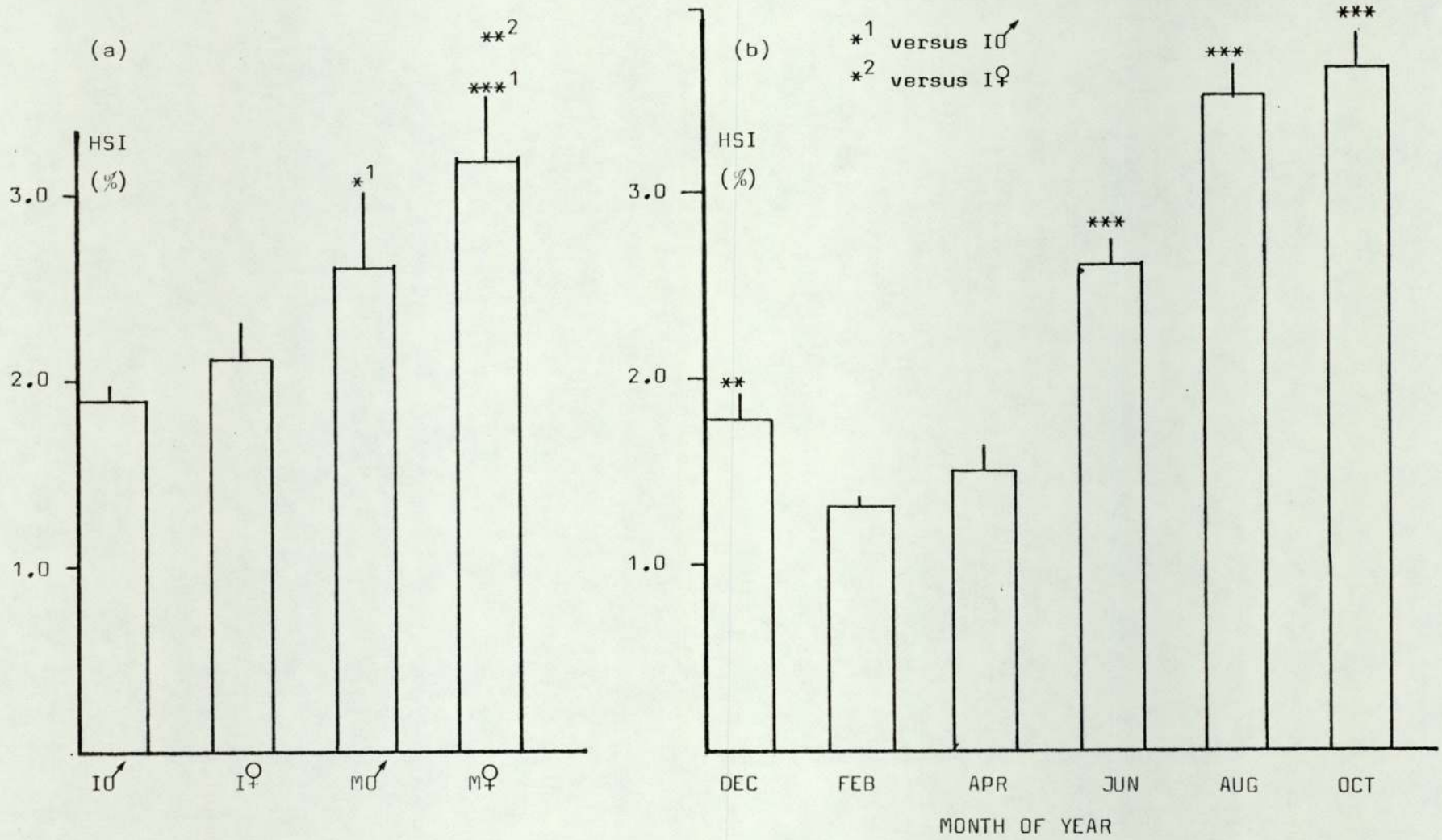


FIG. 3.44. (a) COMPARISON OF MEAN HEPATOSOMATIC INDEX (HSI %) IN MATURE AND IMMATURE MALE AND FEMALE FISH SAMPLED IN DECEMBER AT ASTON FISH CULTURE UNIT, AND

(b) IN MATURING FEMALE FISH SAMPLED AT DIFFERENT TIMES OF THE YEAR ON A COMMERCIAL FARM.

3.7.3. Conclusions.

There is a distinct relationship between HSI and the time of year. The HSI is lowest in late winter/early spring, and highest in autumn/early winter. The HSI may have a direct effect on the vitellogenic response. Although in immature fish there is little significant difference in HSI with weight at a particular time, there is a significantly increased HSI with the degree of maturity, with the greatest HSI observed in mature female fish.

3.8. Effects of Varying Proportion or Nature of Vehicle on Vitellogenic Response.

The aim of these experiments was to determine whether different ratio's of arachis/ethanol, or vehicle had any influence on the serum hormone profile of injected E2, or the vitellogenic response. In the first experiment different ratio's of arachis/ethanol were used from that normally used of 4:1. Secondly, the steroid was injected in a saline-type vehicle.

3.8.1. Materials and Methods.

The effects of different ratios of arachis/ethanol as vehicle on serum hormone profile of injected E2 : 40 immature trout with a mean weight of 70 g were selected and individually tagged. They were distributed randomly between 2 tanks of System B and allowed to acclimatise for 6 days.

Groups of 5 fish were injected with E2 at 5 $\mu g\ g^{-1}$ body weight, suspended in arachis/ethanol emulsion at ratios of 4:1, 2:1, 1:1, 1:2 and 1:4. 3 each of the remaining 15 fish received vehicle only, in the ratios above.

All fish were bled 1,2,5,7,9 and 21 days after injection. The serum samples were stored at $-20^{o}C$ until assay for E2.

Effects on serum hormone profile of injected E2 and the vitellogenic response, when saline is used as vehicle : From the same stock, 16 fish of the same mean weight were selected and allowed to acclimatise in System A for 7 days. After tagging and basal sampling, 8 fish were injected with E2 in 0.8% saline containing 1% EtOH. The dose was 5.0 $\mu g\ g^{-1}$ body weight and the final injection volume 0.3 ml. The steroid was first partially dissolved in the ethanol and then mixed with the saline in a sonic bath at 4°C for ½ hour. The 8 control fish received the same volume of vehicle. All fish were bled 0,1,2,5,7,10,14, 21,28 and 39 days after injection, and the serum stored at -20°C until assay for E2 and calcium.

3.8.2. Results.

Effects of different ratios of arachis/ethanol on serum hormone profile of injected E2 : All treated groups demonstrated gross significant changes in serum levels of E2 following injection, when compared with control fish (Tables 3.32. & 3.33. & Fig. 3.45.). A maximum mean peak E2 value was recorded in that group where the normal 4:1 arachis/ethanol ratio was used (87.5 $\pm$ 19.3 ng ml^{-1}) 2 days after injection. A minimum mean peak E2 level of 20.0 $\pm$ 5.9 ng ml^{-1} was observed in the 1:4 arachis/ethanol ratio group at the same time. There was a significant ($P < 0.01$) negative correlation between the peak mean serum E2 level observed for each group and the amount of ethanol in vehicle emulsion.

TIME (DAYS)	0	2	5	7	9	21
Arachis/ethanol						
4:1	0.5	87.5	22.5	7.6	4.0	0.5
± S.E.	0.2	19.3	4.0	2.33	1.1	0.1
2:1	0.7	49.0	18.5	6.0	1.5	0.6
± S.E.	0.3	5.1	2.6	2.2	0.9	0.3
1:1	0.2	39.5	12.0	5.0	2.0	0.55
± S.E.	0.1	8.9	3.2	1.3	1.0	0.3
1:2	0.6	25.0	13.0	9.0	1.5	0.7
± S.E.	0.2	4.5	2.9	2.3	0.5	0.4
1:4	0.45	20.0	5.0	2.0	0.5	0.6
± S.E.	0.2	5.9	1.1	1.0	0.3	0.2

n = 4

TABLE 3.32. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) IN GROUPS OF FISH INJECTED WITH OESTRADIOL-17β (E2 5 μg g^{-1}) IN DIFFERENT RATIOS OF ARACHIS/ETHANOL VEHICLE.

TIME (DAYS)	0	2	5	7	9	21
Arachis/ethanol						
4:1	0.7	1.2	1.1	0.9	0.4	0.5
± S.E.	0.4	0.9	0.4	0.5	0.1	0.2
2:1	0.6	1.3	1.3	0.9	0.5	0.2
± S.E.	0.2	0.7	0.8	0.4	0.2	0.1
1:1	0.45	1.4	1.1	0.8	0.5	0.6
± S.E.	0.2	0.5	0.5	0.3	0.3	0.2
1:2	0.55	2.1	1.5	1.2	0.9	0.9
± S.E.	0.3	1.1	0.8	0.17	0.4	0.2
1:4	0.9	0.9	0.9	1.1	0.9	0.4
± S.E.	0.4	0.4	0.5	0.3	0.2	0.1

n = 4

TABLE 3.33. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17B (E2 ng ml^{-1}) IN CONTROL FISH INJECTED WITH DIFFERENT RATIOS OF ARACHIS/ETHANOL VEHICLE (0.2 ml).

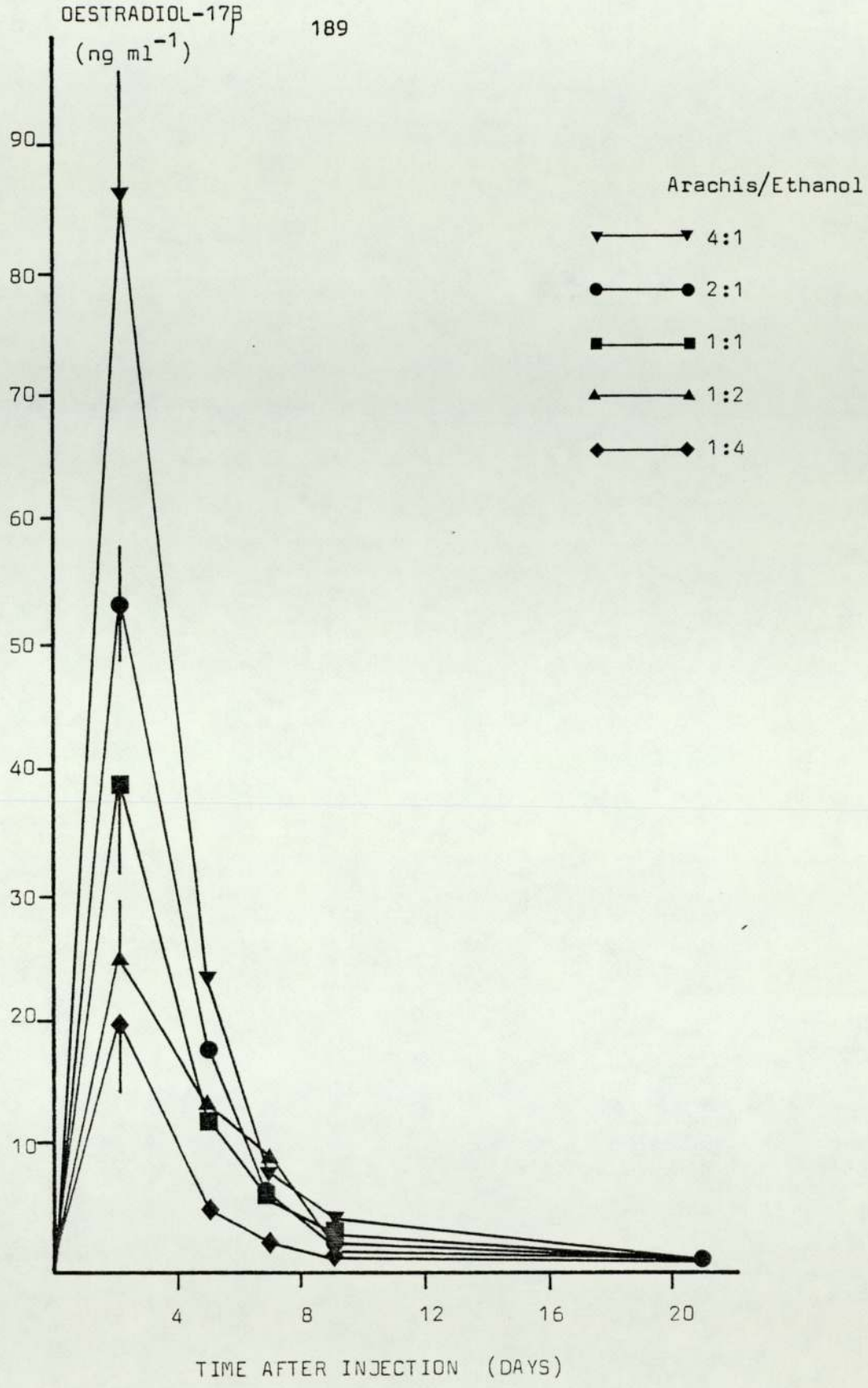


FIG. 3.45. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) FOLLOWING INJECTION OF GROUPS OF FISH WITH OESTRADIOL-17β (E2 5 μg g^{-1}) IN DIFFERENT RATIOS OF ARACHIS:ETHANOL VEHICLE.

Effects on serum hormone profile and vitellogenic response using saline as vehicle : Significant changes in serum levels of E2 and TCa were observed in treated fish during the course of the experiment. Serum E2 levels rose significantly ($P < 0.001$) in treated fish from basal levels of 0.7 ± 0.2 to a peak of 94.75 ± 9.5 ng ml^{-1} 1 day after injection, before returning to control levels after 10 days. No such similar changes were observed in control fish (Table 3.34. & Fig. 3.46.).

Significant increases in TCa were observed in treated fish, rising from basal values of 11.02 ± 0.1 mg $100ml^{-1}$ to a peak of 58.7 ± 7.2 mg $100ml^{-1}$ 21 days after injection (Table 3.35. & Fig. 3.47.). Control fish showed no significant changes.

TIME (DAYS)	0	1	2	5	7
CONTROL	0.5	1.1	1.2	0.9	0.7
± S.E.	0.2	0.4	0.6	0.3	0.4
TREATED	0.7	94.75***	45.0***	7.5**	2.5*
± S.E.	0.2	9.5	6.8	2.7	1.2

TIME (DAYS)	10	14	21	28	39
CONTROL	0.5	0.6	0.5	0.7	0.6
± S.E.	0.2	0.4	0.2	0.2	0.3
TREATED	1.5	1.0	0.7	0.8	0.5
± S.E.	0.5	0.5	0.3	0.4	0.1

n = 8 * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$)

TABLE 3.34. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) IN FISH INJECTED WITH OESTRADIOL-17β (E2 5 μg g^{-1}) IN SALINE AS VEHICLE.

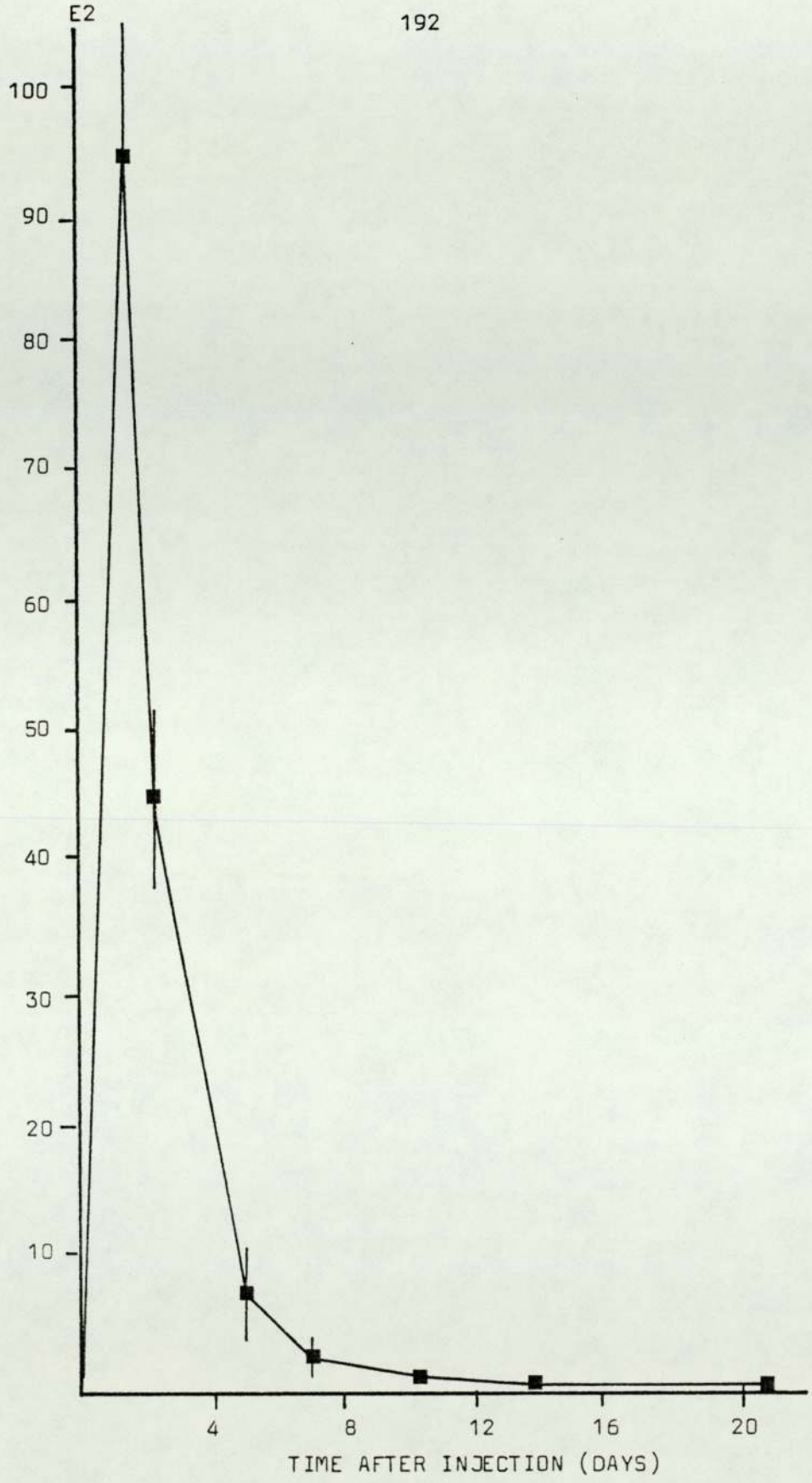


FIG. 3.46. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) IN FISH INJECTED WITH OESTRADIOL-17β (E2 5 μg g^{-1}) IN SALINE AS VEHICLE.

TIME (DAYS)	0	1	2	5	7
CONTROL	10.95	10.7	10.5	10.4	11.01
± S.E.	0.2	0.3	0.2	0.2	0.4
TREATED	11.02	10.5	10.0	12.1	15.0*
± S.E.	0.1	0.3	0.25	0.9	1.2

TIME (DAYS)	10	14	21	28	39
CONTROL	11.2	11.2	12.1	11.49	11.5
± S.E.	0.9	0.7	1.1	0.7	0.5
TREATED	24.4**	37.9***	58.7***	46.2***	41.3**
± S.E.	3.7	5.2	7.2	5.9	6.5

n = 8 * ($P < 0.05$) ** ($P < 0.01$) *** ($P < 0.001$)

TABLE 3.35. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) IN FISH INJECTED WITH OESTRADIOL-17β (E2 5 $\mu g\ g^{-1}$) IN SALINE AS VEHICLE.

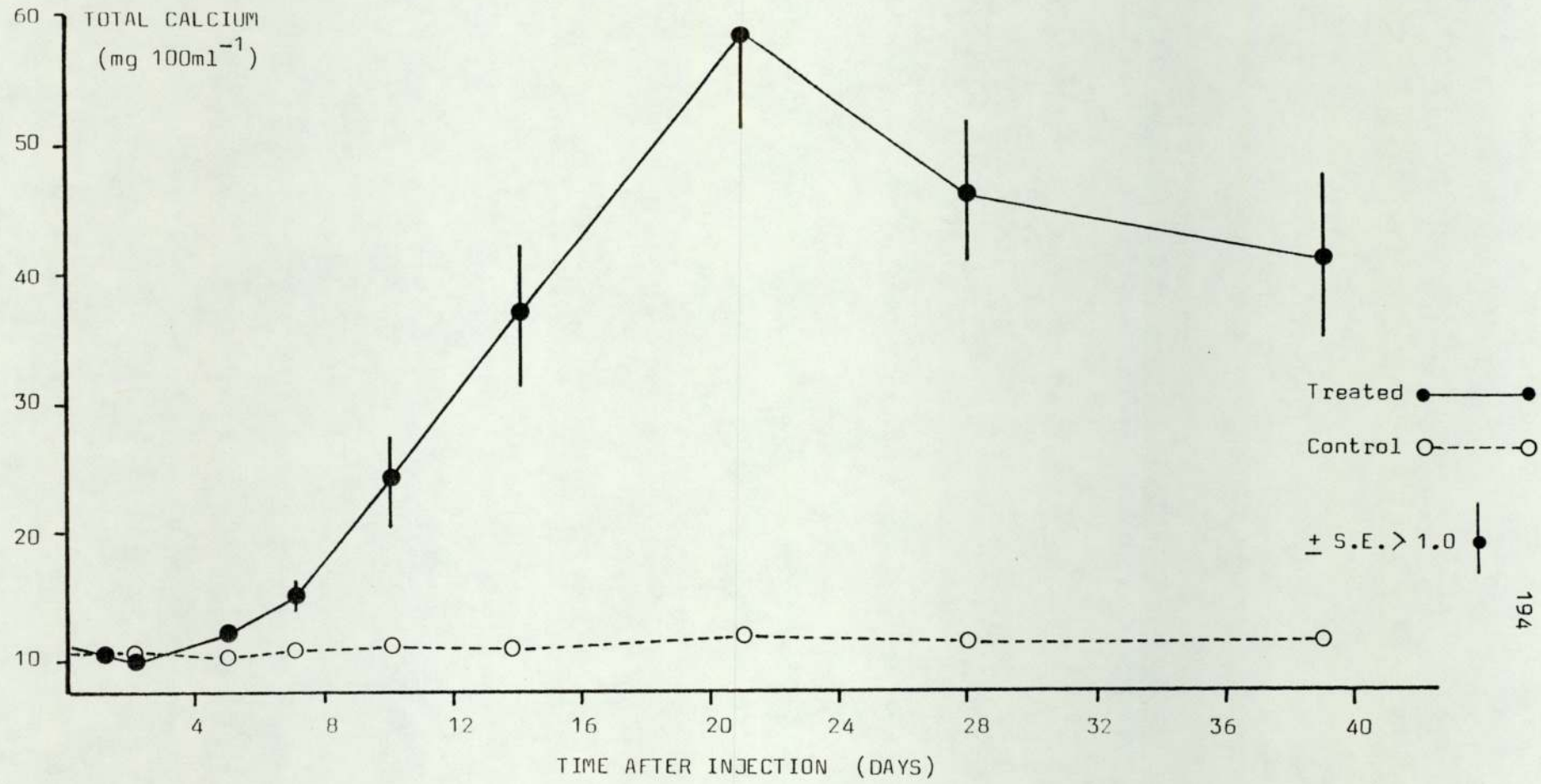


FIG. 3.47. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml^{-1}) IN FISH INJECTED WITH OESTRADIOL-17β (E2 5 μg g^{-1}) IN SALINE AS VEHICLE.

3.8.3. Conclusions.

The amount of ethanol in the vehicle significantly reduces the amount of E2 determined in the serum of the treated fish 2 days after injection (Fig. 3.48.). In fish injected with saline as vehicle, although not significantly different from arachis/ethanol (4:1) in mean peak levels recorded, the profile suggests that the steroid is taken up quicker. Or conversely, a slower release of steroid from arachis emulsion occurs, when compared with oil/ ethanol vehicle (Fig. 3.49.).

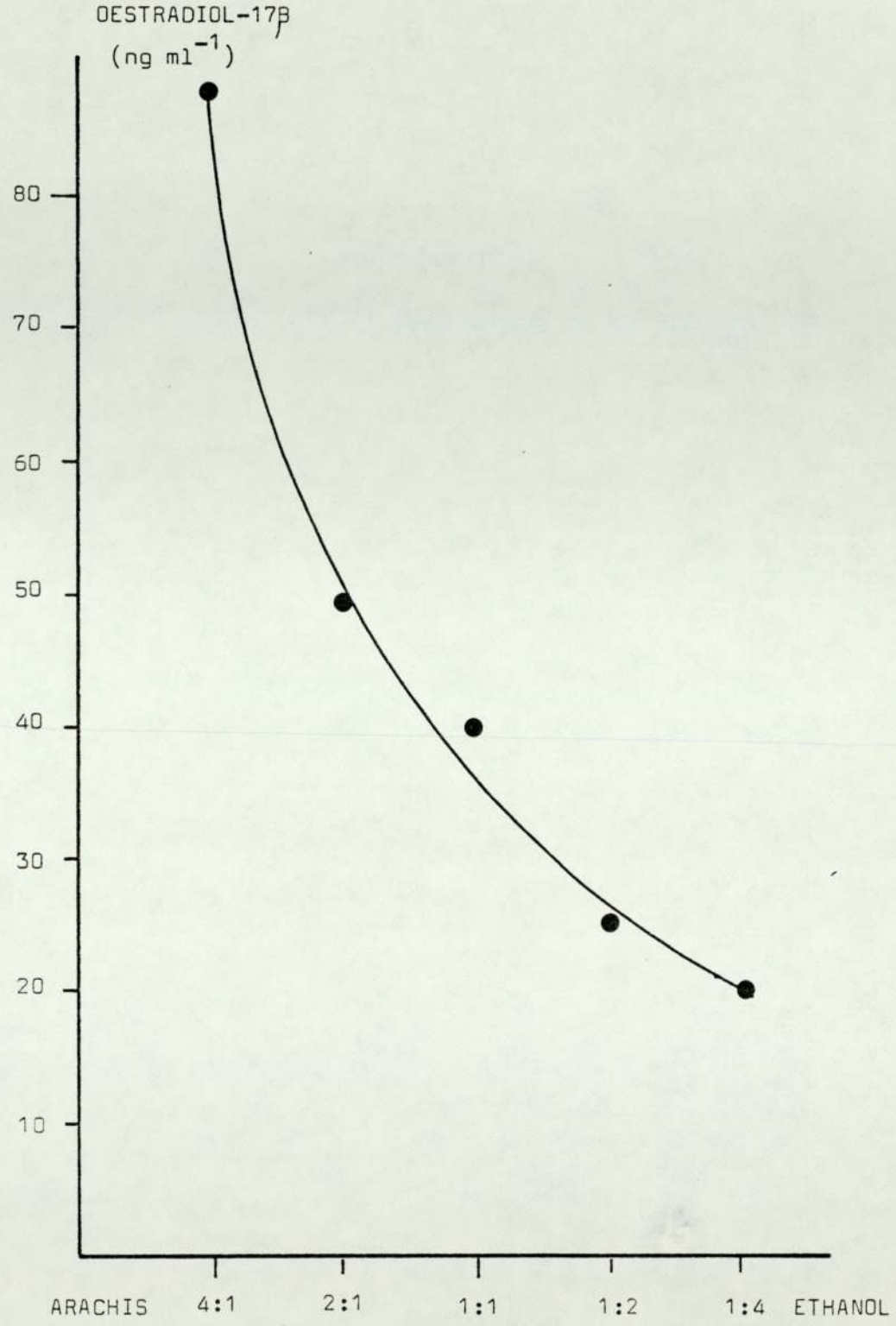


FIG. 3.48. VARIATION IN 2 DAY PEAK MEAN SERUM LEVELS OF OESTRADIOL -17β AFTER INJECTION OF GROUPS OF FISH WITH THE SAME DOSE OF OESTRADIOL-17β IN DIFFERENT RATIOS OF ARACHIS/ ETHANOL VEHICLE.

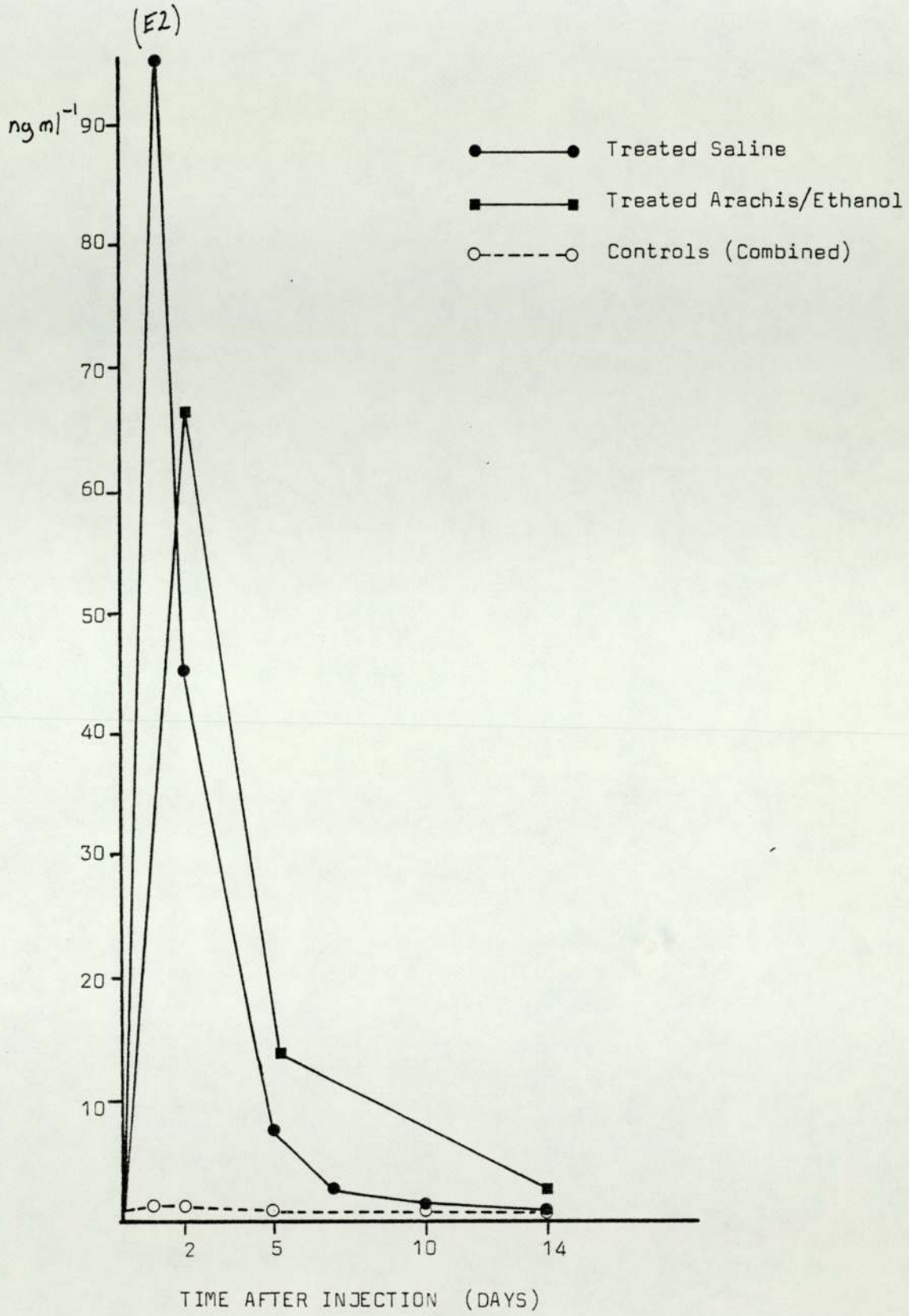


FIG. 3.49. COMPARISON OF UPTAKE AND CLEARANCE OF THE SAME DOSE OF OESTRADIOL-17β (E2 5 μg g^{-1}) INJECTED IN SALINE AS VEHICLE OR ARACHIS/ETHANOL (4:1).

3.9. Summary of Results.

1. Oestradiol-17β caused elevation in serum levels of total calcium and phosphoprotein phosphorus in rainbow trout.

2. Priming with oestradiol-17β was not necessary and trout liver can respond to a single injection of this hormone, although on reinjection a much more rapid secondary response occurred.

3. The vitellogenic response to a specific dose of oestradiol-17β varied both with the time of the injection in the year and with the dose of oestradiol-17β administered.

4. The dose of injected oestradiol-17β was correlated with the peak level of calcium, HSI and GSI, but was negatively correlated with growth.

5. The size and/or age of fish can greatly affect the vitellogenic response and the serum hormone profile of injected oestrádiol-17β varied with the vehicle used.

3.10. Discussion.

The results clearly demonstrate that oestradiol-17B causes elevations in serum levels of calcium and phosphoprotein phosphorus, both of which are constituents of the egg protein or vitellogenin complex (See Chapter 4). A summary of the sequence of changes in oestradiol-17β, total calcium and phosphoprotein phosphorus over the first 11 hours following oestrogen treatment (Fig. 3.50.) shows that oestradiol-17β was taken up into the serum immediately after its injection into the intraperitoneal cavity. However, the increases in serum levels of total calcium and phosphoprotein phosphorus did not occur until at least 11 hours after oestradiol-17β injection. In other studies on amphibians, it has been shown that vitellogenin can be detected in the liver 12 but not 9 hours after the administration of oestradiol-17β (Wittliff & Kenney, 1973; Zelson & Wittliff, 1973). The results from the present work indicate that in trout the release of the complete vitellogenin moiety also occurs after approximately 11 hours. In a more recent study, van Bohemen et al., (1981a) were unable to detect vitellogenin in the liver, but suggested this was indicative of a low storage and high secretion rate.

The long-term sequence of changes in calcium and phosphoprotein phosphorus, summarised in Fig. 3.51., further demonstrate that the uptake of oestradiol-17β was linear over the first 48 hours at which point the peak level was observed. Very few other workers have investigated in detail, serum changes in oestradiol-17β following injection with this hormone and in these studies only 1 or 2 post-treatment samples have been taken.

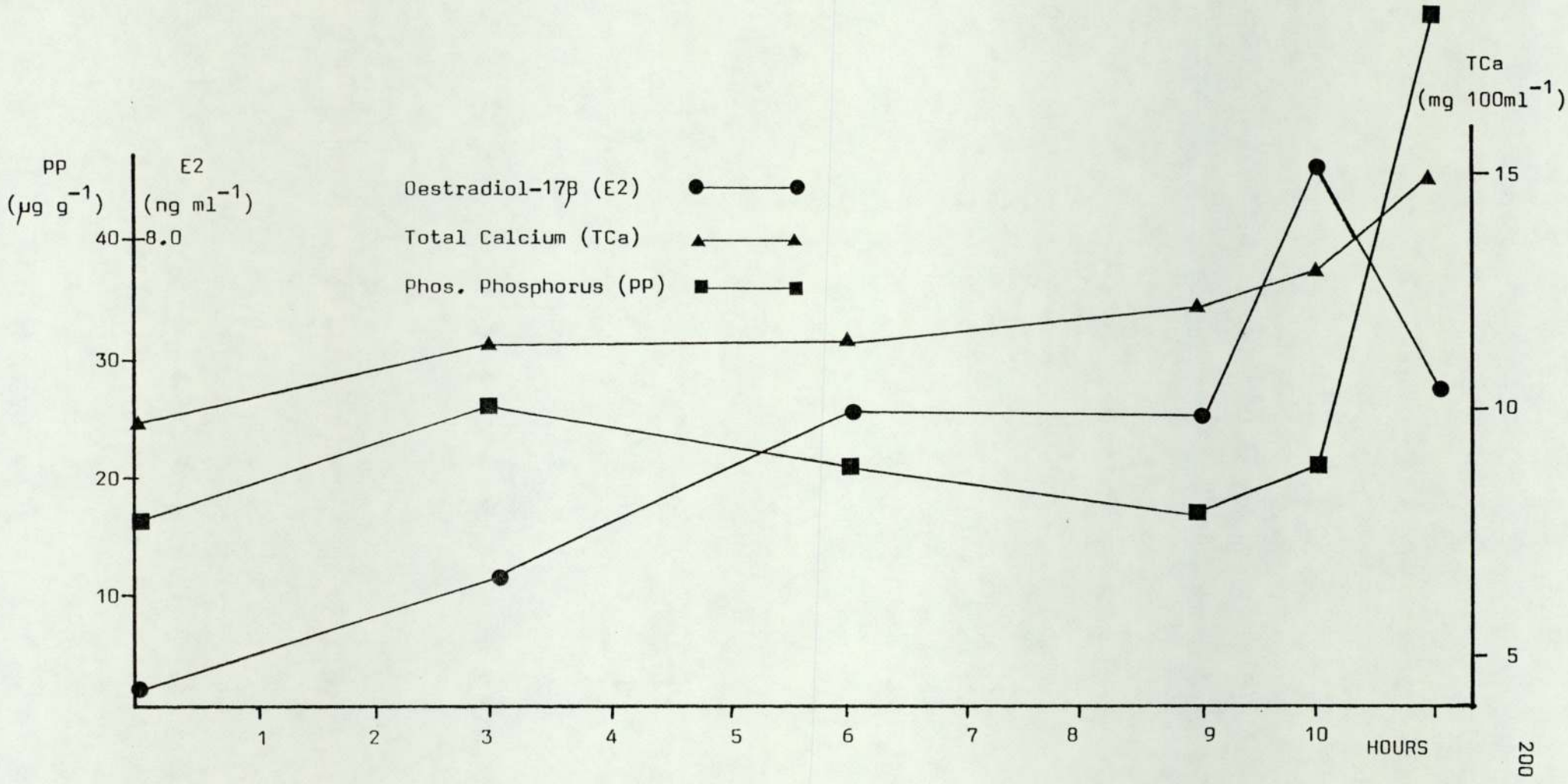


FIG. 3.50. SUMMARY OF SHORT-TERM CHANGES IN TOTAL CALCIUM, PHOSPHOPROTEIN PHOSPHORUS AND OESTRADIOL-17β FOLLOWING A SECOND INJECTION OF OESTRADIOL-17β AT A DOSE OF 5 μg g^{-1}.

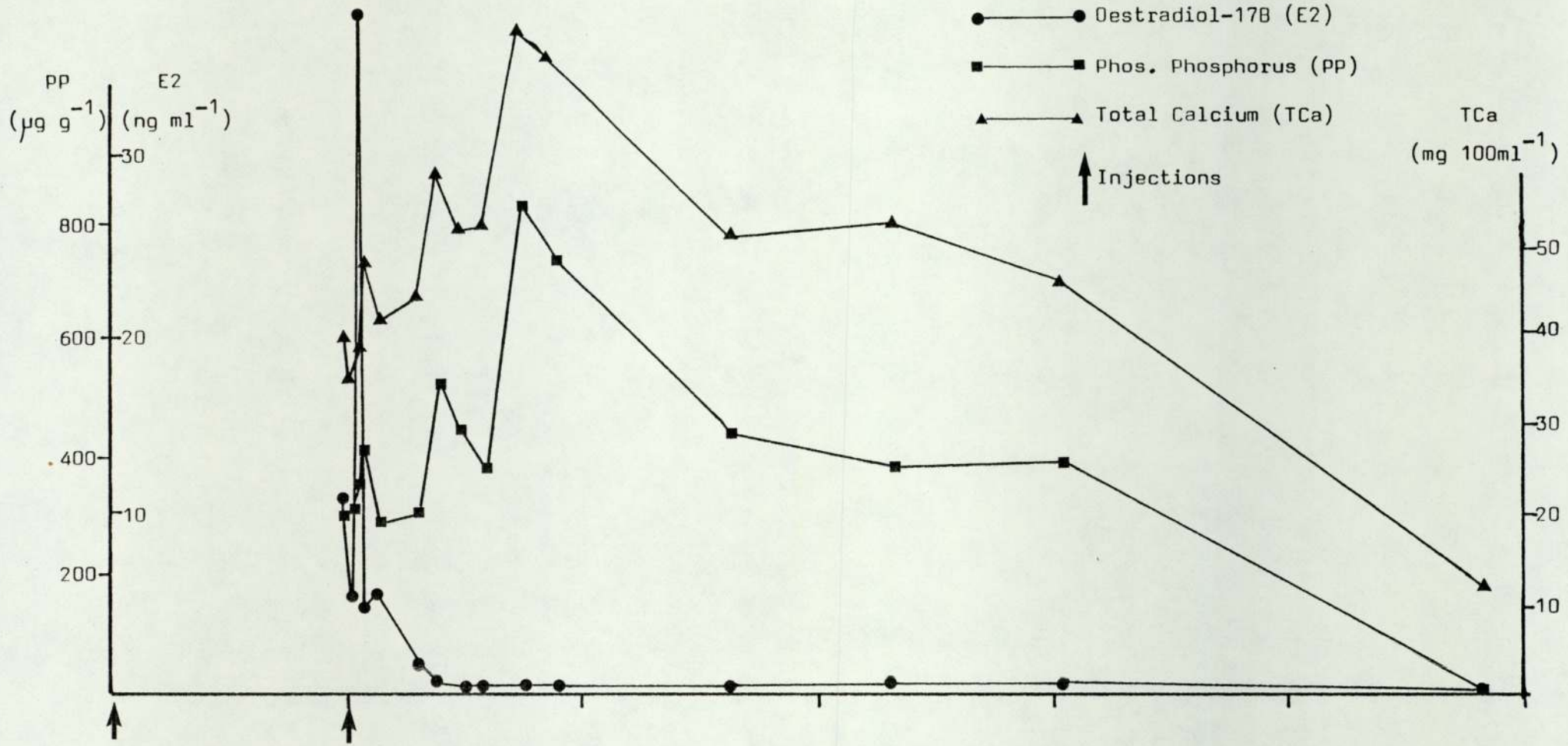


FIG. 3.51. SUMMARY OF LONG-TERM CHANGES IN TOTAL CALCIUM, PHOSPHOPROTEIN PHOSPHORUS AND OESTRADIOL-17β FOLLOWING A SECOND INJECTION OF OESTRADIOL-17β AT A DOSE OF 5 µg g^{-1} BODY WEIGHT.

However, in one investigation using Tilapia a peak of 100 ng $100ml^{-1}$ of oestradiol-17β was observed 5 hours after an injection of oestradiol-17β in sesame oil, and this subsequently fell to 9 ng ml^{-1} after 1 day (Terkatin-Shimony & Yaron, 1978). Consequently, these authors considered the levels to be pharmacological for 1 day and physiological for 4 days. Despite the fact that both the methodology and fish species used in this study were quite different from those of the present work, the results are remarkably similar. After 2 days the serum levels of oestradiol-17β fell rapidly and reached near-control values after 7-11 days. This rapid decline in serum levels of oestradiol-17β, following the initial linear increase also corresponds with the data of Johnstone et al., (1978) who showed that the half-life of oestradiol-17β in trout serum was approximately 12 hours. Clearly, weekly injections of oestrogen would be required if they were being used to maintain physiological levels of this hormone over an extended period. However, the long-term changes in total calcium and phosphoprotein phosphorus after only 2 injections with oestradiol-17β were so pronounced that weekly treatments would appear unnecessary if the objective is to provoke a vitellogenic response.

Although the increase in serum levels of oestradiol-17β was both rapid and short-lived, the highest levels of total calcium and phosphoprotein phosphorus did not occur until 21 days after the 2nd injection and had not returned to basal levels until 135 days. The peak levels of calcium and phosphorus following oestrogenization observed in similar studies on other fish, show considerable variation, as do the doses of hormone used and methods of

administration (See Table 3.38.). Thus, in the killifish and goldfish following treatment with oestradiol benzoate at a dose of 5 $\mu g\ g^{-1}$ total calcium levels increased 8-fold (Mugiya & Watabe, 1977). This compares very closely with the present data where total calcium increased from 10 mg ml^{-1} to a peak of 75 mg $100ml^{-1}$ and at the same time levels of phosphoprotein phosphorus increased from approximately 10 to 832 $\mu g\ ml^{-1}$. The concomittant and equivalent increases in total calcium and phosphoprotein phosphorus suggest there is 1 mole of calcium for every mole of phosphoprotein phosphorus in trout vitellogenin; this has previously been noted in amphibians (Wallace, 1970) (Also see chapter 4 for further discussion of structure of vitellogenin). It would appear that the liver of some fish species (eg trout and goldfish) is very sensitive to oestrogens, whereas in others (eg dogfish) there is a poor vitellogenic response (ie synthesis of vitellogenin by the liver after oestradiol-17β challenge). This may ultimately reflect the different relative requirements of the ovaries of different fish species for specific amounts of vitellogenin or differences in the uptake or utilisation of the complex.

It is of considerable commercial significance that only 2 injections of oestradiol-17β are required to initiate a long-lasting synthesis of vitellogenin by the liver, even though changes in oestradiol-17β are short-lived. One of the aims of this study was to investigate whether hormonal injections could be used to manipulate the vitellogenic response in trout. Clearly only 2 injections to broodstock would be a practical means of treatment and would involve only limited handling of the fish. A similar

SPECIES	DOSE	MEASURED PARAMETER		AUTHOR(s)
Fundulus		Ca^{2+} 4 mEq/L	20 mEq/L	Fleming et al., 1964
Killifish	5 $\mu g\ g^{-1}$	Ca^{2+} 10 mEq/L	80 mEq/L	Mugiya & Watabe, 1977
Goldfish		5 mEq/L	35 mEq/L	
Goldfish	Added to food @ 1 mg/g	Ca^{2+} 62.3	555.3 ug ml^{-1}	Hori et al., 1979
		P 223.8	1197.0 ug ml^{-1}	
Tilapia	1.25 x 10^{-5} Kg^{-1}	Ca^{2+} 10	15 mg 100ml^{-1}	Terkatin-Shimony & Yaron, 1978
		Protein 3	4 g 100ml^{-1}	
Tilapia		Ca^{2+} 12	3 mg 100ml^{-1}	Yaron et al., 1977
Dogfish	3 mg Kg^{-1}	Ca^{2+} 8	15 mg 100ml^{-1}	Craik, 1978a
		Protein 2	10 mg 100ml^{-1}	
Eel	3 $\mu g\ g^{-1}$	Ca^{2+} 7.4	142 mEq/L	Olivereau & Olivereau, 1979

TABLE 3.38. VARIATION IN SPECIES, DOSE OF OESTROGEN AND MEASURED PARAMETERS IN SOME STUDIES OF VITELLOGENESIS IN FISH.

phenomenon has been observed during the normal spawning cycle where levels of total calcium and phosphoprotein phosphorus continued to rise for several months after the maximum levels of oestradiol-17β had been reached (Whitehead et al., 1978a; Scott et al., 1980b); often the highest levels of phosphoprotein phosphorus and calcium were observed at the time of spawning when serum oestradiol-17β was at basal levels. The levels of total calcium and phosphoprotein phosphorus observed after oestrogenization in this study were slightly higher than those which have been reported for the normal seasonal cycle (Whitehead et al., 1978a; Scott et al., 1980b). However, one must take into account the fact that when immature female or male fish are treated with oestradiol-17β, the vitellogenin they produce may not be taken up by ovarian tissue and consequently may build up in the serum; also, oestradiol-17β is constantly being produced during the normal cycle even when overall serum levels are falling. It is possible that there are differences in the metabolism of vitellogenin in immature and mature fish similar to those reported for Xenopus (Wallace & Jared, 1968). These authors showed that the physiological half-life of vitellogenin was approximately 2 days in maturing female toads, whereas in ovariectomised or male animals it was 40 days. If we take the 21-day peak figure of calcium (75 mg $100ml^{-1}$) as representative of maximum vitellogenesis and the $t_{\frac{1}{2}}$ of vitellogenin in immature trout to be similar to that of amphibians ie 40 days, one would expect the calcium level to fall to a basal level of 10 mg $100ml^{-1}$ in 141 days ie $3\times t_{\frac{1}{2}}$ periods plus the initial 21 days to reach a peak vitellogenin production. Thus, it would appear that after the second injection of oestradiol-17β,

in immature fish the hormone is taken up into the serum within 3 hours, reaches a peak 2 days later, before falling to basal levels after 7 to 11 days. Subsequently, vitellogenin is released into the blood at about 11 hours, reaching a maximum rate of synthesis after 2 or 3 days which continues for up to 3 weeks, at which point vitellogenin synthesis stops and vitellogenin is lost as a function of its $t_{\frac{1}{2}}$ which is thought to be approximately 40 days in length.

Somewhat surprisingly, the results show that a single injection is sufficient to initiate synthesis of vitellogenin by the liver in immature male and female trout, suggesting that priming with the hormone is unnecessary. In these fish the oestradiol-17β was also quickly taken up into the blood, reaching a peak value at 1 day before falling to control levels 12 days after injection. However, the vitellogenic response to this injection occurred much more slowly. Total serum calcium levels fell after 24 hours and were only marginally increased 5 days after the injection. The peak levels for calcium occurred at 22 days, as with the double injection although the maximum serum concentration attained was reduced; in addition, calcium levels fell to basal after 60 rather than 141 days. These data suggest either that the half-life for vitellogenin is approximately 20 days in immature fish which have not previously been exposed to oestradiol-17β, or that vitellogenin secretion was not maintained for as long a period of time. However, when primed fish are reinjected with the same dose of oestradiol-17β, a much more rapid secondary response occurred (Fig. 3.52.). By comparison, total calcium increased by 3 mg $100ml^{-1}$ in 2 days with a slightly higher peak (50.5 mg $100ml^{-1}$) after 21 days. This may

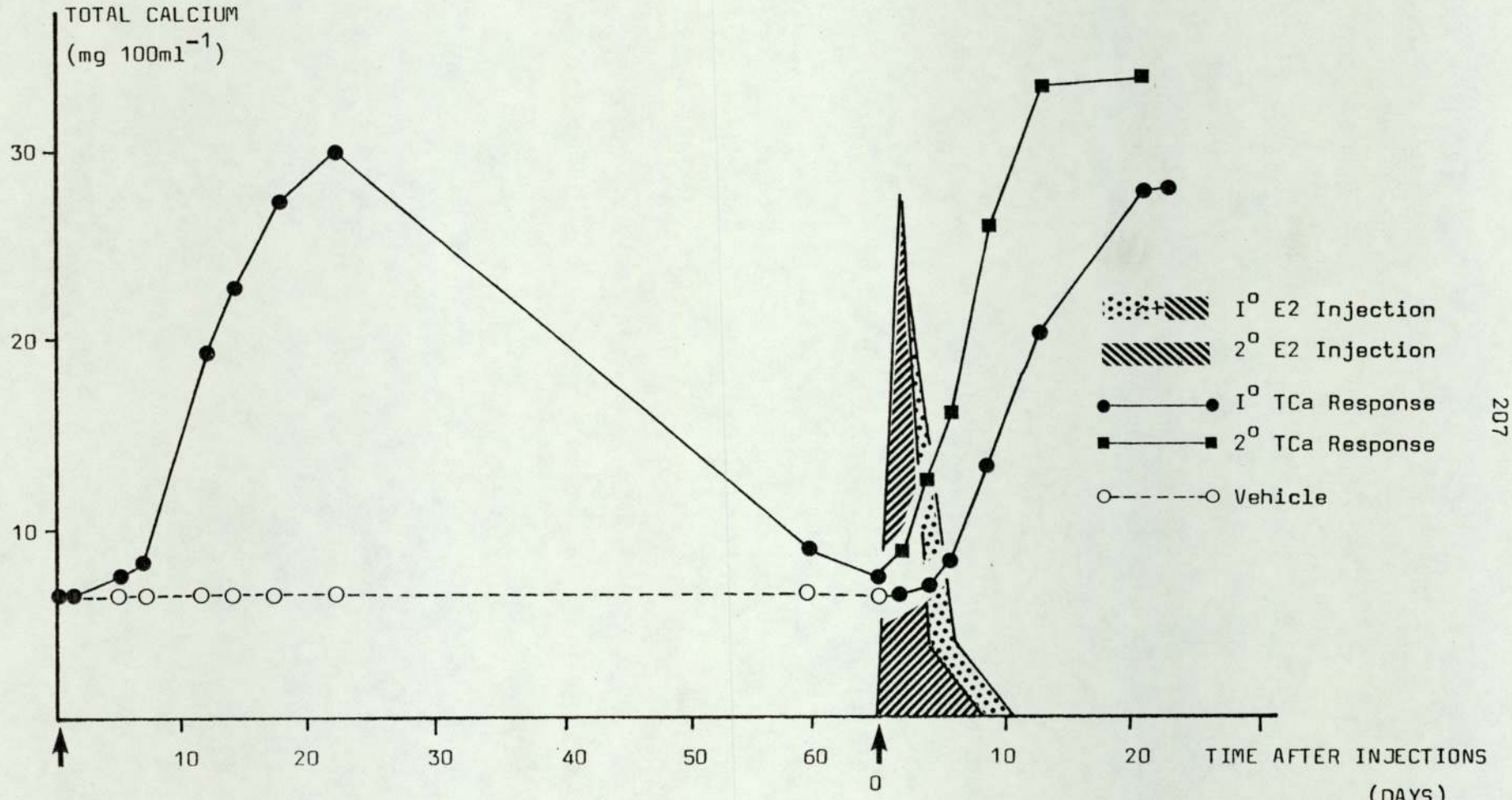


FIG. 3.52. SUMMARY OF PRIMARY AND SECONDARY RESPONSES TO THE SAME DOSE OF OESTRADIOL-17β (5 μg g^{-1})

IN PRIMED AND UNPRIMED GROUPS OF FISH.

in part explain why some workers have used either priming or multiple doses of oestrogens when investigating the response of the liver to this hormone (de Vlaming et al., 1977; Terkatin-Shimony & Yaron, 1978; Yaron et al., 1977). Although the phenomenon of primary and secondary induction has been previously recognised in birds (Beuving & Gruber, 1971) and in amphibians (Clemens, 1974; Ryffel et al., 1977; Knowland, 1980), only van Bohemen et al., (1982b) have reported a similar phenomenon in fish, and surprisingly oestrone was chosen to prime the liver in this study. After secondary induction, the lag-period between treatment and appearance of vitellogenin was virtually eliminated. Measurement of this lag-period in birds, which is considerably shorter than in amphibians (See Tata, 1978b; Knowland, 1980) has proved difficult because experiments have been carried out both in vivo and in vitro, and with assays of different sensitivity. Different lag-periods are also a feature of the induction of chicken egg-white proteins such as ovalbumin and conalbumin, often the time depending on whether it follows primary or secondary hormone treatment, or whether the hormone used is oestrogen or progesterone (Tata, 1978b). The difference between primary and secondary lag-periods was approximately 3 days in this work, where total calcium was used as an indirect estimate of vitellogenin.

The reason(s) for the differences in primary and secondary responses is still poorly understood. It may be explained by the rates at which the polyribosomes engaged in synthesising vitellogenin become functional (Tata, 1978b). During primary

induction a temporal gap has been observed between the accumulation of mRNA and immunoprecipitable polysomes, whereas after secondary induction the rate of appearance of vitellogenin is primarily a function of the rate at which mRNA appears in the cytoplasm (Tata, 1978b). It has also been noted that both primary and secondary inductions are dependant on de novo transcription of the vitellogenin gene (Farmer et al., unpublished data reported by Tata, 1978b). It was also reported in the same study that in Xenopus some rate-limiting mechanisms, or factors essential for specific translation of vitellogenin mRNA have to be established during the lag-period preceding primary induction. Such factors must therefore be relatively permanently 'imprinted' so that secondary stimulation then only involves a replenishment of the mRNA initially formed during primary stimulation, but subsequently degraded. This mechanism could be explained by one or more of the following reasons, which have previously been reported by Tata (1978a): firstly, a requirement for specific initiation or elongation factor(s); secondly, a requirement for special tRNA's, especially in view of the high serine content of the phosvitin moiety; and/or lastly, the establishment of a stable rough endoplasmic reticulum which preferentially facilitates both translocation of vitellogenin mRNA and post-translocational modifications of the nascent protein (Lewis et al., 1976). It is possible that the increase in the level of oestrogen receptor which follows primary induction with oestradiol-17β is maintained for a long time and that this reservoir of receptors is partly responsible for the more rapid secondary response (Westley & Knowland, 1978). It is known that the ultimate response is determined by both the concentration of

oestrogen receptors (Cidlowski & Muldoon, 1972; Lesniak & Roth, 1976; Schneider & Gschwendt, 1977) and their affinity coefficient for oestradiol (Westley & Knowland, 1978; Weichman & Notides, 1980). If increased and then stable levels of oestrogen receptor were established after primary induction, on reinduction with oestradiol -17β effectively more vitellogenin-committed hepatocytes would be activated (Bergink et al., 1974; Wallace & Bergink, 1974). The relative specificity of the oestrogen receptor and the effects of other hormones on the primary and secondary vitellogenic response are discussed below (See Chapter 5).

In a recent study van Bohemen et al., (1982b) administered oestrone daily over a period of 7 days to prime ovariectomised trout (Salmo gairdnerii). Immediately after this period the same fish were treated with oestradiol-17β and the vitellogenic response to this hormone compared to that seen after priming with saline or testosterone. Not surprisingly the oestrone-primed fish demonstrated a greater response, but detailed comparisons with the present work are difficult since no control group primed with oestradiol-17β was included. Thus, the present study is the first to unequivocally demonstrate the phenomenon of primary and secondary vitellogenic responses to oestradiol-17β in fish.

The finding that the time of year the fish are treated with oestradiol-17β significantly affects the vitellogenic response, both in terms of the amount of vitellogenin and the rate at which it is produced is of considerable physiological significance (Fig. 3.53.). The levels of hormone in the serum after injection with oestradiol-17B were the same in October as those in January,

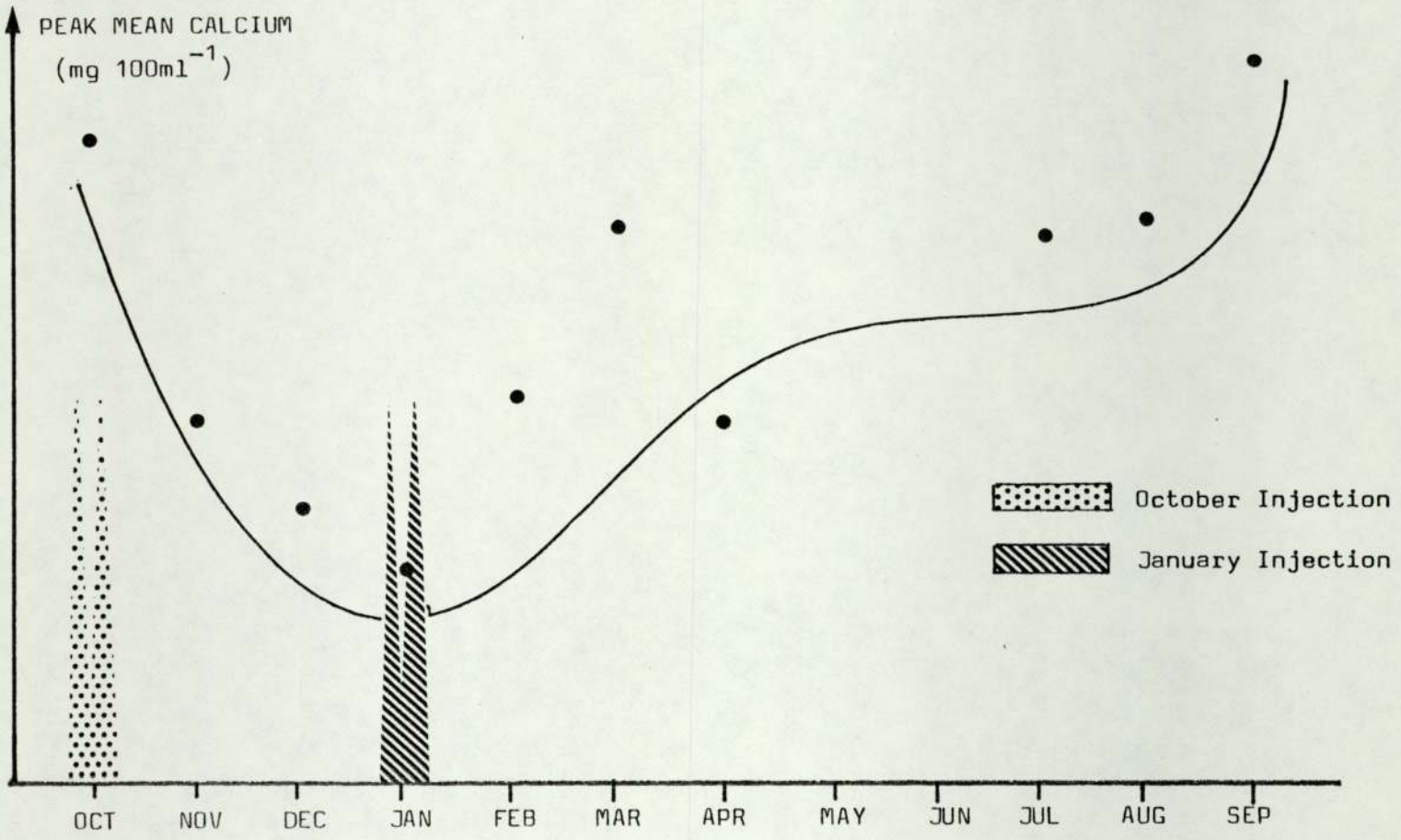


FIG. 3.53. SUMMARY OF SEASOANL VARIATION IN VITELLOGENIC RESPONSE AS EXPRESSED BY PEAK MEAN TOTAL CALCIUM OBSERVED AT EACH MONTH'S INJECTION.

despite the fact that the maximum and minimum vitellogenic responses occurred during these 2 months respectively. However, measurement of the serum levels of oestradiol-17β may only represent the relationship between uptake and clearance of the hormone and not be representative of physiological activity. Furthermore, it does not take into account either the levels or the stability of steroid-receptor interactions. It may be that degradation of the oestrogen-receptor complex is not as rapid in October as it is in January. As previously discussed, there are seasonal variations and sex differences in enzyme systems in the liver which are involved with the hepatic metabolism of steroids (Hansson et al., 1979; Hansson & Gustafsson, 1981; Koivasaari et al., 1981) and these variations may explain the seasonally different vitellogenic responses. Thus, 17-hydroxysteroid oxido-reductase activity has been shown to increase significantly during the spawning period of male but not female trout, whereas 6β-hydroxylase activity was significantly lower in maturing female trout when compared to juvenile and mature trout of both sexes. It is possible that the enhanced synthesis of vitellogenin in the liver of maturing female fish may interfere with the microsomal metabolism of steroids in the liver (Hansson & Gustafsson, 1981). Whether any such variations occur in immature fish is however not clear from these studies. However, it is likely that the differential rates of hepatic metabolism of steroids may, by indirectly controlling overall circulating serum levels, have profound effects on reproductive function.

Such differences in liver function are also indicated by the seasonal variation in hepatosomatic index of both immature and

maturing fish. In maturing female fish the lowest HSI was found in March, and the highest in September (Fig. 3.54.). Similar seasonal variations in HSI have been observed in the female trout during the normal seasonal cycle (van Bohemen et al., 1981b) where the HSI was lowest in March and highest in December. Seasonal relationships between the liver weight and body energy stores have also been described in a number of species (Love, 1970; Shul'man, 1974; Delahunty & de Vlaming, 1980). Thus, HSI values in many fish species are found to be highest during the prespawning period and lowest in post-spawning fish (Wingfield & Grimm, 1977; Htun-Han, 1978; Delahunty & de Vlaming, 1980; van Bohemen et al., 1981b). It has also been observed that there were no significant differences between the HSI of different weight fish at any particular time of the year and so HSI is an appropriate expression of liver size (Delahunty & de Vlaming, 1980). Considering the large quantities of yolk sequestered during ovarian recrudescence, the production of vitellogenin by the liver would ultimately have a severe drain on that organ's resources. Inverse correlations between liver and gonad weights have been found in several fish species (Zahnd, 1959; Larson, 1974; Wooton et al., 1978; Delahunty & de Vlaming, 1980). However, the correlation between liver weights and gonadal activity depends on the energetic requirements for recrudescence, feeding habits and food availability for the species in question (Shul'man, 1974; Htun-Han, 1978; Wooton, 1978). Despite its clear association with reproduction the HSI was also observed to vary seasonally in immature fish where gonadal recrudescence is minimal or absent. Undoubtedly, in the wild state, trout build up energy stores in times of plenty in

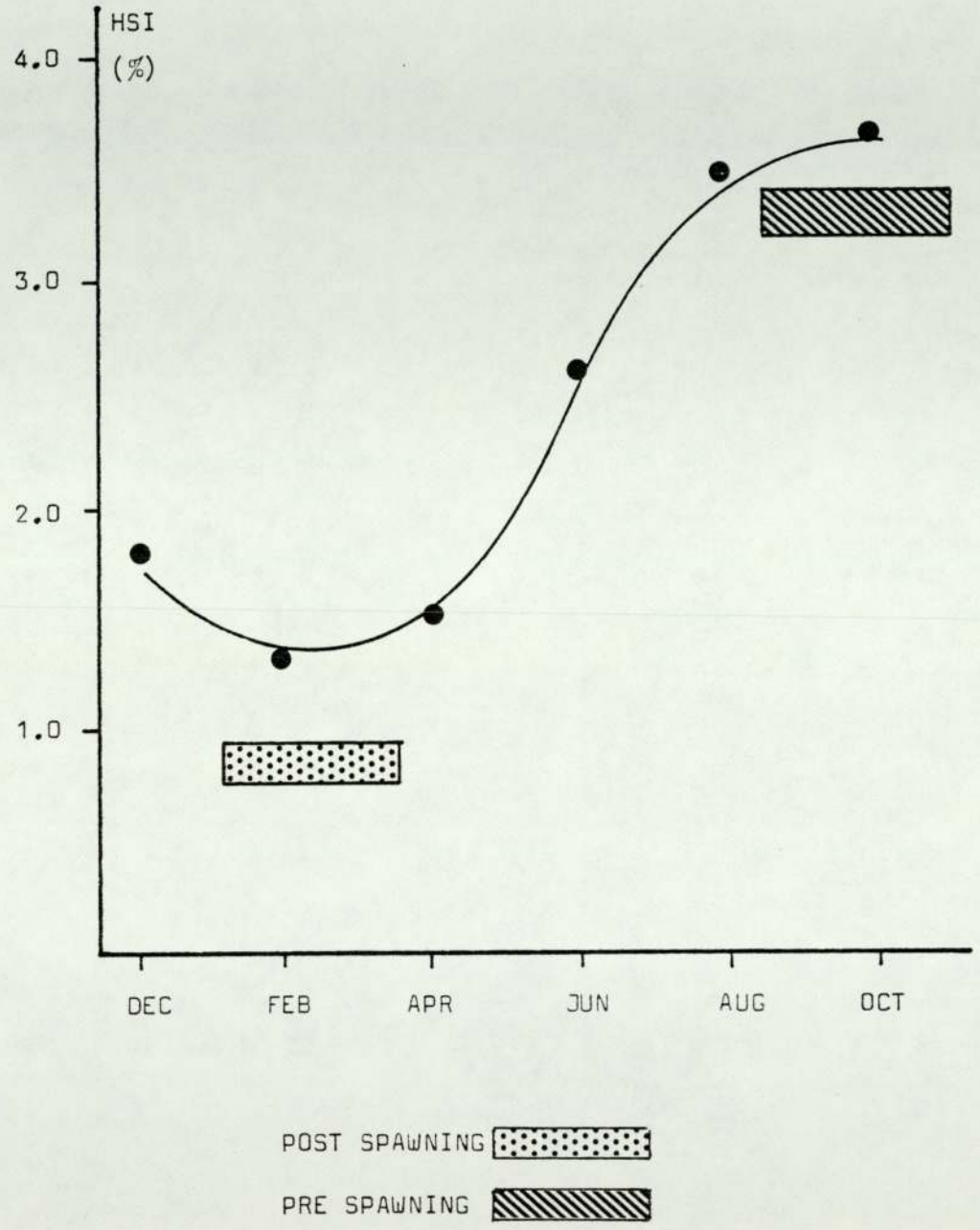


FIG. 3.54. SUMMARY OF SEASONAL VARIATION IN HEPATOSOMATIC INDEX (HSI%) IN MATURING FEMALE FISH DURING THE NORMAL SEASOANL CYCLE.

preparation for the winter months when food is at a minimum. However, the demonstration that HSI varied in fish under constant conditions, fed pelleted food to ration, suggests that trout may be adapted physiologically to amass stores during the summer or early autumn months. As such, this may also indicate that this change in HSI is not primarily linked to reproduction since it occurs in immature fish (Eliassen & Vahl, 1982a; 1982b). Certainly, it is unclear what effect these differences in HSI may have on vitellogenesis. The true effect of HSI on this response would be revealed by an investigation of vitellogenin production by liver slices cultured in vitro from fish 25 days after treatment with oestradiol-17β. It might be mentioned that preliminary data from in vivo experiments suggest that the vitellogenic response of post-spawned females is much reduced. Furthermore, the results reveal that the larger the fish the greater the vitellogenic response. In this instance the peak level of calcium and rate of synthesis are correlated with the weight of liver and not the HSI, since at the same point in time the HSI in different weight fish may not be significantly different.

Initially, experimental results suggested that the liver of trout in January was refractory to treatment with oestradiol-17β. However, later results clearly demonstrate a response by the liver to injected oestradiol-17β in both October and January, although there were marked differences in sensitivity to the hormone. Although doses between 0.01 and 40.0 $\mu g\ g^{-1}$ gave significant increases in October, in January the 3 lowest doses had no effects (0.01, 0.1 and 0.5 $\mu g\ g^{-1}$), even though similar serum levels of injected oestradiol-17β were observed during both these 2 months.

Furthermore, maximal vitellogenic responses of the same order as those seen in October were also obtained in January if doses of 20.0 $\mu g\ g^{-1}$ were used. Possibly, these differences in sensitivity constitute a form of refractory period similar to that which has been reported for other fish species (Sehgal & Sundararaj, 1970; Sundararaj & Sehgal, 1970a; 1970b; Baggerman, 1972; Kaya, 1973; Egami & Hosokawa, 1973). In many teleosts a period of gonadal insensitivity or refractoriness to environmental factors which would normally elicit ovarian development follows the breeding season. This ultimately prevents breeding at a time when the young may not survive, and may also provide a rest period for resources to be built up for the subsequent spawning cycle. Clearly, the failure of aseasonal gonadal development is equally as important as the cueing of normal development to coincide with favourable environmental conditions. Although it is unlikely that serum levels of oestradiol-17β would be high in this post-spawning period in trout, these data suggest that serum levels would need to be far higher than normal physiological values in order for the liver to respond and produce further amounts of vitellogenin. As such, this phenomenon may effectively form part of such a refractory period in this species. The presence of a refractory period demonstrates that the neuroendocrine-gonadal axis is not simply a passive system driven by the annual changes in environmental factors, but suggests that there is an underlying endogenous rhythm, or internal clock, which has to be synchronised with environmental change for reproduction to be initiated.

It has previously been reported that the dose of oestradiol-17β

is correlated with the vitellogenic response in amphibians (Wallace & Jared, 1968). These authors found that the rate of maximum synthesis and the time to reach the maximum level were directly proportional to the oestrogen administered. This is at variance with the present work where the highest dose did not give the greatest response. However, this is probably due to the toxicity of oestradiol-17β at high doses, since similar observations have been made by other authors working with Xenopus (Skipper & Hamilton, 1977) and trout (van Bohemen et al., 1981a). After the administration of different amounts of oestradiol-17β there was a significant correlation between dose and HSI. These results, together with histological data (See Chapter 6) provide confirmation that trout, like other oviparous species, synthesise vitellogenin in the liver. A comparison of fish injected with different doses of oestradiol-17β in October and January showed the HSI of all experimental fish except the 2 highest doses treatment groups were significantly higher than respective January ones. Pang & Balbontin (1978), found no effect of oestradiol benzoate on HSI in killifish at doses of 0.5 or 20.0 $\mu g\ g^{-1}$. Similarly, de Vlaming (1977) was unable to demonstrate any changes of HSI in 30-55g goldfish treated with 25 or 50μg of oestradiol per day, for 12 days. However, HSI more than doubled in the European eel 15 days after treatment with high doses of oestradiol-17β and was still increasing up to 78 days after treatment (Olivereau & Olivereau, 1979). A similar increase was observed in maturing Japanese eels treated with oestrogenic and gonadotrophic hormones (Ochiai et al., 1974), and in rainbow trout (van Bohemen et al., 1981b). Indeed oestrogens in general stimulate liver hypertrophy, for example in

Oryzias latipes, an implanted oestrone pellet resulted in a paler and softer liver after 7-9 days (Egami, 1955) and in the present work the higher doses of oestradiol-17β produced an externally visible swelling in the front belly region. The increase in HSI after oestradiol-17β treatment, supported by the observation that this increase is proportional to the dose administered, is clearly the result of the liver's increased metabolic activity during vitellogenin synthesis.

As well as having direct effects on the liver, one must also consider that oestradiol-17β, especially at high doses, may have direct or indirect effects on the ovary, which ultimately lead to the initiation of endogenous vitellogenesis, or the uptake of vitellogenin. Thus it is of interest to note that the present results show a significant correlation between the injected dose of oestradiol-17β and GSI in the female fish in both October and January. However, in October's treatment with oestradiol-17β, no group had a GSI significantly above that of controls. Furthermore, in January's treatments the 2 treated groups with significantly elevated GSI values, showed a reduction in mean body weight during the course of the experiment. This might suggest an anomaly in the results, in that gonad weight was unaffected, but GSI increased because the fish lost weight. Such difficulties have also been noted for other fish and it has been suggested that GSI is not an accurate measure of relative gonad size, or ovarian activity especially if there is an appreciable weight range of fish (Delahunty & de Vlaming, 1980). Furthermore, the relationship of gonad weight to body weight may vary between populations of the same

species as well as between different species. Even if fish of similar size are used, and the GSI considered to be a valid estimate of ovarian activity or size, it is suggested that histological examinations are essential in order to establish true gonadal activity (de Vlaming & Shing, 1977; de Vlaming & Vodicnik, 1978; Vodicnik et al., 1979). Without histological evidence it is difficult, in this present study, to describe what, if any effect, the injected oestradiol-17β has had on gonadal tissue. Some workers have noted a correlation between GSI and oestradiol-17β during the normal reproductive cycle (de Vlaming, 1977; Yaron et al., 1977; Lambert et al., 1978) and also after oestrogen stimulation (Olivereau & Olivereau, 1979). However, in both situations either no correlation, or negative ones have also been reported (Ho & Vanstone, 1961; Simon & Reinboth, 1974). The wide range of effects would appear to be due to differences in dose of oestradiol-17β, length of treatment, and relative maturity and species of fish.

Since oestrogens are recognised as being responsible for the maintenance of ovarian integrity in female fish, it might be expected that oestradiol-17β treatment would have some effects on the GSI. There is clearly a specific selection for vitellogenin by the presumptive vitellogenic oocytes, using a micropinocytotic process (Wallace et al., 1970; Wallace & Bergink, 1974). However, it is not clear which hormones are involved in controlling ovarian uptake of the complex. In a number of in vivo studies investigating the isolation and purification of gonadotrophins, Idler & Co-workers examined some aspects of vitellogenin uptake with hypophysectomised Winter flounder (Campbell & Idler, 1976; Ng & Idler, 1978a; 1978b; Idler & Ng, 1979). They observed

that the pituitary gland contained a factor(s), which was capable of stimulating the uptake of labelled vitellogenin from the blood into oocytes. It was not certain, however, whether this factor acted directly on the oocytes or indirectly via steroidogenesis. By studying this phenomenon *in vitro* with trout oocytes, it was subsequently found that pituitary gonadotrophin acts directly on oocytes in stimulating uptake without any steroidal facilitation (Campbell, 1978). Another study using immature trout further demonstrated that whole salmon pituitary extract, but not purified gonadotrophin, was capable of stimulating vitellogenin uptake (Upadhay *et al.*, 1978).

Further work on the purification of gonadotrophin using affinity chromatography on Concanavalin A sepharose (Con-A Sepharose), by Idler & Co. workers has demonstrated 2 distinct gonadotrophins from American plaice, winter flounder, carp and salmon (Campbell & Idler, 1976; 1977; Campbell, 1978; Ng & Idler, 1978a; 1978b; Idler & Ng, 1979; Ng & Idler, 1979). The pituitary gonadotrophins present in these 4 species were divided into a fraction that does not bind to the sepharose gel (Con A-1) and one that does (Con A-2). However, the biological activities of these 2 fractions are less separable, for example the Con A-2/glycoprotein rich fraction has recently been shown to affect vitellogenesis as well as maturation and ovulation. Thus, one hormone may have all the activities classically associated with gonadotrophin(s) in fish (Idler & Ng, 1979; Ng & Idler, 1979). Thus the existence of a gonadotrophic hormone which solely effects the uptake of vitellogenin by the ovary remains to be demonstrated.

A further major difficulty in the purification of fish gonadotrophin(s) for use with <u>in vitro</u> and bioassays is the likely contamination with thyroid stimulating hormone (TSH), since in fish like mammals, the chemical structure is similar to that of gonadotrophin(s) (Fontaine, 1969). Thus when investigating the biological activity of purified gonadotrophins, contamination with TSH could cause spurious results, especially since it has been suggested in goldfish that thyroid hormones are necessary for the completion of vitellogenesis (Hurlburt, 1977). Since no bioassay to determine TSH activity of fish gonadotrophins has been established, it is impossible to gauge the purity of pituitary preparations. It is of some interest to note that mammalian TSH binds to Con-A sepharose, equivalent to Idler & Co. workers Con A-2 gonadotrophic fraction. Furthermore, there is evidence from this (see Chapter 5) and other studies that other hormones may be involved in the uptake and/or synthesis of vitellogenin. It has been observed in goldfish that oestradiol-17β initiates the early stages of yolk accumulation and pregnenolone controls the latter stages (Khoo, 1979). Thus, since the role of fish TSH cannot be discounted in affecting uptake, it is of some significance that fish fed with a T_4 treated diet and then injected with oestradiol-17β showed much reduced serum vitellogenin levels when compared with fish on a control diet. In teleosts, as in elasmobranchs, the likely involvement of thyroid hormones in reproduction has long been recognised, though their precise role has not as yet been established (Pickford & Atz, 1957; Dodd & Matty, 1964; Dodd, 1975; Young, 1980). Evidence derives mainly from the close relationship that exists between thyroid and reproductive cycles (Bromage &

Sage, 1968; Ichikawa et al., 1974; White & Henderson, 1977; Osborn & Simpson, 1978). There is no doubt that the thyroid has an important role in the reproductive physiology of the teleost fish and that it may act on several different targets including the pituitary, gonads and liver, although the nature of its actions and the identity of specific targets and tissues has yet to be realised. There is some evidence to suggest that thyroxine enhances the ovarian uptake of vitellogenin (Lewis & Dodd, 1974; Hurlburt, 1977). Such an effect would explain the casual observation of lowered vitellogenin levels in fish which were pre-treated with a T_4 diet. Unfortunately, no histological examination of the ovary was taken to support this contention. However, a similar effect of thyroxine on the vitellogenic response was also noted by Bailey (1957) working with goldfish. He observed marked changes in total calcium and phosphorus in 250 g mature goldfish injected with 0.1-0.5 mg of oestradiol-17β. However, when thyroxine was administered at the same time as oestradiol-17β, no such changes were observed and no explanation for this phenomenon was offered. It is possible, however, that the increases in T_3 and T_4 observed in spring may have had some effect on the seasonal variation in response to oestradiol-17β injection. Certainly the serum T_3 levels recorded in the spring for the coho salmon are the highest concentrations found in any non-experimental vertebrate (Leatherland & Sonstegard, 1980). It may be that T_3 and T_4 have a more generalised metabolic influence rather than a specific effect on vitellogenic uptake.

Of profound importance in this work is the proportion of injected hormone which is absorbed and thus available to exert a vitellogenic

action. These data demonstrate that the higher the injected dose the lower is the proportion of uptake of steroid into the serum. It may be that there is a maximum rate at which the steroid can be taken up from the peritoneal cavity. It is also possible that at higher dose levels more hormone is lost by leakage from the injection site. In a study where 7 daily injections of oestradiol-17B were given to trout, only 0.05% of the total amount of steroid administered, remained one day after the last injection (van Bohemen et al., 1981a), these results are similar to those reported by Terkatin-Shimony & Yaron (1978). The relatively low serum levels shortly after treatment reported in these and the present work suggest that the clearance of oestradiol-17B is very rapid. Absolute discrepancies in clearance rate between this and other work may have occurred as a result of the vehicle used for injecting the steroid, since van Bohemen et al., used a saline vehicle. When an emulsion is used and the steroid is pre-dissolved in ethanol, the rate of uptake is partly dependant on the dissolution rate, as well as the dose. Thus the release would be slower and more controlled. Our experience with saline or oil only is that the steroid is only partly dissolved and treatment with such a heterogenous mixture could result in error. Thus, in real terms, the actual dose of hormone administered using saline, oil or oil/ethanol as vehicle may be subject to considerable variation.

It is evident from the results so far discussed that a single or double injection of oestradiol-17β at a dose of approximately 5 $\mu g\ g^{-1}$ in oil/ethanol is an effective method of inducing vitellogenin production in trout. Such responses could be used to influence the quality of eggs and the control of their production in

commercially farmed trout stocks. One problem faced by the trout farmer is the question of poor quality eggs from first-stripping fish. Such eggs from broodstock spawning for the first time are usually very small and unsaleable as eggs; furthermore, there may be problems with differential growth of the hatched alevins from the eggs especially if they are mixed with those derived from the eggs of broodstock at their 2nd or 3rd spawning. Clearly there would be significant economic advantages if a farmer could obtain eggs of normal size from first stripping fish by using simple hormonal manipulations. Currently, an investigation is being made of the effect of a single injection of oestradiol-17B on the size and quality of eggs at their first spawning if given to prospective broodstock at 1 year of age (ie 1 year before 1st stripping).

It is possible that the smaller size of first stripping eggs when compared with subsequent spawnings may be due to differences in primary and secondary responses to oestradiol-17β. Thus fish undergoing ovarian recrudescence for the first time may be more sensitive to changes in oestradiol-17β at the onset, if they have previously been primed with oestradiol-17B. Another possibility might be the use of oestradiol-17β as a supplement to the endogenous steroid production during the 8 or 9 months before the time of ovulation in trout. Treatment with oestradiol should increase the maximum amount of vitellogenin available from the onset of maturation and may subsequently improve egg quality. If oestrogen treatment were to be used commercially, the present results on the use of different types and proportions of vehicle indicate that further work is necessary on the different methods of application of this hormone, including administration of the steroid in the diet.

The results of this first series of experiments indicate that the physiology of vitellogenesis in trout is similar to that previously reported for other oviparous species. Certainly the apparent dissimilarities between fish, amphibians and birds are no greater than those that exist between different species of the same phylogenetic groups. Possibly of more importance will be studies of the structure of specific vitellogenins where more fundamental differences are likely to be uncovered. This is examined in more detail in the next chapter.

CHAPTER 4.

THE NATURE OF VITELLOGENIN IN TROUT SERUM AND A COMPARISON OF METHODS OF DETERMINATION.

4.1. Introduction.

A feature common to all oviparous vertebrates is the relatively large size of their eggs, necessary because of the demand this form of reproduction has on supplying an adequate energy store for the subsequent independent development of the young. Although the accumulation of glycogen and lipids (triglycerides, neutral lipids and fatty acids) supports a portion of the embryos' development, by far the majority of the embryos' energy requirements are met by the assimilation of the yolk-proteins lipovitellin and phosvitin. The presence of a large, heterogenous, female-specific protein in the serum during ovarian maturation and after oestrogen stimulation in oviparous species has now been recognised as the precursor to these yolk-proteins and this has been given the name vitellogenin (Pan et al., 1969). The origin of this large lipoglycophosphoprotein is the liver (Zahnd, 1959; Aida et al., 1973a, 1973b; Peute et al., 1978) from where the complex is transported to the ovary, sequestered by the oocytes in a specific micropinocytotic process (Droller & Roth, 1966; Korfsmeier, 1966; Anderson, 1968; Uhlrich, 1969) and subsequently broken down into its individual yolk proteins by proteolytic cleavage (Wallace et al., 1970). Much of the data on the nature of vitellogenin and its physiology arise from studies on amphibians and birds (Wallace, 1978), although recent evidence has shown that a similar scheme exists in teleosts (de Vlaming et al., 1980). However, there is further evidence to suggest that teleosts may differ in several ways to other oviparous groups, most especially with respect to the nature of the component parts that combine to make this large protein-complex.

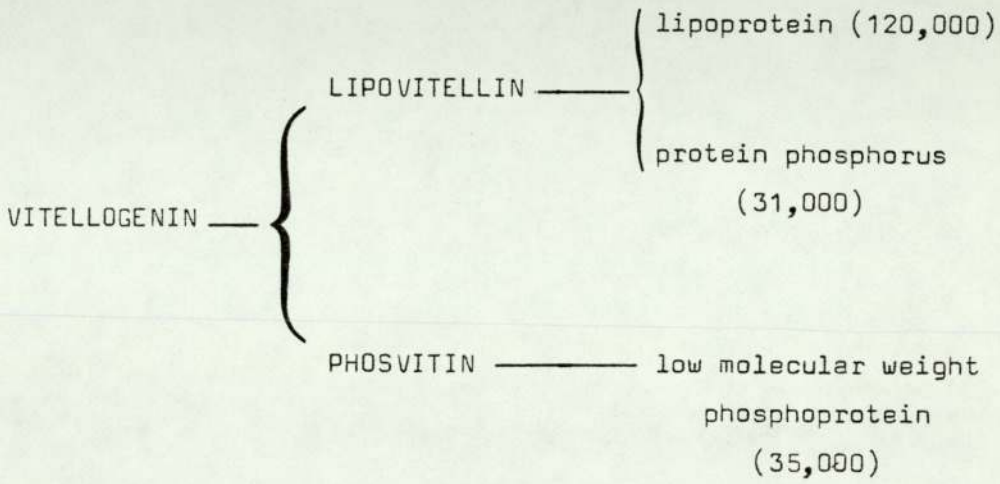
The presence of large amounts of phosphorus bound to the phosvitin portion of the complex, has been exploited as an indirect estimate of serum levels of vitellogenin in fish (Whitehead et al., 1978a; Campbell & Idler, 1980; Scott et al., 1980b). Chromatographic and electrophoretic procedures to characterize vitellogenin have also been used, mainly in work on amphibians and birds (Urist et al., 1958; Wallace et al., 1966; Christmann et al., 1977) but also more recently with teleosts (Campbell & Idler, 1980; de Vlaming et al., 1980; Hara et al., 1980) and such techniques have shown that the vitellogenins derived from different species show marked heterogeneity. Furthermore, there may even be multiple forms of vitellogenin in the same species (Wallace, 1978; de Vlaming et al., 1980; Wiley & Wallace, 1981). Among the oviparous vertebrates, the teleosts appear to hold a position of uniqueness both with respect to the structure of vitellogenin and to its physiology (Wallace, 1978). Thus, there are reductions in the phosphorylation of the complex in this group and also possibly the amounts of calcium which, like phosphorus, is also bound to the vitellogenin molecule (Hori et al., 1979. See Chapter 3). In addition, teleost vitellogenin appears highly susceptible to denaturation as demonstrated by its distinct lack of homogeneity after electrophoresis and chromatography. Indeed, though electrophoresis is an extremely sensitive method of determining serum levels of vitellogenin, the heterogeneity of the vitellogenin molecule has restricted its use to qualitative rather than quantitative procedures, although it has been used more recently to estimate absolute levels in rainbow trout (van Bohemen et al., 1981b). Similarly, traditional chromatographic methods that can adequately precipitate amphibian and bird vitellogenins fail to do so with

teleosts, and a more complicated approach is necessary (Wallace, 1978). With these factors in mind the present work includes a detailed comparison of the indirect methods of estimating vitellogenin ie. phosphoprotein phosphorus and calcium determinations, with a specific homologous radioimmunoassay for (intact) trout vitellogenin. Previously, other RIA methods to determine vitellogenin have relied on egg yolk-fractions or degradation products of the vitellogenin complex for their immunological cross-reactivity (Idler et al., 1979; Campbell & Idler, 1980). However, variation in the degree of protein denaturation in preparing the antigen especially in the serum samples to be measured could cause inconsistent results as a result of variable immunological responses. The use of intact trout vitellogenin as antigen to raise specific antisera with which to determine the serum levels of this large complex in the same animal must clearly be of some advantage.

Furthermore, by using the heterogenetic behaviour of vitellogenin under electrophoretic and chromatographic separation the phosphorus and calcium components could be related to the different fractions of the vitellogenin molecule. In this way, the relative sensitivity, accuracy and repeatability of the different methods could be assessed.

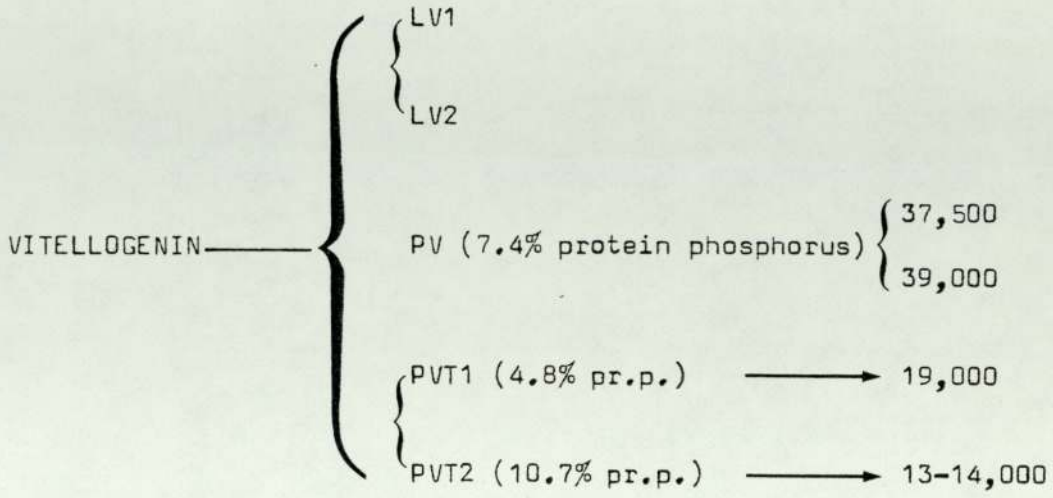
Although egg-yolk proteins, specifically phosvitin and lipovitellin, have been intensively studied and chemically well-characterised over the last 40 years, it has only recently been demonstrated that they originate from a common precursor (Follett & Redshaw, 1974; Wallace, 1978). Thus, studies of the nature of vitellogenin in hen-yolk (Joubert & Cook, 1958a, 1958b) and a number of other animal

species including trout (Fujii, 1960) showed that there is approximately twice as much lipovitellin as phosvitin in the vitellogenin molecule. Subsequently, the molecular weight of amphibian vitellogenin was shown to be approximately 400,000 (Wallace & Bergink, 1974; Wallace, 1978), existing as a dimer in the serum, with a monomeric weight of approximately 200,000 (See below).



However, recently it has been shown that the mRNA's coding for vitellogenins are composed of 2 families, each having at least 2 distinct sets of sequences (Wahli et al., 1979). Thus, in the amphibian model at least, it would seem that 4 different vitellogenin molecules could be produced. Subsequently, Wiley & Wallace (1981) in an extensive study of the structure of vitellogenins in Xenopus have further characterized the vitellogenic molecule into 5 major polypeptide fractions, the 3 already identified above, plus 2 completely new ones which they named the phosvette fractions. Furthermore, they divided each of the 2 lipovitellin fractions (LV1

& LV2) into 3 species, and the phosvitin proper fraction (PV) into 2 (See below).



PVT = Phosvette LV = Lipovitellin PV = Phosvitin

These authors suggest that these multiple yolk-proteins are the cleavage products of multiple vitellogenins and that the phosvitins and phosvettes represent alternate cleavage products arising from homologous regions of different parent vitellogenin molecules.

The recent evidence for multiple vitellogenins and yolk proteins may well explain the conflicting data regarding the size and number of the different components of the vitellogenin complex. The size and heterogeneity of this molecule together with the uniqueness of its constituent parts make it an extremely unusual protein, even more so in its multiplicity. For example phosvitin, which contains approximately 79% of the phosphorus in amphibian vitellogenin, is made up of 40% serine (esterified to phosphate) to which the majority

of the phosphorus is attached. The study of Wiley & Wallace (1981) indicates that insertions and deletions must have occurred to account for the observed multiplicity of vitellogenins. The high phosphorus content of these proteins has long been recognised as characteristic of vertebrate vitellogenins (Fujii, 1960) and acid-insoluble ^{32}P has been used as a specific indicator of vitellogenin (Wallace & Jared, 1968; Ansari et al., 1971). Because of the high levels of phosphorus in vitellogenin and the ease with which it is incorporated it is intended in this study to label trout vitellogenin with P^{32} by injecting a fish actively synthesising vitellogenin with the radioactive tracer. Thus, it would be possible to trace the intact vitellogenin and phosvitin fraction during different chromatographic procedures. Many studies of vitellogenesis have used serum levels of phosphoprotein phosphorus as indicators of vitellogenin (see Chapter 3; Ho & Vanstone, 1961; Emmersen & Emmersen, 1976; Emmersen & Petersen, 1976; Craik, 1978a; 1978b; Whitehead et al., 1978a; Elliott et al., 1979; Emmersen et al., 1979; Hori et al., 1979; Korsgaard & Petersen, 1979; Campbell & Idler, 1980). If one can calculate the % of phosphorus to be found in the specific vitellogenin under investigation, then if the serum levels of phosphorus are determined, one can estimate the serum vitellogenin levels by multiplying by the appropriate conversion factor. In the absence of further information on the levels of phosphoprotein phosphorus in trout, in our assay, phosphoprotein phosphorus levels are multiplied by 71.4, assuming 1.4% protein phosphorus as in other vertebrates. However, the proportion of phosphorus appears to vary greatly throughout the different vertebrate classes, and phosvitin in teleosts is both smaller and less phosphorylated (Jared & Wallace, 1968). For example,

in gold fish, cod and salmon there is 0.79, 0.75 and 1.6% of phosphorus respectively (Plack et al., 1971; Idler et al., 1979; de Vlaming et al., 1980). Clearly, the proportion of phosphoprotein phosphorus in the trout vitellogenin molecule must be established if the method of estimating vitellogenin by its phosphoprotein phosphorus content is to be of any real value. This can only be achieved by correlating serum values for phosphoprotein phosphorus with parallel determinations of vitellogenin by RIA of the same samples.

Apart from the level of phosphorylation (Fujii, 1960), teleost phosvitin varies in several other respects to other oviparous forms. For example, trout phosvitin is soluble in acid, whereas amphibian, reptilian, chicken and lamprey phosvitins show different degrees of acid insolubility. This acid solubility has been noted in other teleost species (Schmidt et al., 1965). Furthermore, in some teleosts the associated egg proteins are assembled in a soluble granular form, whereas amphibian yolk-proteins are laid down as insoluble crystals or yolk-platelets within the developing oocyte (Wallace, 1978). The reasons for the solubility of teleost vitellogenin may be related to a lower degree of phosphorylation in this group. It has been suggested that dephosphorylation of phosvitin occurs in the ovaries of teleosts at the onset of oogenesis (Mano, 1970) and considerably less protein-kinase (phosphorylating) enzyme has been found in trout and killifish ovaries when compared with amphibians (Jared & Wallace, 1968). The physiological reason for the greater solubility of vitellogenin may be related to the rapid absorption of water which occurs at this time, a phenomenon first observed by Fulton in 1898, which appears

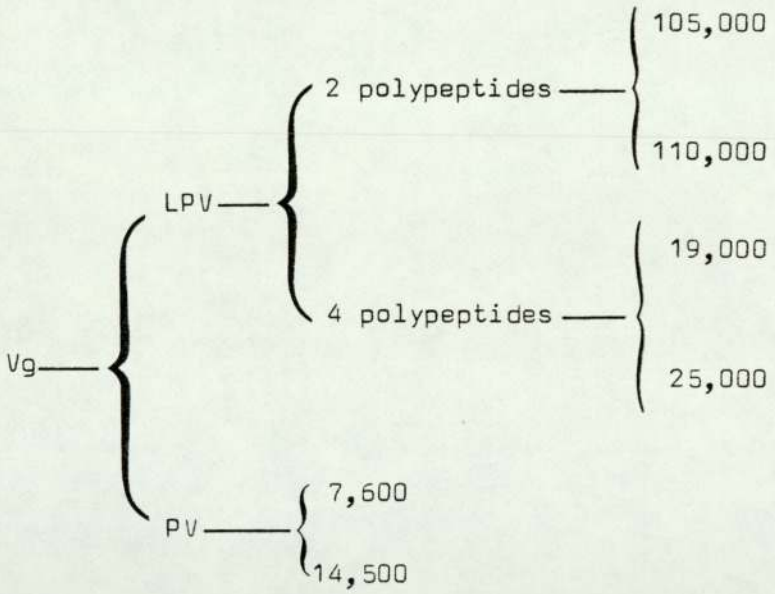
unique to most teleosts. After the accumulation of yolk-protein the oocytes of many teleosts undergo a second, relatively rapid enlargement concommittant with nuclear maturation. The relatively great accession of a watery fluid from outside the egg dissolves the yolk-spheres and is associated with the dissolution of the germinal vesicle and rearrangement of the chromatin for fertilisation, marking the completion of ovarian growth. This rapid physical or physico-chemical change is so pronounced in some marine species that the loss in density makes the eggs pelagic which provides a means of egg dispersal. It occurs to a lesser degree in demersal ovipositors like the trout, with a smaller quantity of fluid being absorbed and with a minimal effect on the yolk granules.

Another feature of phosvitins is their chromatographic heterogeneity, and it has been noted that phosphoprotein phosphorus (or P^{32}) values do not parallel absorbancy at 280μ in the phosvitin region, a feature recognised by other studies (Mecham & Olcott, 1949; Wallace, 1963; Barman et al., 1964). It was concluded that the phosphorylation system in teleosts may be less active and the resulting yolk proteins incompletely and heterogenously phosphorylated (Jared & Wallace, 1968). Furthermore, Mano & Lipmann(1966) have resolved discrete phosvitin subfractions from the ovary of several teleosts which appear to represent a similar protein with different levels of phosphorylation. This suggests a similarity with the phosvitins and phosvettes of the amphibian Xenopus which have also been shown to have different degrees of phosphorylation (Wiley & Wallace, 1981). Evidence that vitellogenins may also exist in more than one form in teleosts, has recently been demonstrated in the goldfish (de Vlaming et al., 1980). Different forms of vitellogenin may have separate

physiological roles either in their nutritional value to the egg, in their speed of uptake or in their availability at different stages of the reproductive cycle. For example, vitellogenin(s) produced in male fish after treatment with high doses of oestradiol-17β could conceivably be structurally different to vitellogenin(s) produced by female fish during normal maturation. Furthermore, the ratio of phosphoprotein phosphorus to vitellogenin (RIA) may not remain the same throughout the reproductive cycle especially if the cleavage of different parent molecules results in differentially phosphorylated yolk-proteins. There is evidence that TCA precipitation of the serum proteins, used as the first step in the method of phosphoprotein phosphorus determination, may not fully precipitate all the phosphorus component and consequently low estimates may result (de Vlaming et al., 1980). The same authors observe that this is particularly the case if a proteolytic inhibitor (eg PMSF) is not added to the serum at sampling to prevent denaturation of the intact molecule. Thus, the method used for phosphoprotein phosphorus in this study is compared with the homologous RIA not only to correlate absolute levels, but also to investigate whether the ratio of phosphorus to vitellogenin varies at different stages of the reproductive cycle and after oestrogen treatment.

Like phosvitin, the lipovitellin fraction in teleosts shows some differences when compared to other oviparous species. For example, teleost lipovitellin is more soluble in dilute saline and more heterogenous than the corresponding molecule from the hen, frog, dogfish and cuttlefish (Jared & Wallace, 1968). Furthermore, some of the components derived from yolk preparations seem to have neither the characteristics of lipovitellin nor phosvitin (Jared & Wallace,

1968) and one of these, the β component found in salmonids, contains no lipid or protein phosphorus (Markert & Vanstone, 1971). A recent study investigating the structure of vitellogenin in the goldfish has split the previously identified LV1 and LV2 (Lipovitellin) portions of the complex into 6 polypeptide species (de Vlaming et al., 1980). Furthermore, the same authors demonstrated that in goldfish, vitellogenin exists in 3 molecular weight forms, though normally as a dimer of 380,000 approximate molecular weight, with 15.4% protein nitrogen and 0.79% protein phosphorus (See below).



It was shown that the true nature of the intact vitellogenin molecule could only be determined if a proteolytic inhibitor (eg PMSF) was added prior to electrophoresis or chromatography (de Vlaming et al., 1980). Without this inhibitor large amounts of a lipovitellin-like protein (330,000) were isolated instead of the

vitellogenin dimer. Interestingly, Hori et al., (1979) found the molecular weight of the trout vitellogenin in its dimeric form was 330,000 and this was without the use of proteolytic inhibitors. However, such are the differences in vitellogenin between oviparous species that it is unlikely that the molecular weight of trout vitellogenin would be identical to that of goldfish, despite a similar structure. Indeed, the molecular weight of the standard used in the present radioimmunoassay was found to be approximately 500,000, with the monomer just less than half this figure (Sumpter, 1981). Similar figures of 600,000 (dimer) and 220,000 (monomer) have previously been observed in trout, and it has been tentatively suggested that vitellogenin monomers may combine into greater multiples than 2 (Hara & Hirai, 1978).

The observation that vitellogenin usually exists as a dimer but readily splits into its monomeric form, may lead to innacuracies when using a RIA to estimate serum levels of vitellogenin if a proteolytic inhibitor is not used. For example, if the antibodies were raised against the intact parent molecule, subsequent denaturation in vitro may artificially increase the number of antigenic sites and thus cause overestimation. However, this would depend on how easily in this case, trout vitellogenin splits up and the conditions under which the samples are taken and stored before assay. In view of this potential difficulty a comparison was made of the effects of the duration of the storage of the serum samples on vitellogenin levels.

The homologous radioimmunoassay used in this study is extremely

sensitive, measuring as little as 10 ng of vitellogenin. This enables it to be used with samples from male fish at different states of maturity and after oestradiol-17β treatment. One property attributed to vitellogenin is that it only occurs naturally in females but with much increased levels during ovarian maturation. This is somewhat surprising since males are clearly capable of producing the protein in response to oestradiol-17β treatment and there is evidence to suggest that in some teleosts males do possess oestrogens, sometimes at levels higher than females at a similar stage of development (Schreck et al., 1973; Schreck & Hopwood, 1974; Terkatin-Shimony & Yaron, 1978). Furthermore, other hormones, including testosterone in some teleosts, have been shown to have a certain degree of vitellogenic potency (Hori et al., 1979; See Chapter 5). However, in one electrophoretic study of vitellogenesis using oestrogen-treated male rainbow trout, it would appear that some vitellogenin-like protein is present in male fish at the time of injection (Campbell & Idler, 1980). However, the authors were unwilling to classify this as vitellogenin even though it develops electrophoretically coincident with vitellogenin bands from male serum after oestradiol-17β treatment. Although polyacrylamide-gel electrophoresis (PAGE) is an extremely sensitive method, in the majority of studies of oviparous vitellogenesis it has only been used qualitatively because of the tendency of vitellogenins to break up in vitro. However, because PAGE is so sensitive, very small serum volumes are used, in order to avoid overloading the gels with high serum levels of vitellogenin. Thus, the relative sensitivity is poor and small serum samples of a few microlitres may not visualise very low vitellogenin levels, as for example are present in males. A failure to realise that vitellogenins might be

present, albeit at very low levels together with the relative insensitivity of PAGE and other methods used, may be the reason for the reported absence of vitellogenin in most studies of male teleosts. The difference in sensitivities of these methods and the effects on the interpretation of results will be investigated in the present work.

If measurements of vitellogenin by RIA or by determination of phosphoprotein phosphorus content may be dependant on preservation of the intact vitellogenin molecule, then the indirect estimation of vitellogenin using calcium measurement may prove more reliable. Increased levels of serum calcium coincident with spawning and after oestrogen treatment have been noted in many studies of oviparous reproduction (Hess, 1928; Bailey, 1957; Fleming & Meier, 1961; Ho & Vanstone, 1961; Urist & Schjeide, 1961; Clark & Fleming, 1963; Booke, 1964; Fleming et al., 1964; Woodhead, 1968, 1969; Balbontin et al., 1978; Whitehead et al., 1978; Scott et al., 1980b). Bailey (1957) suggested that calcium is bound to the serum protein vitellin and hence these increased levels of calcium reflect an increase in the bound or unfilterable fraction (See Chapter 3). Clearly it is unlikely that such high levels of calcium in the serum (up to 100mg100 ml^{-1}) would be in the free form, since high levels of this component would lead to severe ionic disturbances (Hoar, 1973). Subsequently, it has been demonstrated that calcium is bound to the vitellogenin molecule in Xenopus and is present in an equivalent ratio with protein phosphate groups (Wallace, 1970). However, it was observed that the proportion of calcium in goldfish vitellogenin appeared to be about that of that in Xenopus, although these low levels are thought to be related to the relative ease with which calcium is

lost from the vitellogenin molecule in teleosts (Wallace, 1970; Ansari et al., 1971; Hori et al., 1979). Differences in the relative proportions of calcium in vitellogenins of different species further emphasises the heterogeneity of this large complex. The origin of vitellogenin-bound calcium varies with the degree of organisation of bone and thus in cellular-boned fish (eg eel) the calcium is derived from the bone (Lopez et al., 1976) whereas in acellular-boned fish such as the killifish, it comes from the scales (Mugiya & Watabe, 1977). Functionally, this divalent cation is thought to solubilize the vitellogenin complex in the serum as well as provide a source of this substance for the developing embryo. Although it is not entirely certain whether all or only a proportion of this calcium enters the oocyte when vitellogenin is taken up, it may be the forerunner to a mechanism for producing a calcareous-shelled egg which is widely found in higher oviparous species. Thus in the present work a correlation will be made between changes in calcium, phosphoprotein phosphorus and those of vitellogenin (RIA), to evaluate the potential accuracy of measurement of calcium as an index of vitellogenin. Furthermore, it is intended to show conclusively that it is the bound fraction that increases during maturation and after oestrogen stimulation, and that the protein to which this calcium is bound is vitellogenin.

Of all the many different characteristics attributed to vitellogenins, their large molecular weight is probably the most unique. However, the heterogeneity of this protein, a consequence of its large molecular weight, has complicated the identification, purification and characterization of this lipoglycophosphoprotein,

to such a degree that few studies have made direct measurements of vitellogenin, but have relied on indirect estimates by determining phosphoprotein phosphorus and calcium levels in the serum. The use of these indirect methods has in turn relied on data from essentially bird and amphibian studies on vitellogenesis, whereas more recent data with fish suggest there may be distinct and significant differences in the levels of these elements in teleosts. Subsequently, this series of experiments was designed to compare the sensitivity, accuracy and reliability of the different methods available to measure vitellogenin in rainbow trout and to show if indirect methods can give valid estimates of vitellogenin in teleosts, in that they truly reflect changes in this large protein complex.

4.2. A Comparison of Direct (Homologous RIA) and Indirect (Phosphoprotein phosphorus and calcium) Methods of Vitellogenin Determination in Trout Serum.

The aim of this series of experiments was to compare both the absolute levels and the profile of changes in vitellogenin determined by using the different methods outlined in section 2.2.4.. Thus, in serum samples taken from a variety of fish, levels of vitellogenin determined by specific homologous radioimmunoassay for intact trout vitellogenin, are correlated both with total serum calcium and estimated vitellogenin (by multiplying phosphoprotein phosphorus levels by 71.4).

4.2.1. Materials and Methods.

Serum samples were taken from the following groups :-

(a) Immature male and female fish (less than 1 yr old).

(b) Female fish after a previous spawning, but before the onset of the next.

(c) Female fish at different times during ovarian maturation.

(d) Male fish at different times after induction of vitellogenesis by injection with E2 (5 $\mu g\ g^{-1}$).

The serum samples were assayed for vitellogenin by radioimmunoassay (VgRIA), total calcium (TCa), phosphoprotein phosphorus (PP) and estimated vitellogenin (VgE) calculated from PP by multiplying by 71.4. Correlations of these determinations were made by the method of least squares fit.

In addition, a group of 5 fish were selected at random, weighed, tagged and injected with E2 at a dose of 5 $\mu g\ g^{-1}$ body weight. These

fish were blood sampled at the time of injection and subsequently at 2,5,8,11,21,36 and 48 days thereafter. The more frequent sampling procedure provided a detailed investigation of the differences in sensitivity of the different methods of vitellogenin determination.

4.2.2. Results.

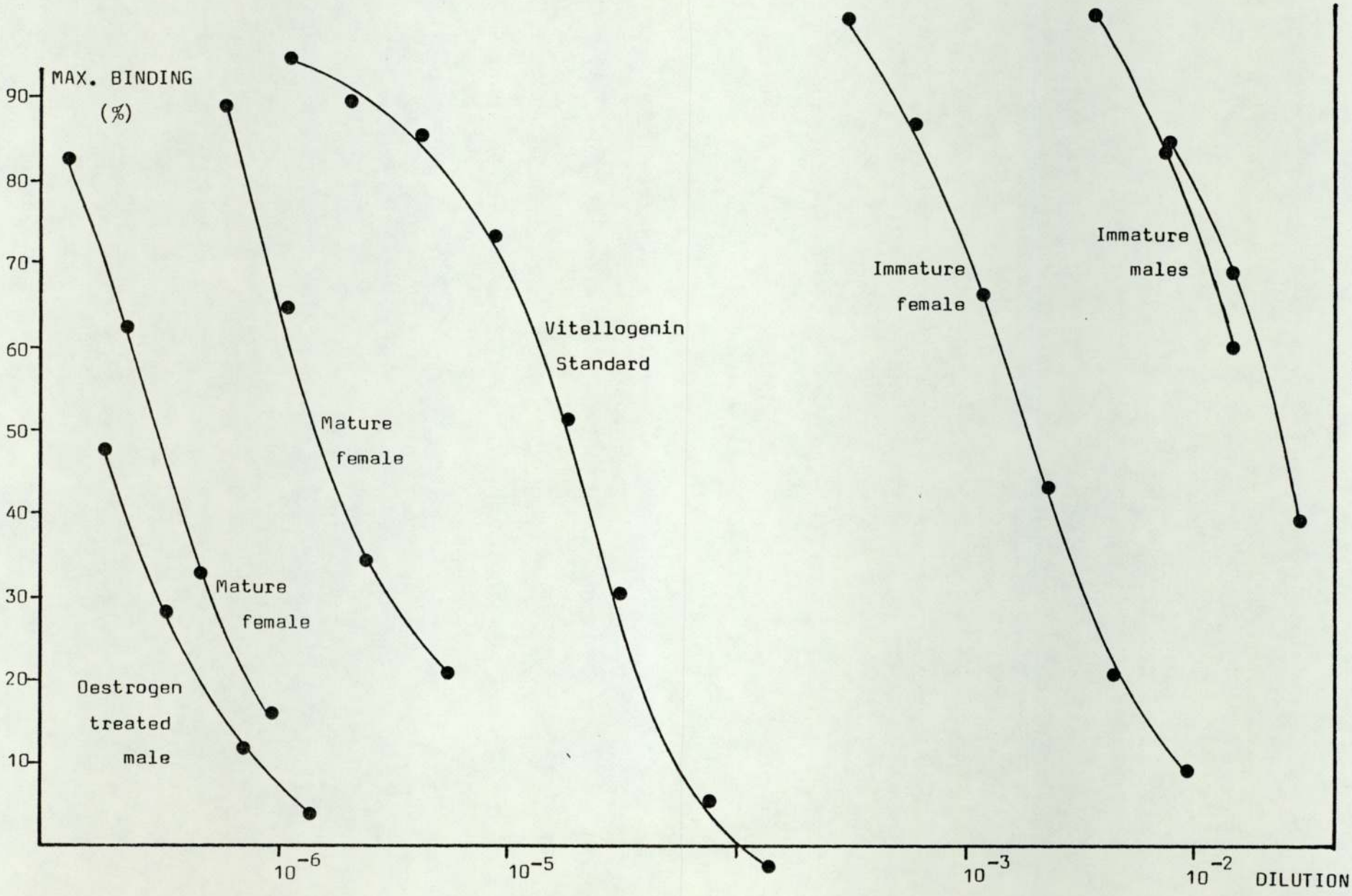
Determination of serum VgRIA levels in male and female trout of different age and maturity demonstrate a 10^6 fold range in absolute levels (Table 4.1. & Fig. 4.1.). Surprisingly, vitellogenin was found in some untreated 1yr male fish at levels not too dissimilar from those of immature females (See Table 4.1.). However, in other male fish studied (not shown in Table 4.1.) VgRIA was undetectable. These immature male fish demonstrated the lowest levels (circa 130-290 ng ml^{-1}) with immature females of a similar age and size having slightly higher levels (0.86 - 1.45 $\mu g\ ml^{-1}$). Serum vitellogenin levels in immature female fish, between spawnings, were significantly ($P < 0.001$) higher than immature females which had not previously reached maturity. In mature female fish and male fish injected with E2, very high levels of vitellogenin were observed up to a maximum of 215 mg ml^{-1}. Serial dilutions of the serum samples from all the above groups of fish showed complete parallelism with the vitellogenin standard used in the assay (Fig. 4.1.).

Some individual values for TCa, PP and VgE are compared in Table 4.1.. VgE levels as determined indirectly via PP levels, are approximately half the levels determined by RIA (VgRIA).

TABLE 4.1. VITELLOGENIN LEVELS (MEASURED BY RIA) IN IMMATURE AND MATURE FEMALES, AND IN IMMATURE AND OESTRADIOL -17β TREATED MALE TROUT; COMPARED WITH ESTIMATED VITELLOGENIN (DETERMINED INDIRECTLY BY PHOSPHOPROTEIN PHOSPHORUS) AND TOTAL SERUM CALCIUM.

	Vg (RIA)	Ca^{2+} (mg $100ml^{-1}$)	PP (μg ml^{-1})	VgE(mg ml^{-1})
Immature ♂	130.0 (ng ml^{-1})	8.18	5.9	0.42
	170.0 "	8.2	7.4	0.53
	290.0 "	8.3	7.5	0.54
Immature ♀	0.86 (μg ml^{-1})	8.25	10.2	0.73
	1.05 "	8.4	14.0	1.0
	1.45 "	8.37	8.9	0.64
Immature ♀	255.0 (μg ml^{-1})	13.79	16.3	1.2
	390.0 "	12.61	18.1	1.3
	800.0 "	11.63	21.8	1.56
Mature ♀	58.0 (mg ml^{-1})	44.32	379.0	27.2
	126.0 "	72.3	732.0	52.3
	140.0 "	83.4	868.0	62.1
	175.0 "	101.09	1193.0	85.2
	215.0 "	117.98	1297.0	92.6
Oestradiol Injected ♂	170.0 (mg ml^{-1})	77.08	754.0	53.9
	200.0 "	99.44	1147.0	81.9
	205.0 "	76.22	766.0	54.7

FIG. 4.1. PARALLELISM OF STANDARD CURVE FOR VITELLOGENIN RIA WITH SERIAL DILUTIONS OF SERUM FROM IMMATURE AND MATURE FEMALE AND IMMATURE AND OESTRADIOL-17β INJECTED MALE TROUT.


MAX. BINDING
(%)
90
80
70
60
50
40
30
20
10
Oestrogen
treated
male
Mature
female
Mature
female
Vitellogenin
Standard
Immature
female
Immature
males
10^{-6}
10^{-5}
10^{-3}
10^{-2}
DILUTION

The differences in sensitivity of the methods used is shown in the results on Table 4.2.. Mean serum levels of VgRIA, increased significantly ($P < 0.01$) from a basal level of 0.09 ± 0.03 mg ml^{-1} to 5.07 ± 1.5 mg ml^{-1} after only 2 days; reaching a peak of 85.0 ± 16.3 mg ml^{-1} 36 days after the injection. In contrast, serum levels of VgE rose significantly ($P < 0.001$) from 0.71 ± 0.3 mg ml^{-1} at the time of injection, to 4.21 ± 0.5 mg ml^{-1} after 11 days, at a time when VgRIA was determined at 22.3 ± 2.3 mg ml^{-1}. However, mean serum levels of VgE reached a peak of 45.7 ± 9.5 mg ml^{-1} at 36 days, at the same time as VgRIA. Mean TCa levels fell over the first 2 days from a basal value of 10.4 ± 0.2 to 9.9 ± 0.3 mg $100ml^{-1}$, rising significantly ($P < 0.05$) to 12.5 ± 0.7 mg ml^{-1} after 8 days and reaching a peak of 61.0 ± 8.3 mg $100ml^{-1}$ after 36 days. Serum levels of PP rose significantly ($P < 0.001$) from a basal value of 9.9 ± 3.8 μg ml^{-1} to a peak of 640.0 ± 160.0 μg ml^{-1} also 36 days after injection (Fig. 4.2.).

The ratio of VgRIA to TCa varied from 0.9:1 at 5 days, to 1.4:1 at 36 days, whereas the ratio of VgRIA to VgE varied from 0.1:1 at basal to 1.9:1 at 36 days. The ratio of TCa to PP was approximately 1:1. The differences in sensitivity of the different methods may be partly responsible for the variation in these ratio's. These VgRIA determinations were carried out on serum that had been stored at $-20^{o}C$ for up to 2 months.

Serum VgRIA levels were significantly correlated ($P < 0.001$; $r=0.98$; $n=8$) with VgE levels after E2 injection. Thus, although there was a 2-fold difference in absolute values between VgRIA and VgE methods, the profile of changes was clearly correlated.

Similarly, TCa levels were significantly correlated with PP ($P < 0.001$; $r=0.98$; $n=89$) and VgRIA ($P < 0.001$; $r=0.96$; $n=8$) in male fish, after treatment with E2 (Fig. 4.2.).

Serum levels of TCa were significantly correlated with VgRIA ($P < 0.001$; $r=0.99$; $n=9$) during the normal reproductive cycle of female fish (Fig. 4.3.). It ought to be mentioned that the analyses for vitellogenin RIA were performed on samples that had been stored for up to 9 months with repeated freezing.

	DAYS AFTER INJECTION							
	0	2	5	8	11	21	36	48
VgRIA (mg ml^{-1})	0.09	5.07	8.9	14.0	22.35	46.8	85.0	77.5
$\pm$ S.E.	0.03	1.5	2.2	3.0	2.3	8.4	16.3	20.2
Calcium (TCa mg $100ml^{-1}$)	10.4	9.9	10.0	12.5	14.9	33.1	61.0	55.5
$\pm$ S.E.	0.2	0.3	0.4	0.7	1.0	6.7	8.3	7.5
VgE (mg ml^{-1})	0.71	0.87	1.1	1.44	4.21	18.9	45.7	36.8
$\pm$ S.E.	0.3	0.1	0.4	0.2	0.5	3.0	9.5	11.2
PP (μg ml^{-1})	9.9	12.2	15.1	20.2	58.9	264.0	640.0	514.5
$\pm$ S.E.	3.8	4.1	4.3	2.6	6.5	77.7	160.8	133.5

TABLE 4.2. COMPARISON OF SENSITIVITY OF DIRECT (VgRIA mg ml^{-1}) AND INDIRECT (VgE, PP & TCa) METHODS FOR DETERMINATION OF VITELLOGENIN IN TROUT SERUM TAKEN AT DIFFERENT TIMES AFTER INJECTION WITH OESTRADIOL-17β (E2 5 μg g^{-1}).

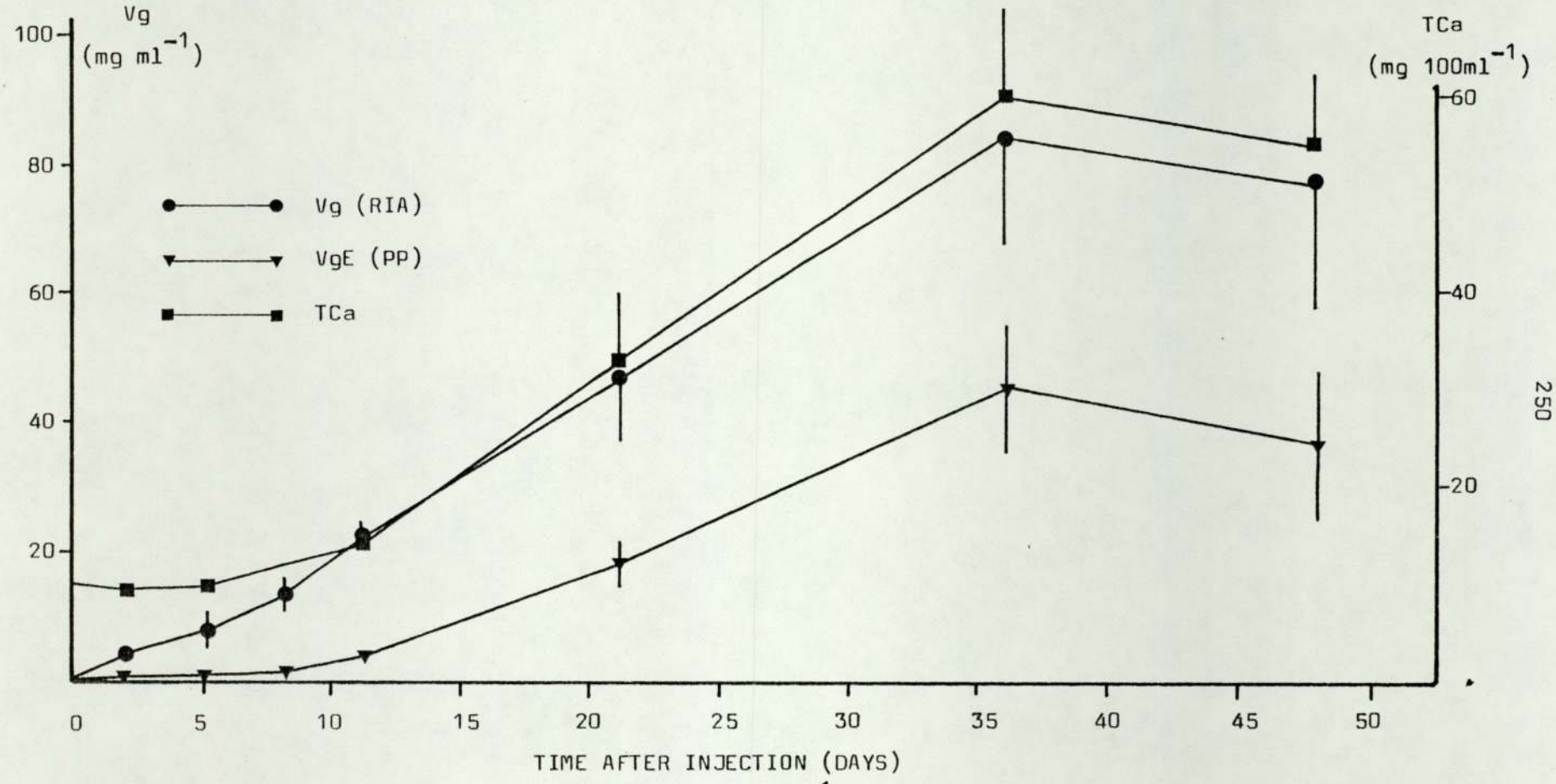


FIG. 4.2. COMPARISON OF SENSITIVITY OF DIRECT (VgRIA mg ml^{-1}) AND INDIRECT (VgE, PP & TCa) METHODS FOR DETERMINATION OF VITELLOGENIN IN TROUT SERUM TAKEN AT DIFFERENT TIMES AFTER INJECTION WITH OESTRADIOL-17β (E2 5 μg g^{-1}).

	MONTH					
	JUN	JUL	AUG	SEP	NOV	DEC
Vitellogenin (VgRIA mg ml^{-1})	6.6	44.3	128.6	195.0	42.0	1.1
± S.E.	1.7	6.0	31.5	23.0	14.5	0.2
Total Calcium (TCa mg $100ml^{-1}$)	15.0	23.5	60.5	103.5	27.0	12.0
± S.E.	0.5	1.5	13.0	11.0	7.5	0.7

TABLE 4.3. COMPARISON OF VITELLOGENIN LEVELS AS DETERMINED BY RIA (VgRIA mg ml^{-1}) WITH MEASUREMENT OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) AS AN INDIRECT ESTIMATE OF THE PROTEIN.

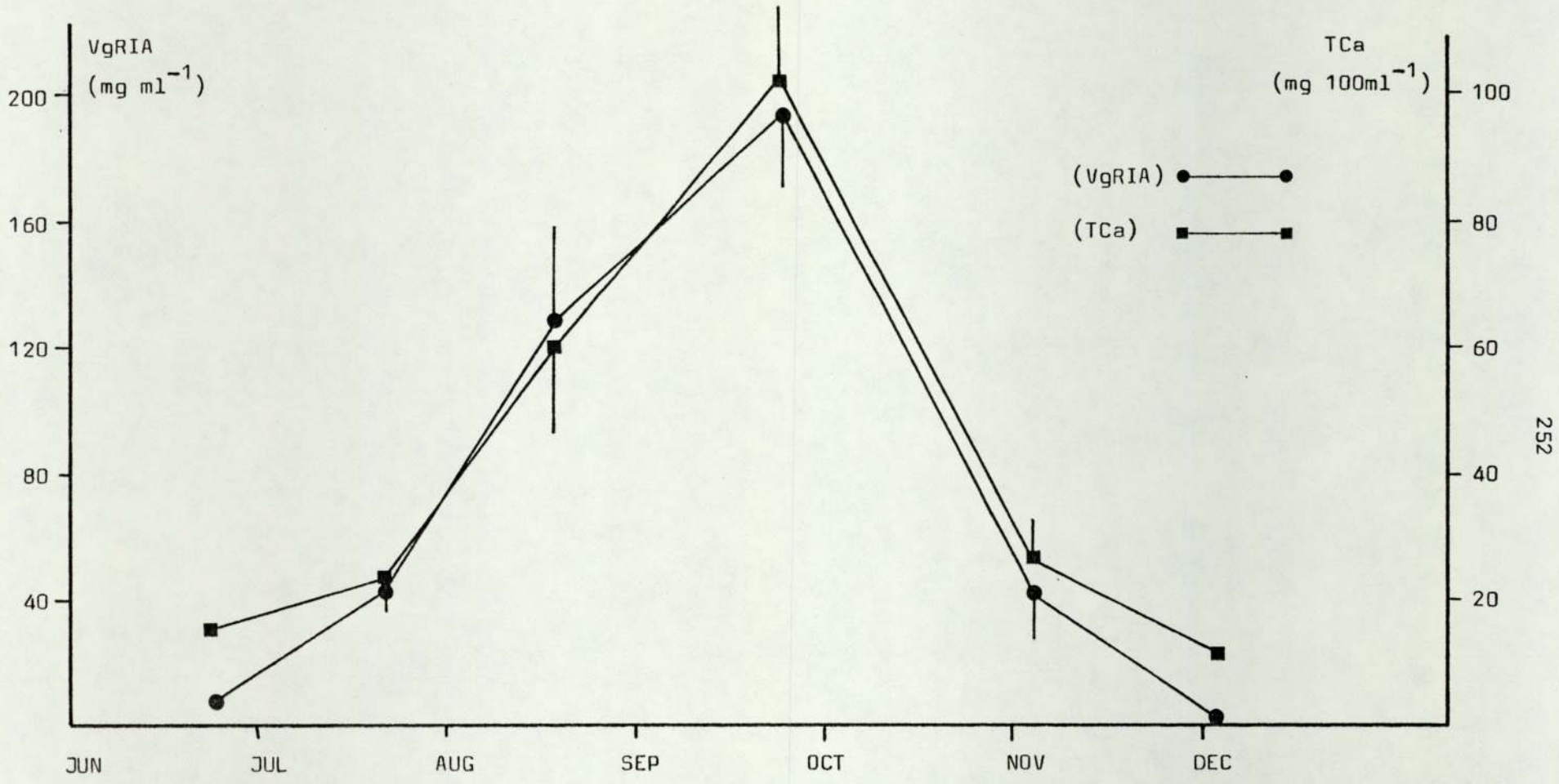


FIG. 4.3. COMPARISON OF VITELLOGENIN DETERMINATIONS DIRECTLY BY RIA (VgRIA mg ml^{-1}) AND INDIRECTLY VIA TOTAL CALCIUM (TCa mg 100ml^{-1}) IN RAINBOW TROUT SERUM TAKEN DURING THE REPRODUCTIVE CYCLE ($P < 0.001$; $r = 0.99$).

4.3. A Comparison of the Serum Protein Profile of Immature Male fish Treated with Oestradiol-17β (E2) and Mature Female Serum, using Polyacrylamide-Gel Electrophoresis.

The aim of this series of experiments was to investigate by electrophoresis the lipid and protein character of the vitellogenin molecule in E2-treated immature male fish and mature female fish. This method, although used qualitatively in this work, is very sensitive, and it was a further aim to see if vitellogenin could be identified in immature male fish providing sufficient volumes of serum were added.

4.3.1. Materials and Methods.

The methods used are as outlined in Chapter 2 (See 2.2.4.4.). A group of immature rainbow trout were selected at random and injected with E2 at a dose of 5 $\mu g\ g^{-1}$ in arachis/ethanol suspension. All fish were tagged and blood sampled at the time of injection and at 2,7,9,21 and 28 days thereafter. A similar group of fish injected with vehicle alone acted as controls. At the end of the experiment, the fish were sacrificed and the male fish identified by examination of the gonads under binocular microscope. The serum samples taken from male fish during the course of the experiment were run on the Shandon gel electrophoresis system and compared with male (control) sera. Serum samples were also taken from immature and mature female trout and similarly treated for comparison. Gel-columns were stained with either Sudan black (lipid-staining), fast-green (protein) or alizarin-redS (calcium) by the methods outlined in 2.2.4.4.. The stained portions of the fixed gels were further quantified with a linear laser/white light densitometer. A sample

of the vitellogenin used as standard in the radioimmunoassay was run on the columns for reference and then stained with fast-green.

4.3.2. Results.

The sudan-black stained gels of serum samples from E2-treated male fish were distinguished from the controls by the appearance of 2 new bands (V3, R_f 0.07 - 0.084; V2, R_f 0.15 - 0.18) and by an increase in density of an existing band (V1, R_f 0.29 - 0.39) which was variably present in all fish tested (Fig. 4.4.). A similar lipid pattern was observed in serum samples taken from mature female fish (Fig. 4.4.). This was confirmed by the quantification of these stained gels on the densitometer (Fig. 4.5.). No such change in banding was observed in control fish (Fig. 4.6.).

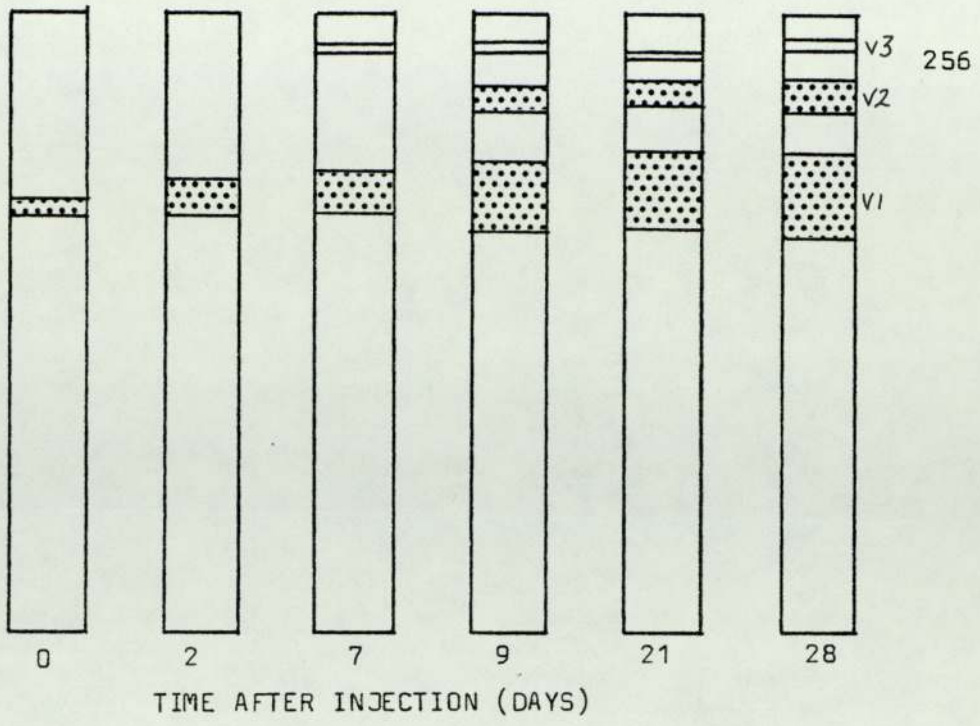


FIG. 4.4. (a) ELECTROPHORETIC SUDAN-BLACK STAINED SERUM PROFILE OF A MALE FISH INJECTED WITH OESTRADIOL-17β ($5\ \mu g\ g^{-1}$).

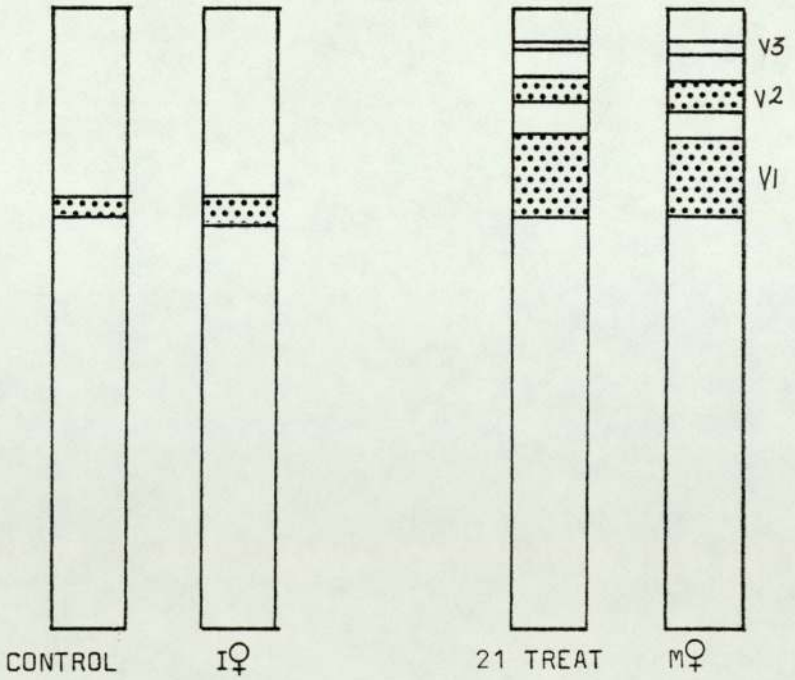


FIG. 4.4. (b) COMPARISON OF SERUM PROTEIN PATTERN OF CONTROL WITH IMMATURE FEMALE RAINBOW TROUT, AND 21 DAY E2-TREATED FISH SAMPLE WITH MATURE FEMALE TROUT.

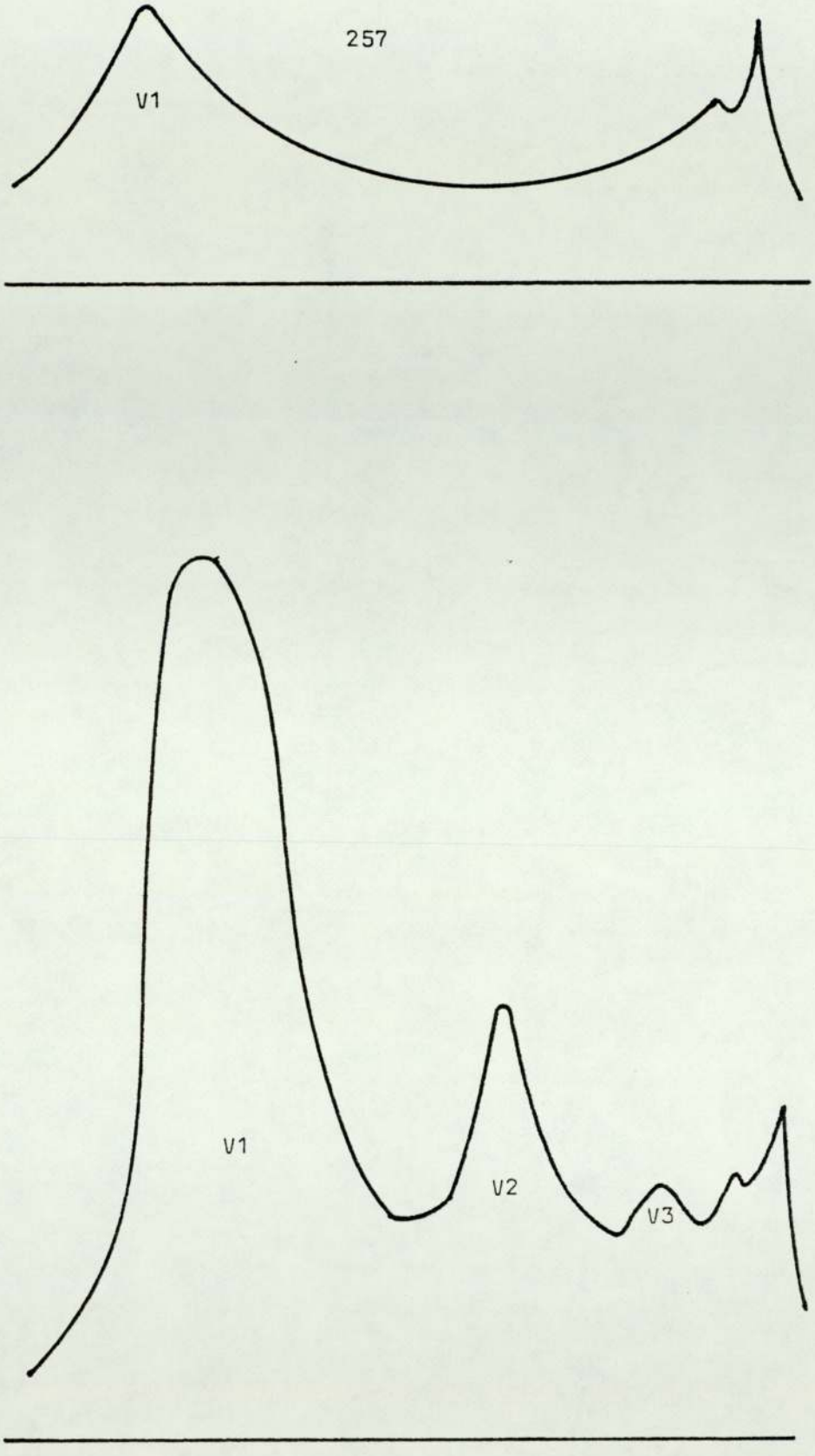


FIG. 4.5. DENSITOMETER TRACE OF GELS FOR SERUM TAKEN FROM TREATED FISH AT BASAL (TOP) AND 21 DAYS AFTER INJECTION (BOTTOM).

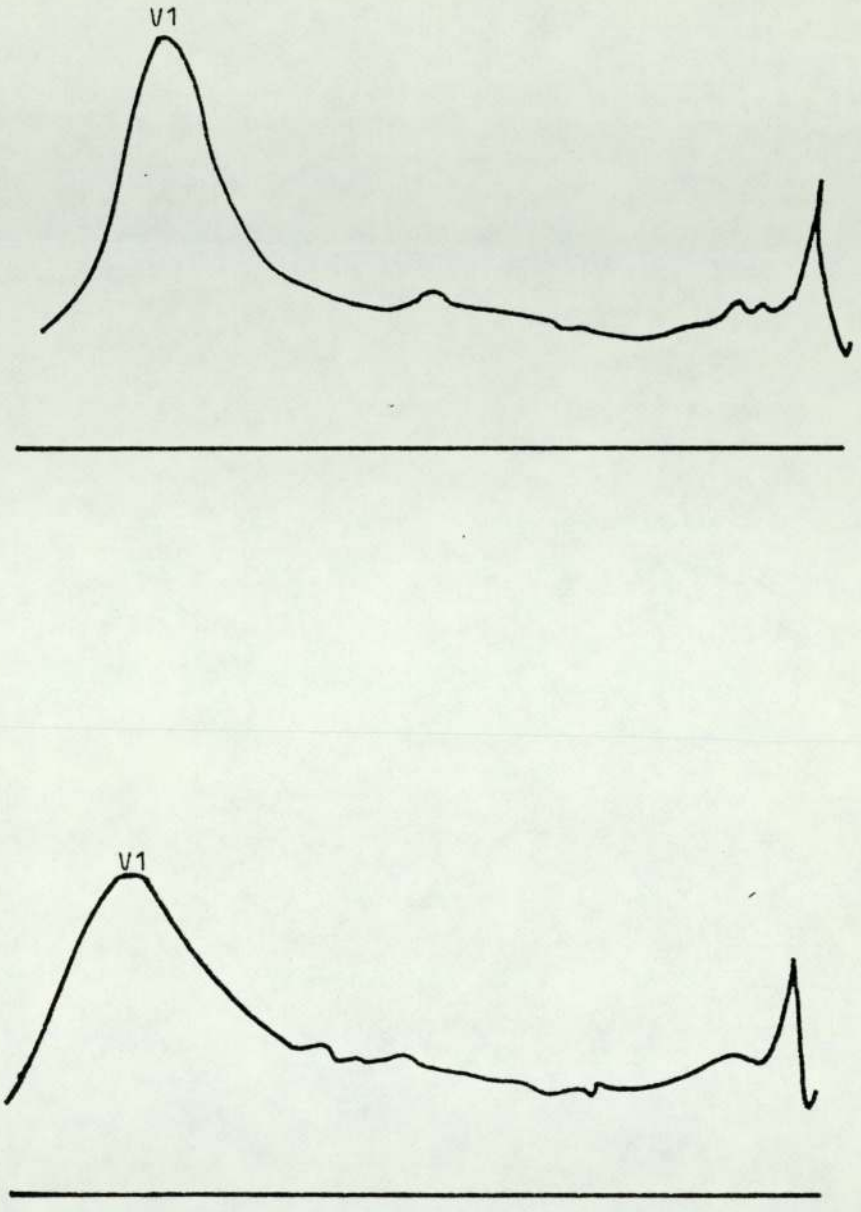


FIG. 4.6. DENSITOMETER TRACE OF SERUM FROM CONTROL FISH TAKEN AT ZERO TIME (TOP) AND 21 DAYS AFTER INJECTION WITH EMULSION ONLY (BOTTOM).

The fast-green stained gels from serum samples taken from E2-treated fish were marked by the appearance of one new band, corresponding to the V1 band above, which was not seen in control fish. This was identical to the stained gel on which the vitellogenin standard had been run (Fig. 4.7). The fast-green also marked the position of the albumin and globulin fractions, present in treated and control fish, though reduced in those fish producing vitellogenin (Fig. 4.7.).

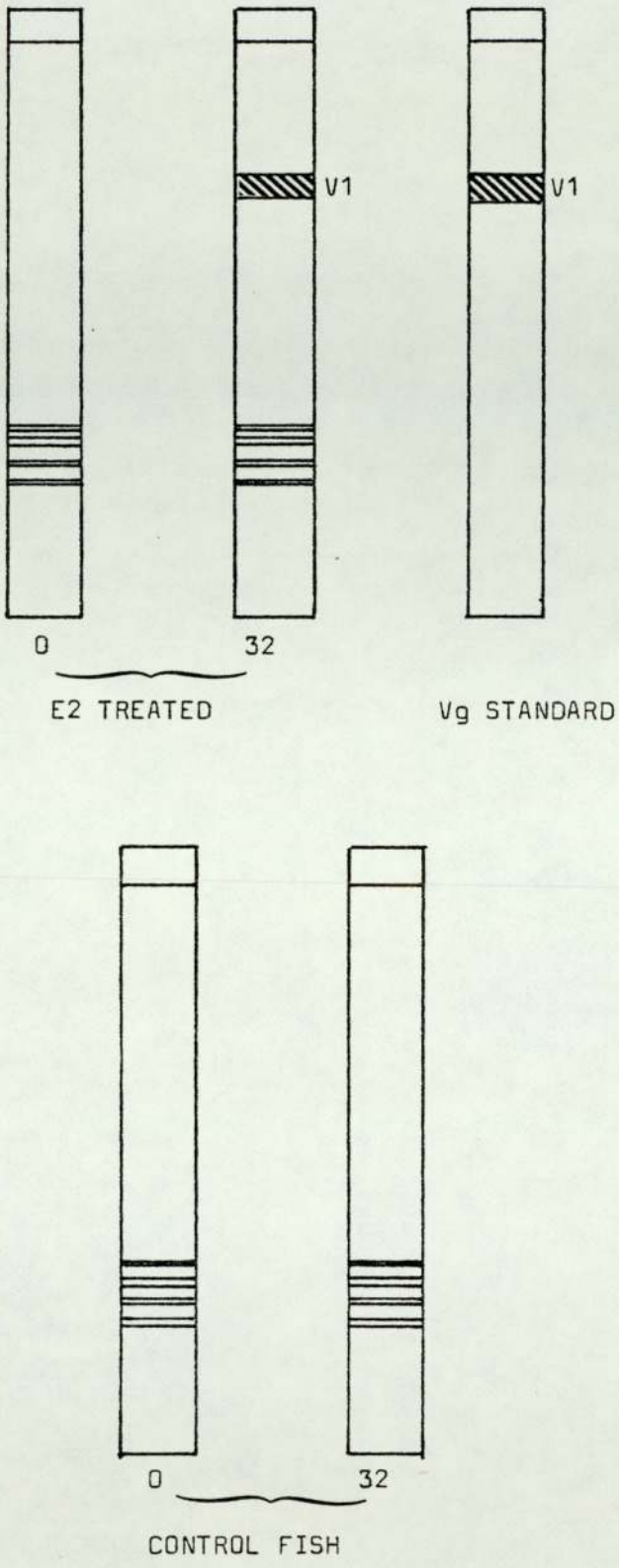
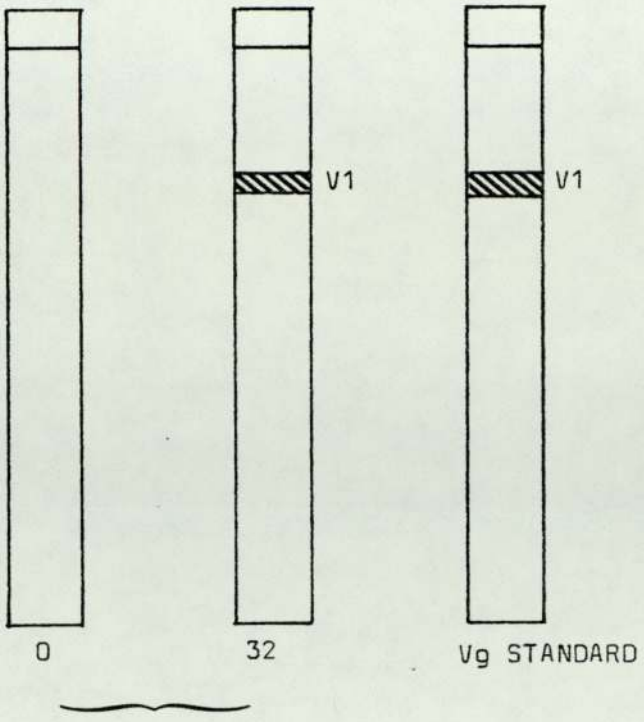


FIG. 4.7. FAST-GREEN STAINED GELS OF SERUM FROM TREATED AND CONTROL FISH AT 0 AND 32 DAYS AFTER INJECTION WITH OESTRADIOL-17β (E2 5 $\mu g\ g^{-1}$) COMPARED WITH INTACT VITELLOGENIN STANDARD.

The gels stained with alizarin redS (which marks calcium) showed a similar banding to those stained with fast-green, in that a band was identified coincident with the V1 fraction. Thus, both the serum sample from the 32-day E2-treated fish and the vitellogenin standard revealed single bands corresponding to this V1 fraction (R_f 0.27). Control sera taken from vehicle injected fish at zero time and 32 days post injection were unstained with alizarin redS (Fig. 4.8.).



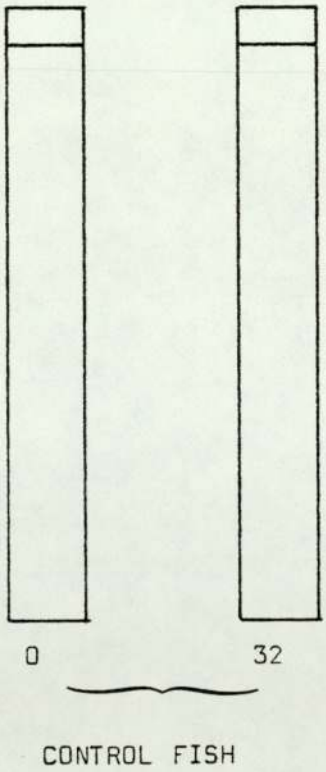


FIG. 4.8. ALIZARIN RED (CALCIUM) STAINED GELS OF SERUM FROM TREATED AND CONTROL FISH AT ZERO TIME AND 32 DAYS AFTER INJECTION WITH OESTRADIOL-17β (E2 5 $\mu g\ g^{-1}$), COMPARED WITH INTACT VITELLOGENIN STANDARD.

4.4. Characterization of the Vitellogenin Molecule and some of its Components by Sephadex Chromatography.

Using present knowledge of the structure of amphibian and avian vitellogenins, the aim of this series of experiments was to demonstrate that calcium and phosphorus are also bound to the trout vitellogenin molecule, the synthesis of which is induced by oestradiol-17β. Thus, measurement of vitellogenin indirectly by determinations of total calcium or phosphoprotein phosphorus could be further validated.

4.4.1. Materials and Methods.

Further studies of the structure of vitellogenin were made using 10cm sephadex columns (internal diameter 1.5cm). 0.5ml serum samples were loaded into the columns and eluted with 0.05M phosphate buffered saline (pH 7.05) containing 0.02% sodium cyide in Analar water. 0.5ml fractions were collected in small plastic tubes (LP3, Luckham's Ltd., U.K.), stoppered and stored at 4°C until assay on completion of the elution (< 2 hours). The fractions were assayed for calcium using the Corning analyser and the protein elution profiles determined at 280μm on a UV spectrophotometer (SP 800). Serum samples were taken from fish actively synthesising vitellogenin and those that weren't, for comparison.

I^{125} labelled trout vitellogenin (0.2ml = 24,000 cpm; See 2.2.4.1.) was added to 0.3ml of elution buffer, eluted through the column and the whole fractions collected (0.5ml) for counting on a gamma counter (Gammaset) for 100 seconds. The fraction number was

plotted against cpm and compared with the above profiles. Similarly, I^{125} labelled sodium iodide was eluted through the column as a marker for free iodine and treated as above.

Tritiated calcium (Ca^{45}; 0.1ml = 10,000 dpm, Radiochemical Centre, Amersham) was also eluted through the column. 0.1ml was added to 0.4ml buffer and o.5ml fractions were collected in scintillation vials containing 10ml scintillant (PPO). The vials were counted on a β-counter (Tricarb) for 10 minutes each against scintillant + 0.5 ml buffer as background. Fraction number was plotted against dpm. This tritiated calcium acted as a marker for free calcium in the serum, as distinct from the unionised protein-bound calcium.

Approximately 21 days after a second injection with E2 (5 $\mu g\ g^{-1}$) immature trout were injected ip with 1.5ml labelled phosphorus (P^{32}) as orthophosphate in dilute HCl (pH 2-3). The aim of this experiment was that the fish would incorporate the P^{32} into the vitellogenin complex and especially into the phosvitin moiety. The fish was bled after 5 days and 0.5ml of serum eluted by the procedure above. Fractions were collected in scintillation vials to which had been added 10ml scintillant (PPO). After leaving overnight at $4^{o}C$ the vials were counted for 10 minutes each and dpm plotted against fraction number and compared with the profiles above. Labelled P^{32} as orthophosphate was also eluted through the column on its own to act as a marker for free phosphorus.

4.4.2. Results.

Spectrophotometric analysis of the eluted fractions from control sera demonstrated 2 small protein peaks eluting at 4.4 and 5.2ml and another one at 7.5ml (Fig. 4.9.). TCa analysis of the same fractions revealed a single peak of calcium at 7.0ml (Fig. 4.10.). β-counting of the collected fractions after Ca^{45} elution similarly demonstrated a single peak which also eluted at 7.0ml (Fig. 4.10.).

Analysis of fractions from an E2-treated fish demonstrated 4 protein peaks, the most significant of which was the presence of a new high mw protein which eluted at 3.2ml and a large increase in the protein eluting at 5.2ml, which was also present in control fish. There was no changes in the protein eluting at 7.5ml, although a further new protein eluting at 6.4ml was also observed after E2-treatment (Fig. 4.9.).

TCa analysis of fractions from an E2-treated fish demonstrated the appearance of a new peak of calcium which eluted at 3.5ml, coincident with the new protein, in addition to sub-peaks at 4.5, 5.5 and 7.0ml respectively (Fig. 4.10.). This last peak at 7.0ml was also present in control fish and in the elution of Ca^{45} (Fig. 4.10.).

Counting of the fractions collected after eluting 0.5ml of the labelled intact vitellogenin, demonstrated a large peak eluting at 3.0ml and a second much smaller peak at 7.5ml (Fig. 4.11.). This latter peak represents I^{125} that has become detached from the vitellogenin molecule as shown by the counting of the fractions collected after eluting sodium $iodide^{125}$; this gave a single peak at 8.0ml (Fig. 4.11.).

Counting of the fractions collected from the elution of 0.5ml of serum taken from fish injected with P^{32} during active vitellogenesis demonstrated 3 peaks. The first peak eluted at 2.8ml, the second which was much larger eluted at 5.2ml, whereas the third, a very small peak, eluted at 7.6ml (Fig. 4.12.); the latter corresponding to free P^{32} as shown by the elution of orthophosphate on its own (Fig. 4.12.).

FIG. 4.9. FRACTION ABSORBANCE (80nm) AFTER ELUTION ON SEPHADEX G-25, OF SERUM TAKEN FROM AN E2-TREATED FISH 21 days AFTER INJECTION AND COMPARED WITH VEHICLE-INJECTED CONTROL.

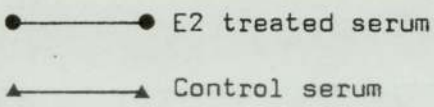


FIG. 4.10. TOTAL CALCIUM VALUES IN COLLECTED FRACTIONS FROM 21 DAY E2-TREATED SERUM AND VEHICLE INJECTED CONTROL, COMPARED WITH THE RADIOACTIVITY IN FRACTIONS COLLECTED AFTER ELUTION OF Ca^{45} ON THE SAME COLUMN.

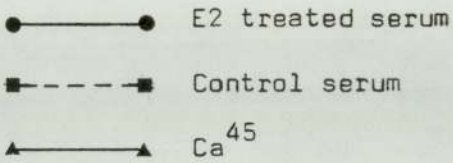


FIG. 4.11. PROFILE OF RADIOACTIVITY (I^{125}) IN COLLECTED FRACTIONS FOLLOWING THE ELUTION OF INTACT IODINATED VITELLOGENIN ASSAY STANDARD, COMPARED WITH THE ELUTION OF SODIUM IODIDE.

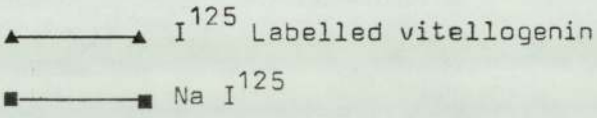
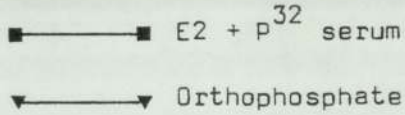


FIG. 4.12. PROFILE OF RADIOACTIVITY (P^{32}) IN FRACTIONS COLLECTED AFTER THE ELUTION OF SERUM TAKEN FROM A P^{32} INJECTED TROUT, INJECTED PREVIOUSLY WITH E2, COMPARED TO THE ELUTION OF ORTHOPHOSPHATE.



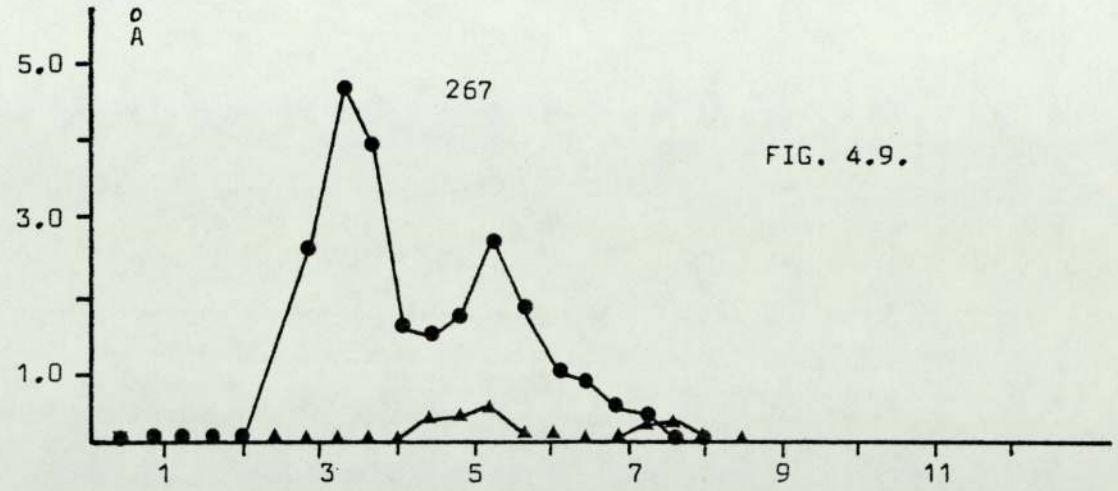


FIG. 4.9.

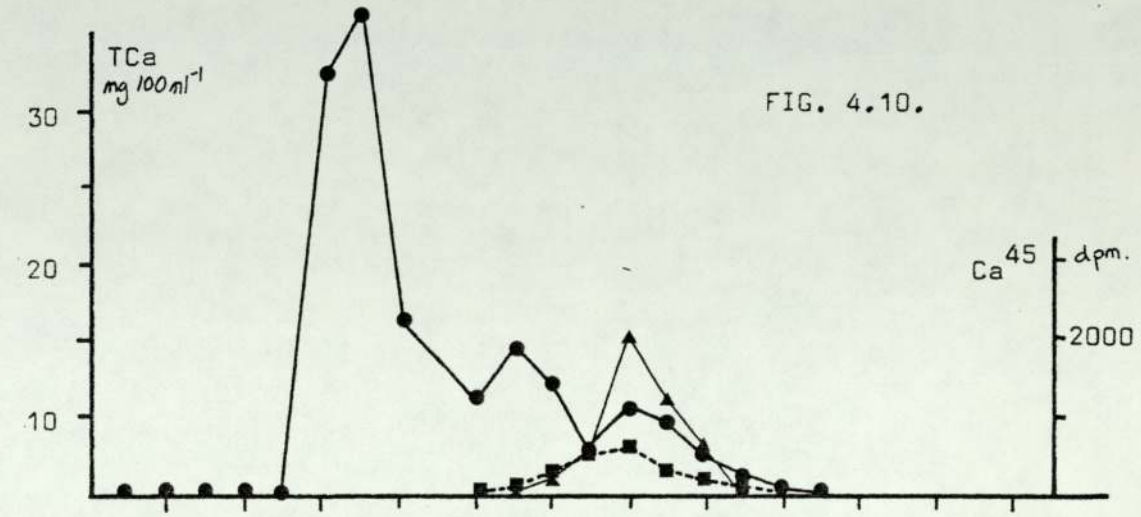


FIG. 4.10.

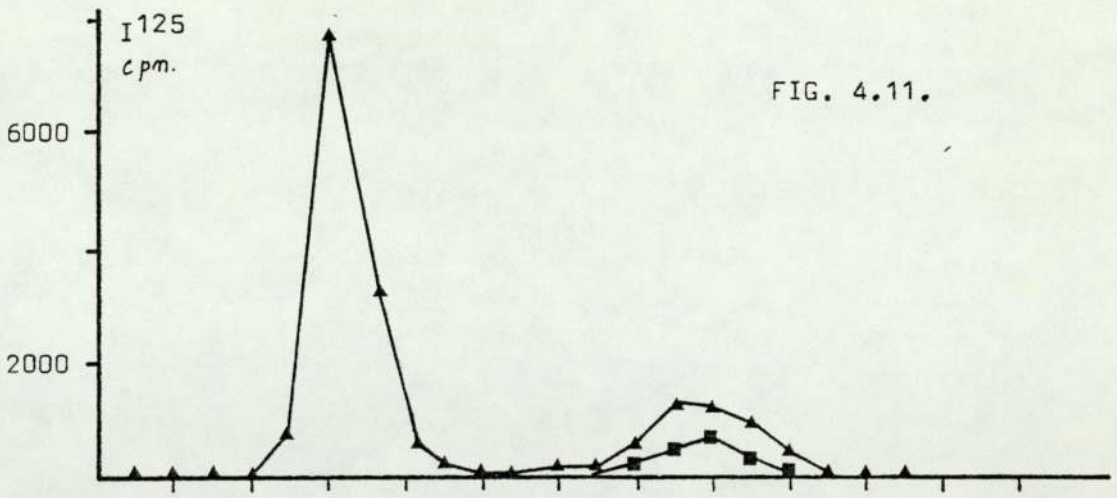


FIG. 4.11.

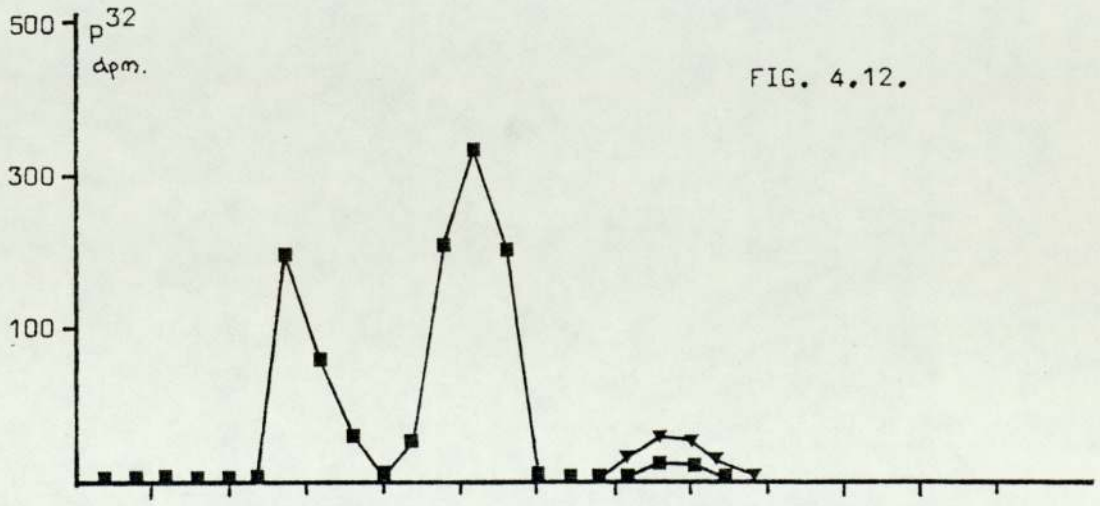


FIG. 4.12.

Summary of results :

(a) Determinations of vitellogenin by RIA (VgRIA) in fish of different sex and stages of maturity indicate a 200,000 fold increase in Vg levels during sexual maturity in female trout and also the presence of low, but detectable levels in immature male fish.

(b) Serum VgRIA levels were correlated with the profile of changes in total serum calcium (TCa), phosphoprotein phosphorus (PP) and hence estimated vitellogenin (VgE) after oestradiol-17β (E2) injection, and with TCa during the normal reproductive cycle of the female.

(c) Due probably to a greater sensitivity of RIA determinations, VgRIA levels were not correlated with the other parameters over the first 8 days following E2 injection.

(d) VgE levels were 0.5 times the levels shown by RIA in samples taken from maturing female fish which had been stored for 9 months, and 0.7 times the levels in fish after E2 injection, which were determined after 2 months storage.

(e) Sudan-black stained PAGE gels of serum from E2 injected or mature female fish showed the appearance of 2 new bands (V2 & V3) and an increase in a band seen in immature fish (V1).

(f) Fast-green stained PAGE gels of serum from E2 treated or mature fish demonstrated the appearance of one band in the serum coincident with the position of V1 above and the vitellogenin assay standard (VgS).

(g) In alizarin-red stained gels of serum from E2 treated fish one new band appeared coincident with V1 and VgS.

(h) Sephadex-gel filtration of serum from 21 day post-E2 treated fish on G25 columns revealed the appearance of 2 new high molecular weight proteins eluting at 3.2ml (P1) and 5.2ml (P2) with relatively more protein associated with P1 in fresh samples.

(i) Calcium analyses of these fractions similarly revealed 2 new peaks eluting at 3.5ml (P1) and 5.5ml (P2), which indicated that calcium is bound to these new proteins as shown by elution of immature serum and free calcium (Ca^{45}).

(j) Counting of P^{32}-injected vitellogenin synthesising fish also revealed 2 peaks coeluting with P1 and P2, with relatively more phosphorus associated with the smaller molecular weight P2.

(k) P1 was identified as intact Vg since iodinated RIA standard (VgI) eluted at 3ml.

4.5. Discussion.

The results clearly demonstrate that the elevations in serum calcium and phosphoprotein phosphorus induced by treatment with oestradiol-17B, reported here and in Chapter 3, occur as a direct consequence of the binding of these two elements to the vitellogenin molecule. Furthermore, the significant correlations of these changes with those of real serum vitellogenin (VgRIA) after both oestradiol-17B injection and during the normal reproductive cycle, confirm that calcium and phosphoprotein phosphorus can be used to monitor changes in this large yolk-precursor.

In all assays of vitellogenin using RIA, serial dilution of serum samples taken from fish of either sex and varied maturity showed full parallelism with the assay standard, indicating the assay was measuring vitellogenin. The standard curves for all vitellogenin RIA's conducted during this work showed minimal interassay variation. The highest vitellogenin levels recorded were from mature females (215 mg ml^{-1}) and oestradiol-17B injected males (205 mg ml^{-1}) Using the same assay, maximum values of 100 mg ml^{-1} of vitellogenin have been reported by Sumpter (1981) with this complex representing about 90% of the total blood protein. Similar studies with other oviparous species have shown that vitellogenin represents about 60% of the blood protein in *Xenopus* (Wallace, 1970) and up to 80% in the turtle (Gapp *et al.*, 1979); it would also appear from electrophoretic evidence that at the height of vitellogenesis, vitellogenin is synthesised at the expense of other proteins (Plack *et al.*, 1971; Hori *et al.*, 1979; Campbell & Idler, 1980). Although the maximum blood protein levels reported for salmonids are

approximately 120 mg ml^{-1} (Alexander, 1977) and are somewhat lower than those reported here, the values recorded in most other studies are extremely variable (Plack et al., 1971; Crim & Idler, 1978; Gapp et al., 1979; Idler et al., 1979; Campbell & Idler, 1980; Ho et al., 1981). Thus, in one study the maximum level in the brown trout was 85.0 $\mu g\ ml^{-1}$ (Crim & Idler, 1978) whereas in the silver eel, there was a maximum value of 800 $\mu g\ \mu l^{-1}$ (800 mg ml^{-1}) (Burzawa-Gerard, 1982). It has subsequently been shown that the method used in Crim & Idler's (1978) study, which uses purified yolk lipophosphoprotein as standard and antigen, gives a gross under-estimation (40×) (Idler et al., 1979; Campbell & Idler, 1980). Using a suitably modified form of this assay, resting levels of vitellogenin in female Atlantic salmon before their first spawning were estimated at 5.2 $\mu g\ ml^{-1}$, rising to 6.0 mg ml^{-1}, just prior to spawning (Idler et al., 1979). These levels are still more than an order of magnitude lower than those reported here although this might be due to species differences. However, using another RIA developed for rainbow trout with antibodies raised against intact PMSF preserved vitellogenin (although yolk-lipovitellin was used as label), serum levels of vitellogenin were found to rise from basal values of 30 $\mu g\ ml^{-1}$ to around 20 mg ml^{-1}, 13 days after treatment with oestradiol-17B at a dose of 5 $\mu g\ g^{-1}$ (Campbell & Idler, 1980). This agrees very closely with the present data, where serum levels of vitellogenin (RIA) rose from basal values of 90 $\mu g\ ml^{-1}$ at the time of injection of oestradiol-17β, to 22.35 mg ml^{-1} 11 days later. Furthermore, such mg ml^{-1} levels must be present in maturing female rainbow trout to account for the 1g day^{-1} growth increase in gonad observed during vitellogenesis (See Chapter 7). Similar maximum

egg protein levels of 32 mg ml^{-1} have been observed in both maturing female and oestrogen-injected cod, by an immunodiffusion method (Plack et al., 1971). One important difference in the present work relates to the vitellogenin levels of immature female fish and mature females in ovarian quiescence. Thus, vitellogenin levels in immature female trout before their first maturation are in the $\mu g\ ml^{-1}$ range, whereas during post-spawning quiescence the resting levels are found almost in the mg ml^{-1} range. Although this may be due to an increased half-life of vitellogenin occurring as a result of the absence of ovarian uptake of vitellogenin in quiescent fish during this period, it is more likely related to an enhanced secondary response in fish which have previously spawned (See Chapter 3). Thus, once the liver is sensitised to oestradiol-17β (1^{o} response), the liver may subsequently be more sensitive to the basal tonic levels of this hormone in post-spawning quiescent female fish. This suggests that once initiated, vitellogenin production in females is continuous, and it is only the rate of synthesis which changes.

Despite the wide acceptance of vitellogenin as a female specific protein (Follett & Redshaw, 1974; Tata, 1978a; Wallace, 1978) levels as high as 300 ng ml^{-1} were found in some immature males. In contrast, in many other studies in birds, reptiles and fish using similar radioimmunoassay or immunoreactive techniques, there have been no reports of vitellogenin in male serum (Markert & Vanstone, 1971; Plack et al., 1971; Aida et al., 1973; Redshaw & Follett, 1976; Craik, 1978a; Crim & Idler, 1978; Gapp et al., 1979; Idler et al., 1979; Campbell & Idler, 1980; Ho et al., 1981). Only Sumpter (1981),

who used the same assay as that used here has shown that levels of up to 200 $\mu g\ ml^{-1}$ are present in male trout. Sumpter (1981) also reported that the vitellogenin immunoreactivity of pooled male plasmas, eluted on Sepharose 6B, appeared as a discrete peak in the expected position of authentic intact vitellogenin, strongly suggesting that these findings were not due to the presence of proteins other than vitellogenin. The surprising finding of vitellogenin in males is supported by the polyacrylamide-gel electrophoresis (PAGE) results, where the electrophoretic profiles of serum from oestradiol-17B treated males were exactly the same as those from mature females. The PAGE-gels of vitellogenic sera, stained with sudan-black, were marked by the appearance of three bands : two of these were new (V2 & V3), whereas the third (V1) was also present in small quantities in immature male serum before treatment with oestradiol-17β. Furthermore, vitellogenin assay standard, developed with fast-green stain, had the same mobility (R_f) as the V1 fraction. In a similar study, Campbell & Idler (1980), showed an increase in a protein in male trout injected with oestradiol -17β, which was already present before treatment. Clearly, male trout and probably the males of all other oviparous species are capable of synthesising vitellogenin provided they are treated with oestrogens (Ho & Vanstone, 1961; Urist & Schjeide, 1961; Plack et al., 1971; Aida et al., 1973; Follett & Redshaw, 1974; Hickey & Wallace, 1974; Emmersen & Petersen, 1976; de Vlaming et al., 1977; Wallace, 1978; Elliott et al., 1979, 1980; Ho et al., 1981; See also Chapter 3). However, none of these investigations offer any real suggestion concerning the reason or physiological significance of naturally occurring vitellogenin in males. Two recent independent studies

have reported that androgens, albeit at pharmacological levels, induce vitellogenesis in fish (Hori et al., 1979; Le Menn et al., 1979). However, such levels of testosterone would not be found either in immature or even mature male fish, and furthermore, 11-ketotestosterone, the other major androgen in fish (Idler et al., 1960), has not been shown to stimulate vitellogenesis. Whether such reported actions are due to the aromatisation of androgens to oestrogens (See Chapter 5), or due to the lack of receptor specificity is not known, since evidence for both is provided (Hori et al., 1979; Le Menn et al., 1979). It is more likely that the appearance of vitellogenin in male fish of some species, is due to the presence of oestrogens often at higher levels than females of a similar age and maturity (Schreck et al., 1973; Schreck & Hopwood, 1974; Terkatin-Shimony & Yaron, 1978).

In view of the sensitivity of male fish to oestradiol-17β and the presence of this hormone in most oviparous males, it is surprising that there have been no other reports of vitellogenin in males, especially during studies of the seasonal hormonal changes. The absence of such data may be the result of the relative insensitivity of other methods of determination of vitellogenin when compared to RIA. It is possible that vitellogenin in males is unique to fish, since other equally sensitive RIA's have failed to detect vitellogenin in males of other oviparous groups (Gapp et al., 1979; Ho et al., 1981). These results indicate that the other indirect assays which are used to monitor vitellogenin levels are unable to demonstrate any significant changes in this complex until levels of vitellogenin of parallel samples reach mg ml^{-1} proportions as determined by RIA.

Thus, indirect methods can only be used with confidence to detect vitellogenin in mature females or oestradiol-17β treated fish, and would not appear sensitive enough for studies of immature fish of either sex.

Many of the problems related to the measurement of vitellogenin by RIA appear to be due to the difficulties in preparing a pure intact vitellogenin for use as standard antigen and label in the assays. Thus, some assays use proteins isolated from yolk as both standard, antigen and label, others use the intact vitellogenin as standard and antigen but an iodinated fraction of that molecule as label, and still others including the present assay, use the intact vitellogenin molecule as standard, antigen and label. This has resulted in the considerable variation in levels observed. However, this does not account for the apparent two-fold difference in some of the VgRIA values observed between the results of this study, and those of Sumpter (1981), for the same assay was used in both investigations. There are also two-fold differences in parallel samples of vitellogenin levels as determined by RIA and those estimated by phosphoprotein phosphorus. Furthermore, there are similar discrepancies between VgRIA and calcium levels in this and Sumpter's (1981) study. Thus, Sumpter (1981) showed that the ratio of vitellogenin (mg ml^{-1}) to calcium (mg $100ml^{-1}$) was approximately 1.17:1, whereas samples taken from the reproductive cycle in this work had a ratio of 1.9:1. However, these particular samples had been stored for up to 9 months, with repeated defrosting and refreezing. An effect of thawing is supported by a ratio of 1.4:1 in samples which had been stored for a shorter period without

defrosting. Also, a ratio of 1.3:1 was observed in other samples taken from maturing female trout (See Chapter 7), which were assayed within a month of sampling.

Studies of the character of fish vitellogenins have confirmed that the intact molecule usually exists as a dimer under normal physiological conditions (Hickey & Wallace, 1974; Hara & Hirai, 1978; Hori et al., 1979; de Vlaming et al., 1980), but because of the relative ease with which vitellogenins break-up and their heterogenous behaviour under in vitro conditions (Wallace, 1978), it has been suggested that proteolytic inhibitors (like phenyl methyl sulfonyl fluoride - PMSF) must be used when collecting samples for purification procedures (Hickey & Wallace, 1974; Campbell & Idler, 1980; de Vlaming et al., 1980). Similarly, when using RIA methods where the intact vitellogenin molecule is used to raise antibodies, it has been suggested that in the absence of inhibitors, proteolysis of vitellogenin in the sample may artificially increase the number of antigens in vitro, and consequently higher estimates might occur. It is interesting to note therefore, that in samples subjected to conditions under which breakdown would be likely to occur (9 months with repeated thawing and refreezing), high levels of VgRIA were observed (relative to Ca^{2+} which is very stable even under these conditions - See Materials and Methods), whereas samples stored at $-20^{o}C$ for only 2 months until assay, showed lower levels relative to calcium. It is conceivable that a greater denaturation in the long-stored samples may have caused greater over estimation. Thus, one must examine critically determinations of vitellogenin in serum samples taken without such proteolytic inhibitors. It is

also important to appreciate that such differences may also affect phosphoprotein phosphorus determinations as well as RIA of vitellogenin (de Vlaming et al., 1980), since it is probable that a considerable proportion of phosvitin could also detach itself from vitellogenin without the use of such inhibitors. Since free phosvitin, unlike the intact molecule, is not fully precipitated by TCa (de Vlaming et al., 1980) and constitutes the first step in the assay for phosphoprotein phosphorus, it is possible that the increased levels of free phosvitin may lead to lower levels of total phosphoprotein phosphorus and hence an underestimate of vitellogenin (VgE). The results from this work suggest that trout vitellogenin probably contains 1.3 - 1.4% phosphorus and therefore the estimates of vitellogenin (VgE) calculated from phosphoprotein phosphorus by using 71.4 (assuming 1.4% PP) as a conversion factor, were approximately correct. The amount of phosphorus in vitellogenin in this study is calculated from the VgRIA:TCa ratio (mg ml^{-1}:mg $100ml^{-1}$) which gives a value of 1.3 - 1.4% calcium in trout vitellogenin. Since results elsewhere in this study (See Chapter 3) indicate that phosphorus and calcium coexist in the vitellogenin molecule in a 1:1 ratio, as similarly proposed for Xenopus by Wallace (1970), then the phosphoprotein phosphorus content must be approximately the same. However, it has already been discussed that the RIA may overestimate vitellogenin levels and the Vg:TCa ratio (& PP) may subsequently be less than 1.3%, in which case VgE values would be underestimated.

Although there are differences in absolute levels of vitellogenin, it is important the emphasise the highly significant correlations

between the profiles of these different values as estimated by direct and indirect procedures. However, over the first ten days following oestradiol-17B injection, when there was a 244 fold increase in VgRIA levels, changes in TCa and VgE were virtually undetectable with only 1.4 and 5 fold increases respectively. Indeed, after only 2 days VgRIA rose from 0.9 mg ml^{-1} to 5.07 mg ml^{-1}, over which period calcium levels actually fell 0.5 mg ml^{-1}. The difference in sensitivity between the methods may account for the apparent discrepancies in the literature relating to the time of onset of vitellogenesis in the maturation of female oviparous species. Also, in studies investigating the 'lag-period' between oestradiol-17β injection and the onset of vitellogenin production, the sensitivity of the assay is the determining factor (Tata, 1978; Knowland, 1980). Thus in the present work, measurements of vitellogenin by RIA suggest a 'lag-period' of a few hours, whereas determinations of calcium would suggest this period after oestradiol -17β treatment was approximately 8 days (See also Chapter 3). In the same way, 'lag-periods' of 2-4 hours have been reported for the chicken (Gruber et al., 1976), 6-8 hours for the turtle (Ho et al., 1981), 9-12 hours for male frogs (Clemens et al., 1975) and 24-28 hours for the cod (Plack et al., 1971). Thus, the use of this highly sensitive and specific radioimmunoassay to intact trout vitellogenin has some distinct advantages over other methods. However, the ability of some methods to monitor many of the seasonal changes in vitellogenin, together with the relative simplicity of for example calcium determinations and their reproducibility after extended storage makes such methods applicable to long-term studies of vitellogenesis.

Since evidence from other studies has indicated that calcium and phosphorus were bound to the vitellogenin molecule (Bailey, 1957; Wallace, 1978), the determinations of these two elements have been used to monitor changes in vitellogenin in the absence of a reliable RIA technique. The results from the present study clearly show these indirect determinations were justified. Thus, in oestrogen-treated fish, two new peaks of calcium appeared (when compared with immature male serum and Ca^{45}), one which coeluted with iodinated intact vitellogenin standard at 3.0 - 3.5ml (P1), and the other peak eluting at 5.5ml (P2). These peaks in calcium were clearly associated with the two new protein peaks which appeared in the same serum, the higher and lower molecular weight peaks eluting at 3.2 (P1) and 5.2ml (P2) respectively. Similarly, the serum from the fish injected with oestradiol-17B and subsequently with P^{32} eluted two peaks of high activity, coincident with P1 and P2 with relatively more activity associated with P2. The calcium is believed to be bound to the vitellogenin molecule to increase the solubility of the protein in the serum (Wallace, 1978) and in addition provides a source of this valuable element for the embryo (Dacke, 1979). In view of its close relationship with vitellogenin, it is surprising that there is no published evidence to support the uptake of this ion into the egg. The high levels of phosphorus in all vitellogenins are due to the inclusion of phosvitins in this complex, and most especially serine, to which the majority of the phosphorus is attached. Some recent evidence has suggested that some of the phosphorus in phosvitin may be used as an energy source at final maturation (Craik, 1982), at a time when many teleost eggs undergo a rapid expansion due to the uptake of water (Fulton,

1898; Wallace, 1978). This evidence is based on theoretical calculations of the energy required to take up this water against the energy released by the level of dephosphorylation (Craik, 1982).

The chromatographic and electrophoretic methods used in this work were designed to deomstrate that the increase in calcium and phosphorus levels observed in vitellogenic females, occurs as a result of binding to this protein. Also, to recognise its protein/lipid character on PAGE gels, in both cases by reference to iodinated assay standard and in the latter case by reference to unlabelled RIA standard vitellogenin. It is clear from the present results, that serum from oestradiol-17β-treated fish contains two new high molecular weight proteins (P1 & P2 in this study). P1 is tentatively characterised as the dimeric vitellogenin, since it coeluted in the same position as iodinated RIA standard, and it had much of the labelled P^{32} associated with it. Furthermore, heating of the serum before elution, results in an increase in P2 at the expense of P1. The smaller molecular weight protein is relatively high in phosphorus which suggests that it is phosvitin-like, especially since phosvitin easily detaches from the intact molecule (de Vlaming et al., 1980). However, it has been reported that phosvitins do not readily absorb light at 280nm (Jared & Wallace, 1968; Wallace, 1978), which P2 did and this protein also contained considerable amounts of calcium. P2 could therefore possibly represent the monomeric vitellogenin, probably together with other breakdown products including phosvitin; this would also in the P2 eluate account for the relatively high phosphorus levels. Using different procedures, similar multiple peaks have been found by other workers during their attempts to isolate intact teleost

vitellogenins (Hickey & Wallace, 1974; Hara & Hirai, 1978; Hori et al., 1979; de Vlaming et al., 1980) especially when proteolytic inhibitors were not used in the collection vessels. Although amphibian vitellogenins have been well characterised (Wiley & Wallace, 1981), recent investigations into the nature of teleost vitellogenins have found that accepted techniques for the precipitation of amphibian and avian vitellogenins do not work very well with fish (Wallace, 1978). Thus, with the development of other methods and the careful use of proteolytic inhibitors, recent studies have been able to successfully isolate a single vitellogenin peak, purify it and in some cases characterise its structure (Hickey & Wallace, 1974; Emmersen & Petersen, 1976; Hara & Hirai, 1978; Hori et al., 1979; Idler et al., 1979; de Vlaming et al., 1980; Sumpter, 1981; Wallace & Selman, 1982).

In many of these studies, the purified vitellogenins have been subjected to native and SDS-gel electrophoresis for comparison with vitellogenic serum in the attempt to understand more of the size and structure of their components. In this study PAGE electrophoresis of serum from a 21-day post-oestradiol-17β treated fish, revealed two new sudan black stained bands and a large increase in an existing one. Similar results were reported in two other studies on goldfish where both purified vitellogenin (de Vlaming et al., 1980) and serum from oestradiol-17β treated fish (Hori et al., 1979) gave three bands associated with vitellogenin when subjected to electrophoresis on native PAGE-gels and stained with Coomassie-blue or amido-black. Similarly, in another study with rainbow trout, three bands could also be distinguished in serum from

oestradiol-17β treated male fish (Campbell & Idler, 1980). Using 7.5% gels Hori et al., (1979), concluded that his band I (that with the lowest R_f value, equivalent to V3 in this study) was a dimer of band III (higher $R_f \equiv$ V1). This proposition was supported by : firstly, the molecular weight of I was approximately twice that of III; secondly, I and III had similar banding patterns under SDS-gel electrophoresis; and lastly, I and III formed a single precipitin line to antisera raised to their mixture. Although band II was not identified, de Vlaming et al., (1980) using purified goldfish vitellogenin also observed three bands which were related to those of Hori et al., (1979). Furthermore, by measuring the R_f values of these proteins on different % native gels, by Ferguson plot analysis, the authors suggested that band II was possibly a minor form of vitellogenin because it had the same intercept at zero mobility as band III and was slightly smaller. De Vlaming et al., (1980) also concluded that band III was a monomer of band I, since band I had twice the free electrophoretic mobility as either of the other two. This suggests that the V1 reported here may be a monomer of V3, although further proof is clearly required. If this is the case, then the relative intensity of the staining of the three bands, indicates that under these conditions a very small proportion of the dimer (V3) remains intact.

In contrast, the same gel-samples stained with fast-green dye revealed only a single protein band, with an R_f value the same as V1 from sudan-black stained gels and vitellogenin assay standard, from which one might conclude that V1 is the dimer and not the monomer as suggested above. This anomally may however, be as a

result of differences in methodology. The gels which were stained with fast-green were smaller than the sudan-black stained gels and consequently less sample was used to avoid tailing and over-loading of the columns. Even with sudan-black, V2 and V3 bands were much less intensely stained than V1, and it may be that there was insufficient material to properly visualise bands V2 and V3. It is noticeable in the work of Campbell & Idler (1980), where imido-black (protein) was used, that the two bands with the lower mobility were also faintly stained. It may be that at the concentrations used, dyes for lipid stain more intensely than protein stains. This is supported by the observation that there was no V1 band in immature fish after fast-green staining. Alizarin-red (calcium) stained gels on which vitellogenic serum had been electrophoresed, also demonstrated a single weak-staining band with an R_f value equivalent to V1, confirming an association of this ion with this oestradiol-induced protein.

Thus, by in vitro assay, chromatographic and electrophoretic techniques, these results demonstrate that as a result of their close association with trout vitellogenin, both total calcium and phosphoprotein phosphorus determinations may be used as indirect estimates of gross changes in serum vitellogenin. However, the reduced sensitivity of these methods when compared with RIA, indicate that some changes may be missed if only these procedures are used. In addition, care must be taken in the collection and storage of samples, due to the heterogenous behaviour of vitellogenin in vitro. Chromatographic and electrophoretic results confirmed the heterogeneity of trout vitellogenin as in other vertebrates.

Clearly the heterogeneity of vitellogenins has occurred as a result of their physiological role as a complete food supply for the developing embryo. In order to store this food supply, the growing oocytes must pass through certain developmental and morphological changes as this large molecule is sequestered from the blood. Whilst the next chapter is something of a digression in this context, the histological development of the follicles will be subsequently investigated, together with the hormonal mechanisms controlling these processes. In any study of hormonal control mechanisms, the physiological roles of all the hormones involved must be established. Although the results of this and the previous chapter have demonstrated a role for oestradiol-17β in the direct control of vitellogenesis, recent contrary reports have suggested roles for other hormones including testosterone in this process in fish. Thus, the next chapter investigates whether other hormones have vitellogenic potency in trout.

CHAPTER 5.

THE EFFECTS OF SEX STEROIDS ON VITELLOGENESIS.

5.1. Introduction.

It is now well established that in oviparous vertebrates vitellogenin synthesis is regulated by the ovarian hormone oestradiol -17B (Tata, 1978; Wallace, 1978). This finding, however, is primarily based on observations in avian and amphibian species and until very recently and evidence for oestradiol-17β regulated vitellogenin synthesis in teleosts was largely circumstantial (Campbell & Idler, 1976). Thus, it is known that the administration of oestradiol-17β into certain teleosts leads to elevations of vitellogenin in the serum (See Chapter 3; Bailey, 1949; Ho & Vanstone, 1961; Plack et al., 1971; Aida et al., 1973; Campbell & Idler, 1976; Elliott et al., 1979, 1980). Also, increases in serum levels of oestradiol-17β have been correlated with rising levels of vitellogenin in female fish during the reproductive cycle (Eleftheriou et al., 1966; Schreck & Hopwood, 1974; Wingfield & Grimm, 1977; Lambert et al., 1978; Whitehead et al., 1978a; Scott et al., 1980b; Bromage et al., 1982b). However, it is possible that under normal physiological conditions other sex-steroids in addition to oestradiol-17β may be involved in the induction of hepatic vitellogenin synthesis. Certainly, a number of other steroids including oestrone (Urist & Schjeide, 1961; Utter & Ridgeway, 1967; Terkatin-Shimony & Yaron, 1978; van Bohemen et al., 1982a), oestriol (Terkatin-Shimony & Yaron, 1978), testosterone in fish (Le Menn & Lamy, 1976; Hori et al., 1979; Le Menn, 1979; Le Menn et al., 1980) and progesterone in the oviparous lizard (Yaron & Widzer, 1978) have been shown to possess some vitellogenic potency. In view of this deficiency in the literature, the present experiment was designed to investigate whether other sex-steroids besides

oestradiol-17β are able to induce hepatic vitellogenesis in trout. Such information may provide an understanding of the role of these steroids during the vitellogenic and maturational phases of the reproductive cycle in this species.

Oestrogens have been detected in extracts of ovarian tissue from various species (Barr, 1968), but of these oestradiol-17β and oestrone have been found more often than oestriol (Katz et al., 1971). Although it has been assumed that oestradiol-17β is likely to be the most important of the oestrogens physiologically (Yaron et al., 1977; See Chapters 3 & 7) there is evidence that oestrone has some vitellogenic potency (Terkatin-Shimony & Yaron, 1978; van Bohemen et al., 1982a) and since this oestrogen occurs naturally in rainbow trout, it was concluded by van Bohemen & Co workers that the role of oestrone in any investigation of the physiology of vitellogenesis cannot be ignored in this species. In one study of the effects of the different oestrogens on vitellogenesis oestriol was found to be the most potent inducer, but the reason for this is not known, since although oestriol has been detected in some species (Lupo & Chieffi, 1963; Lupo & Chieffi, 1965; Eleftheriou et al., 1966) it does not appear to occur naturally in the majority of fish species including trout (Lambert & van Oordt, 1982). Thus in the present study it was intended to look at the relative potency of these three oestrogens in inducing vitellogenin synthesis by the liver, using oestradiol-17B as a reference for all other steroids administered.

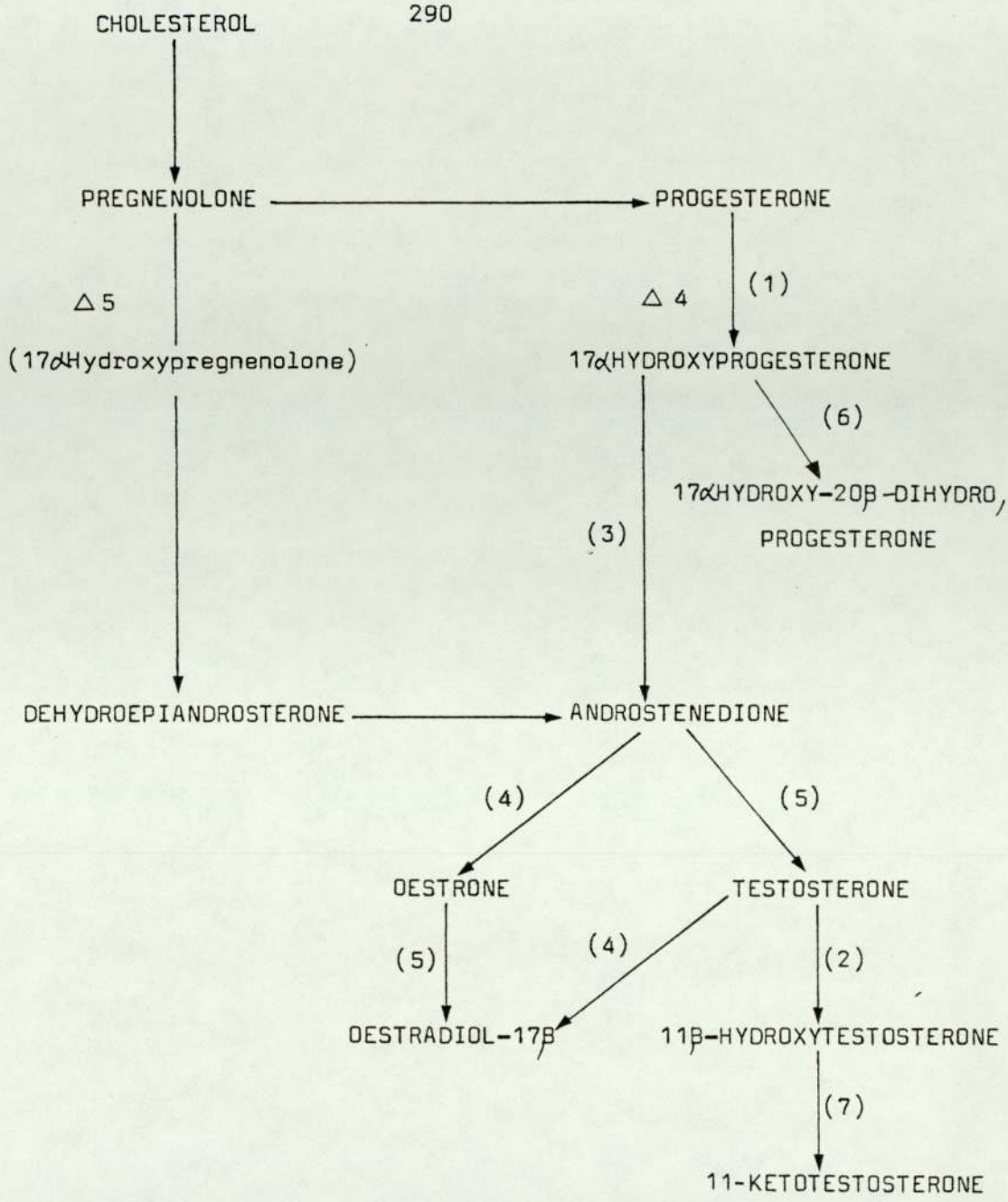
Although oestradiol-17β has been observed to stimulate the hepatic synthesis of yolk proteins and raise the serum yolk-protein levels, it does not appear to stimulate the incorporation of vitellogenin into

the oocyte (Campbell & Idler, 1976). Evidence regarding the effects of oestrogens on the ovary of a number of fish species seem contradictory. Thus, oestrogens have been shown to cause ovarian degeneration (Tavolga, 1949; Egami, 1954), stimulation (Svardson, 1943; Kawamoto, 1950) and both stimulation (of oogonia) and retardation (of primary oocytes) in the same species (Bullough, 1942). In a more recent study of the effects of a number of steroids (Khoo, 1979) it was observed that all three oestrogens induced the formation of yolk vesicles in the ovaries of hypophysectomised goldfish. This is somewhat surprising since 'vesicular yolk' (See Chapter 6) is chemically distinct to vitellogenin which is synthesised in the liver and carried in the blood to the oocyte, where it is incorporated as yolk granules. In view of these conflicting data, an investigation will be made of the effects of these oestrogens on ovarian weight and histology.

In association with oestrogens many studies have isolated other steroids from ovarian extracts of fish including testosterone and progesterone (Gottfried, 1964; Barr, 1968; Katz et al., 1971; Ozon, 1972). Furthermore, somewhat surprisingly, it has been observed that testosterone and 11-ketotestosterone are two of the major biosynthetic products of in vitro incubations of ovarian tissues (Eckstein & Katz, 1971; Colombo et al., 1972, 1973; Lambert & Pot, 1975). Also, high serum testosterone levels have been observed in several female teleosts during the reproductive cycle reaching peak levels just before ovulation; these levels are far higher than those seen in mature males (Campbell & Idler, 1976; Wingfield & Grimm, 1977; Scott et al., 1980b; Stuart-Kregor et al., 1981). Since

testosterone is converted to oestradiol-17β by the aromatase enzyme (See Diagram 5.1.) several authors have concluded that testosterone acts as a precursor in oestrogen synthesis (Campbell & Idler, 1976; Wingfield & Grimm, 1977; Scott et al., 1982a). It has also been suggested that there may even be a preferential synthesis of oestradiol-17B via testosterone, rather than via oestrone (Scott & Baynes, 1982). Whether these high androgen levels are simply acting as precursors or whether they have a physiological role is not really known. There is evidence from mammalian studies that testosterone plays a part in the control of atresia (Magoffin & Erickson, 1981). Furthermore, testosterone injection has also been shown to induce atresia in second growth-phase oocytes (vitellogenic) of Oncorhynchus gorbuscha (Yamazaki, 1972). Other studies of the effects of testosterone on the teleost ovary have shown that androgens are so inhibitory to ovarian function that masculinisation tends to occur. However, the primary effects on the developing ovary would appear to involve the inhibition of oogenesis and vitellogenesis and that this inhibition is followed by degeneration of the oocytes (See Dodd, 1960). In the light of these data the effects of testosterone on the ovary are investigated in the present study.

It has already been mentioned that progestagens in addition to oestrogens and androgens have also been isolated from teleost ovaries. However, progestagens have not been shown to have any vitellogenic action in one study in the goldfish (Khoo, 1975) and furthermore, recent evidence indicates that these steroids, especially 17α hydroxy-20β-dihydroprogesterone (17α20β) are most likely involved in the later stages of ovarian development ie. final



ENZYME SYSTEMS

(1) 17α-Hydroxylase

(2) 11β-Hydroxylase

(3) C21-C19, demolase

(4) Aromatase

(5) 17-hydroxysteroid dehydrogenase (HSD)

(6) 20β-HSD

(7) 11β-HSD

Diagram5.1. STEROID BIOSYNTHETIC PATHWAYS IN THE RAINBOW TROUT (AFTER KIME, 1982, PERSONAL COMMUNICATION).

maturation and ovulation (Jalabert, 1976; Scott et al., 1982) rather than with vitellogenesis. Thus, just before ovulation, increases in serum levels of 17α-hydroxyprogesterone (the precursor to 17α20B) have been observed in the rainbow trout (Scott et al., 1982) followed by a rapid increase in 17α20B around the time of spawning (Kagawa et al., 1981; Scott et al., 1982; Scott & Baynes, 1982; Scott & Sumpter, 1982a). Due to the importance these hormones play in maturation in salmonids and since studies in other vertebrates suggest some stimulation of vitellogenin by progesterone (Yaron & Widzer, 1978) the effects of 17α-hydroxyprogesterone and 17α20B will also be investigated in the present work.

Since many of the steroids on the biosynthetic pathway in fish play important roles as precursors to physiologically active hormones, steroids on the Δ4 and Δ 5 pathways other than those already chosen were also selected for the present investigation. In a study using hypophysectomised goldfish for example, pregnenolone (at the beginning of Δ 5 and Δ 4 pathways) has been observed to induce the formation of yolk granules (Khoo, 1979). Thus it is conceivable that some of these hormones may be physiologically active in their own rights and their effects on vitellogenesis, ovarian weight and histology will be monitored.

Thus, although a role for oestradiol-17β in the control of vitellogenesis in teleosts has now been recognised (Wallace, 1978; de Vlaming et al., 1980; See also Dodd & Sumpter, 1982), such is the diversity of teleost species, that other steroids may have similar effects. Certainly during vitellogenesis in female trout, serum levels of oestrone and testosterone show marked changes and it

could be argued that the high levels of oestrogens at this time are merely the inactive metabolites of other physiologically active steroids. Furthermore, many of the endocrine changes overlap the different phases of ovarian development and their physiological significance is difficult to interpret. Clearly, in this overall investigation into the hormonal mechanisms controlling vitellogenesis in trout, all those hormones capable of inducing hepatic vitellogenesis must be identified. Although it is likely that oestradiol-17β is primarily responsible for the induction of vitellogenin in this species, other hormones, more especially oestrone, may play important secondary roles in the successful development of the ovary.

5.2. Materials and Methods.

In this experiment, the effects of oestrone (E1), oestradiol-17β (E2), oestriol (E3), pregnenolone (P5), testosterone (T), androstenedione (AD), dehydroepiandrosterone (DHA), 17α-hydroxyprogesterone (17αOHP4), 17α-hydroxypregnenolone (17αOHP5) and 17α-hydroxy-20β dihydroprogesterone (17α20B) on vitellogenesis, HSI and GSI were studied.

110 one-year+ rainbow trout, with a mean wt of 50g were selected, weighed, individually tagged and allowed to acclimatise in system A for a week. 10 groups of 10 fish were bled and injected with one of the above steroids. All steroids were obtained from Sigma U.K. (Poole, Dorset) and initially dissolved in a minimum quantity of ethanol, held in an ultrasonic bath for 30 seconds and made up to the correct volume with Arachis oil to give a final injection volume of 200 μl. The steroids were injected intraperitoneally at a dose of 5 $\mu g\ g^{-1}$ body weight. The remaining group of 10 fish were injected with the same volume of vehicle only as controls. All fish were bled 1,3,22 and 36 days after injection. Serum samples were assayed for vitellogenin by RIA and total serum calcium.

38 days after the initial injection all groups were reinjected with the same steroid at the same dose. Those fish injected with E1, E2, E3, P5, T and controls were bled 0,6,18 and 25 days after the second injection. Whereas, the remaining 5 treated groups and controls were bled 0,6,12 and 21 days after the second injection. On day 26 after the second injection, all fish were sacrificed, weighed and segregated into treatment and sex. The hepatosomatic and gonadosomatic indices were determined and gonadal and pituitary tissues taken for histology (See Diagram 5.2.).

(DAY 0) 11 groups of 10 fish blood sampled and injected with one of the following

E1; E2; E3; P5; AD; DHA; 17αOHP4; 17αOHP5; 17α20β; C.

(DAY 1) . . . . . BLOOD SAMPLED

(DAY 3) . . . . . BLOOD SAMPLED

(DAY 22). . . . . BLOOD SAMPLED

(DAY 25). . . . . BLOOD SAMPLED

(DAY 36). . . . . BLOOD SAMPLED

(DAY 38) all groups bled and reinjected with same dose as above :

	GROUP A	GROUP B
	C; E1; E2; E3; P5; T.	AD; DHA; 17αOHP4; 17αOHP5; 17α20β; C.
(DAY 6)	BLOOD SAMPLED	BLOOD SAMPLED
(DAY 12).		BLOOD SAMPLED
(DAY 18).	BLOOD SAMPLED	
(DAY 21).		BLOOD SAMPLED
(DAY 25).	BLOOD SAMPLED	

(DAY 26) All fish sacrificed - HSI & GSI's determined, pituitary & ovarian tissue taken for histology.

DIAGRAM 5.2. PROTOCOL OF INJECTION AND BLOOD SAMPLING.

5.3. Results.

After the initial injection, only 3 treated groups (oestrone E1, oestradiol-17β E2 and oestriol E3) showed significant increases in total serum calcium, whereas 7 treatment groups (E1, E2, E3, pregnenolone P5, androstenedione AD, dehydroepiandrosterone DHA and 17α-hydroxy-20β dihydroprogesterone 17α20β) demonstrated significant increases in serum vitellogenin.

Total serum calcium (TCa) : In all but 2 treatment groups (E2 & 17α20β) there was a significant decrease in the mean TCa levels one day after injection when compared to controls. Those fish treated with P5, DHA and 17α-hydroxyprogesterone (17αOHP4) were significantly lower ($P < 0.05$; $P < 0.01$; $P < 0.001$) than control fish 3 days after injection, and the 17α20β treated group significantly ($P < 0.01$ & $P < 0.05$) lower than control fish 22 and 25 days after injection. Testosterone (T) and 17αOHP4 treated groups were significantly ($P < 0.05$; $P < 0.01$) lower than control fish, 36 days after the initial injection (Table 5.1.).

TCa in E1 treated fish rose significantly ($P < 0.05$) from a basal mean value of 8.28 ± 0.1 to a peak of 10.9 ± 1.0 mg $100ml^{-1}$ after 25 days, before falling to 10.3 ± 0.4 mg $100ml^{-1}$ ($P < 0.01$) after 36 days TCa levels in E2 treated fish rose significantly ($P < 0.001$) from a mean basal value of 8.28 ± 0.1 to a peak of 13.0 ± 0.2 mg $100ml^{-1}$ 22 days after injection, falling to 8.9 ± 0.4 mg $100ml^{-1}$ after 36 days. In E3 treated fish, mean TCa rose significantly ($P < 0.001$) from a basal value of 8.28 ± 0.1 to a peak of 14.5 ± 0.9 mg $100ml^{-1}$ after 22 days and was still significantly ($P < 0.001$) above control levels of

8.9 ± 0.2 at 11.5 ± 0.6 mg $100ml^{-1}$ 36 days after injection. TCa in control fish rose from a mean of 8.28 ± 0.1 to 8.9 ± 0.2 mg $100ml^{-1}$ after 36 days (Table 5.1. & Fig. 5.1.).

HORMONE	BASAL	1	3	22	25	36
E1	8.28	7.89*	7.9	10.7*	10.9*	10.3**
± S.E.	0.1	0.2	0.4	0.9	1.0	0.4
E2	8.28	8.7	8.3	13.0***	11.4***	8.9
± S.E.	0.1	0.6	0.4	0.2	0.3	0.4
E3	8.28	7.9***	7.7	14.5***	12.9	11.5***
± S.E.	0.1	0.1	0.2	0.9	2.1	0.6
P5	8.28	7.8***	7.3*	9.1	8.6	8.5
± S.E.	0.1	0.1	0.3	0.6	0.4	0.2
TEST.	8.28	7.2***	7.2	8.1	8.4	8.2*
± S.E.	0.1	0.2	0.7	0.5	0.2	0.2
CONTROL	8.28	8.5	8.1	8.6	8.7	8.9
± S.E.	0.1	0.1	0.1	0.1	0.1	0.2

TABLE 5.1. CHANGES IN TOTAL SERUM CALCIUM (mg $100ml^{-1}$) FOLLOWING A SINGLE INJECTION OF EITHER OESTRONE (E1), OESTRADIOL-17β (E2), OESTRIOL (E3), PREGNENOLONE (P5) OR TESTOSTERONE (T) AT A DOSE OF 5 $\mu g\ g^{-1}$ COMPARED TO VEHICLE-INJECTED CONTROLS.

CONTD.....

HORMONE	BASAL	1	3	22	25	36
AD	8.28	7.5**	7.2*	9.0	8.6	8.2
± S.E.	0.1	0.3	0.4	0.5	0.2	0.3
DHA	8.28	7.7***	6.7**	9.0	8.5	9.4
± S.E.	0.1	0.1	0.6	0.5	0.3	0.2
17αOHP5	8.28	7.6*	7.4	8.0*	8.6	8.4
± S.E.	0.1	0.4	0.6	0.2	0.4	0.3
17αOHP4	8.28	7.7*	6.7***	8.4	8.1	8.1**
± S.E.	0.1	0.3	0.2	0.2	0.3	0.1
17α20β	8.28	7.8	8.3	8.0**	8.0*	8.8
± S.E.	0.1	0.6	1.6	0.2	0.3	0.4
CONTROL	8.28	8.5	8.1	8.6	8.7	8.9
± S.E.	0.1	0.1	0.1	0.1	0.1	0.2

TABLE 5.1. CHANGES IN TOTAL SERUM CALCIUM (mg $100ml^{-1}$) FOLLOWING A SINGLE INJECTION OF EITHER ANDROSTENEDIONE (AD), DEHYDROEPIANDROSTERONE (DHA), 17α-HYDROXYPROGESTERONE (17αOHP4), 17α-HYDROXYPREGNENOLONE (17αOHP5) OR 17α-HYDROXY-20β DIHYDROPROGESTERONE (17α20B) AT A DOSE OF 5 $\mu g\ g^{-1}$ COMPARED TO VEHICLE-INJECTED CONTROLS.

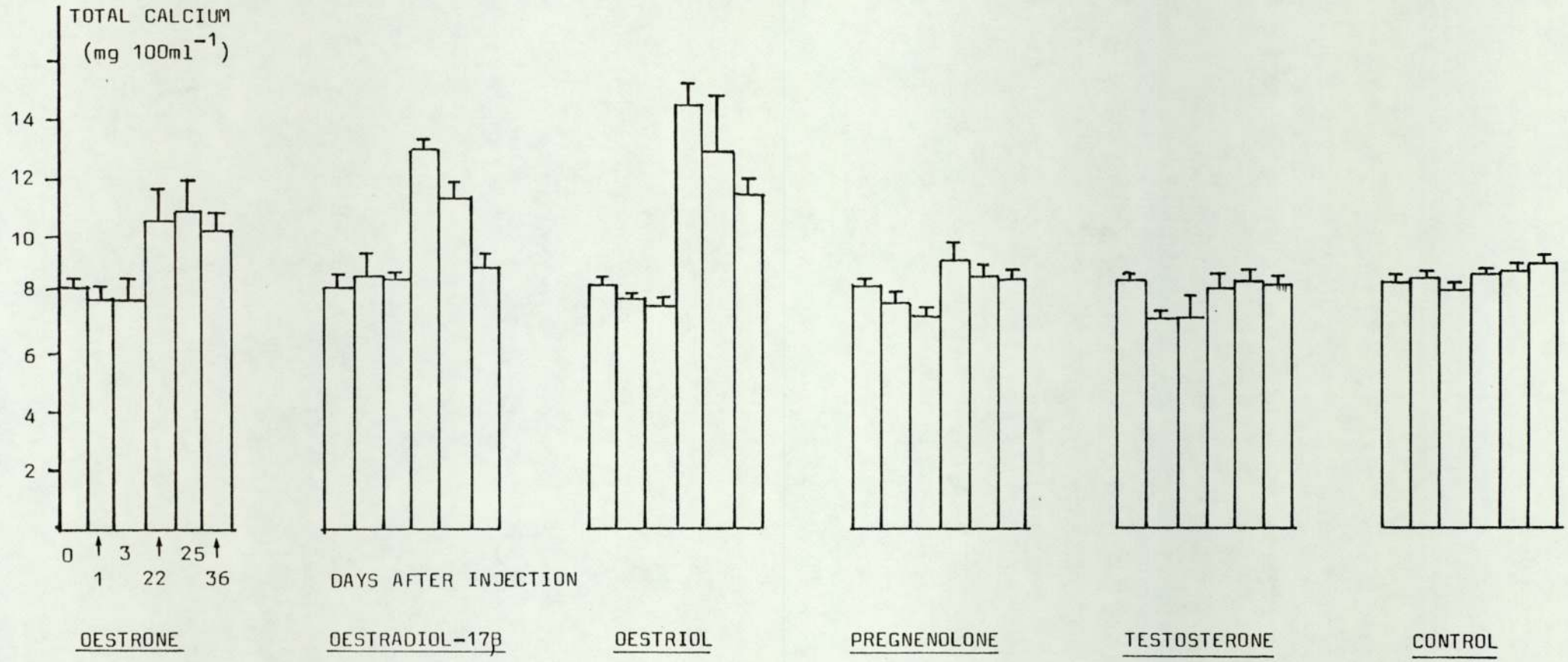


FIG. 5.1. CHANGES IN TOTAL SERUM CALCIUM (mg 100ml^{-1}) FOLLOWING A SINGLE INJECTION OF EITHER OESTRONE (E1), OESTRADIOL-17β (E2), OESTRIOL (E3), PREGNENOLONE (P5) OR TESTOSTERONE (T) AT A DOSE OF 5 μg g^{-1} COMPARED TO VEHICLE-INJECTED CONTROLS.

CONTD.....

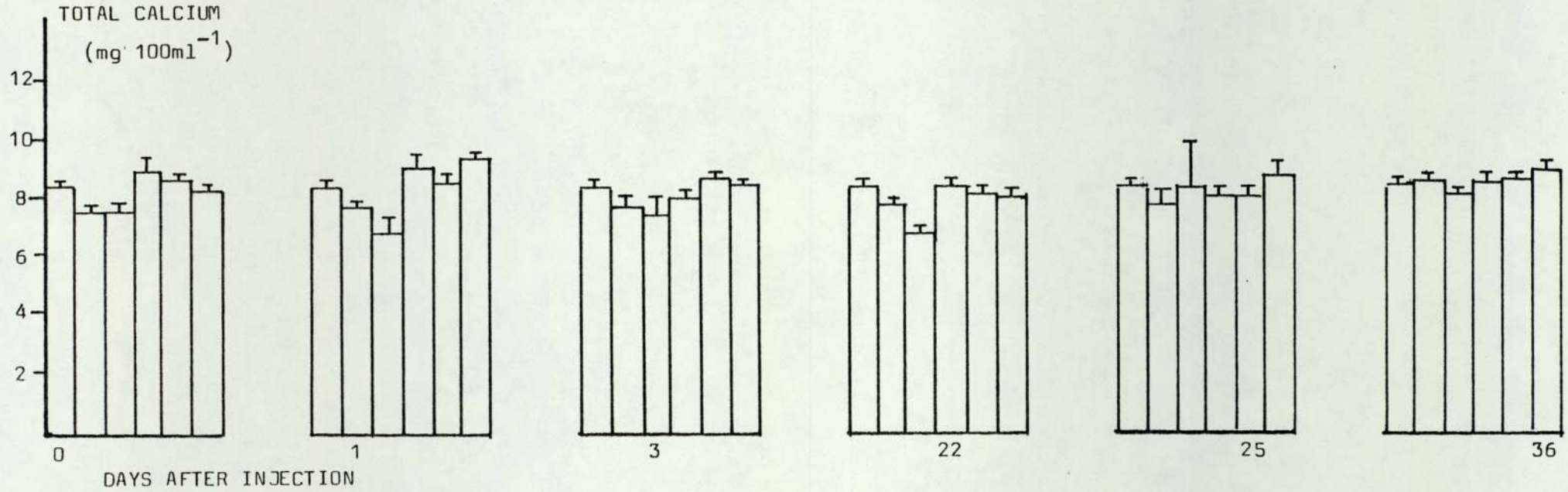


FIG. 5.1. CHANGES IN TOTAL SERUM CALCIUM (mg 100ml^{-1}) FOLLOWING A SINGLE INJECTION OF EITHER ANDROSTENEDIONE (AD), DEHYDROEPIANDROSTERONE (DHA), 17α-HYDROXYPROGESTERONE (17αOHP4), 17α-HYDROXYPREGNENOLONE (17αOHP5) OR 17α-HYDROXY-20β DIHYDROPROGESTERONE (17α20B) AT A DOSE OF 5 μg g^{-1} COMPARED TO VEHICLE-INJECTED CONTROLS.

Serum Vitellogenin (VgRIA) : The mean basal VgRIA level in all fish was 1.55 ± 0.4 $\mu g\ ml^{-1}$. In E1, E2 and E3-treated groups VgRIA levels had almost doubled after one day, rising to 2.5 ± 0.4, 2.4 ± 0.7 and 2.5 ± 0.1 $\mu g\ ml^{-1}$, reaching peak mean levels of 3,010 ± 606, 4,500 ± 500 and 7,500 ± 1,185 $\mu g\ ml^{-1}$ after 25, 22 and 22 days respectively. VgRIA levels in all 3 groups were still significantly above control levels of 20.25 ± 7.0 $\mu g\ ml^{-1}$ at 1,700 ± 239, 529 ± 91.1 and 4,050 ± 922 $\mu g\ ml^{-1}$ respectively after 36 days.

Mean VgRIA levels in P5-treated fish rose significantly ($P < 0.001$) from 1.55 ± 0.4 $\mu g\ ml^{-1}$ to a peak mean level of 1,970 ± 152.0 $\mu g\ ml^{-1}$ after 22 days before falling to 61.0 ± 24.6 $\mu g\ ml^{-1}$ after 36 days. There were no significant changes in those groups treated with T, 17αOHP5 and 17αOHP4.

Mean serum VgRIA levels in AD and DHA-treated fish rose significantly ($P < 0.001$ & $P < 0.01$) from basal mean levels of 1.55 ± 0.4 to peak levels of 810 ± 61.3 and 910 ± 333 $\mu g\ ml^{-1}$ after 22 days, before falling to 300 ± 27.5 and 25.0 ± 7.6 $\mu g\ ml^{-1}$ respectively.

There was a significant ($P < 0.001$) increase in the mean serum VgRIA levels of the group treated with 17α20B, rising from a basal value of 1.55 ± 0.4 $\mu g\ ml^{-1}$ to a peak of 57.5 ± 7.6 $\mu g\ ml^{-1}$ after 22 days, falling to 32.0 ± 7.1 $\mu g\ ml^{-1}$ after 36 days (Table 5.2. & Fig. 5.2.).

HORMONE	BASAL	1	2	22	25	36
E1	1.55	2.5***	945.0***	2250***	3010***	1700***
± S.E.	0.4	0.4	45.1	538	606	239
E2	1.55	2.4**	850***	4500***	3400***	529***
± S.E.	0.4	0.7	66.8	500	690	91.1
E3	1.55	2.5***	1875***	7500***	4900***	4050***
± S.E.	0.4	0.1	59.2	1185	1035	922
P5	1.55	1.15*	49.5***	1970***	222*	61.0
± S.E.	0.4	0.2	4.5	152	59.3	24.6
T	1.55	1.2	18.9	33.7	22.5	15.0
± S.E.	0.4	0.3	18.0	10.5	7.3	6.4
CONTROL	1.55	0.54	8.2	18.3	13.7	20.25
± S.E.	0.4	0.2	1.7	5.2	4.6	7.0

TABLE 5.2. CHANGES IN VITELLOGENIN ($\mu g\ ml^{-1}$) AS MEASURED BY RADIOIMMUNOASSAY IN GROUPS OF FISH INJECTED WITH EITHER OESTRONE (E1), OESTRADIOL-17β (E2), OESTRIOL (E3), PREGNENOLONE (P5) OR TESTOSTERONE (T) AT A DOSE OF 5 $\mu g\ g^{-1}$ COMPARED WITH VEHICLE-INJECTED CONTROLS.

CONTD.....

HORMONE	BASAL	1	3	22	25	36
AD	1.55	1.6	40.0*	810***	725***	300***
± S.E.	0.4	0.5	-	61.3	50.0	27.5
DHA	1.55	1.83	31.3	910**	793**	25.0
± S.E.	0.4	1.2	11.8	333	231	7.6
17αOHP5	1.55	1.25	24.0	37.5	20.0	15.75
± S.E.	0.4	0.3	6.7	8.1	3.9	4.6
17αOHP4	1.55	3.1	37.8	81.48	30.09	9.6
± S.E.	0.4	1.2	15.2	32.3	14.1	2.4
17α20β	1.55	0.4	34.5***	57.5***	45.0**	32.0
± S.E.	0.4	0.1	1.5	7.6	8.2	7.1
CONTROL	1.55	0.54	8.2	18.2	13.7	20.25
± S.E.	0.4	0.2	1.7	5.2	4.6	7.0

TABLE 5.2. CHANGES IN VITELLOGENIN ($\mu g\ ml^{-1}$) AS MEASURED BY RADIOIMMUNOASSAY IN GROUPS OF FISH INJECTED WITH EITHER ANDROSTENEDIONE (AD), DEHYDROEPIANDROSTERONE (DHA), 17α-HYDROXYPROGESTERONE (17αOHP4), 17α-HYDROXYPREGNENOLONE (17αOHP5) OR 17α-HYDROXY-20β DIHYDROPROGESTERONE (17α20β) AT A DOSE OF 5 $\mu g\ g^{-1}$ COMPARED WITH VEHICLE-INJECTED CONTROLS.

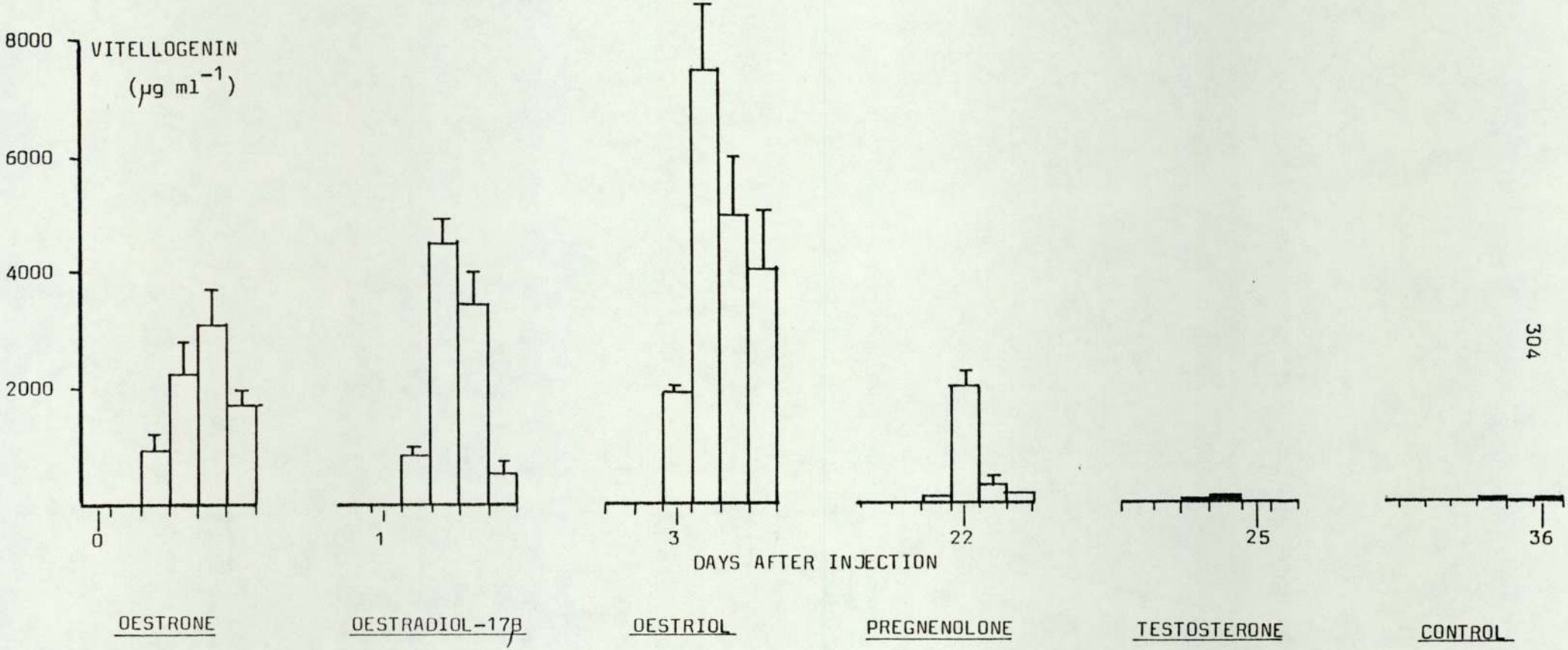


FIG. 5.2. CHANGES IN VITELLOGENIN (µg ml^{-1}) AS MEASURED BY RADIOIMMUNOASSAY IN GROUPS OF FISH INJECTED WITH EITHER OESTRONE (E1), OESTRADIOL-17β (E2), OESTRIOL (E3), PREGNENOLONE (P5) OR TESTOSTERONE (T) AT A DOSE OF 5 µg g^{-1} COMPARED WITH VEHICLE-INJECTED CONTROLS.

CONTD.....

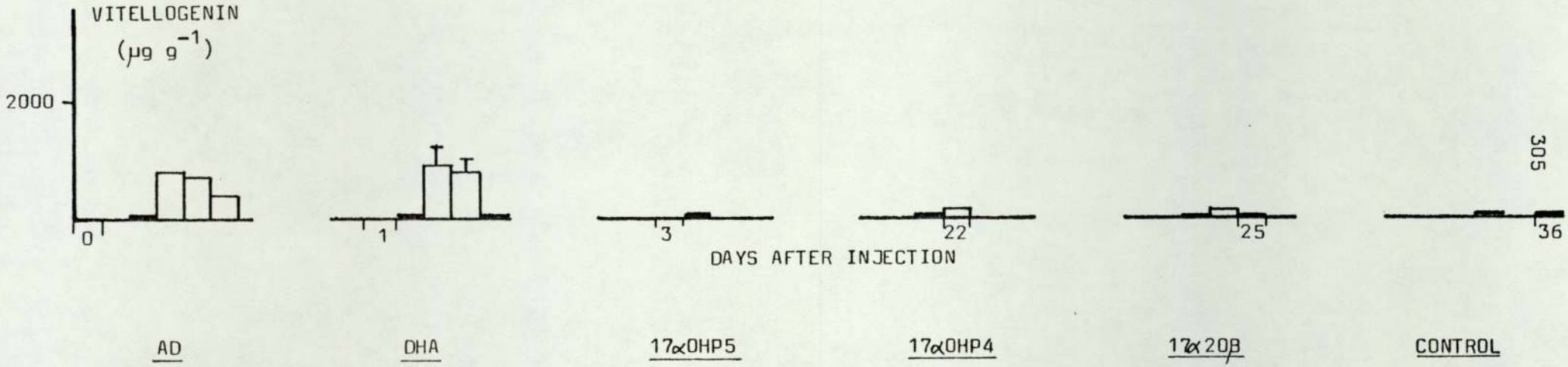


FIG. 5.2. CHANGES IN VITELLOGENIN (µg ml^{-1}) AS MEASURED BY RADIOIMMUNOASSAY IN GROUPS OF FISH INJECTED WITH EITHER ANDROSTENEDIONE (AD), DEHYDROEPIANDROSTERONE (DHA), 17α-HYDROXYPROGESTERONE (17αOHP4), 17α-HYDROXYPREGNENOLONE (17αOHP5) OR 17αHYDROXY-20β, DIHYDROPROGESTERONE (17α20β) AT A DOSE OF 5 µg g^{-1} COMPARED WITH VEHICLE-INJECTED CONTROLS.

Reinjection : On reinjection of each group with the same dose of steroid 38 days after the initial injection, E1, E2 and E3-treated groups demonstrated much more rapid secondary vitellogenic responses as indicated by changes in total serum calcium (Table 5.3. & Fig. 5.3.). Thus mean TCa levels in E1-treated fish rose significantly ($P < 0.001$) from basal levels of 9.89 $\pm$ 0.4 mg $100ml^{-1}$ to peak mean levels of 18.8 $\pm$ 2.2 mg $100ml^{-1}$ after 25 days. Mean TCa levels in E2-treated fish rose significantly ($P < 0.001$) from a mean basal level of 9.1 $\pm$ 0.3 mg $100ml^{-1}$ to peak mean levels of 18.5 $\pm$ 1.8 mg $100ml^{-1}$. In E3-treated fish mean TCa levels rose significantly ($P < 0.001$) from 10.5 $\pm$ 0.5 mg $100ml^{-1}$ to a peak mean level of 29.01 $\pm$ 4.5 mg $100ml^{-1}$ after 18 days. Basal TCa levels in both E1 and E3-treated groups were significantly higher ($P < 0.05$) than basal levels in control fish at 8.8 $\pm$ 0.3 mg $100ml^{-1}$ (Table 5.3. & Fig. 5.4.).

In P5 and 17α20B-treated groups, mean TCa levels rose significantly ($P < 0.05$) from basal values of 8.7 $\pm$ 0.3 and 8.8 $\pm$ 0.2 mg $100ml^{-1}$ to peak mean levels of 9.7 $\pm$ 0.4 and 9.5 $\pm$ 0.4 mg $100ml^{-1}$ respectively. Other treated groups and control fish showed no such similar changes in serum levels of TCa. (Tables 5.3. & 5.4. and Figs. 5.4.).

These serum samples were not assayed for VgRIA.

HORMONE	BASAL	6	18	25
E1	9.89*	13.34***	18.5***	18.8***
± S.E.	0.4	0.6	1.8	2.2
E2	9.1	14.1***	18.5***	10.02***
± S.E.	0.3	0.4	1.8	0.3
E3	10.5*	15.2***	29.01***	28.12***
± S.E.	0.5	1.1	4.5	4.8
P5	8.7	9.45	9.7*	9.25
± S.E.	0.3	0.75	0.4	0.6
T	8.3	8.39	8.4	8.68
± S.E.	0.3	0.2	0.4	0.9
CONTROL	8.8	8.6	8.5	8.4
± S.E.	0.3	0.3	0.2	0.2

TABLE 5.3. CHANGES IN TOTAL SERUM CALCIUM (mg $100ml^{-1}$) FOLLOWING REINJECTION WITH EITHER OESTRONE (E1), OESTRADIOL-17β (E2), OESTRIOL (E3), PREGNENOLONE (P5) OR TESTOSTERONE (T) AT A DOSE OF 5 $\mu g\ g^{-1}$ AFTER PREVIOUS INJECTION WITH THE SAME HORMONE 38 DAYS EARLIER.

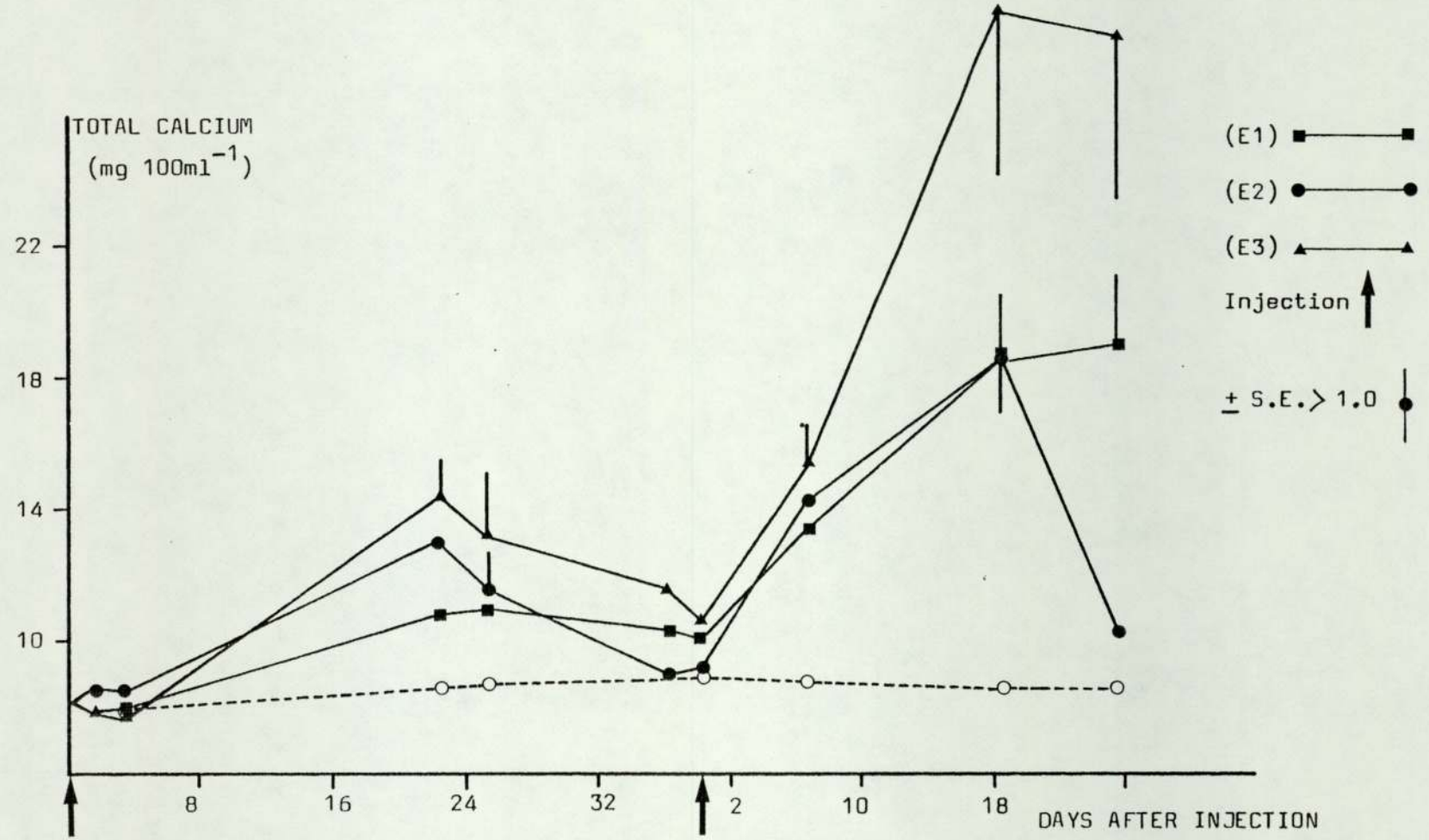


FIG. 5.3. COMPARISON OF PRIMARY AND SECONDARY VITELLOGENIC RESPONSES TO INITIAL AND SUBSEQUENT INJECTION OF OESTRONE (E1), OESTRADIOL-17β (E2) AND OESTRIOL (E3) IN GROUPS OF TROUT.

HORMONE	BASAL	6	12	21
AD	8.4	8.8	9.7	8.97
± S.E.	0.2	0.3	0.7	0.3
DHA	9.2	9.1	9.01	8.7
± S.E.	0.1	0.4	0.2	0.2
17αOHP4	8.3	8.5	8.83	8.73
± S.E.	0.3	0.2	0.1	0.1
17αOHP5	8.2	8.6	9.3	9.29
± S.E.	0.2	0.3	0.3	0.8
17α20β	8.8	9.2	9.5*	8.6
± S.E.	0.2	0.1	0.4	0.5
CONTROL	8.8	8.6	8.5	8.4
± S.E.	0.3	0.3	0.2	0.3

TABLE 5.4. CHANGES IN TOTAL SERUM CALCIUM (mg $100ml^{-1}$) FOLLOWING REINJECTION WITH EITHER ANDROSTENEDIONE (AD), DEHYDROEPIANDROSTERONE (DHA), 17α-HYDROXYPROGESTERONE (17αOHP4), 17α-HYDROXYPREGNENOLONE (17αOHP5) OR 17α-HYDROXY-20β DIHYDROPROGESTERONE (17α20β) AT A DOSE OF 5 $\mu g\ g^{-1}$ AFTER PREVIOUS INJECTION WITH THE SAME HORMONE 38 DAYS EARLIER.

FIG. 5.4. CHANGES IN TOTAL SERUM CALCIUM (mg $100ml^{-1}$) FOLLOWING REINJECTION WITH EITHER OESTRONE (E1), OESTRADIOL-17β (E2), OESTRIOL (E3), PREGNENOLONE (P5) OR TESTOSTERONE (T) AT A DOSE OF 5 $\mu g\ g^{-1}$ AFTER PREVIOUS INJECTION WITH THE SAME HORMONE 38 DAYS EARLIER.

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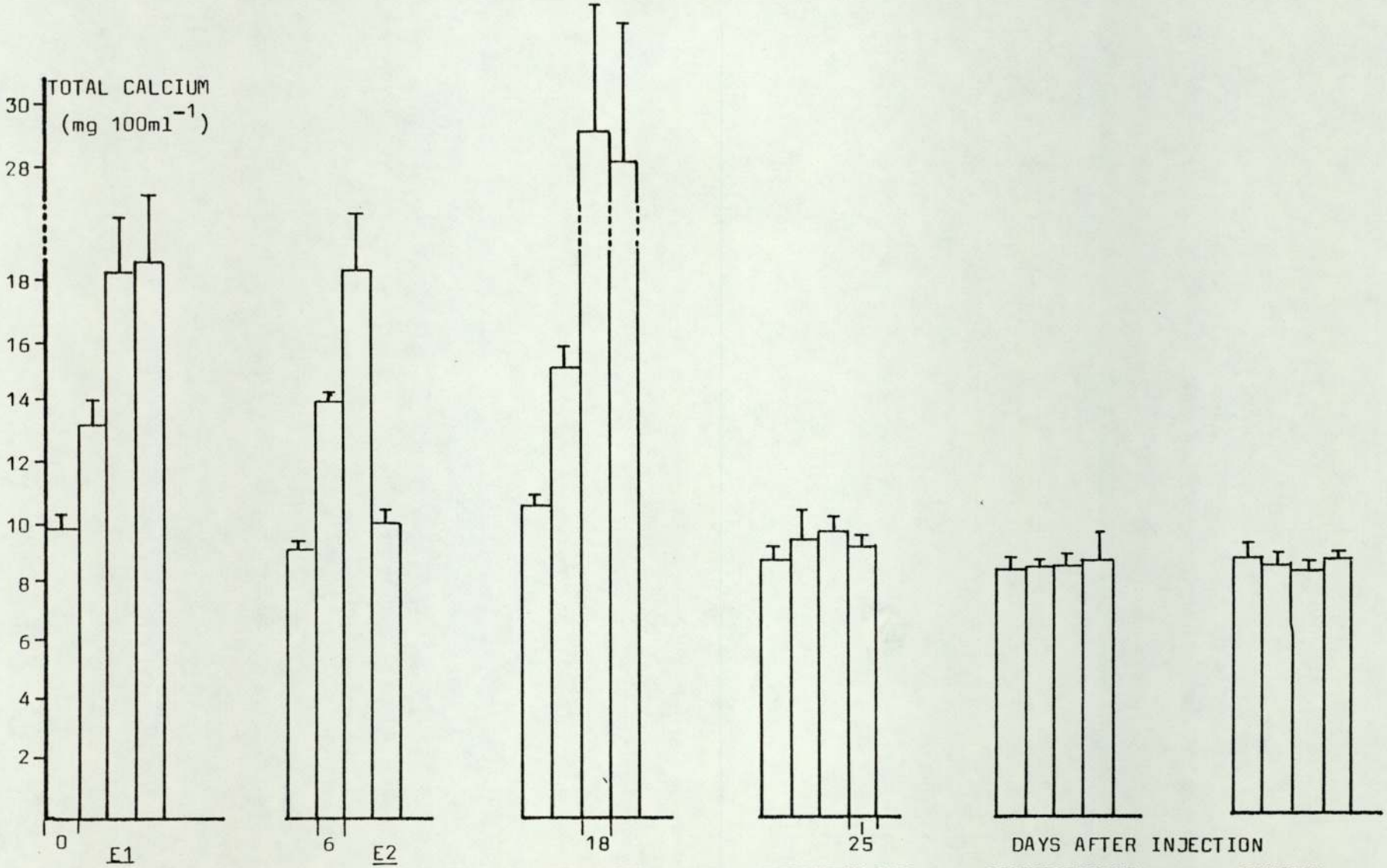

FIG. 5.4. LEGEND OPPOSITE.

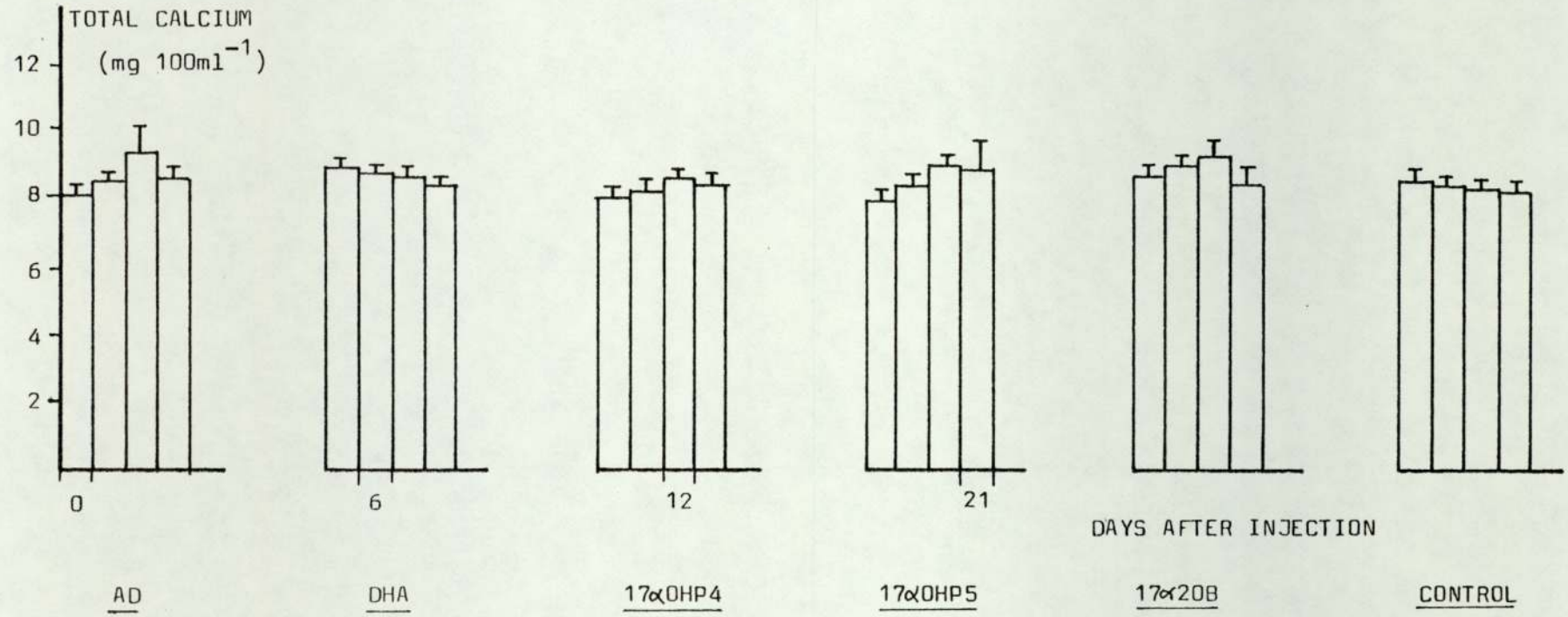

FIG. 5.4. CHANGES IN TOTAL SERUM CALCIUM (mg 100ml^{-1}) FOLLOWING REINJECTION WITH EITHER ANDROSTENEDIONE (AD), DEHYDROEPIANDROSTERONE (DHA), 17α-HYDROXYPROGESTERONE (17αOHP4), 17α-HYDROXYPREGNENOLONE (17αOHP5) OR 17α-HYDROXY-20β-DIHYDROPROGESTERONE (17α20β) AT A DOSE OF 5 μg g^{-1} AFTER PREVIOUS INJECTION WITH THE SAME HORMONE 38 DAYS EARLIER.

Hepatosomatic (HSI) and Gonadosomatic (GSI) indices : At the end of the experiment when the fish were sacrificed, there was very little difference in the HSI of male and female fish between treatment groups when compared to controls.

The HSI in female fish treated with AD and 17α20β was significantly ($P < 0.01$) lower than the HSI in control female fish. The HSI in male fish treated with P5 was significantly ($P < 0.05$) lower than the HSI in male control fish. Maximum HSI's in male fish was found in 17α20β-treated fish at 1.75 $\pm$ 0.15%, and the maximum in female fish was 2.03 $\pm$ 0.18% in E3-treated group. Minimum HSI's in male and female fish were found in the P5 and 17α20β-treated groups respectively, with values of 1.24 $\pm$ 0.09 and 1.31 $\pm$ 0.03% (Table 5.5. & Fig. 5.6.).

Similarly, there was little difference between treatments in the GSI of male fish, when compared to controls apart from the 17α20β-treated group which were significantly ($P < 0.05$) higher than the control value of 0.04 $\pm$ 0.01% at 0.06 $\pm$ 0.007%. Minimum GSI in male fish was found in 17α20B-treated fish at 0.03 $\pm$ 0.01% and the maximum mean GSI in E2-treated fish at 0.82 $\pm$ 0.7%.

However, in female fish the GSI was significantly above the control value of 0.12 $\pm$ 0.006% in fish treated with E1 ($P < 0.01$) at 0.21 $\pm$ 0.02%, E2 ($P < 0.001$) at 0.22 $\pm$ 0.02%, P5 ($P < 0.01$) at 0.17 $\pm$ 0.02%, AD ($P < 0.001$) at 0.2 $\pm$ 0.01%, DHA ($P < 0.001$) at 0.174 $\pm$ 0.01%, 17αOHP4 ($P < 0.001$) at 0.2 $\pm$ 0.02%, 17αOHP5 ($P < 0.05$) at 0.18 $\pm$ 0.02% and 17α20β ($P < 0.01$) at 0.19 $\pm$ 0.02%. A maximum GSI in female fish of 0.22 $\pm$ 0.02% was found in the E2-treated group and a minimum GSI of 0.12 $\pm$ 0.006% found in the controls (Table 5.5. & Fig. 5.6.).

HORMONE	HSI (Mean + S.E.)		GSI (Mean + S.E.)	
	♂	♀	♂	♀
E1	1.51	1.66	0.31	0.21***
± S.E.	0.05	0.14	0.17	0.02
E2	1.68	1.97	0.82	0.22***
± S.E.	0.16	0.12	0.7	0.02
E3	1.73	2.03	0.18	0.197
± S.E.	0.09	0.18	0.09	0.04
P5	1.24*	1.94	0.043	0.17**
± S.E.	0.09	0.46	0.01	0.02
T	1.58	1.58	0.057	0.22
± S.E.	0.19	0.21	0.004	0.33
CONTROL	1.59	1.87	0.04	0.12
± S.E.	0.14	0.18	0.01	0.006

TABLE 5.5. MEAN HSI AND GSI 25 DAYS AFTER REINJECTION WITH EITHER OESTRONE (E1), OESTRADIOL-17β (E2), OESTRIOL (E3), PREGNENOLONE (P5), TESTOSTERONE (T) COMPARED WITH VEHICLE-INJECTED CONTROLS.

CONTD.....

HORMONE	HSI (Mean + S.E.)		GSI (Mean + S.E.)	
	♂	♀	♂	♀
AD	1.49	1.43**	0.05	0.2***
± S.E.	0.05	0.04	0.01	0.01
DHA	1.5	1.56	0.053	0.174***
± S.E.	0.16	0.07	0.01	0.01
17αOHP4	1.48	1.56	0.04	0.2***
± S.E.	0.05	0.09	0.001	0.02
17αOHP5	1.63	1.44	0.06*	0.18*
± S.E.	0.12	0.16	0.007	0.02
17α20β	1.75	1.31**	0.03	0.19**
± S.E.	0.15	0.03	0.01	0.02
CONTROL	1.59	1.87	0.04	0.12
± S.E.	0.14	0.18	0.01	0.006

TABLE 5.5. CHANGES IN HSI AND GSI IN GROUPS OF FISH 25 DAYS AFTER REINJECTION WITH EITHER ANDROSTENEDIONE (AD), DEHYDROEPIANDROSTERONE (DHA), 17α-HYDROXYPROGESTERONE (17αOHP4), 17α-HYDROXYPREGNENOLONE (17αOHP5) OR 17α-HYDROXY-20β-DIHYDROPROGESTERONE (17α20β) COMPARED WITH VEHICLE-INJECTED CONTROLS.

FIG. 5.5. MEAN HSI AND GSI 25 DAYS AFTER REINJECTION WITH EITHER OESTRONE (E1), OESTRADIOL-17β (E2), OESTRIOL (E3), PREGNENOLONE (P5) OR TESTOSTERONE (T) COMPARED WITH VEHICLE-INJECTED CONTROLS.

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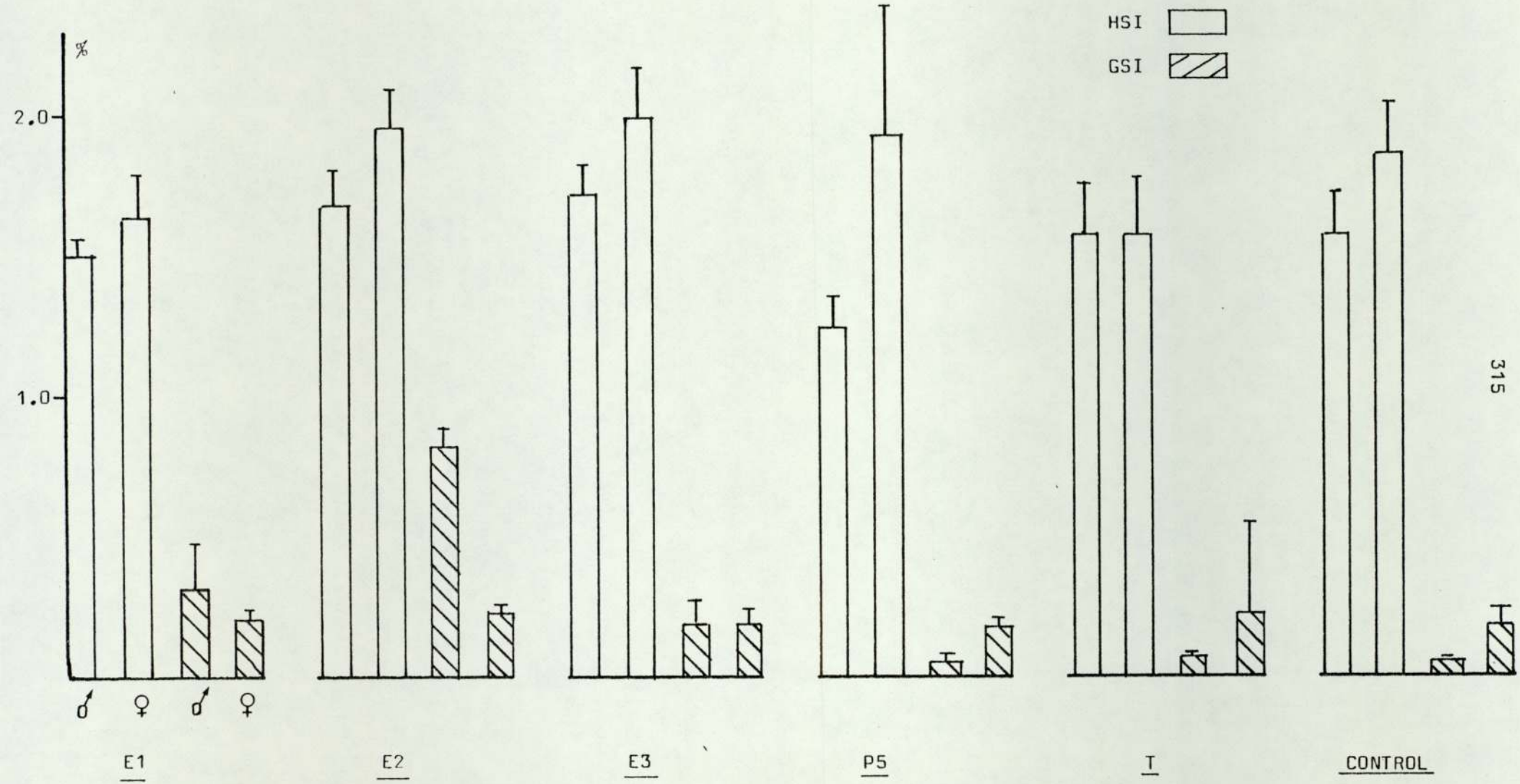


FIG. 5.5. LEGEND OPPOSITE.

FIG. 5.5. CHANGES IN HSI AND GSI IN GROUPS OF FISH 25 DAYS AFTER REINJECTION WITH EITHER ANDROSTENEDIONE (AD), DEHYDROEPIANDROSTERONE (DHA), 17α-HYDROXYPROGESTERONE (17αOHP4), 17α-HYDROXYPREGNENOLONE (17αOHP5) OR 17α-HYDROXY-20β-DIHYDROPROGESTERONE (17α20β) COMPARED WITH VEHICLE-INJECTED CONTROLS.

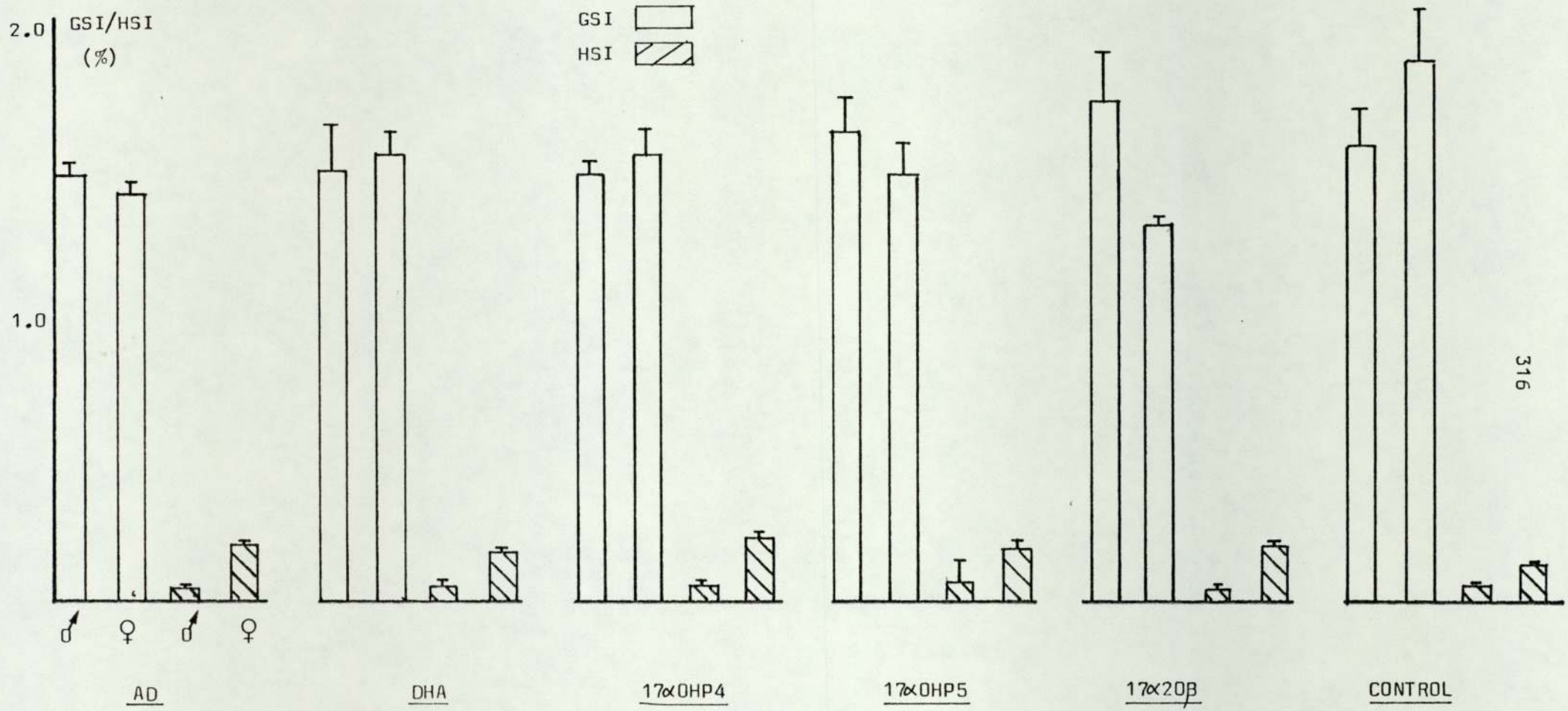


FIG. 5.5. LEGEND OPPOSITE.

Effects of Sex Steroids on Ovarian Histology : Under light microscopy, there were no apparent differences in the stage of development between the different treatment groups. All ovarian sections examined were in the primary growth phase (See Chapter 6) and were not significantly different from control sections.

5.4. Discussion.

The results clearly demonstrate that the liver receptor mechanism responsible for the activation of vitellogenesis shows a distinct preference for oestrogens. Thus, fish injected with either oestrone, oestradiol-17β or oestriol demonstrated variable responses as shown by significant increases in serum levels of vitellogenin (RIA) and total calcium. Subsequently, on reinjection with the same three steroids, more rapid secondary responses were observed as demonstrated by increases in total calcium. On both occasions, oestriol had twice the vitellogenic potency of either of the other two oestrogens. Androstenedione and dehydroepiandrosterone treated groups whilst significantly raising vitellogenin levels, had no effect on calcium after the first injection. The skin colouration darkened considerably in these two groups. Pregnenolone treated fish showed a small, although significant vitellogenic response after the first and second injection. All other treatment groups showed variable and inconsistent responses when compared to controls and oestrogen-treated fish. In all treated groups the GSI was significantly raised above controls (except oestriol and testosterone treated fish) whereas there were no significant increases in HSI.

Of the ten steroids tested in the present work, only the three oestrogens: oestrone, oestradiol-17β and oestriol, androstenedione, dehydroepiandrosterone and pregnenolone had any capacity to raise serum vitellogenin levels in this study. In other comparative studies of the effects of oestrone and oestradiol-17β on vitellogenesis, oestrone has generally been found to have a reduced

vitellogenic action when compared to the effects of oestradiol-17B (Redshaw et al., 1969; van Bohemen et al., 1981a). Thus it was observed that oestrone had approximately 5% of the potency of oestradiol-17B when ovariectomised trout were injected with similar doses of the two steroids (van Bohemen et al., 1981a). In contrast, the present results demonstrate that oestrone was almost as potent at inducing vitellogenesis in this species as oestradiol-17B and the apparent discrepancy may be due to some conversion of oestrone to oestradiol-17β in the intact fish used in this study. It has also been shown that when oestrone and oestradiol-17β were injected together into the same fish, the resulting vitellogenic response was greater than the sum of the individual responses when these steroids were injected alone (van Bohemen et al., 1982a). In the same study they noted in fish treated with both steroids, that whilst the serum vitellogenin levels were not correlated with serum oestradiol-17β levels, they were correlated with the sum of these two oestrogens. Since oestrone levels as high as 30 ng ml^{-1} have been observed during the reproductive cycle in rainbow trout (van Bohemen et al., 1981b) a physiological role for this hormone in vitellogenesis has been proposed. It was demonstrated by the same group that after a primary vitellogenic response to a single oestrone injection, subsequent oestradiol-17B treatment in the same fish, yields a more rapid secondary vitellogenic response (van Bohemen et al., 1982b). Since these authors have also observed a small rise in oestrone early in the reproductive cycle of rainbow trout (van Bohemen et al., 1981b), they have suggested that oestrone may prime the liver so that the increased levels of oestradiol-17β that follow, produce a greater secondary vitellogenic response

(van Bohemen et al., 1982b). In view of these findings, the changes in serum levels of oestrone will be studied in the present investigation of the mechanisms controlling vitellogenesis in this species (See Chapter 7).

The vitellogenic response to oestradiol-17β is well documented in Chapter 3, and although the response here is noticeably lower, this may be due to the smaller size of fish and the time of injection (Spring). Also, unlike other results elsewhere in this study (Chapter 3), there was no effect of oestradiol-17β, or the other oestrogens, on the HSI. Previous investigations have demonstrated a correlation between the dose of oestradiol-17β and HSI after treatment in trout (Chapter 3) which is supported by similar findings of increased HSI after oestrogen treatment in other teleosts (Egami, 1955; Oguro, 1956; McBride & van Overbeeke, 1971; Aida et al., 1973a; van Bohemen et al., 1981a). This increase in HSI is due to an increase in the rough endoplasmic reticulum and Golgi systems as a result of oestrogen-induced vitellogenin synthesis (Aida et al., 1973a; van Bohemen et al., 1981b). The absence of significantly increased HSI after oestradiol-17β treatment in this study may be a reflection of the poor vitellogenic response that was observed.

In contrast, the GSI in oestradiol-17β and oestrone treated females were significantly raised in both cases. Although other workers have observed a causal increase in GSI after oestradiol-17β treatment (Olivereau & Olivereau, 1979) and a correlation between increasing serum levels of oestradiol-17β and GSI during the reproductive cycle (de Vlaming, 1977; Yaron et al., 1977; Lambert

et al., 1978) the reported effects of oestrogen treatments on GSI and ovarian histology in the literature are equivocal (See Dodd, 1960; Ho & Vanstone, 1961; Simon & Reinboth, 1974). Thus, whether oestrogens are observed to be stimulatory or not seems to depend, amongst other things on the age, sex and species of fish investigated. However, in the present study, all females in the treated groups (apart from oestriol and testosterone) had significantly increased GSI when compared to controls. Since the growth rates of all treated groups (not reported here) were lower than those of the control group it is possible that the sudden depression in somatic growth due to treatment, artificially increased the gonad:somite ratio. It has previously been observed that GSI alone is not always a valid index of gonadal activity and should be supported by histological examinations (Delahunty & de Vlaming, 1980). This anomaly of increased GSI as a result of decreased growth is supported by the lack of any histological evidence to demonstrate an effect of these hormones on ovarian development. All three oestrogens have been observed to induce the formation of vesicles when the ovaries of hypophysectomised goldfish were studied histologically (Khoo, 1979; See also Chapter 6). However, the dissimilarity between Khoo's (1979) and the present data may be due to the higher dose and frequency of treatment since 10 $\mu g\ g^{-1}$ was given every 3 days for a month in Khoo's study. The appearance of vesicles in immature trout ovaries after oestradiol-17β treatment has also been observed in this laboratory, when the dose and duration of treatment was also greater than the present work. Whether in this case the effect of oestradiol-17β on the ovary was primary or via feedback on the

pituitary is not certain. It has been recognised that the initiation of vesicle formation requires a pituitary stimulus and that oestrogen alone would be insufficient (Vivien, 1939; Barr, 1968; Upadhyay et al., 1978). Furthermore, falling serum levels of oestradiol-17β observed towards the end of maturation in female trout are believed to feedback on the pituitary and trigger the increase in gonadotrophin which is seen just before, during and after ovulation (See Chapter 7). Thus, falling serum oestradiol-17B levels after injection in intact fish, may be sufficient to cause a surge in gonadotrophin which initiates vesicle formation in these fish. Clearly, the number and frequency of these triggers (ie. oestradiol-17β injections) to the pituitary, may have a direct bearing on whether any response occurs. However, there are numerous reports that demonstrate that steroid treatment of immature salmonids and other teleosts causes an increase in the pituitary content of gonadotrophin, but no release (Crim & Peter, 1978; Olivereau & Chambolle, 1978; Crim & Evans, 1979; Olivereau & Olivereau, 1979; Crim et al., 1981). It has been suggested that oestrogenic steroids may act on the hypothalamo-hypophysial axis to stimulate gonadotrophin synthesis in sexually immature teleosts and that this may be part of the mechanism for the onset of sexual maturity or gonadal recrudescence (Peter, 1982). Whatever the effect of oestrogens on immature fish, a role for oestradiol-17B in the direct control of vitellogenesis in teleosts is now well established and changes in the serum levels of this hormone during the reproductive cycle of female trout, will be investigated further (See Chapter 7).

Of the three oestrogens, it was surprising that oestriol, which is not synthesised naturally in rainbow trout (Lambert & van Bohemen,

1979), was the most potent at inducing vitellogenin synthesis. Similarly, oestriol was found to have the greatest vitellogenic action in a study of the effects of oestrogens in Tilapia where it was concluded that its high potency could be attributed to the oestrogen per se rather than its conversion to oestradiol-17β (Terkatin-Shimony & Yaron, 1978). However, according to the criteria laid down by Redshaw et al., (1969), a hormone needs to be both synthesised and released from the ovary into the blood, as well as having vitellogenic potency, in order to play a physiological role in vitellogenesis in oviparous vertebrates. Thus, since oestriol is not synthesised or released by trout ovaries, this oestrogen can be of little significance in the physiology of vitellogenesis in this species.

In contrast to observations in other studies (Hori et al., 1979; Le Menn, 1979; Le Menn & Lamy, 1979) testosterone did not have any vitellogenic potency in this work. Similarly, in another study of the effects of testosterone on vitellogenesis in ovariectomised rainbow trout, no effects on serum vitellogenin levels were observed with similar doses to those used here (van Bohemen et al., 1981c). These authors suggested that since those studies observing a vitellogenic action in testosterone used intact fish, then a conversion of this hormone by the aromatase enzyme to oestradiol-17β may have accounted for its apparent physiological action, whereas ovariectomised fish were unable to carry out this conversion. Indeed Hori et al., (1979) noted a stimulation in NADPH cytochrome c reductase one week after methyltestosterone treatment and since this enzyme is involved in steroid metabolism this supports the

aromatisation of androgens to oestrogens in these studies. However, since there is evidence that the brain of some teleosts, including trout, has high aromatase activity (Lambert & van Oordt, 1982), and since no effect of testosterone was observed in intact fish in the present study, the vitellogenic action of this steroid reported by other workers may be related to the pharmacological levels administered. This is supported by a study of the effects of testosterone on the inhibition of vitellogenesis in the turtle which revealed an inverse correlation between the dose of testosterone and the reduction in the vitellogenic response (Ho et al., 1981). They concluded that whilst at lower doses testosterone inhibited vitellogenesis, at higher doses some aromatisation to oestrogens occurred which subsequently masked any inhibition. In view of the reports in the literature of high levels of testosterone in females during the reproductive cycle, serum changes in this hormone will be investigated in trout held under closely controlled environmental conditions.

Since oestrone and oestradiol-17β are the two major biosynthetic products from in vitro incubations of trout ovary with androstenedione, then conversion of this steroid by aromatase to oestrogens could also have accounted for its vitellogenic action in this study. This is supported by the observation that androstenedione had no vitellogenic potency in ovariectomised rainbow trout when given in similar doses (ven Bohemen et al., 1982a). Similarly the significant increase in vitellogenin (RIA) following dehydroepiandrosterone treatment may have also been due to a conversion of this steroid, initially to androstenedione and subsequently to oestrogen.

However, a conversion to oestrogen does not account for the observation that both these steroids initially caused a decrease in serum calcium levels and caused no significant increase in this element even after a second injection. Similarly Hori et al., (1979) found that whilst vitellogenin could be detected immunologically and electrophoretically after androstenedione treatment, there was no effect on calcium in intact goldfish. It is possible that these two steroids had a direct effect on calcium metabolism which prevented a mobilization of this element from the scales. Although there are few reports of the direct effects of androgens on serum calcium, decreased levels have been observed in male Atlantic salmon spawning in fresh water, and it has been suggested that these fish were unable to regulate serum calcium levels which reflected their lower osmotic environment (Dacke, 1979). The darkening of the skin observed in androstenedione and dehydroepiandrosterone treated fish is similar to one of the reported changes that occurs in maturing male salmonids and it may be that these two hormones have an effect on ion regulation, possibly related to changes in osmotic permeability of the skin. However, although serum changes in androstenedione are observed during reproduction, the low vitellogenic response observed with high doses of this steroid and also dehydroepiandrosterone, make them of lesser significance in the present investigation.

Thus, of the steroids tested only oestrone and oestradiol-17β are of further consideration due to their high vitellogenic action and synthesis and release by the ovaries of female rainbow trout. Also, serum changes in testosterone will be monitored, not because

of any proven vitellogenic potency in trout, but because no physiological role has as yet been demonstrated for the high levels of this hormone which are observed in female trout during maturation. It is possible that a close study of the morphological changes that are occurring in the ovary during this period may provide evidence of a role for testosterone in the reproduction of female teleosts.

CHAPTER 6.

THE HISTOLOGY AND STRUCTURE OF THE OVARY.

6.1. Introduction.

Although the production of vitellogenin by the liver is of considerable importance in female oviparous species, the acquisition of this large yolk-precursor by the ovary and its subsequent conversion to the yolk proteins within the oocyte is crucial to the successful development of the ovary. However, the specific process by which serum vitellogenin is sequestered by the oocyte represents only one of the four recognised phases of oocyte growth. The first of these is marked by the formation of multiple nucleoli and yolk-nuclei (Hubbard, 1894) and the second by the formation of yolk-vesicles (Konopacka, 1935). The third phase, that of the assimilation of vitellogenin into spheres or granules (Marza et al., 1937; Korfsmeier, 1966) is followed by a period of maturation characterized by the solubilization of the yolk and the breakdown of the germinal vesicle (Fulton, 1898). There is a further phase in the cycle of ovarian development in annually-breeding teleosts which includes ovulation and a period of oogonial proliferation. It was the aim of this study to identify these different phases histologically in rainbow trout, as a reference to a study of the endocrine changes that occur during reproductive cycles in which there was a close control of all environmental conditions (See Chapter 7). In this way, it was possible to relate changes in the hormonal environment to the different morphological stages of development and provide a greater understanding of the mechanisms involved in the control of the different phases of ovarian growth.

It is somewhat surprising amongst teleost species, which as a group account for almost half of all recognised vertebrate species

(Bond, 1979), that the mechanisms for ovarian growth are quite similar, with the greatest diversity being observed in the recruitment of oocytes and the relative timing of events. However, teleost ovaries show striking differences in gross morphology. In some species the ovary is a closed sac, communicating only with its duct (the cystovarian condition) whereas in others the follicles are exposed to the body cavity and the ducts exist as short funnels (gymnovarian) (Dodd & Sumpter, 1982). In the latter condition, to which trout belong, the eggs are shed into the body cavity and subsequently may pass into a cavity that opens to the exterior through an orifice on the urinogenital papilla (van den Hurk & Peute, 1979). In the rainbow trout the paired ovaries are situated ventrolaterally to the swim bladder in the dorsal part of the coelom with each ovary being suspended in the body cavity along its dorsal side. The ovaries which are bounded by a peritoneal membrane consist of ovigerous folds or lamellae, transversely arranged along its longitudinal axis, and these are covered by coelomic or germinal epithelium. However, since most teleosts are seasonal breeders, marked seasonal differences in ovarian structure have been observed and subsequently, a number of authors have divided the cycle of ovarian changes into well-defined developmental stages (Diag. 1; Gokhale, 1957; Polder, 1961; van den Hurk & Peute, 1979; Khoo, 1979; Kagawa et al., 1981).

In early development, teleost ovaries consist of stroma and oogonia, and in the first stage of ovarian development, a mitotic proliferation of these oogonia occurs. The oogonia, which are small rounded cells with clear cytoplasm, a relatively large nucleus and a prominent single nucleolus, occur either singly or more

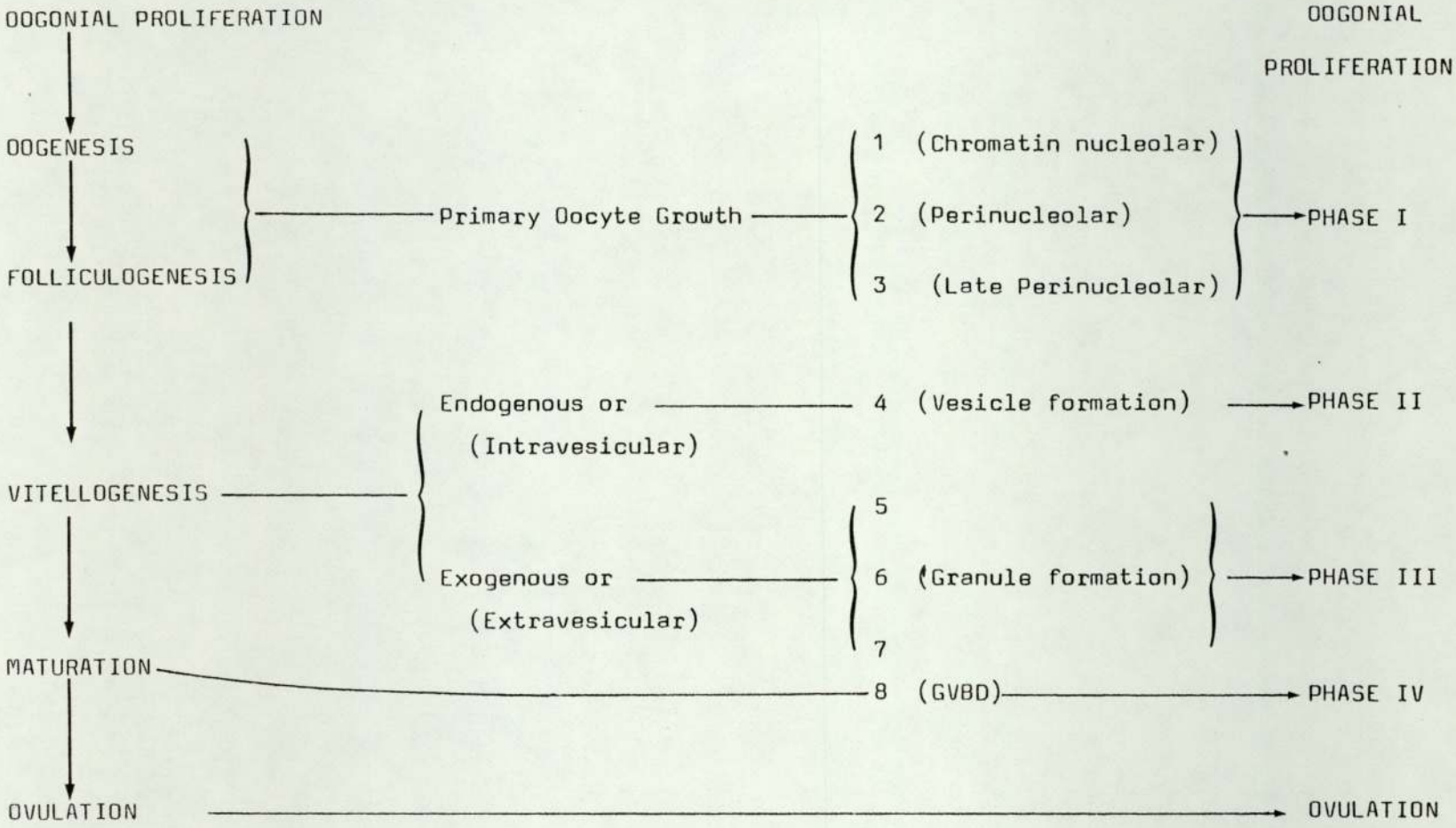


Diagram 6.1. SUMMARY OF THE DIFFERENT NOMENCLATURE USED TO DESCRIBE THE MORPHOLOGICAL CHANGES THAT OCCUR DURING THE REPRODUCTIVE CYCLE OF ANNUALLY BREEDING TELEOSTS.

usually in nests (Mathews, 1938; Barr, 1963; Braekevelt & McMillan, 1967). Oogonia are found in the majority of teleosts at all stages of the reproductive cycle, but undergo periodic mitotic division (Franchi et al., 1962). The timing of this oogonial proliferation in adult fish has been correlated with the reproductive cycle and in annually-breeding teleosts it has been shown to reach a peak during the immediate post-spawning period (Yamazaki, 1965; Braekevelt & McMillan, 1967; de Vlaming, 1972). In other teleost species with different reproductive strategies, oogonial proliferation may be observed at any time and be virtually continuous or occur in waves throughout the year (Tokarz, 1978).

The cyclical nature of oogonial proliferation suggests that it is controlled by hormonal mechanisms (Hoar, 1969; de Vlaming, 1974; Tokarz, 1978) and the few data available indicate that it is primarily the pituitary gland which may be involved in the control of this process. Thus, it has been shown that oogonial mitoses are inhibited by hypophysectomy (Barr, 1963; Dadzie & Hyder, 1976) and subsequently restored by either implanting whole pituitary glands or injecting pituitary extract (Yamazaki, 1965). A gonadotrophin involvement is also suggested from the present study (See Chapter 7), for during the post-spawning period both oogonial mitoses and gonadotrophin values are at their highest levels. Although these high gonadotrophin levels may be related to a diminution of steroid feedback after spawning (See Chapter 7), it is possible that they are involved in the initiation of the early developmental stages for the next cycle. Whether this pituitary involvement in oogonial multiplication is primary, or via gonadotrophin-induced steroid synthesis is unclear, since there is some evidence for a direct

effect of oestrogens on oogonial mitosis. Thus, Bullough (1942), showed that oestrone treatment increased mitotic activity in the minnow during the post-spawning period. In view of these findings and the fact that oestrone is a naturally occurring hormone in many fish species (See Tamaoki, 1980) it is surprising that so few authors have studied serum oestrone levels during this period of ovarian recrudescence in teleosts (See Section 7.1.; Cedard et al., 1961; Eleftheriou et al., 1966; van Bohemen et al., 1982c). This omission is remedied in the present study where changes in the levels of this oestrogen, together with oestradiol-17β are monitored over the complete reproductive cycle (See Chapter 7). Although oestradiol-17B levels are known to be low during the immediate pre- and post-spawning period (Fostier et al., 1978; Whitehead et al., 1978a; Scott et al., 1980b) this may not be the case for oestrone.

Following mitosis, the oogonia move rapidly into oogenesis, which is the meiotic transformation of oogonia into oocytes (Eggert, 1931; Yamazaki, 1965; Tokarz, 1978), but unlike oogonial mitosis, oogenesis seems to be independent of any pituitary influence (Khoo, 1975). Furthermore, oestrogens have been shown to have inhibitory or regressive effects both on oogenesis and also the primary phase of oocyte growth which follows (Berkowitz, 1941; Egami, 1954; See Dodd, 1960). This primary phase of oocyte growth has been arbitrarily divided into three stages : (1) the chromatin nucleolar stage, (2) the perinucleolar stage, and (3) the late perinucleolar stage (Yamamoto, 1956; Yamamoto & Yamazaki, 1961; van den Hurk & Peute, 1979; Khoo, 1979). The meiotic transformation is accompanied by a movement of the primary oocytes away from the oogonial nests to become associated with follicular cells and thus initiating

folliculogenesis (Moser, 1967).

In the first stage the oocyte consists of a scant cytoplasm with a centrally-located nucleus and a large basophilic nucleolus. In the next stage, the germinal vesicle increases in size and multiple nucleoli are observed in the periphery (perinucleolar). This stage is also marked by the appearance of extensive aggregations of basophilic and electron-dense material in the perinucleolar cytoplasm. These 'yolk-nuclei' or Balbiani bodies, first described in teleosts by Hubbard (1894), initially appear in the juxtanuclear region but during the late-perinucleolar stage migrate to the periphery where its components are dispersed. Evidence suggests that this material, which is extruded from the nucleus, contains the necessary materials for the formation, multiplication and accumulation of organelles required within the oocyte prior to yolk deposition (Guraya, 1979; Toury et al., 1977). The oocytes increase in size during this primary growth phase at the end of which they reach a 'critical size', beyond which they will not proceed without gonadotrophic stimulation (Vivien, 1939; Barr, 1968). It is during this first growth phase that the various cells that constitute the different follicular layers, develop their close association with the oocytes in the process known as folliculogenesis, such that the oocyte now lies within its definite follicle and is surrounded by a single layer of granulosa cells, a layer of thecal cells, and externally by a layer of epithelial cells. This is closely followed by the extension of numerous microvilli from the oocyte surface, around which the chorion or vitelline membrane starts to accumulate.

The granulosa which is the first follicular structure to be clearly

recognised in folliculogenesis usually consists of a single layer of cells (Nicholls & Maple, 1972; Guraya et al., 1977) surrounding small previtellogenic oocytes usually of stage 1 (See Diagram 6.1.). As the oocyte develops the granulosa cell height increases, reaching a maximum of approximately 7.5μ in the trout (van den Hurk & Peute, 1979); at this point intercellular spaces can also be recognised (Flugel, 1967). These spaces may be important in the passage of vitellogenin from the capillaries to the oocyte (Anderson, 1967; Abraham et al., 1982; Selman & Wallace, 1982). Initially, the granulosa cells are in close contact with the oocyte and a degree of contact is maintained with the developing oocyte by interdigitating cytoplasmic processes which intermesh with the microvilli which extend from the oocyte surface. Studies with the electron microscope have revealed that the granulosa cells have a full complement of all the organelles necessary for protein synthesis (ie rough endoplasmic reticulum and mitochondria with lamellar cristae) (Hoar & Nagahama, 1978). Thus, it has been suggested that these cells are responsible for the production of some phospholipids, which are transported to the oocyte (Guraya, 1965), and also of proteins which are incorporated into the zona pellucida (Wourms, 1976; Wourms & Sheldon, 1976; Hoar & Nagahama, 1978). The zona pellucida is formed between the oocyte surface and the granulosa during the growth of the follicle and shows many variations in its development and structure in the growing oocytes of different teleost species. Thus, depending on the fish species as well as on the stage of oocyte growth in the same species it forms either a monopartite, bipartite or tripartite structure (Guraya, 1978). The reasons for the extensive zonal development of the pellucida especially in oviparous species is

believed to be related to the physicochemical properties of water into which the eggs are shed (Guraya, 1978).

The granulosa cells are separated from the rich capillary plexus and flattened fibro-blast like thecal cells by the basal lamina which is a thick glycoprotein layer containing collagen fibres (Anderson, 1967). The follicular theca is divided arbitrarily into two layers, the externa and interna and these layers have a smooth muscle -like appearance, similarly containing collagen, which confers strength to the growing follicles. However, in addition, recent cytochemical studies have indicated that some cells in the theca, by their high enzyme activity and histology, and known as special thecal cells (STC), are probably the main follicular steroid-synthesising cells of the ovary (Nicholls & Maple, 1972; Saidapur & Nadkarni, 1976; Hoar & Nagahama, 1978; van den Hurk & Peute, 1979; Kagawa et al., 1981). Although the various cells that constitute the different follicular layers have been well characterized by light and electron microscopy (Christensen, 1975; Nagahama et al., 1976; Hoar & Nagahama, 1978; van den Hurk & Peute, 1979) there is much equivocal evidence as to their precise roles in oocyte development. Certain morphological criteria were laid down by Hoar (1965) which suggested that it was either the granulosa cells or the corpora lutea (ie atretic follicles or sites which develop an endocrine function) which are the most likely sites of steroid biosynthesis, rather than the thecal or stromal cells. Although these sites have been confirmed as sources of steroids in some teleosts (Lofts & Bern, 1972; Khoo, 1975; Guraya, 1976) it has also been shown in other studies that thecal and stromal cells do possess steroidogenic activity (Bara, 1965; Nicholls & Maple, 1972; Saidapur & Nadkarni, 1976; Hoar & Nagahama,

1978; van den Hurk & Peute, 1979; Kagawa et al., 1981). Furthermore, in some species, including trout, corpora atretica do not appear to show any endocrine activity (Lambert & van Oordt, 1965; Lambert, 1966, 1970; Yaron, 1971; van den Hurk & Peute, 1979) and in others the granulosa cells appear to be involved in protein rather than steroid synthesis (Hoar & Nagahama, 1978).

A cytochemical feature of cells which synthesise steroids is the presence of enzymes involved in steroid biosynthesis and in particular 3β-hydroxysteroid dehydrogenase (3βHSD) which is the most commonly used marker of steroidogenic function (Savard et al., 1963). Using such techniques, granulosa cells have been observed to show 3BHSD activity in a number of species including trout although the intensity of the reaction shows marked variation (Lambert & van Oordt, 1974; Nagahama et al., 1976; van den Hurk & Peute, 1979; Lambert & van Bohemen, 1979). 3βHSD activity is at its strongest at the onset of and during vitellogenesis at a time when ovarian oestrogen synthesis is increasing (van Bohemen & Lambert, 1978) which suggests a role for these cells during this phase of development. However, at the electron microscopic level no such evidence for steroidogenesis can be demonstrated in granulosa of most species, for these cells have rough endoplasmic reticulum and mitochondria with lamellar cristae which are generally considered as characteristic of protein synthesis. Thus, it would appear that the granulosa cells may have a dual role : being protein- and steroid-synthetic, possibly at different times of the year. However, in these studies above on rainbow trout, the 3βHSD activity, although positively determined in granulosa tissue, is relatively weak when compared to the activity of the special thecal cells and is severely decreased or lost shortly before

ovulation. The timing of this reduction in 3βHSD steroidogenic activity is also supported by histological evidence which reveals degeneration of granulosa cells shortly after ovulation. Since this period is very important with regard to circulating levels of hormones in these fish, most especially the progestagens (Scott et al., 1982), other steroidogenic tissue(s), most probably the STC, may be responsible for the rapid increase in these hormones at this time. Thus it is of interest to note that the increase in 17α-hydroxy-20β-dihydroprogesterone (17α20β) observed at ovulation in the char, has been correlated with an increase in number and steroidogenic activity of STC (Kagawa et al., 1981). Furthermore, a high rate of 17α-hydroxyprogesterone synthesis (the immediate precursor to 17α20β; See Chapter 5) has been observed during this period in trout concomitant with the peak in activity of these STC in this species (van den Hurk & Peute, 1979). In the trout, these STC, seen just before and after spawning, belong to the theca externa (van den Hurk & Peute, 1979) whereas in other species they belong to the theca interna and are cytologically similar to the steroid-producing Leydig cells of the testes (See Dodd & Sumpter, 1982). However, STC have not been identified in all teleost species and whilst they are found throughout the cycle in some fish, eg mackerel, in others like the char they are only observed in mature or post-ovulated follicles (Kagawa et al., 1981).

The only other tissues that might be involved in steroidogenesis at this time are atretic follicles, possibly acting as functional corpora lutea (Lambert, 1970; See also Khoo, 1975). However, due to the lack of critical evidence of their true endocrine function in teleosts, the term 'corpus luteum' may be inappropriate. In the

mammalian ovary the corpus luteum characteristically produces progestagens and is under pituitary control. Certainly if corpora lutea were of primary importance in steroid biosynthesis, then the incidence of atresia would need to be of sufficient magnitude and regularity to account for the cyclical and consistent nature of the various endocrine changes which are observed during the reproductive cycle. Although it has proved difficult to assess this problem fully and to cover all the different reproductive strategies employed by many species, the current data available on the incidence of atresia in teleosts appears to be to the contrary. The proportion of previtellogenic follicles becoming atretic appears very low (Lehri, 1968; Wiebe, 1968), if it occurs at all in healthy well-fed animals (Yaron, 1971) although this may be due to their apparent disappearance from the ovary without leaving any recognisable traces (Hoar, 1965). Furthermore, atresia in gravid fish is often lower than might be expected (Bara, 1965; Lambert, 1970; Yaron, 1971) although the proportion of vitellogenic and mature follicles is higher than previtellogenic numbers (Rastogi, 1966). In contrast to smaller follicles, larger ones go through quite distinct phases of degeneration (Bretschneider & Duyvene-de-Wit, 1947; Hoar, 1965). Thus, in rainbow trout four stages of atretic degeneration of mature vitellogenic follicles have been described (van den Hurk & Peute, 1979). However, the observations that various external environmental factors and nutrition can all influence atresia may explain some of the conflicting reports regarding the extent of atresia in some species. Thus in one investigation of the brook trout, it was estimated that 40% of developing vitellogenic follicles became atretic (Vladykov, 1956) whereas in another study on the same species only

3-5% of the oocytes were atretic (Henderson, 1963b). Marked reductions in the incidence of atresia would, by increasing the number of developing oocytes in the ovary, ie the fecundity, be of considerable economic significance to broodstock management. Thus, the levels of atresia at different stages of development will be assessed in the present work. Furthermore, it would appear that in most teleosts, including trout, atresia is merely an adaptive device for disposing of moribund ova (van den Hurk & Peute, 1979) and it is the granulosa and/or special thecal cells of the follicle which are primarily responsible for the production of steroids. Thus, an investigation of the roles, these various follicular cells may have in ovarian development, was facilitated by means of an electron microscopical study in the present work.

Following folliculogenesis, the initiation of secondary oocyte growth is marked by the appearance of yolk vesicles within the ooplasm (Konopacka, 1935). These vesicles which contain PAS-positive chromophobic material (van den Hurk & Peute, 1979; Khoo, 1979) increase in size and number as they move peripherally within the ooplasm. There is considerable evidence that this material, known as intravesicular or endogenous yolk is synthesised within the oocyte itself (Korfsmeier, 1966; Norrevang, 1968; Upadhyay et al., 1978). However, the classification of this material as yolk is anomalous since a number of studies suggest that these vesicles give rise to cortical alveoli which are found adjacent to the vitelline membrane at later stages of development (Yamamoto, 1955; Osanai, 1956; Malone & Hisaoka, 1963). The cortical alveoli fuse with the vitelline membrane and release their glycoprotein contents into the perivitelline space during the cortical reaction at fertilization (Wallace & Selman,

1981). Since the intravesicular material is lost to the developing embryo and is chemically different to vitellogenin synthesised in the liver (Khoo, 1979) then it is wrong to label this phase as endogenous vitellogenesis. The use of a highly specific radioimmunoassay in this study will reveal whether hepatically-produced vitellogenin is present during this stage of development. If serum vitellogenin is present during this intravesicular stage, the chemical differences between the intravesicular material and the hepatic yolk laid down in the next phase may be due to either a multiplicity of vitellogenins or to some alteration in the uptake and transformation of vitellogenin within the oocyte.

The next recognised growth phase has commonly been termed that of 'true vitellogenesis' (Wallace & Selman, 1981), since it occurs during the period of vitellogenin synthesis by the liver. This 'extra-vesicular' or exogenous yolk formation is characterised by the presence of small eosinophilic yolk granules and this phase is subdivided into three stages depending on the amount of granular yolk present. At first these granules appear in the periphery of the ooplasm (Stage 5) and then aggregate and migrate towards the centre of the ovum (Stage 6). Finally, prior to maturation, the entire ooplasm is filled with granular yolk. The mechanisms controlling the synthesis and release of vitellogenin by the liver under the influence of oestrogens are discussed more fully above (See Chapter 3).

Although the production of vitellogenin is important, without a mechanism to get it into the oocyte, successful ovarian development is impossible. A number of studies have investigated the transfer

of this large protein from the blood to the forming yolk spheres in fish and amphibia (Korfsmeier, 1966; te Heesen & Engels, 1973; Wallace, 1978) and the endocrine mechanisms that control it (Campbell & Idler, 1976; Campbell, 1978; Crim & Idler, 1978; Ng & Idler, 1978). Thus it has been shown that various putative gonadotrophin preparations or extracts stimulate uptake of vitellogenin apparently by increasing micropinocytotic activity at the inner surface of the vitelline membrane (Upadhyay et al., 1978; Abraham et al., 1982; Wallace & Selman, 1981). Several ultrastructural studies have further shown that material incorporated by the micropinocytes is transferred to the yolk granules forming within the peripheral ooplasm (Droller & Roth, 1966; Anderson, 1968; Ulrich, 1969; Schackley & King, 1977). Evidence suggests that vitellogenin probably reaches the oocyte, from the capillary plexus, via the interstitial spaces in the granulosa cells (Abraham et al., 1982; Kagawa et al., 1981; Selman & Wallace, 1982) and then passes through (Selman & Wallace, 1982) or along the surface of the microvilli (Abraham et al., 1982) where it is subsequently pinocytosed. Once in the oocyte the vitellogenin is broken down into its components and laid down as a soluble lipovitellin/phosvitin complex (Wallace, 1973). As such, this phase of growth might be more properly called the 'vitellogenolytic' phase since vitellogenin is certainly not synthesised within the oocyte. Although these granules initially maintain their integrity, in the majority of teleosts at maturation the granules fuse to form a single soluble mass seen characteristically as the transparent eggs of many teleosts (Fulton, 1898; Wallace & Selman, 1981).

Morphologically, the simultaneous processes that occur with the resumption of meiosis (maturation), happen over a relatively short period of time. Maturation, the final phase of secondary oocyte growth, is marked by the clearing of the yolk, breakdown of the germinal vesicle and a rapid though variable hydration, causing an increase in oocyte volume, a phenomenon first observed by Fulton (1898), but subsequently documented by other authors (Clemens & Grant, 1964; Hirose et al., 1976; Wallace, 1978; Craik, 1982). More recently the hormonal mechanisms controlling maturation and expulsion of the mature oocyte from the follicle (ovulation) have become more clearly understood (See Chapter 7; Scott et al., 1982). Once the follicles rupture and release the mature oocytes, the tough chorion and the vitelline membrane are all that remain around the egg. However, during oocyte growth one follicle cell becomes highly specialised and maintains cellular contact with the vitelline membrane by means of a thick cytoplasmic extension. This micropylar cell, first described by Mark (1890) acts as the micropyle and allows the passage of a single sperm at fertilization (Szollosi & Billard, 1974).

In order that the maturation of oocytes is a synchronised event there is a dynamic organisation of ovarian function, so that the majority of developing oocytes pass through all the different phases of growth at approximately the same time. In the trout like many other species the onset of the reproductive cycle is marked by the initiation of the second growth phase brought about by the changing external conditions and the hormonal environment. Thus, although primary oocytes are observed very early in gonad development, it is generally accepted that because of its requirement for a pituitary

trigger, secondary oocyte growth is not seen until the spring/ summer before the first spawning, which usually occurs in the third year of growth in female rainbow trout. However, in other fish there is evidence of a dummy or practice-run (Hickling, 1935; Trout, 1957; Woodhead & Woodhead, 1965; Davis, 1977; Eliassen & Vahl, 1982) before the first spawning as indicated by increases in gonad weight and volume and increase in oocyte diameter in immature fish. Thus, in the present study an investigation is also made of the hormonal and/or morphological changes which occur in the Whitebrook-strain of fish in their second or pre-spawning year. Clearly, if a 'dummy-run' could be artificially induced, possibly by the application of hormones, then it might be possible to produce good quality eggs a year in advance of those from natural stocks. This would be of considerable economic advantage to the broodstock farmer. Such advantages will not be gained until there is a fuller understanding of the morphological and hormonal changes that occur during the reproductive cycle of the female rainbow trout.

Thus, although the mechanisms of oocyte growth in teleosts are very similar, there is a considerable diversity in the strategies used to bring about successful ovarian development (Tokarz, 1978). There is also considerable variation in the way oocytes are recruited to the ovarian cycle and also in the role of atresia in these processes. Subsequently, such differences are reflected in the considerable variation between species in the number and size of eggs produced and also in the numbers of eggs per spawning and of spawnings per year. Whether such differences are shown between different strains of the same species is not known. However, by looking at these changes in three strains of trout, differences in

development might be seen which could account for variations in egg size or quality and fecundity. These might be used to some advantage in commercial broodstock management, where the careful selection of strains on the basis of egg quality and fecundity would be of considerable economic significance.

6.2. Materials and Methods.

The ovarian tissues used in this study were collected from three strains of rainbow trout held both under the closely controlled conditions detailed in Chapter 7 together with fish held under natural conditions in outdoor ponds.

Tissue was taken monthly from freshly sacrificed fish, after weighing the whole animal, at different stages of the reproductive cycle. In addition, the ovaries and liver were weighed for GSI and HSI determinations. Tissue was then cut into small pieces and placed in one of the fixatives for light microscopy detailed in 6.2.1.. For electron microscopy, individual oocytes were teased away from the ovarian lamellae and placed in a prefixative (A or B) for 2 hours, before post-fixation in osmium tetroxide (See 6.2.2.).

Stained sections were examined under the light microscope (Zeiss, Photomicroscope) and the different morphological phases of development identified. Under the elctron microscope (AE1 EMGB) a more detailed examination was made of the different follicular cell types in order to assess their possible roles in ovarian growth, together with the outer regions and follicular envelopes of the oocyte with the aim of investigating the uptake of material.

6.2.1. Light microscopy.

Materials :

The following fixatives -

(a) Bouins - Picric acid (saturated aqueous solution) - 75ml
Formalin (40% HCHO) - 25ml
Acetic acid (glacial) - 5ml

(b) Baker's Neutral Formalin - Formalin (40%) - 10ml
10% $CaCl_2$ (anhydrous) - 10ml
Powdered $CaCO_3$ (excess)

(c) Smith's Dichromate - $K_2Cr_2O_7$ - 0.5g
* Formalin (40%) - 10ml
Acetic acid (glacial) - 2.5ml
Distilled water -100ml

* Formalin added just prior to use

General -

(d) Ethanol; absolute; 95%; 70%; 50%; 30%.

(e) Chloroform.

(f) 1% celloidin in methyl benzoate ('Gurr's', Hopkins and Williams).

(g) Benzene, Xylene (B.D.H.).

(h) Wax - 'Paramat' ('Gurr's', Hopkins and Williams).

(i) Stains :- Ehrlich's Haematoxylin; Eosin and Mallory's triple stain.

(j) Coverslips, slides etc.

Methods :

1. Using Bouin's :-

This method is essentially that described by Pantin (1960) with the following modifications.

(a) Fixation varied with stage of maturity of ovarian tissue. No longer than 48 hours when fully immature, less than 12 hours in very yolky oocytes. Longer than this caused brittleness and difficulties in sectioning.

(b) Dehydration was again adjusted to suit the amount of yolk present. Immature ovary had several changes over 72 hours (to remove picric) whereas yolky oocytes had 3 changes over 24 hours. Dehydration was continued through 90% alcohol up to 95%. Some residual water gives better histological results with yolk-laden eggs (Rugh, 1962).

2. Using Neutral Formalin :-

The method used was that described by Pantin (1960) with similar considerations as regards dehydration above. This method was not used as extensively with mature oocytes, since Bouin's gave more consistent results.

3. Using Smith's Dichromate :-

This was a double embedding technique, a modification of Peterfi's celloidin-paraffin method, having all the advantages of double embedding but much quicker (Pantin, 1960). This method proved inconsistent and did not always give good results. Strict adeherence should be given to the time

schedule as outlined by Pantin (1960). When preparing the fixative the formalin must be added immediately before use, since the dichomate and formalin denature each other. Methyl benzoate was found superior to benzene for clearing the ovarian tissue.

Staining :

All sections were cut at 6-10 mμ, depending on yolk-content, and stained using haematoxylin and eosin and Mallory's triple-stain. Stained preparations were mounted under DPX and examined under a light microscope (Photomicroscope, Carl Zeiss, W. Germany).

6.2.2. Electron Microscopy.

Materials in addition to above :

(a) Pre-fixative A (Szollosi et al., 1978)

2.5% Gluteraldehyde

0.5% Paraformaldehyde

in 0.15M Cacodylate buffer at pH 7.2 containing 0.1% potassium ferricyanide.

(b) Pre-fixative B (Upadhyay et al., 1978)

1.5% Paraformaldehyde

2.5% Gluteraldehyde

0.1% Picric acid

in 0.15M Cacodylate buffer at pH 7.3.

(c) Cacodylate buffer - 0.15M.

(d) Spurr's Resin.

(e) Uranyl acetate stain and Lead citrate stain.

Method :

(a) Fixation - Prefixative A gave consistently better results. Prefixed for 2 hours.

(b) Immersed in excess cacodylate buffer overnight at $4^{o}C$.

(c) Fixed in 1% Osimium tetroxide for 1 hour.

(d) Leave in excess buffer overnight at $4^{o}C$.

(e) Dehydrate through a series of alcohols up to absolute - greater than 15 minutes each.

(f) Place in Spurr's resin/EtoH (50:50) overnight.

(g) Place in Spurr's resin until pieces sink.

(h) Embed in Spurr's resin in gelatine capsules.

(i) Section on ultramicrotome (LKB Ultrotome) and stain with uranyl acetate and lead citrate.

(j) Prepared sections were examined under the electron microscope (AE1 EMGB).

6.3. Results.

The primary aim of this study was to identify the different stages of ovarian development during the reproductive cycle of female trout and to act as a reference to the studies of the endocrine changes that occur under the closely controlled environmental regimes outlined in Chapter 7. Thus, the endocrine events which are related to the ovarian changes under these regimes will be recorded and discussed in Chapter 7. Using light microscopy it was a further aim to investigate whether there are any differences in these morphological changes between different strains of trout or in the levels of atresia which could be related to egg quality or fecundity. Electron microscopy was used to obtain evidence of the function of the different follicular layers in ovarian development.

During the course of this study, gross changes were observed in ovarian morphology. All stages of oocyte development were recognised histologically, apart from those associated with final maturation.

Light Microscopy :

Oogonia and Primary Oocytes : Thus, in ovarian tissue taken from immature rainbow trout and adult female trout during the period of gonadal quiescence after spawning, oogonia and oocytes in the first three stages of oocyte growth were present (See Plate 6.1.). Primary oocytes were observed quite early in ovarian development and were identified in 3 month-old fry. Whether there was any turnover of these oocytes in the time before the spring of their first spawning year was not established, although there was no evidence of

atresia during this period. Primary stage oocytes were found in all ovarian tissues irrespective of season, though their numbers were greatly reduced relative to more developed stages as the ovary matured.

Stage 1 : The proportion of stage 1 to stage 2 and 3 oocytes seemed low, suggesting that in development oocytes pass through this stage very quickly. Those stage 1 oocytes identified were not much larger than oogonia and had a single prominent nucleolus.

Stage 2 : In stage 2 oocytes, the amount of 'yolk-nuclei' material (Balbiani bodies) seemed extremely variable, almost filling the entire ooplasm in some cases.

Stage 3 : As the oocytes increased in size and progressed to stage 3, these yolk nuclei dispersed until all that remained in the largest previtellogenic oocytes was a faint ring around the periphery. In some of these larger primary oocytes the peripheral nucleoli (perinucleolar) observed in stages 2 and early stage 3, were subsequently scattered throughout the nucleus.

Secondary Oocytes : Stage 4 oocytes were recognised by the appearance of vesicles containing blue-staining material (with Mallory's) in ovaries taken early in the reproductive cycle from maturing female fish. These vesicles first appeared as a ring within the ooplasm in early stage 4, but concomitant with the growth of the cell they were eventually scattered throughout the oocyte (See Plate 6.2.). The presence of large numbers of hollow vacuoles were also noted and identified as fat or lipid bodies which had lost their

contents during staining. Stage 4 oocytes were observed in the ovaries of fish at the onset of maturation, at which time evidence of the next stage were also identified. Stage 4 oocytes were also observed in Whitebrook strain fish for an extended period, from the summer of their second year until the spring of their third year. This strain did not spawn until the end of their third year.

PLATE 6.1. SECTION OF OVARY TAKEN DURING PRIMARY GROWTH PHASE OF OOCYTE SHOWING PERINUCLEOLAR AND YOLK-NUCLEUS FORMATION (STAGES 1,2 & 3).

Og oogonium; 2 Stage 2 oocyte; 3 Stage 3 or 'previtellogenic'oocyte; yn yolk nuclei; no nucleoli.

Fixed in Bouin's and stained with Haematoxylin and Eosin.

Magnification x 40

PLATE 6.2. SECTION OF OVARY TAKEN DURING VESICLE FORMATION SHOWING EARLY AND LATE-STAGE 4 OOCYTES.

E4 early Stage 4 oocyte; L4 late Stage 4; ve vesicles; vu vacuoles; g granulosa; nu nucleus.

Fixed in Bouin's and stained with Mallory's trichrome.

Magnification x 100

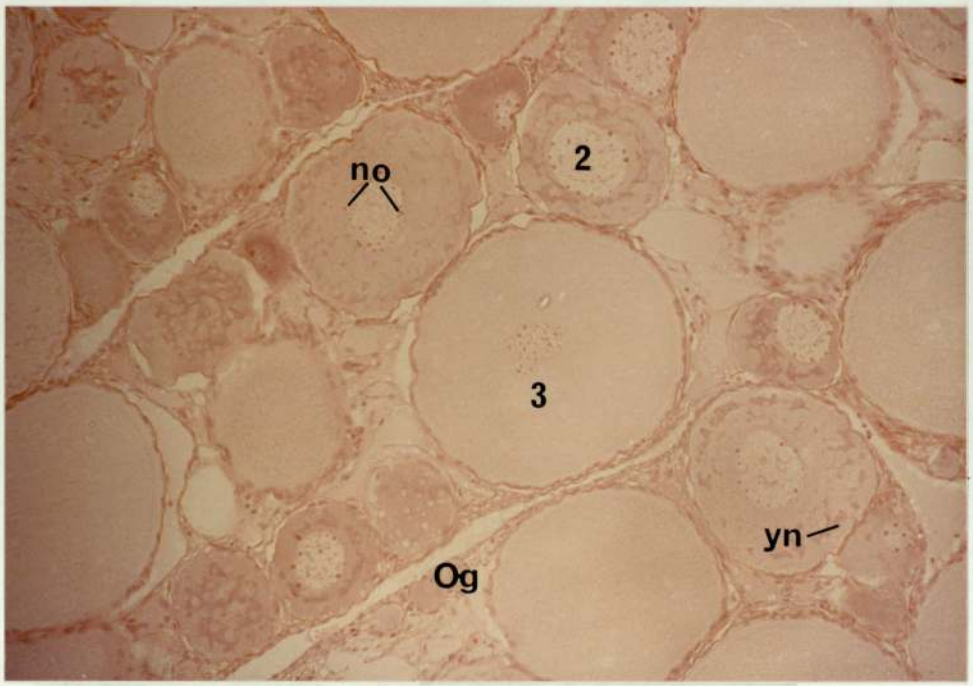


PLATE 6.1.

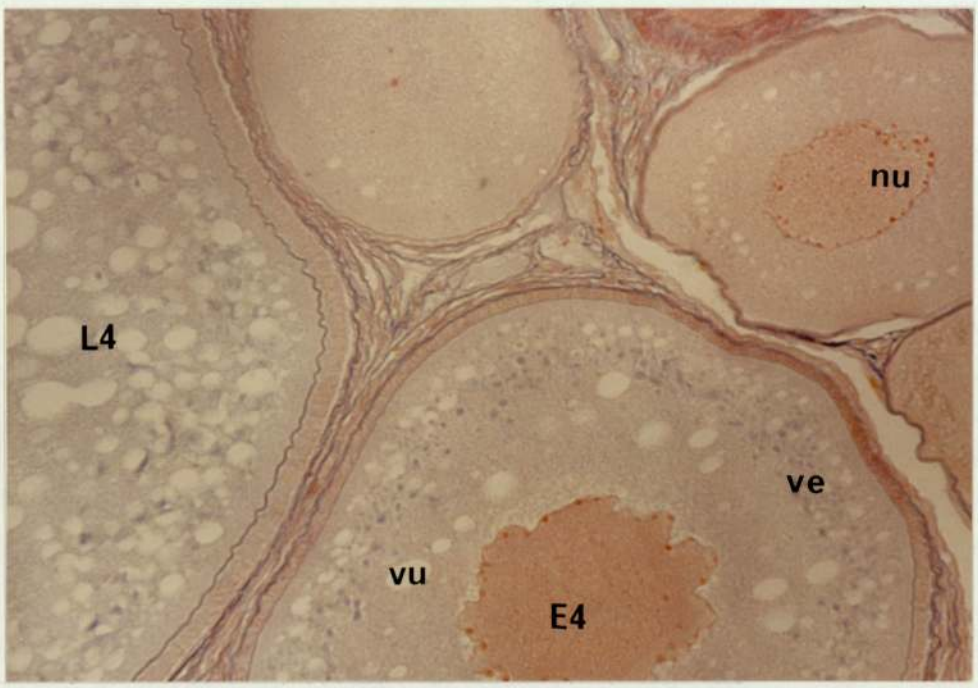


PLATE 6.2.

Stages 5,6 and 7 : The next phase of development was marked by the appearance and accumulation of orange-staining (Mallory's) granules which first appeared as small spheres in the periphery of the ooplasm (Stage 5; See Plate 6.3.). This phase (5,6 and 7) was recognised as the period when true vitellogenin, synthesised in the liver, is taken up by the oocytes and assimilated into yolk spheres. These yolk-granules or spheres appeared to increase in size and number and by stage 6 (Plate 6.3.) filled the centre of the oocyte around the nucleus. Stage 5 and 6 oocytes were often seen at the same time as each other and sometimes in conjunction with stage 4 oocytes, indicating a degree of differential growth during this phase. Eventually, at the expense of the vesicles the oocytes were completely filled with yolk granules of various sizes (stage 7; See Plate 6.4.).

Development of follicle cells and zonal layers : During stages 4,5, 6 and 7 gross changes were also observed in the follicle cells and the zonal layers between these and the oocyte. At stage 4 the granulosa cells were clearly distinguishable as a single layer of pink-staining (Mallory's) columnar cells surrounding the oocyte and the basal lamina and zona pellucida (no striated appearance) both as blue-staining bands (Plate 6.2.). By stage 5 and 6 the zona radiata was now distinguishable, being slightly thicker, red-staining with Mallory's and a highly striated appearance, together with an outer more homogenous layer (Plate 6.3.). By stage 7 the zona radiata appeared as a wide, deep-red staining, striated layer, with a thin more diffuse layer between it and the granulosa. The thecal layers appeared stratified at this stage with layers of deep-blue staining tissue (collagen possibly) interspersed by other red-staining cells (thecal and stromal cells; Plate 6.4.). As in other stages the

single layer of granulosa cells were also clearly recognisable.

Due to the problems that occurred with sectioning and staining large yolky-oocytes no stage 8 oocytes/eggs were successfully mounted for examination although they were recognised from eggs taken from mature animals under binocular microscope.

PLATE 6.3. SECTION THROUGH VITELLOGENIC FOLLICLES SHOWING THE FORMATION (STAGE 5) AND ACCUMULATION (STAGE 6) OF HEPATICALLY SYNTHESISED VITELLOGENIN INTO YOLK SPHERES OR GRANULES.

5 Stage 5 oocyte; 6 Stage 6 oocyte; yg yolk granules; ve vesicles; th theca; gr granulosa; zr zona radiata;

Fixed in Bouin's and stained Mallory's trichrome.

Magnification x 100

PLATE 6.4. SECTION OF AN OOCYTE AT THE COMPLETION OF VITELLOGENIN UPTAKE SHOWING OOCYTE FILLED WITH YOLK-SPHERES AND WELL-DEVELOPED FOLLICULAR LAYERS (STAGE 7).

yg yolk granules; zr zona radiata; zre diffuse layers; gr granulosa; th theca; Cp capillary.

Fixed in Bouin's and stained Mallory's trichrome.

Magnification x 200

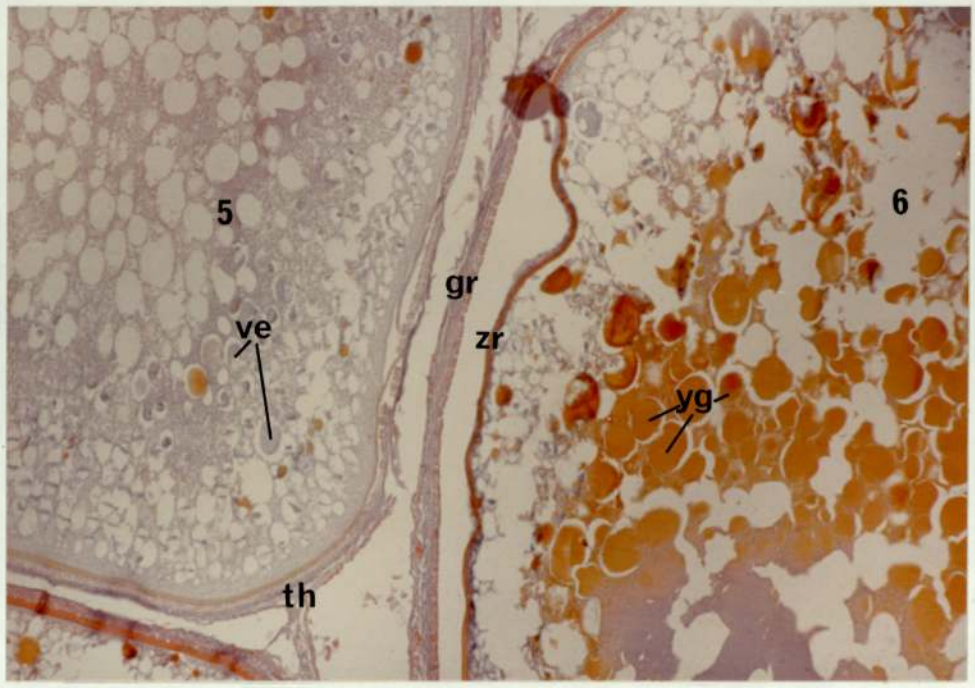


PLATE 6.3.

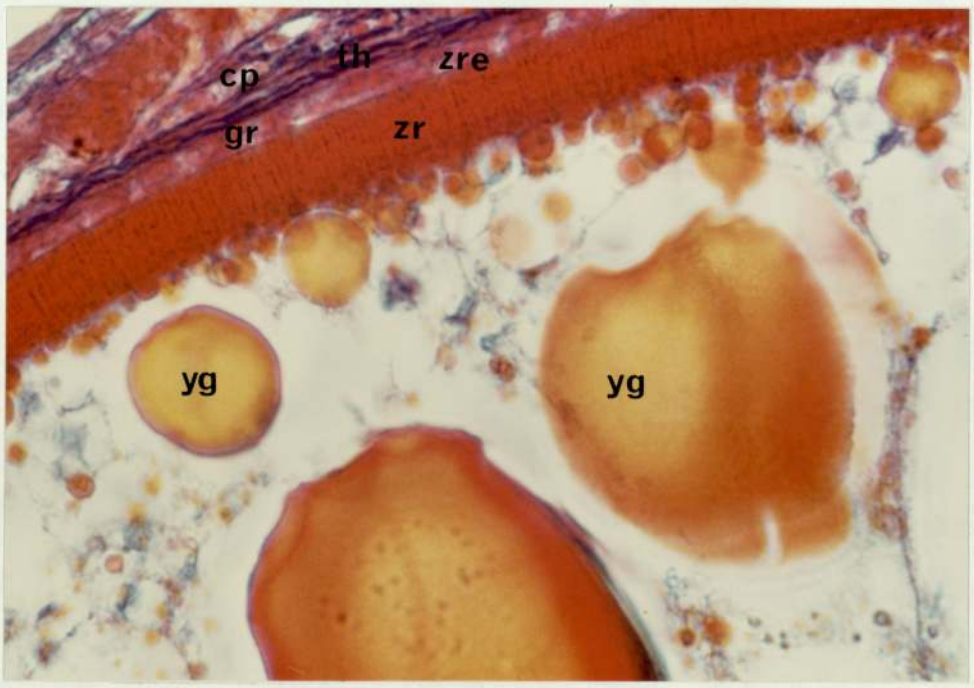


PLATE 6.4.

Electron Microscopy :

Follicular layers : Under high power magnification (X5000) the thecal cells (theca folliculi), basal lamina (membrana propria folliculi) and granulosa cells (folliculi) were all recognised (Plate 6.5.).

Stage 4 : The thecal cells were flattened fibroblast-like cells interspersed by large numbers of collagen bundles (See Plate 6.6.). Although some rough endoplasmic reticulum (RER) was observed in the thecal layers, indicative of protein synthesis, there was no evidence of any STC or steroid synthetic activity at this stage.

The basal lamina (or basement membrane), which separates the thecal cells from the granulosa, had a stratified appearance and also contained smaller amounts of collagen (See Plate 6.7.).

The granulosa cells contained large nuclei and large amounts of RER together with mitochondria with lamellar aristae, indicative of protein synthesis by these cells at this stage (See Plate 6.7.).

Stage 5 : Granulosa cells at this stage also contained large amounts of RER suggestive of protein synthesis, but also contained some mitochondria which appeared to have tubular cristae, the latter being usually found in cells synthesising steroids (See Plate 6.8.).

PLATE 6.5. SECTION THROUGH FOLLICLE CELLS OF A TROUT OOCYTE AT STAGE 4.

Tc thecal cells; Bl basal lamina; Gc granulosal cells.

Uranyl acetate and lead citrate stain.

Magnification × 5000

PLATE 6.6. DETAIL OF COLLAGEN FIBRES ARRANGED IN BUNDLES AND SCATTERED THROUGHOUT THE THECA.

Tc thecal cells; Cb collagen bundles;

Uranyl acetate and lead citrate stain.

Magnification × 50000

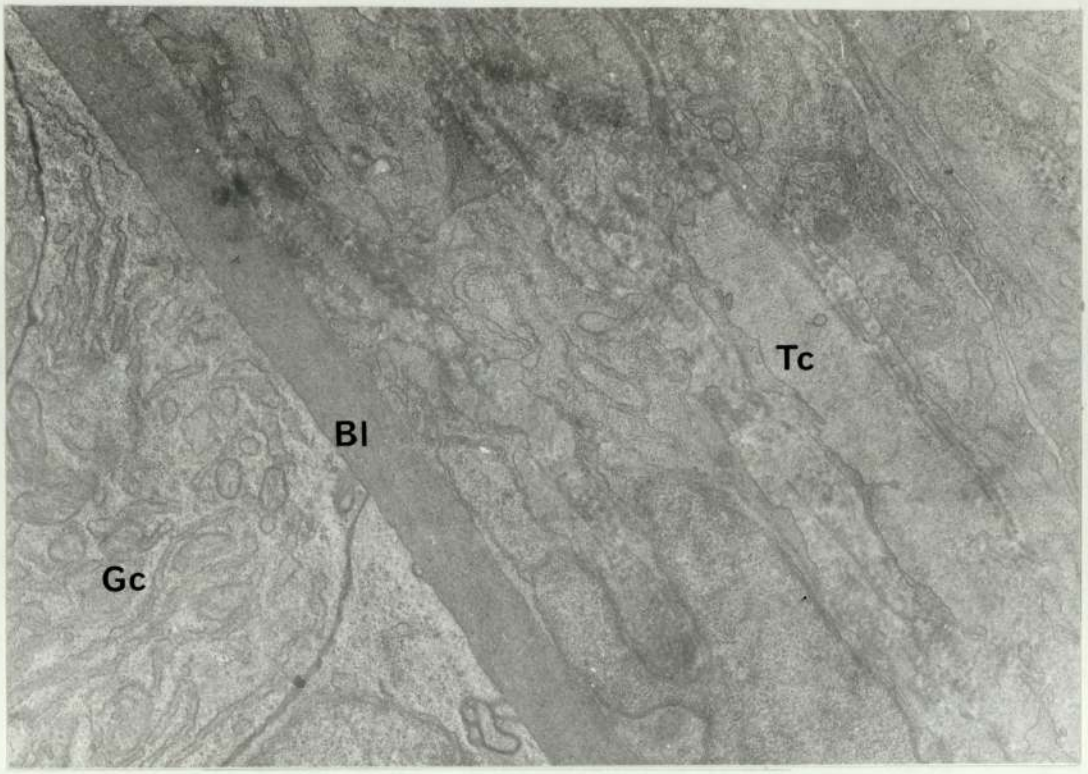


PLATE 6.5.

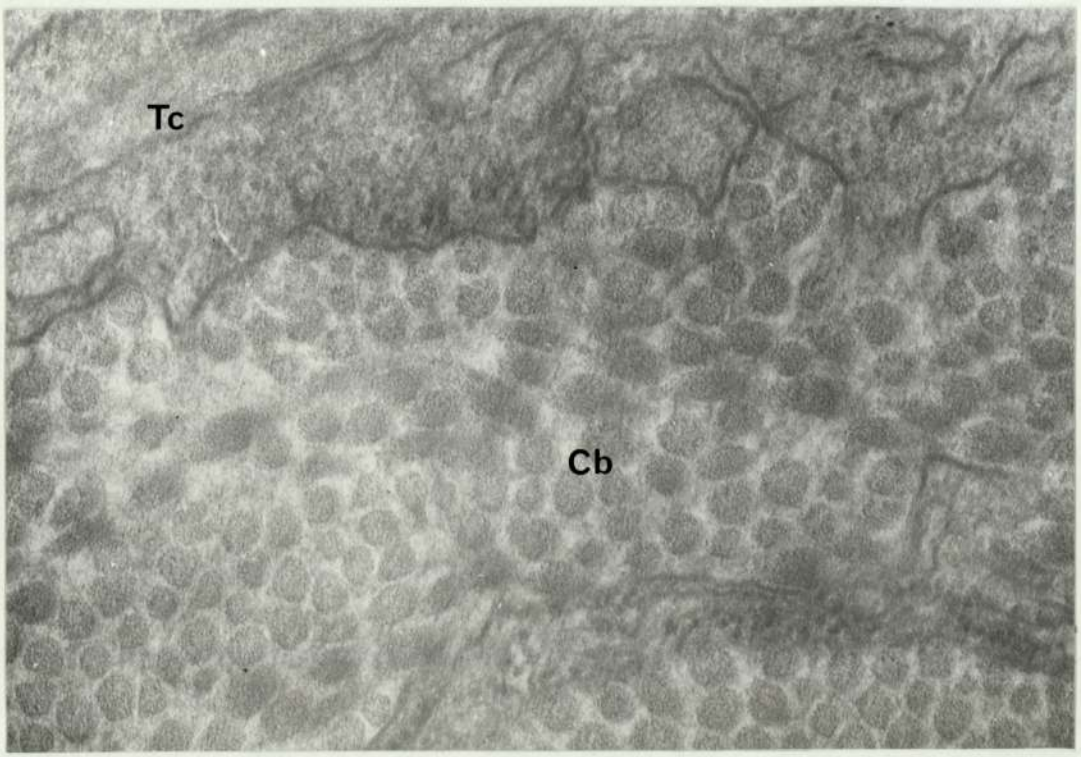


PLATE 6.6.

PLATE 6.7. DETAIL OF SEPARATION OF THECAL TISSUE FROM GRANULOSA BY BASAL LAMINA OR BASEMENT MEMBRANE (STAGE 4 OOCYTE).

Tc thecal cell; Cb collagen bundles;

Gc granulosal cell; nu nucleus;

mc mitochondria (lamellar cristae).

Uranyl acetate and lead citrate stain.

Magnification X 30,000

PLATE 6.8. DETAIL OF GRANULOSA CELL FROM VITELLOGENIC OOCYTE (STAGE 5).

Bl basal lamina; er endoplasmic reticulum;

nu nucleus; mc mitochondria.

Uranyl acetate and lead citrate stain.

Magnification X 12,500

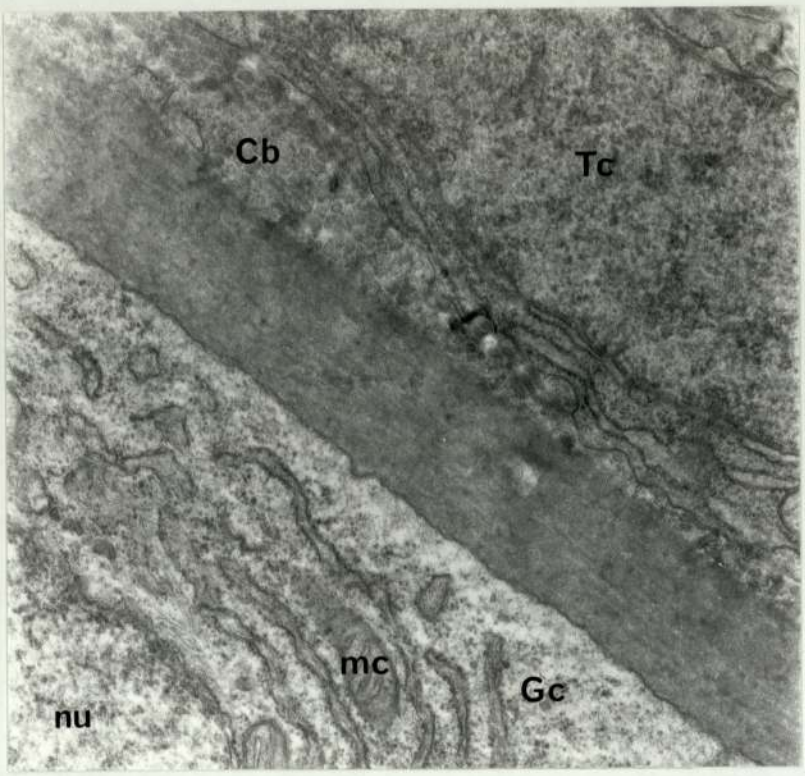


PLATE 6.7.

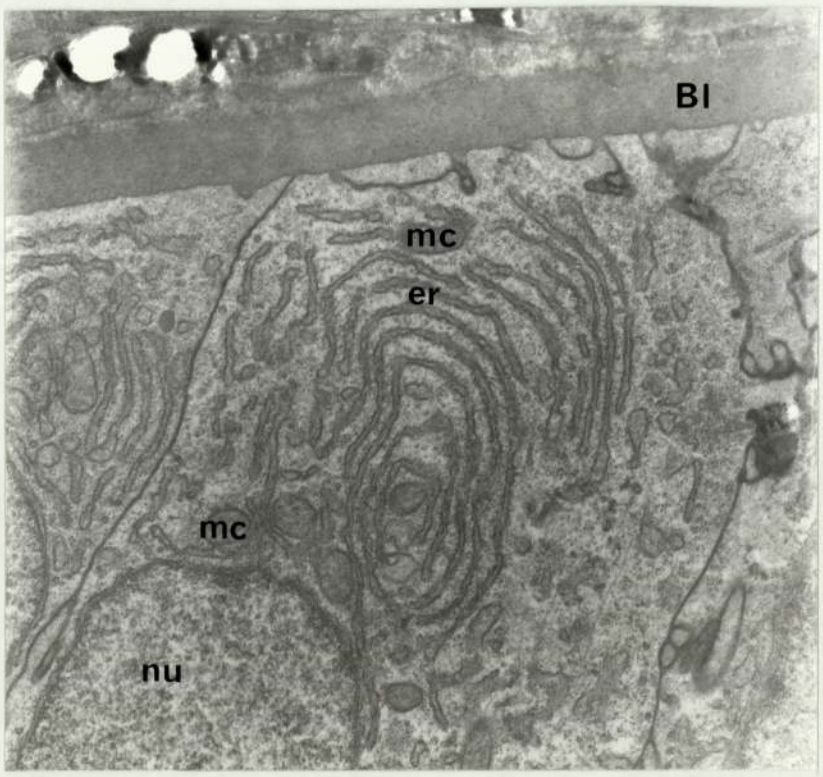


PLATE 6.8.

A detailed examination of the zonal layers in stage 5 oocytes revealed two distinct layers. Thus, a veryelectron dense zip-like layer was recognised as the developing chorion or vitelline membrane, together with numerous microvilli which were observed projecting from the oocyte surface, through the chorion, towards the granulosa (See Plate 6.9.). These microvilli, which grow taller and thinner during development to account for the increase in size of the chorion are responsible for the striated appearance of this zonal layer, seen more characteristically in light microscopy and labelled the zona radiata. Vesicles were identified in stage 5 oocytes, together with lipid bodies and mitochondria with lamella cristae. The ooplasm also contained numerous rod-like structures and what appeared as numerous fuzzy- or bristle-coated vesicles at the base of the microvilli (See Plate 6.9.).

A 10-fold magnification of the portion of the oocyte/microvilli interface marked on Plate 6.9. revealed what appeared to be pinocytotic vesicles being pinched off at the base of the microvilli (See Plate 6.10.). Thus, these small fuzzy-coated (not as clear in Plate 6.10.) vesicles were observed just 'budding-off' almost complete, and formed. These were taken to represent the uptake of true hepatic vitellogenin. All studies of granule material under the electron microscope were found to be of little value, firstly because they were so large that the whole screen was filled by uncontrasted material of the same tone, and secondly because the material disintegrated in the electron column.

Similar to the problems observed with preparing large yolky-oocytes for light microscopy, considerable difficulties were

encountered with oocytes over 1.5 mm in diameter. With larger oocytes it was very difficult to remove the alcohol completely, which resulted in brittleness in the embedding medium, making it impossible to section. Thus, no later stage oocytes or follicular tissues were examined which may have revealed the role of STC cells and/or granulosa cells at later stages in development.

PLATE 6.9. SECTION OF VITELLOGENIC OOCYTE (STAGE 5) SHOWING INTERMESHING OF MICROVILLI ON OOCYTE SURFACE WITH GRANULOSA.

Gc granulosal cell; Oo oocyte; mv microvilli; CH developing chorion; ve vesicles; pv pinocytotic vesicles; zr zona radiata; zp zona pellucida.

Uranyl acetate and lead citrate stain.

Magnification x 7500

PLATE 6.10. DETAIL OF PINOCYTOTIC ACTIVITY AT THE BASE OF THE MICROVILLI IN A VITELLOGENIC OOCYTE (STAGE 5).

Ch developing chorion; pv pinocytotic vesicles; pvf pinocytotic vesicle just forming; Oo oocyte.

Uranyl acetate and lead citrate stain.

X 10 magnification of boxed section on Plate 6.5.

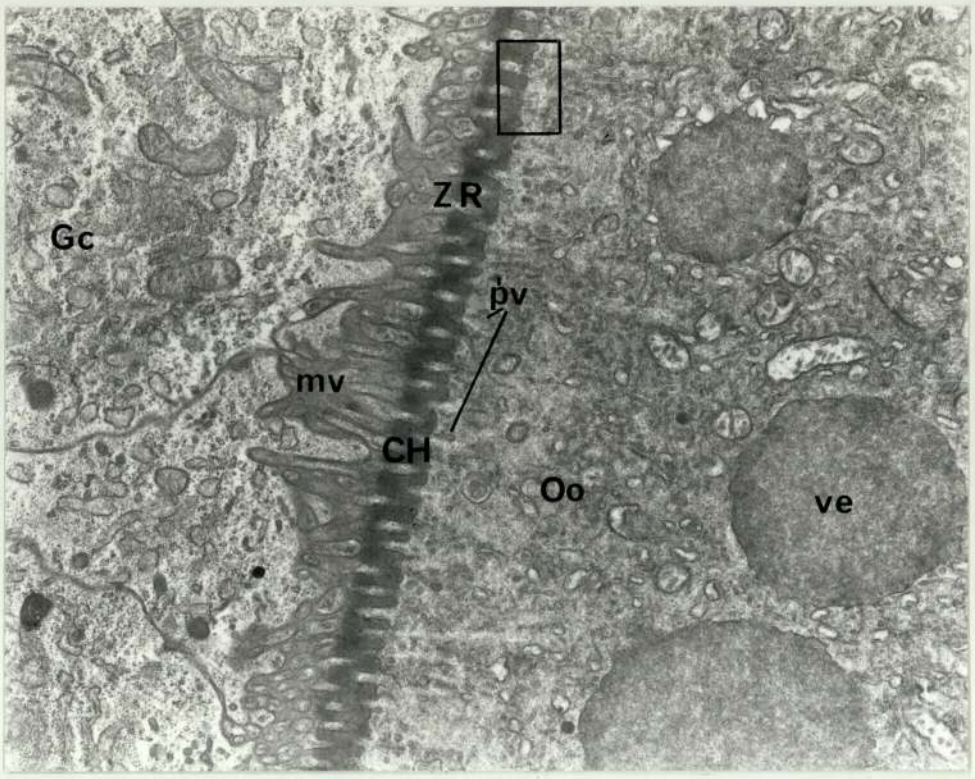


PLATE 6.9.

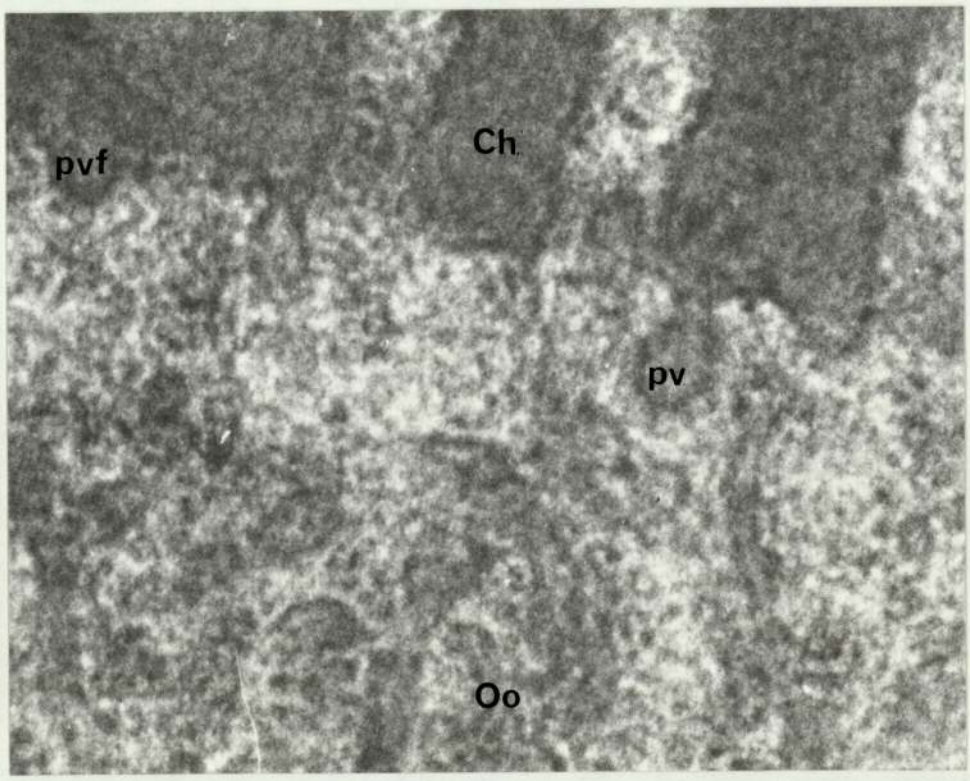


PLATE 6.10.

6.4. Discussion.

The present work confirms the existence of four phases of ovarian development in the trout. The stages observed in this study were similar to those reported for other teleosts. There were no differences in the overall development between the three strains. Thus, the primary phase of oocyte growth, marked by the perinucleolar nature of the nucleus and the dense-staining nuclei, are followed by the formation of vesicles and then granules during vitellogenesis, culminating in the maturation of oocytes and their expulsion as eggs.

The results also demonstrated that most of the ovaries taken from trout at the onset of ovarian recrudescence which contained vesicle stage (4) oocytes, also contained stage 5 or even stage 6 oocytes. However, stage 4 oocytes were observed in ovarian tissue for almost nine months in the whitebrook strain, during the period preceding the Spring of their first year of maturation. Clearly, the differences between intravesicular and extravesicular stages in the rainbow trout are not as distinct as reported in other studies of ovarian development in teleosts (Gokhale, 1957; Polder, 1961; van den Hurk & Peute, 1979; Kagawa et al., 1981). The chemical nature of this intravesicular material, seen as blue-staining vesicles in stage 4 in this study, has been characterised by several authors in an attempt to either associate or dissociate this material from the hepatically synthesised vitellogenin (Guraya, 1965; Korfsmeier, 1966; Khoo, 1979). Collectively, these histochemical studies confirm that the vesicular material contains mucopolysaccharides and/or glycoproteins (Aketa, 1954; Yamamoto, 1956; Guraya, 1965; Korfsmeier, 1966; Khoo, 1979).

In the present work this vesicular material (blue -staining with Mallory's), was clearly distinct from the deep-orange staining granular material, presumably exogenous yolk, which appeared in stages 5,6 and 7. Thus, chemically this vesicular material is quite unlike both the protein, phospholipid and neutral lipid material which is assimilated into the yolk spheres or granules (Khoo, 1979), and also the vitellogenin isolated from the serum of oestrogenised or mature female fish (Chapter 4). Since the vesicular material is chemically distinct from vitellogenin and because most other studies have failed to detect vitellogenin in the serum at the time when the vesicles are formed, its origin has subsequently been attributed to other cellular components of the follicle (Aketa, 1954; Nath, 1960; Guraya, 1965). Since the granulosa cells have been shown to possess protein-synthetic characteristics by electron microscopy (Hoar & Nagahama, 1978; van den Hurk & Peute, 1979) it has been suggested that these cells may be responsible for the formation of vesicular material. The present results similarly show that these cells have protein-synthetic organelles at stage 4, although it seems more likely that they synthesise the proteins which are incorporated into the chorion (or zona radiata) rather than the oocyte (Wourms, 1976; Wourms & Sheldon, 1976; Hoar & Nagahama, 1978). In the electron microscopical study of stage 5 oocytes the developing chorion could be seen as an electron-dense zip-like structure which formed around the base of the microvilli which were themselves projecting from the oocyte surface. As the oocyte grows so this chorion greatly increases in thickness and under light microscopy appears striated due to the microvilli which traverse this layer in order to maintain their close contact with the granulosa. Once

expelled as an egg the chorion forms the only major protection, together with the vitelline membrane which shrinks away from the chorion and a perivitelline space is formed.

There is some evidence that this vesicular material is produced autosynthetically by the oocyte (Korfsmeier, 1966; Norrevang, 1968; Upadhyay et al., 1978) and the ooplasm has been found to contain the necessary organelles for protein synthesis. The results from this study showed large numbers of mitochondria with lamellar cristae in the ooplasm close to developing vesicles which would support this evidence. Thus, an autoradiographic study of zebrafish oocytes demonstrated that vesicles rapidly incorporated tritiated histidine and glucose (Korfsmeier, 1966) leading the authors to conclude that this material is synthesised within the oocyte. At the ultrastructural level evidence that the Golgi complexes give rise to vesicles in goldfish has been provided by Yamamoto & Onozato (1965) and later by Gupta & Yamamoto (1971). A number of studies have indicated that in fish these vesicles subsequently give rise to cortical alveoli (Yamamoto, 1956a, 1956b; Malone & Hisaoka, 1963; Khoo, 1979) and towards the end of vitellogenesis these alveoli fuse with the vitelline membrane and at the time of fertilization release their glycoprotein contents into the perivitelline space (Monroy, 1965; Wallace & Selman, 1981). This is also supported by evidence that endoplasmic reticulum and Golgi elements are involved in the formation of cortical alveoli in the pipefish (Anderson, 1968). So it appears that in the rainbow trout and probably other teleosts, that the appearance of this intravesicular material represents the early development of cortical alveoli and is not related in any way to true

yolk deposition, which follows this vesicular phase of development.

The present results, however, question the distinction that has been made between this phase of true yolk deposition and the period of vesicle formation previously made by other authors (van den Hurk & Peute, 1979; Forberg, 1981; Kagawa et al., 1981). Certainly in this species there is histological evidence of deep orange-staining (Mallory's) yolk granules during this period of vesicle formation (blue-staining) at the beginning of ovarian recrudescence. Also, these results show that the serum levels of vitellogenin, as shown by sensitive radioimmunoassay, have already increased above basal values during this period (See Chapter 7). Thus, the intravesicular phase of development should not be called 'endogenous vitellogenesis' and this phase does not exist as a distinct phase of development for any significant length of time in female fish at the onset of ovarian recrudescence.

It has been recognised that some form of pituitary trigger is required to initiate the secondary growth phase, which is marked histologically by the development of stage 3 oocytes into stage 4 (Vivien, 1939; Barr, 1968). Also, it is recognised that once this process has begun, spawning invariably occurs within one year (Barr, 1968). However, other workers have noted that this role does not always apply and thus, in one study early 'vitellogenic' oocytes were found to be present in the dab throughout the reproductive cycle (Htun-Han, 1978). Also, it has been observed that oocytes at the vesicular stage can often be found throughout the year long reproductive cycle (Forberg, 1981) and this author concluded that either vesicle formation is not as cyclical as the later stages of

secondary growth of the oocyte or that the ovarian development which precedes the first spawning, lasts for longer than one year. This hypothesis receives support from the present study of ovaries taken from the Whitebrook strain fish (See Chapter 7) which contained stage 4 oocytes from the June of their second year to the Spring of their third year at which point they spawned for the first time. However, there was also histological evidence of stage 5 oocytes in some of the ovaries taken from fish in the Winter of their second year, at the time when they would normally spawn one year later. There is similar evidence in other species, which indicate that some oocytes in immature animals enter stages similar to older maturing females, but subsequently reorganise their vitellogenic structures at the time when the older fish were spawning (Yamamoto, 1956; Gokhale, 1957). Whether the present data constitute any evidence for a 'dummy-run' or practice-run will be discussed more fully in relation to the endocrine changes in Chapter 7. However, concomitant with these histological changes in this strain, there were elevations in those serum parameters which would commonly be associated with maturation in other fish. If it were a 'dummy-run' then it might be expected that such fish would reorganise or resorb their oocytes at the theoretical time of spawning in readiness for the first proper cycle. In the histological examination of the ovaries of this strain, no evidence for atresia was recorded at this time, although the literature suggests that this size oocyte may disappear with very little trace (Byskov, 1978) and the frequency of sampling may have caused us to miss this evidence. Atresia during this extended vesicular phase could also have accounted for a continual recruitment of primary oocytes into stage 4 throughout the nine

months, but no evidence for this occurrence was observed. Indeed in all the ovarian samples taken, no atretic follicle was seen at any stage of development, although no study was made of the post ovulated ovary, since oocytes of this size are very difficult to section. These data would suggest that atresia plays no significant role in the reduction of oocytes ie. the fecundity at any stage of the cycle in trout, which are maintained on an adequate diet under farmed conditions.

Following stage 4 oocytes those entering and passing through stages 5,6 and 7 showed no unusual characteristics. These stages were recognised by deep-orange staining spheres which first appeared in the periphery and then increased in number and size until they completely filled the oocyte. The increase in size and number of these granules could be correlated with increases in serum vitellogenin and oestradiol-17β levels together with increases in GSI and oocyte diameter (See Chapter 7). Evidence that this granular material is derived from vitellogenin synthesised in the liver, is now well established (Wallace, 1978; van den Hurk & Peute, 1979; Dodd & Sumpter, 1982). Interestingly, close to the outer surface of the oocyte, at the base of the microvilli, the ooplasm was marked by the presence of micropinocytotic vesicles, which were first observed in sections from ovaries which still contained stage 4 oocytes. Numerous studies have demonstrated the transfer of protein from the blood to the forming yolk spheres (Droller & Roth, 1966; Korfsmeier, 1966; Anderson, 1968; Ulrich, 1969; Schackley & King, 1977) and thus Korfsmeier (1966) detected labelled proteins firstly in the liver and subsequently in the peripheral yolk spheres, within the growing

oocyte of vitellogenic females injected with tritiated amino-acids. Also, labelled protein continued to accumulate within the yolk-spheres, long after the labelled free amino-acids were cleared from the blood. Evidence of a route for vitellogenin from the capillaries to the oocyte has been provided by more recent studies using the electron-dense marker horse-radish peroxidase (HRP) (Abraham et al., 1982; Selman & Wallace, 1982). Thus, after injection of this marker, staining is first observed in the capillaries and then the precapillary spaces of the stroma. Subsequently, it is found in the basement membrane, the region surrounding the granulosa, and then passes between the intergranulosal cell spaces to the microvilli which are themselves projecting through the developing chorion. At this point HRP (and probably vitellogenin) can be seen to pass along the surface (Abraham et al., 1982) or through these microvilli (Selman & Wallace, 1982) to the oocyte where staining is subsequently taken up by fuzzy-coated micropinocytotic vesicles. In other vertebrates (Wallace, 1978) pituitary gonadotrophin(s) have been shown to stimulate the uptake of vitellogenin from the blood into vitellogenic ovaries or oocytes. Although there is evidence for such an occurrence in fish there is not full agreement for the precise mechanism of control (Campbell & Idler, 1976; Campbell, 1978; Crim & Idler, 1978; Ng & Idler, 1978). Thus, pituitary extract has been shown to stimulate uptake of vitellogenin in the trout, apparently by stimulating extensive micropinocytotic activity at the oocyte surface (Upadhyay et al ., 1978). It has even been proposed that the increases in the activity and number of micropinocytes can be used as a bioassay to test putative gonadotrophin preparations (Abraham et al., 1982). The final link providing evidence for the transfer of the material held within micropinocytes to the forming yolk-granules has come from

a number of studies (Droller & Roth, 1966; Anderson, 1968; Ulrich, 1969; Schackley & King, 1977; Selman & Wallace, 1982). Thus at a stage when serum levels of vitellogenin were significantly raised, and granules were forming in the oocyte, the present results show a close association of the microvilli with the granulosa and numerous fuzzy-coated micropinocytes at the oocyte surface at this time (See also Chapter 7). This supports the evidence discussed above for the uptake and assimilation of vitellogenin into the developing oocytes.

Apart from the detailed examination of the granulosa and zonal layers around the oocyte at stage 5, electron microscopic sections revealed other details of the cellular nature of the other follicular layers during the early stages of ovarian development. Since no investigation was made of the later stages (6,7 and 8), it was not expected that any evidence of STC would be observed, as previous evidence has shown that these special steroidogenic cells of the theca occur at about the time of ovulation (van den Hurk & Peute, 1979). Throughout the current investigation the theca cells were smooth-muscle and fibroblastic-like in appearance and were interspersed by large numbers of collagen bundles. The smooth muscle-like appearance of the theca is a recognised criteria of all vertebrate classes (Szollosi et al., 1978) and it has been shown that prostaglandins $F_2\alpha$ can act on these cells in vitro to induce ovulation in trout oocytes (Jalabert & Szollosi, 1978). These thecal cells are also responsible for the phagocytotic digestion of the majority of the collagen bundles after ovulation. This second reduction in size and weight of the ovary prepares the ovarian

lamellae for the subsequent phases of ovarian development that constitute the next reproductive cycle.

Thus, these results confirm the gross morphological changes that must occur within the oocyte and the ovary as a whole to account for the assimilation of food materials for the future development of the embryo and alevins. Interestingly, these results demonstrate in one strain of fish, evidence of either a 'dummy-run' or of an extended vesicle stage of development which has not previously been reported in other species and needs further investigation. Furthermore, in fish recrudescing for a second time, the vesicular phase appears almost indistinguishable from the granular stages, and it may be that it is only during their first spawning year that a distinct vesicular phase can be recognised.

CHAPTER 7.

NEUROENDOCRINE MECHANISMS INVOLVED IN THE CONTROL OF OVARIAN DEVELOPMENT AND VITELLOGENESIS IN THE RAINBOW TROUT.

7.1. Introduction.

Reproduction in female teleosts is a complex sequence of events involving oogenesis, folliculogenesis, vitellogenesis, oocyte maturation, ovulation and oviposition and consequently the organisation of these events into a reproductive cycle has necessitated the evolution of mechanisms to coordinate the sequence and timing of these processes, such that spawning occurs at the most propitious time of year for both young and adults. Since teleosts are genetically predetermined to spawn at a time when environmental conditions are favourable to the future survival of their young, it is understandable that they should use specific environmental cues to control these reproductive mechanisms (de Vlaming, 1974). Furthermore, there is much evidence available to show that transduction of these environmental cues is mediated by the endocrine system and it is the sequence of changes in the hormonal environment that directly controls the different phases of ovarian development and maturation and subsequently the timing of ovulation (Dodd & Sumpter, 1982).

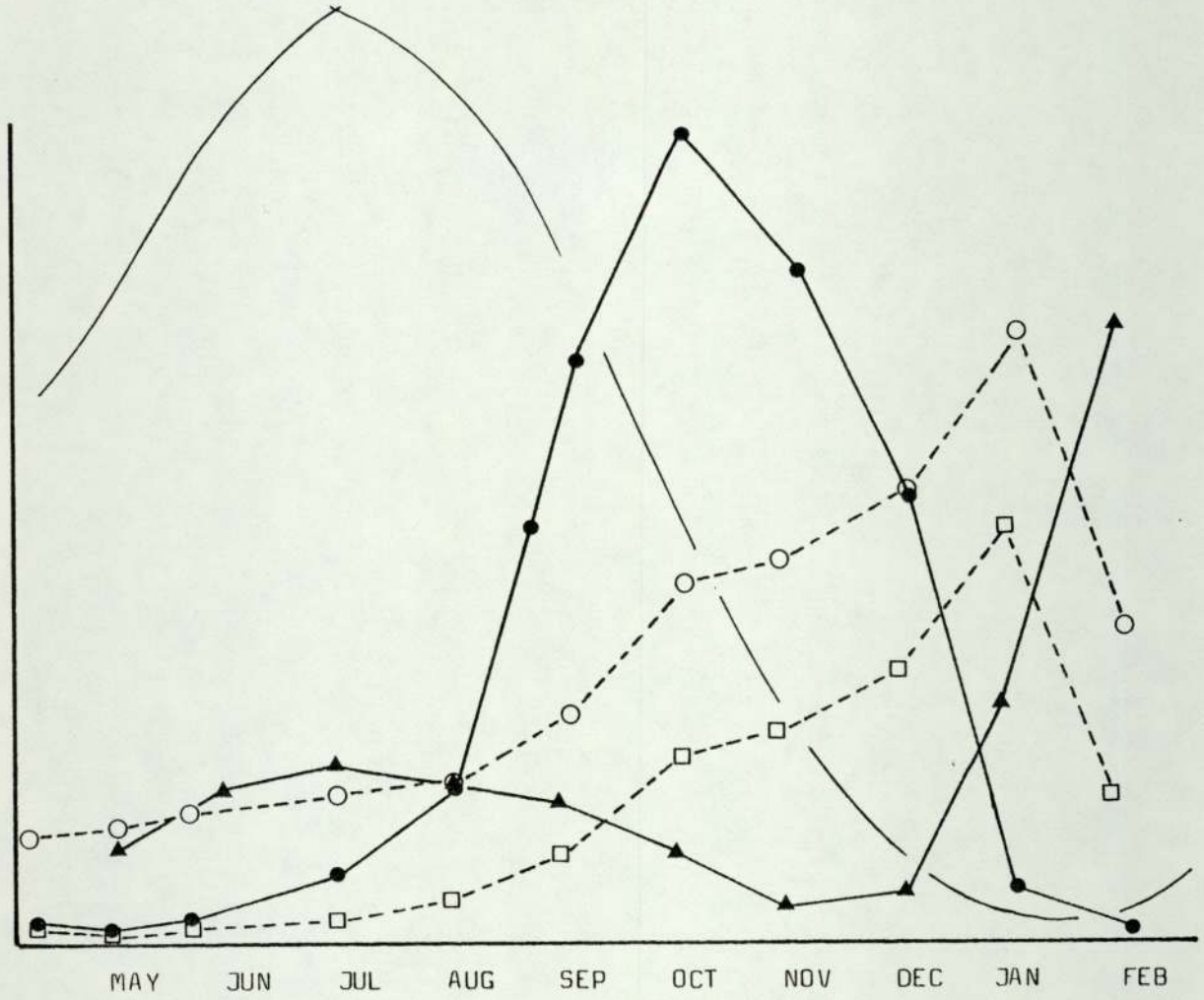
Of the 20,000 known extant species of teleosts, reproductive habits have only been considered in some 300 species (Breder & Rosen, 1966) and in only 50 of these has the physiology of this process been investigated (Htun-Han, 1977). Furthermore, most scientific attention has been focussed on species which are farmed for food or convenient for research purposes. Even with such concentrated effort there are few studies available on the hormonal and environmental mechanisms controlling reproduction in teleosts. Essentially, this is due to the variety of species used, to the

differing nature of the environmental cues and lastly to the difficulty in adequately controlling all environmental conditions. Although it is now well established that the environment can be effectively used to control the timing of egg production, no studies have closely monitored the changing hormonal environment and correlated these with specific phases of ovarian development under closely defined environmental conditions. Thus, the primary aim of this series of experiments was to monitor the sequential hormone changes during the reproductive cycle in female rainbow trout, held under an artificially controlled environment and relate these, by histological examination, to specific stages of ovarian growth. Only such a study can provide information concerning the mechanisms which control the different phases of ovarian development. At the present time much attention has been devoted to controlling just the final event of reproduction in salmonids, that of ovulation. However, manipulation of stages of reproduction other than ovulation is likely to be equally important in our understanding of the determination of oocyte development and ultimately the quality of the egg and hatched fry. Such modification will only become possible with a greater understanding of all stages of development.

Although the timing of ovulation, marked by the production of ripe eggs, is more well-defined than the initiation of gonadal recrudescence, there is little understanding of the mechanisms controlling either of these events; this is primarily due to the equivocal role for gonadotrophin(s) in these processes. There is histological evidence of 2 periods of secretory activity in the cells of the nucleus <u>lateralis tuberis</u> (NLT) one occurring in spring

and the other coincident with the time of spawning. The NLT is one of several aggregations (nuclei) of neurosecretary cells found in the teleost hypothalamus (See Batten & Ball, 1977). It is thought that the neurosecretions these cells produce contains releasing factors which are carried to the pituitary via the main axons of the preopticohypophyseal tract (Leatherland et al., 1966; Dubois et al., 1978; Ekengren & Terlou, 1978). Few workers have attempted to purify and identify these factors in teleosts (Billenstein, 1962; Follenius, 1963) although a partial characterization of carp gonadotrophin-releasing hormone was reported by Breton et al., (1975) and more recently has been shown in a number of teleost species to be distinct from mammalian counterparts (King & Millar, 1979, 1980). These specific releasing factors (eg gonadotrophin releasing hormone) are responsible for the release of pituitary hormones (eg gonadotrophin) from the pituitary glands. At present considerable doubt exists as to the number of gonadotrophin-secreting type, cells in the pituitary gland. Some studies have found only one type, some 2 and others both one and 2 gonadotrophs in the same species, but at different points in the reproductive cycle (See Dodd & Sumpter, 1982). These discordant results may reflect a real situation based on species differences or they may be due to temporal differences in the appearance of one or 2 cell types.

In parallel, doubt also exists as to the number of gonadotrophins produced by the pituitary gland and a number of studies in salmonids have revealed 2 increases in gonadotrophin, one in spring at about the time of initiation of vitellogenesis, and a further, much larger peak, around the time of spawning (See Dig. 7.1.;



DIAG. 7.1. SCHEMATIC REPRESENTATION OF CHANGES IN GONADOTROPHIN (▲——▲), OESTRADIOL-17B (●——●), TOTAL CALCIUM (⊖- - - -⊖) AND PHOSPHOPROTEIN PHOSPHORUS (□- - - -□) HELD UNDER ARTIFICIAL NORMAL SEASONAL CYCLE, AT A CONSTANT 9°C WITH SPAWNING IN JANUARY (Redrawn from Whitehead et al., 1978a, 1978b).

Whitehead et al., 1978a, 1978b; Bromage et al., 1982b). However, a number of other investigations have failed to demonstrate a significant rise in serum gonadotrophin during the early stages of sexual maturation in trout and carp (Crim et al., 1975; Billard et al., 1978; Crim & Idler, 1978). Whether one or 2 peaks in gonadotrophin do occur during the reproductive cycle of teleosts, may not be fully resolved until the number and character of gonadotrophins are themselves clearly identified.

There has been considerable scientific effort directed towards the isolation and purification of teleost gonadotrophin(s) both for the development of in vitro and bio-assays, and in order to resolve the disparity of the number of gonadotrophin(s) in fish. Two gonadotrophins have been isolated by Idler & Co. workers from 4 species of teleosts using affinity chromatography on Con-A sepharose (Campbell & Idler, 1976, 1977; Campbell, 1978; Ng & Idler, 1978a, 1978b; Idler & Ng, 1979; Ng & Idler, 1979). The crude pituitary preparation was divided into 2 fractions, one that does not bind to the gel (Con A-I) and one that does (Con A-II) depending on the degree of glycosylation of the 2 fractions; the Con A-II fraction being rich in glycoproteins. It was at first believed that their biological activities were also distinct and that the Con A-I fraction was vitellogenic and the Con A-II fraction solely maturational. Subsequently, this distinction in activity seems less clear since the Con A-II fraction has been shown to affect vitellogenesis as well as maturation and ovulation, all the activities traditionally associated with gonadotrophin (Idler & Ng, 1979; Ng & Idler, 1979). Without pure preparations of known

biological activity the relative potency or effects of the various preparations to-date cannot be fully explained. It is possible that a single heterogenous hormone exists, rather than 2 physiologically distinct gonadotrophins, since heterogeneity is a recognised feature of mammalian pituitary hormones (Lehninger, 1975).

Without unequivocal evidence to the contrary, it is difficult to imagine that gonadal recrudescence is not initiated by gonadotrophin, although the absolute levels of this hormone may not be as important as the daily rhythm of its release. Such a mechanism was proposed for teleosts and amphibians by O'Connor (1972) and since confirmed for the goldfish by Hontela & Peter (1978) and the trout by Zohar et al., (1982). These authors have shown that there is a daily rhythm of gonadotrophin secretion, involving pulsatile release of this hormone and that it is possibly changes in this rhythm which triggers and subsequently controls reproductive development. Such 'episodic' forms of control have been well documented in sheep (Lincoln, 1976) but not in other vertebrates. In Zohars' study it was observed that small peaks of gonadotrophin were superimposed on the basal level of the hormone in March, at about the time of initiation of gonadal recrudescence and also towards the end of the cycle. Thus it may be that the apparent differences in the literature regarding the appearance of a primary peak in gonadotrophin in spring may be due to the time of sampling in relation to this episodic secretion, rather than real differences in assay sensitivity or the presence of more than one gonadotrophin.

The increase in gonadotrophin just before spawning has been shown by the majority of studies to occur at approximately the same time

as the previously elevated serum levels of oestradiol-17B start to fall (See Fig. 7.1.). This rise in gonadotrophin is thought to be involved in the final stages of maturation and directly involved with the process of ovulation. The high levels and rate at which they are attained may be partly due to a feedback mechanism, since it occurs at a time when serum oestradiol-17B levels are falling (de Vlaming, 1974; Pandey & Hoar, 1972; Breton et al., 1975 ; Ueda & Takahashi, 1977). It is also possible that these high levels of gonadotrophin may be important in determining early ovarian development for the next years cycle. Mitotic divisions of oogonia are characteristic of the immediate post-spawning period in cyclical breeding teleosts, and the cyclical nature of this oogonial proliferation has suggested a possible hormonal involvement (See reviews by Hoar, 1969; Dodd, 1975; de Vlaming, 1974). However, at present the data is somewhat equivocal in that there is evidence for and against pituitary involvement and some to suggest a secondary steroidal influence (See Chapter 6 and Dodd & Sumpter, 1982). Again this may not be fully resolved until the effects of purified gonadotrophins on oogonial mitosis are studied as opposed to crude pituitary extracts.

Although there are considerable difficulties in the interpretation of the gonadotrophin data available, the findings regarding the changes and roles for serum oestradiol-17β are much more clear. Mainly this is due to recent improvements in the techniques used for the estimation of circulating levels of this and other steroids. Thus, serum levels of oestradiol-17β have been observed to start to increase around mid-June during the normal reproductive cycle of the

rainbow trout, most probably as a result of stimulation by gonadotrophin (Fig. 7.1.) and reach a peak in late Autumn, several months before spawning. Subsequently, serum oestradiol-17β levels have been shown to fall before spawning by which time they are at, or very near, basal levels (de Vlaming, 1974; Pandey & Hoar, 1972; Breton et al., 1975 ; Ueda & Takahashi, 1976; Lambert et al., 1978; Whitehead et al., 1978a; Scott et al., 1980b). Thus, serum levels of oestradiol-17β are high at times of maximum vitellogenin production by the liver (Eleftheriou et al., 1966; Wingfield & Grimm, 1977; Fostier et al., 1978; van Bohemen et al., 1981c) and there is much evidence to implicate this steroid in the control of vitellogenin production in teleosts (See Chapter 3). However, very little consideration has been given to other oestrogens. Although oestriol has not been positively identified in trout, oestrone which has, has only been determined in 3 studies on teleost reproduction (Cedard et al., 1961; Eleftheriou et al., 1966; van Bohemen et al., 1981c) even though it is known to have significant vitellogenic effects (Urist & Schjeide, 1961; Terkatin-Shimony & Yaron, 1978; van Bohemen et al., 1982b). Although oestrone is not as potent as oestradiol-17β when administered artificially (Terkatin-Shimony & Yaron, 1978) it has been shown to be present in similar serum levels as oestradiol-17β in those studies where it has been measured during the complete reproductive cycle (Cedard et al., 1961; Eleftheriou et al., 1966; van Bohemen et al., 1981c). Further study of the vitellogenic effects of oestrone in trout by van Bohemen et al., (1982a) led these authors to conclude that serum levels of oestrone cannot be ignored when investigating the physiology of exogenous vitellogenesis in rainbow trout. Furthermore, in female trout the normal pathway for biosynthesis

of oestradiol-17β goes via the aromatisation of androstenedione to oestrone, which is subsequently converted to oestradiol-17β by 17β-hydroxysteroid dehydrogenase (17βHSD) (See Chapter 5). With this in mind, determinations of this hormone were included in the present investigation. Furthermore, by using more than one strain in our experiments, we would be able to observe whether there are any significant intraspecific differences in the levels or profile of the hormones measured. With the oestrogens, such differences could possibly be related to differences in the amounts of vitellogenin produced or sequestered by the ovary, both factors which might be important in the growth of the egg and the determination of egg quality. If differences in egg quality could be related to the serum levels of vitellogenin in different strains of fish, then hormonal manipulation of these levels would be of considerable economic significance to the commercial egg producer.

Certainly at present there is very little evidence of the changes in serum vitellogenin levels accompanying sexual maturation in female teleosts (Crim & Idler, 1978; van Bohemen et al., 1981b) apart from indirect estimates of vitellogenin levels involving measurement of serum calcium and/or phosphorus (Whitehead et al., 1978a; Scott et al., 1980b; Campbell & Idler, 1980; Dodd & Sumpter, 1982). Thus, following initial increases in serum oestradiol-17β in early summer in female rainbow trout, concomitant increases in total calcium and phosphoprotein phosphorus have been observed (See Dig. 7.1. and Scott et al., 1980b). The vitellogenin molecule contains high proportions of both calcium and phosphorus ions (See Chapter 4; Campbell & Idler, 1980). The levels of these 2 serum

components increase steadily as the ovary matures and continue to rise up to the time of spawning, several months after serum levels of oestradiol-17β have begun to fall. Similar changes in phosphorus and calcium coincident with vitellogenesis were reported as early as 1897 (Miescher) and 1928 (Hess et al.,) although techniques at that time were relatively insensitive. Similar changes have since been demonstrated by other workers (Fontaine et al., 1950; Garrod & Newell, 1958; Fleming et al., 1964; Woodhead, 1968; Scott et al., 1980b) during normal reproductive cycles and after artificial administration of oestradiol-17β (See Chapter 3; Aida et al., 1973a; Elliott et al., 1979, 1980; van Bohemen et al., 1981a). These increased levels of vitellogenin obviously reflect the incorporation of exogenous yolk by the developing oocyte. Although these changes in calcium and phosphorus clearly mirror the changes in vitellogenin production, direct measurements of this complex may be more revealing. Thus in the present work a highly sensitive homologous radioimmunoassay for intact trout vitellogenin was used to investigate specific changes in vitellogenin during the reproductive cycle. For comparison with other work a parallel study was made of the changes in serum calcium levels (See Chapter 4). The improved sensitivity of this method should enable very small changes in synthesis of vitellogenin to be detected and provide a fuller description of the nature of the mechanisms which are responsible for the initiation of vitellogenesis.

Furthermore, there is little evidence for the presence of strain differences in the levels of vitellogenin observed during the normal reproductive cycle. There are no data of vitellogenin levels in a particular strain during subsequent reproductive cycles. Some

strains of fish tend to have larger eggs than other strains, and all strains have smaller eggs in their first spawning year. Whether these differences in egg size are related to fecundity or the levels of vitellogenin produced is not known. In order to understand this problem more fully, it was intended that the serum levels of vitellogenin should be compared between different strains of fish of the same age, and between different aged fish of the same strain. If differences in serum levels of vitellogenin can be related to differences in egg quality, then such data may be used as a method of broodstock selection on commercial fish farms.

Although a role for oestrogen(s) in controlling hepatic vitellogenesis in the female reproductive cycle is not surprising, the observation that serum testosterone levels rise in parallel with oestradiol-17β during the female cycle is more difficult to interpret (Schreck & Hopwood, 1974; Campbell et al., 1976; Sumpter & Dodd, 1979). Furthermore, the levels of testosterone in females often reach far higher values than those found in sexually mature males (Campbell & Idler, 1976; Stuart-Kregor et al., 1980) and at a time around spawning when serum oestrogen levels are at basal levels. Although increases in testosterone have been reported, there has been little attempt to determine the function of such changes. Since testosterone can undergo aromatisation to oestradiol-17β, it has been suggested that a decrease in the activity of the necessary aromatase enzyme, whilst probably serving to lower the serum levels of oestradiol-17β causes an increase in serum testosterone levels. However, the fact that testosterone production is not discontinued by inhibition of 17βHSD (See Chapter 5) suggests that this steroid

may have a physiological role. There is evidence from mammalian studies that androgens play a major part in the control of follicular atresia (Magoffin & Erickson, 1981). Thus it was intended that serum levels of testosterone would be determined and to relate these to any histological evidence for atresia.

Not only is there difficulty in the interpretation of the role of testosterone in reproduction, there is now considerable evidence to indicate the involvement of other hormones such as the adrenocorticoids, progestagens, and the thyroid hormones in these processes. Whether the disparity in the action of these different hormones is due to a lowered specificity of hormone action in lower vertebrates remains to be investigated (See Chapter 5). However, investigations of the importance of progestagens and corticosteroids have concentrated on their possible involvement in final maturation and/or ovulation and during this time, like thyroid hormones, they appear to work in conjunction with gonadotrophin. Thus, Sundararaj & Goswami (1977) although agreeing with Jalabert (1976) that follicular maturation is ultimately under the control of pituitary gonadotrophins, also suggest that in some species (eg catfish), some form of corticosteroidal facilitation is necessary. At concentrations of 1 $\mu g\ ml^{-1}$ or less, cortisol significantly enhances gonadotrophin-induced germinal vesicle breakdown <u>in vitro</u>, in oocytes from rainbow trout, goldfish and pike (Jalabert, 1976). It has also been suggested that gonadotrophin may act directly on the interenals, stimulating the secretion of cortisol, 11-deoxycortisol and 11-deoxycorticosterone (Sundararaj & Goswami, 1966, 1971; Goswami & Sundararaj, 1974). Other authors have observed a

possible link between the 11-deoxycorticosteroids and maturation in fish (Colombo et al., 1973; Goetz, 1978) suggesting they act as local hormones facilitating the actions of gonadotrophins. Furthermore, it has been demonstrated that 11-deoxycorticosteroids can be synthesised by teleost ovaries and subsequently possess a physiological role in oocyte maturation and ovulation (Colombo et al., 1973). It is possible that whilst maturation and ovulation are both ultimately under gonadotrophic control, they may be controlled by different mediators. Thus there is evidence for the involvement of the progestagen 17αhydroxy-20β-dihydroprogesterone (17α20β) in the control of oocyte maturation and follicular detachment and of prostaglandins and/or catecholamines, via stimulation of α-adrenergic receptors, in the process of ovulation (Jalabert, 1976). Certainly, until very recently, (See Scott et al., 1982), few studies have looked in detail at the endocrine changes around the time of spawning. Furthermore, some studies have shown that the various hormone levels determined in groups of trout around the time of spawning can be extremely variable because they are changing very rapidly and because of the asynchony of the time of spawning amongst a population. Thus, in this present work blood samples were taken at more regular intervals around the time of spawning.

Also, in teleosts the likely involvement of thyroid hormones in reproduction has long been recognised, though their precise role is not yet established (See Chapter 3; Dodd & Matty, 1964; Dodd & Sumpter, 1982; Young, 1980). There is a close correlation between the thyroid and reproductive cycle and much of the evidence points to an

involvement of thyroid hormones in vitellogenesis, probably in participation with gonadotrophins in stimulating uptake (Ichikawa et al., 1974; Hurlburt, 1977; White & Henderson, 1977; Osborn & Simpson, 1978). However, a direct involvement, of thyroxine on the liver, cannot be discounted since the HSI in thyroxine-treated fish was elevated in the study by Hurlburt (1977).

It appears that the general involvement of these other hormones varies with the species concerned. In salmonids, whereas a role for the progestagens in final maturation and ovulation is clear, the role of corticosteroids may be secondary and possibly related to the stress of spawning. However, in other species such as the catfish, the corticosteroids are of primary importance in these processes. The selection of these different hormonal mediators may be related to the different cues or triggers these species use to bring about final maturation and spawning. Overall, it would seem that these other hormones, thyroid included, are of limited physiological significance individually but act in conjunction or very closely with pituitary gonadotrophin(s).

Apart from the endocrinological changes observed during maturation, various biochemical and morphological changes occur which are important in the provision of the considerable amounts of energy and material required by the developing ovary. Thus, an increase in the gonadosomatic index and oocyte diameter or weight has been observed in a number of fish species concurrent with ovarian development (Henderson, 1963a; Braekvelt & McMillan, 1967; Billard et al., 1978; de Vlaming & Vodicnik, 1978; Lambert et al., 1978; van den Hurk & Peute, 1979; Delahunty & de Vlaming, 1980; van Bohemen et al., 1981b;

Eliassen & Vahl, 1982a). Furthermore, several investigations have made detailed histological studies of the specific stages of ovarian development throughout the reproductive cycle (See Chapter 5; Braekvelt & McMillan, 1967; de Vlaming & Vodicnik, 1978; Bieniarz et al., 1978; van den Hurk & Peute, 1979; Wallace & Selman, 1981). However, of the limited number of studies that have considered the endocrine events that occur in association with these morphological changes (Billard et al., 1978; Lambert et al., 1978; van den Hurk & Peute, 1979) no one study has considered all the endocrine events. Little attempt has been made to correlate separate endocrine events with each other, and/or with associated morphological changes. Thus, it has been virtually impossible to assess the physiological significance of these data. Thus, in the present work the hormonal changes are correlated with each other, and with the various morphological changes that occur during specific phases of ovarian development. Furthermore, it was intended that the HSI and growth rates of our experimental fish would be determined, since both these parameters have been shown to vary during the period of gonadal recrudescence (Delahunty & de Vlaming, 1980). The HSI varies both as a function of its increased activity in producing vitellogenin and in the build up of energy resources during the summer months, whereas growth rate falls as the fish matures, as a result of the redirection of food materials into the growth of the ovary.

Although the endocrine system directly controls the various specific components of reproduction, the environment is ultimately responsible for the overall control of the timing of these processes such that their proper sequence in ovarian development leads to spawning at the correct time of year. Of all the environmental

factors known to affect reproductive habits, photoperiod, temperature, rainfall, pheromones, nutrition and salinity, it is the photoperiod which exerts the major influence in salmonids (de Vlaming, 1974). Thus, accelerated light regimes have been shown to advance spawning in both rainbow and brook trout (Hoover, 1937; Hoover & Hubbard, 1937; Hazard & Eddy, 1951; Corson, 1955; Nomura, 1962; Kunesh et al., 1974; Whitehead et al., 1978a) and salmon (MacQuarrie et al., 1978) and conversely, spawning delayed in trout when extended photoperiods were used (Allison, 1951; Whitehead et al., 1981). Previous studies indicate that a long photoperiod followed by a short one, are the major functional components of the photoperiod regime in female rainbow trout and that long photoperiods encountered early in the year advance spawning (See Dodd & Sumpter, 1982).

Since daylength modifies the endocrine events in trout it would be possible to investigate the endocrine changes outlined above very closely by using well defined artificial photoperiods. Thus, in the present work two artificial photoperiod regimes designed to advance spawning were planned, since a parallel objective was to obtain mid-summer eggs for the farm where the experiments were carried out. Although mid-summer spawning of trout has been previously achieved in this laboratory on an experimental scale, this series of experiments enabled us to examine its application commercially. Furthermore, the availability of large numbers of fish allowed the sacrifice of some animals for parallel histological investigation, an aspect which had not been possible with previous studies because of the limited numbers of fish and experimental facilities. The use of closely-controlled photoperiods also enabled us to investigate other aspects of the environmental factors of control not yet fully described.

For example, there is some suggestion that there is a refractory period to environmental cues during the post-spawning period, which indicates that photoperiodic control is superimposed on an underlying endogenous ryhthm. Further evidence for an endogenous rhythm results from a study where fish, held on a constant 12L:12D photoperiod, spawned at approximately the same time as control fish under natural conditions (Whitehead et al., 1978a). To investigate whether this endogenous rhythm could or could not be modified by light it was our ultimate intention to maintain Summer-spawning fish on a natural 12 month seasonal cycle, held 6 months out of phase.

The use of different strains of rainbow trout spawning at different times was intended to provide us with further information regarding the mechanism by which the environment initiates gonadal development. At present it is not known whether different strains all begin their maturation in response to a specific environmental cue, ie. length of photoperiod, and that the subsequent differences in spawning times are due to different rates of development or whether this period is identical in the different strains, but the mechanism of photoperiodic stimulation varies, so that late-spawning fish start to mature later in the year. Early spawning fish may respond either to a shorter length of daylight, or fewer daily stimulations with a particular daylength compared to late-spawning strains. The use of 3 strains in this study enabled us to investigate this phenomenon and also to establish whether the differential spawning identity of the strains is maintained under artificial photoperiod. This is of considerable commercial significance since the use of various strains with differing spawning times is a convenient method of spreading the availability of eggs. There would be even greater flexibility if

this genetic form of control could be used in conjunction with photoperiodic manipulation of spawning time.

There is now a considerable amount of information describing the endocrine changes that precede spawning in the female rainbow trout. It is also well established that whilst different genetic strains are used to spread the spawning season, eggs can be produced in any month of the year by manipulating the photoperiod. However, it is essential that the quality of eggs produced both from different strains and 'out of season' spawning be maintained comparable to those normally available 'in season' and with no significant difference in the subsequent performance of the fry. Since the vitellogenin the fish produces essentially determines what goes into the eggs, then an understanding of the mechanisms controlling vitellogenesis is crucial in this study. However, as previously stated above, reproduction is a complex sequence of many events of which vitellogenesis is one component and knowledge of how these other events relate to vitellogenesis is of equal importance. Clearly, a study of the roles all the various hormones play in each of the specific morphological phases of development would be of immense value in our understanding of the factors affecting egg quality and fecundity in female rainbow trout. Such information would enable us to control phases of development, such as vitellogenesis, possibly by the treatment of hormones with the aim of improving egg quality, thereby providing the fish farmer with even greater flexibility and the chance of improving farmed fish stocks.

7.2. Series I Experiment.

The aims of the Series I experiment were two fold : Firstly to advance spawning by approximately 2 months in 3 strains of trout by using a truncated photoperiod regime; secondly to investigate the serum changes in oestrone (E1), oestradiol-17β (E2), testosterone (T), vitellogenin (Vg) and total calcium (TCa), which occur during the different phases of the reproductive cycles in these fish.

7.2.1. Materials and Methods.

The experiment was carried out at a commercial trout-hatchery at Pewsey in Wiltshire. Fish were held in a 7.56 metre (approximate capacity 47,191 litres) diameter circular glass-reinforced concrete tank with a water flow of 260 litres per minute from a borehole supply out of chalk through greensand, with a constant temperature of 10 ± 1°C (other water quality parameters are shown in Table 7.1.). The tank was enclosed in a wooden framework and lightproofed with industrial grade black polyethylene sheet. Access was gained via a large hinged, lightproof doorway. Artificial light was supplied by 3 single daylight tubes controlled by a 24 hour time clock, adjusted as described below.

The tank was stocked with approximately 1200 mixed sex rainbow trout of a mean weight of 1.5 kg. The majority of these fish were 2+ years old Caribou strain (California), but numbers of 2+ years old Grampian (Scotland) and 1+ year old Whitebrook (Wales) strains were also included.

The fish were held under a normal photoperiod until 30th April (1980) when they were introduced to the experimental tank and an

Quality Parameters	ppm
Oxygen (O_2)	6.9
BOD	7.7
Alkalinity (as $CaCO_3$)	245
Total Hardness (as $CaCO_3$)	435
Ammonia (NH_3)	1.42
Nitrite (NO_2)	0.045
Nitrate (NO_3)	7.1
pH	7.2
Magnesium	0.8
Iron	0.07
Al, Cu, Mn, Ni, Zn, Pb, Co	$<$ 0.1
Na	9.3
K	4.4
Chloride	14.9
Fluoride	0.24
Phosphate (PO_4)	8.1
Phehols	$<$ 0.1
Thiocyanate	$<$ 0.1
Silicate	10.6

TABLE 7.1. TYPICAL ANALYSIS OF WATER QUALITY IN EXPERIMENTAL PHOTOPERIOD TANK USED IN THESE EXPERIMENTS.

artificial photoperiod of 18 hours light and 6 hours dark (18L:6D). On the 22nd June of the same year the photoperiod was changed to short days (ie 6L:18D) (See Fig. 7.1.). At the same time 12 fish from each strain were selected at random, blood sampled, individually tagged and returned to the tank. The fish were bled monthly until December of the same year, by which time all maturing female fish had spawned.

The serum samples were assayed for oestrone (E1), oestradiol-17β (E2), testosterone (T), vitellogenin (Vg) and total calcium (TCa). Non-tagged fish of each of the 3 strains were selected at random each month, blood sampled and ovarian tissue taken for histology. The oocyte diameter was determined after fixation in Bouins, by counting the number of oocytes in a 5cm strip and taking the mean of 10 counts. Where oocyte diameter was estimated from the eggs of ovulated fish, determinations were made after water hardening, since the softness of unfertilized eggs may lead to considerable error.

Under natural conditions at this location, the Caribou, Grampian and Whitebrook strains spawn in November, December and January respectively. As no corresponding groups of fish were maintained on an artificial natural light cycle, the times of spawning of the experimental fish were compared with similar stocks held in outdoor ponds.

Statistical analysis. Basal and maximum values of the serum parameters determined were compared using either the student's t-test or F-test if the variances were dissimilar. The relationships between serum components and morphological changes

were investigated by calculating the correlation coefficient (r) by the method of least-squares fit. The results of these tests are outlined below (See Section 7.2.2.1.).

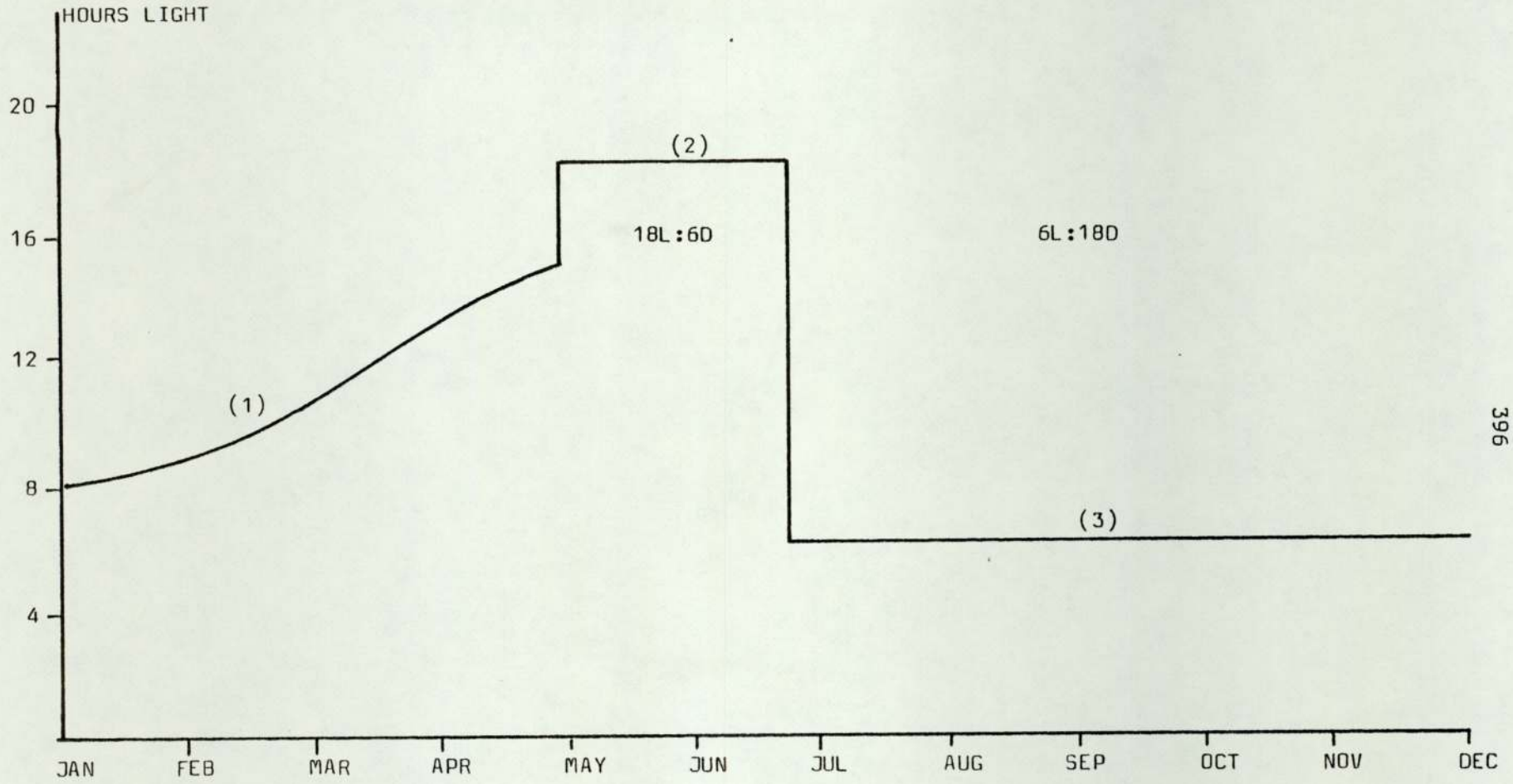


FIG. 7.1. PHOTOPERIOD REGIME FOR SERIES I EXPERIMENT. (1) NORMAL SEASONAL PHOTOPERIOD UNTIL 30 APRIL. (2) SWITCHED TO 18hrs LIGHT/6hrs DARK (18L:6D). (3) 21 JUNE SWITCHED TO 6D:18L.

7.2.2. Results.

Under the artificial photoperiod, spawning was advanced by 6 weeks when compared with similar stocks and strains held in outdoor ponds. In the experimental tank the Caribou strain fish were the first to spawn on the 20th September, followed by the Grampians on the 24th October. No Whitebrook strain female fish reached full sexual maturity during this year. The artificial photoperiod produced no adverse physiological or behavioural changes in experimental fish when compared to those held under natural conditions, although the eggs were smaller (2.9 $\pm$ 0.91 as opposed to approximately 4.5mm); stripping commenced in the Caribou strain held outdoors on the 8th November (Fig. 7.2.).

As the ovary recrudesced in the Caribou and Grampian strain fish, gross changes were observed in oestrone (E1), oestradiol-17β (E2), testosterone (T), vitellogenin (Vg), total calcium (TCa) and mean oocyte diameter (OOD). Some changes in these serum parameters were also observed in Whitebrook fish, even though full maturity was not achieved. Ovarian changes were subsequently confirmed by histology.

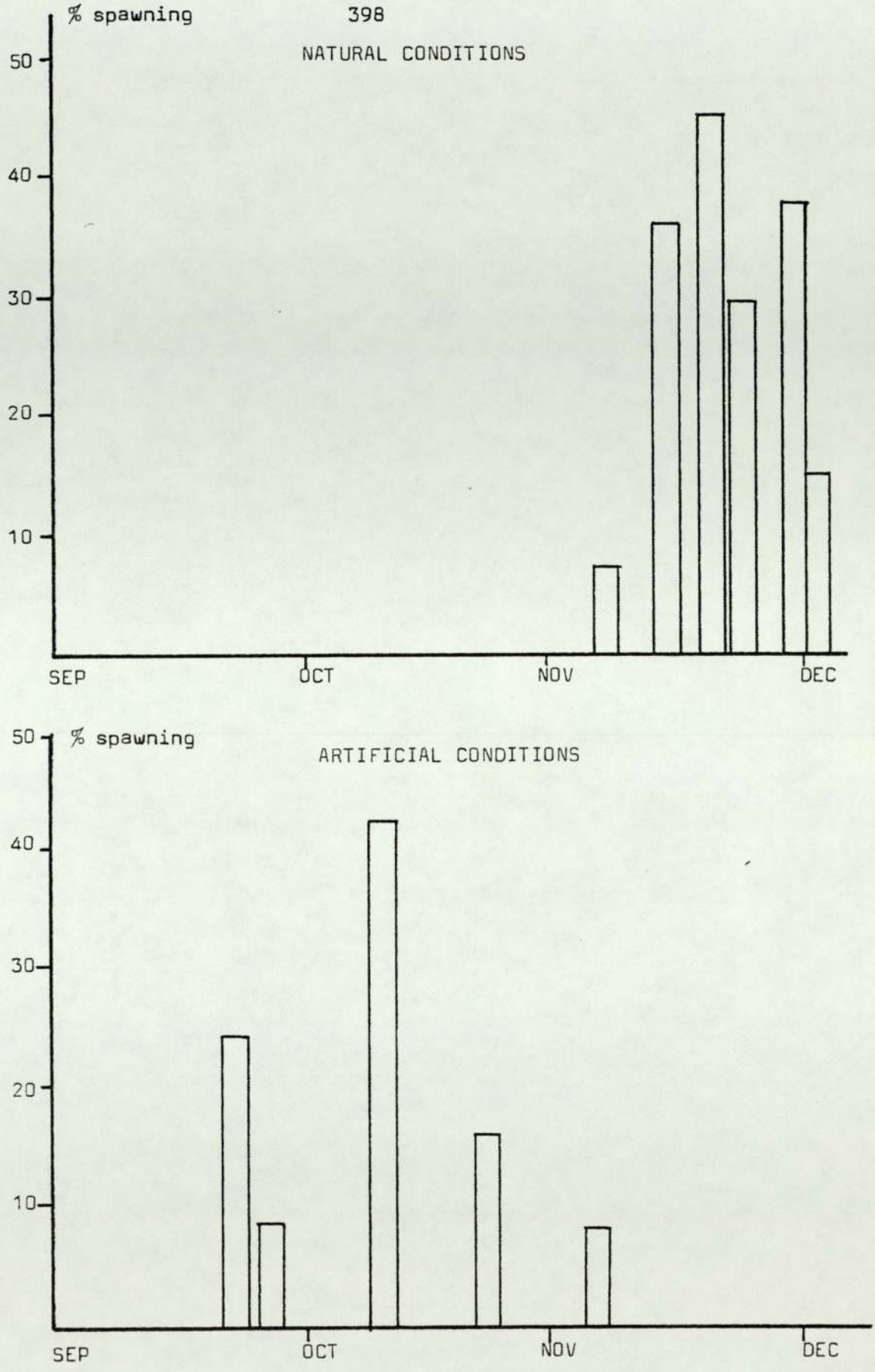


FIG. 7.2. COMPARISON OF SPAWNING TIMES IN CARIBOU AND GRAMPIAN STRAINS UNDER NATURAL CONDITIONS (TOP) AND UNDER ARTIFICIAL PHOTOPERIOD (BOTTOM) IN SERIES I EXPERIMENT.

Changes in serum Oestrone (E1) : Mean serum levels of E1 in Caribou fish rose significantly ($P < 0.05$) from 9.3 $\pm$ 4.5 ng ml^{-1} in June to a peak of 31.0 $\pm$ 7.9 ng ml^{-1} in August one month prior to the commencement of spawning, but they were undetectable one month later when eggs were strippable. In Grampian fish, mean serum E1 rose significantly ($P < 0.001$) from 8.6 $\pm$ 2.5 ng ml^{-1} in June to a peak of 26.0 $\pm$ 3.5 ng ml^{-1} one month later in July, some 2 months prior to spawning, falling to 3.7 $\pm$ 0.9 ng ml^{-1} in November. In Whitebrook fish, 2 increases of serum E1 were observed with levels of 5.9 $\pm$ 2.2 and 6.0 $\pm$ 2.9 ng ml^{-1} in July and November respectively, although neither were significantly different from the mean June value of 3.9 $\pm$ 0.6, or October level of 2.8 $\pm$ 2.2 ng ml^{-1}. (See Table 7.2. & Fig. 7.3.).

		JUN 21	JUL 21	AUG 19	SEP 24	NOV 4	DEC 4
CARIBOU	MEAN	9.3	24.6	31.0	0.0	1.7	0.0
	± S.E.	4.5	3.7	7.9	-	1.5	-
	(n)	(10)	(8)	(6)	(6)	(6)	(6)
GRAMPIAN	MEAN	8.6	26.0	23.6	11.5	3.7	5.9
	± S.E.	2.5	3.5	4.4	5.4	0.9	3.1
	(n)	(10)	(9)	(7)	(7)	(6)	(6)
WHITEBROOK	MEAN	3.9	5.9	2.0	0.6	6.0	2.8
	± S.E.	0.6	2.2	1.7	0.5	2.9	2.2
	(n)	(8)	(10)	(9)	(8)	(6)	(6)

TABLE 7.2. CHANGES IN MEAN SERUM LEVELS OF OESTRONE (E1 ng ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD IN SERIES I EXPERIMENT.

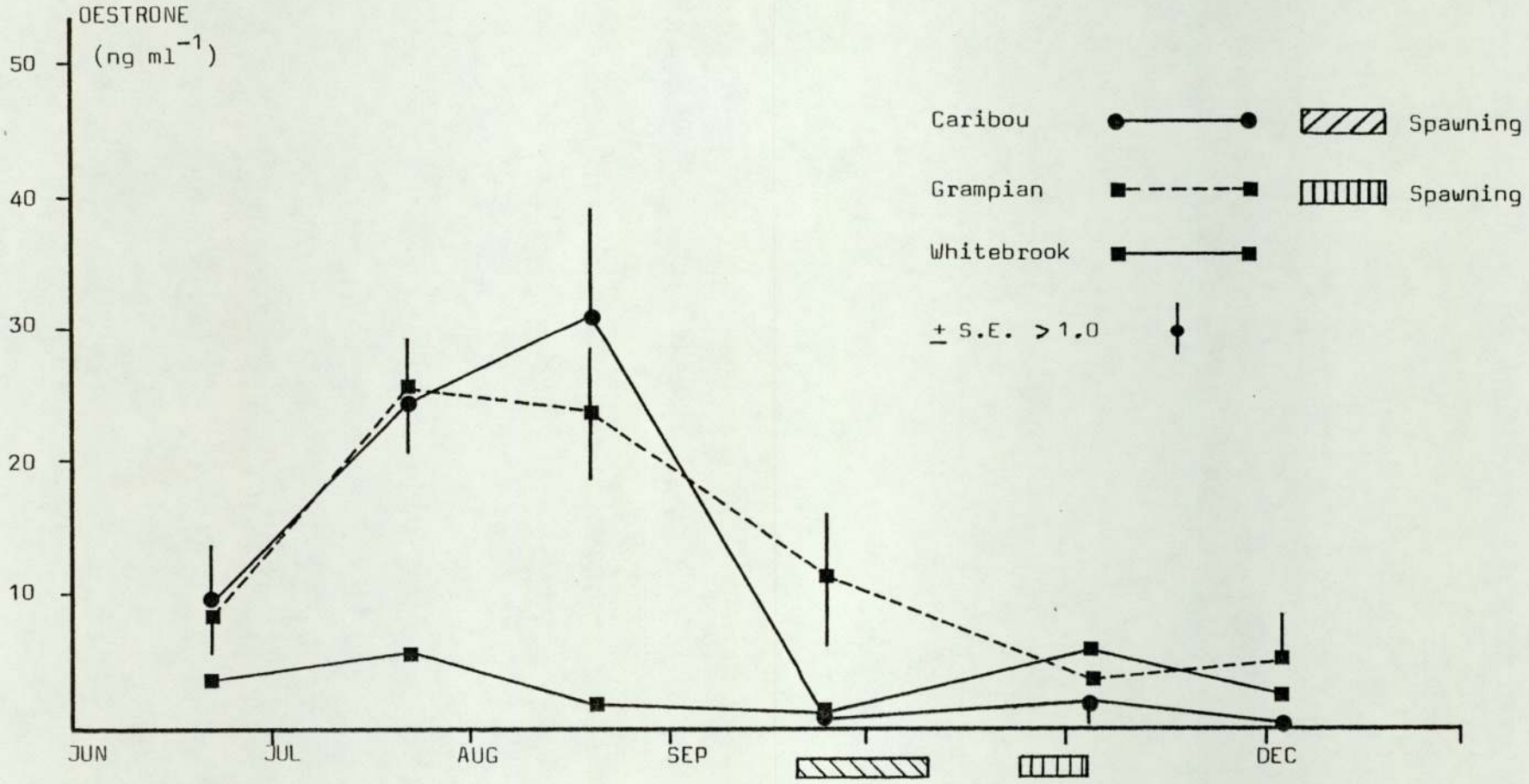


FIG. 7.3. CHANGES IN MEAN SERUM LEVELS OF OESTRONE (E1 ng ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD IN SERIES I EXPERIMENT.

Changes in serum Oestradiol-17B (E2): Serum levels of E2 in Caribou fish rose significantly ($P < 0.001$) from a mean basal value of 7.8 $\pm$ 2.5 ng ml^{-1} in June to a peak mean level of 47.3 $\pm$ 5.6 ng ml^{-1} in August, one month prior to spawning. Serum levels of E2 in Caribou fish fell to near basal levels with a mean of 4.7 $\pm$ 3.0 ng ml^{-1} at the time of spawning in September. In Grampian fish serum levels of E2 rose significantly ($P < 0.01$) from a mean of 7.3 $\pm$ 3.4 ng ml^{-1} in June to a peak of 28.7 $\pm$ 5.2 ng ml^{-1} in September, one month before spawning, before returning to near basal levels of 4.2 $\pm$ 1.2 ng ml^{-1} in November. Surprisingly, serum E2 levels in Whitebrook fish rose significantly ($P < 0.01$) from a mean basal value of 1.5 $\pm$ 0.6 ng ml^{-1} in June, reaching a peak of 9.1 $\pm$ 2.4 ng ml^{-1} in August, before falling to 0.7 $\pm$ 0.5 ng ml^{-1} one month later (See Table 7.3. & Fig. 7.4.).

		JUN 21	JUL 21	AUG 19	SEP 24	NOV 4	DEC 4
CARIBOU	MEAN	7.8	29.1	47.3	4.7	0.9	1.4
	± S.E.	2.5	1.8	5.6	3.0	0.7	1.1
	(n)	(12)	(9)	(6)	(6)	(6)	(6)
GRAMPIAN	MEAN	7.3	20.9	22.8	28.7	4.2	4.1
	± S.E.	3.4	1.7	4.5	5.2	1.2	1.1
	(n)	(12)	(11)	(9)	(8)	(8)	(8)
WHITEBROOK	MEAN	1.5	5.4	9.1	0.7	0.8	1.4
	± S.E.	0.6	1.3	2.4	0.5	0.4	0.7
	(n)	(12)	(10)	(9)	(10)	(6)	(6)

TABLE 7.3. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD IN SERIES I EXPERIMENT.

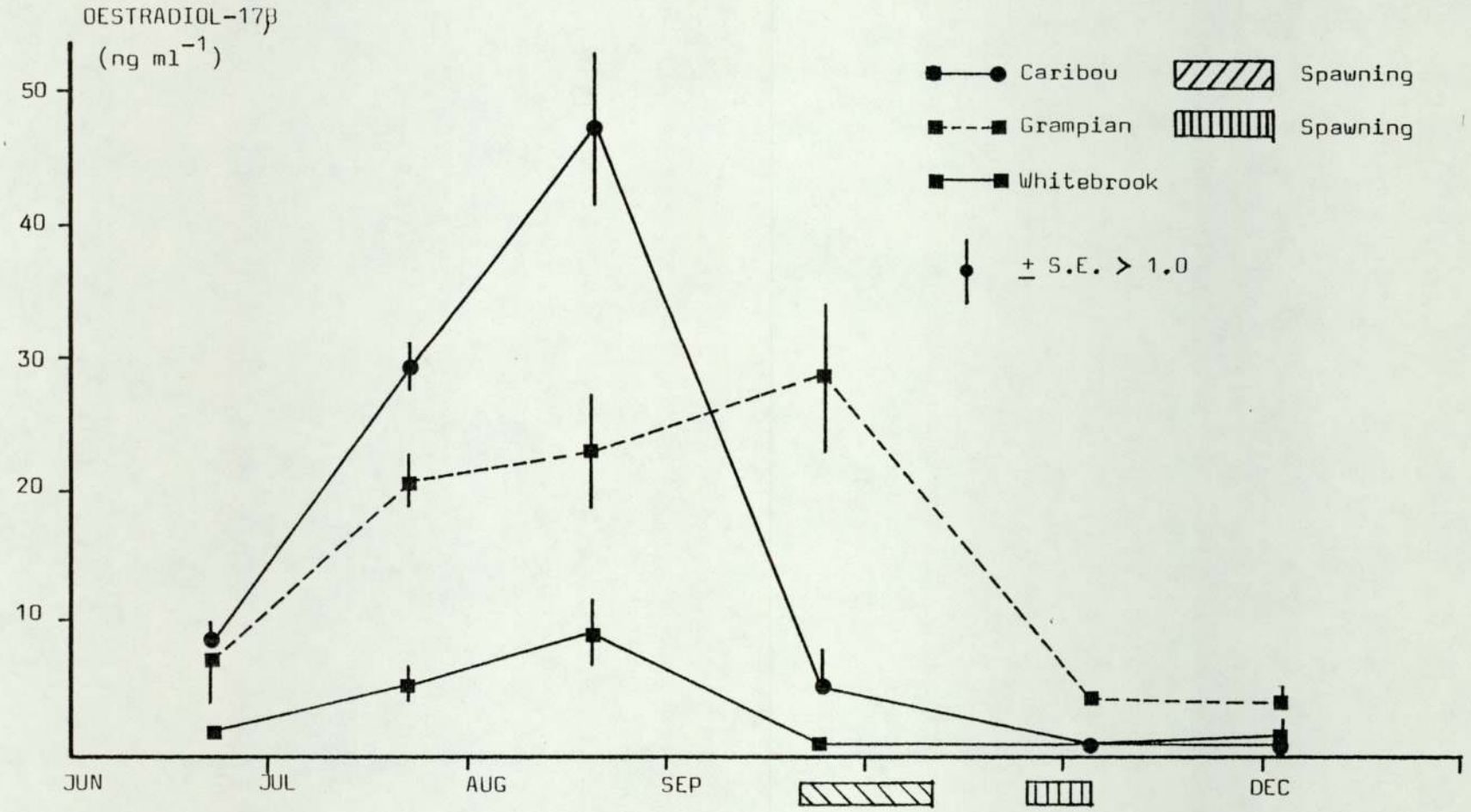


FIG. 7.4. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD IN SERIES I EXPERIMENT.

Changes in serum Testosterone (T) : Mean serum T levels in Caribou and Grampian strains rose significantly ($P < 0.001$) from 10.8 ± 3.4 and 9.8 ± 1.6 ng ml^{-1} in June respectively to peak mean levels of 149.3 ± 32.8 and 204.0 ± 4.0 ng ml^{-1} in September at the time of spawning in Caribou fish. Mean serum T levels in Grampian fish were still high at 157.0 ± 33.5 ng ml^{-1} one month after spawning in November, falling to 8.3 ± 6.5 ng ml^{-1} in December, compared with levels in Caribou of 4.0 ± 1.0 ng ml^{-1} at the same time. In Whitebrook fish mean serum T levels rose significantly ($P < 0.001$) from 3.0 ± 1.0 ng ml^{-1} in June to a peak mean level of 11.7 ± 1.1 ng ml^{-1} in September, before falling to a mean level of 2.2 ± 0.5 ng ml^{-1} in December. (See Table 7.4. & Fig. 7.5.).

		JUN 21	JUL 21	AUG 19	SEP 24	NOV 4	DEC 4
CARIBOU	MEAN	10.8	24.8	62.3	149.3	72.7	4.0
	± S.E.	3.4	4.0	17.4	32.8	13.6	1.0
	(n)	(9)	(6)	(6)	(6)	(6)	(6)
GRAMPIAN	MEAN	9.8	37.1	132.7	204.0	157.0	8.3
	± S.E.	1.6	8.6	22.5	4.0	33.5	6.5
	(n)	(8)	(9)	(7)	(6)	(6)	(6)
WHITEBROOK	MEAN	3.0	2.3	6.2	11.7	3.3	2.2
	± S.E.	1.0	0.5	1.5	1.1	0.5	0.5
	(n)	(8)	(8)	(8)	(6)	(6)	(6)

TABLE 7.4. CHANGES IN MEAN SERUM LEVELS OF TESTOSTERONE (T ng ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD IN SERIES I EXPERIMENT.

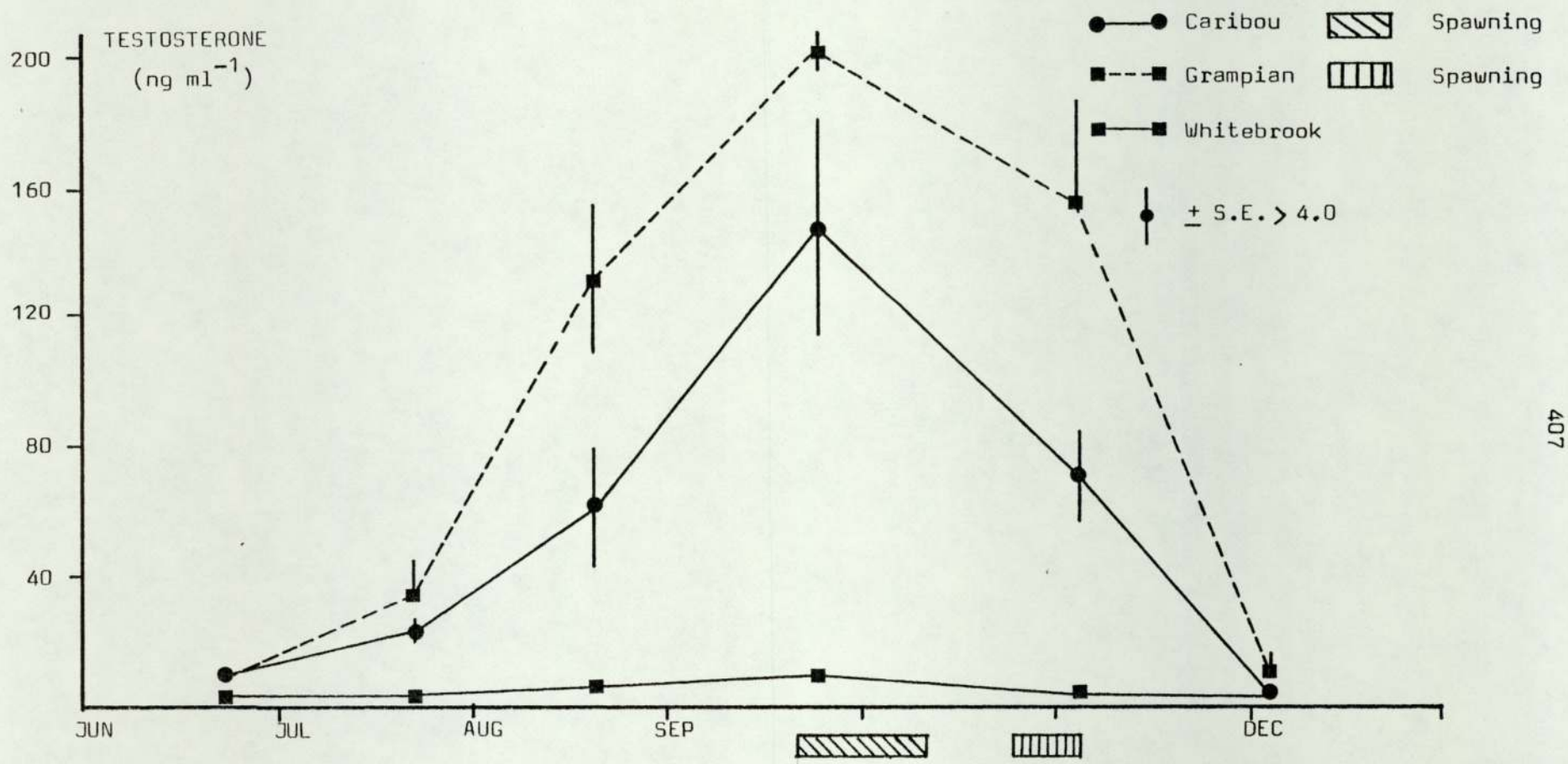


FIG. 7.5. CHANGES IN MEAN SERUM LEVELS OF TESTOSTERONE (T ng ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD IN SERIES I EXPERIMENT.

Changes in serum Vitellogenin (Vg) : Mean serum levels of Vg in Caribou and Grampian strains rose significantly ($P < 0.001$) from similar values of 6.7 ± 0.8 and 5.4 ± 0.4 mg ml^{-1} in June respectively, to peak mean levels of 195.3 ± 11.6 and 99.0 ± 13.1 mg ml^{-1} in September at the time of spawning in Caribou fish. At the end of the experiment in December, mean serum Vg levels had fallen to 1.4 ± 0.3 and 7.6 ± 0.6 mg ml^{-1} in the Caribou and Grampian strains respectively. Serum Vg levels in Whitebrook fish rose from a mean June value of 0.5 ± 0.2 mg ml^{-1} to a peak of 1.7 ± 0.7 mg $100ml^{-1}$ in November (See Table 7.5. & Fig. 7.6.).

		JUN 21	JUL 21	AUG 19	SEP 24	NOV 4	DEC 4
CARIBOU	MEAN	6.7	44.3	128.7	195.3	36.7	1.4
	± S.E.	0.8	5.7	30.6	11.6	7.7	0.3
	(n)	(12)	(9)	(6)	(10)	(8)	(9)
GRAMPIAN	MEAN	5.4	32.2	61.9	99.0	50.0	7.6
	± S.E.	0.4	3.3	9.0	13.1	7.2	0.6
	(n)	(12)	(11)	(10)	(10)	(8)	(8)
WHITEBROOK	MEAN	0.5	1.33	1.42	1.1	1.7	0.3
	± S.E.	0.2	0.3	0.4	0.2	0.7	0.1
	(n)	(12)	(10)	(9)	(10)	(6)	(6)

TABLE 7.5. CHANGES IN MEAN SERUM LEVELS OF VITELLOGENIN (Vg mg ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD IN SERIES I EXPERIMENT.

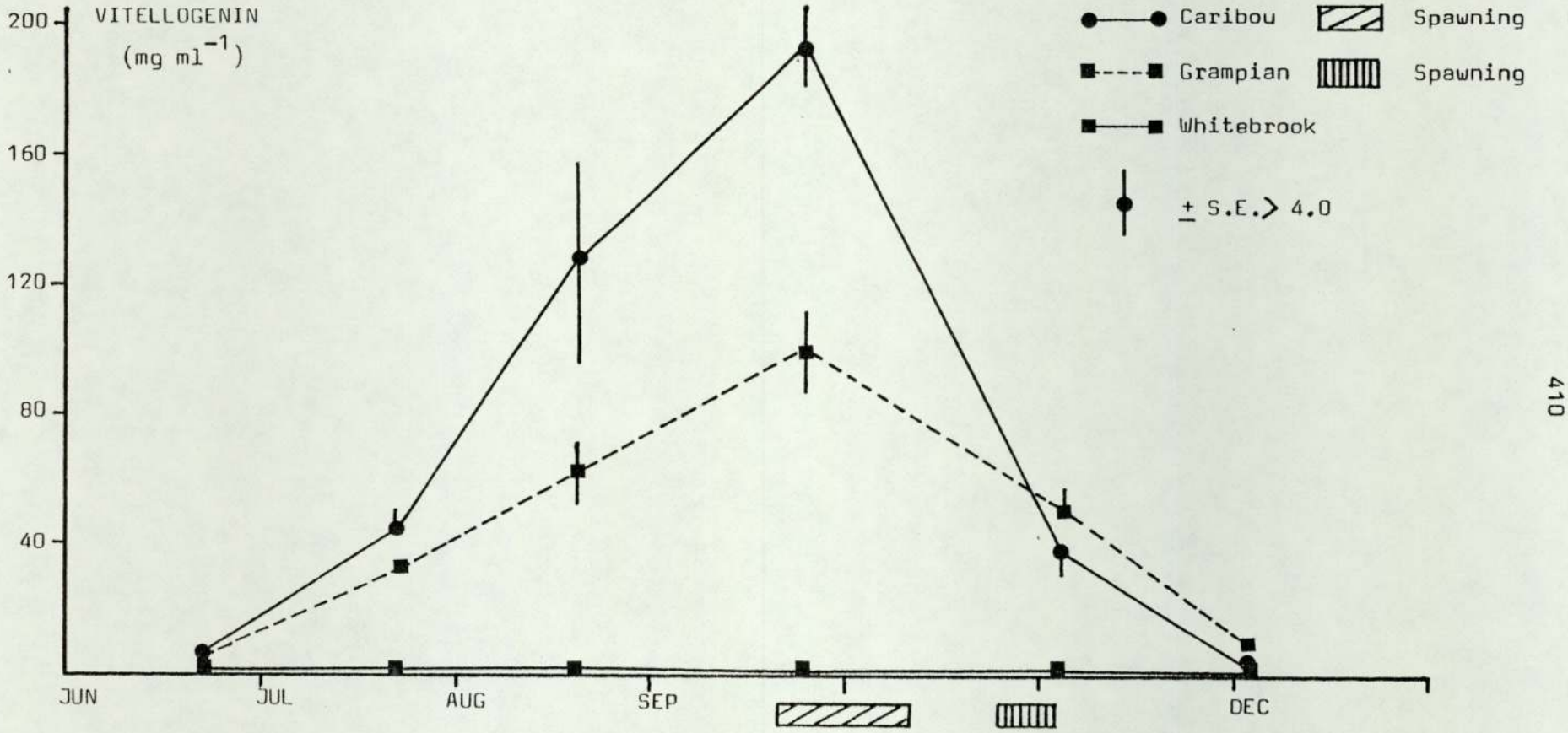


FIG. 7.6. CHANGES IN MEAN SERUM LEVELS OF VITELLOGENIN (Vg mg ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD IN SERIES I EXPERIMENT.

Changes in serum Total Calcium (TCa) : Concomitant with changes in Vg, mean serum TCa levels in June were similar in Caribou and Grampian strains at 14.9 ± 0.6 and 14.9 ± 0.3 mg $100ml^{-1}$ respectively, rising significantly ($P < 0.001$) to 108.7 ± 5.0 and 66.5 ± 12.2 mg $100ml^{-1}$ in September. Peaks in TCa (and Vg) occurred at the time of spawning in Caribou fish and one month before spawning in Grampian fish, though in the latter instance this may have been due to sampling times. Serum TCa levels fell to basal levels of 12.7 ± 0.4 and near basal levels of 17.2 ± 4.2 mg $100ml^{-1}$ in Caribou and Grampian strains in December. In Whitebrook fish, mean TCa levels rose significantly ($P < 0.05$) from 12.7 ± 0.6 mg $100ml^{-1}$ in June, to a peak of 15.9 ± 0.9 mg $100ml^{-1}$ in November (See Table 7.6. & Fig. 7.7.).

		JUN 21	JUL 21	AUG 19	SEP 24	NOV 4	DEC 4
CARIBOU	MEAN	14.9	21.4	77.4	108.7	42.0	12.7
	± S.E.	0.6	1.9	10.9	5.0	16.0	0.4
	(n)	(12)	(9)	(6)	(10)	(8)	(9)
GRAMPIAN	MEAN	14.9	20.0	43.7	66.5	35.6	17.2
	± S.E.	0.3	0.6	5.2	12.2	5.0	4.2
	(n)	(12)	(11)	(10)	(10)	(8)	(8)
WHITEBROOK	MEAN	12.7	13.5	14.4	14.3	15.9	11.4
	± S.E.	0.6	0.5	0.5	0.7	0.9	0.7
	(n)	(12)	(10)	(9)	(10)	(6)	(6)

TABLE 7.6. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD IN SERIES I EXPERIMENT.

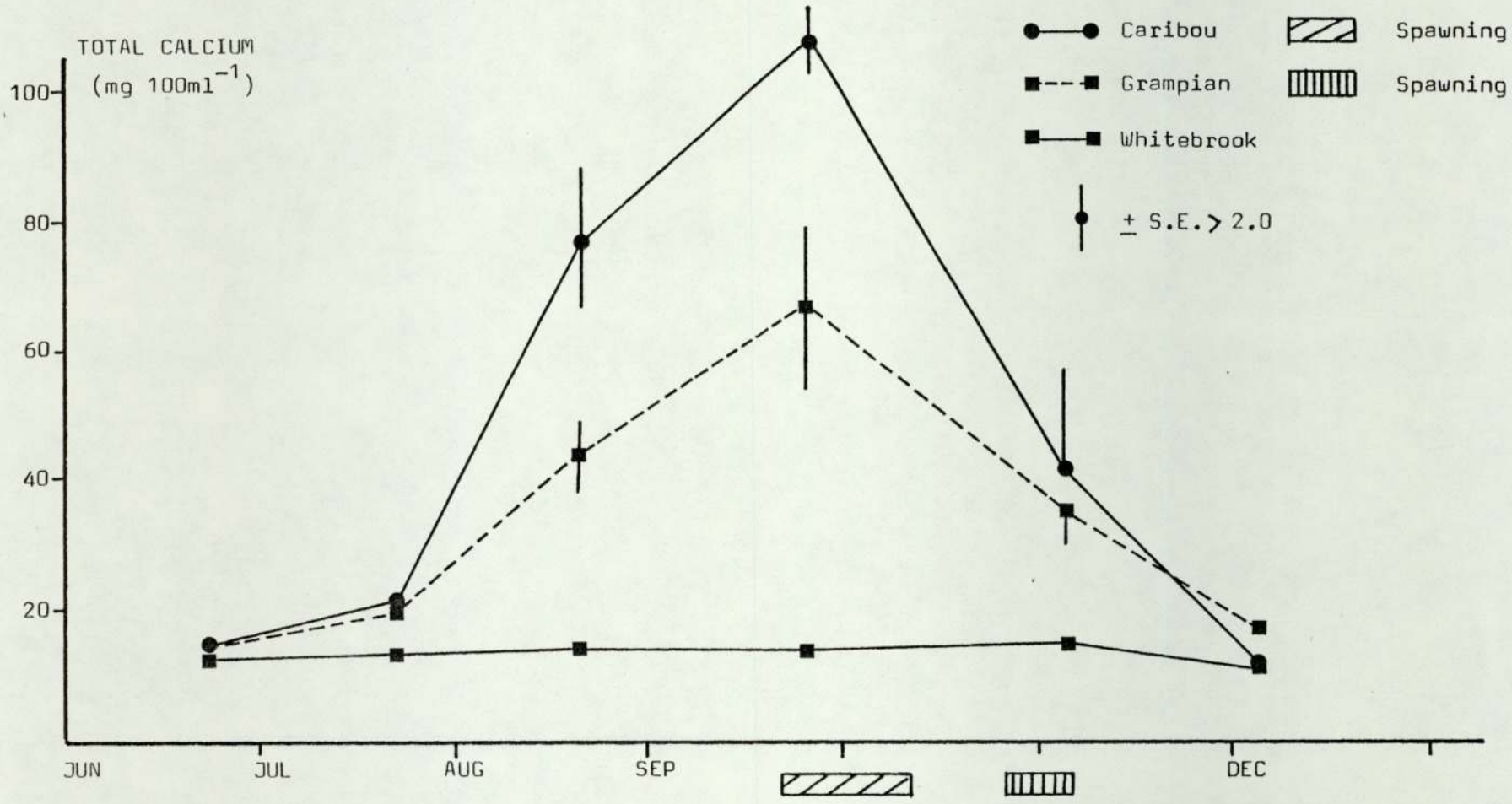


FIG. 7.7. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD IN SERIES I EXPERIMENT.

Changes in mean oocyte diameter (OOD) : The OOD increased steadily with ovarian recrudescence in both Caribou and Grampian fish. Thus, in Caribou fish OOD increased from 0.96 ± 0.03 mm at the first exogenous phase (Stage 5 - previous chapter) in June, to 2.9 ± 0.91 mm (water hardened) in September when ripe eggs could be stripped from the abdomen by gentle pressure. Similarly, OOD in Grampian fish rose from 0.913 ± 0.04 mm in June reaching 3.5 ± 0.13 mm at the time of spawning. The August and September histological specimens for the Grampian fish were not satisfactorily sectioned and hence the stage of development was unfortunately not determined.

In the Whitebrook fish sampled throughout Series I, histological examination indicated oocyte development up to Stage 4 (endogenous phase) in all months monitored, and in December some oocytes of this strain had progressed as far as Stage 5 (See Chapter 6). However, these fish did not spawn until the late summer/early autumn of the following year. The OOD increased from 0.368 ± 0.02 in June to an initial peak of 0.613 ± 0.04 mm in August, before falling to 0.251 ± 0.01 mm in November (See Table 7.7. & Fig. 7.8.).

TABLE 7.7. CHANGES IN MEAN OOCYTE DIAMETER (OOD (mm)) AND OVARIAN DEVELOPMENT IN THREE STRAINS OF RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD IN SERIES I EXPERIMENT.

		JUN 21	JUL 21	AUG 19	SEP 24	NOV 4	DEC 4	
CARIBOU	OOD (mm)	0.964	1.285	1.741	2.9	-	-	
	± S.E.	0.03	0.09	0.10	0.91			
	DEV. ST. OF OVARY	5	6	6/7	8			
	(n)	(1)	(1)	(1)	(3)			
GRAMPIAN	OOD (mm)	0.913	1.503	1.65	2.212	3.5	-	
	± S.E.	0.04	0.07	0.12	0.09	0.13		
	DEV. ST. OF OVARY	5	6	-	-	8		
	(n)	(1)	(1)	(1)	(1)	(3)		
WHITEBROOK	OOD (mm)	0.368	0.44	0.613	0.313	0.251	0.475	
	± S.E.	0.02	0.03	0.04	0.04	0.01	0.01	
	DEV. ST. OF OVARY	3/4	3/4	3/4	3/4	3/4	3/4/5	(see Ch. VI)
	(n)	(1)	(1)	(1)	(1)	(1)	(1)	

TABLE 7.7.

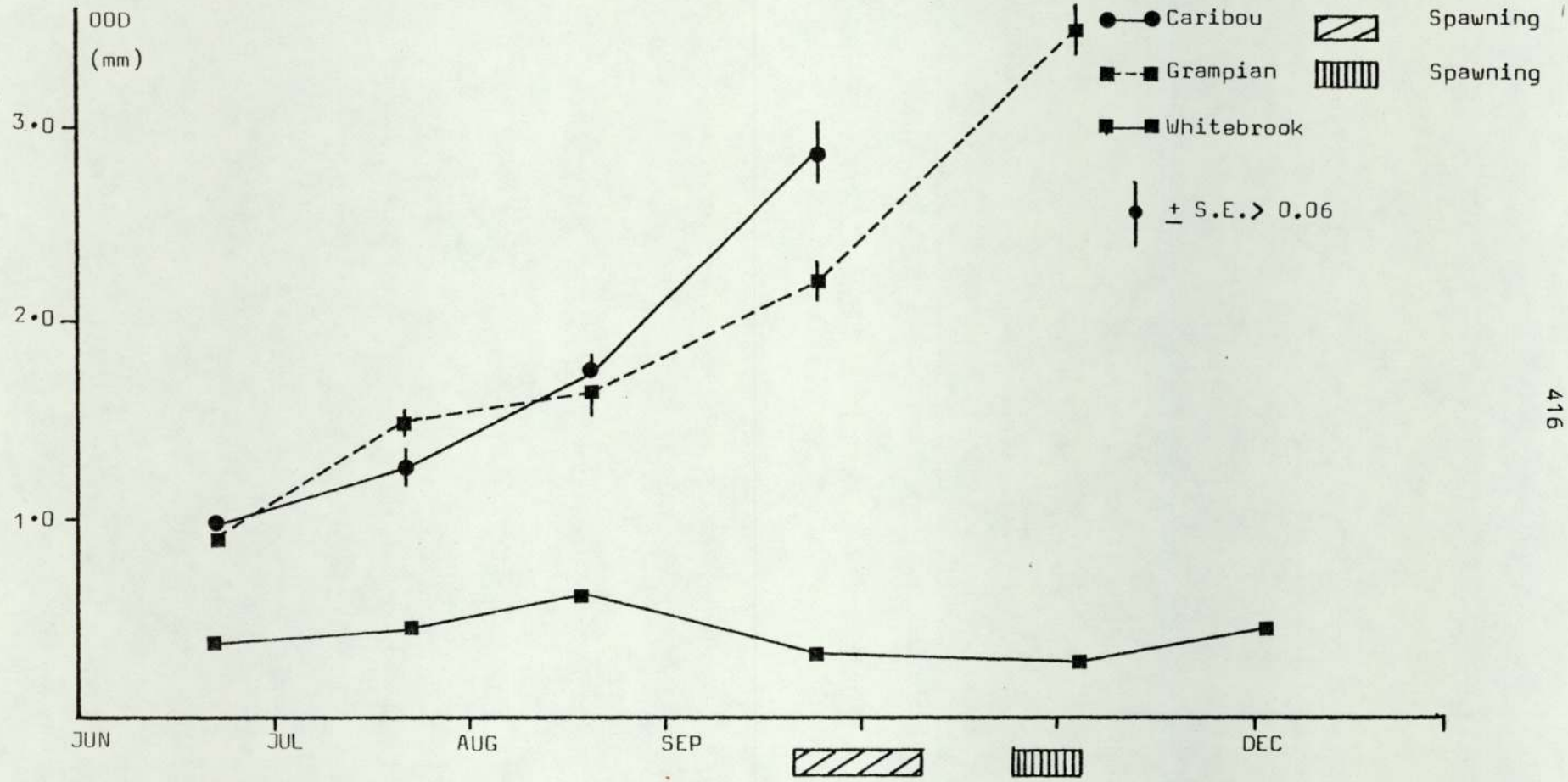


FIG. 7.8. CHANGES IN MEAN OOCYTE DIAMETER (OOD (mm)) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD REGIME IN SERIES I EXPERIMENT.

7.2.2.1. Interrelationships between Oestrone, Oestradiol-17β, Testosterone, Vitellogenin, Total Calcium and Mean Oocyte Diameter.

The timing of the above changes for the Caribou, Grampian and Whitebrook strains are summarised in Figures 7.9., 7.10. and 7.11. respectively. The possible relationships between these components were investigated by statistical correlation for

1) the exogenous vitellogenic phase from June until one month before spawning (established by histological examination).

and for 2) the complete cycle, from June until December.

These correlations will now be considered for each strain in turn :-

Caribou strain : Over the course of the complete cycle, mean serum Vg levels were significantly correlated ($P < 0.001$, $r=0.98$) with TCa; serum E2 levels were significantly correlated ($P < 0.001$, $r=0.973$) with E1 levels over the same period (see Fig. 7.9.).

Over the exogenous phase of development, June until August, mean serum Vg levels were significantly correlated with OOD ($P < 0.01$, $r=0.97$) and E2 ($P < 0.01$, $r=0.965$) (see Fig. 7.12.). Over the same period OOD was significantly correlated with serum E2 levels ($P < 0.001$, $r=0.99$) and with serum E1 ($P < 0.05$, $r=0.949$) (see Fig. 7.12.).

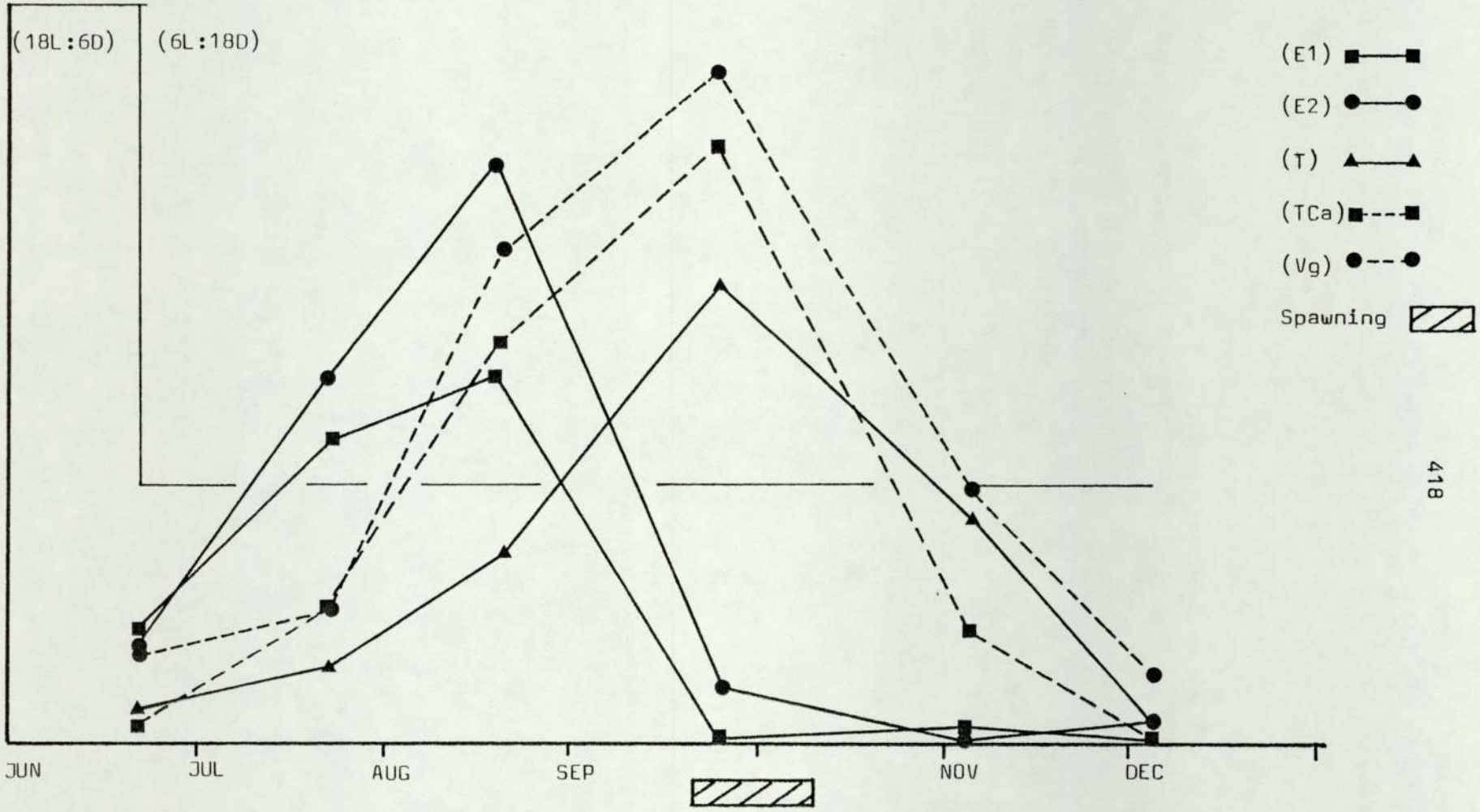


FIG. 7.9. SUMMARY OF SEQUENTIAL CHANGES IN OESTRADIOL-17β (E2), OESTRONE (E1), TESTOSTERONE (T), TOTAL CALCIUM (TCa) AND VITELLOGENIN (Vg) IN CARIBOU STRAIN RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD REGIME IN SERIES I EXPERIMENT. (VERTICAL AXIS COMPOSITE, OF SAME SCALE TO PREVIOUS FIGURES).

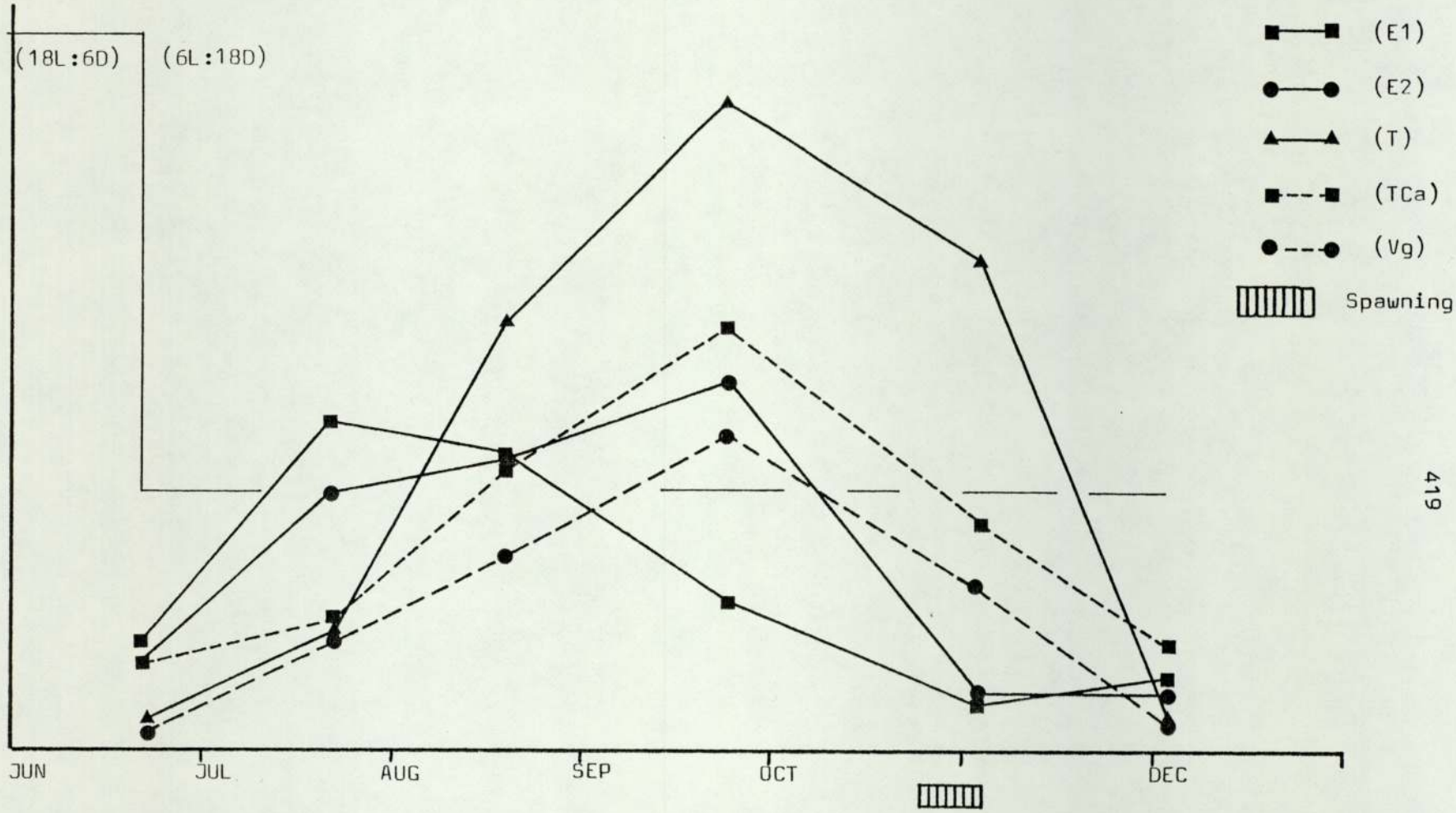


FIG. 7.10. SUMMARY OF SEQUENTIAL CHANGES IN OESTRADIOL-17β (E2), OESTRONE (E1), TESTOSTERONE (T), TOTAL CALCIUM (TCa) AND VITELLOGENIN (Vg) IN GRAMPIAN STRAIN RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD REGIME IN SERIES I EXPERIMENT. (VERTICAL AXIS COMPOSITE, OF SAME SCALE TO PREVIOUS FIGURES).

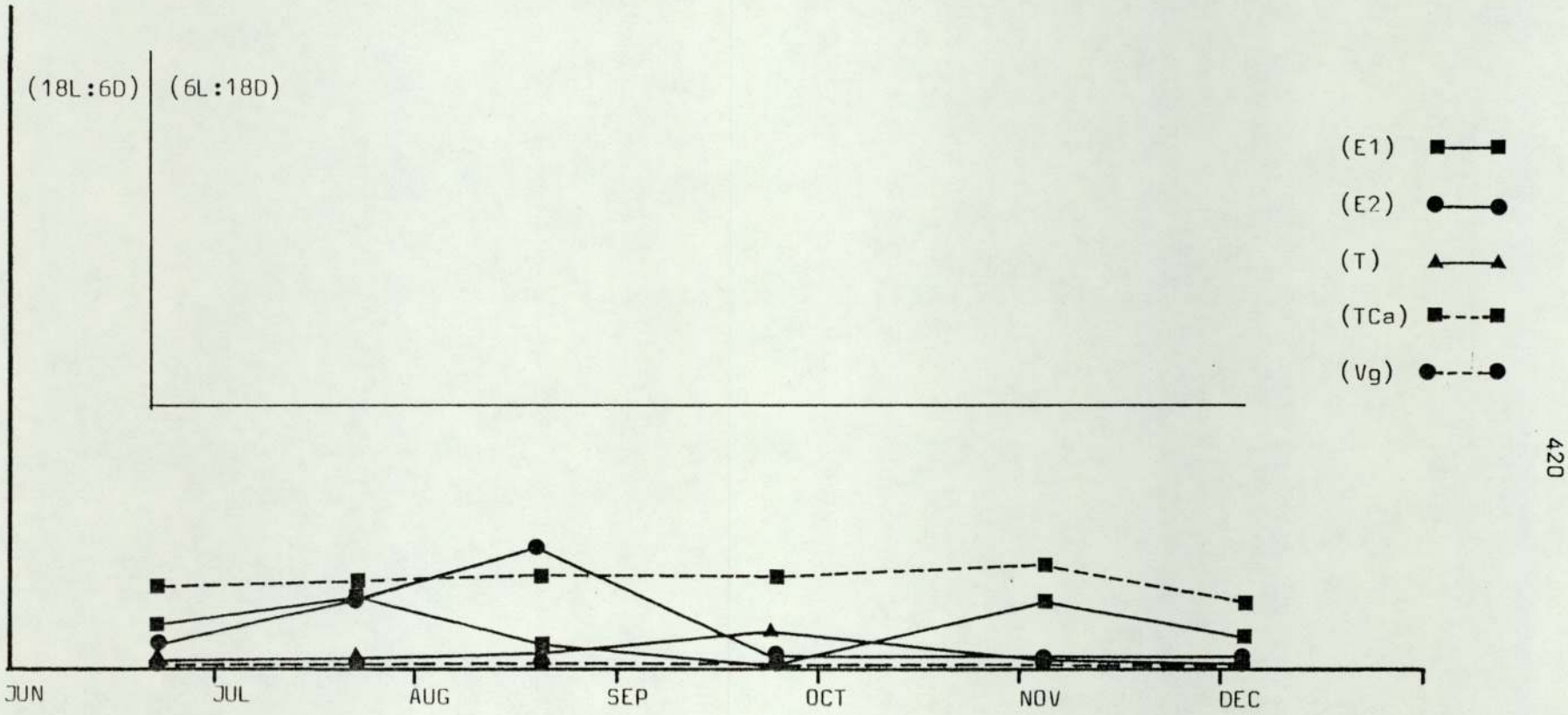


FIG. 7.11. SUMMARY OF SEQUENTIAL CHANGES IN OESTRADIOL-17β (E2), OESTRONE (E1), TESTOSTERONE (T), TOTAL CALCIUM (TCa) AND VITELLOGENIN (Vg) IN WHITEBROOK STRAIN RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD REGIME IN SERIES I EXPERIMENT. (VERTICAL AXIS COMPOSITE, OF SAME SCALE TO PREVIOUS FIGURES).

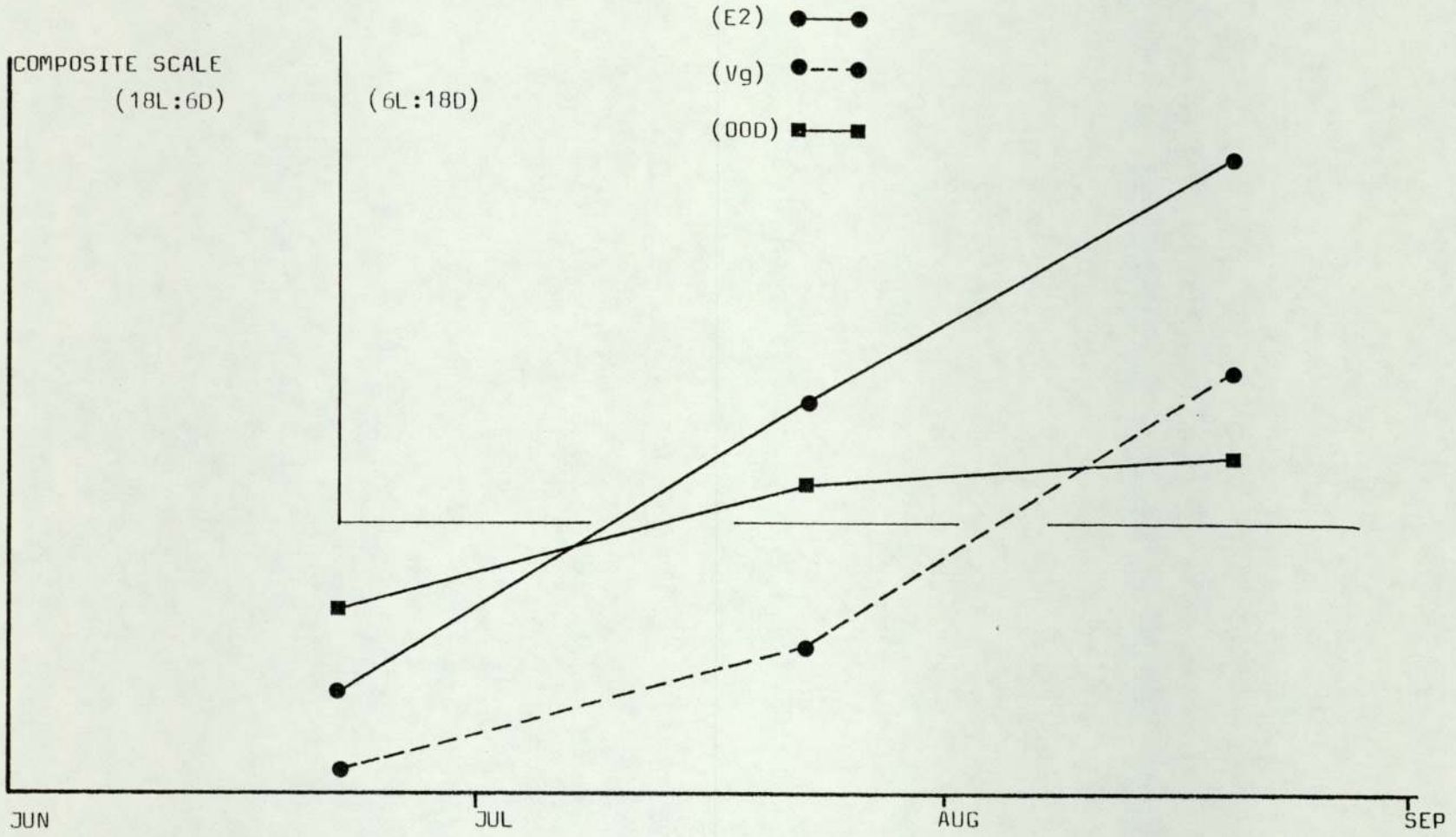


FIG. 7.12. CORRELATIONS OF MEAN SERUM VITELLOGENIN (Vg ng ml^{-1}) WITH MEAN OOCYTE DIAMETER (OOD mm, $P < 0.01$, $r = 0.97$) AND OESTRADIOL-17β (E2 ng ml^{-1}, $P < 0.01$, $r = 0.965$) IN CARIBOU STRAIN RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD REGIME IN SERIES I EXPERIMENT.

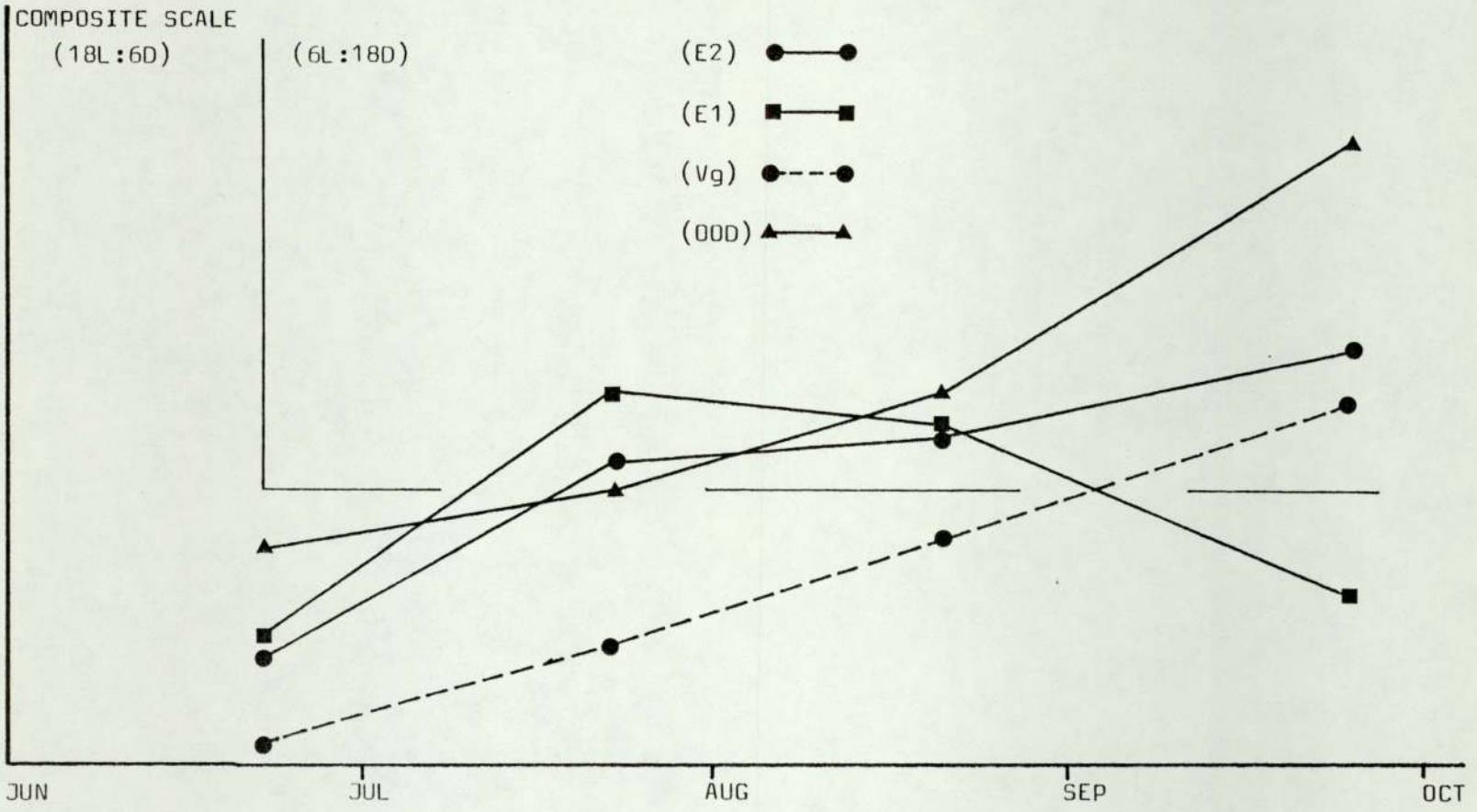


FIG. 7.13. CORRELATIONS OF MEAN SERUM VITELLOGENIN (Vg ng ml^{-1}) WITH MEAN OOCYTE DIAMETER (OOD mm, $P < 0.001$, $r = 0.978$) AND OESTRADIOL-17β (E2 ng ml^{-1}, $P < 0.01$, $r = 0.925$); AND OESTRADIOL-17β WITH OESTRONE (E1 ng ml^{-1}, $P < 0.01$, $r = 0.971$) IN GRAMPIAN STRAIN RAINBOW TROUT HELD UNDER ARTIFICIAL PHOTOPERIOD IN SERIES I EXPERIMENT.

Grampian strain : Over the course of the complete cycle, mean serum Vg levels were significantly correlated with TCa levels ($P < 0.001$, $r=0.98$) and serum E2 levels ($P < 0.05$, $r=0.758$) (see Fig. 7.10.).

There were significant correlations during the exogenous phase of development (June - September) of serum Vg levels with OOD ($P < 0.001$, $r=0.978$) and with E2 levels ($P < 0.01$, $r=0.925$). Furthermore, over the same period, serum levels of E2 and E1 were correlated ($P < 0.01$, $r=0.971$) (see Fig. 7.13.).

Whitebrook strain : Mean serum Vg levels were also significantly correlated with mean TCa levels ($P < 0.001$, $r=0.926$) throughout the period of the investigation, even though full maturity was not achieved. There were no other significant correlations (see Fig. 7.14.).

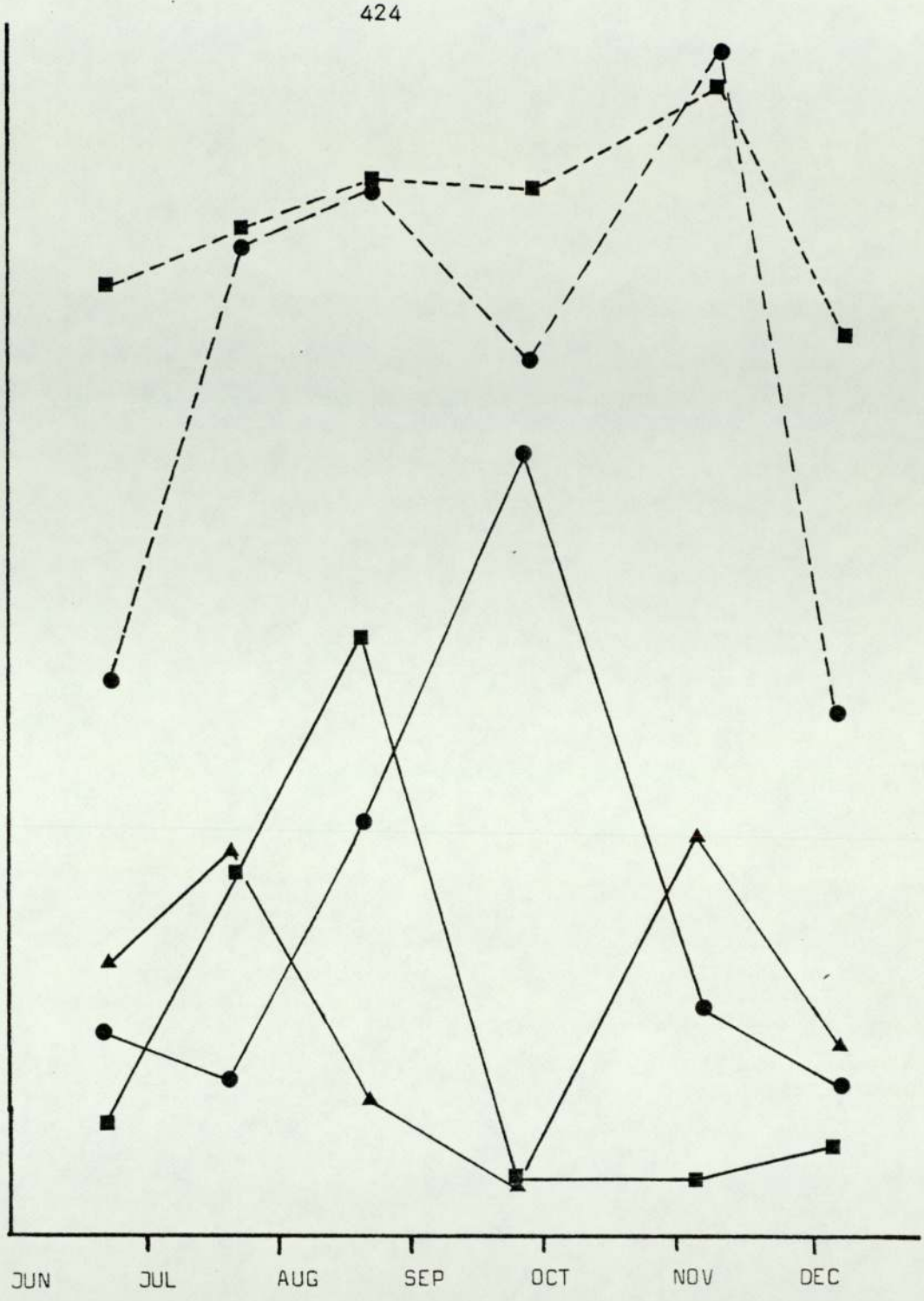


FIG. 7.14. SUMMARY OF CHANGES IN OESTRADIOL-17β (■——■), OESTRONE (▲——▲) AND TESTOSTERONE (●——●, BOTTOM); AND TOTAL CALCIUM (■– – –■) AND VITELLOGENIN (●– – –● TOP), IN IMMATURE WHITEBROOK FISH UNDER THE ARTIFICIAL PHOTOPERIOD IN SERIES I EXPERIMENT (VERTICAL AXIS COMPOSITE, 4 × PREVIOUS SCALES).

7.3. Series II Experiment.

The aims of this experiment were firstly to obtain mid/late-Summer eggs using three strains of trout which normally spawn in November, December and January, and secondly to further study the sequence of changes in serum levels of E1, E2, T, Vg and TCa in relation to ovarian growth and development.

7.3.1. Materials and Methods.

The same stock and facilities were used as in Series I, although further fish were tagged to replace those fish that had been sacrificed for histology, had lost tags or which were recognisable as males after their first maturation. Fish which were spawning under natural conditions at the commencement of this experiment were added to the tank to maintain viable production numbers of broodstock in the tank.

The photoperiod in the experimental tank was altered to a normal yearly seasonal cycle, compressed into 6 months, commencing on the 1st December (Fig. 7.15.). All tagged fish were bled monthly and weighed. The serum samples obtained were assayed for oestrone (E1), oestradiol-17β (E2), testosterone (T), vitellogenin (Vg) and total calcium (TCa). Fish from each of the 3 strains were selected at random, weighed and the GSI and HSI determined. Ovarian tissue was taken, fixed by methods described in 2.3. and the OOD determined. The % wet weight of ovarian tissue was also determined.

Statistical Analysis. Basal and maximum values of the serum parameters determined were compared using either the students t-test

or the F-test, if variances were dissimilar. The relationships between serum components and the morphological changes were investigated by calculating the correlation coefficient (r) by the method of the least squares fit. The results of these tests are outlined in the results below (Section 7.3.2.1.).

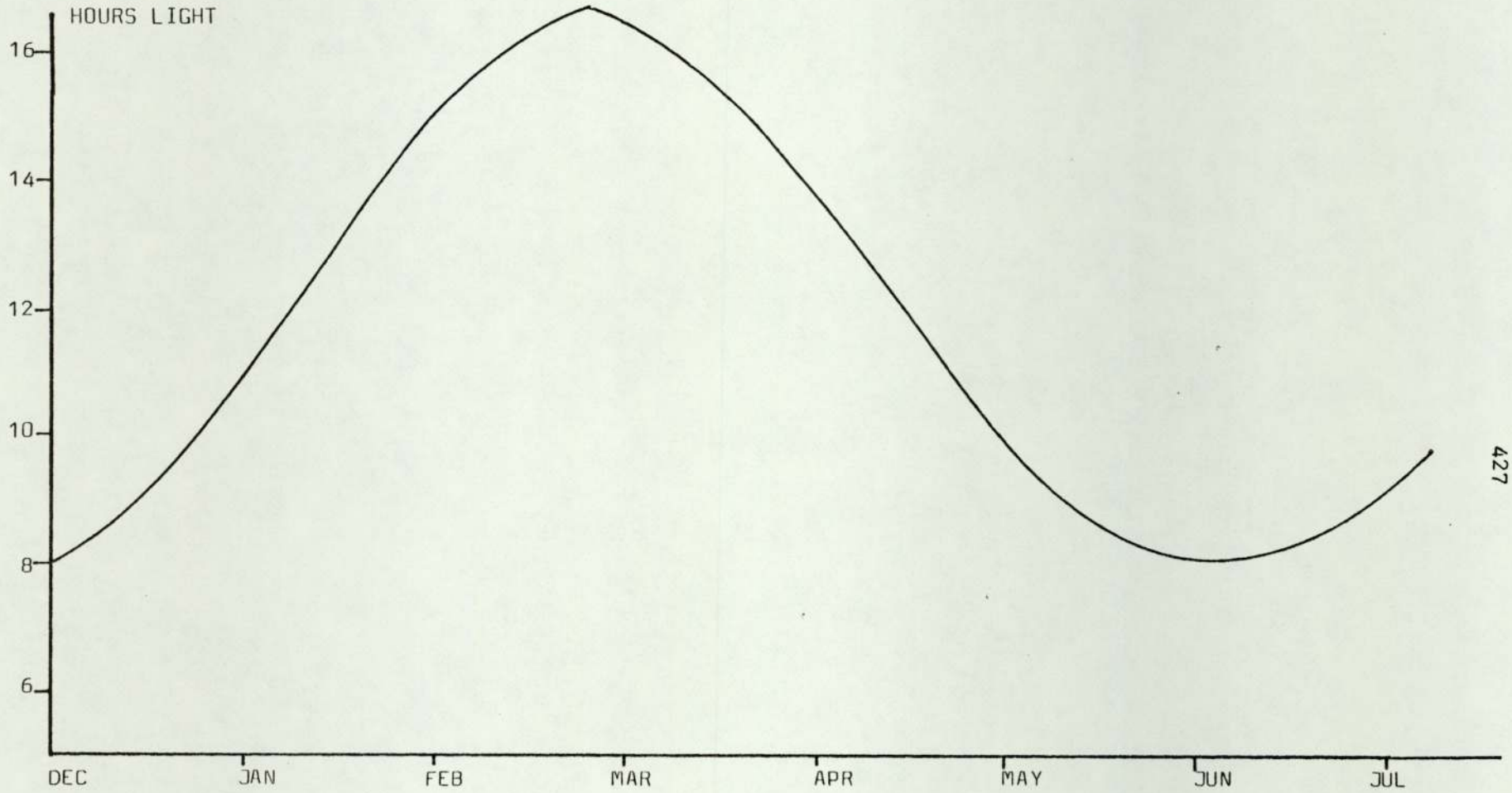


FIG. 7.15. PHOTOPERIOD IN SERIES II EXPERIMENT – NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS.

7.3.2. Results.

Under this 6 month compressed photoperiod, the Caribou stock, introduced the previous April, commenced their second spawning on July 2nd. This was followed in late August by the spawning of Grampian strain fish which had been initially stocked at the same time as the Caribou. The stripping of the original fish, from Series I, which were now spawning for the first time, commenced in September. The groups of Caribou, Grampian and Whitebrook added in December at the beginning of Series II commenced spawning in late August. Although experimental fish demonstrated no obvious physiological disorders, the eggs were again smaller than those of parallel Caribou, Grampian and Whitebrook stocks held under ambient conditions. Over the course of the experiment, gross changes were observed in all serum parameters studied. There were also marked increases in GSI, OOD, % wet weight of ovary and HSI in all 3 strains as the ovary developed. These are considered in detail below :-

Changes in serum Oestrone (E1) : In all 3 strains, there were 2 peaks in serum levels of E1, one early and one late in the reproductive cycles. The serum levels of E1 in both Caribou and Grampian, but not Whitebrook, fish fell to basal values at or before spawning.

Thus in Caribou fish, serum levels of E1, undetectable in December, January and February rose to a peak of 51.3 ± 10.5 ng ml^{-1} in March, achieved a second peak of 41.8 ± 5.9 ng ml^{-1} in early June before falling significantly ($P < 0.001$) to 1.7 ± 1.3 ng ml^{-1} at the time of spawning in early July.

In Grampian fish, serum E1 fell significantly ($P < 0.05$) from 5.85 $\pm$ 3.06 ng ml^{-1} at the beginning of the experiment in December, to 1.0 $\pm$ 0.2 ng ml^{-1} in February, before rising significantly ($P < 0.001$) to the first peak of 32.3 $\pm$ 4.9 ng ml^{-1} one month later in March. Mean serum E1 levels subsequently fell to 22.5 $\pm$ 3.5 ng ml^{-1} in May, reaching a second peak of 32.7 $\pm$ 6.1 ng ml^{-1} in late June approximately one month before spawning. Serum E1 levels had returned to basal ($P < 0.001$) by mid-July prior to spawning in this strain.

In Whitebrook fish, serum levels of E1 rose significantly ($P < 0.01$) from a mean of 2.04 $\pm$ 0.9 ng ml^{-1} in February to 25.8 $\pm$ 6.2 ng ml^{-1} in March. Serum levels of E1 rose significantly ($P < 0.01$) again from a mean of 17.9 $\pm$ 3.7 ng ml^{-1} in May to 31.1 $\pm$ 0.5 ng ml^{-1} at the end of June and 31.0 $\pm$ 0.6 ng ml^{-1} in mid-July. Both these latter peaks were significantly ($P < 0.001$) raised above the mean January level of 2.0 $\pm$ 1.2 ng ml^{-1}. In August, just prior to spawning, serum levels of E1 fell to 11.3 $\pm$ 2.6 ng ml^{-1}, and had returned to basal ($P < 0.001$) by October. (see Table 7.8. & Fig. 7.16.).

		DEC 4	JAN 23	FEB 17	MAR 25	MAY 12	JUN 3
CARIBOU	MEAN	0.0	0.0	0.0	51.3	40.0	41.8
	± S.E.				10.5	3.3	5.9
GRAMPIAN	MEAN	5.85	2.5	1.0	32.3	22.5	27.0
	± S.E.	3.05	0.5	0.2	4.9	3.5	2.5
WHITEBROOK	MEAN	2.8	2.0	2.04	25.8	17.9	22.3
	± S.E.	2.2	1.2	0.9	6.2	3.7	2.7

TABLE 7.8. CHANGES IN MEAN SERUM LEVELS OF OESTRONE (E1 ng ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

CONTD.....

		JUN 25	JUL 2	JUL 10	JUL 16	AUG 28	OCT 9
CARIBOU	MEAN	29.2	1.7	0.8	0.0		
	± S.E.	5.8	1.3	0.8			
GRAMPIAN	MEAN	32.7	23.4	-	2.0	2.5	
	± S.E.	6.1	4.7		1.0	1.0	
WHITEBROOK	MEAN	31.1	25.3	31.0	25.6	11.3	0.8
	± S.E.	0.5	0.9	0.6	0.4	2.6	0.8

TABLE 7.8. CHANGES IN MEAN SERUM LEVELS OF OESTRONE (E1 ng ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

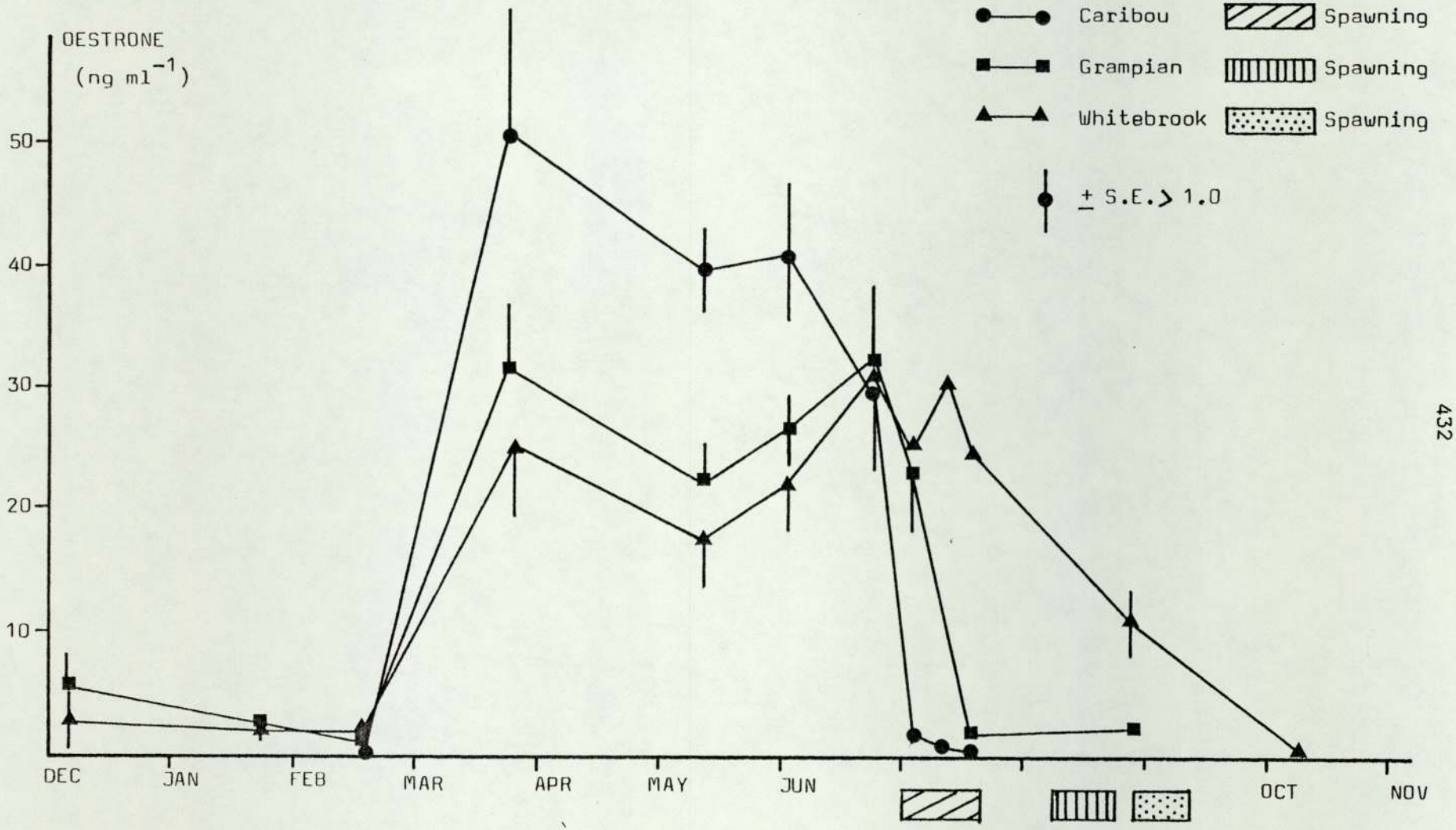


FIG. 7.16. CHANGES IN MEAN SERUM LEVELS OF OESTRONE (E1 ng ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

Changes in Oestradiol-17β (E2) : Serum levels of E2 in Caribou fish rose significantly ($P < 0.001$) from a mean of 1.3 ± 1.0 ng ml^{-1} in January, to a peak of 42.8 ± 4.4 ng ml^{-1} at the beginning of June, before falling significantly ($P < 0.001$) to 4.0 ± 1.2 ng ml^{-1} at the time of spawning in early July.

In Grampian fish, serum levels of E2 rose significantly ($P < 0.001$) from a mean of 1.7 ± 0.1 ng ml^{-1} in February, to a peak mean of 57.0 ± 1.0 ng ml^{-1} in mid-July, falling significantly ($P < 0.001$) to 4.2 ± 2.6 ng ml^{-1} in late August just after spawning. Serum levels of E2 returned to basal levels of 1.7 ± 0.1 ng ml^{-1} in October.

Serum levels of E2 in Whitebrook fish, rose significantly ($P < 0.001$) from a mean of 2.1 ± 0.8 ng ml^{-1} in January to a peak of 33.3 ± 4.4 ng ml^{-1} in mid-July, before falling to 26.5 ± 4.7 ng ml^{-1} in late August, just before spawning in this strain. Serum E2 levels returned to basal levels with a mean of 2.1 ± 0.4 ng ml^{-1} by early November. (see Table 7.9. & Fig. 7.17.).

		DEC 4	JAN 23	FEB 17	MAR 25	MAY 12	JUN 3	JUN 25
CARIBOU	MEAN	1.4	1.3	2.2	29.3	31.6	42.8	20.9
	± S.E.	1.1	1.0	1.0	1.6	1.7	4.4	2.7
GRAMPIAN	MEAN	7.1	1.6	1.7	10.5	22.0	34.3	42.5
	± S.E.	3.1	0.1	0.1	3.7	3.5	5.0	5.0
WHITEBROOK	MEAN	1.4	2.1	2.2	7.8	15.5	24.3	28.7
	± S.E.	0.7	0.8	0.7	1.3	1.4	3.2	3.2

TABLE 7.9. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

Contd....

		JUL 2	JUL 10	JUL 16	AUG 28	OCT 9	NOV 10
CARIBOU	MEAN	4.0	5.3	1.6			
	± S.E.	1.2	0.9	0.4			
GRAMPIAN	MEAN	43.8	57.0	34.4	4.2	1.7	
	± S.E.	12.1	1.0	5.6	2.6	0.1	
WHITEBROOK	MEAN	31.0	-	33.3	26.5	3.7	3.1
	± S.E.	5.8		4.4	4.7	1.4	0.4

TABLE 7.9. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

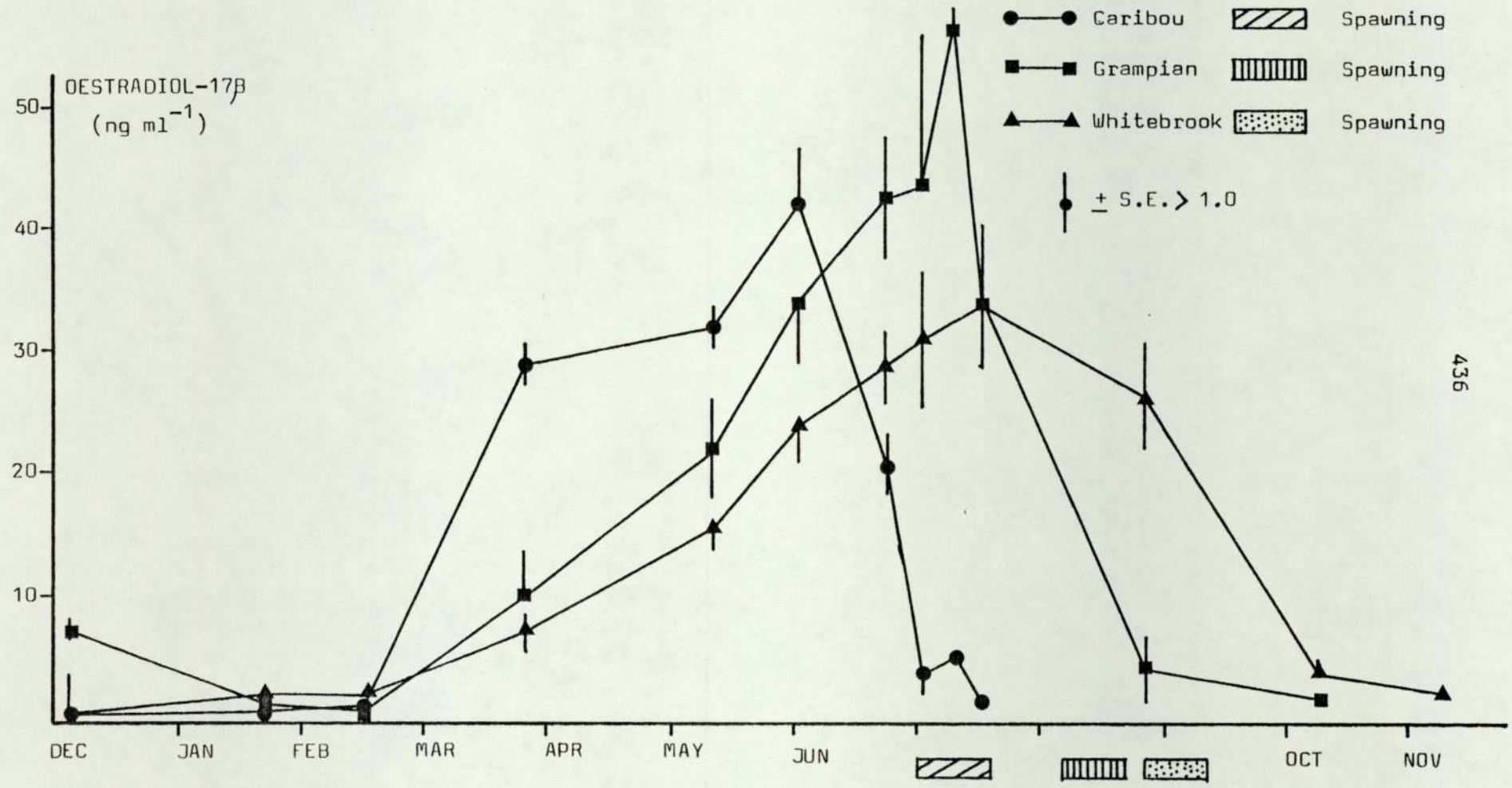


FIG. 7.17. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

Changes in Serum Testosterone (T) : Serum levels of T in all 3 strains increased steadily from March onwards, at first concommittant with E2, but subsequently reaching a rapid peak at or near the time of spawning.

Thus in Caribou fish, serum levels of T rose significantly ($P < 0.001$) from a mean of 4.7 ± 0.8 ng ml^{-1} in February, to a peak of 198.0 ± 9.2 ng ml^{-1} in early July at the time of spawning. Serum levels of T had returned ($P < 0.001$) to near basal levels of 6.9 ± 1.4 ng ml^{-1} by late August.

Similarly, in Grampian fish, serum T levels rose significantly ($P < 0.001$) from a mean of 2.4 ± 0.8 ng ml^{-1} in February, to a peak of 200.5 ± 0.5 ng ml^{-1} in mid-July, just before spawning, falling significantly ($P < 0.001$) to 1.6 ± 0.5 ng ml^{-1} in October.

Serum levels of T in Whitebrook fish increased significantly ($P < 0.001$) from 4.6 ± 1.4 ng ml^{-1} in January, to a peak of 230.0 ± 10.0 ng ml^{-1} in mid-July 6 weeks before spawning, but remained significantly ($P < 0.001$) raised at 189.3 ± 13.6 ng ml^{-1} until late August just before spawning; Subsequently they fell to 4.1 ± 1.2 ng ml^{-1} in November. (see Table 7.10. & Fig. 7.18.).

		DEC 4	JAN 23	FEB 17	MAR 25	MAY 12	JUN 3	JUN 25
CARIBOU	MEAN	4.0	4.8	4.7	9.3	81.3	157.6	192.0
	± S.E.	1.0	0.5	0.8	0.5	19.5	11.9	6.2
GRAMPIAN	MEAN	8.3	4.5	2.4	7.4	46.8	81.3	131.0
	± S.E.	6.5	1.1	0.8	1.2	3.0	5.9	23.7
WHITEBROOK	MEAN	2.2	4.6	5.2	7.9	29.3	44.3	78.7
	± S.E.	0.5	1.4	1.1	1.6	5.1	8.7	26.9

TABLE 7.10. CHANGES IN MEAN SERUM LEVELS OF TESTOSTERONE (T ng ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

CONTD.....

		JUL 2	JUL 10	JUL 16	AUG 28	OCT 9	NOV 10
CARIBOU	MEAN	198.0	89.3	24.0	6.9	5.5	
	± S.E.	2.0	25.9	10.0	1.4	0.8	
GRAMPIAN	MEAN	153.3	199.7	200.5	88.2	1.6	
	± S.E.	29.1	1.2	0.5	8.2	0.5	
WHITEBROOK	MEAN	139.0	-	230.0	189.3	50.0	4.1
	± S.E.	3.8		10.0	13.6	13.5	1.2

TABLE 7.10. CHANGES IN MEAN SERUM LEVELS OF TESTOSTERONE (T ng ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

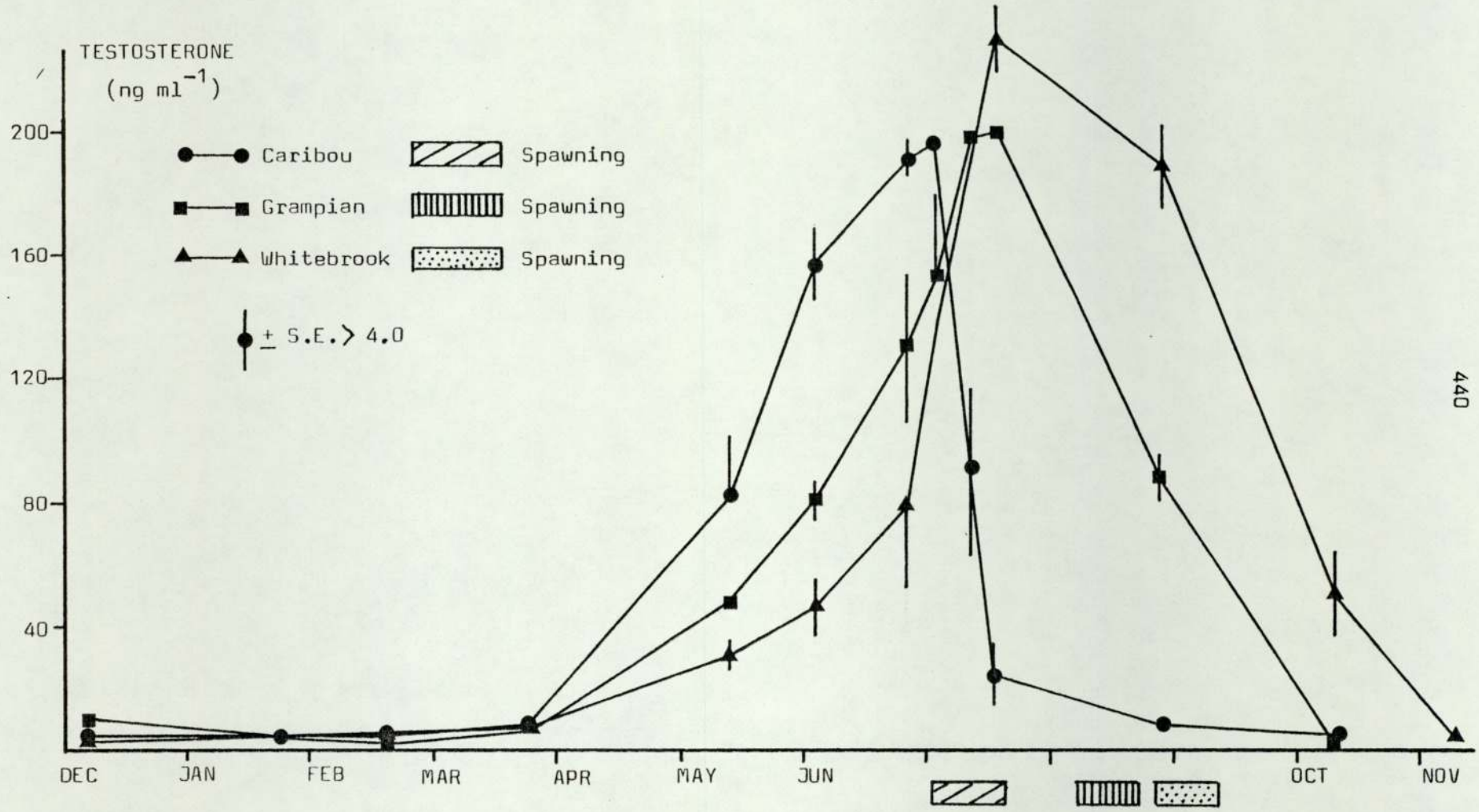


FIG. 7.18. CHANGES IN MEAN SERUM LEVELS OF TESTOSTERONE (T ng ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

Changes in Serum Vitellogenin (Vg) : As observed in Series I, changes in Vg were concomitant with those in TCa in all 3 strains. Thus, in Caribou fish, serum levels of Vg increased significantly ($P < 0.001$) from 1.3 ± 0.3 in December to a peak mean value of 77.1 ± 9.3 mg ml^{-1} in early June, falling to 63.1 ± 5.7 mg ml^{-1} at the time of spawning in early July.

In Grampian fish, serum levels of Vg rose significantly ($P < 0.001$) from a mean of 3.5 ± 0.9 mg ml^{-1} in February, to a peak of 44.0 ± 6.3 mg ml^{-1} in early July, before falling to 33.3 ± 3.5 mg ml^{-1} at the end of July just prior to spawning in this strain.

Serum levels of Vg in Whitebrook fish rose significantly ($P < 0.001$) from a mean of 0.3 ± 0.1 mg ml^{-1} in December, to a peak of 63.2 ± 8.3 mg ml^{-1} in early July, before falling to a mean of 61.8 ± 7.6 mg ml^{-1} at the end of July, one month before spawning. Unfortunately, it was only possible to assay samples taken up to July 16th, sampling for serum Vg by RIA. (see Table 7.11. & Fig 7.19.).

		DEC 4	JAN 23	FEB 17	MAR 25	MAY 12
CARIBOU	MEAN	1.3	1.5	1.5	7.5	39.5
	± S.E.	0.3	0.3	0.4	0.9	3.5
GRAMPIAN	MEAN	7.3	4.1	3.5	10.9	28.7
	± S.E.	3.1	1.9	0.9	2.8	3.4
WHITEBROOK	MEAN	0.3	1.56	2.4	4.7	16.1
	± S.E.	0.1	0.37	0.9	0.9	2.7

TABLE 7.11. CHANGES IN MEAN SERUM LEVELS OF VITELLOGENIN (Vg mg ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

Contd.....

		JUN 3	JUN 25	JUL 2	JUL 10	JUL 16
CARIBOU	MEAN	77.1	66.4	63.1	54.0	46.5
	± S.E.	9.9	8.2	5.7	5.6	5.0
GRAMPIAN	MEAN	38.0	42.8	44.0	36.0	33.3
	± S.E.	7.9	8.4	6.3	3.8	3.5
WHITEBROOK	MEAN	28.0	34.5	63.2	-	61.8
	± S.E.	5.1	3.1	8.3		7.6

TABLE 7.11. CHANGES IN MEAN SERUM LEVELS OF VITELLOGENIN (Vg mg ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

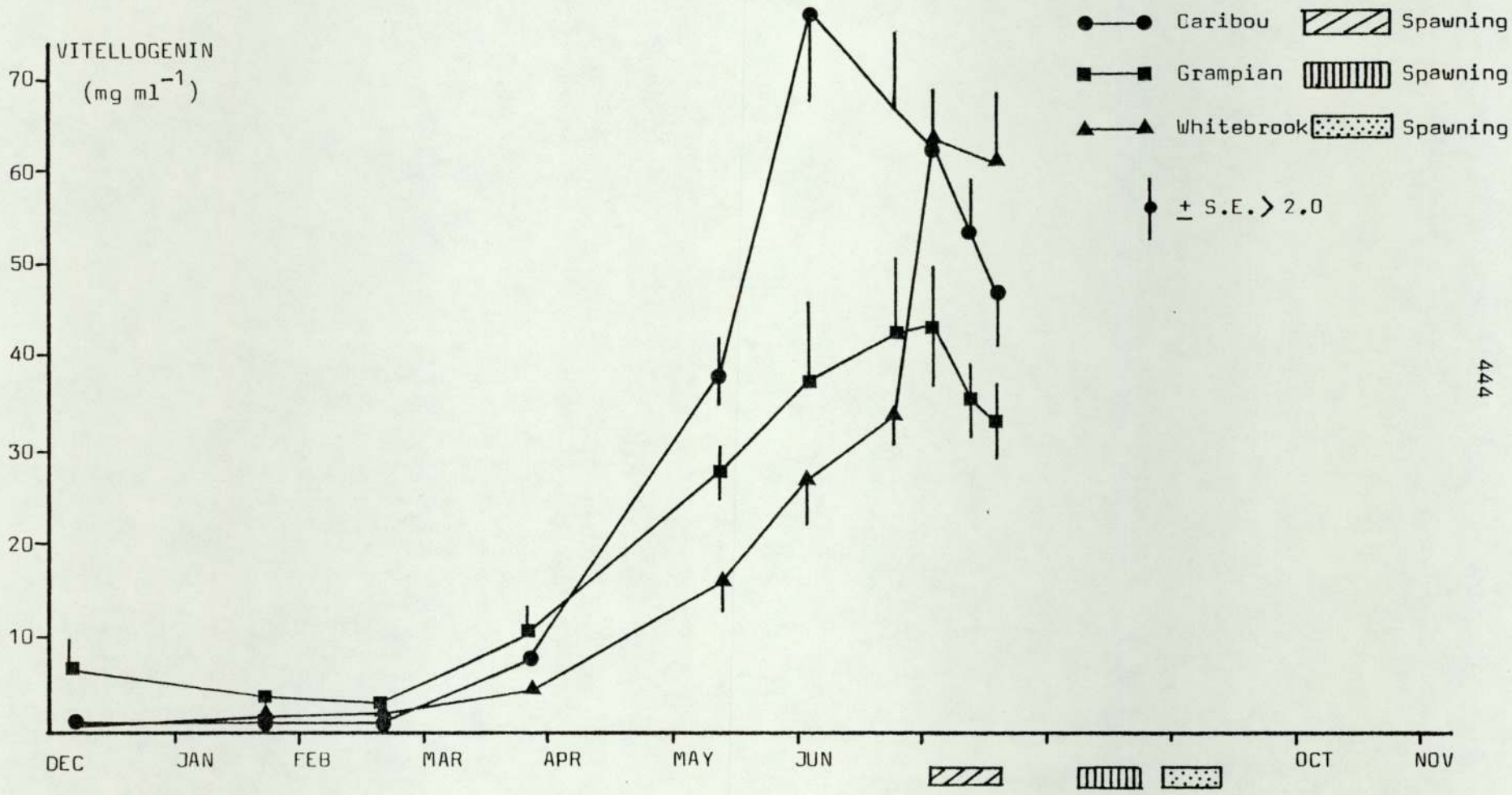


FIG. 7.19. CHANGES IN MEAN SERUM LEVELS OF VITELLOGENIN (Vg mg ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

Changes in Total Serum Calcium (TCa) : Peak mean serum levels of TCa in this series were reduced in Caribou and Grampian strains when compared with the levels observed in the Series I experiment. Thus, serum TCa levels in Caribou fish increased significantly ($P < 0.001$) from a mean value of 12.1 $\pm$ 0.4 mg $100ml^{-1}$ in February, to a peak mean value of 54.7 $\pm$ 9.0 mg $100ml^{-1}$ in early July, one month before spawning. Serum TCa levels in Caribou fish had returned ($P < 0.01$) to a near basal value of 12.2 $\pm$ 0.7 mg $100ml^{-1}$ by late August.

In Grampian fish TCa levels rose significantly ($P < 0.01$) from a basal value of 11.1 $\pm$ 0.4 mg $100ml^{-1}$ in February, to a peak mean level of 28.3 $\pm$ 6.6 mg $100ml^{-1}$ in early July, again approximately one month prior to spawning. Serum TCa returned to basal levels of 11.2 $\pm$ 0.7 mg $100ml^{-1}$ 2 months later in October.

In Whitebrook fish, TCa levels rose significantly ($P < 0.001$) from a mean of 13.6 $\pm$ 0.3 mg $100ml^{-1}$ in March, to a peak of 52.5 $\pm$ 5.2 mg $100ml^{-1}$ at the end of August just before spawning, before falling to near basal levels ($P < 0.001$) of 15.0 $\pm$ 1.7 mg $100ml^{-1}$ in November. (see Table 7.12. & Fig. 7.20.).

		DEC 4	JAN 23	FEB 17	MAR 25	MAY 12	JUN 3	JUN 25
CARIBOU	MEAN	12.1	12.5	12.1	14.5	32.6	54.7	50.3
	± S.E.	0.5	0.4	0.4	0.7	2.1	9.0	7.9
GRAMPIAN	MEAN	14.9	13.7	11.1	13.2	22.5	26.7	25.2
	± S.E.	0.3	0.5	0.4	0.6	2.4	6.0	4.9
WHITEBROOK	MEAN	11.4	13.7	12.7	13.6	15.4	25.8	31.0
	± S.E.	0.7	0.6	0.4	0.3	1.5	1.0	4.0

TABLE 7.12. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

Contd.....

		JUL 2	JUL 10	JUL 16	AUG 28	OCT 9	NOV 10
CARIBOU	MEAN	46.6	42.5	39.5	12.2	12.9	
	± S.E.	7.5	2.1	1.5	0.7	0.3	
GRAMPIAN	MEAN	28.3	26.6	24.5	15.0	11.2	
	± S.E.	6.6	6.1	1.5	0.6	0.7	
WHITEBROOK	MEAN	44.6	-	41.5	52.5	26.6	15.0
	± S.E.	7.5		9.3	5.2	5.0	1.7

TABLE 7.12. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg $100ml^{-1}$) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

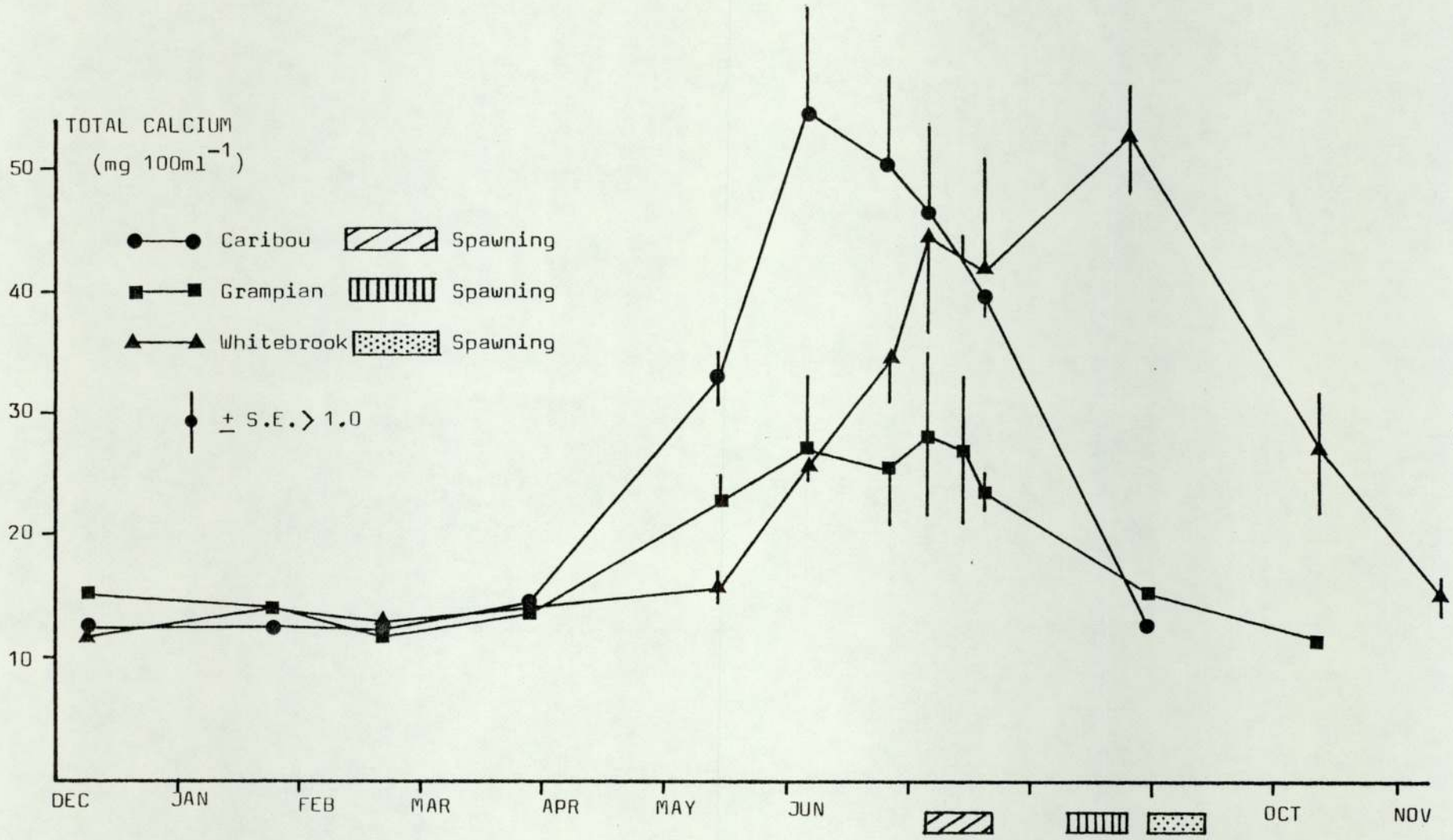


FIG. 7.20. CHANGES IN MEAN SERUM LEVELS OF TOTAL CALCIUM (TCa mg 100ml^{-1}) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

Changes in Gonadosomatic Index (GSI) : In Caribou fish the GSI increased from 0.4% in February to a peak mean value of 18.1 ± 3.0% in early July when the fish were stripped (Table 7.13.). In Grampian fish the GSI increased from 0.3% in February to a peak of 12.68 ± 2.1%, approximately one month before spawning (Table 7.14.). The GSI in Whitebrook fish increased from 0.4% in March to 9.4 ± 1.7% when the last fish were sampled in mid-July (Table 7.15. & See Fig. 7.21.).

Changes in Mean Oocyte Diameter (OOD) : The OOD in Caribou fish increased from 0.42 ± 0.02 mm in January, to a peak mean value of 4.0 ± 0.05 mm just before spawning, and was at 3.9 ± 0.1 mm when the fish were stripped (Table 7.13.). Over a similar period, OOD in Grampian fish increased from 0.44 ± 0.05 mm in February to a peak mean diameter of 4.2 ± 0.17 mm in mid-July one month before spawning (Table 7.14.). Mean OOD increased from a January value of 0.6 ± 0.03 mm in Whitebrook fish to 2.333 ± 0.3 mm in mid-July some 2 months before spawning (Table 7.15. & See Fig. 7.22.).

Changes in % Wet Weight of Ovary (%WW) : The %WW increased steadily with ovarian recrudescence rising from a February value of 65.0 ± 2.6% in Caribou fish, to a peak mean value of 80.2 ± 0.8% in late June, just prior to spawning (Table 7.13.). In Grampian fish the %WW increased from 60.8 ± 0.6% in February, to a peak of 81.2 ± 11.6% in mid-July (Table 7.14.). The %WW in Whitebrook fish rose from 69.5 ± 1.1% in February to a mean of 80.75 ± 4.5% in mid-July at the last sampling (Table 7.15. & See Fig. 7.23.).

Changes in Developmental stage of Oocyte : Histological examination of the ovary of all 3 strains demonstrated a progression through

successive developmental stages as outlined in more detail in the previous chapter (See Tables 7.13., 7.14. & 7.15.). The relationship of specific stages of development with the changing hormonal environment is considered further in the discussion.

Changes in Hepatosomatic Index (HSI) : In all 3 strains the HSI was lowest in February/March and increased as maturity progressed. The highest HSI was noted in Whitebrook fish in January. In Caribou fish HSI increased from 1.3% in February to a peak mean value of 2.65 ± 0.09% in late June, just prior to spawning (Table 7.13.). Similarly, in Grampian fish, the HSI increased from 1.1% in March to a peak of 2.55 ± 0.19% in mid-July one month before spawning in early August (Table 7.14.). In Whitebrook fish HSI fell from 3.65% in January to 1.079% in February before rising to 2.2 ± 0.16% in mid-July (Table 7.15. & See Fig. 7.24.).

Changes in Body Weight (BW) : Mean body weight increased steadily throughout the experiment in all 3 strains. At the beginning of the experiment the mean weights of Grampian, Caribou and Whitebrook fish were 2.13 ± 0.13, 1.929 ± 0.1 and 1.732 ± 0.11 Kg respectively, rising to 3.10 ± 0.17, 2.597 ± 0.37 and 2.471 ± 0.19 Kg in late June (Tables 7.13., 7.14. & 7.15., See Fig. 7.15.). Growth rates were highest at mid-cycle and fell as full maturity, ie spawning, approached.

	JAN 23	FEB 17	MAR 25	MAY 12	JUN 3	JUN 25	JUL 2
Developmental stage of oocyte	4	4/5	5	6	7	7	8
GSI (%)	0.49	0.4	0.6	3.28	8.6	17.2	18.1
± S.E.				0.6	0.9	2.1	3.0
OOD (mm)	0.42	0.796	1.174	2.252	3.928	4.0	3.9
± S.E.	0.02	0.04	0.05	0.18	0.09	0.05	0.1

TABLE 7.13. CHANGES IN MEAN OOCYTE DIAMETER (OOD mm), MEAN GONADOSOMATIC INDEX (GSI %) AND DEVELOPMENTAL STAGE OF OOCYTE IN CARIBOU STRAIN HELD UNDER NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

CONTD.....

	JAN 23	FEB 17	MAR 25	MAY 12	JUN 3	JUN 25	JUL 2
% Wet Weight of Ovary	-	65.0	66.03	67.9	69.7	80.2	-
± S.E.		2.6	1.9	3.1	1.3	0.8	
HSI (%)	2.2	1.3	2.1	2.5	2.6	2.65	2.5
± S.E.				0.15	0.03	0.09	0.17
Mean Body Wt (Kg)	1.928	2.147	2.197	2.483	2.550	2.597	-
± S.E.	0.1	0.15	0.17	0.15	0.152	0.37	

TABLE 7.13. CHANGES IN MEAN WET WEIGHT OF OVARY (WW %), MEAN HEPATOSOMATIC INDEX (HSI %) AND MEAN BODY WEIGHT (BW Kg) IN CARIBOU STRAIN HELD UNDER NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

	JAN 23	FEB 17	MAR 25	MAY 12	JUN 3	JUN 25	JUL 2	JUL 10
Developmental stage of oocyte		3/4	4/5	6				
GSI (%)	-	0.3	0.58	2.54	4.85	8.0	9.45	12.68
± S.E.				0.4	1.2	0.9	1.5	2.1
OOD (mm)		0.44	0.8597	1.8	2.35	3.7	4.16	4.2
± S.E.		0.05	0.3	0.096	0.12	0.25	0.3	0.17

TABLE 7.14. CHANGES IN MEAN OOCYTE DIAMETER (OOD mm), MEAN GONADOSOMATIC INDEX (GSI %) AND DEVELOPMENTAL STAGE OF OOCYTE IN GRAMPIAN STRAIN HELD UNDER NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

CONTD.....

	JAN 23	FEB 17	MAR 25	MAY 12	JUN 3	JUN 25	JUL 2	JUL 10
% Wet Weight of Ovary		60.8	60.5	73.6	75.3	76.8	-	81.2
± S.E.		0.6	0.5	2.4	7.7	4.2		11.6
HSI (%)	2.0	1.9	1.1	2.3	2.47	2.5	2.45	2.55
± S.E.				0.1	0.1	0.15	0.2	0.19
Mean Body Wt (Kg)	2.13	2.34	2.4	2.98	3.05	3.10		
± S.E.	0.13	0.18	0.25	0.2	0.2	0.17		

TABLE 7.14. CHANGES IN MEAN WET WEIGHT OF OVARY (WW %), MEAN HEPATOSOMATIC INDEX (HSI %) AND MEAN BODY WEIGHT (BW Kg) IN GRAMPIAN STRAIN HELD UNDER NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

	JAN 23	FEB 17	MAR 25	MAY 12	JUN 3	JUN 25	JUL 2	JUL 10
Developmental Stage of Oocyte			4/5	5	5/6	6	6	6
GSI (%)	0.49	0.46	0.4	1.05	2.1	3.5	5.2	9.4
± S.E.				0.2	0.2	0.5	1.1	1.7
OOD (mm)	0.6	0.690	1.1	1.545	1.729	1.966	2.08	2.333
± S.E.	0.03	0.03	0.04	0.069	0.19	0.19	0.28	0.3

TABLE 7.15. CHANGES IN MEAN OOCYTE DIAMETER (OOD mm), MEAN GONADOSOMATIC INDEX (GSI %) AND DEVELOPMENTAL STAGE OF OOCYTE IN WHITEBROOK STRAIN HELD UNDER NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

CONTD.....

	JAN 23	FEB 17	MAR 25	MAY 12	JUN 3	JUN 25	JUL 2	JUL 10
% Wet Weight of Ovary		69.5	72.3	77.5	78.7	79.5	-	80.75
± S.E.		1.1	0.7	4.5	12.0	7.3		4.5
HSI (%)	3.65	1.079	1.3	1.5	2.0	2.09	2.15	2.2
± S.E.				0.05	0.15	0.09	0.17	0.16
Mean Body Wt (Kg)	1.732	1.856	2.013	2.291	2.383	2.471	-	-
± S.E.	0.11	0.09	0.25	0.2	0.168	0.19		

TABLE 7.15. CHANGES IN MEAN WET WEIGHT OF OVARY (WW %), MEAN HEPATOSOMATIC INDEX (HSI %) AND MEAN BODY WEIGHT (BW Kg) IN WHITEBROOK STRAIN HELD UNDER NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

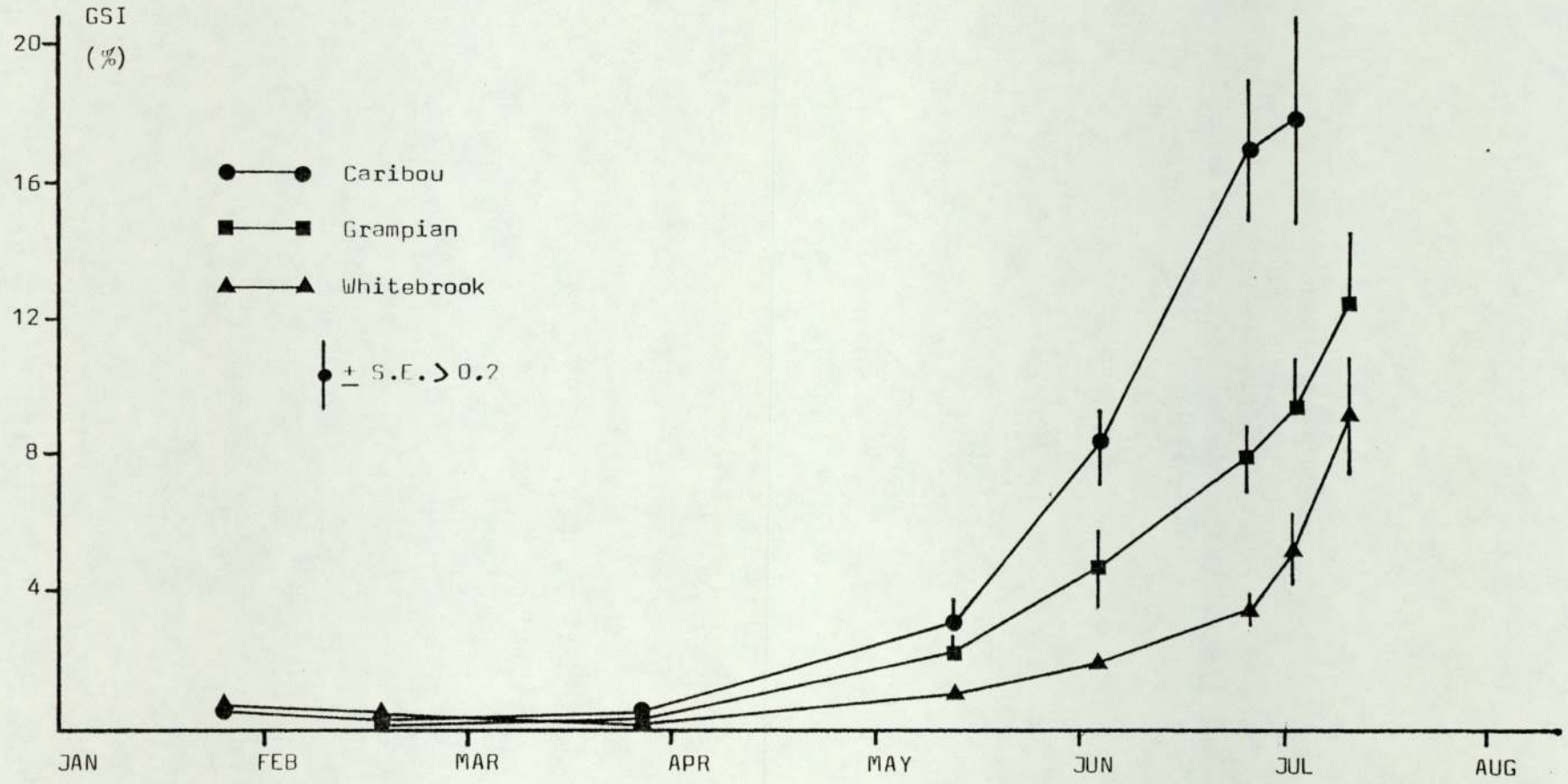


FIG. 7.21. CHANGES IN GONADOSOMATIC INDEX (GSI %) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

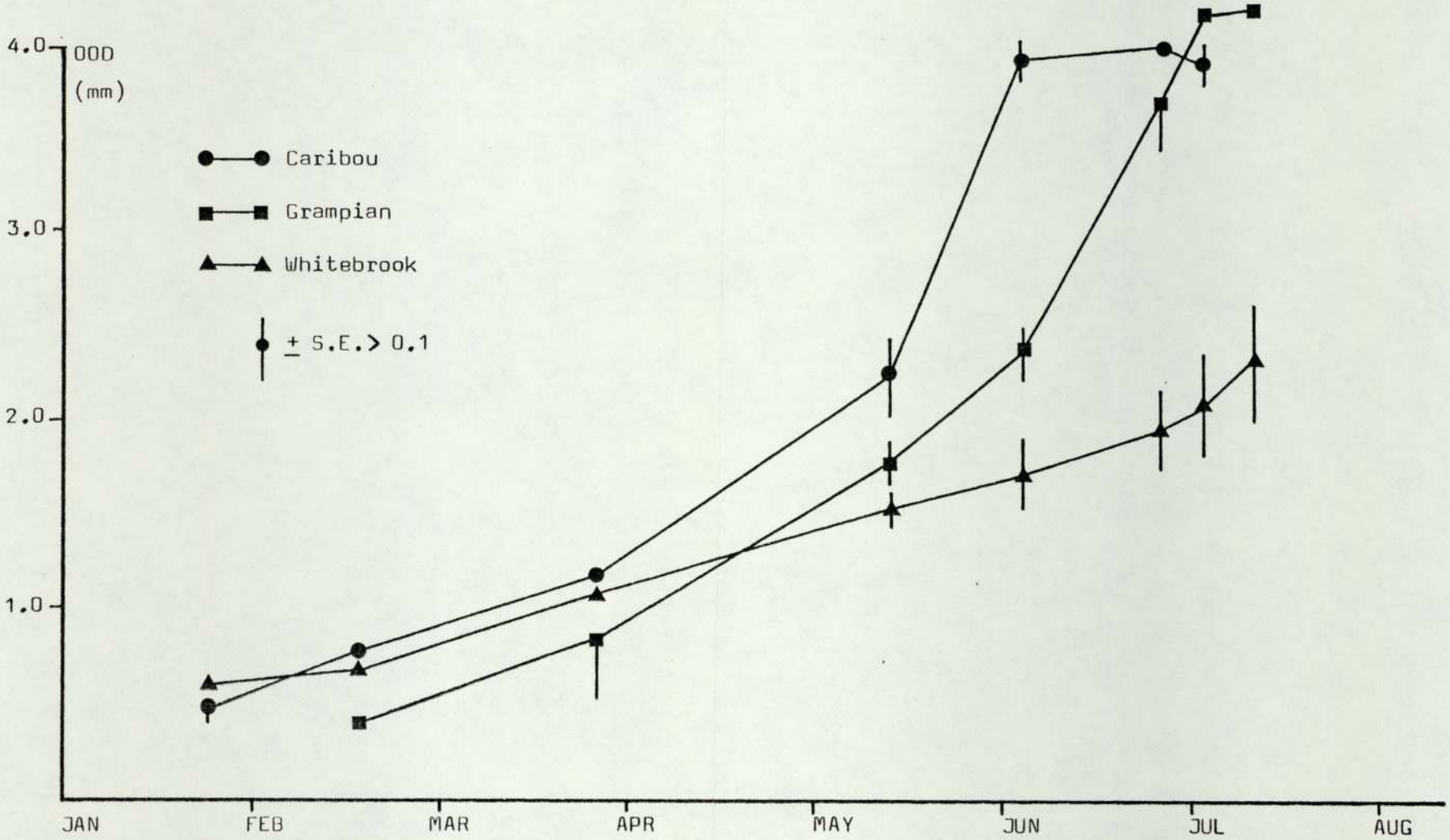


FIG. 7.22. CHANGES IN MEAN OOCYTE DIAMETER (OOD mm) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

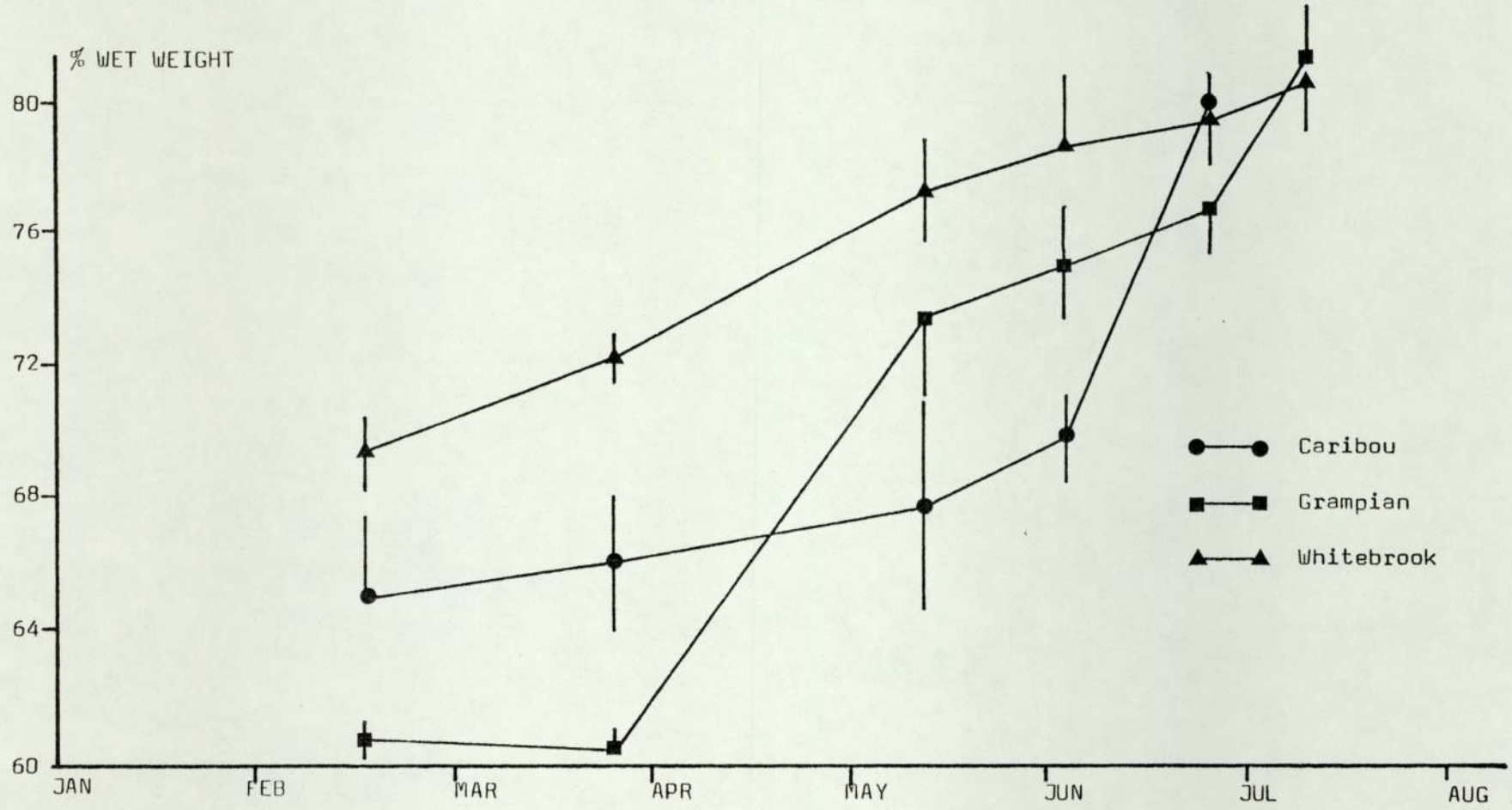


FIG. 7.23 CHANGES IN MEAN % WET WEIGHT OF OVARY IN THREE STRAINS OF RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

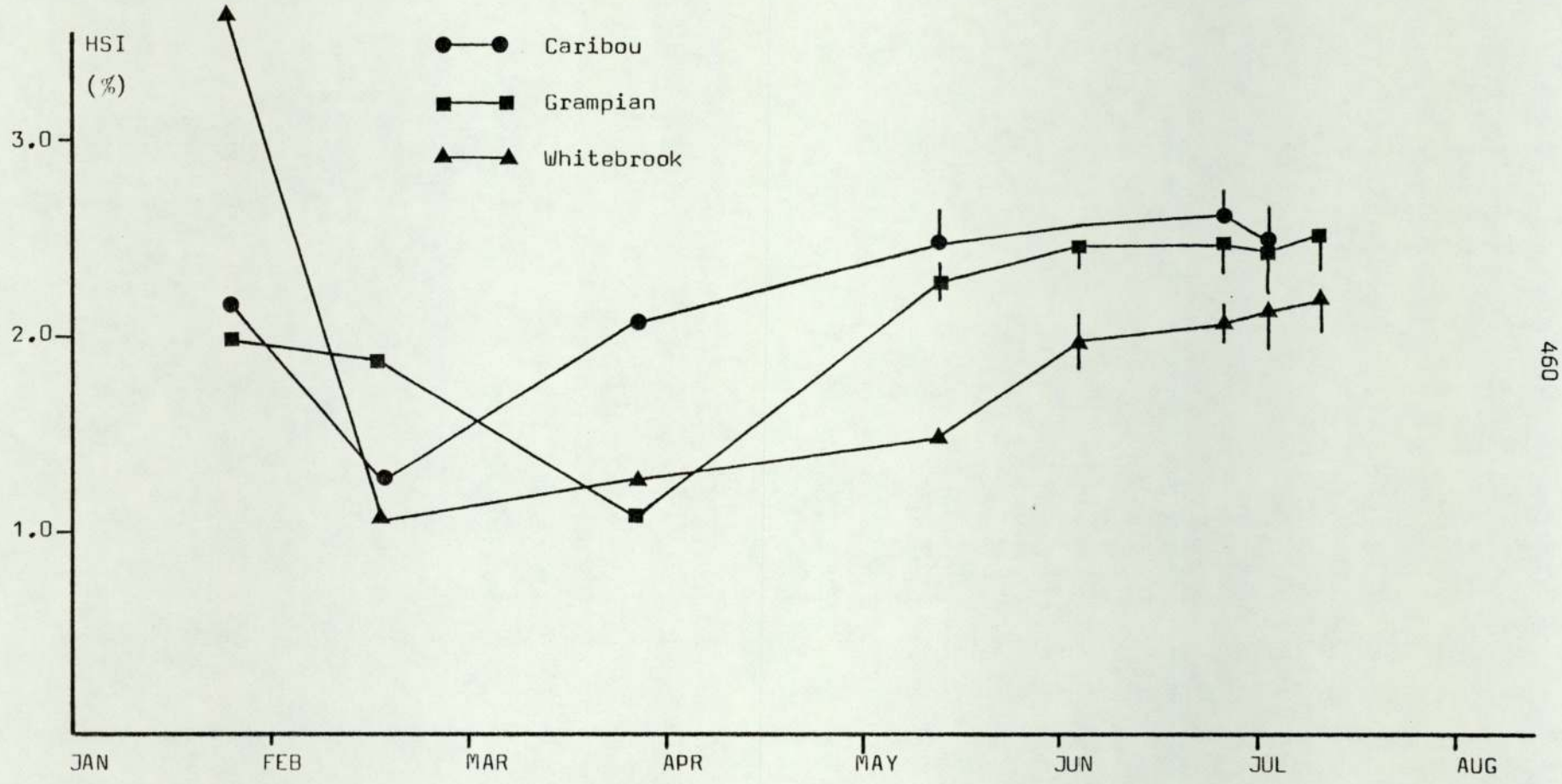


FIG. 7.24. CHANGES IN HEPATOSOMATIC INDEX (HSI %) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

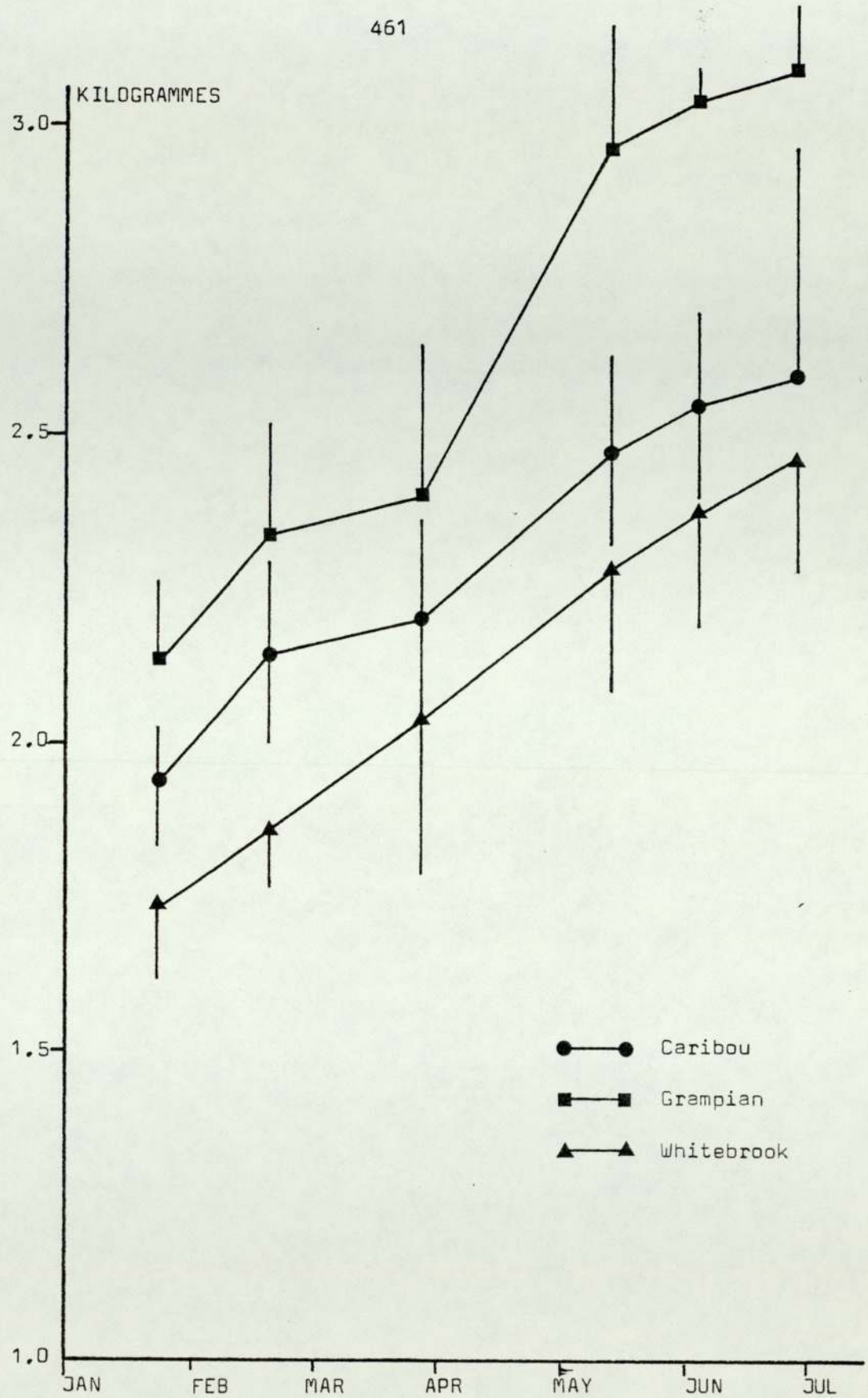


FIG. 7.25. CHANGES IN MEAN BODY WEIGHT (Kg) IN THREE STRAINS OF RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

7.3.2.1. Interrelationships between Oestrone, Oestradiol-17β, Testosterone, Vitellogenin, Total Calcium and the Morphological parameters.

The timing of the different serum changes in the Caribou, Grampian and Whitebrook strains are shown in Figs. 7.26., 7.28. & 7.31. respectively. The relationships between the different parameters were investigated throughout the cycle by calculating the Correlation coefficient (r) by the method of least-squares fit. Thus correlations were determined over the complete cycle, the 'endogenous' phase of development before incorporation of exogenous yolk (See previous chapter) and during hepatic vitellogenesis. These interrelationships are considered for each stage and strain in more detail below :-

Caribou strain : Over the course of the complete cycle mean serum levels of Vg were significantly correlated with TCa ($P < 0.001$, $r=0.999$), OOD ($P < 0.001$, $r=0.984$), GSI ($P < 0.01$, $r=0.834$) and HSI ($P < 0.05$, $r=0.78$) (See Fig. 7.26. & 7.27.).

Over the exogenous phase of development (March-July), serum E2 levels were significantly correlated with serum Vg ($P < 0.01$, $r=0.85$) and HSI ($P < 0.01$, $r=0.98$).

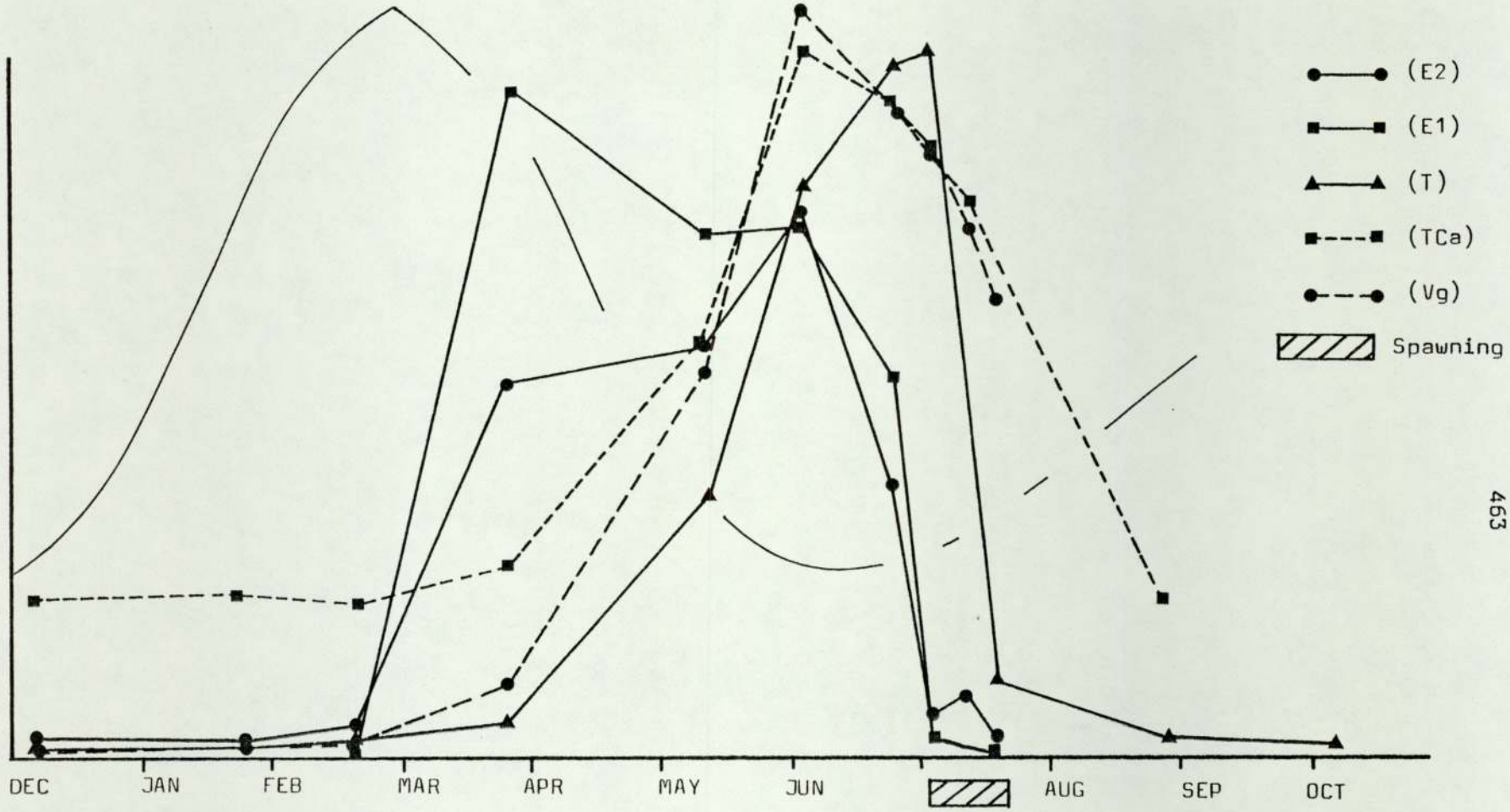


FIG. 7.26. SUMMARY OF SEQUENTIAL CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}), OESTRONE (E1, ng ml^{-1}), TESTOSTERONE (T ng ml^{-1}), TOTAL CALCIUM (TCa mg $100ml^{-1}$) AND VITELLOGENIN (Vg mg ml^{-1}) IN CARIBOU STRAIN RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT. (VERTICAL AXIS COMPOSITE, OF SAME SCALE TO PREVIOUS FIGURES).

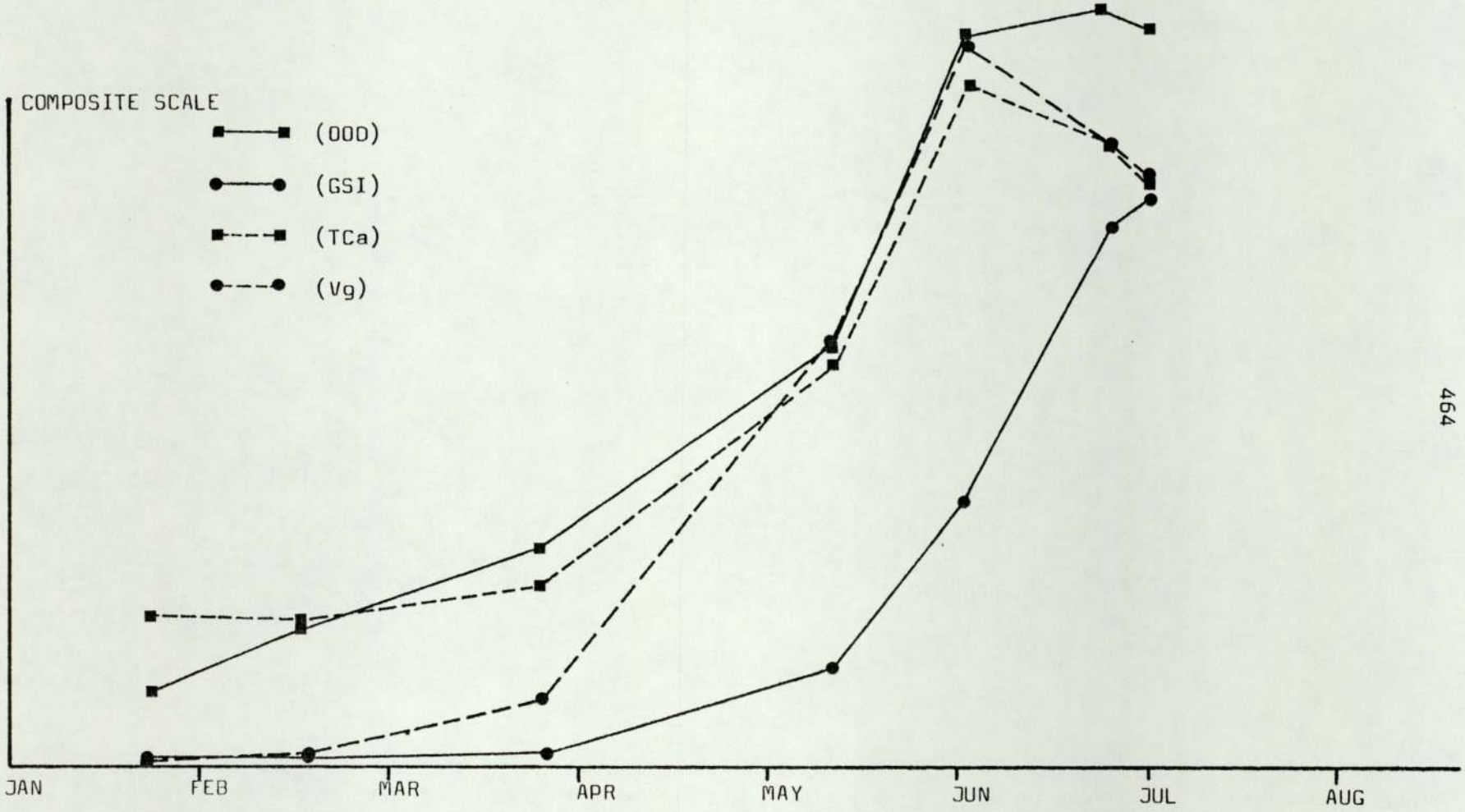
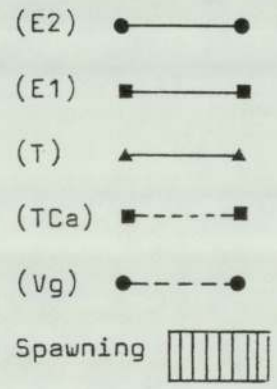


FIG. 7.27. CORRELATIONS OF MEAN SERUM VITELLOGENIN (Vg mg ml^{-1}) WITH TOTAL CALCIUM (TCa mg $100ml^{-1}$, $P < 0.001$, $r=0.999$), MEAN OOCYTE DIAMETER (OOD mm, $P < 0.001$, $r=0.984$) AND GONADOSOMATIC INDEX (GSI %, $P < 0.01$, $r=0.834$) IN CARIBOU STRAIN RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II.

Grampian strain : Over the course of the complete cycle, mean serum Vg levels were significantly correlated with TCa ($P < 0.001$, $r=0.98$), OOD ($P < 0.001$, $r=0.9$), GSI ($P < 0.05$, $r=0.98$) and E2 ($P < 0.001$, $r=0.86$) (See Figs. 7.28.& 7.29.).

Over the exogenous phase of the experiment (March-mid-July), serum E2 levels were significantly correlated with Vg ($P < 0.01$, $r=0.93$), OOD ($P < 0.001$, $r=0.99$) and HSI ($P < 0.05$, $r=0.7$) (See Fig. 7.30.).

FIG. 7.28. SUMMARY OF SEQUENTIAL CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}), OESTRONE (E1 ng ml^{-1}), TESTOSTERONE (T ng ml^{-1}), TOTAL CALCIUM (TCa mg $100ml^{-1}$) AND VITELLOGENIN (Vg mg ml^{-1}) IN GRAMPIAN STRAIN RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT. (VERTICAL AXIS COMPOSITE, OF SAME SCALE TO PREVIOUS FIGURES).



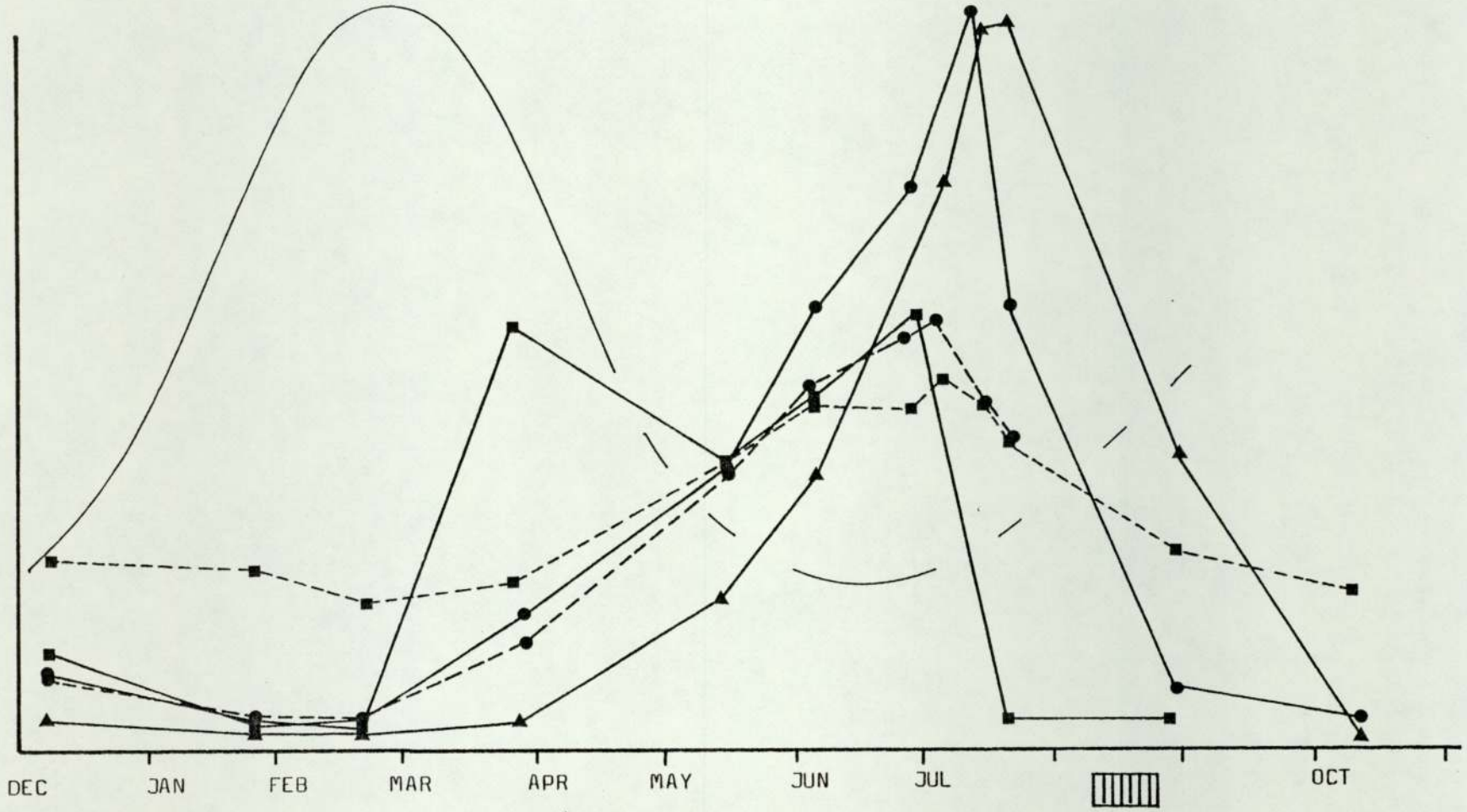


FIG. 7.28. LEGEND OPPOSITE.

FIG. 7.29. CORRELATIONS OF MEAN SERUM VITELLOGENIN LEVELS (Vg mg ml^{-1}) WITH TOTAL CALCIUM (TCa mg $100ml^{-1}$, $P < 0.001$, $r=0.98$), MEAN OOCYTE DIAMETER (OOD mm, $P < 0.001$, $r=0.9$) AND GONADOSOMATIC INDEX (GSI %, $P < 0.05$, $r=0.8$) IN GRAMPIAN STRAIN RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.

(VERTICAL AXIS COMPOSITE, OF SAME SCALE TO PREVIOUS FIGURES).

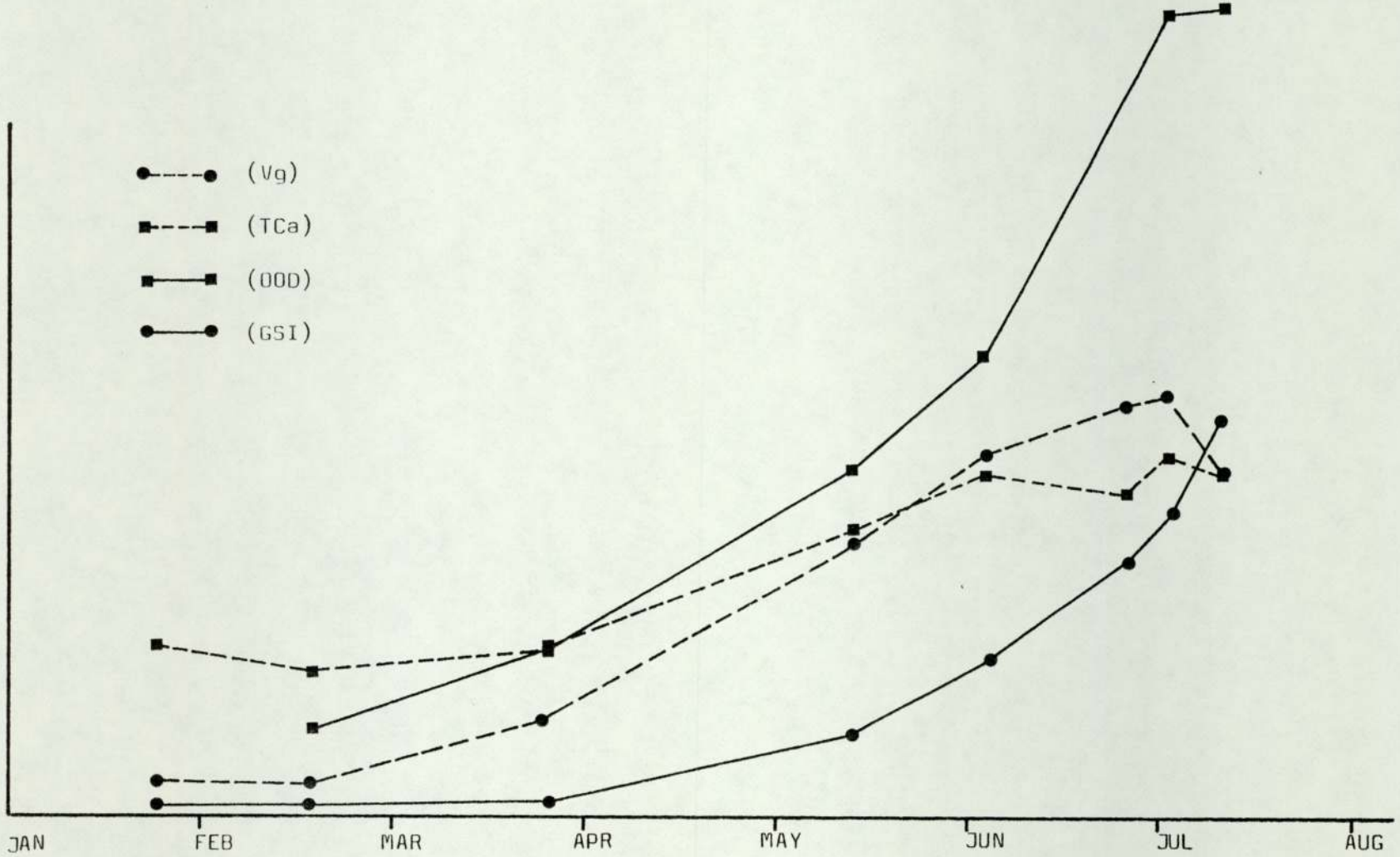


FIG. 7.29. LEGEND OPPOSITE.

FIG. 7.30. CORRELATIONS OF SERUM OESTRADIOL-17β (E2 ng ml^{-1}) WITH MEAN OOCYTE DIAMETER (OOD mm, $P > 0.001$, $r=0.99$), HEPATOSOMATIC INDEX)HSI %, $P > 0.05$, $r=0.7$) AND VITELLOGENIN (Vg mg ml^{-1}, $P > 0.01$, $r=0.93$) DURING VITELLOGENESIS IN GRAMPIAN STRAIN RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.
(VERTICAL AXIS COMPOSITE, OF SAME SCALE TO PREVIOUS FIGURES).

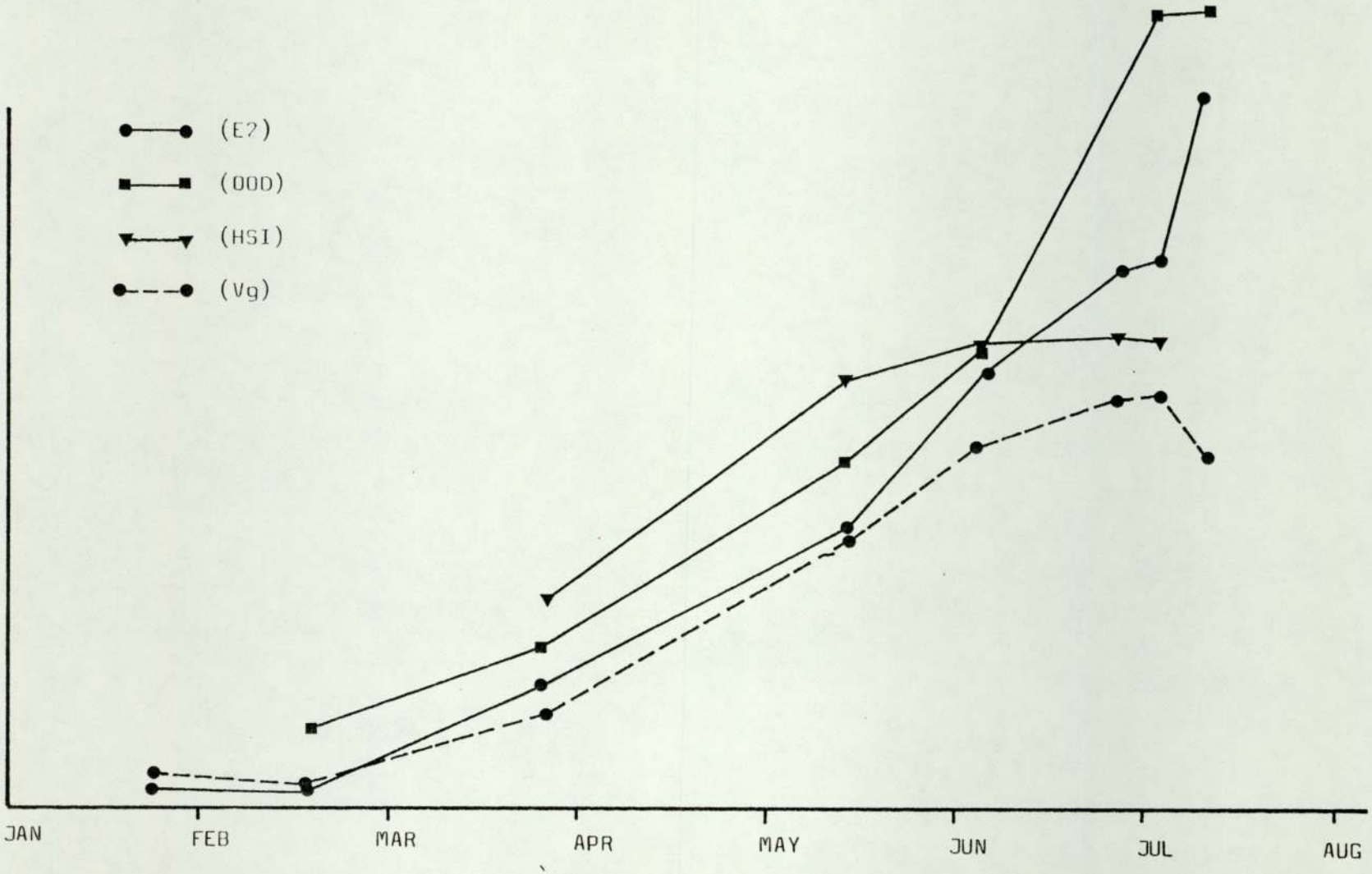


FIG. 7.30. LEGEND OPPOSITE.

Whitebrook strain : Over the complete cycle from December to August, TCa levels were significantly correlated with Vg levels ($P < 0.001$, $r=0.99$), OOD ($P < 0.01$, $r=0.85$), GSI ($P < 0.001$, $r=0.92$) and serum E2 ($P < 0.001$, $r=0.87$) (See Figs. 7.31. & 7.32.).

Serum E2 levels were significantly correlated with TCa ($P < 0.01$, $r=0.88$), OOD ($P < 0.001$, $r=0.99$) and HSI ($P < 0.05$, $r=0.88$) over the exogenous vitellogenic phase from May until August (See Fig. 7.33.).

FIG. 7.31. SUMMARY OF SEQUENTIAL CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}), OESTRONE (E1 ng ml^{-1}), TESTOSTERONE (T ng ml^{-1}), TOTAL CALCIUM (TCa mg $100ml^{-1}$) AND VITELLOGENIN (Vg mg ml^{-1}) IN WHITEBROOK STRAIN RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT. (VERTICAL AXIS COMPOSITE, OF SAME SCALE TO PREVIOUS FIGURES).

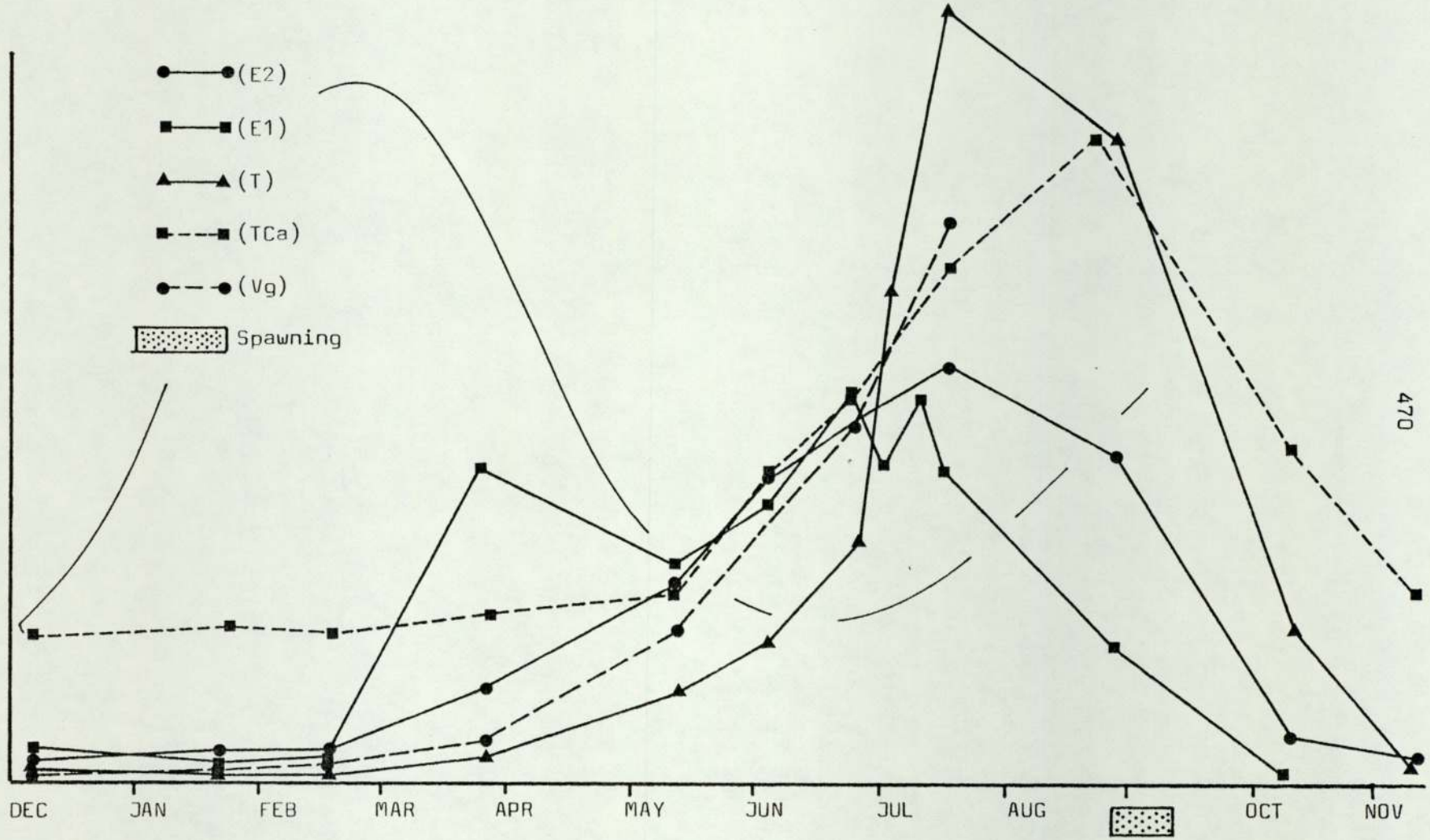


FIG. 7.31. LEGEND OPPOSITE.

FIG. 7.31. CORRELATION OF MEAN SERUM CALCIUM (TCa mg $100ml^{-1}$) WITH VITELLOGENIN (Vg mg ml^{-1}, $P < 0.001$, $r=0.99$), MEAN OOCYTE DIAMETER (OOD mm, $P < 0.01$, $r=0.85$) AND GONADOSOMATIC INDEX (GSI %, $P < 0.001$, $r=0.92$) IN WHITEBROOK STRAIN RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.
(VERTICAL AXIS COMPOSITE, OF SAME SCALE TO PREVIOUS FIGURES).

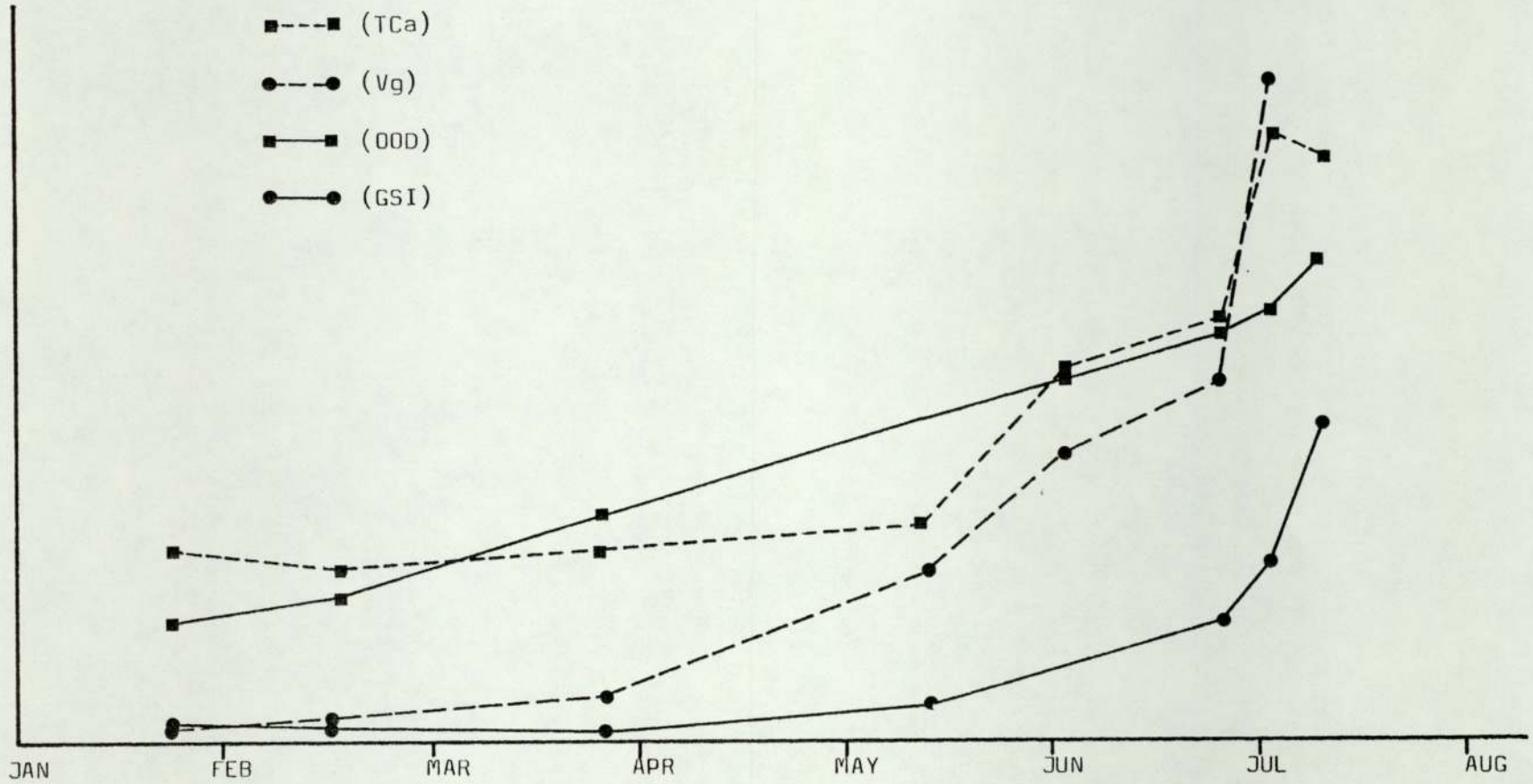


FIG. 7.32. LEGEND OPPOSITE.

FIG. 7.33. CORRELATION OF MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}) WITH TOTAL CALCIUM (TCa mg $100ml^{-1}$, $P > 0.01$, $r=0.88$), MEAN OOCYTE DIAMETER (OOD mm, $P > 0.001$, $r=0.99$) AND HEPATOSOMATIC INDEX (HSI %, $P > 0.05$, $r=0.88$) DURING VITELLOGENESIS IN WHITEBROOK STRAIN RAINBOW TROUT HELD UNDER A NORMAL SEASONAL CYCLE COMPRESSED INTO 6 MONTHS IN SERIES II EXPERIMENT.
(VERTICAL AXIS COMPOSITE, OF SAME SCALE TO PREVIOUS FIGURES).

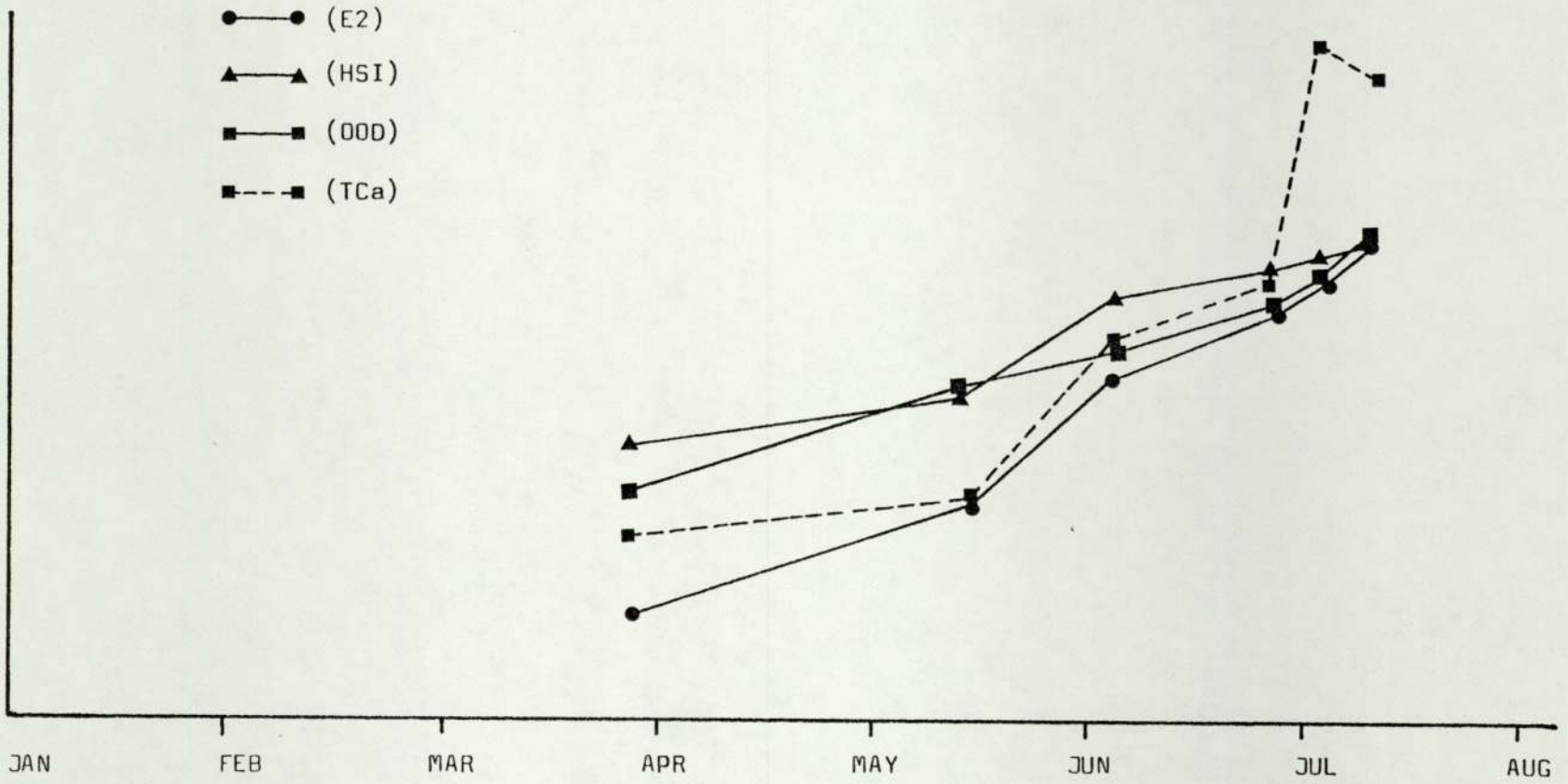


FIG. 7.33. LEGEND OPPOSITE.

7.4. Discussion.

As maturation proceeded, the increase in size and gross reorganisation of the morphology of the ovary were accompanied by marked changes in serum levels of oestrone, oestradiol-17B, testosterone, vitellogenin and total calcium. There were similar sequences and profiles of changes in all three strains of rainbow trout held under both photoperiod regimes of constant length and compressed natural seasonal cycle.

After the initial concomitant increases in oestrone, oestradiol-17B, testosterone and vitellogenin (including total calcium) seen at the onset of gonadal development, the first major change was a rapid increase in the serum levels of both oestrogens. Over the first few weeks of development, serum oestrone levels increased much more rapidly and reached their peak levels earlier than oestradiol-17β. Furthermore, under the 6 month-compressed seasonal cycle, oestrone levels demonstrated a second peak later in the cycle, approximately one month before spawning, at about the same time as maximum oestradiol-17β levels were reached. The high serum levels of oestrone recorded in all three strains, and the previously reported vitellogenic action of this hormone (See Chapter 5; van Bohemen et al., 1982b) suggests that it may play a role in the control of vitellogenesis especially during the earlier stages of this process. A study by van Bohemen et al., (1981c) of the serum levels of oestrone in trout during the reproductive cycle, observed a similar increase in oestrone early in development. Since the same group also found that oestrone injections (1^{o}) were effective in priming the liver for subsequent oestradiol-17β

treatment, they proposed that the early increase in oestrone enabled the liver to provide an enhanced secondary response to oestradiol-17β later in the annual cycle (van Bohemen et al., 1982a, 1982b). However, although this may occur during the first year of maturation, it would be of little consequence in subsequent years since any increase in oestradiol-17β would cause a secondary response, without oestrone, as vitellogenesis had already been evoked during the previous spawning. It has been reported that the injection of oestrone and oestradiol-17β together in trout can cause a greater vitellogenic response, than the sum of their individual responses (van Bohemen et al., 1982a, 1982b). This suggests that it is the total oestrogen presence during the reproductive cycle that is physiologically responsible for the production and maintenance of vitellogenin synthesis, rather than oestrone or oestradiol-17β alone. However, there has been no confirmation of this report in other fish.

Although oestradiol-17β may play a subordinate role at the beginning of maturation, as suggested by its presence at lower levels than oestrone, during the period of maximum vitellogenesis it becomes the major circulating oestrogen. A role for oestradiol -17β in the regulation of vitellogenesis in other oviparous vertebrates is now well established (Tata, 1978; Wallace, 1978) and the present and other results (See Chapter 3) confirm the importance of this hormone in this process in teleosts. Thus, injection of oestradiol-17β increases serum levels of vitellogenin in several species (See Chapter 3; Bailey, 1957; Plack et al., 1971; de Vlaming et al., 1980; Elliott et al., 1980) and serum levels of oestradiol-17β have been correlated with serum vitellogenin

during the reproductive cycles of trout (Scott et al., 1980b; Bromage et al., 1982b). The observations in the present study that, increases in serum levels of oestradiol-17β followed by rises in vitellogenin and a peak of this hormone was followed by maximum vitellogenin levels, are indicative of a causal relationship between the two. Thus, in this work, serum levels of oestradiol-17β were correlated with serum vitellogenin (and total calcium) levels in all three strains during the period of vitellogenesis. It is noticeable that serum levels of vitellogenin continued to rise whilst both oestradiol-17β and oestrone levels were falling, and furthermore, vitellogenin reached a peak at the time when these oestrogens had returned to basal levels. A similar sequence of events has been observed in other studies (Whitehead et al., 1978a; Scott et al., 1980b) strongly suggesting that vitellogenesis continues even after oestrogen synthesis and release has stopped. This is supported by results here (See Chapter 3; Elliott et al., 1979) where total serum calcium and phosphoprotein phosphorus levels were significantly raised for almost 5 months after injection, although blood levels of the steroid had long since fallen to basal values. However, in this experiment, immature male and female trout were used and the protracted elevation in vitellogenin levels may have been due to a long half-life in these animals. It has been shown in Xenopus that the half-life of vitellogenin is only a matter of days in maturing females due to rapid uptake by the ovary, whereas in immature males the half-life was almost 40 days (See Chapter 3). In the present work serum levels of oestradiol-17β and vitellogenin were both significantly correlated with the hepatosomatic index (HSI) during this period of vitellogenesis, suggesting that in fish,

like other oviparous vertebrates, vitellogenin is synthesised in the liver (See Wallace, 1978).

Following the rapid increases in both oestrone and oestradiol-17β, serum levels of vitellogenin began to rise as the ovary developed, reaching a peak either just before or at about the time of spawning. Evidence that these increased serum levels of vitellogenin were primarily responsible for the growth of the gonad was shown by measurement of gonadosomatic index (GSI), oocyte diameter and by the detailed histological examination of all three strains of trout (See also Chapter 6). Thus, serum levels of vitellogenin were correlated with both the GSI and mean oocyte diameter throughout the reproductive cycles of these fish. In order to account for the rapid increase of GSI during vitellogenesis, it was calculated on a dry weight basis that the ovary must sequester approximately 1g of yolk per day. This growth was reflected in the 25-fold increases in surface area of individual oocytes and their 600-fold increases in volume. It is perhaps significant that a 1mm reduction in the diameter of an oocyte effectively halves the oocyte volume. Since the eggs obtained from commercial broodstocks often vary by more than 0.5mm, the consequences of such drastic reductions in egg volume to the subsequent development and performance of the embryo or fry may be considerable. Certainly the eggs from the fish under both these experimental regimes were smaller than those from corresponding groups held outdoors, which may be a reflection of the 'advancement' in spawning time and the shorter period available for egg growth in these fish. Although there were no apparent differences in performance of these eggs through the eyed-stage to swim-up, no

examination was made of their subsequent growth. Currently, trout eggs are sold mainly on the basis of their size, and although there is no evidence that larger eggs are of better quality, it is generally accepted that they produce bigger and faster growing fry. It is conceivable that if any advancements in spawning time reduce the size of the eggs, then delays in spawning may increase their size and thus be of considerable value to the egg producer. Preliminary evidence in this laboratory suggests that this may be the case, and the effects of delayed spawning on egg size and quality is currently being investigated further.

At the onset of gonadal development when oestrone, oestradiol-17β, testosterone, vitellogenin and calcium were all increasing slowly, a histological examination of ovarian tissue revealed a predominance of vesicle stage 4 oocytes and a smaller proportion beginning to show signs of stage 5 granular inclusions (See also Chapter 6). This was confirmed by electron microscopy which demonstrated the presence of micropinocytosis at the developing oocyte surface. Thus, it would appear that at early stages of development, when vitellogenin can only confidently be detected by RIA, that the uptake of this large precursor has already begun. As both GSI and oocyte diameter rose, the amount of granular material within the oocytes increased, until they were completely full. Since the increase in mean oocyte diameter was correlated with increases in serum vitellogenin and this material is itself induced by oestradiol-17β, it may be possible to influence the size of eggs by treatment with oestrogen at a time of year in advance of the natural appearance of this hormone. If a significant effect of artificially increased vitellogenin levels on egg size can be

provided by only one or two injections of oestradiol-17B and/or oestrone, such a technique would be of both commercial importance and applicability to the fish farming industry.

The most striking observations under the long to short artificial photoperiod regime (Series I) were the histological (See also Chapter 6) and endocrinological changes in the immature Whitebrook strain fish during this period. At the time of the switch from long day (18L : 6D) to short day (6L : 18D) at the time of the Summer solstice, the ovaries taken from the Whitebrook strain contained a majority of primary phase oocytes, but also a few of the secondary growth phase. As the experiment progressed, the oocyte diameter doubled and there was an increase in the proportion of stage 4 vesicle-containing oocytes. By December, some stage 5 oocytes were detected, indicating that vitellogenin was being accumulated in some oocytes. Whether these stage 5 oocytes were subsequently resorbed by atresia was impossible to determine due to the infrequency of sampling. Concomitant with the histological changes in immature Whitebrooks, increases in oestrone, oestradiol-17B, testosterone, vitellogenin and total calcium were observed. Although the absolute levels of the various serum parameters were lower than those seen in the other two mature strains, the sequence of the changes in these hormones mirrored those in the Caribou and Grampians. Furthermore, the significant increases in serum levels of vitellogenin that were observed, may have subsequently produced the stage 5 oocytes which were seen in December. It is possible that the morphological and endocrinological changes seen in immature fish of this strain, represent a 'dummy- or practice-run' as far as reproductive development is concerned.

There is similar evidence of 'practice-runs' one year or more before the first spawning in other fish (Hickling, 1935; Trout, 1957; Woodhead & Woodhead, 1965; Davis, 1977; Eliassen & Vahl, 1982). Thus, an increase in ovarian weight has been observed in immature cod (Eliassen & Vahl, 1982) and non-spawning catfish (Davis, 1977). Also, Hickling (1935), noted that the egg diameter increased in immature hake at the time when older mature fish spawned. Furthermore, there are data from other species which indicate that some oocytes in immature animals entered stages similar to older maturing females, but subsequently these vitellogenic oocytes disappeared at the time when older fish were undergoing spawning (Yamamoto, 1956; Gokhale, 1957). It has been recognised in teleosts that the initiation of the secondary growth phase requires a pituitary trigger and that once initiated, spawning usually occurs within a year (Vivien, 1939; Barr, 1968). In the Whitebrook strain, initiation of the secondary growth phase did not result in spawning within a year, and also it is not known whether a pituitary trigger caused this development since the samples were not assayed for gonadotrophin. There is evidence from studies on other fish, that early vitellogenic oocytes were present throughout the reproductive cycle (Htun-Han, 1978; Forberg, 1982). Working with the capelin, Forberg (1982) concluded that either vesicle-stage 4 was not as cyclical as the later stages of secondary growth, or that the ovarian development which precedes the first spawning lasts for longer than a year. The present results could equally be interpreted in this way, since vesicle-stage 4 oocytes were observed for more than six months in immature Whitebrook and there was no evidence to indicate that it was not this population of oocytes that eventually matured at the end of the 6-month seasonal cycle.

Whatever the explanation for the changes exhibited by immature Whitebrook fish, it is probable that they are directly related to the mechanism(s) which determine the onset of maturation or puberty in trout. A number of studies have observed that the administration of oestrogenic steroids to immature fish can cause increases in the cytological activity of the pituitary and gonadotrophin content (Olivereau & Chambolle, 1978; Olivereau & Olivereau, 1979; Crim et al., 1981). Thus, it has been proposed that there are sufficient oestrogenic steroids in immature fish to act on the hypothalamo-hypophysial axis and to stimulate gonadotrophin synthesis by means of a positive feedback (Peter, 1982). This store of gonadotrophin may then only require a releasing factor to trigger its release and LHRH has been shown to exert this effect on gonadotrophin stored in immature pituitary after testosterone treatment (Crim & Evans, 1980). Thus, the observed effects in Whitebrook fish may have been due to fluctuations in oestrogen levels positively feeding back on the pituitary. Clearly it may be possible to mimic this 'practice-run' one year earlier than normally observed, possibly by the use of hormones, and thus obtain eggs of better quality one year in advance of the corresponding control fish.

Although the present results demonstrate that the profile of serum changes in vitellogenin can account for the increases in GSI and oocyte diameter, the changes in vitellogenin were more closely correlated with the serum levels of testosterone than either of the oestrogens. Thus, serum testosterone levels started to increase at the same time as the oestrogens, but unlike oestrone and oestradiol-17β, increased only gradually during the

early stages of vitellogenesis. However, following the initial peak in oestrone and rapid increase in oestradiol-17β, testosterone levels began to rise more quickly and reached a peak just prior to spawning at a time when both oestrogens had returned to basal values. High levels of testosterone in female teleosts have also been reported by other workers (Wingfield & Grimm, 1977; Campbell & Idler, 1976; Scott et al., 1980b); levels which are often far higher than those found in mature male fish of the same species. Although maximum testosterone levels occurred at the height of vitellogenesis, they are unlikely to have any physiological role in vitellogenesis in this species due to the poor vitellogenic potency of this hormone (See Chapter 5). It is more likely that the increase in levels of testosterone are related to increases in oestradiol-17β, since oestradiol-17β is synthesised either via oestrone after aromatisation of androstenedione, or via testosterone via the aromatase enzyme. Thus, high levels of testosterone and oestrone may both be due to their immediate position to oestradiol-17β on the biosynthetic pathway. There is some evidence that a deactivation of aromatase before spawning whilst primarily blocking any further synthesis of oestrogens also causes an indirect increase in testosterone as a result of its position as the primary end product of steroid synthesis. This could account for the very rapid increase in testosterone during the latter stages of development st the same time as the levels of both oestrogens were falling. A more recent study which investigated the endocrine changes in rainbow trout at weekly intervals up to and after ovulation, observed that testosterone levels reached a peak about 8 days before spawning, at which point they rapidly decreased (Scott

& Sumpter, 1982a). The sudden drop in testosterone levels at this stage was attributed, by these authors, to a switch in production from testosterone to progestagenic steroids which are known to be involved in final maturation and ovulation in salmonids (Jalabert, 1976; Kagawa et al., 1981; Scott et al., 1982; Scott & Sumpter, 1982a, 1982b). Thus, if high levels of testosterone do have a physiological role, it is likely that it is during the latter stages of vitellogenesis and/or before final maturation and ovulation. There is some evidence from mammalian studies that testosterone is important in regulating atresia (Magoffin & Erickson, 1981) and testosterone has been shown to induce atresia in fish (Yamazaki, 1971). It may be possible that the maintenance of high, albeit falling, levels of testosterone even after ovulation accelerates the resorption of any remaining oocytes, in preparation for the next cycle. However, it would not appear that the timing of the changes in trout support such a role in this group of fish. It is possible that the preliminary stages of atresia begin before ovulation in some oocytes and that the reduced levels of testosterone after ovulation are sufficient to maintain the degenerative processes involved. Evidence relating to the effects of androgens on vitellogenesis suggest that they are uniformly inhibitory (Dodd, 1960). Thus, it is possible that a combination of the increasing testosterone and falling oestrogen levels cause an inhibition of vitellogenesis in the developing oocytes in preparation for the changes that occur during final maturation. As such, this would be quite separate from the negative feedback on the pituitary caused by falling oestrogen levels, which cause an increase in gonadotrophin just prior to ovulation (Fostier et al., 1978; Bromage

et al., 1982b; Scott & Sumpter, 1982a). Of all the photoperiod regimes used to manipulate reproduction in salmonids, none have prevented ovulation, which suggests there may be some factor that ultimately limits the growth of oocytes by stopping vitellogenesis and bringing about final maturation of the oocyte and ovulation.

During the course of these experiments, some degree of variation in the maximum serum levels of oestrone, oestradiol-17β, testosterone, vitellogenin and total calcium was observed not only between strains, but also between fish of different age from the same strain. However, no differences in these endocrine changes could be attributed to any obvious differences in performance of the broodstock under the artificial photoperiod, the eggs they produced, or the subsequent development of the fry. Furthermore, the profiles and sequences of these endocrine changes were virtually identical to each other and the only difference to other reports of these changes in the same fish, was in their duration (Bromagé et al., 1982b; Scott et al., 1980b; Scott & Sumpter, 1982b). However, these differences in vitellogenin and calcium levels were more pronounced, and there appeared to be an inverse correlation between the serum levels of vitellogenin and the size of the eggs produced. Thus, under both experimental photoperiods, although the maximum serum vitellogenin levels in Caribou strain were twice as high as those observed in Grampian of a similar age, the eggs in Caribou fish were smaller. Also, under both experimental photoperiods, serum levels of oestradiol-17β in Caribou strain fish were approximately the same in both cases, but the eggs from the second spawning were larger and the vitellogenin levels lower. Since serum levels of vitellogenin represent the difference between the

rate of synthesis and the rate of uptake by the ovary in maturing fish, it is possible that lower serum vitellogenin levels are indicative of an increased uptake rather than a lower rate of synthesis of this protein. Thus, it would appear that different strains of a similar age may produce different sized eggs and that the ovaries of older fish may also become more efficient at the uptake of vitellogenin the more frequently they recrudesce, although the importance of differences in fecundity in these strains must also be stressed. Clearly the use of oestradiol-17β or oestrone to increase vitellogenin levels may not be sufficient on their own to affect egg size or quality. Thus, other hormones possibly of pituitary origin may be required to stimulate the uptake of these increased levels into the ovary, since purified gonadotrophin(s) and/or whole pituitary extracts have been shown to stimulate the uptake of vitellogenin in fish (See Dodd & Sumpter, 1982).

Although the sequence of changes in these various sex-steroids are responsible for the direct control of the different phases of ovarian development, these changes are believed to be initiated and controlled by the hormones from the pituitary. Thus, there is evidence from a number of studies in salmonids of increases in gonadotrophin at about the time of the onset of gonadal development as well as during final oocyte maturation and ovulation (Billard et al., 1978; Whitehead et al., 1978c; Bromage et al., 1982b). However, not all studies have observed changes in gonadotrophin at these times and it has been suggested that the initiation of

gonadal development does not need a pituitary signal (Peter & Crim, 1979). The reason for these differences may be partly due to the time of blood sampling since it has been shown that there is a daily pattern of release of gonadotrophin in trout (Zohar, 1982). At present there is no agreement regarding the number of gonadotrophins in teleosts (Dodd & Sumpter, 1982; See also 7.1. and 3.10.). This complicates the interpretation of data and thus it is not clear whether the two increases in gonadotrophin(s) detected in some studies represent separate vitellogenic (non-glycoprotein Con AI type) and maturational (glycoprotein Con AII type) gonadotrophins, or a single multifunctional hormone. However, the physiological role attributed to this initial peak in gonadotrophin is to cause the biosynthesis and release of oestrogens by the ovary (Billard et al., 1978) thus initiating vitellogenesis and also to increase the number and activity of the micropinocytes at the oocyte surface, which then brings about the uptake of this protein into the oocytes (Wallace, 1978; Abraham et al., 1982). However, in all studies where gonadotrophin levels have been monitored, low levels have been recorded during the period of maximum gonadal growth. It is possible that only low levels or the intermittent episodic secretions of gonadotrophin are sufficient to maintain micropinocytosis during this period. There is some evidence in the guppy, that the size of the micropinocytes become larger during late vitellogenesis and it was concluded that this represented the uptake of different extracellular materials (Droller & Roth, 1966). However, it may also be possible that another hormone may be responsible for the production of this second type of pinocyte, although no evidence for such an occurrence was observed in the

present work.

There have been reports that TSH or the thyroid hormones are involved with vitellogenesis, or more specifically, with the uptake of vitellogenin by the ovary. As TSH is a glycoprotein and very similar structurally to gonadotrophin (Fontaine, 1969) separation procedures for gonadotrophin could possibly be contaminated with TSH. Interestingly, Idler & Co workers' Con AII gonadotrophin preparation has also been shown to have some effect on increased uptake of vitellogenin, although contamination by TSH may have accounted for this action. Certainly the present study has shown that oestradiol-17B treated fish, fed a T_4 treated diet have reduced vitellogenin levels in the serum. One explanation of this was that thyroid hormone enhanced the uptake of vitellogenin from the serum. Until homologous assays are available which equivocally measure gonadotrophin and TSH, questions relating to the number of gonadotrophins and the involvement of other pituitary hormones in vitellogenesis will not be resolved. There is much evidence in the literature that 'purified gonadotrophin(s)' do have different biological actions (Campbell & Idler, 1976; Hirose, 1976; Jalabert, 1976; Goetz & Bergman, 1977; Campbell, 1978). Clearly there are factor(s) in the teleost pituitary that affect reproduction other than the purified gonadotrophin preparations that are currently available.

In all species of fish investigated to date, the initiation and subsequent manipulation of the various reproductive processes by environmental cues are achieved as a result of neuroendocrine changes involving the hypothalamus, the pituitary hormones and the sex steroids (de Vlaming et al., 1974; Dodd & Sumpter, 1982).

Thus, there is considerable evidence that the hormonal changes which directly control these processes are ultimately regulated at least in part, if not completely, by environmental factors (de Vlaming, 1972; de Vlaming, 1974; Dodd & Sumpter, 1982). Of all the environmental factors known to affect reproduction in fish it appears that it is the photoperiod that exerts the major influence in salmonids (Hazard & Eddy, 1951; Allison, 1951; Nomura, 1962; Henderson, 1963; Carlson & Hale, 1973; Kunnesh et al., 1974; McQuarrie et al., 1978; Whitehead et al., 1978b; Peter & Crim, 1979; Bromage et al., 1982b). However, although the response of the neuroendocrine system to these cues is well documented, it is less clear firstly how salmonids measure the changing photoperiod and secondly which portion of the light/dark cycle constitutes the definitive cue(s). One possible hypothesis is that these fish simply add up the number of hours light they receive and above an appropriate threshold physiological response triggers the onset of gonadal development (Farner & Follett, 1966). However, this simple 'hour-glass' theory does not appear to be an adequate explanation as far as trout are concerned since it has been shown using constant light cycles of different lengths that different groups spawn after receiving widely differing total numbers of hours of light (Whitehead et al., 1978b; Bromage et al., 1982b). It may be that the rate of change of light is the important photoperiodic determinant although photoperiods of constant length have been shown to be as equally effective in modifying spawning times as seasonally changing light regimes (Whitehead & Bromage, 1980). In the present work, a long (18L:6D) followed by a short (6L:18D) day produced similar results to a 6 month seasonally changing photoperiod and

thus it would appear that it is not the rate of change in daylength which is of importance, but the amount of light perceived per day, and its relationship to photosensitive phases in their reproductive rhythms (Bromage et al., 1982b).

Other workers have similarly shown that compressed seasonal cycles, or long days before the Summer solstice followed by short days accelerate gonadal development and advance spawning (Whitehead et al., 1978b; Whitehead & Bromage, 1980). Conversely, extended seasonal cycle, or short days early in the year followed by long days later in the year tend to delay spawning (Bromage et al., 1982a). The use of different strains in this study aimed to increase our understanding of how fish measure the photoperiod and respond to it and also to see if the different endocrine and physiological processes in the reproductive cycle have been altered to account for the widely different spawning times of the three strains (Caribou spawn in November; Grampian - December; Whitebrook - January).

Little is known about how different strains of the same species respond to the same environmental cues. For example, it is possible that gonadal development is initiated simultaneously in all three strains by the same cue and that it is differences in the rates at which these mature which leads to differences in spawning time. An alternative explanation is that the endocrine events are the same in the three strains, but the separation in spawning times occurs as a result of the triggering of gonadal development by photoperiods of different length. For example, it is possible that an earlier-spawning strain requires only 13 hours daylight for this

to be perceived as a long day and for gonadal development to be initiated, whereas the later-spawning strain may require 15 hours, a daylength not reached until later under a seasonally changing photoperiod. In this case the difference in spawning time is represented by the time it takes for the normal seasonal changing daylength to increase from 13 to 15 hours light (about 4 weeks) and the former strain spawns one month earlier. Clearly the faster the rate of changes between these daylengths, the closer the spawning times should be. Thus, under a 6 month compressed cycle one would expect this difference in the onset of the endocrine changes in the three strains to be halved. If such an interpretation were correct, one would expect there to be a synchronisation of spawning if the switch was made directly to 18L:6D. However, if after such a constant regime, spawning time was still separated, it would suggest that the environmental trigger is more or less the same for each strain, but that each strain requires different numbers of daily stimulations of that daylength.

At the time of the switch from long (18L:6D) to short day (6L:18D) at the beginning of Series I, the serum levels of all the sex steroids and vitellogenin and also the histological development of the ovary of both Caribou and Grampian strains were identical (Whitebrook fish did not mature until the following cycle). Thus it would appear that under this photoperiod, development was initiated at the same time. However, subsequent to the switch to short-day their rates of development, as demonstrated by endocrine and histological changes were different and the earlier-spawning Caribou strain responded more rapidly to the short day. If these had the same daylength cue and matured at different rates, one

would expect this difference to have shown itself at the first sampling. Thus, these results support the proposition of a similar cue in the different strains, but with the earlier strain needing less 'long-day' stimuli to initiate gonadal development or conversely fewer 'short-day' stimuli to curtail the latter stages of gonadal development than does the later spawning strain. These results confirm a requirement for short-days (or absence of long-day) later in the cycle. Such a requirement has also been indicated in a further study on trout (Bromage et al., 1982a, 1982b).

Under the second artificial photoperiod, all three strains matured and maintained their spawning identity by one month, even though they were under a compressed 6 month seasonal cycle. This indicates that gonadal development started at approximately the same time and the rates of change in measured serum parameters were different. However, if one looks critically at the Feb-Mar period when the first changes in all three strains take place, it is evident that the Caribou started before the Grampian, which began development before the Whitebrook; and in each one the infrequency of sampling probably masked the precise points of flexion in the three strains. Monthly sampling under a 6 month regime is probably only as effective as bi-monthly sampling under a normal seasonal photoperiod. It must also be appreciated that since the maximum levels in all three strains were not identical, the rates would be expected to be different. These results would also suggest that in rainbow trout it is the number of daily stimulations of a particular photoperiodic cue that may be the

determining factor in the initiation of gonadal development. Preliminary results for the Series III experiment, where these strains were held under a 12 month seasonal cycle which had its shortest day on 21st June, and longest day on 21st January (See Figs. 7.34. & 7.35.), support these results. Thus, the initiation of reproduction in Caribou fish, shown by significant increases in oestradiol-17β, testosterone and calcium began 2 months before similar changes were observed in Whitebrook fish. Subsequently, the Caribou strain spawned 2 months earlier than the Whitebrook fish at the end of this photoperiod. Another study investigating these same aspects in two strains of rainbow trout (Caribou and Christiansen) have similarly shown that the separation in the initiation of gonadal development is the same as the separation in spawning time, and that the duration and sequence of endocrine changes are identical in the two different strains (Scott & Sumpter, 1982b). These authors have concluded that the different strains respond to different photoperiodic cues or daylength, which the present results do not disprove, and is currently being investigated further.

Thus, increases in both these hormones are correlated with increases in vitellogenin, HSI, GSI and oocyte diameter during the period of rapid ovarian growth when vitellogenin is sequestered from the blood. This also opens up the possibility of improving egg quality or size for the fish farmer, by treatment with these hormones. Furthermore, both these present results and those from Series III (not fully reported here) reveal that photoperiod manipulation can be used successfully on a commercial scale, to

radically alter the spawning time of different strains of female rainbow trout and thus provide a greatly increased availability of eggs probably of better quality.

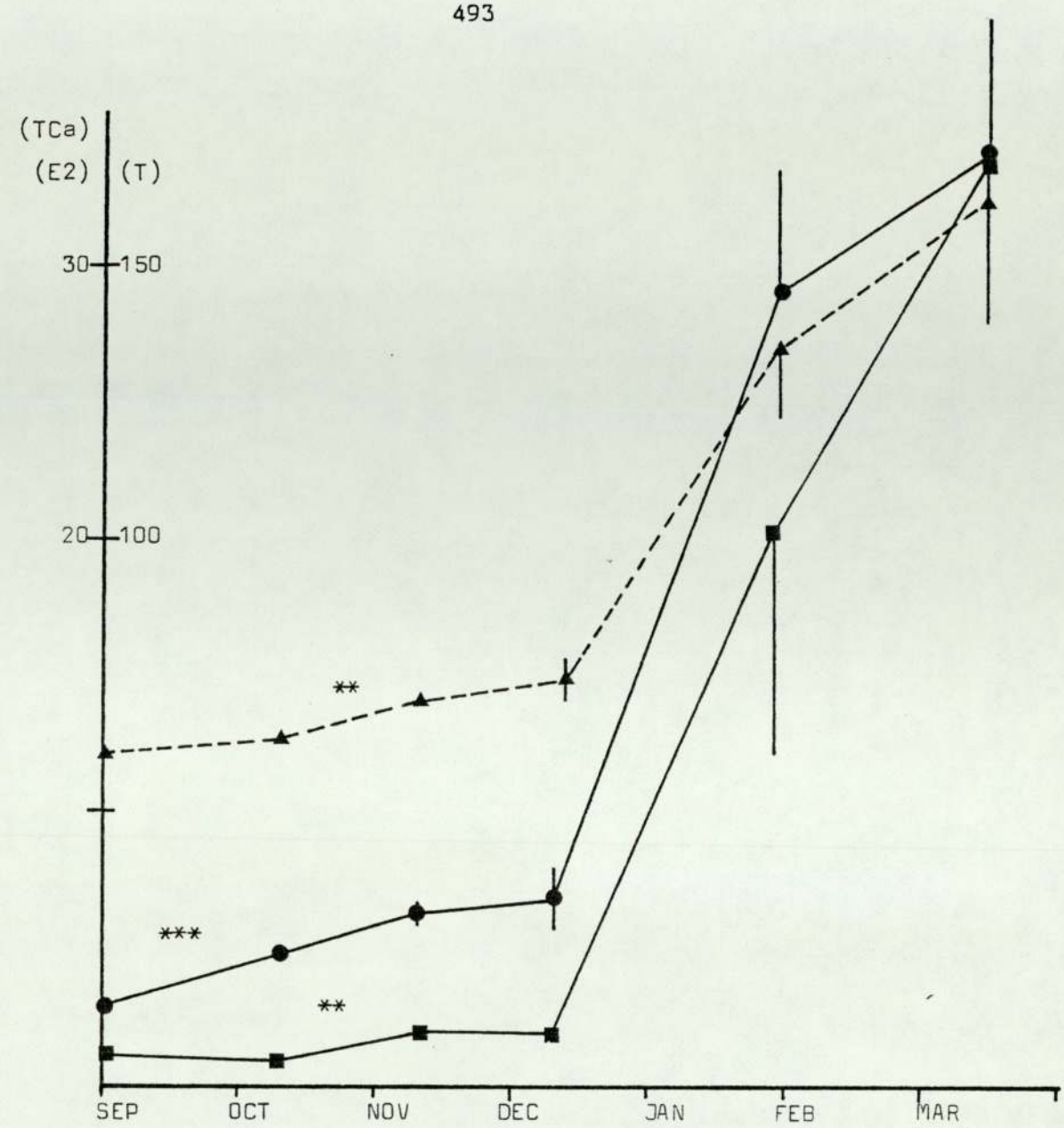


FIG. 7.34. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}), TESTOSTERONE (T ng ml^{-1}) AND TOTAL CALCIUM (TCa mg $100ml^{-1}$) IN CARIBOU (EARLY-SPAWNING) STRAIN RAINBOW TROUT IN SERIES III EXPERIMENT.

(1) Serum oestradiol-17β rose significantly from 3.1 ± 0.014 ng ml^{-1} in late August to 5.0 ± 0.5 ng ml^{-1} in early October ($P < 0.001$; n=7). ●——●

(2) Serum testosterone increased significantly from 5.1 ± 0.6 ng ml^{-1} in early October to 9.75 ± 0.9 ng ml^{-1} in early November ($P < 0.01$; n=10). ■——■

(3) Similarly, total serum calcium increased significantly from 12.9 ± 0.3 mg $100ml^{-1}$ in October to 14.1 ± 0.4 mg $100ml^{-1}$ in early November ($P < 0.01$; n=28). ▲- - -▲

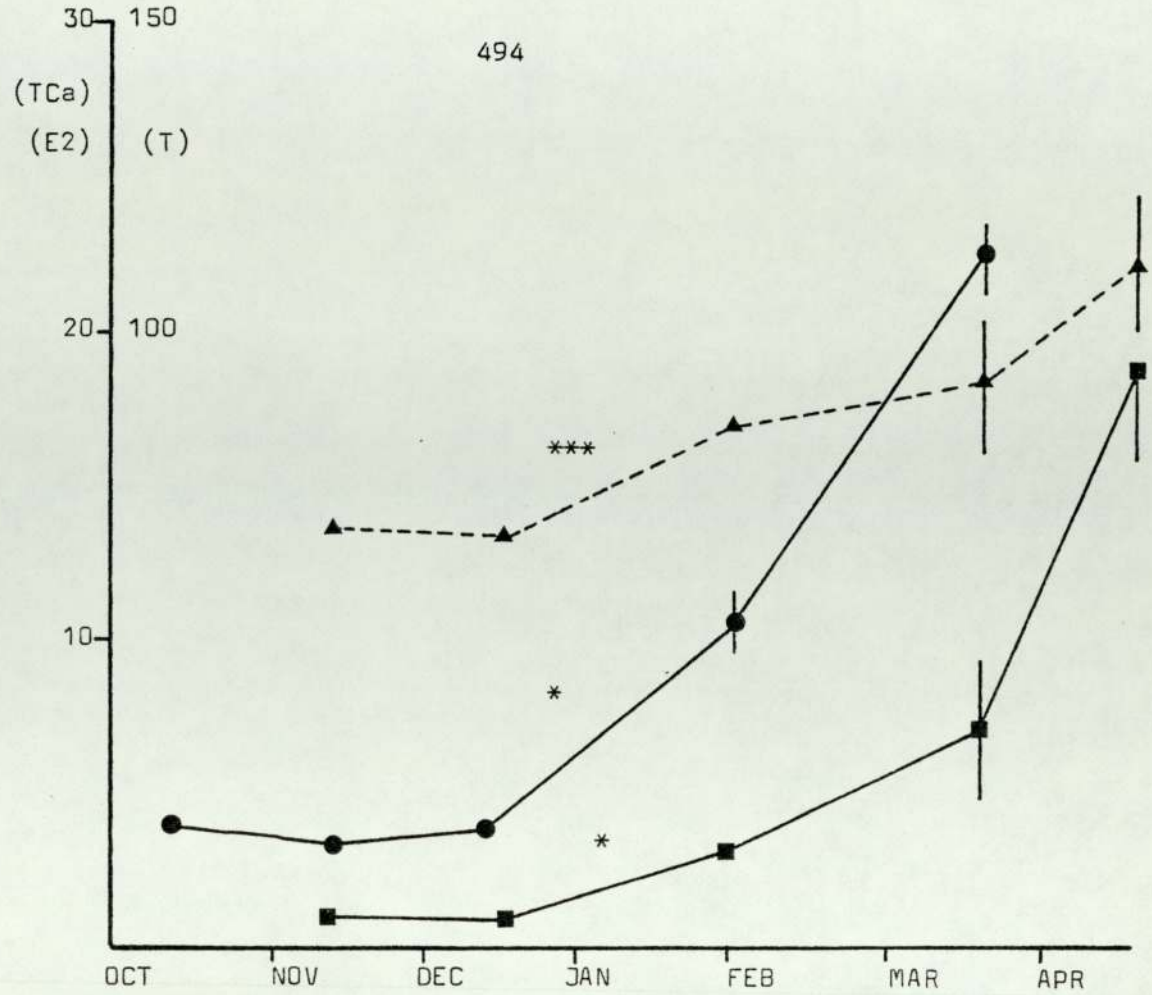


FIG. 7.35. CHANGES IN MEAN SERUM LEVELS OF OESTRADIOL-17β (E2 ng ml^{-1}), TESTOSTERONE (T ng ml^{-1}) AND TOTAL CALCIUM (TCa mg $100ml^{-1}$) IN WHITEBROOK (LATE-SPAWNING) STRAIN RAINBOW TROUT IN SERIES III EXPERIMENT. PHOTOPERIOD: 12 MONTH NORMAL SEASONAL CYCLE HELD 6 MONTHS OUT OF PHASE, WITH SHORTEST DAY 21st JUNE.

(1) Serum oestradiol-17β increased significantly from 3.9 ± 0.03 ng ml^{-1} in December to 10.5 ± 1.5 ng ml^{-1} in January ($P < 0.05$; n=5). ●——●

(2) Serum testosterone increased significantly from 4.1 ± 0.04 ng ml^{-1} in December to 17.6 ± 2.7 ng ml^{-1} in January ($P < 0.05$; n=5). ■——■

(3) Total serum calcium increased significantly from 13.2 ± 0.1 mg $100ml^{-1}$ in December to 17.2 ± 0.6 mg $100ml^{-1}$ in January ($P < 0.001$; n=5). ▲– –▲

CHAPTER 8.

GENERAL SUMMARY

AND CONCLUSIONS.

1. There is currently an increasing demand for rainbow trout culture in the U.K. to become self-sufficient in its requirements for good quality eggs and fry. Because of the threat of disease, there has been a steady decline in the numbers of eggs imported from abroad, to supplement home-produced eggs. Whilst the U.K. industry produces adequate quantities of good quality eggs for a limited period of the year, during the peak spawning time, outside this period the quality and supply are both poor and erratic. Furthermore, little is presently known about what actually determines quality in an egg or what mechanisms decide these factors in oocyte development.

2. The successful development of the ovary, which leads to the production of eggs, is brought about by a complex sequence of events initiated and subsequently modified by changes in the external and hormonal environments. An understanding of how these environmental and endocrine changes modify the different phases of oocyte growth and especially the synthesis and uptake of yolk, may provide the necessary information to control and/or improve the quality of egg supplies.

3. This thesis investigated these problems by monitoring the changes in serum levels of oestrone, oestradiol-17β, testosterone and vitellogenin during the reproductive cycle of three strains of female rainbow trout, held under closely controlled environmental conditions and related these changes to the different phases of ovarian development by histological examination. Thus, any variation in egg quality in the three strains that may have occurred could have been reflected in differences in the endocrinological

or histological events. In parallel, an investigation was made of the effects of the injection of oestrogens and other hormones, on the production of vitellogenin and ovarian development, to see if any changes observed in the normal cycle could be mimicked by hormonal treatment.

4. During the reproductive cycles of all three maturing strains, the primary event was an increase in the serum levels of both oestrogens. Although serum levels of oestrone initially predominated over those of oestradiol-17β, the latter hormone was the major circulating oestrogen during the later stages of vitellogenesis. The increases in both oestrogens were significantly correlated with those of serum vitellogenin and HSI during this phase of vitellogenesis, supporting a role for both these hormones in the hepatic synthesis of vitellogenin in this species.

5. Following the rise in oestrogens, consequent increases in serum vitellogenin and total calcium were observed, which reached peak levels just before, or at about the time of spawning, when serum levels of both oestrone and oestradiol-17β had returned to basal. Changes in vitellogenin and calcium were significantly correlated with the increases in both gonadosomatic index and oocyte diameter, indicating that the vitellogenin produced by the liver is primarily responsible for the rapid increase in gonad size during this period.

6. The use of both sensitive homologous RIA for serum vitellogenin and an electron microscopical study of micropinocytotic activity at the oocyte surface indicated that the uptake of this yolk-precursor starts much earlier than other studies have previously reported.

Light microscopy confirmed the accumulation of vitellogenin during the period of oocyte development and prior to final maturation, oocytes were seen to be full of granular-yolk material.

7. Initial small rises in serum testosterone, increased much more rapidly towards the end of vitellogenesis, possibly as a result of decreased aromatase activity and falling oestrogen levels. High levels of testosterone at this time may inhibit or stop the uptake of vitellogenin, thus preparing the oocyte for final maturation.

8. Although no differences in egg size or quality could be attributed to differences in either endocrine levels or profiles in the three strains, the size of egg was inversely related to the levels of vitellogenin. This would suggest that the uptake of vitellogenin may be of greater importance in this particular aspect of egg quality, rather than the absolute levels produced.

9. The modification of the timing of these endocrine and histological changes by the two artificial photoperiods, was clearly demonstrated by the advanced spawning times in all three strains. The similar responses of the three strains under both the compressed six-month seasonal cycle and the long followed by short days, indicates that it is not the number of hours light received or the rate of change of daylength which is of importance in photoperiod measurement in trout, but the amount of light received per day and its relationship to photosensitive phases in their reproductive rhythms. The results also demonstrate that different strains maintain their separate spawning times by responding to different photoperiodic cues rather than by any modification of the profile of endocrine changes. However, this difference in photoperiodic cue

may not necessarily be in the absolute daylength, but more likely a variable requirement for differing numbers of daily stimulations of that daylength.

10. The eggs from both advancing regimes were smaller than eggs taken from similar fish held under natural conditions which may be a reflection of the 'shorter time' for ovarian development. Conversely, it may be possible to increase egg size if spawning is delayed, by using extended photoperiods.

11. Similar, although much reduced, endocrinological and histological changes were observed in immature Whitebrook strain fish, one year before the natural spawning time. Thus, increases in oestrone, oestradiol-17β, testosterone and vitellogenin were recorded, together with a limited development of second-growth phase oocytes (Stage 5), which together did not result in spawning. It is possible that this species have a practice-run for reproductive development in their prespawning year, a phenomenon demonstrated by some other teleost species.

12. Treatment of immature trout with a single injection of oestradiol-17β resulted in marked and long-lasting increases in serum levels of vitellogenin, total calcium and phosphoprotein phosphorus, similar to those seen during the reproductive cycle of female fish. The duration and height of this response was proportional to the dose administered. Trout treated for a second time demonstrated a much more rapid and increased secondary response. Also the vitellogenic response varied significantly with the time of year, with the liver more sensitive to the same dose of oestradiol-17β in late Summer/early Autumn, than at other times of the year.

A minimum response was observed at the time of year when fish normally spawn, and this phenomenon may be related to some form of refractory period which regulates the onset of reproductive development.

13. Treatment with oestrone and oestriol also yielded significant increases in vitellogenin and total calcium, with oestriol the most potent and oestrone having a similar action to oestradiol-17β. The vitellogenic action of the naturally occurring oestrone, confirms its physiological role in vitellogenesis in this species.

14. The height and long-lasting nature of the vitellogenic response after only one or two injections with oestradiol-17β, indicate that hormonal treatments may have a significant role to play in the control of egg quality and production in salmonid culture :

(a) By supplementing naturally occurring oestrogens either during the normal cycle to increase egg size, or in conjunction with photoperiods designed to advance spawning, to avoid reduced egg size.

(b) To prime fish of one year old or younger, so that at the onset of gonadal development a more rapid secondary response occurs to naturally increasing oestrogens.

(c) To induce the dummy- or practice-run in fish of one year old or younger, with the possibility of obtaining good quality eggs from broodstock of a younger age.

Currently, the use of such hormonal manipulations is still

experimental, although the results from the limited commercial trials carried out so far, appear promising.

15. The combination of the use of artificial photoperiods to provide a greater spread of egg supplies, and hormonal treatment to modify the quality of eggs is of some economic significance to the fish farming industry and may offer a much more flexible approach to broodstock management.

APPENDICES.

APPENDIX I

EXPERIMENTAL RECIRCULATION SYSTEMS.

Both experimental systems at Aston University used in this study, systems A and B (Fig. I.1. & Fig. I.2.) were almost entirely recirculatory. Each system was made up with approximately 79 litres hr^{-1} from a chilled central header tank for recovery of losses due to spillage and evaporation with a minimal overflow. Each system had its own header (Supaglass, Morecambe) to which water was returned (Plastic chemical pump, PV21, Beresford and Son Ltd., Kitts Green, Birmingham) from the filter at 2,040 litres $hour^{-1}$. Each system had three 720 litre experimental tanks (Supaglass) with an internal diameter of 90 cms, and maximum and minimum depths of 36 and 30 cm respectively. Each tank had a tangential water supply of 510 $\pm$ 50 litres hr^{-1}, with a 4 cm central drain (Fig. I.3.). The three tanks emptied into a common waste pipe ($1\frac{1}{4}$" ABS., Plastic construction, Tysley Rd., Birmingham) which in turn drained into a faecal trap.

On system A the trap was of a lamellar type (Fig. I.4.) in which the greatest surface area was offered to the faeces and a high retention time for solids. On system B the trap was somewhat simpler, in that solids entered at a low level and water was taken off at a higher level (Fig. I.5.). Both traps were cleaned periodically, monthly on system A by draining the tank, and daily on B by removing the central pipe. The water from either faecal trap then went to the filter.

The filters used were of the downflow gravel type where ammonia loaded water was set against vitrifying bacteria occupying the surface of the gravel. Filtered water was pumped to the header from the bottom of each filter.

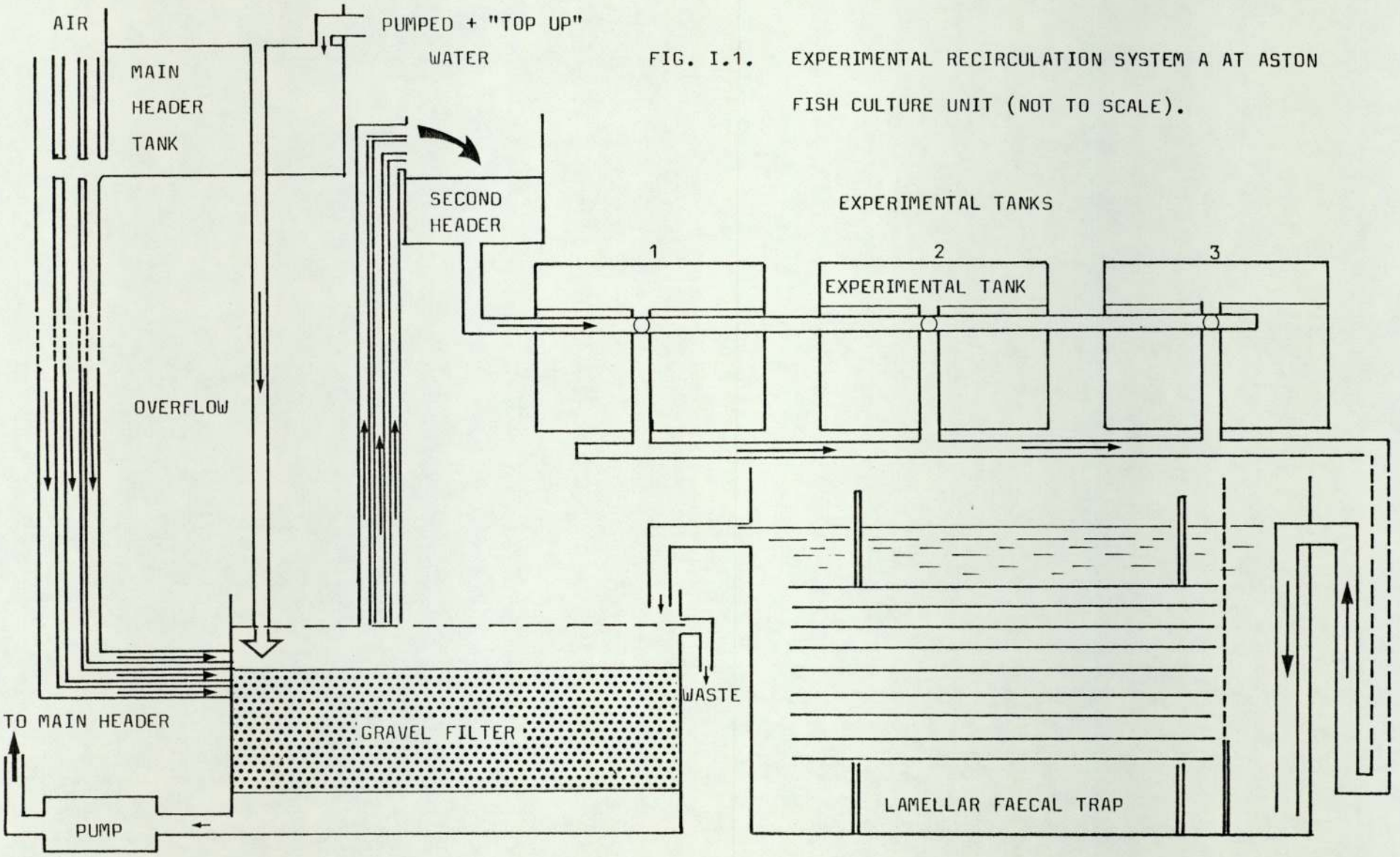


FIG. I.1. EXPERIMENTAL RECIRCULATION SYSTEM A AT ASTON FISH CULTURE UNIT (NOT TO SCALE).

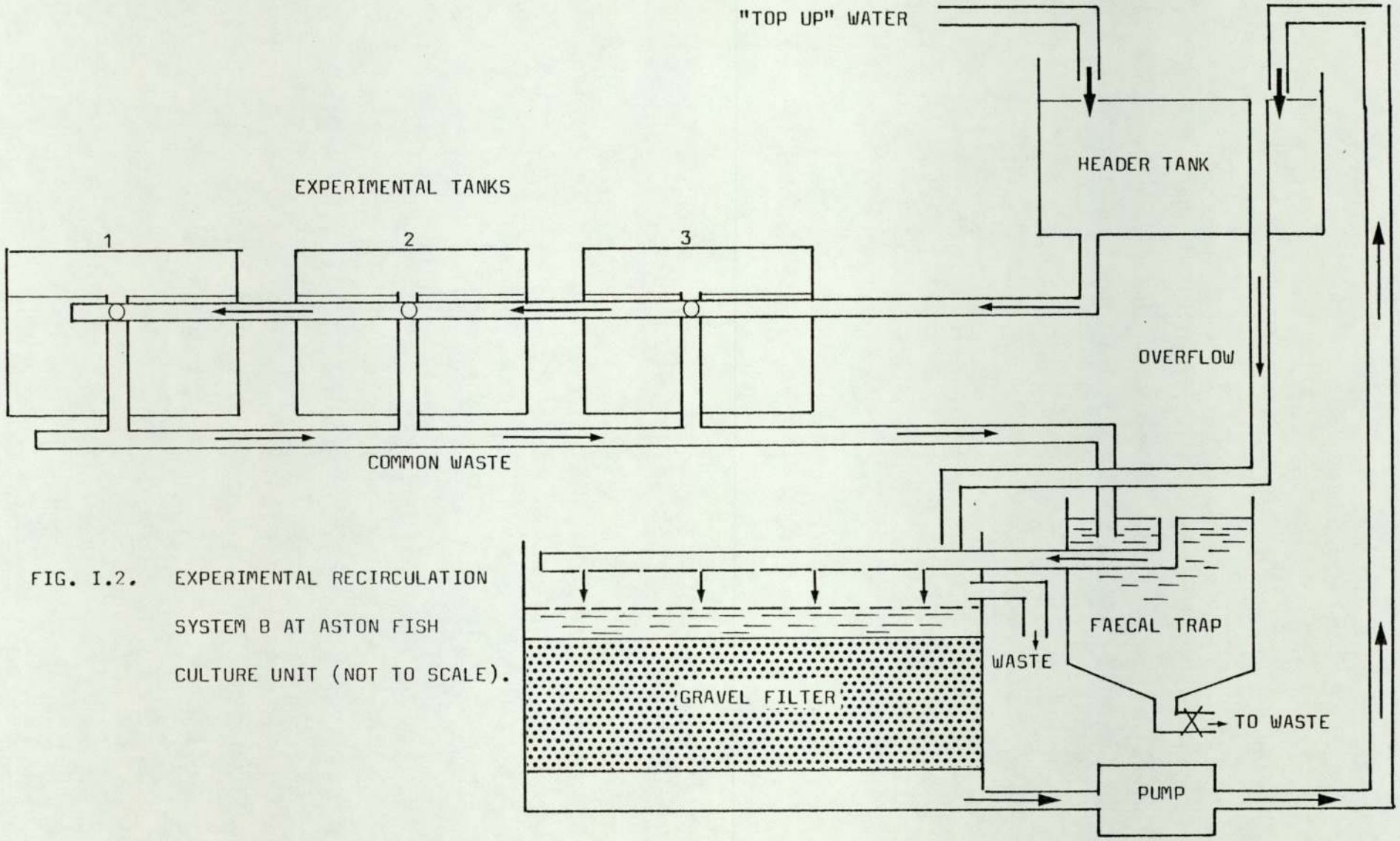
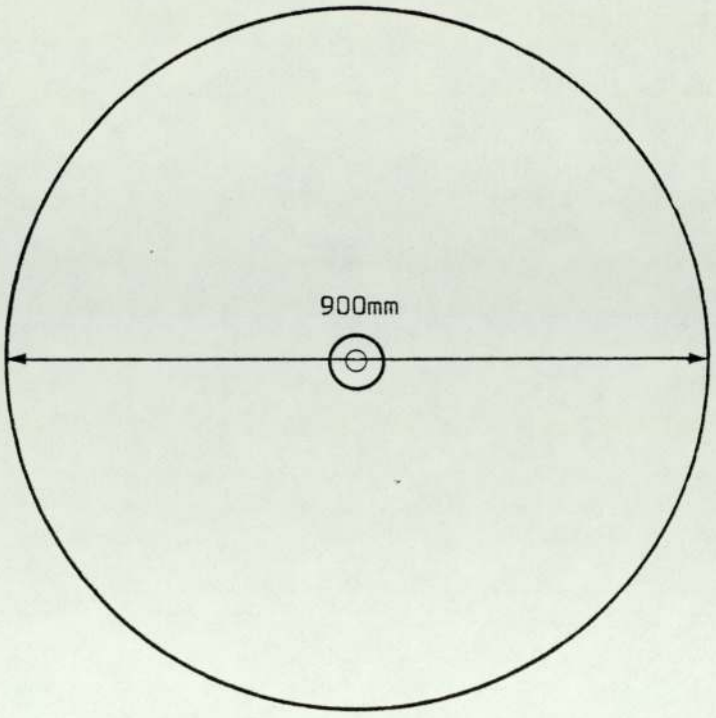
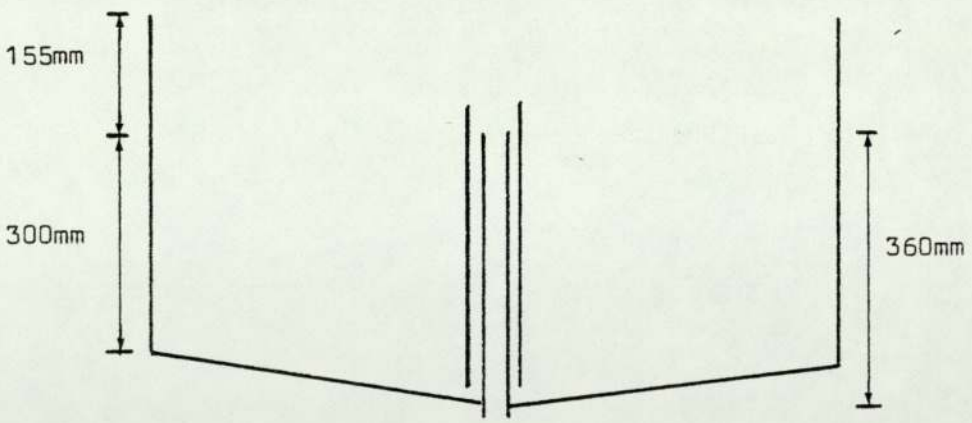


FIG. I.2. EXPERIMENTAL RECIRCULATION SYSTEM B AT ASTON FISH CULTURE UNIT (NOT TO SCALE).



PLAN VIEW

Scale 10mm = 100mm



VERTICAL SECTION

FIG. I.3. PLAN VIEW AND VERTICAL SECTION OF AN INDIVIDUAL TANK USED IN THE EXPERIMENTS CARRIED OUT AT ASTON FISH CULTURE UNIT.

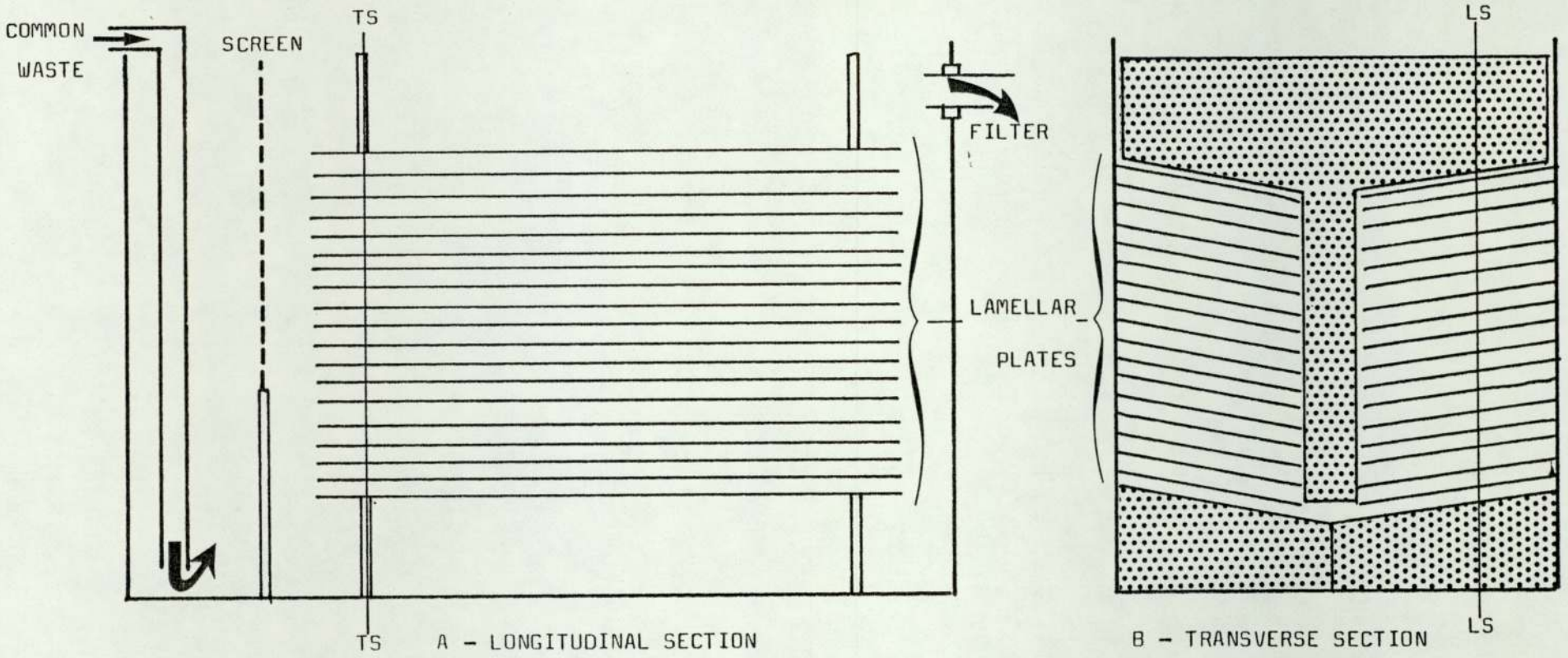


FIG. I.4. TRANSVERSE AND LONGITUDINAL SECTIONS OF THE LAMELLAR-TYPE FAECAL TRAP USED ON SYSTEM A (NOT TO SCALE).

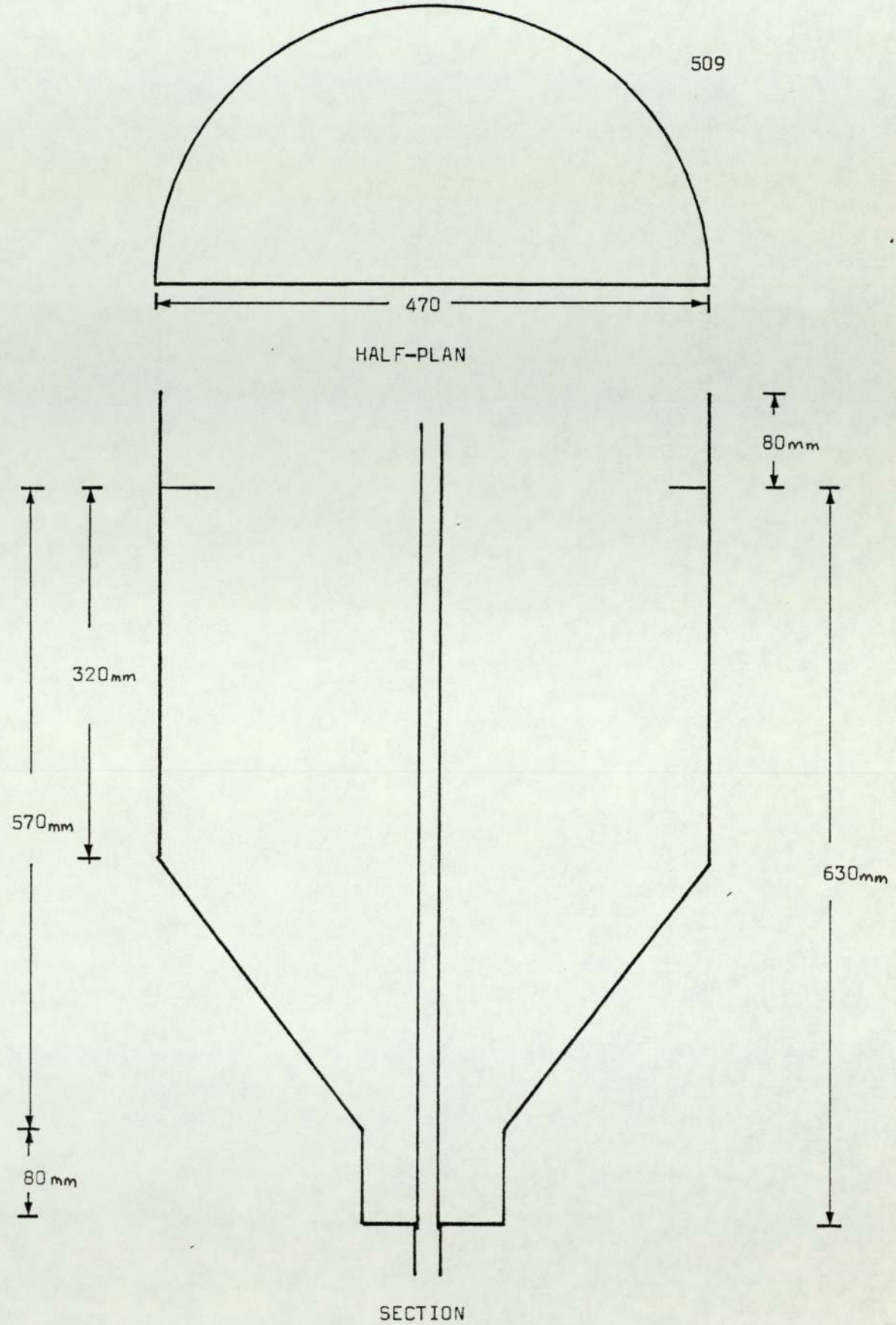


FIG. I.5. HALF-PLAN AND VERTICAL SECTION OF FAECAL TRAP USED ON SYSTEM B (NOT TO SCALE).

APPENDIX II

WATER QUALITY PARAMETERS IN THE FISH HOLDING FACILITY AT THE UNIVERSITY OF ASTON.

All experimental systems at the Universities' Fish Culture Unit are run on Birminghams' tap water, being pumped from the Elan Valley in Wales. The water quality was monitored routinely in the two systems throughout the three years of this study. Samples were taken every few days for determination of temperature, pH, ammonia, nitrates, dissolved oxygen and water hardness.

The dissolved oxygen was determined by the Winkler method and expressed in mg litre^{-1}. In theory, the white precipitate of manganous hydroxide produced by the action of alkaline potassium iodide on manganous chloride or sulphate, is converted by the dissolved oxygen into a brown precipitate of manganic hydroxide. The addition of sulphuric acid liberates free iodine which is titrated against this sulphate with starch as indicator. At no time during the course of an experiment did the dissolved oxygen fall below 7 mgL^{-1}, well above the suggested minimum level for rainbow trout of 5 mgL^{-1}.

The temperature, measured by mercury thermometer, was taken in the three tanks and a mean recorded. Generally, whilst experiments were in progress, the temperature was maintained at 12^{o}C $\pm$ 2^{o}. A chiller in the Unit maintains Summer temperatures below lethal temperatures and a gas-fired space heater maintains Winter water temperatures well above lower growth-limiting values.

The pH was determined with a meter and fluctuates around a neutral value. At high pH, the NH_4^+ ion is not favoured and subsequently higher concentrations of unionised ammonia develop. The ammonia present in the system is the end product of protein

metabolism, being excreted through the gills and in recycled water could possibly build up to toxic levels. The ammonia in solution exists as ionised (NH_4^+) and unionised forms (NH_3) and it is only the latter which is toxic to fish. The relative proportions of these two depends primarily on pH although the temperature of the water exerts a modifying influence. Ammonia levels were well below toxic levels to rainbow trout throughout this study.

The nitrate levels in the water closely follow those of ammonia since they are formed by the biological oxidation of ammonia by the nitrifying bacteria in the filter bed. Possibly of more importance are nitrites which are an intermediate in the process of nitrification, these can accumulate as a consequence of elevated levels of ammonia or incomplete nitrification. Nitrites may be toxic by converting haemoglobin to methaemoglobin which combines irreversibly with oxygen. Nitrate content was similarly measured with a probe and expressed in ppm.

Water hardness was determined using BDH water hardness tablets. One tablet added to 100 ml water sample and stirred with 2 ml ammonia buffer until dissolved. This is titrated against 0.02 N EDTA, to give the total hardness (ppm). The water was relatively soft averaging approximately 30 ppm.

Tables II.1 and II.2 show measurements of temperature, pH, NH_3, O_2, NO_3+NO_2 and hardness in the two systems used during this study.

TABLE II.1 WATER QUALITY SYSTEM A.

DATE	TEMP (^{o}C)	pH	O_2 (mgL^{-1})	NH_3 (ppm)	NO_3+NO_2 (ppm)	HARDNESS (ppm)
23/10/78	14.1	6.7	11.0	0.1	6.5	24.0
27/10/78	15.5	-	11.1	"	7.0	23.0
30/10/78	15.5	6.9	9.2	"	3.0	22.0
3/11/78	15.8	7.0	9.5	"	2.5	22.0
6/11/78	16.0	6.7	10.0	"	2.4	20.0
10/11/78	14.5	7.0	9.9	"	1.8	22.0
13/11/78	13.0	6.3	9.8	0.1	5.5	24.0
17/11/78	11.5	6.5	10.8	0.1	5.7	26.0
20/11/78	11.0	6.7	10.4	"	4.5	34.0
23/11/78	11.0	6.5	10.1	0.2	4.5	32.0
27/11/78	10.5	6.6	10.8	0.1	3.0	28.0
4/12/78	9.8	6.7	10.6	"	3.0	22.0
8/12/78	9.0	-	11.0	"	3.9	33.0
12/12/78	10.5	6.9	12.5	"	3.1	29.0
15/12/78	9.6	6.7	10.0	0.1	3.5	31.0
21/12/78	8.5	6.6	11.0	0.1	3.8	30.0
2/1/79	8.1	6.7	11.2	0.1	2.9	30.0
5/1/79	8.9	6.6	11.1	0.1	3.5	27.0
8/1/79	9.0	6.4	10.4	0.1	4.0	23.0
12/1/79	7.5	6.4	10.8	0.1	3.8	21.0
15/1/79	8.5	6.6	10.8	0.1	3.5	27.0

TABLE II.1 Contd.....

DATE	TEMP	pH	O_2	NH_3	NO_3+NO_2	HARDNESS
19/1/79	7.9	6.7	11.2	0.1	3.6	25.5
25/1/79	8.0	6.7	10.1	"	3.8	25.5
29/1/79	7.7	6.5	10.8	"	4.3	22.0
2/2/79	8.5	6.5	10.3	"	5.5	22.5
6/2/79	8.5	6.7	10.7	"	5.1	28.5
9/2/79	7.9	6.7	10.7	0.4	5.0	21.0
13/2/79	7.8	6.6	9.8	0.3	6.7	23.5
16/2/79	7.9	6.6	11.4	0.1	4.5	22.0
20/2/79	7.0	6.8	11.0	0.3	3.5	-
23/2/79	8.0	6.7	-	0.1	4.0	23.0
27/2/79	8.5	6.7	10.7	0.2	4.5	23.5
2/3/79	8.5	6.8	-	0.1	4.5	28.5
5/3/79	9.0	6.6	10.3	0.2	4.9	29.0
9/3/79	10.4	6.9	-	0.4	-	26.0
12/3/79	9.5	6.9	9.2	0.5	4.0	23.5
16/3/79	8.6	6.6	-	0.2	4.6	23.5
19/3/79	8.5	6.9	9.3	0.2	4.0	32.5
23/3/79	9.0	6.8	-	0.2	3.5	21.0
27/3/79	9.5	6.8	9.3	0.2	4.7	19.5
2/4/79	10.0	6.7	10.0	0.2	4.5	20.0
6/4/79	10.0	6.8	-	0.3	4.4	-
9/4/79	10.1	6.8	10.2	0.1	3.9	22.0

TABLE II.1 Contd.....

DATE	TEMP	pH	O_2	NH_3	NO_3+NO_2	HARDNESS
1/10/79	14.5	6.7	6.8	0.3	20.0	50.0
9/10/79	18.0	5.2	6.7	0.6	45.0	51.5
17/10/79	13.5	5.4	7.0	0.7	45.0	67.0
25/10/79	13.0	7.0	7.0	0.4	61.0	48.5
30/10/79	13.0	6.8	8.4	0.3	39.0	35.0
7/11/79	12.2	7.2	9.7	0.1	33.0	40.5
12/11/79	10.0	7.1	10.5	0.7	15.0	25.0
20/11/79	10.7	7.1	10.4	0.1	15.0	23.5
28/11/79	11.0	7.0	9.9	0.2	27.0	27.0
3/12/79	11.7	7.1	9.7	0.1	25.0	28.5
11/12/79	10.5	7.2	10.4	0.1	14.0	29.0
28/12/79	10.0	6.7	10.7	"	15.0	26.5
3/1/80	8.7	6.8	10.7	"	17.0	27.0
7/1/80	10.0	6.6	9.8	0.1	20.0	25.5
15/1/80	9.0	6.8	10.6	0.1	24.0	29.5
22/1/80	11.0	7.1	8.2	0.1	65.0	57.0
30/1/80	11.0	7.1	8.4	0.1	13.5	26.5
5/2/80	11.7	6.9	7.1	0.1	65.0	43.0
12/2/80	12.0	7.2	7.4	0.1	80.0	41.0
20/2/80	11.0	6.9	7.8	0.1	12.0	25.5
26/2/80	9.8	7.1	9.6	0.1	9.5	24.0
29/2/80	13.0	6.9	7.1	0.2	20.0	27.0

TABLE II.1 Contd.....

DATE	TEMP	pH	O_2	NH_3	NO_3+NO_2	HARDNESS
20/4/79	13.5	6.7	9.1	0.1	5.0	34.5
27/4/79	12.5	6.3	8.9	0.2	4.0	20.0
2/5/79	9.0	6.8	10.0	0.1	3.8	21.0
11/5/79	11.3	7.1	9.6	0.2	4.2	29.5
15/5/79	12.0	7.1	9.0	0.3	5.2	29.0
18/5/79	10.7	7.0	9.7	0.2	5.0	32.0
24/5/79	12.0	6.9	9.7	0.1	5.0	33.0
1/6/79	11.5	7.0	9.5	0.2	6.2	35.5
4/6/79	12.5	6.9	-	0.2	6.6	31.0
11/6/79	12.5	6.9	8.8	0.2	7.2	32.5
26/6/79	13.4	8.7	9.0	0.2	10.0	35.0
3/7/79	13.5	9.1	7.1	0.2	8.0	26.0
9/7/79	13.6	7.1	6.7	0.2	13.0	32.0
16/7/79	16.2	6.7	7.3	0.6	9.5	29.5
23/7/79	14.5	7.3	6.7	0.5	6.8	21.0
30/7/79	17.2	6.7	6.9	0.3	6.2	21.5
7/8/79	17.0	6.9	6.1	0.5	5.3	21.0
13/8/79	17.2	6.1	6.9	0.5	5.5	20.0
29/8/79	14.0	6.9	6.5	0.3	8.5	23.5
10/9/79	15.5	6.5	7.0	0.2	11.0	31.0
18/9/79	15.0	7.0	7.0	0.2	13.0	36.0
25/9/79	14.0	7.2	7.2	0.2	7.3	32.0

TABLE II.1 Contd.....

DATE	TEMP	pH	O_2	NH_3	NO_3+NO_2	HARDNESS
3/3/80	12.0	6.7	7.1	0.2	12.0	25.0
7/3/80	12.5	7.0	6.5	0.2	12.0	22.0
12/3/80	13.5	6.9	6.3	0.2	15.0	25.5
18/3/80	11.5	6.8	6.7	0.3	13.0	20.5
21/3/80	11.0	6.8	8.0	0.1	15.0	24.5
25/3/80	12.2	7.1	7.3	0.1	16.0	28.5
28/3/80	12.5	6.8	7.5	0.1	16.0	26.5
25/4/80	10.5	7.0	9.8	0.1	13.0	20.5
29/4/80	11.5	6.9	9.6	"	16.0	20.5
2/5/80	11.5	7.2	9.7	"	9.5	21.0
9/5/80	11.5	7.1	9.5	"	-	22.0
15/5/80	12.0	6.9	8.6	0.1	10.0	21.0
20/5/80	12.8	6.8	8.8	"	13.0	20.5
23/5/80	12.0	6.9	8.9	"	11.0	23.5
28/5/80	12.8	6.9	7.9	"	25.0	34.0
31/5/80	12.0	7.2	7.4	"	30.0	34.0
3/6/80	12.7	6.8	7.9	"	37.0	36.0
6/6/80	13.5	6.8	8.2	0.2	34.0	33.0
10/6/80	14.5	7.0	7.2	0.1	8.5	31.5
13/6/80	14.5	7.2	7.8	"	6.8	30.5
25/6/80	13.5	6.8	8.5	"	6.5	22.5
27/6/80	13.3	6.8	6.5	0.2	9.3	24.0

TABLE II.1 Contd.....

DATE	TEMP	pH	O_2	NH_3	NO_3+NO_2	HARDNESS
1/7/80	12.5	6.6	7.7	0.2	15.0	25.5
8/7/80	14.5	6.9	8.4	0.1	14.3	21.0
11/7/80	13.0	6.5	-	0.1	11.0	24.0
15/7/80	13.0	6.4	8.3	0.1	-	27.0
21/7/80	12.7	6.5	7.6	0.1	16.0	25.5
24/7/80	14.5	6.4	-	0.1	15.0	25.0
28/7/80	13.5	6.5	8.6	0.1	15.0	24.0
1/8/80	13.5	6.5	-	0.1	13.0	23.5
6/8/80	13.5	6.5	8.2	0.1	14.0	26.3

TABLE II.2 WATER QUALITY SYSTEM B

DATE	TEMP (^{o}C)	O_2 (mgL^{-1})	pH	NH_3 (ppm)	NO_3+NO_2 (ppm)	HARDNESS (ppm)
28/12/79	12.0	10.7	6.7	0.1	14.0	23.5
3/1/80	10.5	10.9	6.9	"	14.5	23.5
15/4/80	11.5	11.2	6.9	"	6.5	20.5
22/4/80	11.5	10.2	7.1	0.1	5.0	21.5
25/4/80	12.5	9.6	7.1	0.1	7.3	22.5
29/4/80	13.5	9.8	7.0	0.1	5.9	19.5
2/5/80	13.5	9.7	7.2	"	5.5	21.0
9/5/80	11.8	10.2	7.2	0.1	-	19.5
15/5/80	14.5	8.8	7.0	0.3	4.3	20.0
20/5/80	15.0	9.3	7.0	0.1	4.9	19.0
23/5/80	11.5	9.4	7.0	0.1	3.5	22.0
28/5/80	13.0	8.7	7.1	0.2	5.8	30.5
31/5/80	12.5	"	7.3	0.4	10.0	33.0
3/6/80	12.0	8.8	7.0	0.3	18.0	32.0
7/6/80	12.5	10.6	"	0.1	20.0	31.0
10/6/80	12.5	8.6	"	0.2	14.0	32.5
13/6/80	13.0	10.3	7.1	0.1	8.2	31.5
25/6/80	12.7	8.3	6.8	0.1	6.5	22.0
27/6/80	12.0	8.2	6.9	0.4	10.5	24.5
1/7/80	12.5	10.5	6.9	0.1	4.9	21.0

TABLE II.2 Contd.....

DATE	TEMP	O_2	pH	NH_3	NO_3+NO_2	HARDNESS
8/7/80	12.5	10.4	7.1	0.1	5.5	21.0
11/7/80	13.5	-	6.8	"	4.2	22.0
17/7/80	13.0	9.8	6.8	"	-	21.5
21/7/80	13.0	10.0	7.2	"	4.5	22.0
24/7/80	17.0	8.2	7.0	0.1	2.7	20.0
28/7/80	13.5	10.7	7.1	0.1	3.9	22.0
1/8/80	13.5	-	7.0	"	3.5	21.5
6/8/80	13.5	9.4	7.1	"	3.2	22.0
12/8/80	12.7	10.1	6.7	"	9.0	24.0
19/8/80	11.8	10.9	7.3	"	9.0	25.5
27/8/80	12.0	10.2	6.7	"	14.0	30.0
2/9/80	13.0	9.9	6.7	"	18.0	30.5
9/9/80	12.5	9.7	7.1	"	5.6	25.5
16/9/80	13.0	9.7	7.0	"	6.5	22.5
24/9/80	12.0	9.9	6.7	"	10.0	24.5
30/9/80	11.5	10.0	6.9	"	8.5	24.5
20/10/80	12.0	10.1	7.0	0.3	6.0	23.0
24/10/80	11.5	10.0	7.0	0.1	2.5	21.5
28/10/80	11.5	10.0	7.0	0.1	4.0	21.5
31/10/80	10.5	10.3	6.8	0.1	8.0	23.0
2/11/80	10.8	10.2	6.8	0.1	21.0	28.5

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