REPRODUCTION IN MALE SALMONIDS WITH SPECIAL REFERENCE TO THE PROBLEMS AND CONTROL OF MATURATION IN FISH CULTURE

ROBERT HARBIN

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SUMMARY

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Reproduction in male salmonids with special reference to the problems and control of maturation in Fish Culture

Robert HARBIN
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The commercial problems accompanying the sexual maturation of male salmonids include a reduction in growth rate, the appearance of secondary sexual characteristics and changes in body composition. Information is presented on the development and extent of these changes among maturing rainbow trout and the endocrine control system by which such effects are controlled is reviewed.

In particular, the roles of the naturally occurring androgens, testosterone and 11-ketotestosterone, are examined. When administered to immature rainbow trout 11-ketotestosterone was more potent in inducing male secondary sexual characteristics, while testosterone exerted a greater depression of dry matter digestibility. Changes in this latter parameter are suggested to be an important component of the decreased food conversion efficiency observed in mature fish.

The influences of environmental factors upon male sexual maturation are examined and the predominant controlling effect of photoperiod confirmed. Thus under long days male maturation was advanced by two months, while constant short days delayed maturation by four months. Although changes in ration level produced clear differences in growth rate, no differences in either the timing or extent of male maturation were seen in rainbow trout fed at varying levels.

Methods by which male maturation may be controlled are reviewed and evidence is presented on three main approaches: immune manipulation, estrogen induced feminization and high dose androgen sterilization. Injection of gonad extract and adjuvant failed to produce significant changes in the extent of male maturation or gonadal development.

Estrogen treatment of fry produced a partial feminization of rainbow trout, though complete feminization was only achieved together with undesirable side effects at higher steroid doses. High dose androgen treatment produced a delay in male maturation and partial sterility, the extent of which varied between sites. Possible reasons for the variable response to hormonal treatment are examined.

Key words : SALMONIDS, MALE, MATURATION, CONTROL, CULTIVATION.

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CHAPTER 1

GENERAL INTRODUCTION

Salmonid fish cultivation represents a major economic facet of the world's aquaculture effort. Although the controlled farming of fish is an ancient activity, the intensive cultivation of salmon and trout is of relatively recent origins. Salmonid cultivation has been an important part of European aquaculture only since the importation of rainbow trout at the end of the last century. Developments in Denmark, and to a lesser extent, France and Italy, have given rise to the intensive cultivation of rainbow trout for the table and these methods have been widely adopted and refined in the United Kingdom (Drummond Sedgwick, 1973).

Within the U.K. the economic importance of salmonid cultivation is emphasised by statistics showing salmon and trout production to account for over 90% of the value of all farmed species (Table 1·1). Forecasts have suggested that the expansion in intensive trout farming will continue into the 1980's with an annual production level of 15 - 20,000 tonnes/year by the end of the decade (Purdom, 1979). Allied to the expansion of the trout industry, considerable effort is now directed towards the cultivation of salmon in sea cages or pens. The Atlantic salmon (Salmo salar) is an especially important farmed species in Norway (Edwards, 1978) and the Scottish salmon farming industry is developing rapidly, while Pacific salmon, and especially coho salmon (Oncorhynchus kisutch), are farmed in Russia, Canada, U.S.A., Japan and recently France (Donaldson and Hunter, 1982a).

This expansion of salmonid aquaculture has led to the focusing of research interest on a number of areas which may limit production. Thus improvements have been made in the fields of feed formulations, disease control, environmental requirements and hatchery design. One particular area to which much research effort has been directed is that of reproduction. In order to optimize production, it is necessary for the producer to manipulate and control the reproductive cycle of his stock. One especially important aspect of the capacity to influence reproduction among salmonids is the ability to control the sexual maturation of male fish. The attainment of sexual maturation, especially of males, by production stocks of salmon and rainbow trout constitutes an important constraint to the further development and expansion of salmonid aquaculture. Donaldson and Hunter (1982a) have listed the potential management applications of techniques which either result in sterile or largely female populations. (Tables 1•2 and 1•3). The commercial advantages of such techniques result largely because of the removal of male maturation, which poses special problems for the fish farmer.

The nature of these problems and the possible methods by which such manipulation may be accomplished will be examined in this work. As Tables 1•2 and 1•3 illustrate, the advantages of such control would be considerable and would allow producers to maximize production by avoiding the detrimental effects of salmonid sexual maturation.

Table 1•1

Current Production of farmed species in the U.K. (1980)*

Species	Production (tonnes)	Production (value £'000)
Trout	6000	9900
Salmon	700	3850
Eels	200	600
Turbot	50	90
Oysters	-	230
TOTAL	6950	14670

(*data from National Farmers Union)

Table 1•2

Potential management applications of techniques for increasing the proportion of females in salmon culture

A) To increase egg production from a given number of broodstock or hatchery escapement.

B) To reduce size of hatchery escapement required to provide a given egg take.

C) Elimination of precocious male (jack) salmonids.

D) To reduce the cost of growing and maintaining aquaculture broodstock to produce a given egg take.

E) To increase the landed value of the commercial catch where the ovary has a higher value than the testis.

Table 1•3

Potential management applications of techniques for the production of sterile salmonids

A) To maximize growth by diverting resources which would otherwise be utilized for gonadal development.

B) To prevent production of precocious male (jack) salmon.

C) To permit year-round marketing of adult salmonids where sexual maturation and the appearance of secondary sexual characteristics results in mortality and/or reduces the commercial value.

D) To present new management options by preventing the anadromous migration of sea-ranched salmonids.

E) To produce trophy-sized salmonids by extending the life cycle beyond the normal time of spawning and death in Oncorhynchus species.

(from Donaldson and Hunter, 1982a)

Problems of Maturation

Sexual maturation may be defined as the complex of morphological, biochemical and behavioural changes associated with the attainment of full reproductive ability. Whilst the production of functional gametes is the ultimate end point of maturation, other changes accompany and prepare for this event. These other changes may be grouped together under the term secondary sexual characteristics. Among maturing salmonids, and especially male salmonids, these secondary sexual characteristics are especially pronounced and their development, coupled with the other changes accompanying maturation, has a number of adverse effects upon commercial salmonid production. (Bromage et al, 1979; Bye and Lincoln, 1979).

Detrimental changes in appearance occur with the attainment of sexual maturity in salmonids. Darkening and thickening of the skin and the development of the hooked lower jaw or kype renders mature male salmonids less acceptable to the consumer due to their unpleasant appearance (Robertson and Wexler, 1960a). This is further exacerbated by the presence of fungal lesions (Richards and Pickering, 1978) which frequently accompany damage caused by fighting among male fish (Roberts, 1978). Due to carcass changes, the flesh of mature fish is less palatable and appears pale and 'watery' when contrasted with that of immature fish (Davidson and Shostrom, 1936; Lane, 1979).

Both male and female fish show a depression of growth compared with immature fish of similar size (Nomura, 1963; Bye and Lincoln, 1979; Whitehead, 1979). This growth depression is coincident with the time of spawning of both male and female fish, though females recover from these adverse growth effects more fully than do males. Such a change

in growth pattern with maturity reflects a shift towards gonadal growth at the expense of somatic growth. To what extent this reduced growth results from changes in appetite, reduced food conversion or simply the diversion of energy towards gonadal growth remains uncertain. Among mature fish the testes normally contribute between 3 - 8% of body weight, while the female ovary can account for 10 - 14% of body weight (Roberts, 1978). As gonadal growth occurs at the expense of extra muscle and is an item which is discarded when fish are sold 'dressed', the diversion of food resources to the gonad is obviously unwelcome. In the case of a 100 tonne/year producer, gonadal maturation could cost £5 - 10,000 simply in lost production weight.

Changes in skin structure also increase the succeptibility of mature fish to bacterial and fungal attack, especially lesions due to the fungus Saprolegnia (Richards and Pickering, 1978). Disease resistance is frequently lowered and mortalities among spawning fish are frequently high (Roberts and Shepherd, 1974). This is especially the case when salmonids are grown in sea water, when high mortalities of mature males will occur (Drummond Sedgwick, 1973; Edwards, 1978; Gjedrem and Gunnes, 1978). Both male and female Pacific salmon do not survive spawning, whilst in the Atlantic salmon, the majority of fish surviving spawning and returning to sea as kelts are female.

Although both male and female salmonids suffer profound physiological and morphological changes as a result of maturation, it is the male which incurs the most adverse effects and consequently suffers most from these processes.

Special problems associated with male maturation

As well as sexual maturation constituting a more damaging process in the male salmonid, the problems of maturation are further exacerbated in males by virtue of the timing of maturation. Among salmonids it is normal for the males to mature before the females. Thus in rainbow trout the male normally matures in its second winter, while the female does not mature until its third winter. Thus while maturation constitutes a drain on the profitability of salmonid culture, the extent of the loss depends upon the time at which fish are cropped. If rainbow trout are cropped before their second winter, normal male maturation will be avoided. Obviously such a strategy gives a limited production cycle and limits the size of the final product. At present the majority of trout producers in the U.K. produce 'portion-size' fish of approximately 250 g. As this size can normally be attained within two summers, normal male maturation poses no problem.

However, a varying proportion of male trout will mature before the second winter. Maturity may take place during the first year after hatch and in addition some salmon also mature early as parr (Shaw, 1840; Dodd et al., 1978). These mature males, known as precociously mature males, display some of the male secondary sex characteristics as well as showing reduced growth (Murphy, 1980a). Even though maturing at this early stage, they are capable of producing viable gametes (Jones and King, 1950; Thorpe, 1975; Thorpe and Morgan, 1980). The occurrence of precocious males has been widely reported in salmonids including the Atlantic salmon (Orton et al., 1938; Jones, 1940; Saunders and Henderson, 1965), rainbow trout (Johnstone et al., 1978; Schmidt and House, 1979) and Pacific salmon (Robertson, 1957; Gebhards, 1960; Donaldson et al., 1972b;Mackinnon and Donaldson, 1976). Among salmon, a more familiar

manifestation of precocity is the occurrence of grilse, fish which mature after a single sea year (Edwards, 1978).

The extent to which male maturation occurs under culture conditions is difficult to assess with accuracy (Forster - pers. comm.). Estimates depend upon the particular species and stock cultivated, the method of cultivation and the interaction of genetic and environmental factors. While the physiological control of normal reproduction and its relationship to environmental factors will be examined in later chapters, it is perhaps worth mentioning that precocious maturity may have a strong genetic component. Thorpe (1975) reported that the incidence of precocious male maturation among Atlantic salmon smolts varies with parental genotype. Schaffer and Elson (1975) also report that parental sea age influences the grilse/salmon ratio of offspring. The genetic factors involved in precocious maturation are however not simple. Moeller et al. (1976) provided evidence that genetic factors may be involved in the determination of maturation in the rainbow trout, but were not easily explained. The data of Glebe et al. (1980) show an increased proportion of precocious salmon parr when mature male parr are used as parents, but the results were not statistically significant.

Environmental factors have been implicated in precocity among early maturing rainbow trout, Atlantic and Pacific salmon, cultivated in sea cages or pens. (Saunders and Henderson, 1976; Edwards, 1978; Sutterlin et al., 1978). It has been suggested that precocity results from high feed levels associated with increased growth, though the interplay of growth, nutrition and salinity may be more complex. The extent of the commercial concern regarding early maturation has been

highlighted by a recent article (Needham, 1983), pointing out the unpredictable nature of this phenomenon amongst salmon stocks. Thus stocks of different racial origin, from different river systems, show considerable variation in their degree of early maturation. It appears that reducing the feeding rate and protein quality of diets reduced the extent of precocity among salmon and resulted in a fall in the number of grilse.

In addition to the problems posed by precocious maturation in the production of portion sized trout, both fresh and sea-water cultivation of rainbow trout and salmon to larger sizes is clearly limited by the occurrence of male maturation (and if a sufficiently long production cycle is employed, possibly also female maturation). Obviously the extent of the problem and possible approaches to its solution depend upon the type of cultivation practised and the marketing strategy adopted. The situation at Shearwater Fish Farming, where most of the large scale experiments described in this work were carried out, may be judged typical of the industry and provide a useful background to the investigation.

Shearwater Fish Farming

Shearwater Fish Farming Ltd. arose as an offshoot of the British Oxygen Company (BOC) New Venture Secretariat. In 1974 an experimental trout farm was commissioned at Low Plains, Armathwaite, Cumbria, to enable the development and application of high technology methods to fish farming, and especially the development of oxygenation techniques for aquaculture. Since then the activities of the Company have diverged into a wide range of fish farming projects, including turbot rearing at

Wylfa, Anglesey and a mixed salinity trout and salmon farm at Finnarts Bay, Stranraer. In 1980 control of Shearwater Fish Farming passed to Kraft Foods Ltd.

Production policy at present is for the Low Plains site to produce approximately 120 tonnes of portion-sized rainbow trout/year, with a production cycle of approximately 16 months. The second trout farm at Finnarts Bay also produces rainbow trout as well as acting as an experimental production unit for salmon and turbot. However, due to limitations in available water supply, both fresh and sea-water, it appears that a shift towards a smaller tonnage (approximately 60 tonnes/year) of larger rainbow trout in the 1·5 - 2 kg range would seem to be the main future of this site.

The situation at these two farms demonstrates the main features of trout farming practice in the U.K. Thus the bulk of production is aimed at the portion size market with a production cycle of less than two years. Here the main problem posed by male maturation is clearly that of early or precocious maturation.

The development of a market for larger trout, both for the table and for restocking angling waters, extends the production cycle beyond two years and thus normal male maturation becomes a major limiting factor. At Finnarts Bay maturation among male rainbow trout, maintained in mixed salinity water, has led to very high mortalities. If the production cycle is sufficiently extended, then female maturation will also become a problem when the females mature in their third winter.

The developments in the production of larger rainbow trout are paralleled by the approach to salmonid culture adopted in Norway,

where the bulk of production consists of fish larger than 1·5 kg (Edwards, 1978), and in Scotland where the development of cage cultivation of Atlantic salmon is becoming a significant industry. Again in these areas maturation poses a problem in limiting the length of available production cycle, which may be exacerbated by new culture techniques. Edwards reports that a number of Norwegian salmon farms have recently encountered high levels of male maturation after only one year at sea. Similar increases in grilse maturation have been reported to follow improved husbandry (Needham, 1983).

A further area where the control of maturation is of significance is the introduction of alien or new species such as coho salmon, which Unilever recently proposed to introduce for cultivation in Scotland. Fears have been expressed regarding the possible ecological implications of competition between the newcomer and the resident Atlantic salmon stocks, already depleted by netting and poaching. If the introduced fish could be prevented from maturing, then concern regarding possible environmental competition might be reduced.

Aims and Objectives

Clearly, the occurrence of sexual maturation, especially of male fish, is an important constraint upon salmonid aquaculture, which may become more important as developments such as sea cage cultivation expand. The commercial control of maturation and the prevention of its undesirable side effects, such as the changes in appearance and growth rate, are obviously of vital importance to the industry.

In order to examine methods of control, it is first necessary to consider the basic processes which control sexual maturation and in

parallel give rise to the various adverse changes associated with this process. This discussion occupies the initial chapters in this thesis and provides a rationale for the examination of possible control methods.

In the remainder of this thesis the possible control methods by which sexual maturation may be avoided will be examined and as far as possible their use under commercial conditions assessed. While this work was largely directed towards the production situation in operation at Shearwater Fish Farming, it has wider relevance within the fish farming industry.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Contents

GENERAL MATERIALS AND METHODS

Materials and methods having application to more than one section of this thesis are included in this chapter. Any specific materials and methods relevant to a particular experiment will be discussed in the detailed protocol for that experiment.

All experiments were carried out on rainbow trout (Salmo gairdneri, Richardson). The source of fish used in a particular experiment will be specified in the experimental details.

Unless specified otherwise, fish were fed on B.P. Mainstream trout food according to manufacturer's feeding tables (B.P. Nutrition Ltd.).

2·1 Fish handling

2·1·1 Anaesthesia

Any procedures, such as blood sampling, requiring anaesthesia were carried out following immersion of fish in a 1:20,000 solution of Benzocaine (ethyl - 4 - amino - benzoate) (B.D.H. Chemicals Ltd., Poole) for a period of approximately two minutes (Laird and Oswald, 1975). The required amount of Benzocaine was first dissolved in a small volume of acetone (10 ml) and added to the correct volume of water. Anaesthetic was mixed immediately before use.

2·1·2 Blood sampling

Blood samples were withdrawn from the Cuverian duct of anaesthetised fish using a hypodermic syringe (either 1 ml or 5 ml) and a 21G hypodermic needle. Following transfer to a clean plastic tube (Luckhams, LP3 tube), the blood samples were allowed to stand for 10 - 15 minutes at room temperature to allow clotting.

They were then centrifuged at 2,500 RPM for 10 minutes. Serum was pipetted into a clean labelled LP3 tube and stored at -20°C until required for assay.

Few losses were experienced as a result of this method of blood sampling and rapid sampling was possible, limited only by the time taken to anaesthetise the fish.

2•1•3 Tagging

Commercially available tags were used in all experiments in which fish were individually identified. Numbered plastic tags (Charles Neal Ltd., Finchley) were attached by means of a doubled strand of nickel-silver wire inserted through the dorsal musculature, anterior to the dorsal fin, via a pair of hypodermic needles.

Additionally, in some experiments fish were fin clipped as an aid to identification. If it was necessary to distinguish between only two groups, then the adipose fin of one group was clipped. Fin clipping was found to be fairly unsatisfactory in situations where a number of groups were to be distinguished, due to regeneration of pelvic and pectoral fins and the possible occurrence of fin nipping among fish.

2•2 Fish Holding facilities

Depending upon the location at which experiments were performed and the particular experimental design, fish were maintained in a variety of different systems. The particular system used will be specified in the experimental protocols, while the systems are described below.

2•2•1 Aston University

a) Recirculating system

This system utilized a pumped recirculation design, allowing the bulk of the water to be recirculated with only a small volume (approximately 5 - 10%) of fresh ('make-up') water to allow for spillage and evaporation.

A typical system (Diagram 2•2•1•1) consisted of 3 x 1 metre diameter circular fibre glass tanks, gravity fed from a header tank at a flow rate of around 4 litres/minute to each tank. Each tank was connected to a common waste pipe feeding a faecal trap, which allowed settlement of faecal solids.

The outflow from the faecal trap fed a gravity filter bed, which also received some outflow from the header tank. An overflow from the filter bed carried any surplus water to waste. Water from the base of the filter was pumped back to the header tank by means of an electric pump (Beresfords Ltd.) protected by an inline filter.

During the course of experiments, oxygen saturation, ammonia nitrates, pH and temperature were monitored to allow the maintenance of suitable water quality.

At weekly intervals the common waste pipe was cleaned, as was the inline filter. The faecal trap was emptied daily. At approximately monthly intervals, or when indicated by a build-up of ammonia and nitrates, the gravel filter bed was dug over and the gravel washed.

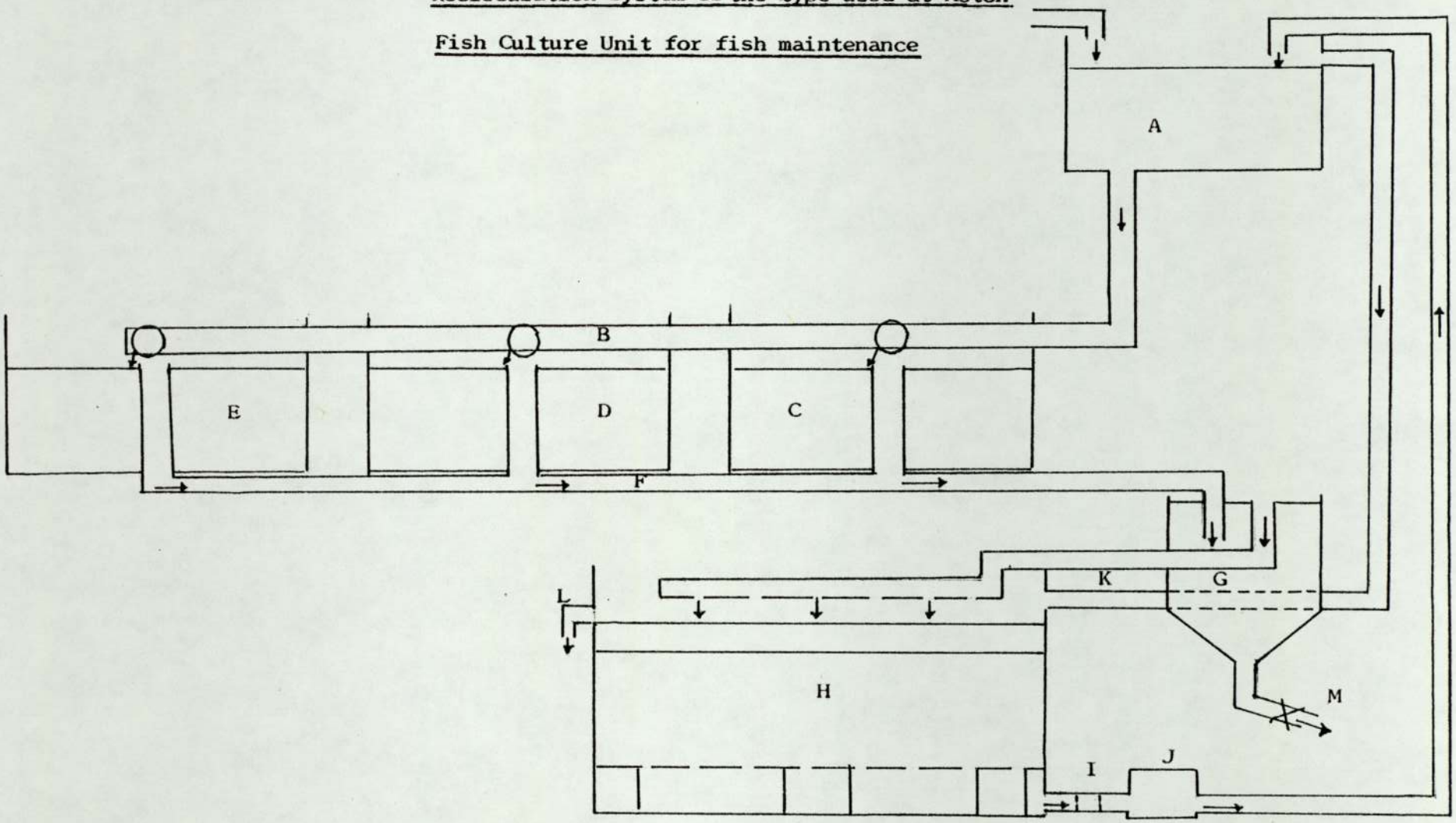
Recirculation system of the type used at Aston Fish Culture Unit for fish maintenance (Diagram 2•2•1•1)

Key

A.	Header tank
B.	Common feed pipe
C,D,E.	Tanks
F.	Common waste pipe
G.	Faecal trap
H.	Biological filter
I.	In line filter
J.	Pump
K.	Overflow from header tank
L.	Overflow from filter
M.	Discharge for faecal trap

Diagram 2·2·1·1

Recirculation System of the type used at Aston Fish Culture Unit for fish maintenance



b) Oxyder system

In certain experiments fish were maintained in an oxyder, a commercially available production unit (Field Stream and Covert Ltd., Meriden). This unit utilized a through flow of mains water of approximately 12 litres/minute. The carrying capacity of the unit was boosted by means of an oxygenating pump drawing air down a pipe and through a venturi. The unit was drained by a sump, protected by screens. A series of movable screens allowed experimental fish to be segregated into groups.

2•2•2 Shearwater Fish Farming

a) Low Plains

Experimental fish were maintained in a variety of systems at Low Plains, depending upon experimental design and size of fish. Early feeding fry were maintained in 400 litre shallow fry tanks, while larger fish were kept in 800 litre square tanks at a flow rate of approximately 60 litres/minute. Water was oxygenated to 100% saturation, at a constant temperature of 9°C and pH was 6•6.

b) Finnarts Bay

At Finnarts Bay, fish were maintained in 800 litre fibre glass tanks. Water supply was fresh water at varying temperature, depending upon time of year (ranging from 3°C to 15°C).

2•3 Assay techniques

2•3•1 Radioimmunoassay of Serum Testosterone

Introduction

The method used was adapted from a protocol supplied with a commercially available antiserum (Steranti Ltd., St. Albans). The method

used a specific antibody, a tritiated tracer and dextran charcoal step for separation of bound and free steroid.

Antiserum (Anti-testosterone)

A commercially available antiserum was used in all assays. (Anti-testosterone-3- CMO, BSA.- Steranti A002). This represented a pool of high titre, high specificity bleeds of immunised New Zealand White Rabbits produced by immunization with testosterone - 3 - O (carboxymethyl) oxime - bovine serum albumin. This was supplied in lyophilised form, which on reconstitution was normally sufficient for 1,000 determinations when used at the recommended dilution.

Reconstitution was effected by the addition of 1 ml of RIA buffer and gentle mixing. This stock solution was dispensed into 100 µl aliquots and stored at -20°C. For use in assays each aliquot was diluted with a further volume of buffer to give a working solution. Each batch of antiserum was tested before use to determine the strength of working solution giving 50% Bound ^{3}H testosterone in the absence of competing, unlabelled steroid.

Tritiated Testosterone Solution

Assay label

High specific activity tritiated testosterone (1, 2, 6, 7 - ^{3}H - testosterone) was supplied by the Radiochemical Co., Amersham). (Specific activity 83 Ci/m Mol.). From this original solution containing 250 µ Ci in 5 ml toluene, a working solution of approximately 20,000 DPM/100 µl was prepared by drying down a 40 µl aliquot and dilution with 20 ml RIA buffer.

Recovery label

Assay label was diluted 1 : 10 with RIA buffer to give recovery label containing approximately 2,000 DPM/100 µl.

Reagents

Scintillation solution

P.P.O (2, 5 diphenyloxazole)	20 g
Tolulene (Analar)	2.5 l.
2 - Ethoxyethanol (Analar)	2.5 l.

RIA Assay Buffer

Sodium Dihydrogen Phosphate ($Na\ H_2PO_4\ 2H_2O$)	4·37 g
Disodium Hydrogen Phosphate ($Na_2\ HPO_4\ 12\ H_2O$)	21·84 g
Sodium Azide (NaN_3)	1·0 g
Sodium Chloride (NaCl)	9·0 g
Gelatine *	1·0 g

Made up to a litre with distilled water after adjusting the pH to 7·0 with NaOH.

(*Dissolve in a little warmed distilled water before addition.)

Testosterone Standard

A stock solution of testosterone supplied by Steranti (S002), containing 10 ng/100 µl testosterone in ethanol, was used to prepare a working standard. 100 µl of the stock solution was diluted with 10 ml acetone giving a working standard of 1 pg/µl testosterone.

Dextran Coated Charcoal

'Separex' Dextran coated charcoal tablets were used to separate bound and free steroid (Steranti). Each tablet produces a fine and homogenous suspension of Dextran coated charcoal when dissolved in 50 ml of RIA buffer.

Preparation of glassware

Assays were carried out in glass tubes (8 x 1 cm). Before use tubes were soaked overnight in 'Decon' (B.D.H. Chemicals Ltd., Poole), transferred to fresh Decon in an ultrasonic bath and cleaned for one hour. Tubes were then washed overnight in running tap water, rinsed in distilled water and dried in a 60°C oven.

Assay Method

Determination of recovery

100 µl aliquots of serum were pipetted into glass tubes and 100 µl of recovery label added to each tube. Tubes were briefly mixed on a 'vortex' mixer and incubated overnight at 4°C.

Two 100 µl aliquots of recovery label were pipetted into two scintillation vials for determination of total recovery counts.

Extraction

2 ml of ethyl acetate (Analar) was added to each tube, which were capped and placed on a rotating mixer for 30 minutes. Tubes were spun for two minutes at 1,000 RPM at 4°C and a 200 µl aliquot removed for assay and one of 400 µl to complete determination of recovery. In the case of samples having very high levels of testosterone, the aliquot taken for assay was reduced to 100 µl.

The portion for assay was pipetted into a clean tube and dried down at 30°C in a vacuum oven. Recovery counts for each sample were determined by counting the 400 µl aliquot in 10 ml scintillant.

Standard Curve

A standard curve was prepared by pipetting out a series of

volumes (0 - 1000µl) of testosterone working standard (1 pg/µl). These were dried down at the same time as the ethyl acetate extracts. They were then assayed as normal samples and the percentage binding of ^{3}H testosterone plotted against the amount of competing steroid on 3 cycle log paper (Chartwell 6232) to give a standard curve for the assay (Diagram 2•3•1•1).

A fresh standard curve was prepared with each assay.

Blanks and Lateral Standards

An ethyl acetate blank was run with each assay. A non-specific binding blank was also run with assays consisting of a tube in which 100 µl of RIA buffer replaced the 100 µl of Antisera in a normal O std tube.

Assay Procedure

100 µl antiserum at working dilution and 100 µl of ^{3}H Testosterone (20,000 DPM approximately) were added to each tube. At the same time two 100 µl aliquots were pipetted into vials for counting to determine Total counts (assay).

Tubes were vortexed briefly and incubated overnight at 4°C. 500 µl 'Separex' Dextran coated charcoal was added and tubes were vortexed. After standing for 15 minutes at 4°C, they were spun at 2,000 RPM for ten minutes at 4°C.

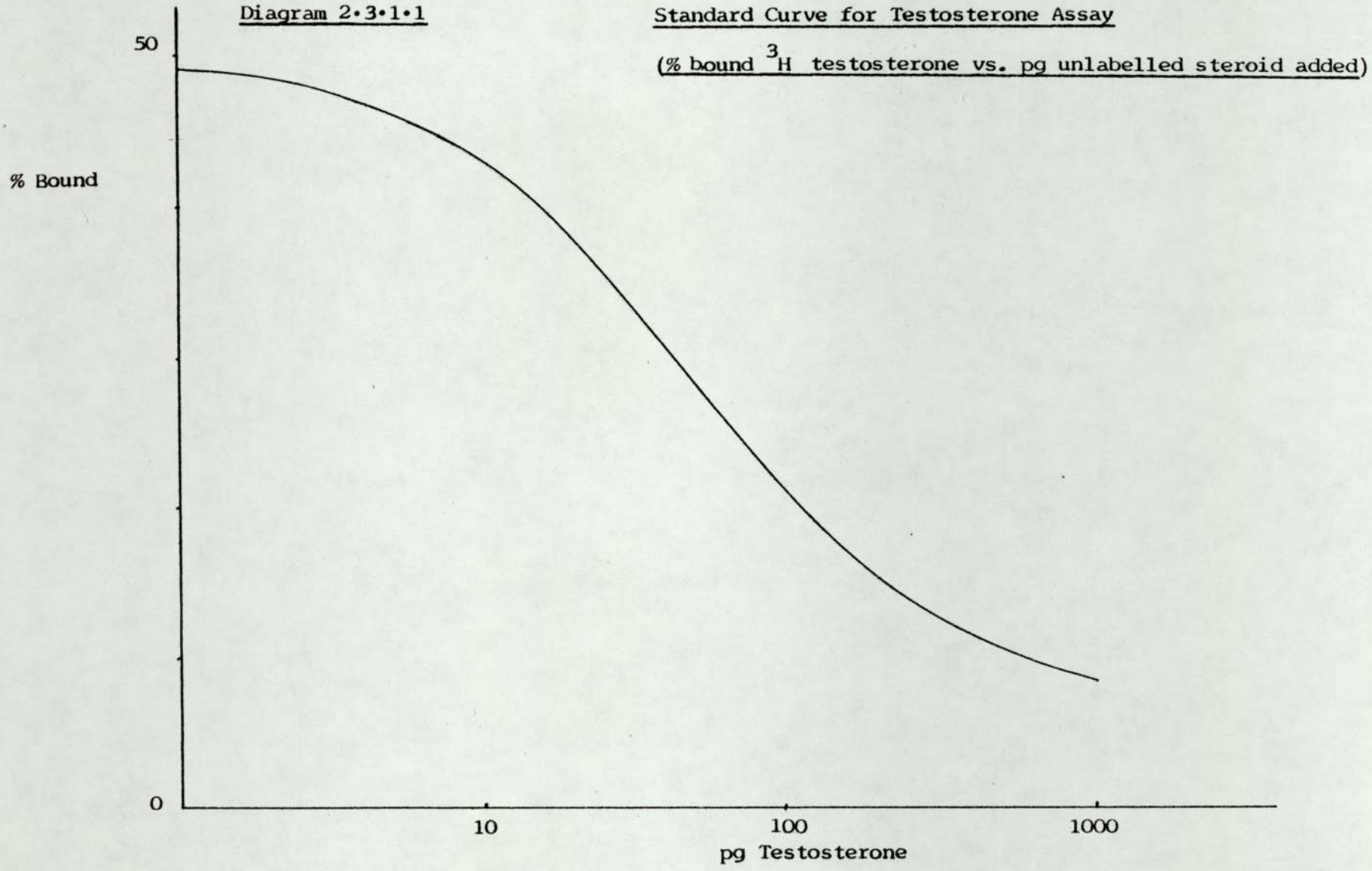
A 400 µl aliquot was pipetted into a scintillation vial and 10 ml scintillation fluid added to all vials.

Vials were counted on a Hewlett Packard Betacounter.

Diagram 2•3•1•1

Standard Curve for Testosterone Assay

(% bound ^{3}H testosterone vs. pg unlabelled steroid added)



Assay Calculations

Recovery

$$\text{DPM in 400 µl Ethyl Acetate} \times \frac{2{\cdot}0}{0{\cdot}4} = \text{DPM Rec.}$$

$$\frac{\text{DPM Rec.}}{\text{DPM Total (rec.)}} \times 100 = \%\ \text{Recovery}$$

Standards

$$\text{DPM in 400 µl} \times \frac{700}{400} = \text{DPM Assay}$$

$$\frac{\text{DPM assay}}{\text{Total DPM (assay)}} \times 100 = \%\ \text{B.}$$

For standard curve plot % B./competing (unlabelled) testosterone (levels of 0, 10, 20, 50, 100, 200, 300, 500, 1000 pg Test.).

Unknowns

Calculate % ^{3}H Testosterone Bound.

Read competing testosterone from Std. curve (pg).

Correct for recovery.

Subtract blank if necessary.

pg/assay = pg/200 µl ethyl acetate.

x 10 = pg/2 ml ethyl acetate/100 µl serum.

x 10 = pg/ml.

÷ 1000 = ng/ml testosterone.

Assay Parameters

Recovery. Recovery was high and ranged from 75 - 95%.

Sensitivity. Sensitivity was of the order of 8 - 15 pg Testosterone.

Inter-assay coefficient of variation. One sample was measured in 8 consecutive assays and the coefficient of variation calculated.

$$\text{Inter-assay C.V.} = \frac{\text{S.D.}}{\text{Mean}} \times 100 = \frac{2{\cdot}65}{22{\cdot}30} \times 100 = 11{\cdot}8\%.$$

Intra-assay coefficient of variation

Determined by measurement of 1 sample 6 times in one assay

= 4•60%.

Cross Reactivity of Antisera. Calculated from displacement at 100pg level.

Steroid	Cross reactivity (%)
Testosterone	100
Methyl Testosterone	19•5
11KT	32•7
5 α-DHT	70
Androsterone	0•24
Dehydroepiandrosterone	0•008
Progesterone	< 0•008
Pregnenolone	
17 β estradiol	
Cortisol	
Cortisone	

Of those steroids naturally present in salmonids only 11 keto-testosterone may be expected to compete to a significant extent with testosterone for binding sites on the antibody. When pooled serum samples were serially diluted and assayed the curve produced paralled the testosterone standard curve, though this parallelism was reduced when samples from mature males were pooled, suggesting some cross reactivity occurs.

In most instances within this work the testosterone assay was used as an index of maturation, rather than to specifically identify the androgens. For use in this context it was felt that the risk of cross reactivity was acceptable.

2•3•2 RIA of Serum Gonadotrophic Hormone (GTH)

RIA of serum GTH was carried out by Dr. B. Breton (Laboratoire de Physiologie des Poissons, Institute National de Recherche Agronomique, 78350 Jouy en Josas, France).

Serum samples were packed in either plastic straws sealed with plasticine or in polypropylene microfuge tubes and sent to France packed in dry ice in a thermos flask.

Method

Samples were assayed using a double antibody method in which separation of bound and free steroid was accomplished by a second heterologous antibody. Antisera to GTH was raised in Guinea Pigs by subcutaneous injection of 250 µg purified trout GTH (tGTH) (Breton et al., 1976) in 500 µl 8% NaCl mixed with Complete Freund's adjuvant. A total of 7 injections were necessary to obtain an antibody capable of binding 50% of labelled hormone at a $\frac{1}{2} \times 10^{-5}$ dilution of antiserum in the absence of competing GTH.

Iodination of purified tGTH was carried out using Iodine -125 (I^{125}) using the chloramine T method of Greenwood et al. (1963) using the same conditions as for carp GTH (Breton et al., 1971).

Sensitivity of the assay was of the order of 7 - 10 pg GTH.

Assay Procedure

Assays were carried out in 500 µl plastic tubes each containing 10,000 cpm of labelled hormone, 20 µl unknown serum or standard GTH solution and antibody at the working dilution of $\frac{1}{2} \times 10^{-5}$. Veronal buffer (0•025 M) at pH 8•6 and containing 2•5% Human Serum Albumin was used in assays. Tubes were incubated for four days at $3^{\circ}C$.

Separation of bound and free labelled GTH was by a double immuno-precipitation reaction involving anti guinea pig γ - globulins raised in rabbits.

50 µl of rabbit sera were added to each tube and allowed to incubate for one hour at room temperature. Before centrifugation 1·5 ml of cold incubation buffer without serum albuminwas added to each tube. Tubes were centrifuged at 3600 G and 4°C for 20 minutes and the supernatant discarded. Tubes were counted on a gamma counter and the results obtained transferred to Logit-log plots to give a linear standard curve from which the value of unknown serum samples could be read.

Results were expressed in ng/ml.

2·4 Histology

Material for histological examination was fixed in either Bouin's Fixative or Phosphate Buffered Formalin (Roberts, 1978).

Bouin's Fixative

Saturated Picric Acid	75 ml
Glacial acetic acid	5 ml
Formalin	25 ml

Phosphate Buffered Formalin (PBF)

Disodium Hydrogen Phosphate ($Na_2 HPO_4$)	1·2 g
Sodium Hydrogen Phosphate ($NaH_2 PO_4$)	0·8 g
Formalin	20 ml
Distilled H_2O	180 ml

Processing

1. Fix 24 hours.
2. Wash H_2O 1 hour.
3. 70% alcohol 4 - 8 hours.
4. 90% alcohol over night.
5. Abs. Alc. 1 2 hours.
6. Abs. Alc. 2 3 hours.
7. Abs. Alc. 3 3 hours.
8. Chloroform overnight.
9. Wax 1 2 hours.
10. Wax 2 2 hours.
11. Wax 3 2 hours.
12. Embed in fresh wax.

Sections were cut at 8 μ on a Spencer Rotary Microtome.
Sections were stained with Haematoxyln and Eosin.

2·5 Determination of Dietary Digestibility

Digestibilities may be determined by either direct or indirect methods. In fish direct determinations are impractical due to the need for quantitative faecal sample collection (Windell et al., 1978a). So an indirect method, using chromic oxide as an inert marker, was used. As a dietary inert substance, chromic oxide (Cr_2O_3) is excreted without digestion or loss.

Chromic oxide was incorporated into the diet fed and faecal samples were collected after a minimum period of feeding, normally 14 days. Faecal samples and diet were assayed for Cr_2O_3 and the percentage dry matter digestibility calculated from the equation:

$$\% \text{ dry matter digestibility} = 100 \times \left(1 - \frac{(C)}{(B)} \times \frac{(1 - B)}{(1 - C)}\right)$$

(Maynard and Loosli, 1969). Where C = g indicator / g dry matter diet

B = g indicator / g dry matter faeces.

2·5·1 Preparation of Chromic oxide diet

Diet was prepared in 3 kg batches, where appropriate. 3 kg of BP 'Mainstream' fry food (00) was weighed and 1% by weight Cr_2O_3 (BDH Chemicals, Poole) added. The powder and fry food were mixed on a 'Hobart' commercial food mixer with the addition of water until it reached a doughy consistency. The mixture was then pelleted using the pelletising attachment of the mixer. The resulting pellets were thoroughly dried in a drying oven overnight with gentle heating from a fan heater. A sample of diet was retained for analysis of Cr_2O_3 content.

2·5·2 Faecal Sampling

Following anaesthesia gentle pressure along the side of the vent was used to expel a faecal sample onto a piece of labelled silver foil. Care was taken to avoid slime or milt contamination. Only the initial portion expelled was used for digestibility determination as it has been reported that larger portions may be unrepresentative and give an artificially low digestibility (Windell et al., 1978a).

Faecal samples were dried overnight at 80°C, powdered and stored in small plastic tubes (Luckhams, LP3).

2·5·3 Chromic Oxide measurement

Determination of chromic oxide in both faeces and food was by the method of Furukawa and Tsukahara (1966). Dried faeces or food was powdered and 50 or 100 mg of each sample weighed accurately. Each

sample was transferred to a dry 100 ml Kjeldahl flask and 5 ml concentrated nitric acid (Analar) added to each. Flasks were gently swirled to wash down any particles adhering to the sides.

After standing for five minutes, flasks were heated on a micro-electric heater in a fume cupboard, with occasional turning to prevent particles adhering to the sides, until a white precipitate develops (approximately 20 minutes).

Flasks were removed from the heater and cooled. 3 ml of Perchloric acid was added to each flask and the flasks reheated until the greenish-white colour changes to a red-orange colour (usually 30 - 40 minutes). To ensure the reaction is complete, samples were heated for a further 10 minutes, after which flasks were removed, cooled slightly and 50 ml of distilled water added to each to terminate the reaction. Following cooling to room temperature, the contents of each flask were transferred to a clean 100 ml volumetric flask and made up to 100 ml with distilled water.

After standing for 10 minutes to precipitate any organic matter, an aliquot from each flask was transferred to a plastic curvette and the optical density at 350 mμ read on a Beckman spectrophotometer zeroed with a distilled water blank.

Calculation

The standard curve for the wet digestion method may be expressed by the equation:

$$Y = 0{\cdot}2089X + 0{\cdot}0032 \qquad \text{Eq(2)}$$

Where Y = Optical Density at 350 mμ.

X = Chromic oxide content of sample (mg/100 ml).

The values obtained for faecal samples and a sample of diet used may then be substituted in equation (1) to calculate the % dry matter digestibility.

2·6 Proximate Analysis

Proximate analysis was carried out on samples of trout muscle. Fish were killed by immersion in a 10 x normal strength solution of Benzocaine for five minutes. A cube of muscle was cut from the dorsal musculature just anterior to the dorsal fin. Skin was removed from the block and it was weighed on a pre-weighed foil. Samples were dried for 48 hours at 80°C and re-weighed. They were then ground up in a mortar and stored in plastic tubes in a dessicator.

2·6·1 Muscle Water

% muscle water was calculated by the equation :

$$\% H_2O = \frac{(\text{weight wet} - \text{weight dry})}{\text{weight wet}} \times 100$$

2·6·2 Protein Determination

Protein was determined by the micro-Kjeldahl method (Pearson, 1973) in which organic nitrogen in protein is converted to ammonia, which is then measured.

Digestion

10 - 20 mg of powdered muscle sample were carefully weighed out and added to a micro-Kjeldahl digestion flask. 1·5 ml of concentrated sulphuric acid and 80 mg of Kjeldahl catalyst were added to each flask. The flask was placed on a digestion rack and heated (if the sample began to char, the heat was reduced to prevent undue frothing). Heating was continued until the digest cleared. At the end of digestion

the flask was allowed to cool and the walls washed down with a small volume of distilled water.

Distillation and Titration

The digest was steam distilled in a Markham still. Steam from a steam generator was run through the apparatus prior to sample loading and the inner vessel washed by automatic siphoning of distilled water from the inner vessel to waste. This procedure was repeated between samples.

Once the apparatus had been washed, the cooled digest was introduced into the inner vessel of the still via the funnel. 0·2g zinc dust and 10 ml NaOH (40%)were added. The flask and stopper were carefully washed out with distilled water into the inner chamber, taking care not to break the liquid seal of the stopper, otherwise ammonia may be lost.

Steam was passed through the reaction mixture with all valves closed and the distillate collected in a conical flask containing 10 ml saturated boric acid solution with a few drops of mixed indicator (methyl red plus bromocresol green). As ammonia was driven into this flask the colour changes from a reddish-brown to a blue green. Distillation was carried out for another five minutes after this colour change.

The boric acid solution was titrated against 0·01 N HCl; the end point of the titration being the loss of the blue green colour and the appearance of a slight pink tinge.

Calculation

The titre of HCl obtained is substituted into the equation to give the % protein : $$\% \text{ Protein} = \frac{0{\cdot}014 \times \text{titre} \times 6{\cdot}25}{\text{sample wt.(g)}}$$

2·6·3 Fat Analysis

The method used was adapted from that of Korn and Macedo (1973). 10 g of Sodium sulphate Na_2SO_4 (BDH Chemicals) were placed in a glass chromatographic column. Samples were powdered, accurately weighed and mixed with 20 g Na_2SO_4 (samples of 0·5 - 0·25 g weight dried muscle were used). This mixture was added to the column. Finally a further 10 g Na_2SO_4 was added.

50 ml of trichlorofluoromethane (Hopkin and Williams) was gently poured on to the column and the valve opened to allow the solvent to flow through. When the solvent front reached the base of the column, the tap was closed for a one-hour retention period.

The tap was opened and the solvent plus fat drained into a pre-weighed 100 ml beaker. The extract was then concentrated on a hotplate in a fume cupboard at about 40°C for 30 - 60 minutes, followed by 10 minutes at 70°C in a drying oven. Finally the beaker plus fat was cooled in a dessicator and re-weighed. The % fat was then calculated by the equation :

$$\% \text{ fat} = \frac{\text{weight of fat (g)}}{\text{weight of sample (g)}} \times 100.$$

CHAPTER 3

REVIEW OF THE CONTROL OF SEXUAL MATURATION IN TELEOSTS WITH SPECIAL REFERENCE TO MALE SALMONIDS

Contents

3·1 Introduction

Sexual maturation in salmonids is, as in all organisms, a closely ordered process, capable of modification by both extrinsic and intrinsic factors. Such 'plasticity' is obviously an adaptive advantage, allowing the synchronization of reproductive effort within the most favourable period. Sexual maturation and its control in teleosts has been extensively reviewed. (Hoar, 1955, 1957, 1965, 1969; Dodd and Wiebe, 1968; Dodd, 1972, 1975; Donaldson, 1973, 1975; de Vlaming, 1974; Fontaine, 1976; Callard et al., 1978c;Peter and Crim, 1979; Peter, 1981, 1982; Billard et al., 1982; Dodd and Sumpter, 1982).

Sexual maturation comprises a group of processes initiating and maintaining gonadal recrudescence, gametogenesis and spawning. In addition to these primary processes, a number of secondary changes occur. These secondary sexual characteristics include changes in colour, morphology and behaviour. The onset and course of sexual maturation is controlled by the endocrine system, modified by the CNS mediated influence of environmental factors (de Vlaming, 1974; Peter and Crim, 1979; Peter, 1981, 1982).

While the individual components of the reproductive system have been intensely studied and a broad outline of the major control pathways obtained (Dodd and Wiebe, 1968; Dodd, 1972, 1975; Donaldson, 1973, 1975; de Vlaming, 1974; Fontaine, 1976; Olivereau, 1977; Peter and Crim, 1979; Peter, 1981, 1982; Dodd and Sumpter, 1982) until recently far less was known of the detailed interrelationships existing between individual components. In part this gap developed through an overreliance on analogy with the mammalian reproductive system, but also due to the lack of specific methods for monitoring

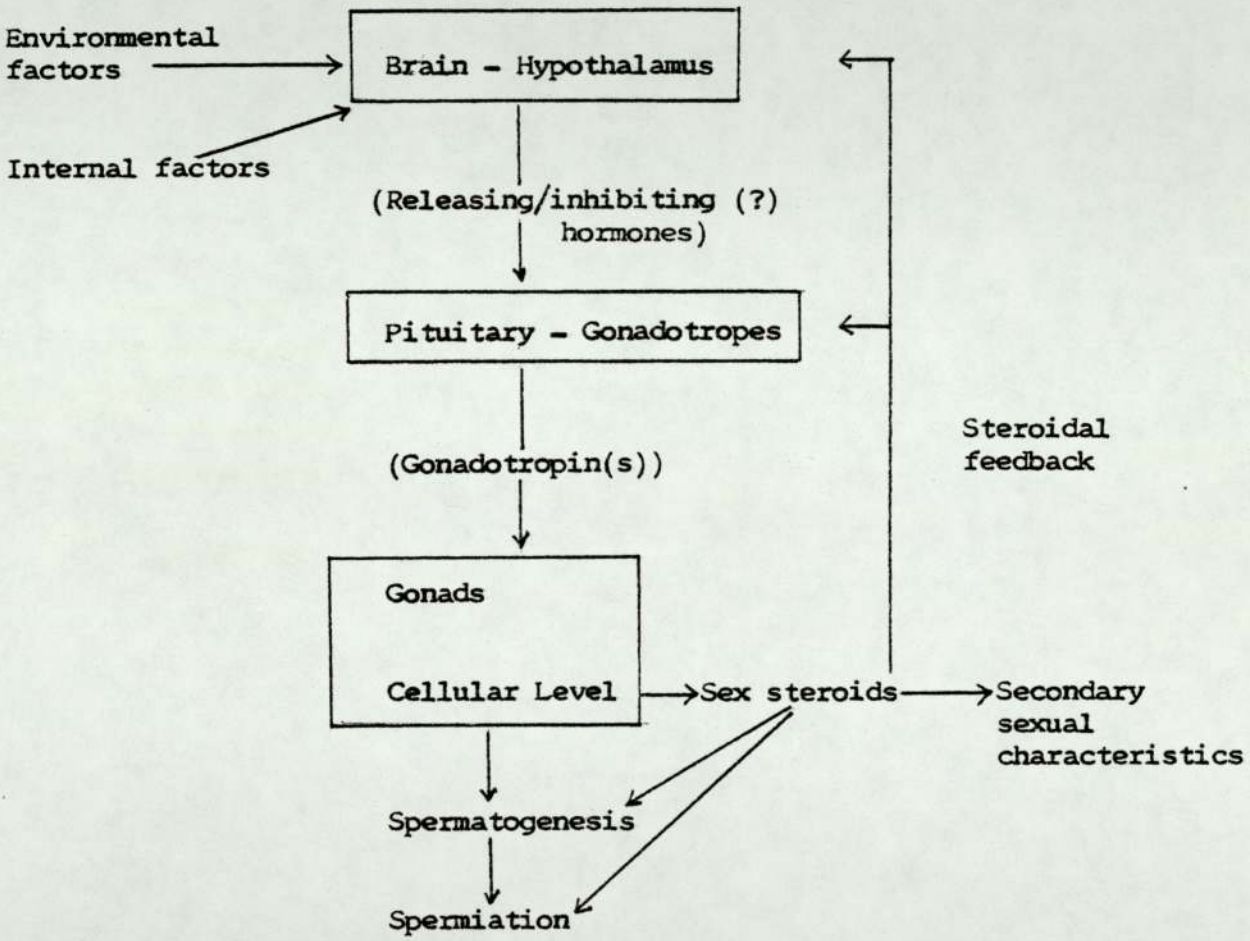
changes in endocrine status. Recent technical developments such as the development of specific radioimmunoassays (Breton et al., 1971, 1976; Sangalang and Freeman, 1977; Simpson and Wright, 1977; Scott et al., 1980a,b) have allowed the elucidation of a number of control pathways. These latest developments, together with a discussion of the individual components of the reproductive system, will be reviewed in this chapter.

The main components of the reproductive system and its endocrine control network are shown in fig. 3•1•1. Environmental influences are mediated through the CNS hypothalamus, which controls the activity of the pituitary gonadotropes by means of gonadotropin releasing and/or inhibiting hormones. Stimulation of pituitary gonadotropes causes synthesis and release of one or more gonadotropin, which acts on the gonad to promote the synthesis of sex steroids (predominantly androgens in the male teleost). Either directly or via sex steroids, gonadotropin brings about the initiation and maintenance of spermatogenesis and sperm release (spermiation). In addition, the production of sex steroids stimulates the development of the secondary sexual characteristics. Changes in steroid levels seem to exert important controlling influences upon the activity of the hypothalamus-pituitary by means of steroidal feedback to these components. This hierarchy of control has been termed the hypothalamic-pituitary-gonadal axis.

This chapter will examine the structure and function of the components of this system and their control. Though the bulk of the work reviewed will refer to the male salmonid, where necessary work relating to non-salmonids and to females will be included in

order to illustrate features of special interest. This discussion will provide a rationale for the discussion in later chapters of methods of manipulating the reproductive system in order to prevent or to curtail sexual maturation.

Fig. 3·1·1. The Reproductive System of teleosts and its endocrine control system - some inter-relationships



3·2 The Hypothalamus and the control of pituitary function

A large body of evidence has demonstrated the overriding importance of the hypothalamus in the control of the mammalian pituitary gland. (Donovan, 1970; Holmes and Ball, 1974). Neurohormones synthesised within the neurones of the hypothalamus are transported to the pituitary, over which they exert a controlling influence by promoting the release of pituitary tropic hormones (Pecile et al., 1965; Pecile and Muller, 1966; Reichlin, 1966; Schally and Kastin, 1966; Muller et al., 1967; Schally et al., 1968, 1969). Thus the release of the two mammalian gonadotropins is under the control of a decapeptide hormone, Luteinizing Hormone Releasing Hormone (LH.RH.) (Schally and Kastin, 1972a, b). This decapeptide is available as a synthetic compound.

The reproductive function of the teleost pituitary is thought to be under similar control (Ball et al., 1965, 1972; Peter, 1970; Breton et al., 1971). Direct evidence for the presence of a hypothalamic neurohormone responsible for the release of teleost gonadotropin has been presented by the demonstration of a stimulatory effect of hypothalamic extracts on pituitary gonadotropin accumulation or release in a number of teleost species including carp (Breton et al., 1971, 1972; Breton and Weil, 1973; Weil et al., 1975), goldfish (Breton et al., 1972; Crim et al., 1976) and rainbow trout (Breton et al, 1972). Such studies support the existence of a gonadotropin releasing factor (GRF) exerting a positive, stimulatory effect upon gonadotropin production. However, a number of these early studies have been criticized as lacking specific controls or failing to consider the

developmental state of the fish used (Peter and Crim, 1979). Few reports have attempted to correlate GRF activity with sexual or gonadal state, though de Vlaming and Vodicnik (1975) reported increased GRF activity in hypothalamic extracts of golden shiner (Notemigonus crysoleucas) exposed to stimulatory temperature and photoperiod regimes.

Attempts to localize such activity within the brain and hypothalamus by immunocytological methods have yielded equivocal results, possibly due to uncertainty regarding the antigenic similarity of teleost GRF and the mammalian LH.RH commonly used as an antigen. Goos and Murathanoglu (1977) recorded LH.RH immunoreactive material in both the telencephalon and anterior hypothalamus of rainbow trout. In the platyfish immunoreactive fibres were observed in the nucleus lateral tuberis (NLT) region of the hypothalamus and in the telencephalon (Schreibman et al, 1979). However, Dubois et al (1979) and Nozaki and Kobayashi (1979) failed to observe such material in rainbow trout brain, though the latter workers obtained positive results in the brain of Japanese eels. In the carp immunoreactive LH.RH like material was observed within the NPO region of the hypothalamus and the antero-lateral hypothalamic region (Pan et al, 1979).

Though the failure to localize LH.RH like material in teleost hypothalamic tissue may be dismissed as resulting from structural dissimilarities between LH.RH and teleost GRF, greater success has resulted from attempts to detect immunoreactive material in or near the pituitary gland. Immunocytological techniques have located LH.RH like material in the neurohypophysial tissue aligned with the proximal pars distalis of rainbow trout (Dubois et al, 1979), platyfish (Schreibman et al, 1979) and Japanese eel (Nozaki and Kobayashi, 1979).

Until further evidence is available the functional significance of a positive demonstration of LH.RH like material in teleost hypothalamic tissue must be viewed with caution.

Functional studies have attempted to localize GRF activity by correlation of cellular activity within the hypothalamus with gonadal and pituitary state. Thus parallel changes in the nuclei, nucleoli and vacuolar structure of cells within the NLT region have resulted from castration and selective lesioning studies. (Peter, 1970; Zambrano, 1971; Holmes and Ball, 1974; Peter and Crim, 1978). In addition, changes within the nucleus preopticus (NPO) have been observed. (Ball and Baker, 1969; Holmes and Ball, 1974; Terlou et al., 1978). Evidence implicating the NPO region of the hypothalamus in the control of pituitary reproductive function must, however, be viewed with caution as the close proximity of fibre tracts of the NPO and NLT (Ekengren, 1973) would allow an indirect pathway to the NPO via the NLT. (Weiss, 1976; Ekengren and Terlou, 1978).

Further selective lesioning studies have implicated the NLT. Dodd et al (1978) showed a decrease in GSI and pituitary GTH content following lesions to the NLT of Atlantic salmon parr. Lesioning of the NLT and NPO of goldfish were shown to be capable of depressing GSI and resulting in ovarian regression (Peter and Crim, 1978), though in this case no changes in serum GTH were observed. Peter (1982) suggests the reason GTH levels did not reflect the decline in gonad size may be that maintenance of the gonad requires a daily phasic release of GTH which was abolished by lesioning. Thus though absolute GTH levels were no different, the daily rhythm of GTH important in gonadal development (Hontela and Peter, 1978) was abolished.

These results implicating the NLT region have been confirmed by chemical lesioning experiments using monosodium L-glutamate (Peter et al., 1980).

Though no general agreement on the functional localization of GRF activity within the teleost hypothalamus and brain can yet be reached, the evidence suggests the NLT is an important area exerting control of the gonadotropic function of the pituitary (Peter, 1982). In addition, the NPO and areas of the telencephalon may be involved in this regulation. Questions still remain regarding the functional role of LH.RH like material in teleosts. Recent work using a radio-immunoassay for mammalian LH.RH has attempted to quantify such activity within the brain and body of the European eel (Dufour et al., 1982). The hypothalamus, optic lobes and mesencephalic components of the midbrain were found to contain 19, 25 and 30 pg of LH.RH like material/mg of fresh tissue. Higher concentrations (112 pg/mg fresh tissue) were found in the pituitary gland. Very high blood levels (40 - several hundred pg/ml) were found in this species, but the material appeared to have only a weak gonadotropic function, raising doubts about its role in the regulation of the pituitary gonadotropes.

In addition to the existence of GRF material in the hypothalamus of teleosts, lesioning and castration studies have provided some evidence for the existence of a gonadotropin release inhibition factor (GRIF). Thus lesioning of the NLT of sexually mature goldfish gave rise to an increase in serum GTH levels and ovulation (Peter et al., 1978). It was later shown that this result was due to damage to the pituitary stalk (Peter and Paulencu, 1980). Similar evidence was produced by Leatherland (1970a) who showed ectopic pituitary grafts

in the stickleback were capable of increased gonadotropic activity, suggesting normal control was by an inhibitory rather than a stimulatory factor. Peter (1982) suggests such an inhibitory control could be lifted immediately prior to spawning to allow the gonadotropin 'surge' associated with ovulation, but this idea, though attractive, does not fit the evidence that LH.RH is capable of causing ovulation in teleosts. The identity of the presumptive GRIF is uncertain, though Peter (1982) suggests dopamine could fulfil such a role.

Transport of the neurosecretory material to the gonadotropes of the pituitary is thought to take place initially via nerve tracts leaving the hypothalamic nuclei (Peter and Fryer, 1981). However, the teleost pituitary lacks a well defined median eminence and portal system which is the accepted pathway through which neuroendocrine messages reach the mammalian adenohypophysis (Holmes and Ball, 1974). Indeed, the vascularization of the teleost pituitary is highly variable, though that of the Atlantic salmon (Fridberg and Ekengren, 1977) and brook trout (Hill and Henderson, 1968) are very similar. Within the salmonids it appears the main route for neurochemical transmission is by neurocrine fibres innervating the well developed perivascular spaces penetrating the anterior pituitary. (Nagahama, 1973; Fridberg and Ekengren, 1977). This contrasts with the situation in the roach (Leuciscus leuciscus), where direct innervation of at least some of the gonadotropes is seen (Ekengren et al, 1978 b).

As yet no teleost GRF has been isolated and chemically structured, though some preparative work has revealed interesting details of the molecular weights and immunological identities of such factors.

Chromatography of carp hypothalamic extracts on sephadex G-25 revealed a peak of stimulatory activity having a molecular weight of less than 5000 (Breton et al, 1975a). (Mammalian LH.RH has a molecular weight of 1183). Similarities in antigenic structure between hypothalamic extracts of a wide range of vertebrates, including teleosts, elasmobranchs, reptiles and birds, suggest GRF from these species differ from mammalian LH.RH and are closer in size and antigenic properties to each other. (King and Millar, 1979, 1980). However, Barnett et al. (1979) have produced results suggesting the presence of a high molecular weight GRF in both hypothalamic and extrahypothalamic extracts of cod capable of eliciting LH.RH responses in rats. Coupled with the already mentioned variability in response to anti-LH.RH, these results suggest some important differences between mammalian LH.RH and teleost GRF.

In spite of these differences, synthetic LH.RH is able to carry out some of the biological functions of teleost GRF. These have included the induction of changes in pituitary cytology, indicating GTH synthesis and release (Kaul and Vollrath, 1974; Lam et al, 1976), ovulation (Hirose and Ishida, 1974; Lam et al., 1975; Aida et al, 1978) and increased serum GTH (Breton et al, 1971; Breton and Weil, 1973; Crim et al, 1976; Crim and Evans, 1980). However, the doses required to produce these responses are generally much higher than those effective in mammals, perhaps due to differences in the active sites of LH.RH and teleost GRF. In some cases negative results have been obtained using LH.RH. Sokolawska et al. (1978) reported negative effects of LH.RH on oocyte maturation in female carp. Synthetic analogues of LH.RH having high mammalian potency have been shown to be more effective than LH.RH in teleosts.

Donaldson et al. (1979 , 1982) have shown LH.RH analogues to be capable of inducing ovulation in salmonids without the normal 'priming' dose of GTH, normally needed for LH.RH to exert such an effect. In addition, LH.RH analogues produced a more prolonged release of GTH than LH.RH (Peter, 1980).

Though an immense amount of work has been directed towards the question of the hypothalamic control of the pituitary gland's gonadotropic function in teleosts, the actual structural identity and cellular localization of teleost GRF is uncertain, as are the detailed interactions between the centres responsible for control and external and internal factors. Of particular interest are the interactions between hypothalamic control centres and steroidal feedback. Steroidal binding sites have been identified in the brain of a number of teleost species including goldfish (Kim et al, 1978a), paradise fish (Davis et al, 1977)and green sunfish (Morrell et al, 1975). Interestingly these steroid binding sites have included sites in the NLT, NPO and telencephalon. In view of the possible functional importance of these areas in the control of GRF activity, it is tempting to postulate a relationship between steroid binding in these areas and their GRF activity. In addition a further interaction between GRF activity and steroidal feedback may be the seasonal modification of the responses of gonadotropes to GRF by steroids. (Crim and Cluett, 1974; Weil et al, 1975, 1978; Goos et al, 1982; van Ree et al., 1982).

3·3 The Pituitary

3·3·1 The Pituitary gonadotropes

Within the teleost pituitary presumptive gonadotropes have been identified by a variety of light and electron microscopical characteristics. Early identification of these gonadotropes has relied upon their tinctorial and histochemical characteristics. Presumptive gonadotropes are typically basophilic cells containing glycoprotein secretory granules, which are periodic acid/Schiffs (PAS) +ve, aldehyde fuchsin (AF) +ve and alcian blue (AB) +ve (Holmes and Ball, 1974).

Gonadotropes appear to be localized within the proximal pars distalis (PPD) of the teleost pituitary (Holmes and Ball, 1974; Doerr-Schott, 1976; Goos et al., 1976). However, in salmonids they may also extend into the rostral pars distalis (RPD) (Cook and Van Overbeeke, 1972; Nagahama, 1973; Ekengren et al., 1978a,c; Peute et al., 1978).

These cells have been shown to undergo cyclic changes of activity during the reproductive cycle in a variety of teleost species (Ball and Baker, 1969), including rainbow trout (Robertson and Wexler, 1962; Peute et al., 1978). Experimental procedures known to affect gonadotropin function, such as castration (McBride and Van Overbeeke, 1969; Febvre and Lafaurie, 1971) and steroid administration (Sage and Bromage, 1970; de Vlaming, 1974; Olivereau and Olivereau, 1979a,b) have produced changes in gonadotrope structure suggestive of functional change. In addition the administration of synthetic LH.RH causes gonadotrope degranulation, and GTH release in salmon (Ekengren et al., 1978a) and goldfish (Kaul and Vollrath, 1974; Lam et al., 1976).

A major cause of contention in teleost reproductive physiology is the number of gonadotropins present within the pituitary gland (Olivereau, 1976; Ekengren et al., 1978a). Two distinct gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) have been isolated in mammals. Each gonadotropin is associated with a particular gonadotrope type; the alpha and beta cells respectively (Holmes and Ball, 1974). The number of gonadotropes present in the teleost pituitary has been cited as evidence for both the view-points that only one GTH is present in teleosts and that two or more GTH's exist in these species. This difference in interpretation reflects differences in gonadotrope characteristics observed by a variety of methods.

Evidence for only a single gonadotrope type has been presented for a number of teleost species, including Cymatogaster aggregata (Leatherland, 1969, 1970b), Heteropneustes fossilis (Baker et al., 1974), Carassius auratus (Kaul and Vollrath, 1974), Poecilia latipinna (Peute et al., 1976), Rutilus rutilus (Ekengren et al., 1978b) and a variety of salmonids (Donaldson, 1973) such as Salmo salar (Ekengren et al., 1978a), Oncorhynchus nerka (McKeown and Leatherland, 1971, 1973; Nagahama, 1973) and Salmo gairdneri (Peute et al., 1978).

However, in a number of teleosts essentially similar methods and techniques have suggested the presence of two types of pituitary gonadotropes. These species include Mugil sp (Olivereau, 1968), Anguilla and Conger spp (Knowles and Vollrath, 1966), Carassius auratus (Leatherland, 1972), Leuciscus rutilus (Bage et al., 1974). Two gonadotrope types have also been postulated in salmonid species including Salmo salar (Olivereau, 1976), Oncorhynchus tshawytscha (Olivereau, 1972), Oncorhynchus kisutch (Chestnut, 1970),

Oncorhynchus nerka (Cook and Van Overbeeke, 1972), Oncorhynchus spp (Olivereau and Ridgway, 1962), Salmo gairdneri and Salmo fario (Olivereau, 1978).

The difficulties of interpretation inherent in such studies are shown by the variable results arising from the use of similar techniques in the same species. Thus Leatherland (1972) reported the presence of two types of gonadotrope in the pituitary of Carassius auratus, while Kaul and Vollrath (1974) found evidence of only a single gonadotrope type in the pituitary of this species. Similar variation exists in the results of Olivereau (1976) and Ekengren (1978a), who suggest the presence of two gonadotropes and a single gonadotrope respectively in the Atlantic salmon. Without knowledge of the identity and functional importance of the cell types studied such differences are difficult to reconcile. The most likely explanation for the variable results would seem to be the existence of a single cell type showing wide morphological variation, reflecting varying activity. (Abraham, 1974; Ekengren et al, 1978a).

The existence of two gonadotropes need not provide a priori evidence for the existence of two teleostean gonadotropins. Equally the existence of only a single gonadotrope does not rule out the possibility that more than one gonadotropin is produced. Recent results have suggested that even if two gonadotropins can be isolated they may both be present in a single cell. Thus antibody to Con A 1 (vitellogenic GTH) and Con A 11 (maturational GTH) (Ng and Idler, 1979) gonadotropic fractions reacted with both gonadotrope cells observed (Van Oordt, 1982).

The main conclusion regarding the number of gonadotrope types in the teleost pituitary is that, unlike mammals, it is not possible to assign distinct functional roles corresponding to the alpha and beta cells of mammals. Further work on the biochemical structure and function of teleost gonadotropin(s) is necessary before the questions posed may be resolved.

3•3•2 Biochemical studies of teleostean gonadotropin(s)

While two mammalian GTH's, LH and FSH, have been isolated and a similar dichotomy of gonadotropin function observed in birds, anuran and urodele amphibia, chelonian and chrocodillian reptiles (Licht *et al.*, 1977), the situation in teleost fish remains unclear. In the above species the gonadotropins isolated have been shown to be glycoprotein hormones having a distinct subunit structure.

A variety of purification techniques have been used to isolate partially purified GTH in a number of teleost species including *Oncorhynchus tshawytscha* (Donaldson *et al.*, 1972a; Donaldson, 1973; Pierce *et al.*, 1976; Breton *et al.*, 1978), *Oncorhynchus keta* (Idler *et al.*, 1975b, c; Yoneda and Yamazaki, 1976; Idler and Ng, 1979) and rainbow trout (Breton *et al.*, 1976). Among non-salmonid species GTH has been purified from the pituitaries of carp (Burzawa-Gerard, 1971, 1974; Burzawa-Gerard and Fontaine, 1972; Idler and Ng, 1979), American flounder and Winter flounder (Ng and Idler, 1979).

Two of the best characterized preparations are carp GTH (cGTH) (Burzawa-Gerard, 1971, 1974) and salmon GTH (SG-G100) (Yamazaki and Donaldson, 1968a, b; Donaldson *et al.*, 1972a). Both are glycoproteins (Donaldson, 1973; Burzawa-Gerard, 1974; Pierce *et al.*, 1976) with a distinct

subunit structure (Burzawa-Gerard, 1971; Donaldson et al, 1972a), features shared by trout GTH (tGTH) (Breton et al, 1976). Interestingly some similarities between these glycoprotein preparations and mammalian LH/FSH have been noted (Pierce et al, 1976; Jolles et al., 1977; Yoneda et al.,1977; Burzawa-Gerard,1982). Marchelidon et al (1978) noted the association of ovine LH beta subunits with cGTH alpha subunits, indicating a degree of structural similarity. Further fractionation of chinook salmon and chum salmon GTH has produced some evidence of male and female specificity (Idler et al, 1975c; Breton et al, 1978), though the differences observed were too slight to suggest the existence of specific male and female gonadotropins within the salmonid pituitary.

These gonadotropin preparations have been shown to be capable of initiating a variety of gonadal responses. Thus SG-G100 induces complete sexual maturation in juvenile male pink salmon (Oncorhynchus gorbuscha) (Funk and Donaldson, 1972) and reinstates oogenesis and vitellogenesis in hypophysectomized catfish (Sundararaj and Anand, 1972; Sundararaj et al, 1972). In addition, it is also capable of inducing the in vitro maturation of oocytes of Salmo gairdneri (Jalabert et al, 1972). cGTH is capable of inducing spermatogenesis and spermiation in hypophysectomized teleosts (Billard et al, 1970; Billard and Escaffre, 1973) as was SG-G100 (Yamazaki and Donaldson, 1968b).

In addition to its biological assay, the development of specific RIA techniques has allowed the direct measurement of GTH activity. RIA techniques for the measurement of cGTH (Breton et al, 1971, 1972; Crim et al, 1976; Hontela and Peter, 1978, 1980) and salmon GTH (Crim et al, 1974, 1975; Breton et al, 1976; Breton and Billard, 1977) have been described.

Mention has already been made of the controversy regarding the number of gonadotropins in the teleost pituitary. The ability of both SG-G100 (Donaldson et al., 1972a;Donaldson, 1973) and cGTH (Billard and Escaffre, 1973) to stimulate complete gametogenesis in hypophysectomized animals suggests only a single GTH is necessary for such development. In addition, Nayyar et al.(1976); Upadhyay (1977) and Funk and Donaldson (1972) have shown either cGTH or SG-G100 to cause complete testicular development in catfish, rainbow trout and chum salmon. However, such a view has been criticised by Ng and Idler (1978). They point out that SG-G100 is only partially purified and report that further purification on Concanavalin-A (Con A) sepharose gave a different result.

Their results suggest the presence of two gonadotropins, one absorbed by Con A sepharose (Con AII) and hence presumably a glycoprotein, and a second unabsorbed (Con AI) fraction, either carbohydrate poor or a non-glycoprotein. To date this duality appears to be present in four species; Oncorhynchus keta (Ng and Idler, 1978; Idler and Ng, 1979), Hippoglossoides platessoides, Pseudopleuronectes americanus (Ng and Idler, 1979) and Cyprinus carpio (Idler and Ng, 1979).

The biological activity of these two fractions has been examined and Con AI appears to be implicated in vitellogenic activity of teleosts, while Con AII has a wide range of maturational activities. Thus Con AI has been designated the vitellogenic GTH, while Con AII is termed the maturational GTH. Further work has shown that Con AII stimulates androgen production (testosterone (T) and 11-ketotestosterone (11 KT)) in hypophysectomized winter flounder and juvenile rainbow trout and that the administration of antibodies to this fraction leads to a fall

in 11-KT levels in male landlocked Atlantic salmon (Ng and Idler, 1980). Antibodies against Con AI fractions resulted in a fall in GSI and inhibition of oogenesis in the winter flounder, while antibodies directed against Con AII fraction did not significantly affect ovarian histology or size. It appears in certain teleost species two GTH fractions, one a glycoprotein and the second a non-glycoprotein, may be present in the pituitary, with a functional division between their roles. While Con AII is active in stimulating oocyte maturation, ovulation, spermiation, steroidogenesis and cyclic adenosine monophosphate (cAMP) production, Con AI acts to stimulate vitellogenin uptake by oocytes and to a lesser extent cAMP production and steroidogenesis. As available RIA's for GTH measure Con AII activity (Ng and Idler, 1978; Peter, 1981) the role of Con AI GTH remains uncertain, especially in the male teleost. However, the fact that testicular development may occur in the absence of large-scale changes in RIA-detectable GTH (Stuart-Kregor et al., 1981) suggests that such a dichotomy may be less well defined in salmonids. The development of further bioassay techniques and the development of specific methods of measurement are necessary before full functional roles can be assigned to these fractions.

3·3·3 Cyclic changes in gonadotropin secretion

While a broad correlation has been established between gonadal state and the activity of the teleost pituitary gonadotropes (Pickford and Atz, 1957; Robertson and Wexler, 1962; McBride and Van Overbeeke, 1969; Peute et al., 1978) precise data regarding cyclic changes in blood and pituitary levels of GTH have depended upon biochemical studies similar to those described. The development of RIA techniques

for teleost GTH has allowed the measurement of changes in blood and pituitary levels of GTH.

Plasma and pituitary GTH levels have been measured in a variety of salmonid species. These include rainbow trout (Salmo gairdneri) (Billard and Breton, 1978 ; Billard et al., 1978; Fostier et al., 1978, 1982; Whitehead et al., 1978a, b, 1979), brown trout (Salmo trutta) (Crim et al., 1975; Billard et al., 1978; Crim and Idler, 1978), Atlantic salmon (Salmo salar) (Crim et al., 1975; Crim and Evans, 1978; Dodd et al., 1978; Stuart-Kregor et al., 1981), pink salmon (Oncorhynchus tshawytscha) (Crim et al., 1975), Sockeye salmon (O. nerka) (Crim et al., 1975) and brook trout (Salvelinus fontinalis) (Crim et al., 1975).

Among non-salmonid species seasonal fluctuations in GTH have been measured in carp (Cyprinus carpio) (Bieniarz et al., 1977; Billard and Breton, 1978; Billard et al., 1978), goldfish (Carassius auratus) (Billard et al., 1978; Hontela and Peter, 1978; Stacey et al., 1979) Cook and Peter, 1980), tench (Tinca tinca)(Billard and Breton, 1978) and roach (Rutilus rutilus) (Escaffre et al., 1976).

In view of the variety of RIA's and species used in these investigations, it is hardly surprising that variations in the seasonal pattern of GTH secretion are observed. Among salmonids there is general agreement that increased levels of GTH are observed at the time of ovulation and spermiation. Among male brown trout, Atlantic salmon and rainbow trout, low plasma GTH levels are seen prior to the onset of spermatogenesis. A slight increase in plasma GTH has been observed at the initiation of spermatogenesis and GTH levels increase throughout the reproductive cycle with high levels at the time of

spermiation (Billard et al., 1978; Crim and Evans, 1978 ; Whitehead et al., 1979). In other salmonids GTH levels do not appear to increase until gonadal development was well advanced (Billard and Breton, 1977; Billard et al., 1978; Crim and Idler, 1978; Fostier et al., 1978). For example, Stuart-Kregor et al. (1981) were unable to demonstrate any progressive plasma GTH increases associated with increasing plasma testosterone and 11-ketotestosterone levels.

This apparent absence of a correlation between GTH and early gonad development has led to suggestions that GTH is not directly implicated in this process (Peter and Crim, 1979). Alternatively, the absence of GTH increases during this period may reflect deficiencies in the GTH assays used (Stuart-Kregor et al., 1981). The question of what GTH assays actually measure is discussed by Dodd and Sumpter (1982). Ng and Idler (1979) suggest that as Con AI (vitellogenic) GTH does not interfere with RIA's of Con AII (maturational) GTH and that Con AI has low androgen synthesising potency in male teleosts (Ng and Idler, 1980), it is likely that the GTH assays used should measure the biologically active GTH in males. However, the results of GTH RIA's need to be viewed with caution. In an earlier report (Idler and Ng, 1979), Con AI (vitellogenic) GTH was shown to have steroidogenic properties in hypophysectomized winter flounder. In addition the observed absence of GTH changes accompanying gonadal development (Stuart-Kregor et al., 1981) requires explanation. Further studies are necessary before the reasons for these differences in results can be assessed.

In addition to seasonal changes in GTH levels, circadian variation in blood and pituitary levels have been observed (O'Connor, 1972; de Vlaming and Vodicnik, 1977a;Hontela and Peter, 1978; Peter and Crim,

1978) in teleosts and it has been suggested (Peter, 1981) that the maintenance of gonadal development is dependent upon these daily fluctuations in GTH. Thus gonadal recrudescence may, during its early stages, be sensitive to short term cyclic fluctuations in GTH levels rather than absolute levels.

The presence of high GTH levels around the time of spermiation has been the subject of further study and it now appears that the interrelationships between GTH, androgens and spermiation is more complex than was originally supposed (Billard et al., 1982; Fostier et al., 1982). This complexity is demonstrated by Sanchez-Rodriguez et al. (1978). While GTH levels fell at the start of spermiation, following a fall in androgen levels they subsequently increased to higher values approximately six weeks after the initiation of spermiation.

The environmental influences regulating these changes in GTH levels will be examined later in this work. The importance of such influences in regulating cyclic changes in GTH have been extensively reviewed (de Vlaming, 1972, 1974; Billard et al., 1978; Peter and Crim, 1979; Peter, 1981; Crim, 1982).

3·4 The Gonad

3·4·1 Testis structure and development

The morphology of the teleost testis has been the subject of a number of reviews (Lofts, 1968; Hoar, 1969; Dodd, 1972; Lofts and Bern, 1972; de Vlaming, 1974) and the teleost testis appears to show greater structural variation than any vertebrate group (Callard et al.,

1978c). In view of this wide variation, this review will be limited to the structure of the salmonid testis.

In salmonids the testes are paired, tubular structures consisting of a mass of seminiferous tubules connecting to a pair of efferent ducts, the vas deferentia via numerous vas efferentia. The anatomy and histology of the salmonid testis has been reviewed (Weisel, 1943; Henderson, 1962, 1967; Oota et al., 1965; van den Hurk, 1973; van den Hurk et al., 1978a, b). Histologically the testis consists of a mass of branched tubules bounded by a basement membrane and surrounded by perilobular cells. Within these tubules germinal cells develop in numerous cysts. In addition somatic cells are present within the tubule and are variously termed Sertoli cells (van den Hurk et al., 1978a, b), lobule boundary cells (Lofts and Marshall, 1957; Henderson, 1962), cyst cells (Roosen-Runge, 1977) or intralobular somatic cells (Billard et al., 1982). Between the lobules lie the interstitial or Leydig cells (Robertson, 1958), analogous to the Leydig cells of the mammalian testis. The steroidogenic role of these cellular elements will be examined later in this section.

The embryonic development of the salmonid testis has been examined (Okada, 1973; Laird et al., 1978; van den Hurk et al., 1980). First signs of testicular development are the appearance of peritoneal folds arising at the base of the kidney. In rainbow trout (at 10°C) these folds first appear at six days pre-hatch (Laird et al., 1978). As has been suggested in birds, mammals and amphibia (Berril and Karp, 1976), germ cells migrate into the developing gonad from mesodermal tissue around the gut. At the time of hatching germinal cells are already visible within the genital ridge, which has become

progressively separated from the dorsal peritoneum (Laird et al., 1978). Initially the early testis consists of spermatogenia surrounded by somatic (Sertoli) cells and seminiferous tubule formation was first noted in rainbow trout at about 250 days post-hatch (van den Hurk et al., 1980). Sexual differentiation of the gonad appears to be complete by day 50 - 64 in the rainbow trout, depending upon temperature (Okada, 1973; Laird et al., 1978; van den Hurk et al., 1980). The course of sex differentiation is thought to be influenced by inducer substances, which may or may not be sex steroid molecules (Yamamoto, 1969; Harrington, 1974). A number of experiments have demonstrated that exogenous steroids can influence sex differentiation (Donaldson and Hunter, 1982a). Recent work has demonstrated the appearance of steroidogenic enzymes in undifferentiated trout gonadal tissue,supporting sex steroids' role in influencing differentiation of the gonad (van den Hurk and Lambert, 1982).

The spermatogenic cycle has been examined in a number of salmonid species, notably the rainbow trout (Oota et al., 1965; Billard and Breton, 1978; van den Hurk et al., 1978a, b). Three stages were distinguished within the process by van den Hurk et al., 1978a, b.

i) A multiplication phase (June - September) during which division of spermatogonia gives rise to secondary spermatogenial cysts.

ii) A maturation phase (September - January) in which division of secondary spermatogonia gives rise to primary and secondary spermatocytes, which in turn give rise to spermatids and eventually spermatozoa.

iii) Functional maturity (January - June) in which spermatozoa

pack the lobules and ducts of the testis. If these cells are not shed naturally or artificially 'stripped' resorption takes place, aided by the Sertoli cells. Early in this stage primary interstitial germ cells invade the tubules and perhaps in conjunction with 'resting' primary spermatogonia form the basis for the next spermatogenic cycle.

This picture corresponds to that presented for the spermatogenic cycle of other salmonids (e.g. Jones, 1940; Weisel, 1943; Henderson, 1962; Hiroi and Yamamoto, 1970; O'Halloran and Idler, 1970).

3·4·2 Cellular sites of steroidogenesis

Androgen and sex steroid synthesis within the salmonid testis has been the subject of a considerable amount of work and no little controversy. Marshall and Lofts (1956), O'Halloran and Idler (1970), Lofts and Bern (1972) and Henderson (1962) have suggested the lobule boundary cells of the teleost testis as the prime steroidogenic cells. This finding is based upon the appearance of these cells but also upon an inability of these workers to observe typical interstitial cells in a variety of teleosts, including salmonids.

Other workers have observed typical interstitial cells, analogous to the mammalian Leydig cell in salmonid testes (Robertson, 1958; Billard et al., 1972; van den Hurk et al., 1978a, b; Oota and Yamamoto, 1976). In a combined histological and histochemical study, van den Hurk et al. (1978a, b) found that the majority of steroidogenic enzyme activity was concentrated in the interstitial cells with only slight activity in the intra lobule somatic cells. This intra lobular

steroidogenic activity was maximal when steroidogenic activity of interstitial cells was low and the authors suggest this local androgen production may be involved in sperm migration and spermiation. A later involvement of the intra lobular cells may be in the resorption of unshed spermatozoa. This question is reviewed by Nagahama et al. (1982). Further support for the idea of the interstitial cells as the prime androgen synthesising site is the observation that these cells undergo cyclic changes associated with the production of steroids (Sundararaj et al., 1971; Nicholls and Graham, 1972; Gresik et al., 1973). Stimulation of the interstitial cells of immature silver eels was observed in response to HCG administration (Sugimoto and Takahashi, 1979).

3·4·3 Biochemical effects of gonadotropin

The importance of the hypothalamus-pituitary system in the control of the testicular cycles in lower vertebrates is well recognised (Wiebe, 1972). Mammalian experiments have suggested that the interaction of GTH (FSH/LH) and gonadal cells involve cAMP, give rise to an increase in steroidogenic enzymes, especially hydroxysteroid dehydrogenases (HSD), and cause elevated androgen production (Turner and Bagnara, 1971).

Much less is known regarding the biochemical effects of GTH upon the teleost testis, though the available evidence suggests it exerts its function by similar mechanisms. Idler et al. (1975a) have demonstrated increased testicular cAMP levels associated with GTH treatment of immature trout. Low levels of cAMP were found in the testes of immature trout and these were rapidly elevated by GTH, though only salmon or carp GTH was effective and LH/FSH was ineffective.

Fontaine et al. (1972) examined the potency of cGTH or SG-G100 on goldfish adenyl cyclase activity and showed that, while both GTHs elevated cAMP production, only cGTH exerted a prolonged effect, suggesting some degree of species specificity of response. Further reports have implicated cAMP in the mediation of teleost GTH induced effects (Fontaine et al., 1970; Menon and Smith, 1971). Interestingly it appears that some gonadal rhythm in sensitivity to GTH may be mediated via cAMP production. Thus Kuo and Watanabe (1978) report that exogenous GTH and prostaglandins were more effective in elevating cAMP levels in Oryzias latipes when applied at the end of the light phase of the photoperiod regime. Similarly Hirose and Donaldson (1972) and Hirose and Hirose (1972) have reported that maximum sensitivity to GTH induced spawning occurs at a similar period as the sensitivity of cAMP production to GTH stimulation.

Stimulation of interstitial cell steroidogenic enzymes by GTH has been noted in teleost testis (Pickford and Atz, 1957; Yamazaki and Donaldson, 1968b, 1969; Hoar, 1969; de Vlaming, 1974). During the reproductive cycle male teleosts show increased testicular levels of HSD and glucose-6-phosphate dehydrogenase (G6PD) enzymes associated with increased GTH levels (van den Hurk et al., 1978a, b).

Direct evidence for an effect of GTH upon the biosynthesis of androgens by the teleost testis was obtained by Ng and Idler (1980). Maturational (Con AII) GTH prepared from winter flounder pituitary reinitiated the production of 11-KT and T production in hypophysectomized winter flounder. In addition this preparation induced spermiation and a reappearance of sex hormone binding protein, lost after hypophysectomy. Vitellogenic (Con AI) GTH was unable to

stimulate androgen production, though it was able to bring about a reappearance of binding protein. An antiserum to Con AII maturational GTH reduced 11-KT production in intact salmon. In juvenile rainbow trout Con AII stimulated androgen production and especially 11-KT in the male and T in the female.

In the plaice (Pleuronectes platessa) androgen production was measured in vitro and the effect of gonadotropin or pituitary extracts studied (Duggan and Bolton, 1982). In testicular tissue from spermiating fish GTH or pituitary treatment resulted in a 680% increase in T production, while a fall in 11-KT production (46%) was observed. In immature testicular tissue, both 11-KT and T production were elevated by SG-G100 indicating a variation in response to GTH depending upon gonadal state.

3•4•4 Sex Steroids

a) Testicular Steroidogenesis

In teleost fish, as in all vertebrates, steroid structure, in contrast to that of peptide hormones, appears to be conservative (Colombo et al., 1977). However, teleost steroidogenesis appears to show several novel features. Both 11-deoxy and 11-oxygenated androgens are synthesised by the teleost testis (Ozon, 1972). The chief testicular androgens produced are testosterone (T), (17β-hydroxy-4-androsten-3-one), 11β Hydroxytestosterone (11βOH), (17-dihydroxy-4-androsten-3-one) and 11-ketotestosterone (11-KT), (17β-hydroxy-4-androsten-3, 11-dione) (Idler et al., 1971). Though both T and 11-KT have been shown to have androgenic properties in vivo the physiological importance of 11βOH is uncertain.

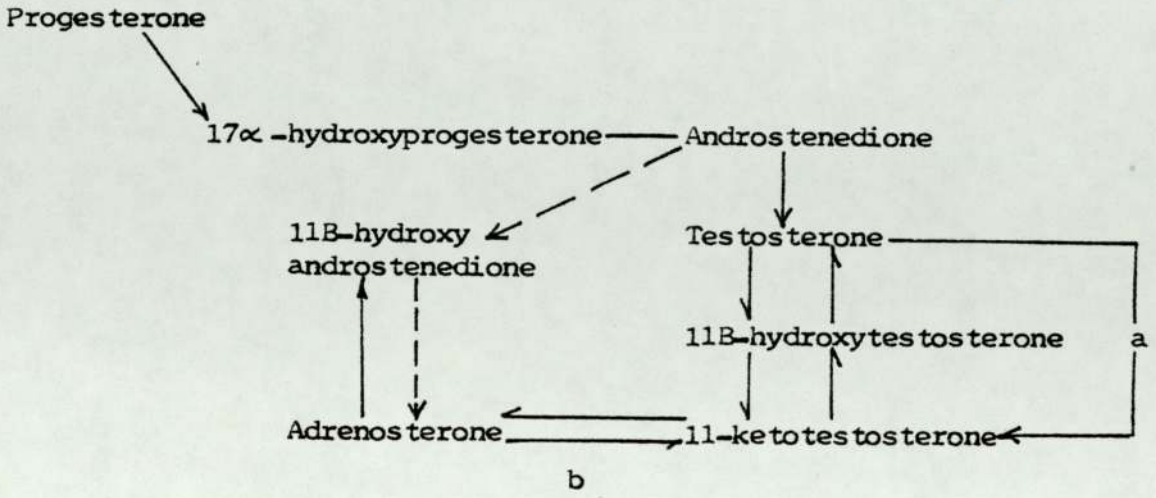
Testosterone was identified in the plasma of both male and female spawned Atlantic salmon (Grajcer and Idler, 1961), while 11-KT was first identified in the plasma of post-spawned male sockeye salmon (Idler et al., 1961a). The presence of these two androgens was confirmed by Schmidt and Idler (1962) and Idler et al. (1971). Higher levels of the two androgens were found in testicular blood, indicating the testicular source of these steroids. 11-KT was shown to be present at higher concentrations than T, in both peripheral and testicular plasma and was suggested to be the main androgen in salmon as its levels increased with sexual maturation, while that of T did not (Idler et al., 1971). 11βOH was also identified in gonadal and interrenal tissue of Atlantic salmon (Idler and McNab, 1967; Idler et al., 1966) and was synthesised by rainbow trout testicular homogenates (Arai and Tamaoki, 1967a, b). Incubation of teleost testicular tissue with a variety of labelled precursors has enabled the elucidation of the testicular steroidogenic pathways (Idler et al., 1966; Arai and Tamaoki, 1967a, b; Idler and McNab, 1967; Freeman and Idler, 1975; Kime, 1980). A broad scheme suggested by the work of Arai and Tamaoki is shown below (Fig. 3·4·4 ·1).

The main pathway of androgen synthesis involves 17α-hydroxy-progesterone and androstenedione. Synthesis of 11-KT is thought to proceed via testosterone and 11β hydroxytestosterone, both of which function as intermediates in this process. It has been suggested (Idler et al., 1966) that in some circumstances 11-KT production may 'bypass' 11βOH and proceed directly from T, though this is not widely accepted. Similarly, another minor route to 11-KT production seems to be by the reduction of adrenosterone (A) to 11-KT (Idler and McNab, 1967). Apart from their roles in androgen biosynthesis, little is known of the physiological roles of 11βOH and A.

Fig. 3•4•4•1

Androgen Biosynthesis by testicular tissue of teleosts

(from Arai and Tamaoki, 1967a, b)



——→ Steps demonstrated in vitro.

— — —→ Steps inferred.

a. Possible bypass pathway suggested by Idler et al. (1966).

b. Reduction of Adrenosterone to 11-KT suggested by Idler and McNab, (1967).

As early methods of steroid assay, such as double derivative analysis, were relatively tedious procedures, requiring large volumes of plasma or tissue extract, knowledge of the roles of androgens in salmonid reproductive physiology has been slow accumulating. However, with the development of analytical techniques, such as RIA, allowing the measurement of steroid levels in small volumes of plasma, the scope for investigation has been greatly extended (Schreck et al., 1972a,b; Sangalang and Freeman, 1977; Simpson and Wright, 1977; Wingfield and Grim, 1977).

The previously observed sex distribution of T and 11-KT has been confirmed in the majority of teleost species examined by such methods. Thus T is present in both male and female teleosts, including winter flounder (Campbell et al., 1976), Atlantic and Pacific salmon (Ozon, 1972; Hunt et al., 1982), plaice (Wingfield and Grim, 1977), cod (Sangalang and Freeman, 1977) and rainbow trout (Simpson and Wright, 1977; Scott et al., 1980a, b).

11-KT appears to be confined in male teleosts. In rainbow trout (Wright, 1976; Scott et al., 1980a), Atlantic salmon (Hunt et al., 1982), salmon parr (Dodd et al., 1978; Stuart-Kregor et al., 1981) and brook trout (Sangalang and Freeman, 1977) 11-KT is confined to males and levels increase at the time of maturity and spermiation. Though Schmidt and Idler (1962) reported the presence of relatively high levels of 11-KT in plasma of post-spawned female salmon, this report remains unconfirmed. Katz and Eckstein (1974), Lambert and Pot (1975) and Colombo et al. (1977) have noted the occurrence of 11-KT in female Tilapia aurea, Poecilia reticulata and Anguilla anguilla respectively. However, the occurrence of 11-KT in female teleosts is sporadic and appears to be unrelated to maturation.

In addition to the production of free androgens, the teleost testis is capable of producing relatively large quantities of steroid glucuronides (Grajcer and Idler, 1961, 1963; Hews and Kime, 1978; Kime, 1979, 1980). These conjugates are normally produced by the liver in mammals and are thought to represent a method of physiologically inactivating free steroids (Hadd and Blickenstaff, 1969). The large amounts of these glucuronides produced by the teleost testis suggest they may play an active role, perhaps in the control of steroidogenesis (Kime, 1980). As conjugates they would not be measured by steroid RIA without prior hydrolysis.

b) Cyclic changes in sex steroid levels

Among immature salmonids low plasma androgen levels have been recorded. In 40 g and 150 - 350 g rainbow trout (immature) plasma levels of 11-KT were less than 1ng/ml (Magri, 1980) and 2 - 4 ng/ml (Wright, 1976). These levels increase markedly with the initiation and development of sexual maturation. Such cyclic changes in androgen levels have been reported in a number of salmonid species. In the rainbow trout (Schreck , 1974; Sanchez Rodriguez et al, 1978) increases in total androgens have been recorded at the time of maturation.

Serum levels of testosterone have been shown to increase as sexual maturity is approached in the Atlantic salmon (Idler et al, 1971; Hunt et al, 1982), salmon parr (Stuart-Kregor et al, 1981; Dodd et al, 1978), rainbow trout (Whitehead et al, 1979; Campbell et al, 1980; Scott et al, 1980a, b), brook trout (Sangalang and Freeman, 1974) and winter flounder (Campbell et al, 1976).

Similarly, serum levels of 11-KT have been shown to increase in rainbow trout (Wright, 1976; Campbell et al., 1980; Scott et al., 1980a), Atlantic salmon (Idler et al., 1971, 1981; Hunt et al., 1982), salmon parr (Dodd et al., 1978; Stuart-Kregor et al., 1981) and brook trout (Sangalang and Freeman, 1974). However, unlike T levels, the increase in 11-KT is only observed in males.

Relatively few studies have followed variations in levels of both androgens within individual fish over the course of the sexual cycle, though Whitehead et al. (1979) have provided such data for seasonal variations in T levels. Only three studies have followed changes in both androgens throughout the sexual cycle of male salmonids (Scott et al., 1980a; Stuart-Kregor et al., 1981; Hunt et al., 1982).

Scott et al. (1980a) showed levels of both T and 11-KT began to increase from basal (2 - 4 ng/ml) levels over the period July - October in male rainbow trout and that T levels reached peak values of 150 ng/ml in November, just prior to spermiation in December. 11-KT levels continued to rise and reached peak values of 250 ng/ml in February, after the initiation of spermiation. Hunt et al. (1982) recorded a different androgen profile in male Atlantic salmon grilse. In these fish 11-KT levels began to increase in early Spring, prior to the increase in T and rose rapidly from mid-August to reach a broad peak in September - October of more than 300 ng/ml. Following the initiation of spermiation in October - November, levels of 11-KT fell sharply to basal levels in December. T levels increased in July - August and showed a sharp increase thereafter to reach peak values of 100 ng/ml in September, just prior to spermiation. T levels fell rapidly to basal at the initiation of spermiation.

In the male Atlantic salmon parr (Stuart-Kregor et al, 1981) levels of both androgens were much lower than those recorded by Scott et al. (1980a) and Hunt et al. (1982). Basal levels of T in immature male parr were 0·69 ng/ml and a significant increase was observed in maturing parr (0·91 ng/ml) with a further increase to 2·81 ng/ml in fully mature fish. Levels of T were significantly lower in regressing parr than in fully mature fish, but remained well above basal. 11-KT levels showed a similar profile, except that the increase observed from immature (0·90 ng/ml) to maturing parr (1·55 ng/ml) was non-significant. Levels in mature fish were significantly higher (5·5 ng/ml) and again a significant fall occurred in regressing males (2·19 ng/ml), though levels remained above basal.

The differences in results observed in the three studies examined above may be related to differences in age and species of experimental fish. It is nevertheless possible to draw certain conclusions. Firstly 11-KT levels in maturing fish are higher in fish approaching spermiation and while T levels fall either prior to or shortly after spermiation, increased 11-KT levels appear more prolonged. Both androgens show a relatively rapid increase towards the time of sexual maturity, though Hunt et al. (1982) observed an early increase in 11-KT levels, not of T levels, in salmon grilse. They point out this early increase in 11-KT coincides with the beginning of testicular gametogenesis and suggest a causal relationship between the two events. An early increase of 11-KT, prior to that observed for T, was not observed in rainbow trout or salmon parr.

The presence of higher levels of 11-KT in mature male salmonids seems to reflect the results of Ng and Idler (1980), who noted

preferential synthesis of 11-KT in immature male rainbow trout and Testosterone in immature female rainbow trout following injection of GTH (Con AII). Similar results have been recorded in non-salmonids. The injection of HCG, which stimulates spermiation *in vivo* in *Sparus auratus*, stimulates the *in vitro* production of 11-KT but depresses the production of T in testicular homogenates (Eckstein *et al*, 1978).

The low androgen levels of mature salmon parr are interesting as they confirm the earlier results of Dodd *et al.* (1978), though whether they reflect differences in the age of maturation or species differences remains unclear. No significant differences were noted between early maturing (1 year) male rainbow trout and normally maturing males (Scott *et al*, 1980a). However, in this experiment the fish showed very rapid growth and this result does not rule out an age related effect upon size, which may in turn influence androgen levels.

One major difference between the reports examined is the relative timing of the increases in serum androgens. Scott *et al* (1980a) found increased T levels prior to 11-KT. T levels peaked earlier and then fell rapidly while 11-KT levels were still rising. Hunt *et al.* (1982) observed an early increase in 11-KT levels, paralleling gonadal growth, and a late increase in both steroids. Again T peaked prior to 11-KT This pattern led Hunt *et al.* to suggest that T was not directly implicated in the early stages of spermatogenesis and spermiation. Spermatogenesis may, however, be sensitive to local hormone levels and results obtained from peripheral blood samples may be misleading. In cyprinids, such as the carp, only very slight increases in serum androgens are recorded

prior to spermiation and throughout the reproductive cycle androgen levels remain low (3 ng/ml). Thus the early stages of spermatogenesis may be sensitive to very low ('tonic') androgen levels (Weil, 1981).

The suggestion that 11-KT is the major androgen in salmonids is supported by a number of experimental observations. The greater peak levels of 11-KT, compared with T, have already been mentioned. 11-KT is also largely confined to male salmonids, while T is present in both males and females and occurs at higher levels in female than in male mature salmonids (Scott et al, 1980b). 11-KT has also been demonstrated to have a greater androgenic potency than T (Idler et al., 1961a,b; Arai, 1967; Hishida and Kawamoto, 1970), though this difference may be related to the relative protein binding in vivo of the two androgens. 11-KT has been reported to compete poorly with T for binding sites on the sex hormone binding protein (SHBP) of trout (Fostier and Breton, 1975).

However, T may still have an important role in the overall control of the reproductive process. Billard (1978) showed T was able to exert a negative feedback effect upon pituitary GTH activity early in the sexual cycle in trout, while 11-KT was effective later in the cycle. It has been suggested that steroid feedback upon the hypothalamus-pituitary axis is dependent upon the aromatization of androgens to estrogens (Callard et al, 1978a, b). As 11-KT is non-aromatizable (Engel, 1975), the feedback effects of T and 11-KT would differ and this has been shown to be the case (Crim et al, 1981).

The role of high T levels in female teleosts is currently the subject of investigation. Testosterone acts as a precursor in the

synthesis of estradiol 17β (E_2) (Lambert and Pot, 1975) and it has been suggested that high T levels in females may reflect an inability of E_2 synthesis to account for all T production (Campbell et al, 1976). Again T may have a role in the feedback control of reproduction in the female. High levels of aromatase activity have been demonstrated in the brains of rainbow trout (Lambert and van Bohemen, 1980) and a seasonal rhythm observed for such activity. Thus increased T levels at a time when E_2 levels are low or falling may help to maintain GTH at a level permitting ovulation.

In addition to seasonal variation in androgen levels, circadian variations in androgen levels may occur. Such variation was shown by Schreck et al. (1972a), though due to the large standard errors these results were not statistically significant. It has been suggested that circadian variation in androgen levels may result from a variation in androgen clearance rates as suggested in the skate (Fletcher et al, 1969).

Though a number of reports have recorded the presence of relatively high levels of E_2 in male teleost plasma (Schreck and Hopwood 1974; Terkatin-Shirmony and Yaron, 1978; Billard and Breton, 1978) these reports were not confirmed by Whitehead et al. (1978) who observed low levels of E2 throughout the reproductive cycle of rainbow trout.

c) The control of testicular steroidogenesis

The control of steroidogenesis within the salmonid testis has been shown to be under the control of GTH, as was directly demonstrated by Ng and Idler (1980). Interestingly no apparent variation in testicular sensitivity to exogenous GTH between mature and immature fish has been

observed (Donaldson et al., 1972 b;Funk and Donaldson, 1972) suggesting no age-related regulation of testicular GTH responses. The preferential synthesis of 11-KT in males and T in female immature trout (Ng and Idler, 1980) suggests a genetic difference in enzyme systems between the sexes. However, in Sparus aurata, a protandrous hermaphroditic teleost, HCG treatment resulted in a stimulation of 11-KT production in both male and female gonadal tissue (Eckstein et al., 1978). On the other hand T synthesis was stimulated in the ovary and depressed in the testis. As the majority of mature fish are female (95%) in this species, the authors suggest T depression may be an important factor in switching development to female elements.

In addition to GTH control of steroidogenesis, it has been suggested that the reported high testicular glucuronide production represents a further control mechanism (Kime, 1980). The major androgens T, 11-KT and 11βOH are all produced both in a free and a conjugated (glucuronide) form by the teleost testis. In trout the main glucuronide produced appears to be T-glucuronide (Hews and Kime, 1978; Kime, 1979, 1980) and it may be that diversion of T precursor represents a mechanism by which 11-KT production is controlled.

It has recently been reported that at the time of spermiation or ovulation in rainbow trout, a shift in steroidogenesis occurs and that the pathway between 17α-hydroxyprogesterone and androstenedione is blocked (Scott and Baynes, 1982). Thus levels of synthesised androgens fall and 17α-hydroxyprogesterone and 17α-hydroxy-20β-dihydroprogesterone (17α-20β) levels rise. This is of interest as 17α-20β has been directly implicated in the process of ovulation (Fostier et al., 1973; Jalabert, 1976; Jalabert et al., 1977) and

these progestagens may play some role in the process of spermiation. The factors responsible for blocking the activity of the C21 desmolase responsible for this shift in steroidogenesis are not yet known.

d) Protein Binding of Sex Steroids

Steroids may interact with a variety of plasma proteins and in the majority of mammalian species the binding of steroids to protein molecules represents a means of physiologically inactivating steroids (Sandberg et al, 1966; Westphal, 1971). It seems similar systems operate in teleosts and elasmobranch fishes. Protein binding of teleost sex steroids seems to involve two distinct systems. A weak binding, transcortin-like, system has been demonstrated in Atlantic salmon plasma, with low affinity for sex steroids (Freeman and Idler, 1966; Idler and Freeman, 1968, 1969; Fostier and Breton, 1975). In addition a high affinity sex hormone binding protein (SHBP), with a high affinity for T and E_2 has been isolated from rainbow trout plasma (Fostier and Breton, 1975). (However, although the terms low and high affinity are used, it is important to stress that even the strongly binding teleost SHBP has a 10 - 100 fold lower binding than mammalian Corticosteroid Binding Protein (CBP)).

The conclusion that protein binding inactivates steroids has been verified in teleosts by the demonstration that unbound steroid was effective in inducing in vitro maturation of trout oocytes, while bound steroid was largely inactive (Fostier and Breton, 1975). In addition, the authors showed that the SHBP of trout had a far greater affinity for T than for 11-KT.

No detailed data exist regarding possible changes in protein binding of androgens, either throughout the sexual cycle or between sexes. However, Idler and Freeman (1968, 1969) noted that in an elasmobranch (thorny skate) SHBP binding of T was of similar magnitude in both sexes and was unaffected by maturity. In mature female rainbow trout Fostier and Breton (1975) report that 73% of testosterone was present in a bound form but present no data on corresponding binding in mature male trout. Some comparative data on the relative binding efficiencies of androgens in intact juvenile trout of both sexes has been reported (Ng and Idler, 1980). A higher ratio of Bound : Free steroid was recorded in female trout (16 and 38) compared with male trout (1·9 and 1·8). Unfortunately as T was used in binding studies in females and 11-KT in males, these differences cannot be attributed to sex differences.

The presence of SHBP in the blood of teleosts appears to be under pituitary control and a disappearance of SHBP follows hypophysectomy in the winter flounder (Ng and Idler, 1980). Both Con AI and Con AII were able to restore this protein in hypophysectomized fish. Thus its disappearance would seem unrelated to blood steroid levels as while Con AII elevates steroid levels Con AI appears not to do so but was still able to stimulate the reappearance of SHBP.

e) The endocrine control of spermatogenesis

Spermatogenesis and spermiation comprise the sequence of cellular transformations by which primordial germ cells are converted to viable gametes and these gametes released. Both processes are under intimate endocrine control (Billard et al, 1982; Fostier et al, 1982).

The hormonal environment in which these events take place has already been examined. It seems that spermatogenesis, especially the later stages, takes place when GTH levels are high and androgen levels either high or rising. As mentioned, some workers have observed initial increases in GTH and androgens early in the spermatogenic cycle which may be related to the cellular processes of spermatogenesis. Spermiation commences at a time when GTH levels are high (Fostier et al, 1982). As spermiation proceeds GTH levels fall, though they may rise subsequently (Sanchez-Rodriguez et al, 1978). Androgens seem to reach peak levels close to the end of spermatogenesis and initiation of spermiation, though peak levels of 11KT occur later than those of T and seem more intimately associated with spermiation. These hormonal changes provide indirect evidence that both spermiation and spermatogenesis are controlled by GTH and/or androgen levels. However, the exact relationship between hormone levels and the initiation and control of these processes remains uncertain as does the extent to which GTH exerts its effects either directly or via androgens.

The administration of exogenous GTH has been shown to be capable of stimulating spermatogenesis in some teleost species (Ahsan and Hoar, 1963; de Vlaming, 1972; Pickford et al, 1972). Induction of spermiation has also been produced by the injection of pituitary extracts ('hypophysation') to mature fish (Pickford and Atz, 1957; Shehadeh, 1975; Zohar and Billard, 1978) and by the administration of exogenous GTH, such as HCG (Clemens and Grant, 1965; Kuo et al., 1973; Carreon et al., 1976). In intact mature trout spermiation has been reinitiated, though only temporarily, by injection of tGTH (Billard, 1982).

Evidence that GTH is required for spermatogenesis and spermiation has been provided by the demonstration of a cessation of these processes following hypophysectomy, though the stage at which these cease seems to vary with species. Due to technical difficulties, hypophysectomy of salmonids is very difficult (Yamazaki, 1976), but has been carried out in a number of teleost species. In the lake chub (Ahsan, 1966b), guppy (Billard, 1969) and Indian catfish (Sundararaj and Nayyar, 1967) hypophysectomy caused a cessation of spermatogenesis and testicular regression. Similarly, hypophysectomy suppressed spermiation in winter flounder (Ng and Idler, 1980), goldfish (Yamamoto and Yamazaki, 1967; Yamazaki and Donaldson, 1968a; Billard, 1976, 1977b), though not in the lake chub (Ahsan, 1966b)or plaice (Barr, 1963).

Replacement therapy with GTH has been attempted but as many of the preparations administered are mammalian hormones (LH, FSH, HCG) results require careful interpretation. Successful stimulation by GTH of spermatogenesis in hypophysectomized teleosts has been reported (Ahsan, 1966b;Sundararaj and Anand, 1972; Billard and Escaffre, 1973; Nayyar et al., 1976). Generally it seems teleostean GTHs are more successful in such stimulation (Ahsan, 1966b;Yamazaki and Donaldson, 1968b; Billard, 1970). Spermiation has also been reinstated in hypophysectomized goldfish by both cGTH (Billard, 1977b) and SG-G100 (Yamazaki and Donaldson, 1968a, 1969). Billard (1982) has suggested that the GTH species specificity requirement for reinstatement of spermiation is much wider than that for spermatogenesis.

As an alternative to hypophysectomy, the requirement for GTH of spermatogenesis and spermiation has been investigated by 'chemical hypophysectomy'. Treatment with methallibure inhibits gonadal function in mammals by selective inhibition of pituitary gonadotrope activity

(Paget et al., 1961; Brown, 1963). Methallibure treatment has been shown to bring about a cessation of spermatogenesis in teleosts (Hoar et al., 1967; Martin and Bromage, 1970; Pandey, 1970; Kapur and Toor, 1978; Hyder et al., 1979). Administration of methallibure to mature male salmon parr was unable to prevent spermatogenesis and spermiation, though it depressed androgen levels (Murphy, 1980a, b).

No direct evidence for the requirement of a second GTH in the control of spermatogenesis and spermiation has been presented. Only Con AII (maturational) GTH was effective in reinstating androgen secretion and spermiation in hypophysectomized winter flounder. Con AI (vitellogenic) GTH had no effect on the reinstatement of androgens or spermiation (Ng and Idler, 1980).

The action of GTH upon spermatogenesis and possibly spermiation are believed to be mediated by androgens. To what extent these two hormone species act independently or in tandem is uncertain. The administration of androgens to intact fish has been shown to be capable of stimulating spermatogenesis (Shehadeh et al., 1973; Fagerlund and McBride, 1977), though when high doses are administered (Hirose and Hibiya, 1968b; Yamazaki, 1976) the opposite effect may be observed. In intact fish endogenous hormones may complicate the interpretation of results. Androgen effects may be mediated via a feedback on GTH production, though Billard (1982) reports on inhibition of spermatogenesis by MT and E_2 treatment in the absence of changes in GTH levels.

In the absence of endogenous hormones, exogenous testosterone has been shown to be capable of stimulating and initiating spermatogenesis in goldfish testicular fragments (Remacle, 1976; Declerq et al., 1977). In hypophysectomized teleosts variable results of exogenous androgen

administration upon spermatogenesis have been reported. In hypophysectomized goldfish androgens were able to initiate and maintain spermatogenesis, though at high doses (Yamazaki and Donaldson, 1968b; Billard, 1974). Similarly, in hypophysectomized Indian catfish spermatogenesis was supported by exogenous androgens, though surprisingly corticosteroids were also effective (Sundararaj and Nayyar, 1967; Nayyar et al., 1976). Generally high doses were required for this effect, perhaps due to the existence of the blood-testis barrier around semeniferous tubules. It has been suggested that in some species only certain stages of spermatogenesis are GTH sensitive (Lofts et al., 1968; Pandey, 1969), though this may reflect the question of a locally effective dose for stimulation.

In hypophysectomized teleosts spermiation seems less androgen dependent (Billard et al., 1982), perhaps due to the already high androgen levels and the requirement for androgens seems to be non-specific; thus corticosteroids may stimulate spermiation in mature goldfish (Billard, 1976). In salmonids the situation is less clear. In the majority of cultured salmonids (salmon and rainbow trout) spermatozoa are not normally shed under artificial conditions in the absence of specific stimuli such as spawning gravel beds. Thus other factors in addition to GTH and androgens may be involved in spermiation. One group of factors which may exert an influence are the prostaglandins. These have been implicated in female sexual behaviour in teleosts (Stacey, 1981), and in male goldfish specific stimuli such as the presence of an ovulating female leads to a rapid increase in plasma GTH and milt production. This rapid increase in GTH may be prostaglandin mediated (Kyle et al., 1979). Such a rapid acting short term response seems likely to form part of the spawning behaviour and spermiation response of male salmonids.

It seems clear that control of both spermatogenesis and spermiation are under at least partial control of pituitary GTH, which may exert all or part of its effects via sex steroids. Both spermatogenesis and spermiation seem to differ in the degree of dependence upon these factors.

3·5 Control of Reproductive Cycles

3·5·1 Environmental factors

Environmental influences upon teleost reproductive cycles have been reviewed by de Vlaming (1972, 1974), Peter and Crim (1979), Peter (1981) and Crim (1982). The particular environmental influences controlling salmonid reproductive activity will be examined in greater detail in a later section. Within salmonids and gasterosteids there is general agreement that photoperiod is the predominant environmental variable regulating reproductive activity, while in cyprinids and gobids temperature becomes the major regulating factor (de Vlaming, 1974).

The importance of photoperiod has been demonstrated by Whitehead et al. (1978a, 1979). Compression of photoperiodic change into a shorter cycle resulted in an acceleration of the endocrine changes associated with maturation and earlier spawning. In male rainbow trout compression of the photoperiodic cycle into either six or nine months resulted in earlier spermiation and an earlier peak of serum testosterone in compressed groups (Whitehead et al., 1979).

Breton and Billard (1977) also observed an effect of shortening photoperiod, though not of constant long or short photoperiods, upon gonadal recrudescence of male rainbow trout. Spermatogenesis was accelerated under shortening photoperiod, with a greater effect at 16°C than at 8°C. Elevated plasma GTH levels were recorded in male trout

under shortening photoperiods and again the greatest increase was recorded at higher temperatures (16°C). Photoperiod has also been implicated in the control of reproductive cycles in Phoxinus phoxinus, Gasterosteus aculeatus and Heteropneustes fossilis (Baggerman, 1969, 1972; Vasal and Sundararaj, 1975; Sundararaj and Vasal, 1976; Scott, 1979).

Similar results have demonstrated the importance of temperature in cyprinids (Gillet and Billard, 1977; Gillet et al., 1977, 1978; Hontela and Peter, 1978; Peter and Crim, 1979; Cook and Peter, 1980) and increased GTH levels reported to accompany temperature-induced gonadal development.

Both light and temperature effects via the CNS act to trigger release of pituitary GTH, which stimulates gonadal development. Temporal variation in sensitivity to GTH (de Vlaming and Vodicnik, 1977b) may modify the response to GTH. In addition sensitivity to circadian variation in GTH (Hontela and Peter, 1978, 1980; Peter et al., 1982) may 'trigger' gonadal development even in the absence of elevated GTH levels.

3•5•2 Steroidal feedback

Steroidal feedback control of reproduction is a well documented concept in mammalian reproductive physiology (Donovan, 1970; Turner and Bagnara, 1971). Similar steroidal feedback on the hypothalamic-pituitary axis operates in teleosts and has been demonstrated in salmonids. Thus castration throughout the sexual cycle of rainbow trout has been shown to induce increases of pituitary GTH secretion indicating a negative feedback effect of sex steroids (Billard et al., 1976, 1977; Billard, 1978). While castration prior to gonadal development caused only a slight increase in plasma GTH, castration

later in the cycle produced greater increases in GTH. At an early stage of testis development GTH levels increased two-fold following castration, while in spermiating rainbow trout a five-fold increase in plasma GTH was observed. These results suggest negative feedback of sex steroids exert a progressive constraining effect upon the pituitary and hypothalamic axis. Exogenous T, E_2 or, later in the cycle, 11-KT, can suppress these GTH increases, indicating the steroidal nature of this feedback. (Interestingly, Billard (1982) reports that hemicastration of rainbow trout results in an increase in weight of the remaining testis, though GTH levels do not increase following hemicastration. He suggests that in addition to a GTH mediated effect on testicular size, there may be a chalone-like factor controlling gonadal growth.).

Evidence of the direct uptake of radioactive steroids has shown androgen and estrogen binding sites in the NLT and the NPO of male Paradise fish (Macropodus opercularis), goldfish (Carassius auratus) and platyfish (Xiphophorus maculatus). (Davis et al, 1977; Kim et al., 1978a, b). Perhaps significantly these areas have been implicated in the control of GTH release in teleosts.

In addition to this negative feedback effect, it appears teleost sex steroids may play a role in positive feedback upon the hypothalamus-pituitary. T administration was thus able to increase pituitary GTH in male and female Atlantic salmon parr and rainbow trout (Crim and Peter, 1978; Crim and Evans, 1979). It was also shown that E_2 and aromatizable androgens, such as T, were the effective steroids in this response (Crim et al, 1981). The synthetic steroid 1, 4, 6-androstatrien - 3, 17 - dione, which blocks aromatase activity and prevents the conversion

of T to estrogens, was shown to be capable of blocking the response to T. T and 11-KT appear to differ in their feedback roles as T is aromatizable, while 11-KT is not and the two steroids represent different inputs to the hypothalamic centres controlling reproduction.

Similar positive feedback is suggested in immature male and female European eels by cytological evidence suggesting increased gonadotrope activity (Olivereau and Chambolle, 1978; Olivereau and Olivereau, 1979a, b). Recent work has examined the possibility that maturational effects of positive feedback by steroids may act to promote sexual maturation, as has been suggested in the mammalian initiation of puberty (Donovan, 1970). T treatment of pituitary gonadotropes has been shown to stimulate GTH production and storage, which could be released by LH.RH (Crim and Evans, 1980). Gielen et al. (1982) showed a similar effect, again in rainbow trout, with T acting to promote synthesis of gonadotrope GTH and released GTH promoting steroid synthesising enzyme activity (3β HSD) in the testis and such an effect has been demonstrated by Goos et al. (1982), Olivereau et al. (1982) and van Ree et al. (1982). It seems such feedback by aromatizable androgens may act upon the hypothalamic-pituitary axis to stimulate GTH secretion in sexually immature teleosts and bring about the initiation of maturity. In view of its sporadic occurrence in male teleosts a direct role for E_2 seems unlikely, though Billard et al. (1978) report a temporary increase in plasma E_2 at the initiation of gametogenesis in rainbow and brown trout.

3•5•3 Other hormonal influences on reproductive cycles

a) Thyroid hormones

The thyroid hormones, thyroxine (T_4) and thyronine (T_3) have been suggested to be capable of exerting an influence on reproduction in teleosts (Sage, 1973). Alterations in thyroid activity have been reported in sexually mature teleosts (Fontaine and Leloup, 1962; Dodd and Matty, 1964) and similar alterations in thyroid activity have resulted from the administration of sex steroids (Sage and Bromage, 1970; van Overbeeke and McBride, 1971). Hunt and Eales (1979) showed that testosterone propionate was capable of either directly or indirectly stimulating thyroid function in immature rainbow trout. However, the changes in thyroidal function associated with sexual maturation seem more likely to reflect the high androgen levels associated with this process and there is little evidence of a direct role of the teleost thyroid in control of sexual activity and maturation. In support of this conclusion Osborn and Simpson (1978) reported no difference between the thyroid hormone status of mature and immature rainbow trout. In addition, Pickering and Christie (1981) report no increase in plasma thyroxine among mature male brown trout.

b) Corticosteroids

Seasonal changes in corticosteroids have been reported in maturing teleosts (Robertson and Wexler, 1960b). However, the interpretation of the significance of these changes is complicated by the occurrence of elevated corticosteroid levels associated with spawning migrations (Hane and Robertson, 1959; Schmidt and Idler, 1962), though such elevated 11-oxygenated corticosteroid levels have also been reported in non-migratory rainbow trout (Robertson *et al.*, 1961) and brown trout

(Pickering and Christie, 1981). Perhaps the strongest role for an involvement of corticosteroids in teleost reproduction comes from *in vitro* work showing corticosteroids to be capable of inducing ovulation in ovary of *Heteropneustes fossilis* (Goswami and Sundararaj, 1971), thus confirming the *in vivo* response in this species (Sundararaj and Goswami, 1966a, b) to corticosteroid induced ovulation.

The literature regarding the identity of steroidal mediators of GTH induced ovulation has been reviewed (Hirose, 1976; Jalabert, 1976; Lam *et al.*, 1978; Iwanatsu, 1980). It seems most likely that a progestagen, 17α 20β, is the most likely mediator of GTH induced ovulation in salmonids.

It has been suggested that cortisol may be catabolized in the teleost liver to 11β-hydroxyandrostenedione and androstenetrione and thus be capable of acting as a precursor for androgen formation (Kime and Hews, 1978b). However, Truscott (1979) failed to find evidence to suggest bilary excretion of 11-KT steroids following the injection of cortisol into immature trout. Thus although corticosteroids may play some role in the regulation of reproductive activity of certain teleosts, it is not yet possible to assign them a function in male salmonids.

c) The Pineal Gland

The pineal gland has been shown to be capable of exerting an influence on gonadal maturation in mammals such as the hamster (Reiter *et al.*, 1975), which rely upon photoperiod to synchronize reproduction. A number of experiments suggest this may be the case in teleosts. Pinealectomy of teleosts has resulted in gonadal either recrudescence

or regression (Fenwick, 1970b; Urasaki, 1976; de Vlaming and Vodicnik, 1977b; Vodicnik et al., 1978) depending upon environmental conditions. However, in other teleosts pinealectomy had no effect on gonadal condition (Peter, 1968; Vodicnik et al., 1978).

Melatonin, a pineal constituent, has been shown to influence gonadal function in either a positive or negative manner depending upon environmental conditions. Extended treatment of teleosts resulted in gonadal regression (Fenwick, 1970a; Urasaki, 1972 ; de Vlaming et al., 1974; Saxena and Anand, 1977).

Thus it seems that, depending upon environmental conditions, the pineal is capable of exerting a modifying influence over the control of reproduction and this role seems to be related to mediating the perception of photoperiod information.

3·6 Conclusions

This review has shown the teleost reproductive system and its associated endocrine control mechanisms to comprise a number of diverse components, the integrated action of which gives rise to the production of viable gametes, the ultimate objective of the process.

In broad outline this system is similar to that described in the mammal. Thus the CNS-hypothalamus controls the activity of pituitary gonadotropes by means of releasing hormones. As yet no teleostean GRF has been isolated and chemically sequenced and questions remain regarding the significance of LH.RH like activity in teleost brains. The release of GTH brings about maturation of gonadal germ cells and their proliferation and development (spermatogenesis), though the exact nature of GTH's effects at the testicular level, and in particular to what extent they involve androgens, remains uncertain.

In particular the possible role of a second (Con AI) GTH in male teleosts poses a number of questions which must remain unanswered until the development of assay techniques allow the measurement of this compound. Until this time it seems GTH activity in male teleosts may be, as in mammals, thought to be limited to glycoprotein GTH (Con AII).

The presence of two androgens, 11-KT and T, in male teleosts appears to be unique to teleosts and certain amphibia. Again the relative roles of the two hormones in spermatogenesis, feedback control and the initiation of male secondary sexual characteristics require further work.

At the testicular level recent work suggests an interesting complexity in the control of steroidogenesis and of the significance of shifts in steroid metabolism and glucuronide formation, while at the opposite end of the control network it is now realised that sex steroids may have an important part to play in the initiation of sexual maturation by means of a positive feedback effect upon the hypothalamic-pituitary axis.

In addition to its scientific interest such a study provides a means of consideration of sites through which the problems of male maturity in salmonids may be approached and provides a rationale for the application of such methods.

CHAPTER 4

COMMERCIALLY IMPORTANT ASPECTS OF SEXUAL MATURATION AND THEIR ENDOCRINE CONTROL

Contents

4•1 Introduction

Salmonid cultivation may be viewed from a reductionist viewpoint as the conversion of relatively low cost nutrients into high value muscle protein with maximum yield in minimum time (Simpson, 1976). Thus growth rate, food conversion and the production of a suitably sized product at the correct time are of paramount importance to the commercial trout or salmon producer.

As already discussed the changes associated with sexual maturation in male salmonids have proved to be a major constraint to the efficient cultivation of salmonids. Deleterious changes in maturing males, allied to the appearance of the male secondary sexual characteristics are of special concern. These changes occur at least one year earlier in male salmonids, and in the case of precociously mature males two years earlier, than the corresponding changes in females. In addition the changes accompanying male maturation are of greater severity than those occurring in maturing female salmonids.

The major deleterious changes of concern to the producer are the reduced growth rates, lowered conversion efficiency and the reduced marketability associated with the unappealing physical appearance of mature male salmonids. Increased mortalities and disease succeptibility also accompany sexual maturation and lead to losses, especially among male production stock. These adverse changes seem to be especially marked when salmonids are cultivated either in sea cages or at mixed salinity sites. For example, at Shearwater Fish Farming's Finnarts Bay site mortalities among maturing males were extremely high and have exceeded 90% (J. A. Ralph, Pers. comm.).

Although these changes are of major commercial importance, little work has been directed towards the detailed mechanisms by which such

effects arise and to their possible importance in culture operations. The significance of these changes and the endocrine mechanisms by which they are brought about will be assessed in the experiments reported in the present chapter.

4·2 The effects of sexual maturation upon various aspects of growth and food conversion in rainbow trout

4·2·1 The effect of sexual maturation upon food conversion and ad libitum food intake in rainbow trout

In view of the relative paucity of data on the effects of sexual maturation on food intake and food conversion in sexually mature salmonids a short-term experiment was carried out at the University of Aston Fish Culture Unit to measure the food intake and conversion efficiency of a group of mature male rainbow trout.

Materials and Methods

Two year old rainbow trout, of approximately 350 g, from a stock reared at the University of Aston Fish Culture Unit were used in this experiment. Twelve mature males and twelve immature trout were selected from a group held in a large holding tank in December. Mature males were identified by the presence of male secondary sexual characteristics and by spermiation with gentle hand pressure. Each group was housed in a separate tank within a recirculating system. After a seven day acclimation period the fish were weighed and then fed twice daily ad libitum for the rest of the experimental period.

After ten days the fish were re-weighed and the conversion efficiency (C.E.) calculated from the weight of food fed and the total weight gain as follows:-

$$\text{Conversion Efficiency (C.E.)} = \frac{\text{food fed}}{\text{weight gain.}}$$

Results

The results are shown in Table 4•2•1•1. Over the experimental period the total weight of immature fish increased from 4•736 kg to 5•242 kg, an increase of 0•506 kg, while the total weight of mature fish increased from 4•195 kg to 4•347 kg, an increase of 0•152 kg.

During this time the experimental fish consumed 0•642 kg of food, while the immature fish consumed 1•004 kg of food. Thus less food was consumed by the mature males, but this may be related to their slightly lower average weight. When the amount consumed was corrected for initial differences in body weight by converting to a % of original body weight, the mature fish were found to have consumed the equivalent of 1•5% of their body weight/day, while the immature fish consumed 2•1% body weight/day. The calculated conversion efficiencies of the mature and immature fish were 4•22 and 1•98 respectively, indicating better utilization of consumed food in the immature group.

Discussion

This short-term experiment revealed differences in both the amount of food consumed and the utilization of food by the two groups. A reduction in food intake was observed in mature males. A similar reduction in ad libitum food intake has been reported in a number of non-salmonid species (Honmans and Vladykov, 1954; Mackinnon, 1972; Lande, 1973). In salmonids, maturing as they do in the winter months, such a fall in food intake would be expected due to low water temperatures. In this experiment the fish were maintained at $10^{o}C \pm 1^{o}C$ for the duration of the experiment.

The interpretation of the significance of a fall in food intake is further complicated in migratory salmonids as prolonged fasting often

Table 4•2•1•1

Weight gain, ad libitum food intake and food conversion efficiency of mature and immature rainbow trout

		Immature (n = 12)	Mature (n = 12)
Day 0	Total weight (kg)	4•736	4•195
	$\bar{x}$ weight (g) $\pm$ SE	394•7 $\pm$ 10•4	349•6 $\pm$ 9•8
Day 10	Total weight (kg)	5•242	4•347
	$\bar{x}$ weight (g) $\pm$ SE	436•8 $\pm$ 12•0	362•3 $\pm$ 11•8
	Total wt. gain (kg)	0•506	0•152
	Total food fed (kg)	1•004	0•642
	Equivalent % B.wt./day*	2•1	1•5
	Food conversion efficiency	1•98	4•22

* % Body weight/day calculated as $\frac{\text{Mean weight of food/day (g)}}{\text{Original mean wt. of fish (g)}} \times 100$

accompanies the spawning migration. In the Atlantic salmon (Salmo salar) this may account for a loss of up to 25% body weight (Mills, 1971). The high post-spawning mortalities associated with spawning among Atlantic salmon have in the past been attributed to this weight loss (Belding, 1934).

In addition to the reduced food intake mature males showed a poor utilization of consumed food, illustrated by the elevated conversion efficiency within this group. Few reports have examined food conversion efficiency at the time of reproductive maturity in salmonids. However, the results from a group of experimental fish maintained at Shearwater Fish Farming's Low Plains site do support the idea that a fall in conversion efficiency occurs and is associated with spawning. In particular both the timings of spawning and the fall in conversion efficiency were shown to be modified by alterations in the photoperiodic cycle under which the fish were maintained (Whitehead, 1979). (Table 4•2•1•2).

Thus the growth reduction occurring among mature male salmonids seems to involve both a reduction in food intake and a reduced food conversion efficiency. The results of Bye and Lincoln (1979) show a reduction in growth of mature male rainbow trout, though no mechanism was suggested to explain this. Further experiments in this chapter will examine these aspects of maturation in greater detail.

4•2•2 The effect of sexual maturation upon growth and total feed digestibility in rainbow trout

In view of the potential importance of changes in conversion efficiency on the growth performance of mature male salmonids an experiment was carried out to determine the effects of maturation upon

Table 4•2•1•2

Estimated conversion efficiencies of rainbow trout under simulated photoperiod cycles (Data from Whitehead, 1979)

Month	Photoperiod cycle			
	Normal 12 month cycle	Normal cycle compressed into 9 months	Normal cycle compressed into 6 months	12 hrs light/ 12 hrs dark cycle
July	1•86	1•48	1•97	1•06
Aug.	1•42	1•57	1•45	2•03
Sept.	1•47	1•57	1•49	1•78
Oct.	1•32	1•41	2•56 (+)	1•65
Nov.	1•39	2•09 (+)	*	2•25
Dec.	2•01 (+)	2•35	*	2•94 (+)
Jan.	7•81	*	*	*

* No conversion efficiency calculated on these dates as a weight loss occurred.

(+) Indicates time of spawning.

growth and to attempt to provide information on the mechanisms which could give rise to a reduction in food conversion efficiency. One way in which conversion efficiency could be adversely affected is via an effect on the ability of fish to digest consumed food, ie. the digestibility of diet. This aspect will be examined in the present experiment.

Materials and Methods

This experiment was carried out using rainbow trout reared at the University of Aston Fish Culture Unit. Fish were examined in mid - late October and any showing the development of male secondary sexual characteristics were tagged, together with a similar number of immature fish and removed to a separate compartment of a large holding tank.

Fish were fed B.P. Mainstream No. 4 diet, twice a day, except for the 14 day period preceding faecal sampling, when they were fed a similar diet but with the addition of 1% chromic oxide (Cr_2O_3) as an inert indicator substance. (The preparation of this diet is described in Chapter 2). Faecal samples were collected on days 48 and 81 by the use of gentle hand pressure alongside the vent. The Cr_2O_3 content of both faeces and diet were determined as described. Any remaining faeces were pooled and used for the determination of faecal protein and estimation of protein digestibility.

During the experimental period fish were periodically weighed and examined for spermiation. The water temperatures during the experiment ranged from 11°C to 6°C.

Results

a) Weight

The mean body weights of mature male and immature fish are shown

in Table 4•2•2•1 and Graph 4•2•2•1. Mature males had a lower mean body weight on all sampling dates, except day 0, when the mean body weight of mature males was slightly higher than that of immature fish (218•9 $\pm$ 10•9 g and 208•6 $\pm$ 14•8 g respectively). On all other sampling dates this was reversed and the immature fish had a higher mean body weight. By day 70 of the experiment the weight difference between the two groups was statistically significant (males 317•4 $\pm$12•9 g : immature fish 392•6 $\pm$ 32•3 g ; $P < 0{\cdot}05$).

The depression in growth is more clearly shown by the percentage change in body weight/day of both groups (Table 4•2•2•2 and Graph 4•2•2•2). Both groups showed a fall in percentage growth/day, probably due to the falling temperatures over the experimental period. The depression in percentage growth was much more marked among the mature fish and was significantly different at all sampling dates, except days 15 - 28.

b) Spermiation

The maximum extent of spermiation among mature males was reached by day 70 of the experiment, when all males were capable of expressing sperm under gentle hand pressure. The extent of spermiation throughout the experimental period is shown in Table 4•2•2•3.

c) Digestibility

Due to the small amounts of faeces collected from each fish, it was necessary to pool samples in order to calculate the total dry matter digestibility. The results of these determinations are shown in Table 4•2•2•4.

Table 4•2•2•1

Mean Body weights of mature and immature rainbow trout

(Mean body weight (g) ± SE)

Day	0	14	28	42	56	70	84
Mature Males n = 9	218•9 ± 10•9	248•2 ± 8•1	271•3 ± 7•5	291•5 ± 9•0	306•3 ± 10•9	317•9 ± 12•9 (*)	313•3 ± 16•1 (*)
Immature fish n = 9	208•6 ± 14•8	253•3 ± 17•4	288•8 ± 19•2	328•0 ± 24•8	346•5 ± 28•1	392•6 ± 32•3	422•5 ± 36•2

t test (*) $P < 0.05$

Table 4•2•2•2

Mean % increase in body weight/day of mature and immature rainbow trout

(Mean % ± SE)

Day	0 - 14	15 - 28	29 - 42	43 - 56	57 - 70	71 - 78
Mature males n = 9	1•03 ± 0•17 (*)	0•70 ± 0•16	0•53 ± 0•11 (*)	0•36 ± 0•09 (*)	0•21 ± 0•08 (**)	0•22 ± 0•05 (**)
Immature fish n = 9	1•56 ± 0•08	1•02 ± 0•07	0•93 ± 0•10	0•65 ± 0•10	0•68 ± 0•09	0•53 ± 0•10

t test (*) $P < 0.05$
mature vs. immature (**) $P < 0.01$

Table 4•2•2•3

The extent of spermiation in male rainbow trout sampled from mid-October

Day	0	14	28	42	56	70	84
Number of fish showing spermiation	-	-	1	2	7	9	9
% number of males	-	-	11	22	78	100	100

Table 4•2•2•4

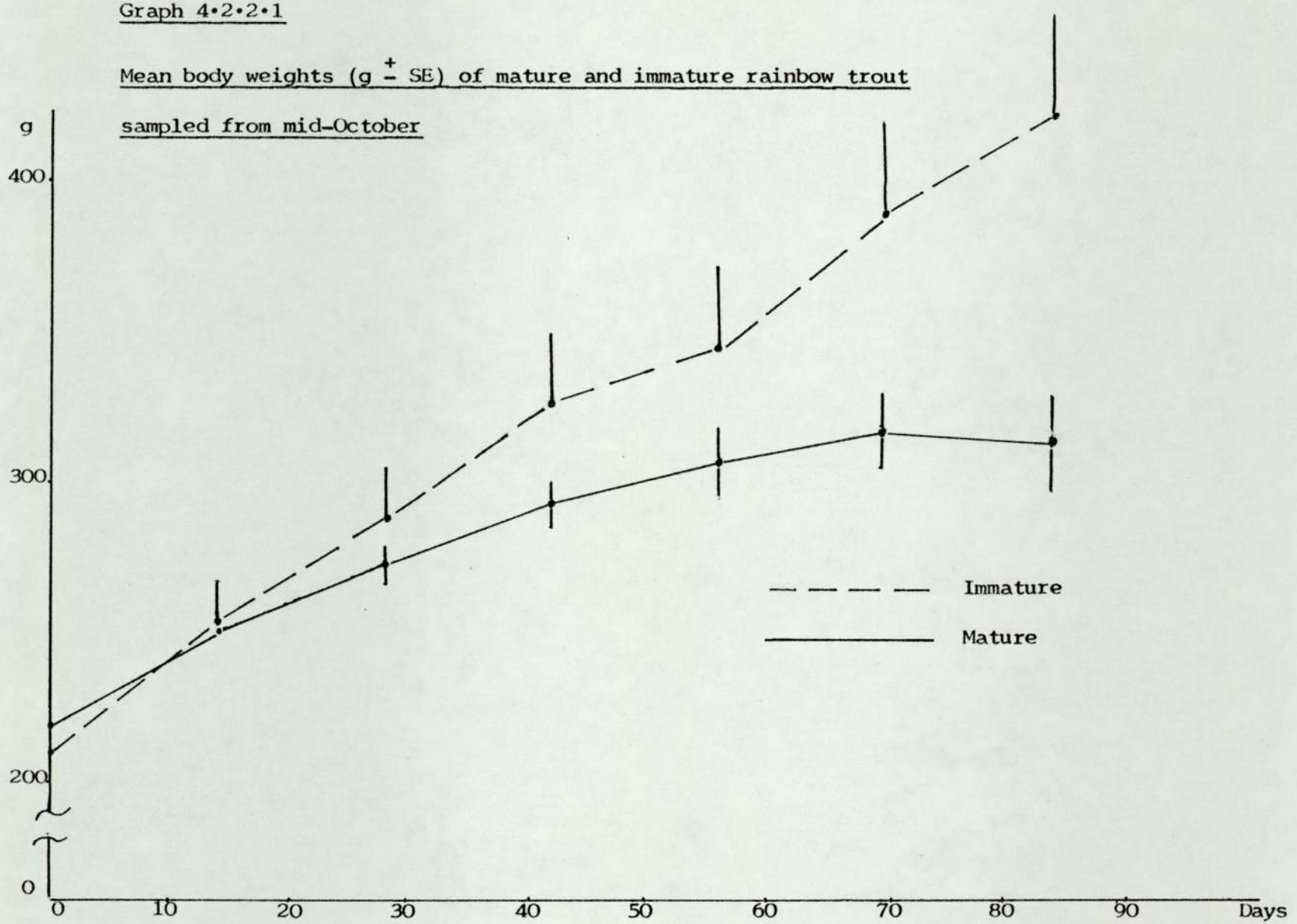
Total dry matter digestibility values of mature and immature rainbow trout

	No. of fish pooled/sample	mg Cr_2O_3 / g faeces	% dry matter digestibility	Mean % dry matter digestibility ± SE
Day 48				
Mature males	2	25•2	64•4	
	2	19•9	54•8	61•9 ± 3•12
	1	27•5	67•1	
	2	23•4	61•5	
Immature fish	1	27•7	67•5	
	3	30•4	70•4	
	2	26•1	65•5	68•7 ± 1•33
	1	31•4	71•3	
	1	29•2	69•3	
Day 81				
Mature males	1	25•3	64•5	
	2	21•1	57•5	
	2	24•8	63•8	63•4 ± 2•25
	2	22•6	60•3	
	2	30•4	70•9	(*)
Immature fish	2	29•7	69•7	
	2	26•3	65•8	
	2	31•7	71•6	69•32 ± 1•07
	1	31•3	71•3	
	2	28•2	68•2	

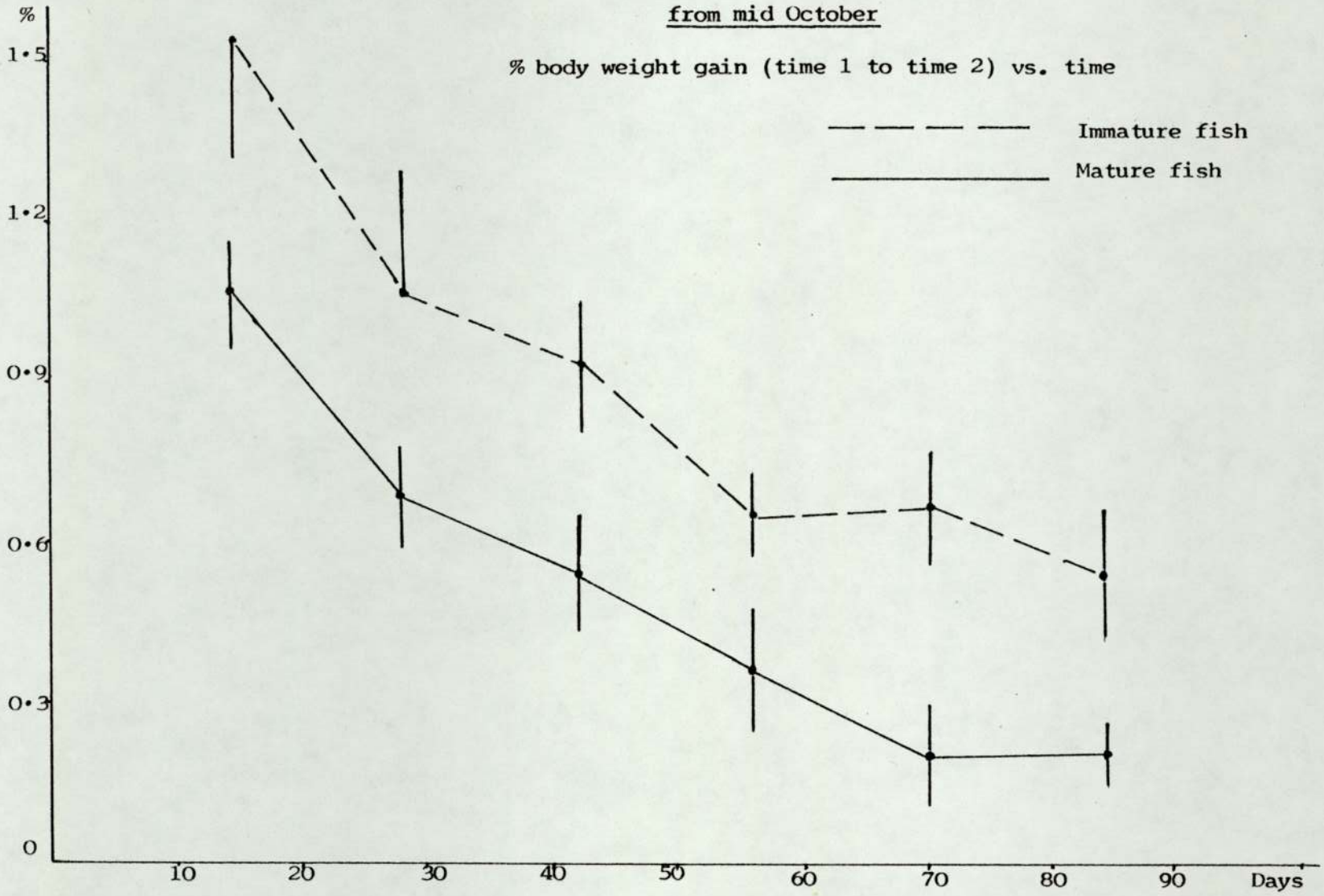
t test (*) $P < 0•05$

Graph 4•2•2•1

Mean body weights (g $\pm$ SE) of mature and immature rainbow trout sampled from mid-October



Graph 4•2•2•2 % weight gain per day for mature and immature rainbow trout sampled from mid October



On both sampling dates the % dry matter digestibility was lower in mature males than in immature fish. On day 48 of the experiment the mean % digestibility was 61·9 $\pm$ 3·12% for mature male fish and 68·7 $\pm$ 1·3% for immature fish. These values were not significantly different ($P < 0{\cdot}1$). Later in the experiment (day 81), samples showed a significantly lower ($P < 0{\cdot}05$) % dry matter digestibility in mature males (63·4 $\pm$ 2·25%) than in immature fish (69·32 $\pm$ 1·07%).

Due to the lack of sufficient amounts of faeces a single estimation of faecal protein was made for each group using pooled samples from the 81 day sampling. The protein digestibility was calculated from the following formula:-

$$\% \text{ protein digestibility} = 100 - 100 \left(\frac{\% \ Cr_2O_3 \text{ food}}{\% \ Cr_2O_3 \text{ faeces}} \times \frac{\% \text{ pr. in faeces}}{\% \text{ pr. in food}} \right)$$

The mean faecal protein of the mature males was 24·5% and that of the immature fish 13·3%; the diet contained 51% protein.

The calculated % protein digestibilities were 92·1% for immature fish and 82·0% for mature males. Thus in addition to the reduction in total dry matter digestibility, mature fish show a reduction in protein digestibility and utilization.

Discussion

Sexual maturation of rainbow trout was seen to be accompanied by a marked depression of growth. In this experiment this was evident before spermiation. Although a less marked reduction in growth rate was observed among immature fish throughout the experimental period, it seems likely this was due to the falling temperatures leading to a reduction in food intake.

Several studies have reported the growth depression occurring among maturing male salmonids (Nomura, 1963; Bye and Lincoln, 1979; Whitehead, 1979). The data presented are similar to that reported by Bye and Lincoln (1979) and Lincoln and Purdom (unpublished). The timing of this depression of growth may be related to increasing levels of androgens occurring over the period prior to spermiation.

The precise reasons for the growth depression accompanying male maturation are unclear, though a number of possible factors have been implicated, including the reduction in conversion efficiency reported in the previous experiment. Measurement of total dry matter digestibility of male fish reported here suggest that this reduced conversion may in part be due to a fall in digestibility. This depression of digestibility was significant in the mature males by day 81 of the experiment. Preliminary measurements of protein digestibility show this parameter to be depressed relative to immature fish, although these were determined using very small amounts of faeces and should be viewed as preliminary data only.

Although a fall in temperature was recorded during the experiment, this would seem to be unrelated to the reduction of digestibility. Windell et al. (1978b) report that reduced temperatures had no effect upon total digestibility, except in very small fish (18 g) at 7°C. As the same authors have reported that ration size only influences digestibility at high ration levels, when a depression of digestibility occurs, the results obtained in the present experiment would not appear to be explained by differences in ration or fish size.

Thus, it seems that at the time of spermiation a fall in both total dry matter digestibility and protein digestibility occurs, which

may be related to the poor growth performance of maturing males. This reduction in digestibility occurs at a time when androgen levels are either high or rising and a possible causal link between these endocrine events and the observed changes will be examined in later experiments.

4•2•3 The effect of sexual maturation upon both dry matter digestibility and serum androgen levels in rainbow trout

In order to determine the effects of sexual maturation on the % dry matter digestibility of male trout and in particular to determine the relationship between serum androgen levels and digestibility, an experiment was carried out at the University of Aston Fish Culture Unit in which individual digestibilities and androgen levels were followed. In order to allow the calculation of the digestibility values from individual fish, three year old fish were used.

Materials and Methods

Three year old male rainbow trout, of approximately 600 g, were selected from a stock maintained at the University of Aston. These male trout were tagged and weighed in early November. Fish were fed B.P.'Mainstream' diet according to manufacturer's recommended levels, except for a 14 day period prior to faecal sampling, when they were fed a diet of corresponding ration and frequency but containing 1% Cr_2O_3 prepared as described.

At monthly intervals fish were weighed and faecal and serum samples collected until March of the following year, after which they were taken at two-monthly intervals. At each sampling fish were checked for spermiation and five fish selected at random for measurement of sperm volume. Serum samples were stored at -20°C and assayed for serum

testosterone levels as described. Digestibilities were calculated and correlated with individual testosterone levels by the method of Parker (1973). During the experimental period water temperatures varied from 10°C in November to a low of 6°C in March, after which they climbed to 15°C in July.

Results

a) Weight

The growth of male fish over the experimental period is shown in Table 4•2•3•1 and Graph 4•2•3•1. Mature male trout grew throughout the experiment, though growth was slight in the early part of the experiment, especially in December - February. However, due to high tag loss within this group it was not possible to monitor individual growth rates. The growth rate of these mature males was markedly less than that of a group of two year old female (immature) fish maintained under identical conditions in the same holding unit (Table 4•2•3•2 and Graph 4•2•3•1).

b) Spermiation

A number of fish were running milt at the time of the first sampling. By day 29 all fish were running under slight hand pressure. Due to tag loss among males it proved impossible to sample the same fish on each occasion so five similar sized fish were chosen at random. The mean volume of sperm collected on each date is shown in Table 4•2•3•3 and Graph 4•2•3•2.

Sperm volume had increased significantly ($P < 0.05$) by day 29 to 14.1 ± 4.8 ml from 1.3 ± 0.7 ml in November. Sperm volumes remained high until after day 95 (12.3 ± 5.7 ml), after which the volume fell

Table 4•2•3•1

Mean Body weights of three year old mature male rainbow trout weighed from November onwards (Mean weight (g) ± SE)

Day	Mean weight ± SE	n
0	599•9 ± 27•0	25
29	632•3 ± 30•3	26
62	647•2 ± 24•4	31
95	655•6 ± 31•5	27
120	691•4 ± 40•1	15
180	720•1 ± 79•2	10
244	752•6 ± 32•9	9

Table 4•2•3•2

Mean Body weights of two year old immature female rainbow trout sampled from November onwards

Day	Mean weight (*) (g)	n
0	329•4	20
25	395•6	20
62	496•1	20
123	640•8	20
170	810•3	20
210	940•7	20

(*) Mean weight of batch of 20 fish.

Table 4•2•3•3

Sperm volume (ml ± SE) collected from a random sample of three year old male rainbow trout vs. time (days)

Day	Volume	n
0	1•3 ± 0•7	5
29	14•1 ± 4•8 (*)	5
62	13•8 ± 7•0	5
95	12•3 ± 5•7	5
120	7•5 ± 1•5	5
180	3•2 ± 1•0 (*)	5
244	0•1 ± 0•0	1

t test (*) $P < 0.05$ for consecutive samples.

Table 4•2•3•4

Mean serum testosterone levels of three year old mature male rainbow trout sampled from November

Mean testosterone (ng/ml ± SE)

Day	Testosterone (ng/ml)	n
0	71•75 ± 5•87	17
29	124•81 ± 12•85 (***)	25
62	26•26 ± 2•68 (***)	29
95	16•88 ± 2•01 (*)	27
120	16•09 ± 2•09	14
180	2•69 ± 0•65 (**)	8
244	5•26 ± 0•74 (*)	8

t test (***) $P < 0.001$ for consecutive samples.
(**) $P < 0.01$
(*) $P < 0.05$

Table 4•2•3•5

Mean dry matter digestibility (% ± SE) of mature male rainbow trout

(Mean serum testosterone values for these fish are also shown, ng/ml ± SE)

Day	Mean digestibility (% ± SE)	n	Mean serum testosterone (ng/ml ± SE)
0	62•50 ± 1•56	11	65•75 ± 5•72
29	60•41 ± 1•91	11	133•25 ± 20•88
62	68•63 ± 1•06 (**)	16	27•99 ± 4•20
95	72•66 ± 0•86 (*)	12	19•66 ± 3•31
120	73•07 ± 1•43	9	14•81 ± 2•38
180	71•36 ± 1•15	8	2•69 ± 0•65
244	68•75 ± 0•95	7	5•60 ± 0•70

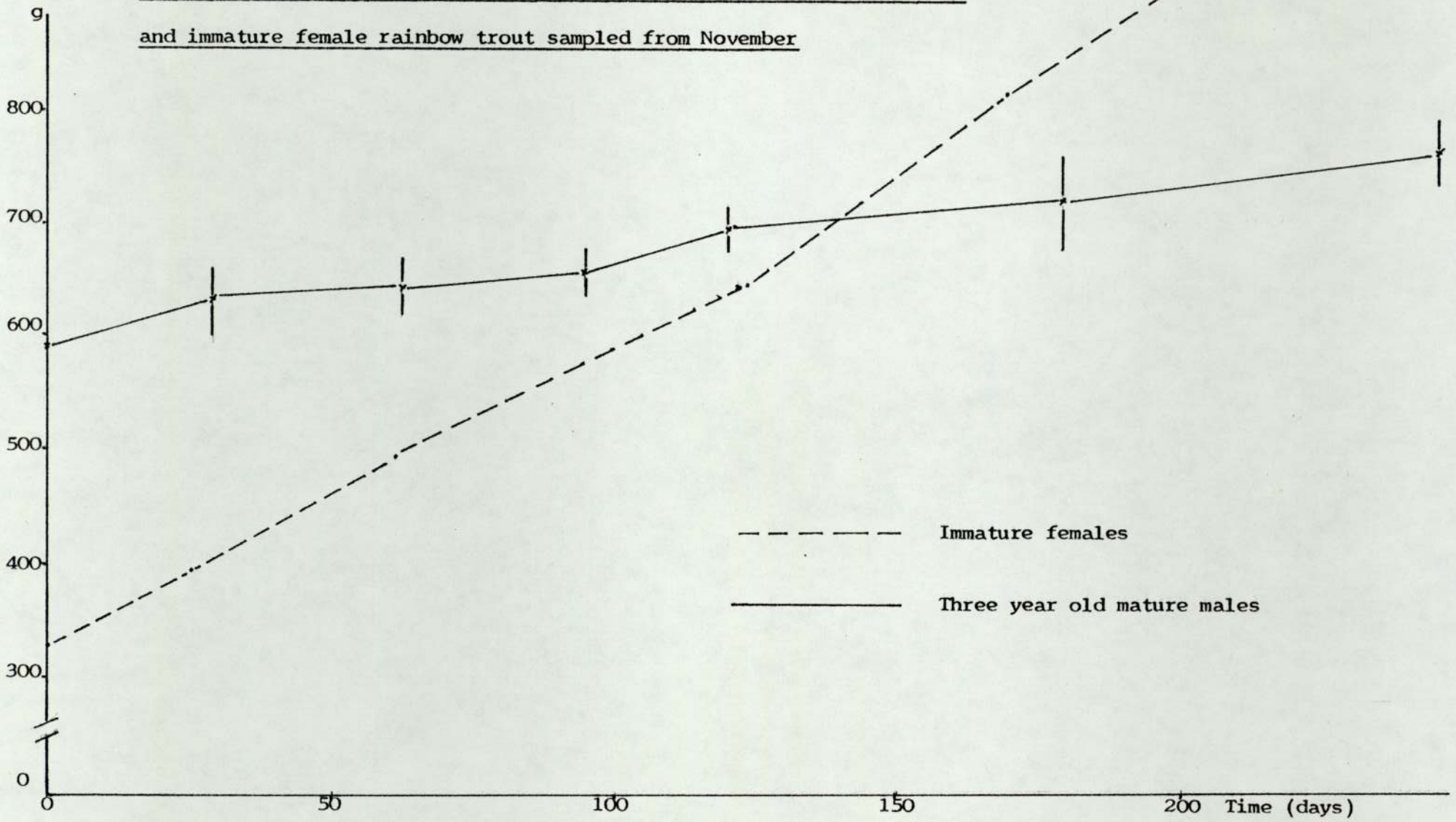
t test samples compared with previous sample, e.g. Day 62 and Day 29.

(**) $P < 0.01$

(*) $P < 0.05$

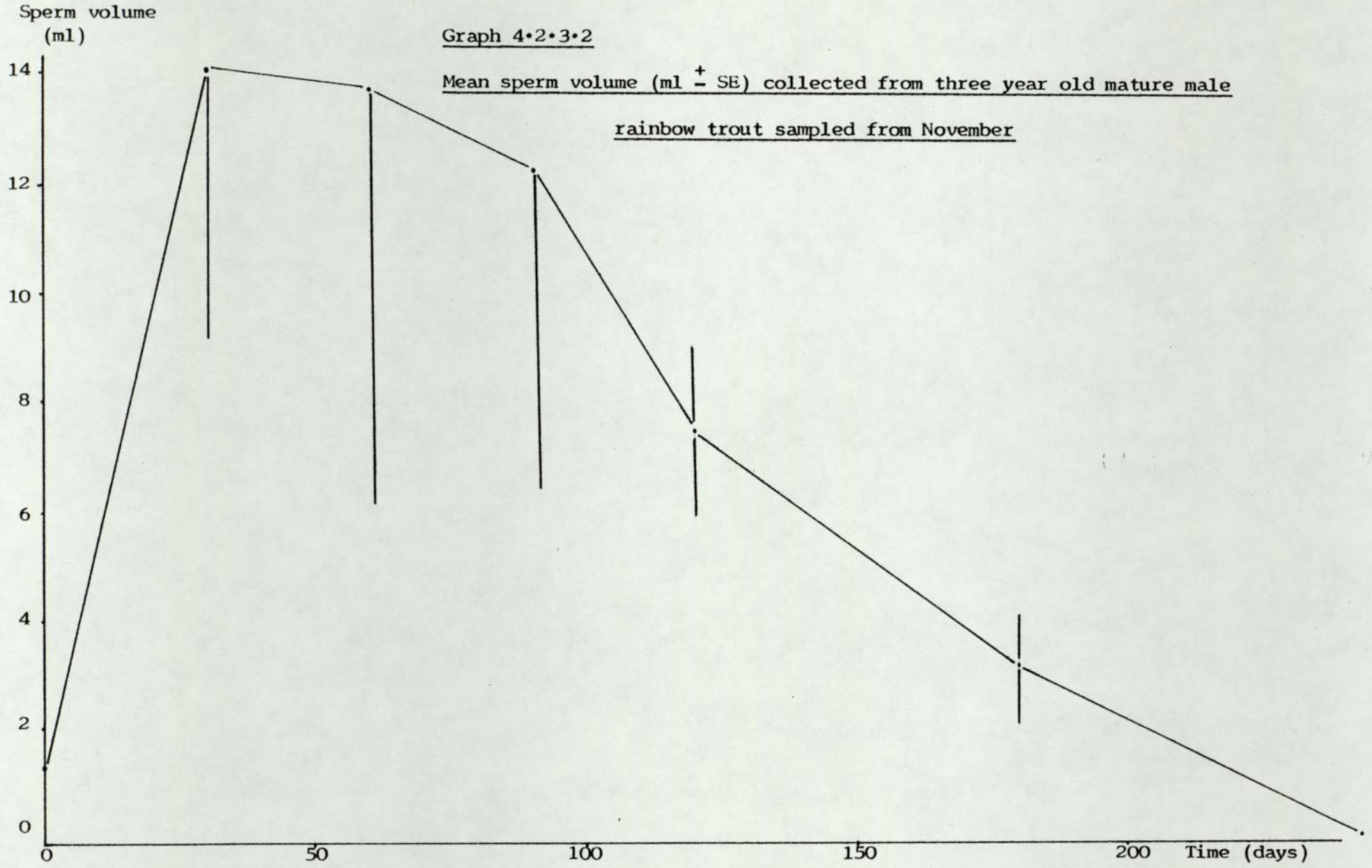
Graph 4•2•3•1

Mean body weight (g $\pm$ SE) of three year old mature male rainbow trout and immature female rainbow trout sampled from November



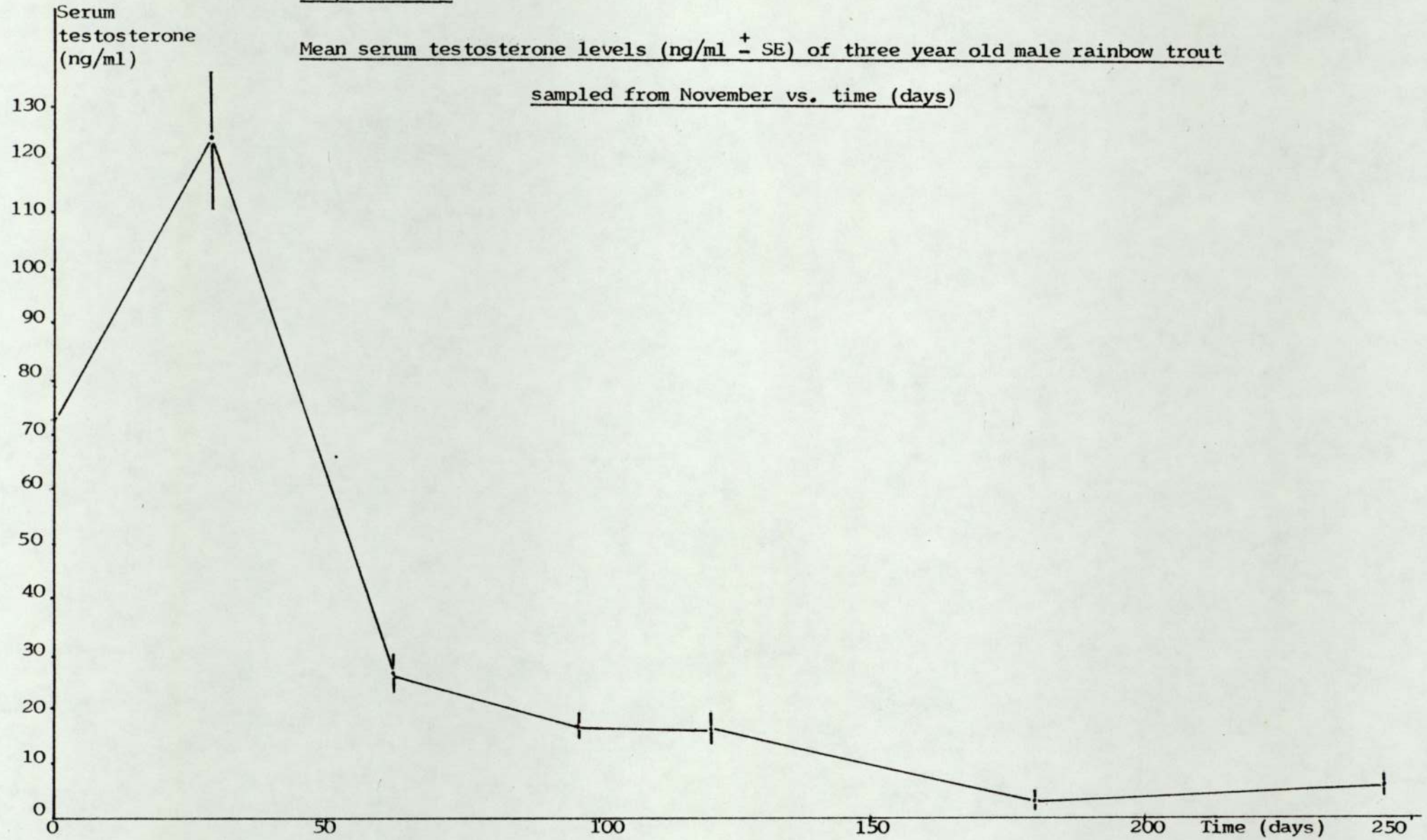
Graph 4•2•3•2

Mean sperm volume (ml ± SE) collected from three year old mature male rainbow trout sampled from November



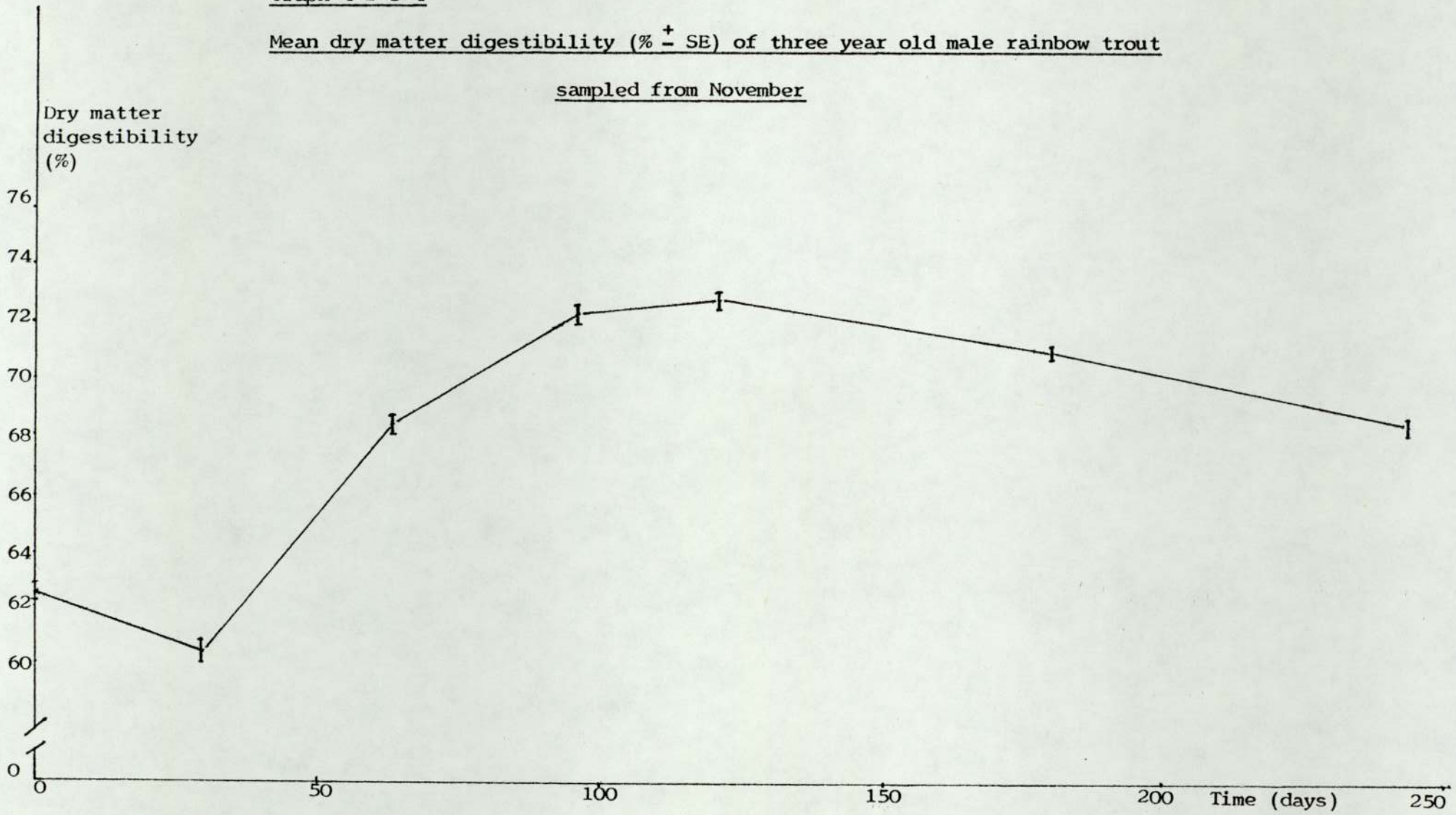
Graph 4•2•3•3

Mean serum testosterone levels (ng/ml ± SE) of three year old male rainbow trout sampled from November vs. time (days)



Graph 4•2•3•4

Mean dry matter digestibility (% ± SE) of three year old male rainbow trout sampled from November



to 7·5 ± 1·5 ml by day 120 and a further significant ($P < 0·05$) fall was observed by day 180 to 3·2 ± 1·0 ml. Thus throughout the experimental period male fish were capable of expelling milt under gentle pressure. It was observed, however, that the milt appeared much thinner later in the experiment, though the composition of this milt, and in particular the spermatocrit, was not studied.

c) Serum Androgen levels

Serum testosterone levels measured in these fish are shown in Table 4·2·3·4 and Graph 4·2·3·3. Serum T levels showed a highly significant ($P < 0·001$) increase from day 0 (71·75 ± 5·87 ng/ml) to day 29 (124·81 ± 12·84 ng/ml). Following this peak a sharp fall occurred to 26·26 ± 2·68 ng/ml ($P < 0·001$) by day 62, with a further significant ($P < 0·05$) fall to 16·88 ± 2·01 ng/ml by day 95. By day 180 mean T levels had fallen significantly ($P < 0·01$) to 2·69 ± 0·65 ng/ml. At the end of the experiment a slight but significant increase in serum testosterone was observed to 5·26 ± 0·74 ng/ml ($P < 0·05$).

d) Dry matter digestibility

Dry matter digestibilities, determined from faecal samples, are shown in Table 4·2·3·5 and Graph 4·2·3·4.

Total dry matter digestibility was initially low in mature male fish (62·50 ± 1·56%) and fell to a minimum on day 29 (60·41 ± 1·91%), coincident with peak levels of serum testosterone. A significant ($P < 0·01$) increase in digestibility was observed between days 29 - 62 (to 68·63 ± 1·06%). Digestibility further increased significantly ($P < 0·05$) between days 62 - 95 (68·63 ± 1·06 to 72·66 ± 0·86%). Mean digestibility remained high until the end of the experiment, when a

slight but non-significant fall in digestibility was observed between days 180 - 244 (71·36 $\pm$ 1·15 to 68·75 $\pm$ 0·95%).

The correlation coefficient (r) for individual digestibility and serum testosterone levels was calculated between days 0 - 95. Over this period this value was 0·495 (n = 57); this value was highly significant ($P < 0·001$) (Parker, 1973).

Discussion

Throughout the experiment mature males showed low growth. This was especially shown at the time of spermiation and subsequent to this event, and only exhibited a slight recovery later. Because three year old fish were used in this experiment, it was not possible to provide a control group, as three year old females were also maturing at this time. Although a direct comparison is obviously invalid, it was noted that the growth of the mature males was poor compared to two year old immature (largely female) trout maintained under equivalent conditions. This difference in growth may be related to the age difference between these groups, but it seems likely that, in view of the results of previous experiments and the noted effect of maturation upon growth, a major component of this difference is due to the effects of sexual maturation.

This reduction in growth occurred at the time of peak levels of serum testosterone and was also coincident with a reduction in total dry matter digestibility. These latter two parameters were shown to share a statistically significant inverse relationship, suggesting that they may be causally related. The reduction in digestibility has also been suggested as playing a part in the depression of food conversion efficiency previously seen in mature males (Whitehead, 1979).

The results of the present experiment do not rule out the possibility that the effect on dry matter digestibility is related to some other non-androgen related factor and this relationship is the object of further study in the next chapter.

4•2•4 The effect of sexual maturation upon the proximate body composition of rainbow trout

In order to collect data regarding changes of possible commercial significance in mature male salmonids the proximate composition, together with a range of tissue parameters, were determined in mature and immature rainbow trout. Surprisingly, in view of the importance of the subject, few studies have examined changes in body composition arising from sexual maturation on non-migratory, as opposed to migratory, salmonids. Such a migration introduces further variables; both physiological and biochemical changes resulting from migration complicate interpretation of the possible effects of sexual maturation.

In addition to males maturing at the normal time (usually in their second winter), a varying proportion of males may mature early. Such precocious fish were recognised by Jones (1940) in his study of salmon parr. They were shown to be capable of participating in the normal spawning activities of adult salmon (Jones and King, 1950). The proximate composition of a number of precociously mature male rainbow trout was also determined, together with that of normally maturing males.

Materials and Methods

Normally maturing, two year old, fish were selected from a stock maintained at the University of Aston Fish Culture Unit in mid - late

January. An equal number of two year old immature fish were selected as controls.

Following anaesthesia, a blood sample was removed, which was divided for hormone assay and determination of haematocrit. The fish were then sacrificed and weighed.

A muscle sample was removed from the dorsal musculature, anterior to the dorsal fin. The skin was removed and this sample used for the determination of tissue water, fats and proteins as described in General Materials and Methods.

The gonads, liver and viscera were dissected free and used to calculate the indices for these organs from the formula:-

$$\text{Organ index} = \frac{\text{organ weight (g)}}{\text{body weight (g)}} \times 100.$$

In the case of the viscera the initial weight of the viscera and the visceral contents were noted separately. In addition a specimen of skin was removed from the dorsal anterior surface and fixed in Buffered neutral formalin and processed for histology. Sections were cut at 8 µm, stained with Haematoxylin and eosin, and used for the estimation of epidermal thickness.

In addition precociously mature male rainbow trout were selected in early February from a batch of one year old fish reared at the University of Aston. These fish were identified by the appearance of secondary sexual characteristics, including darkening, kype development and spermiation. These secondary sexual characteristics were far less pronounced than in normal, two year old, males. In particular kype development was rarely seen and the most reliable characteristic was skin darkening.

Similar parameters were measured in these one year old fish, with the exception of haematocrit and epidermal thickness. Body length was measured in addition to weight to allow the determination of the condition factor (k).

Results

A) Two year old fish

The results of the proximate and organ analyses of two year old fish are shown in Table 4•2•4•1 and Fig. 4•2•4•1 and 4•2•4•2.

a) Body weight

Mature male fish were significantly lighter than immature fish ($P < 0.05$) in January. The mean body weight of mature males was 321.7 ± 13.0 g, while that of immature fish was 390.7 ± 26.49.

b) Organ indices

Gonadosomatic index (GSI). As would be expected highly significant ($P < 0.001$) differences were observed between the mean GSI of mature and immature fish, reflecting the diversion of energy and nutrient reserves towards gonadal growth. The mean GSI of mature males was 2.84 ± 0.30, while that of immature fish (largely female) was 0.15 ± 0.03.

Hepatosomatic index (HSI). No significant difference was found between the mean HSI of mature male or immature rainbow trout, though the mean HSI of mature males was slightly higher (1.70 ± 0.24) than that of immature fish (1.61 ± 0.10).

Viscerosomatic index (VSI). The mean VSI of mature male fish was significantly ($P < 0.01$) lower than that of immature fish. The mean VSI of mature males was 3.92 ± 0.20 compared with 6.32 ± 0.24 in immature fish.

Table 4·2·4·1

Proximate analysis and organ indices of mature and immature two year old rainbow trout

(Mean values ± SE)

	VSI	HSI	GSI	Haematocrit	% muscle water	% muscle protein (a	Food index (b	Testosterone (ng/ml)	Mean body weight (g)	% muscle fat (a
Mature males	3·93 ± 0·20 (11	1·70 ± 0·24 (11	2·84 ± 0·30 (11	35·4 ± 0·85 (11	77·05 ± 0·56 (11	14·74 ± 0·61 (5	1·53 ± 0·36 (11	27·72 ± 2·93 (11	321·7 ± 12·97 (13	3·84 ± 0·29 (9
	(**)	(**)					(*)	(**)	(*)	
Immature fish	6·32 ± 0·24 (10	1·61 ± 0·10 (10	0·15 ± 0·03 (10	34·1 ± 1·03 (10	75·99 ± 0·20 (10	15·70 ± 1·22 (5	2·99 ± 0·47 (10	1·61 ± 0·27 (10	390·7 ± 26·39 (14	3·88 ± 0·42 (8

t test vs. controls (**) $P < 0·01$

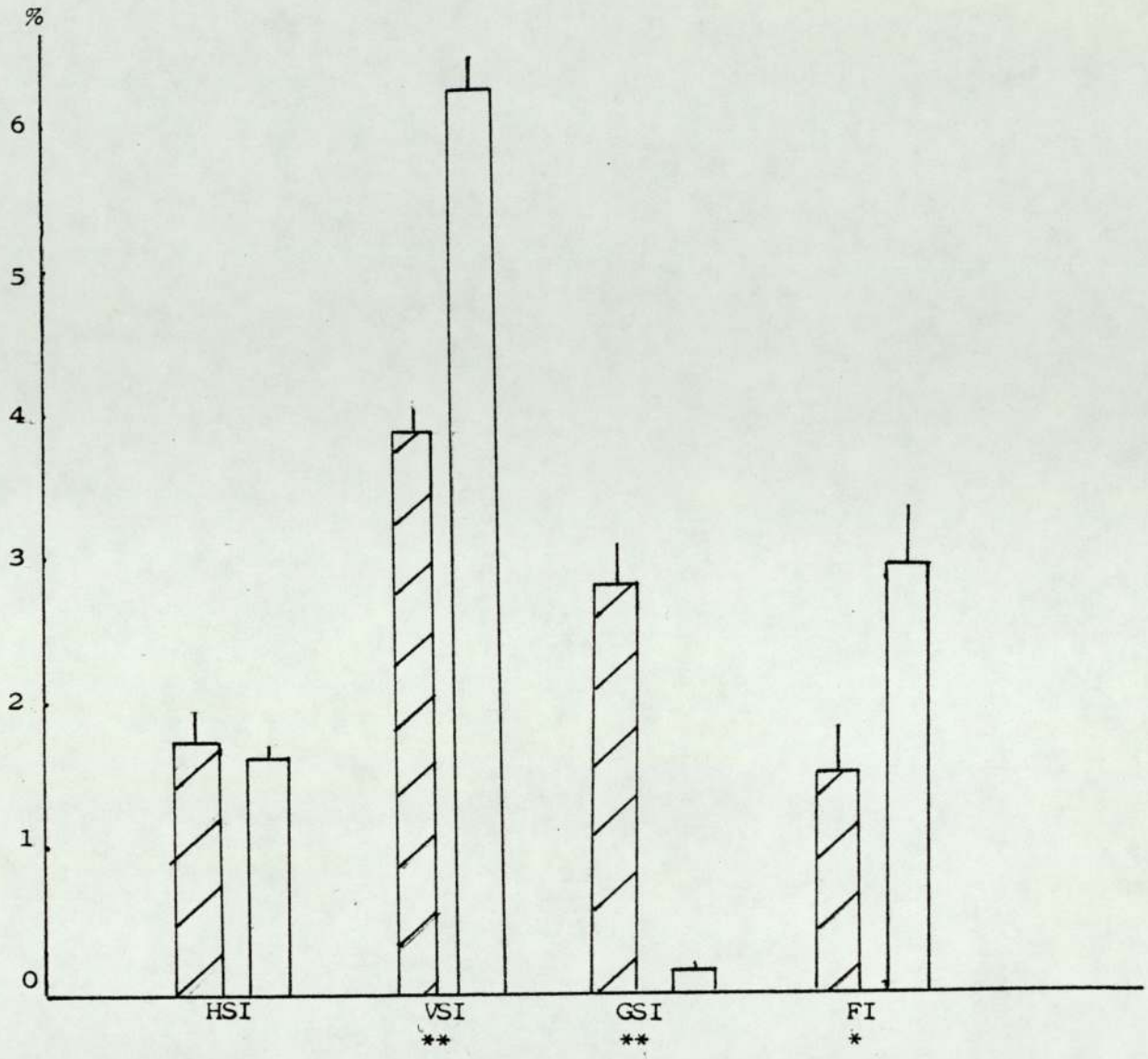
(*) $P < 0·05$

a) calculated as % wet weight.

b) calculated as $\frac{\text{weight visceral contents}}{\text{body weight}} \times 100$

Fig. 4·2·4·1

Organ indices of mature and immature two year old rainbow trout sampled in January



HSI Hepatosomatic index
VSI Viscerosomatic index
GSI Gonadosomatic index
FI Food index

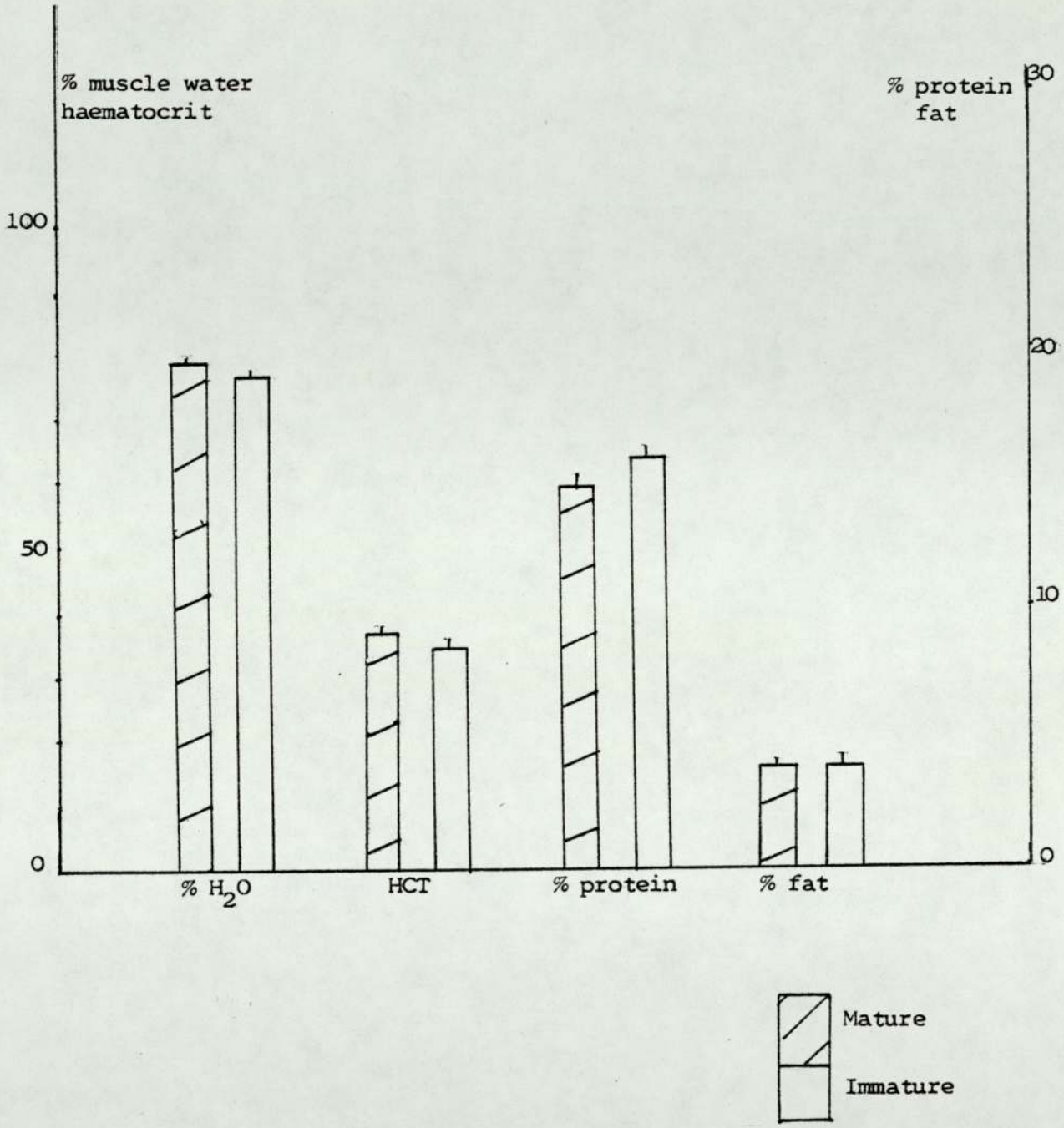
Mature males

Immature

* Pairs differ $P < 0.05$
** " " $P < 0.01$

Fig. 4·2·4·2

Proximate analysis of mature and immature two year old rainbow trout sampled in January



Food index. The food index provided an indicator of the amount of food remaining in the viscera and was calculated from the expression:-

$$\text{Food index} = \frac{\text{weight of visceral contents (g)}}{\text{body weight (g)}} \times 100$$

A highly significant ($P < 0{\cdot}01$) difference was observed between the mean food index of mature males ($1{\cdot}53 \pm 0{\cdot}36$) and immature fish ($2{\cdot}99 \pm 0{\cdot}47$). So mature male fish contained less residual food than immature fish.

c) Proximate Analysis

% muscle water. The mean % water of muscle samples from mature males was higher than that of immature fish. Thus the mean % H_2O of mature fish muscle samples was $77{\cdot}05 \pm 0{\cdot}56\%$ compared with $75{\cdot}99 \pm 0{\cdot}20\%$ in immature muscle samples. However, the mean values were only different at the level of $P < 0{\cdot}1$.

% muscle protein. Although the immature fish had a higher mean % muscle protein (expressed as % wet weight) of $15{\cdot}70 \pm 1{\cdot}22$ compared with mature males ($14{\cdot}74 \pm 0{\cdot}61$) the difference was not statistically significant.

% muscle fat. Similar mean % muscle fat values were found in both groups. Mature males had a mean % muscle fat of $3{\cdot}84 \pm 0{\cdot}29$ and immature fish $3{\cdot}88 \pm 0{\cdot}42$.

d) Haematocrit

No significant difference was observed between the haematocrit of mature ($35{\cdot}4 \pm 0{\cdot}85$) and immature ($34{\cdot}1 \pm 1{\cdot}03$) fish.

e) Serum testosterone levels

Serum testosterone levels were significantly lower ($P < 0.01$) in mature male fish than in immature fish. Mature males had a mean serum testosterone level of 27.72 ± 2.93 ng/ml, while that of the immature fish was 1.61 ± 0.27 ng/ml. The lower mean testosterone level of the mature fish compared with freshly spermiating fish illustrates that these fish had reached maturity earlier than the sampling time.

f) Skin thickness

The results of measurements made on the thickness of the epidermis and preliminary counts of goblet cell numbers are shown in Table 4·2·4·2. A significant increase in skin thickness was observed in mature males, in which the mean epidermal thickness was 210.4 ± 22.6 µm compared with 97.3 ± 4.9 µm in immature fish. Preliminary counts revealed a fall in goblet cell numbers/unit length of epidermis in mature males compared with immature fish (10.08 ± 1.13 and 16.5 ± 2.2; $P < 0.05$).

B) Precociously mature males

The results of the analysis of one year old mature male and immature rainbow trout are shown in Table 4·2·4·3 and Figs. 4·2·4·3 and 4·2·4·4.

a) Body weight and length

Precociously mature males were not significantly different in weight from immature fish. The mean body weight of mature males was 115.9 ± 5.7 g and that of immature fish 117.0 ± 9.1 g. However, mature males were significantly shorter than immature males (19.6 ± 0.04 cm and 21.0 ± 0.5 cm respectively; $P < 0.05$). Mature males were thus shorter and fatter than immature fish. This is shown by the

Table 4•2•4•2

Epidermal thickness and goblet cell numbers in two year old mature male and immature rainbow trout (January)

	Mean epidermal thickness (um)	Goblet cell number (cells/unit length, skin)
Mature males	210•4 ± 22•6 (*) (5	10•08 ± 1•13 (*) (5
Immature fish	97•3 ± 4•90 (5	16•54 ± 2•20 (5

t test (*) $P < 0.05$

Table 4·2·4·3

Proximate Analysis of precociously mature male and immature rainbow trout sampled in February

(Mean values ± SE)

	Mean body weight (g ± SE)	Mean length (cm ± SE)	Mean condition factor (k) (a	Mean GSI	Mean VSI	Mean HSI	% muscle water	% muscle protein	% muscle fat	Serum testosterone (ng/ml)
Precocious males	115·9 ± 5·7	19·6 ± 0·5	1·54 ± 0·03	2·43 ± 0·13	5·15 ± 0·16	1·59 ± 0·08	77·4 ± 0·4	17·3 ± 0·09	2·89 ± 0·27	13·24 ± 1·48
n	(13	(13	(13	(13	(13	(13	(13	(13	(13	(8
		(*)	(**)	(***)	(***)		(**)		(***)	(***)
Immature fish	117·1 ± 9·1	21·0 ± 0·4	1·30 ± 0·03	0·06 ± 0·00	6·65 ± 0·31	1·70 ± 0·40	79·6 ± 0·3	17·06 ± 0·02	1·41 ± 0·10	1·95 ± 0·92
n	(13	(13	(13	(13	(13	(13	(13	(13	(13	(8

t test (***) $P < 0·001$
(**) $P < 0·01$
(*) $P < 0·05$

a) Condition factor (k) calculated as $k = \left(\frac{W}{L^3} \times 100\right)$

Fig. 4·2·4·3

Proximate composition, mean serum testosterone levels and condition factor of precociously mature and immature rainbow trout sampled in February

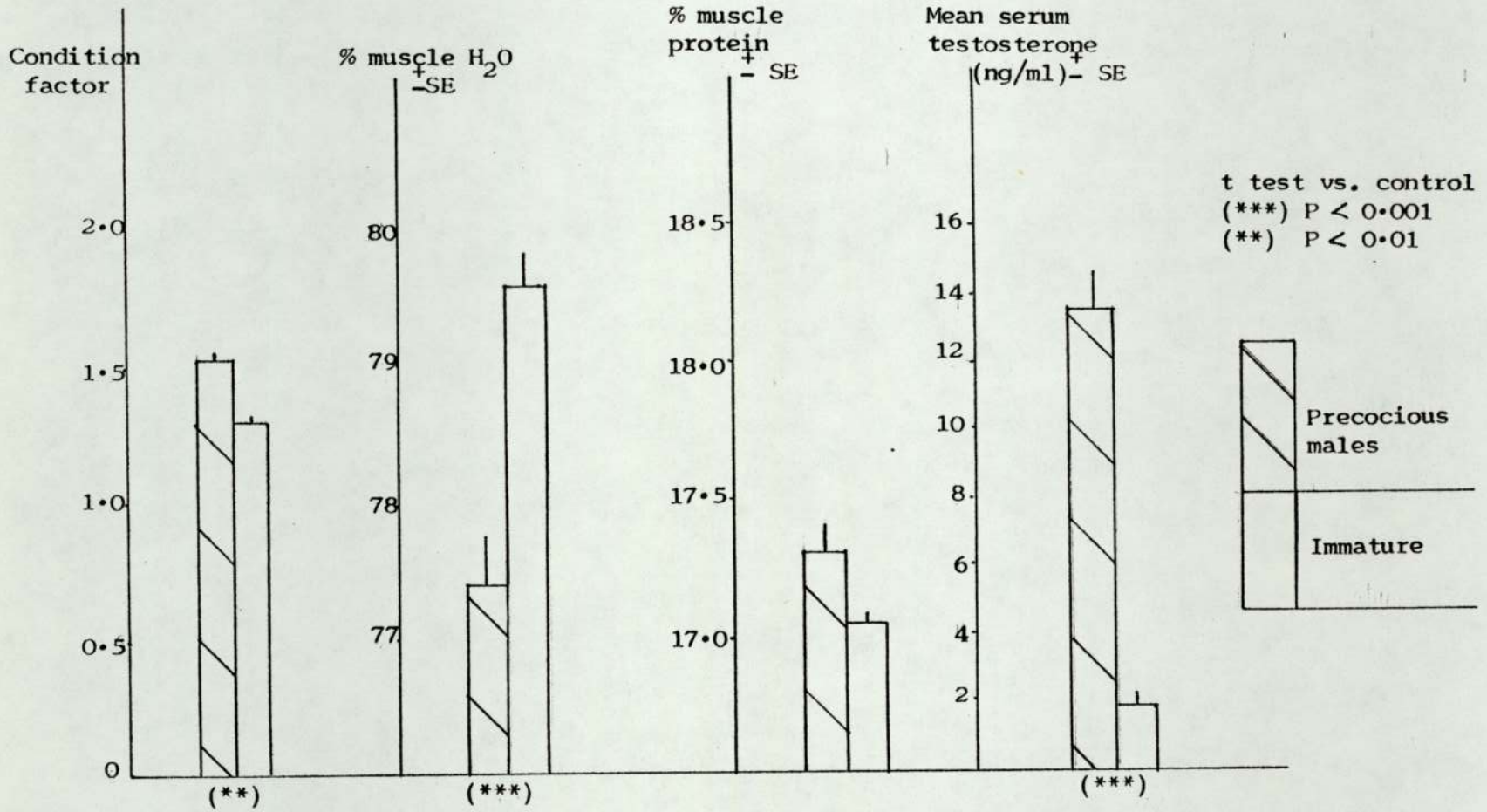
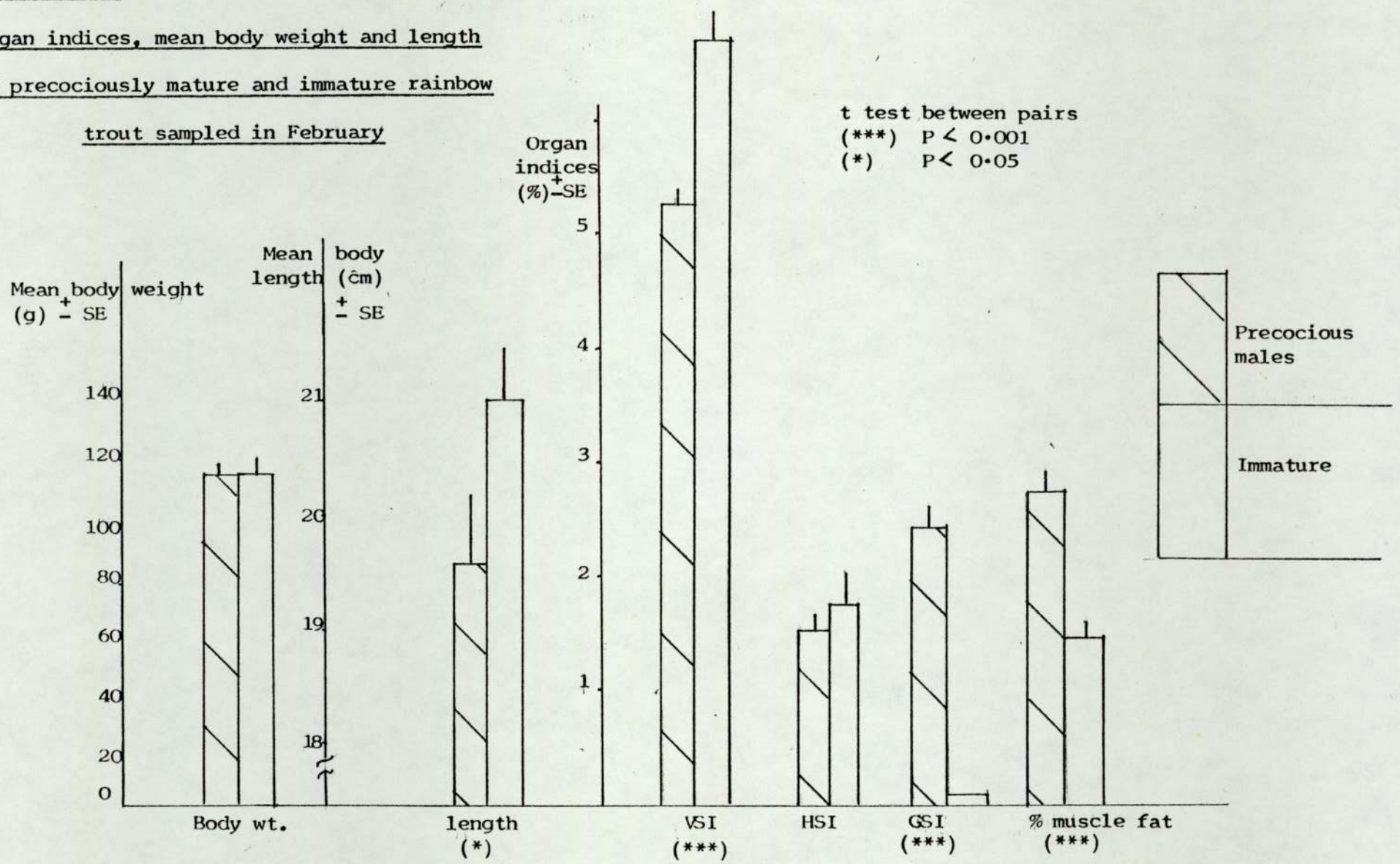


Fig. 4·2·4·4

Organ indices, mean body weight and length of precociously mature and immature rainbow trout sampled in February



significantly ($P < 0{\cdot}01$) higher value of the condition factor (k) in this group ($1{\cdot}54 \pm 0{\cdot}03$) compared with immature trout ($1{\cdot}30 \pm 0{\cdot}03$).

b) Organ indices

GSI. Again, as expected, a highly significant ($P < 0{\cdot}001$) difference was seen between the mean GSI of mature ($2{\cdot}43 \pm 0{\cdot}13$) and immature fish ($0{\cdot}06 \pm 0{\cdot}01$), again reflecting the diversion of material into the development of the gonad in these fish.

HSI. Although the mean HSI of mature male fish was slightly lower ($1{\cdot}59 \pm 0{\cdot}08$) than that of immature fish ($1{\cdot}70 \pm 0{\cdot}4$), no significant difference was observed between mean levels.

VSI. A highly significant ($P < 0{\cdot}001$) difference was found between the mean VSI values of mature ($5{\cdot}15 \pm 0{\cdot}16$) and immature ($6{\cdot}65 \pm 0{\cdot}31$) one year old trout. As in the two year old mature males sexual maturation resulted in a significant reduction in VSI.

c) Proximate analysis

The results of analysis of the composition of one year old mature male and immature fish showed a slightly different pattern to that of two year old mature and immature fish.

% muscle water. A significant ($P < 0{\cdot}01$) depression of mean muscle water content was seen in mature males ($77{\cdot}4 \pm 0{\cdot}4\%$) compared to immature fish ($79{\cdot}6 \pm 0{\cdot}3\%$).

% muscle protein. Muscle protein levels in the two groups were not significantly different with mature fish having a mean % muscle protein value of $17{\cdot}3 \pm 0{\cdot}09\%$ and immature fish $17{\cdot}06 \pm 0{\cdot}02\%$.

% muscle fat. Elevated muscle fat levels were seen in mature male fish (2·89 $\pm$ 0·27%) compared with 1·41 $\pm$ 0·10% in immature fish. This difference was statistically significant ($P < 0{\cdot}001$).

d) Serum testosterone levels

The mean serum testosterone level of mature males was 13·24 $\pm$ 1·48 ng/ml, while that of immature fish was 1·95 $\pm$ 0·92 ng/ml. This difference was statistically significant ($P < 0{\cdot}001$). Thus, it would appear that the serum testosterone levels of these mature fish are low relative to two year old fish. However, it is possible that these fish had already reached full maturity and that at the time of sampling the levels of androgens had begun to fall.

Discussion

Differences were observed in body composition of two year old mature and immature rainbow trout. In addition further differences were observed between early maturing (precocious) males and immature fish of the same age. The changes in body composition in mature fish appeared to vary with age. Thus the pattern of reproduction related changes in the two year old and one year old fish differed.

Among two year old mature males a statistically significant ($P < 0{\cdot}05$) reduction in mean body weight was observed, presumably reflecting the reduced growth performance of maturing salmonids. Additional changes were found in proximate composition of mature male trout. A slight increase in mean muscle water content was observed, though this was non-significant ($P < 0{\cdot}1$).

In his study of three year old rainbow trout Lane (1979) reported increased muscle hydration accompanying the onset of sexual maturation.

However, as Lane used three year old fish of both sexes his results may be in part age-related. Determination of the % muscle water content from a small sample of three year old male rainbow trout gave a higher mean value of 80·4 $\pm$ 1·2% (Harbin, unpublished). Increased muscle hydration with age has also been reported and Templeman and Andrews (1956) have reported that extreme muscle hydration gives rise to the 'jellied' condition in older plaice (Pleuronectes platessa). The role of temperature in muscle hydration in salmonids remains unclear, though a number of workers have reported a variable degree of cold water hydration (Gordon, 1958; Toews and Hickman, 1969; Murphy and Houston, 1977; Mearow and Houston, 1980). However, reduced water temperature seems an unlikely explanation for the observed slight increase in muscle water.

An increase in muscle water among mature males may reflect a reduced food conversion efficiency and a reduction in muscle protein. These findings have been reported in migratory salmonids which have undergone a prolonged fast (Davidson and Shostrom, 1936; Macleod et al., 1958). In the present experiment a slight, though non-significant, decrease was observed among two year old mature males. No difference was seen in the mean muscle fat or protein levels of two year old mature and immature fish.

The observed significant reduction in mean VSI of mature male trout may reflect the high androgen levels associated with maturation. High androgen levels could bring about such an effect in a number of ways. Elevated androgen levels may act directly upon the viscera causing a general atrophy of the viscera (Robertson and Wexler, 1960a; McBride and van Overbeeke, 1971). An alternative effect is the

depletion of fat stores around the viscera. Jensen (1980) suggests that in salmonid species such as Salmo trutta and Salvelinus alpinus the main food reserves lie not in the liver (as in the Gadidae) but in the visceral fat reserves. In these two species a direct correlation was observed between the energy content of the viscera and the visceral dry weight. A significant fall in visceral dry weight was noted towards the time of spawning in maturing, though not in immature, fish, indicative of the use and diversion of energy reserves towards gonadal development.

The depression in the amount of food present in the gut suggests either an increased digestive ability of mature fish or a reduction in ad libitum food intake. The previous experiments showing a decreased food conversion efficiency in mature males and a reduction in ad libitum food intake would seem to support the latter possibility.

The mean epidermal thickness of mature males was significantly greater than that of immature fish. A sexual dimorphism in skin thickness has been reported in brown trout (Salmo trutta) (Pickering, 1977, 1978) and Atlantic salmon (Salmo salar) (Wilkins and Jancsar, 1979). Changes reported in this experiment were more pronounced than those reported by Pickering (1978). Vitamin A deficiency has been suggested as a causal factor in epidermal thickening of mature male salmonids (Smirnov, 1959), though the results of Pickering (1974) indicate that such a cause is unlikely. It may be of significance that the skin of salmonids, despite its poor vascular system, is capable of binding and metabolizing androgens such as testosterone to 5α-dihydrotestosterone (Hay et al., 1976), which are thought to be the active androgens in mammals (King and Mainwaring, 1974). In addition the administration of androgens has been shown to induce

epidermal hyperplasia (Idler et al., 1961a,b; Yamazaki, 1972; McBride and van Overbeeke, 1971; Murphy, 1980a). These observations suggest that androgens are directly implicated in the observed epidermal effects in male sexual maturation.

Goblet cell counts in the epidermis of mature males revealed a fall in goblet cell numbers compared to controls. However, no specific staining method, such as that advocated by Pickering (1978), was used. This fall in goblet cell numbers may be related to the increased mucus production of mature males.

No significant difference was observed between the haematocrit of mature male and immature rainbow trout. Lane (1979) reported a depression of haematocrit accompanying spawning, though again the data referred to three year old fish and were thought to represent part of the general changes in hydration accompanying maturation. However, Lane's results are surprising in view of the finding of Peterson and Shehadeh (1971) that androgen treatment resulted in an increased haematocrit in mullet (Mugil cephalus). Sano (1960) also recorded a fall in the haematocrit of mature male rainbow trout from 46% to 22%. However, to what extent the effect on haematocrit reflects a stress response is uncertain.

Differences were also observed in body composition of precociously mature and immature trout, though a different pattern of responses was seen. Thus precociously mature male trout were not significantly lighter than immature one year old fish, but were significantly shorter and therefore showed a higher condition factor. The absence of a weight difference between the two groups may suggest that, unlike maturation in two year old fish, precocious maturation possibly represents less of an energy drain. However, no information on the

pre-maturation growth of these fish is available and such a suggestion must remain tentative. It is possible that early maturing fish show a faster growth rate prior to maturation and then suffer a similar depression in growth, as may be observed in two year old fish. A similar depression in growth of male Atlantic salmon parr (Salmo salar) has also been reported (Murphy, 1980a).

Proximate analysis of early maturing males shows a reduction in the muscle water content and a significant increase in muscle fat with no apparent difference in muscle protein. These results support the idea of a reduction of severity of the maturation related effects and of catabolic effects. However, like the two year old mature males, a significant depression of VSI occurred, again demonstrating either mobilization of visceral fat reserves and/or androgen induced visceral degeneration. Such a difference in response between the two groups of males might be related to age differences and, as the secondary sexual characteristics were less marked in early maturing fish, possibly they reflect the lower serum androgen levels which were seen in these fish. Some authors have reported the androgen levels of precociously mature male salmon parr to be low (Dodd et al., 1978; Stuart-Kregor et al., 1981). In the present study the serum T levels of precociously mature trout were lower than two year old fish, though no long-term changes are reported. Scott et al. (1980a) report no difference in androgen levels between two year old and early maturing fish, though in this case the fish used were fast growing and it is possible that a size effect was operating.

In the present experiments no significant difference between the GSI of males maturing at one or two years was seen. Schmidt and House (1979) report that the GSIs of precociously mature male rainbow trout

may be lower than normally maturing fish. GSI values of up to 10% (i.e. within the normal range for two year old males) have been reported for precociously mature male salmon parr (Crim and Evans, 1978; Dodd et al., 1978).

Thus, although differences were observed between mature and immature rainbow trout, the response in terms of growth, body composition and organ indices differed with the age at which the males reached maturity. It is suggested this represents age-related differences in energy mobilization for maturation, which in turn may reflect differences in serum androgen profiles.

4·3 The effects of exogenous androgen administration upon maturation related parameters in rainbow trout

The previous experiments have revealed a number of maturation related changes in male rainbow trout, including effects on growth, conversion efficiency, digestibility and proximate body composition. Sexual maturation involves a complex series of hormonal and metabolic changes leading to the production of viable gametes, and in addition appearance of the secondary sexual characteristics outlined above. Although androgens have been implicated, the detailed relationship between hormonal factors and the induction of these changes remains unclear.

In order to collect data relating the endocrine dynamics of sexual maturation to these secondary effects, a series of experiments were carried out in which exogenous steroids were administered to immature rainbow trout.

4•3•1 Effects of 17α-methyltestosterone and testosterone upon secondary sexual characteristics, proximate composition, growth and gonadal development

In this experiment two androgens, testosterone and 17α-methyltestosterone (MT), were incorporated into the diet fed to rainbow trout at a dose of 30 mg/kg diet. Though testosterone is a natural androgen in salmonids, MT is a synthetic androgen of high oral potency (Yamazaki, 1972, 1976).

Materials and Methods

Rainbow trout (approximate mean weight 70 g) were divided into three groups, each of 30 fish. The fish were housed in a recirculating system and each group fed either a steroid treated or control diet. For the first 68 days of the experiment fish were fed at the rate of 2% body weight/day (Bwt/d), following which the ration was reduced to 1% Bwtd. until the termination of the experiment on day 134.

During the experiment fish were regularly weighed, either individually or as a batch. At intervals pooled faecal samples were collected from fish and blood samples removed for hormone assay. Secondary sexual characteristics were assessed visually at the time of sampling and the fish were photographed.

On days 68 and 98 of the experiment five fish from each group were sacrificed for analysis of body composition. Further analysis was carried out on fish sampled at the end of the experiment.

Diet preparation

B.P. 'Mainstream' (00) food was used to prepare all diet used in this experiment. 2 kg batches of diet were weighed out and mixed with

10 g of chromic oxide using the beater attachment of a Hobart industrial food mixer. During mixing water was added until the desired consistency was reached; normally equivalent to 1 volume of water : 2 volumes of diet. This mixture was then pelletized using the mincer attachment and dried using gentle heat in a drying cabinet.

Incorporation of steroid

In order to avoid possible oxidation of steroid by dietary fats, the method of Guerrero (1975) and Johnstone et al. (1978) was used to treat diet. Batches of diet to be treated were defatted by slow efflux of ethanol in glass columns. Defatted diet was then removed to a round bottomed flask and steroid added in sufficient ethanol to cover the diet. The flask was then attached to a rotary evaporator and the ethanol dried off in vacuo, aided by gentle heating (35°C). When almost dry the diet was removed to a drying tray and air dried overnight.

Steroid was added to the diet at the dose of 60 mg/kg diet and so to achieve the required dose rate of 30 mg/kg diet, this was mixed with an equal amount of non-defatted diet.

Control diet was prepared in a similar manner except that steroid was not added prior to the evaporation of ethanol.

Results

a) Growth

The growth of fish throughout the experimental period is shown in Table 4•3•1•1 and Graph 4•3•1•1. Androgen treatment resulted in a depression of growth of treated fish. By day 85 of the experiment the mean weight of control fish was significantly higher ($P < 0{\cdot}01$) than that of either T or MT fed fish.

Table 4•3•1•1

Growth of immature rainbow trout fed methyltestosterone (MT) or testosterone (T) at 30 mg/kg diet or a control diet

Mean weight (g), n = 25

Day	Control	Testosterone	Methyltestosterone
0	71•5 ± 2•14	68•0 ± 3•01	67•5 ± 2•0
10	82•5	71•2	69•5
20	93•2	81•5	71•5
32	122•2	108•5	87•0
42	135•3	113•1	92•2
55	152•3	121•0	97•0
68	168•7 (**)	129•2	104•3
85	178•0 ± 6•0	138•8 ± 6•12 (**)	132•5 ± 5•14
98	186•4	153•4	146•3
120	203•0	166•3	158•4
134	210•0 ± 9•58	171•0 ± 9•75	168•5 ± 7•21

t test vs. control for sample date
(**) P < 0•01

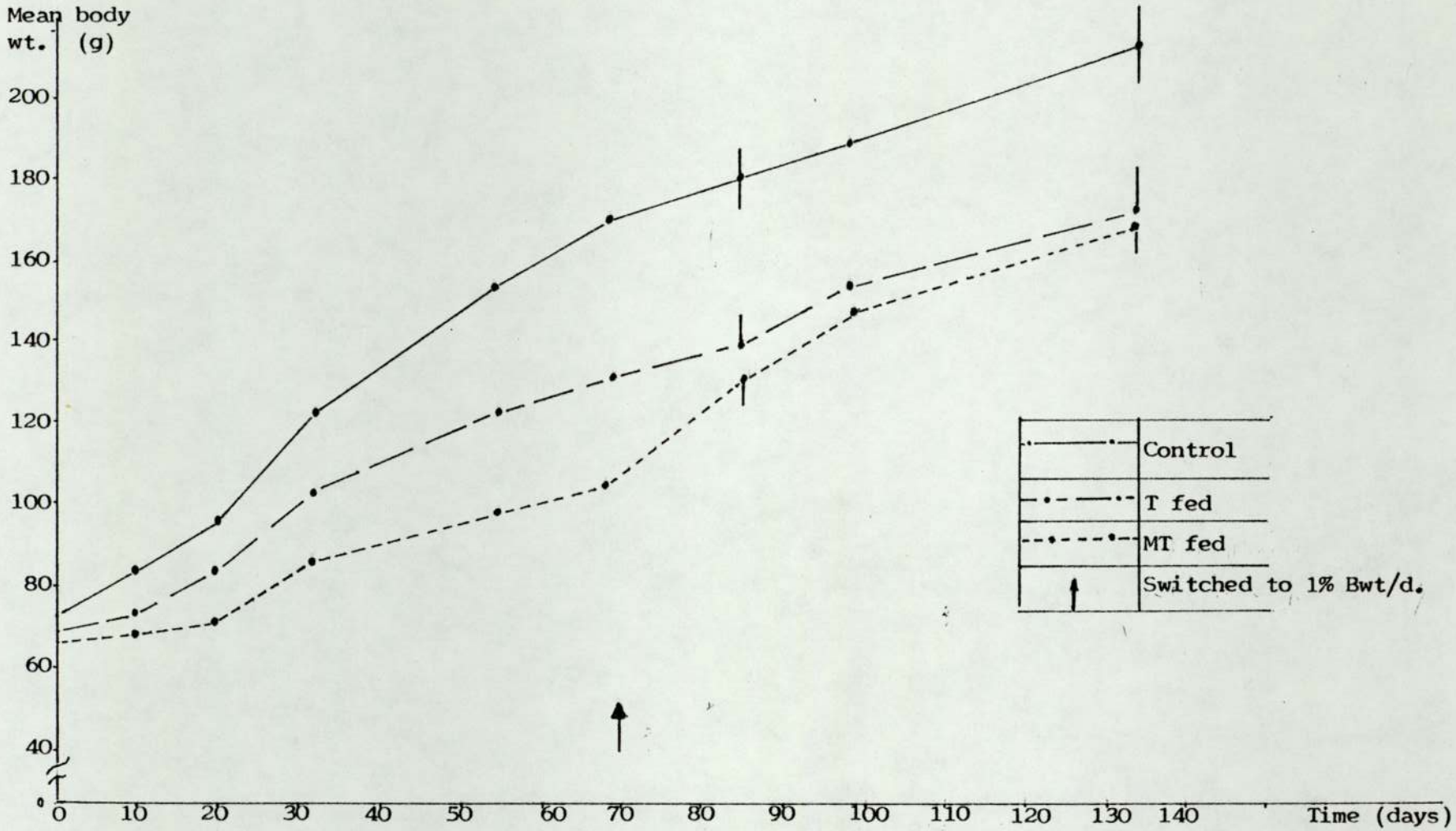
Table 4•3•1•2

Specific growth rate (g/day) of androgen fed (MT or T at 30 mg/kg diet) or control rainbow trout

Days	Control	Testosterone	Methyltestosterone
0–68	1•99	1•32	0•84
69–134	0•38	0•49	0•93

Graph 4•3•1•1

Growth of immature rainbow trout fed MT or T (30 mg/kg diet) or a control diet



Following the reduction in daily ration to 1% Bwt/d on day 68 an improved growth performance was seen in androgen treated fish. This improvement was most marked in the MT treated fish. These data are summarized in Table 4•3•1•2, which shows the specific growth rate (g/day) over the two phases of the experiment. With the reduction in ration size a reduction of growth was seen in the control fish. Although a reduction occurred in the T treated fish, this was less marked than in the controls. The MT fed group showed an actual improvement in growth over this second phase of the experiment.

b) Proximate analysis

The results of the analysis of body composition of samples taken on days 68, 98 and 134 are shown in Tables 4•3•1•3, 4•3•1•4 and 4•3•1•5 and in Figs. 4•3•1•1, 4•3•1•2, 4•3•1•3 and 4•3•1•4.

Day 68. Analysis of samples taken on day 68 of the experiment revealed differences in the carcass composition of treated and control fish. The most marked change was the increase in muscle fat resulting from androgen treatment. In control fish mean muscle fat levels of $1•58 \pm 0•12\%$ were observed, while in testosterone fed and MT fed groups the corresponding values were $2•50 \pm 0•25\%$ and $2•85 \pm 0•37\%$. These differences were significant at the $P < 0•05$ level.

A significant ($P < 0•05$) fall in muscle protein levels was found in testosterone treated ($15•99 \pm 0•51\%$) compared with control fish ($18•38 \pm 0•4\%$). A similar slight, though non-significant ($P < 0•1$), depression of muscle protein was observed following MT treatment ($16•59 \pm 0•81\%$).

No change was observed in the mean % muscle water which was similar in control, testosterone and MT treated groups ($77•5 \pm 0•63\%$, $77•4 \pm 0•63\%$ and $77•1 \pm 0•68\%$ respectively.

Table 4•3•1•3

Proximate analysis of androgen treated and control fish sampled on day 68 of feeding either MT or T (30 mg/kg diet) or a control diet

	Control	Testosterone	Methyltestosterone
% muscle protein	18•38 ± 0•40	15•99 ± 0•51 (**)	16•58 ± 0•81
n	5)	5)	5)
% muscle fat	1•58 ± 0•12	2•50 ± 0•25 (*)	2•85 ± 0•37 (*)
	5)	5)	5)
% H_2O	77•5 ± 0•63	77•4 ± 0•63	77•1 ± 0•68
	5)	5)	5)
VSI	6•23 ± 0•18	5•34 ± 0•29 (*)	5•48 ± 0•25 (*)
	5)	5)	5)
GSI male	0•108 ± 0•005	0•10 ± 0•01	0•24 ± 0•08
	2)	2)	2)
female	0•149 ± 0•008	0•18 ± 0•07	0•14 ± 0•02
	3)	3)	3)
HSI	1•38 ± 0•13	1•52 ± 0•16	1•24 ± 0•17
	5)	5)	5)

t test treated vs. controls
(**) $P < 0•01$
(*) $P < 0•05$

Table 4•3•1•4

Proximate analysis of androgen treated and control fish sampled on day 98 of feeding either MT or T (30 mg/kg diet) or a control diet

	Control	Testosterone	Methyltestosterone
% muscle protein	19•06 ± 0•91	17•7 ± 1•21	17•33 ± 0•47
n	6)	6)	6)
% muscle fat	2•12 ± 0•25	2•09 ± 0•42	2•0 ± 0•18
	6)	6)	6)
% H_2O	76•4 ± 0•39	76•8 ± 0•52	77•9 ± 0•66
	6)	6)	6)
VSI	6•29 ± 0•36	4•80 ± 0•21	4•43 ± 0•26
	6)	6) (*)	6) (*)
GSI male	0•46 ± 0•06	0•23 ± 0•04	0•21 ± 0•01
	2)	2) (*)	2) (*)
female	0•17 ± 0•02	0•18 ± 0•02	0•17 ± 0•03
	4)	4)	4)
HSI	1•36 ± 0•11	1•38 ± 0•13	1•40 ± 0•14
	6)	6)	6)

t test treated vs. controls
(*) $P < 0•05$

Table 4·3·1·5

Proximate analysis of androgen treated and control fish sampled on day 134 of feeding either MT or T (30 mg/kg diet) or a control diet

	Control	Testosterone	Methyltestosterone
% muscle water	76·15 ± 0·28	76·79 ± 0·52	(*) 77·1 ± 0·31
n	9)	9)	10)
VSI	5·30 ± 0·42	4·53 ± 0·35	4·81 ± 0·30
	9)	10)	9)
HSI	1·31 ± 0·06	1·34 ± 0·09	1·31 ± 0·09
	9)	10)	9)
GSI male	4·48 ± 0·9	2·65 ± 0·5	0·38 ± 0·13 (***)
	6)	5)	9)
female	0·36 ± 0·06	0·30 ± 0·09	0·49 ± 0·23
	9)	5)	6)

t test treated vs. control (***) $P < 0·001$
(*) $P < 0·05$

Table 4·3·1·6

Mean serum testosterone levels (ng/ml ± SE) in androgen treated and control rainbow trout

Day	Control	Testosterone	Methyltestosterone (A)
7	3·43 ± 0·63	(***) 44·96 ± 7·27	90·65 ± 21·5
n	5)	5)	5)
35	3·83 ± 1·51	(***) 57·80 ± 6·87	130·8 ± 13·45
	5)	5)	5)
90	5·88 ± 1·51	19·55 ± 7·29	51·95 ± 11·7
	5)	5) (***)	5)
120	5·69 ± 1·10	(**) 9·32 ± 0·60	60·25 ± 6·1
	5)	5)	5)

t test T vs. control
(***) $p < 0·001$
(**) $P < 0·01$

(A) MT samples assayed in Testosterone assay - converted to approximate MT values by multiplying by cross reactivity value of MT in Testosterone RIA.

Fig. 4·3·1·1

Proximate composition of androgen fed (MT or T, 30 mg/kg diet) and control rainbow trout after 68 and 98 days feeding (Mean muscle protein and fat (% ± SE))

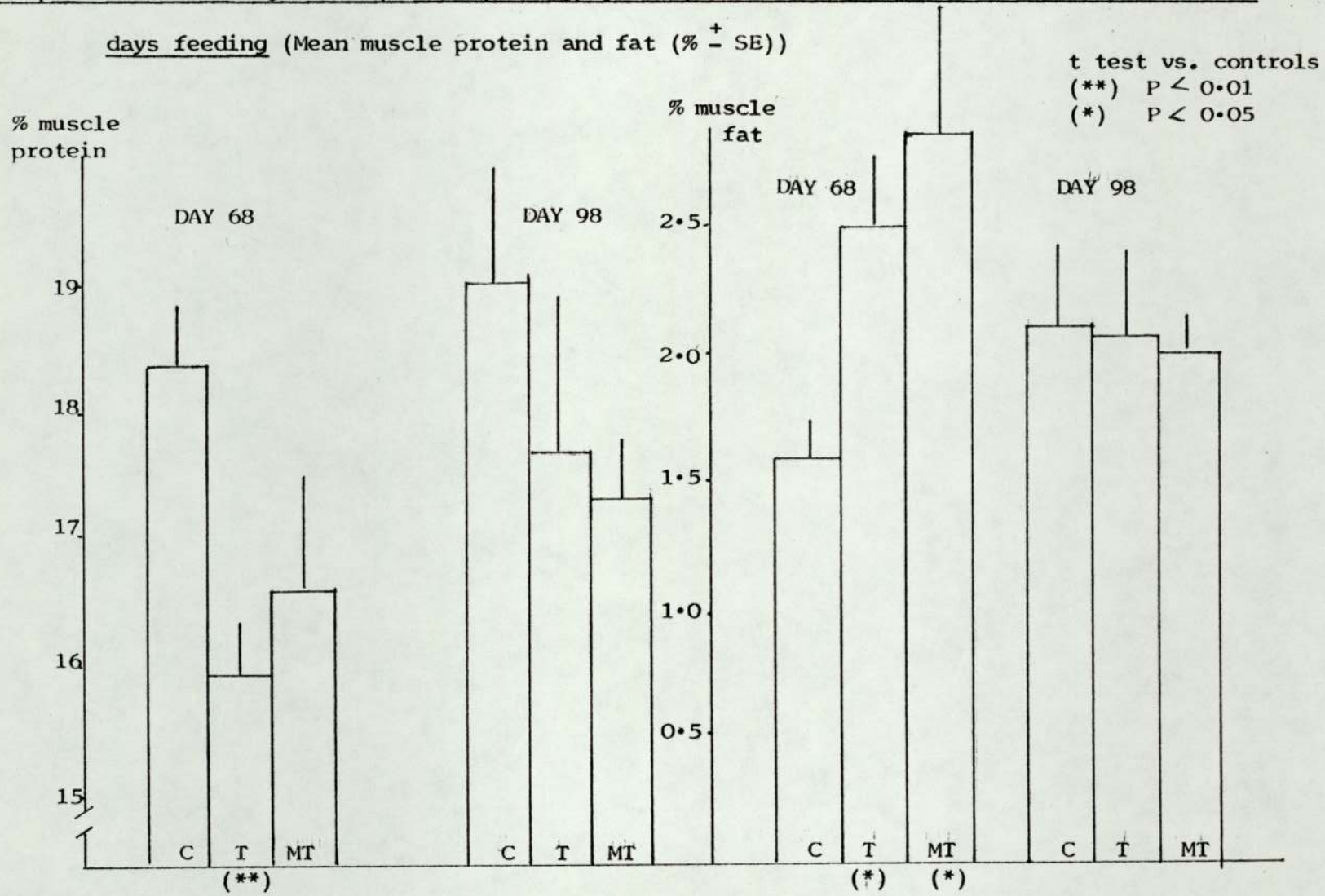


Fig. 4·3·1·2. Mean viscerosomatic indices of androgen fed (MT or T, 30 mg/kg diet) and control rainbow trout after 68, 98 and 134 days feeding

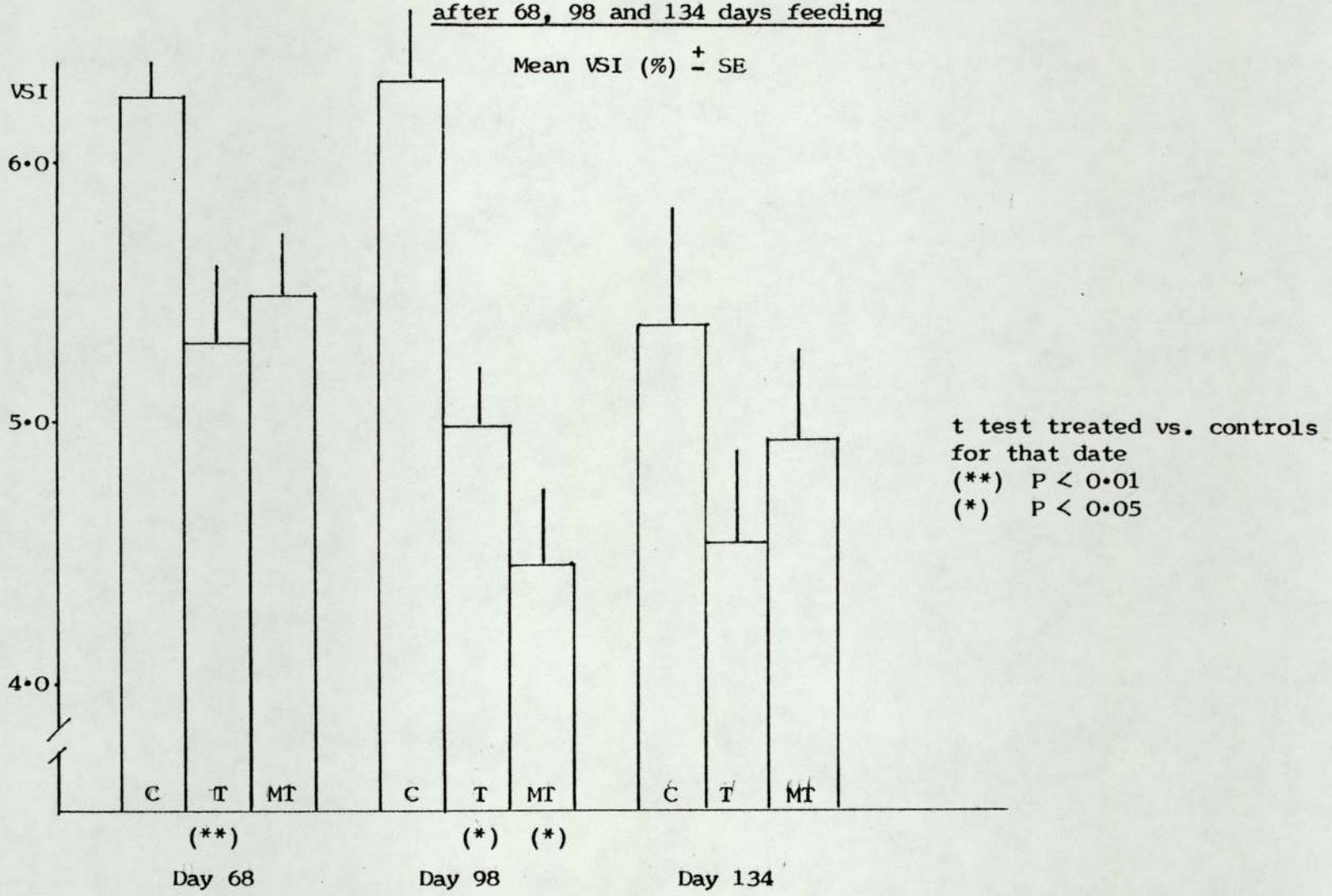


Fig.4•3•1•3 Mean muscle water content of androgen fed (MT or T, 30 mg/kg diet) and control rainbow trout after 68, 98 and 134 days feeding

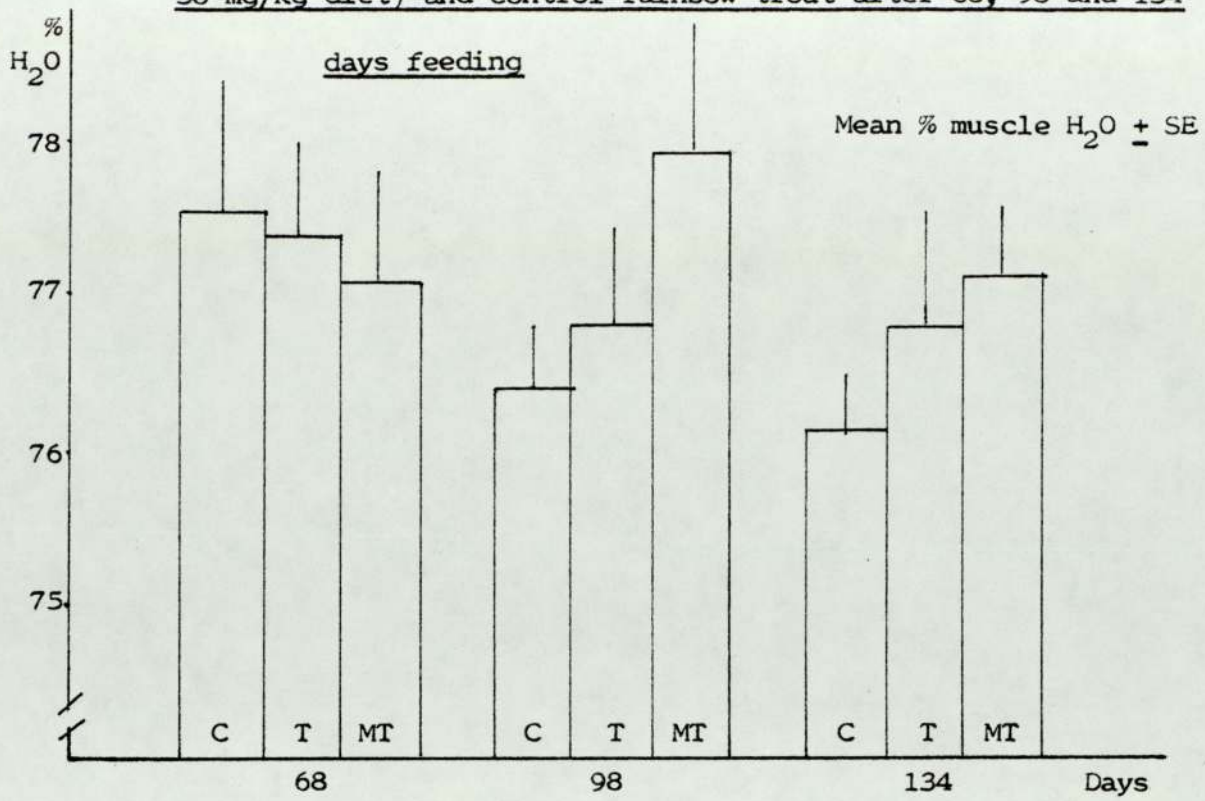
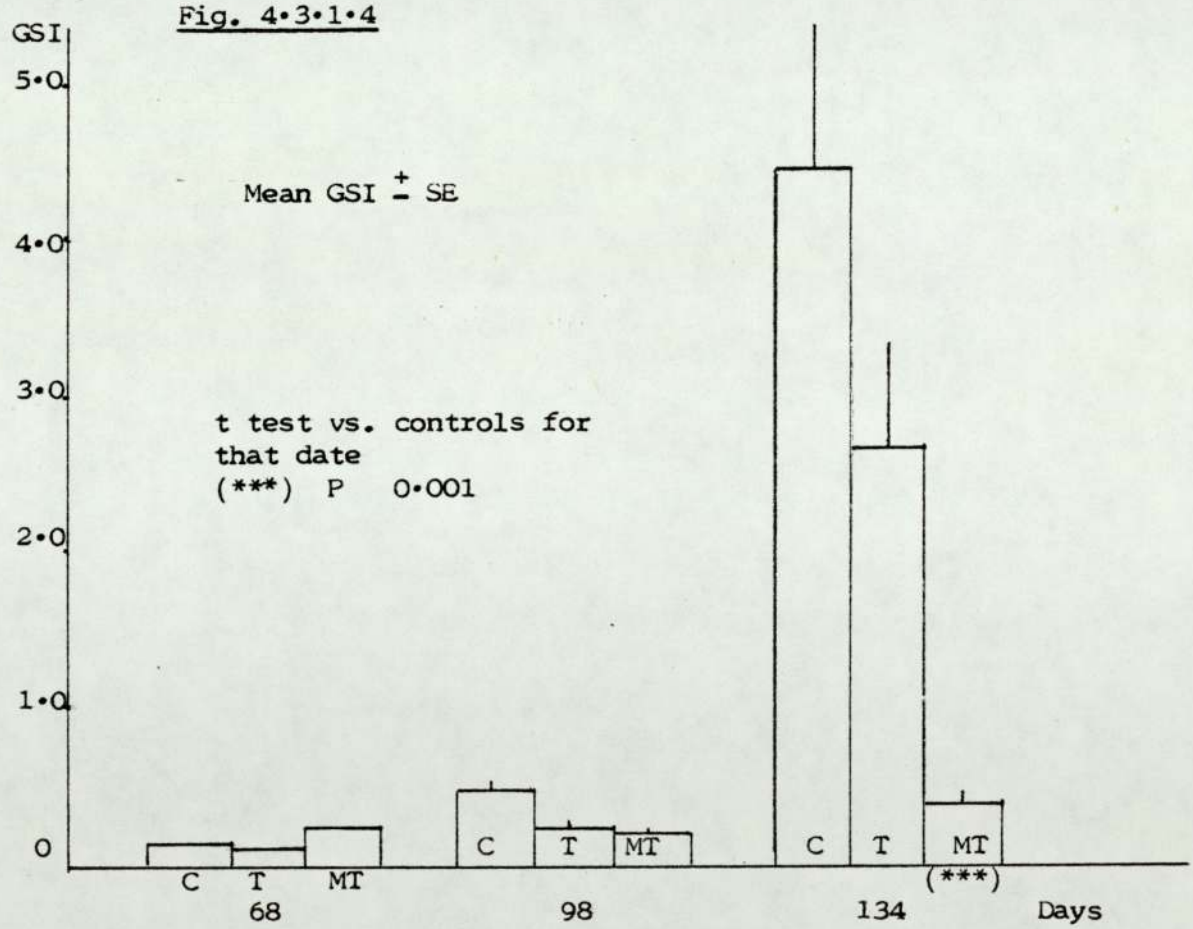
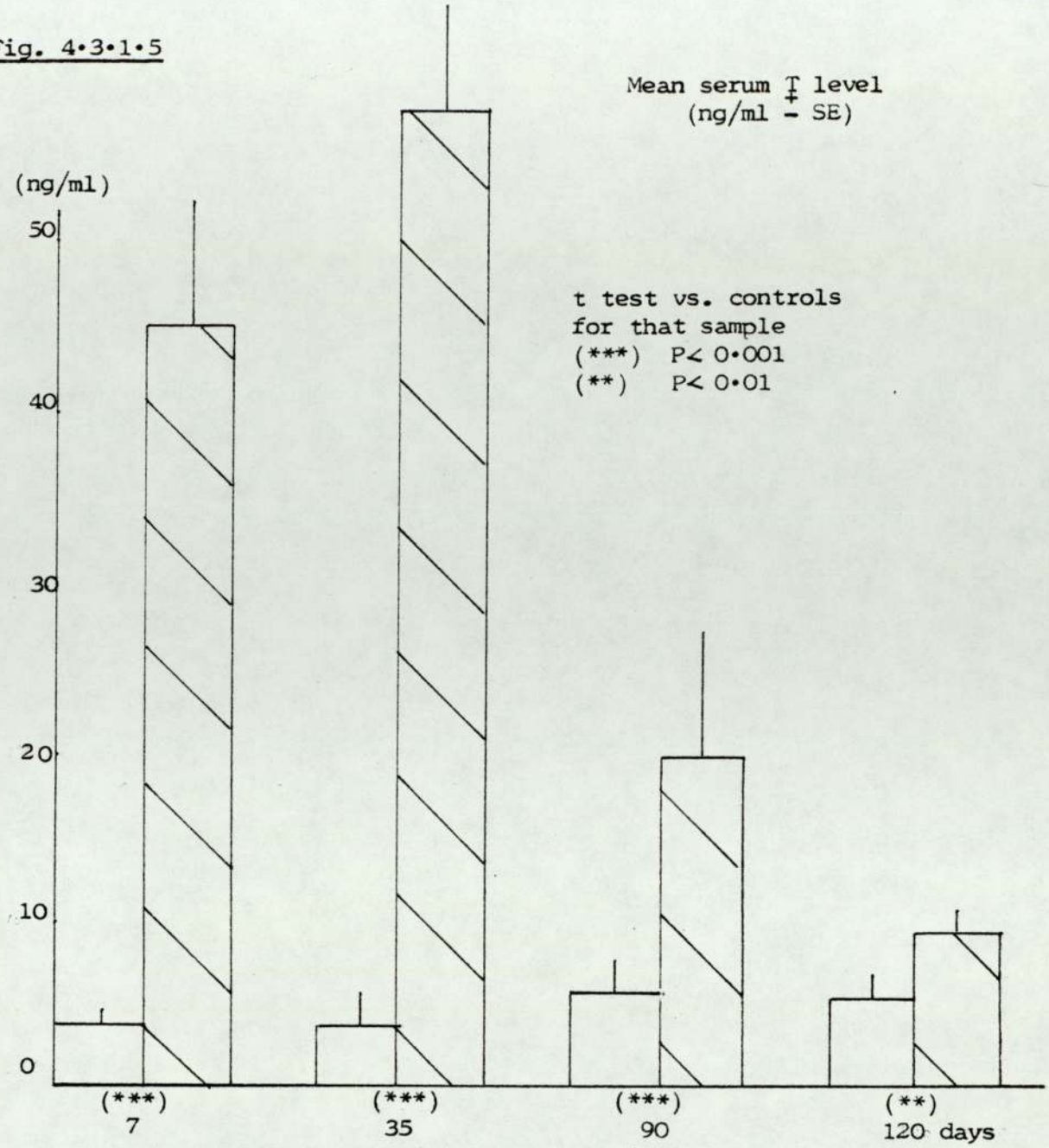


Fig. 4•3•1•4

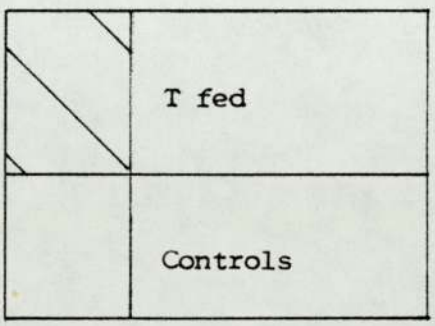


Mean male GSI of androgen fed (MT or T, 30 mg/kg diet) and control rainbow trout after 68, 98 and 134 days feeding

Fig. 4·3·1·5



Mean serum testosterone levels of T fed (30 mg/kg diet) and control rainbow trout sampled at 7, 35, 90 and 120 days feeding



In both androgen treated groups the mean viscerosomatic index (VSI) was depressed from a control mean VSI of 6·23 $\pm$ 0·18 to 5·34 $\pm$ 0·29 (T) and 5·48 $\pm$ 0·25 (MT). These means were significantly different from the control mean value at the $P < 0{\cdot}05$ level. No differences were observed in the mean Gonadosomatic index (GSI) or Hepatosomatic index (HSI) of treated and control fish.

Day 98. Samples taken on day 98 showed a different response to androgen treatment (Table 4·3·1·4), possibly reflecting the reduced steroid intake following the lowering of the daily ration in the second phase of the experiment.

Again mean protein levels of T treated (17·7 $\pm$ 1·21%) and MT treated (17·33 $\pm$ 0·47%) were lower than the corresponding control mean muscle protein value (19·08 $\pm$ 0·91), though these differences were not statistically significant.

Unlike earlier samples the mean muscle fat levels of controls (2·12 $\pm$ 0·25%), T treated (2·09 $\pm$ 0·42%) and MT treated (2·0 $\pm$ 0·18%) fish did not differ significantly.

The mean muscle water content was also not significantly different between control (76·4 $\pm$ 0·39%) and T treated (76·8 $\pm$ 0·52%) groups, though the mean % muscle water of the MT fed group was higher than that of control fish at 77·9 $\pm$ 0·66. This difference was not significant ($P < 0{\cdot}1$).

As in earlier samples the mean VSI was lower among androgen treated fish. The control mean VSI on this day was 6·29 $\pm$ 0·36 compared with T (4·80 $\pm$ 0·21) and MT treated fish (4·43 $\pm$ 0·26). These differences were significant at the $P < 0{\cdot}01$ (T) and $P < 0{\cdot}05$ (MT) levels.

While no difference was observed in mean female GSI between groups a significant difference was seen with regard to male GSI. The mean male GSI of treated fish, 0·23 ± 0·04 (T) and 0·21 ± 0·01 (MT), were significantly lower ($P < 0·05$) than that of control fish (0·46 ± 0·06).

Day 134. At the end of the experiment further fish were sacrificed and various organ parameters determined. No significant differences were observed between the muscle water content of control (76·15 ± 0·31%) and T treated fish (76·79 ± 0·52%), but MT treated fish showed a significantly ($P < 0·05$) higher % muscle water content (77·1 ± 0·31%).

The mean VSI of both T (4·53 ± 0·35) and MT fed (4·81 ± 0·30) fish was significantly lower than that of control fish (5·30 ± 0·42), though these differences were not statistically significant.

As observed in earlier samples no effect of androgen treatment was seen on the mean GSI of female fish but a significant depression ($P < 0·001$) was observed in MT fed males. The control mean male GSI was 4·48 ± 0·9, while MT treated males had a mean GSI of 0·38 ± 0·13. T treated males also showed a lower mean GSI, but this effect was not statistically significant.

c) Steroid levels

Steroid levels in serum samples taken throughout the experiment are shown in Table 4·3·1·6 and Fig. 4·3·1·5. Samples were assayed for serum T as described in General Materials and Methods. Samples from MT treated fish were also assayed using the T assay, simply for comparison. The results of this series should be viewed with caution due to the non-homologous nature of the RIA used.

Samples taken on days 7 and 35 showed high mean T levels (44·96 ± 7·27 ng/ml and 57·80 ± 6·80 ng/ml) in T treated fish. Control mean T values remained low on these dates (3·43 ± 0·63 ng/ml and 3·83 ± 1·57 ng/ml). The differences in control and T treated groups mean T levels were significant at the $P < 0{\cdot}001$ level on both occasions.

Following the reduction in feed rate a fall in mean serum T levels was observed in T treated fish. By day 90 the mean T level of T fed fish had fallen to 19·55 ± 7·29 ng/ml and this fell further to 9·32 ± 0·60 ng/ml by day 120. These levels remained statistically higher than control levels ($P < 0{\cdot}01$).

Bearing in mind the reservations regarding the results of assay of MT treated samples, they seemed to follow a similar pattern, with high levels early in the experiment and a fall following the reduction in feed rate. However, this fall appeared less pronounced.

d) Digestibility values

The % dry matter digestibility values, determined by the Cr_2O_3 method, are shown in Table 4·3·1·7 and Fig. 4·3·1·6. Each value represents the mean of either two or three replicates from pooled samples of faeces collected as described.

Initially on day 43 the mean digestibility values were lower in both androgen treated groups. In the T treated group the mean % dry matter digestibility was 61·50 ± 0·14%, while in the MT fed fish the corresponding value was 58·75 ± 0·64%. The mean digestibility among control fish on this date was 76·25 ± 0·21%.

Samples taken in the second phase of the experiment, following the reduction in feed rate, showed % dry matter digestibility values

Table 4•3•1•7

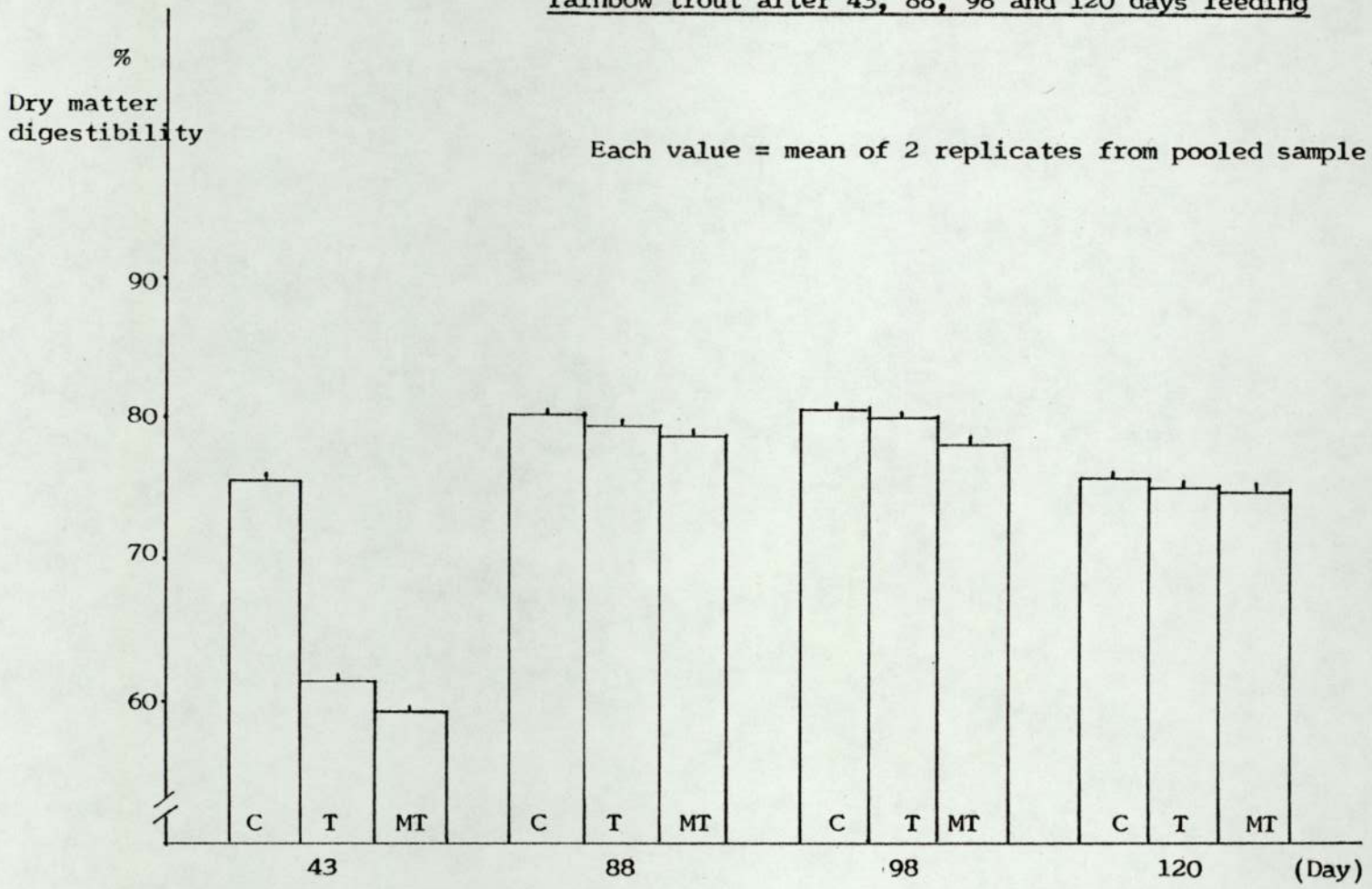
Mean % dry matter digestibility of androgen treated (MT or T, 30 mg/kg diet) and control rainbow trout after 43, 88, 98 and 120 days feeding

Mean % dry matter digestibility ± SE

Day	Control	Testosterone	Methyltestosterone
43	76•25 ± 0•21	61•50 ± 0•14	58•75 ± 0•64
n	2)	2)	2)
88	80•6 ± 0•87	78•30 ± 0•65	77•90 ± 0•40
	3)	3)	3)
98	81•4 ± 1•05	80•00 ± 1•3	76•60 ± 1•35
	3)	3)	3)
120	76•0 ± 0•42	74•7 ± 0•77	74•4 ± 0•49
	2)	2)	2)

(n = number of replicates assayed from pooled sample).

Fig. 4·3·1·6. Mean % dry matter digestibility of androgen treated (MT or T, 30 mg/kg diet) and control rainbow trout after 43, 88, 98 and 120 days feeding



among androgen treated fish to be only slightly less than that of control fish. Thus on day 98 the mean digestibility values were 81·4 $\pm$ 1·05%, 80·0 $\pm$ 1·3% and 76·6 $\pm$ 1·35% for control, T and MT fed fish.

So variations in dry matter digestibility were observed, which appeared to be related to the dose of androgen received. Due to the nature of the sampling programme, no statistical analysis of these results was possible. However, as each pooled sample represented a pooled sample of faeces from all fish in the group, the values obtained should give a reasonable estimate for this parameter.

e) Male secondary sexual characteristics

Treatment with both androgens resulted in the appearance of male secondary sexual characteristics among fish of both sexes. MT was much more potent in promoting these features. The appearance of skin darkening was noted from day 35 of the experiment in both groups. By day 88 the effects of androgen treatment were pronounced, especially among MT fed fish, in which kype development was far more advanced than in T treated fish. The extent of these secondary sexual characteristics are shown in Plates 4·3·1·1 to 4·3·1·4 taken on day 98 of the experiment.

By the end of the experiment all MT fed fish, irrespective of sex, were similar in appearance to mature males. Less extreme effects were seen in T treated fish, though skin darkening was frequently noted. Pronounced kype development was only occasionally noted in T treated fish. Darkening was noted in occasional fish in the control group towards the end of the experiment, probably indicative of the presence of developing males within this group.

Plate 4•3•1•1 (overleaf)

Appearance of control (1) and androgen treated (T or MT, 30 mg/kg diet - numbered (2) and (3) respectively) rainbow trout after 98 days feeding

Plate 4•3•1•2 (overleaf)

Close up of control rainbow trout to show absence of kype development after 98 days feeding control diet


1
2
3


1

Plate 4•3•1•3 (overleaf)

Close up of T fed rainbow trout (30 mg/kg diet) after 98 days feeding treated diet, showing slight darkening and kype development

Plate 4•3•1•4 (overleaf)

Close up of MT fed rainbow trout (30 mg/kg diet) after 98 days feeding treated diet, showing pronounced darkening and kype development


2


3

Discussion

Dietary administration of the androgens, testosterone and 17α - methyltestosterone, at the level of 30 ppm diet, resulted in a depression of growth in both androgen treated groups. This growth depression was greatest with MT treatment. In both cases the effect appeared to be dose-related. A reduction in ration size, from 2% body weight/day to 1% Bwt/d at the end of the first phase of the experiment (day 68) produced an improvement in growth rate among androgen treated fish. Thus in the first phase the specific growth rate (g/day) of control fish was greater than that of T treated fish, which was, in turn, greater than that of MT treated fish. In the second phase of the experiment the specific growth rate was highest in MT treated fish, followed by T treated, then control fish.

The initial depression in growth resulting from androgen treatment may reflect the dose-dependent balance between the androgenic and anabolic activities of androgens. Thus, high doses may give rise to catabolic effects and a reduction in growth, while lower doses promote nitrogen retention and anabolic effects. This dichotomy of response has been recognized in the literature and may in part explain the contradictory nature of the reported responses to androgen treatment (Yamazaki, 1976). High androgen doses caused a growth depression in the guppy (Clemens et al., 1966) and were suggested as the agent responsible for the natural growth depression in mature male guppies (Eversole, 1939). Ashby (1957) reported a similar growth depression due to androgens in brown trout, as did Yamazaki (1976) in the rainbow trout and goldfish. At increased doses the synthetic testosterone analogue, 4 - chlorotestosterone acetate, produced a growth depression

in rainbow trout, while a promotion of growth was observed at lower dose levels (Hirose and Hibiya, 1968b).

Great interest has been directed towards the anabolic response to low androgen doses, with a view towards increasing the commercial production of salmonids. (McBride and Fagerlund, 1973, 1976; Fagerlund and McBride, 1975, 1977; Simpson, 1976; Yamazaki, 1976; Higgs et al., 1977; Saunders et al., 1977; Yu et al., 1979; Fagerlund et al., 1980). Increased growth arising from a combination of increased food intake, higher conversion efficiency and nitrogen retention have been reported in these studies.

In contrast to their role at low levels in anabolism, the mechanisms by which high doses of androgen exert their effects have attracted less attention; in particular the role of steroids in producing the deleterious changes accompanying sexual maturation remains less clear.

Yamazaki (1976) noted a depression of appetite and ad libitum food intake in goldfish fed 30 mg MT/kg diet, though this depression was not sufficiently large to account for the poor growth performance of these fish. Within the present experiment ration size was fixed at constant levels (2·0 and 1·0% Bwt/d) and changes in appetite would not have influenced the growth observed; it was, however, noted that steroid treated fish seemed more reluctant to consume their daily ration than control fish. So changes in growth rate presumably reflect changes in food conversion efficiency and the catabolic effect of androgens.

The reduction in total digestibility observed in androgen fed fish parallels those reported in mature male fish and indicates a possible

mechanism by which high androgen levels may bring about a reduction in food conversion efficiency. The greater effect observed with steroid treatment compared to that seen in mature males may be due to the oral route of administration. Low digestibility values in androgen fed fish were found in the first phase of the experiment, when androgen levels were high. This coincided with a reduction in growth of these fish. No relationship between digestibility and steroid levels was observed in the second phase of the experiment. Following the reduction in daily ration size, and hence the dose of steroid supplied, mean total digestibilities were similar to those of control fish.

The cellular mechanisms by which androgens act to influence conversion efficiency remain unclear, though recent evidence suggests an increased level of proteolytic activity in the alimentary canal of carp fed androgens at the dose of 10 ppm (Lone and Matty, 1981). Further evidence of a direct response of the alimentary canal to dietary steroid administration is the observed hypertrophy of intestinal cells following the administration of low doses of MT in masu salmon (Oncorhynchus masu) (Yamazaki, 1976). It is possible that exogenous steroids may act directly upon the alimentary canal, either promoting or inhibiting enzyme synthesis and/or release, the particular response depending upon the dose administered. Preliminary data from this laboratory suggest a specific effect of maturation and perhaps androgen treatment upon intestinal dipeptidase activity in rainbow trout fed testosterone (30 mg/kg). This work was conducted with Dr. R. Ash of the University of Bradford.

The levels of mucosal glycyl-leucine hydrolase activity, the so-called 'master' dipeptidase (Das and Radhakrishnan, 1973;

Josefsson etal., 1977) were measured in rainbow trout fed either MT, T or a control diet for a period of 12 weeks. In addition activity was assayed in those males that matured. Enzyme activity was assayed by the technique of Ash (1980). (See Appendix 1).

The mucosa from two separate regions were assayed from each fish; the caecal region and the region approximately 2" below the caeca (post-caecal region). The results of the enzyme assays are shown in Table 4·3·1·8 and Fig. 4·3·1·7.

The results indicated a slight, though not significant depression in caecal, though not post-caecal glycyl-leucine hydrolase activity in testosterone fed trout. Mean caecal enzyme levels fell from 71·66 $\pm$ 17·59(Control) to 41·16 $\pm$ 11·26 μ moles/m/g (T). A significant depression of caecal enzyme activity was, however, noted in those male control fish which matured during the experimental period. Mature male control trout had a mean mucosal enzyme level of 28·00 $\pm$ 3·46 μ moles/m/g, while the value for immature control fish was 71·66 $\pm$ 17·59 μ moles/m/g ($P < 0·05$). No difference was noted between the post-caecal mucosal enzyme levels of either T fed (66·61 $\pm$ 27·19) and mature male controls (64·26 $\pm$ 27·06 μ moles/m/g) and immature control fish (69·50 $\pm$ 15·75 μ moles/m/g), though the mean enzyme level of the mucosa from this region among MT fed trout was higher (126·10 $\pm$ 38·3 μ moles/m/g), but not significantly so.

It may be of significance that in rainbow trout the greatest peptide hydrolytic activity is located in the mucosa of the caecal region (Ash, 1980) and this possesses both tryptic and chymotryptic activity (Croston, 1965), and so would seem to be of major importance in protein digestion.

Table 4•3•1•8

Glycyl-leucine hydrolase activity (u mole substrate hydrolysed/m/g wet weight) in the caecal or post caecal regions of the intestine of control (mature or immature) or androgen treated (MT or T, 30 mg/kg diet) rainbow trout

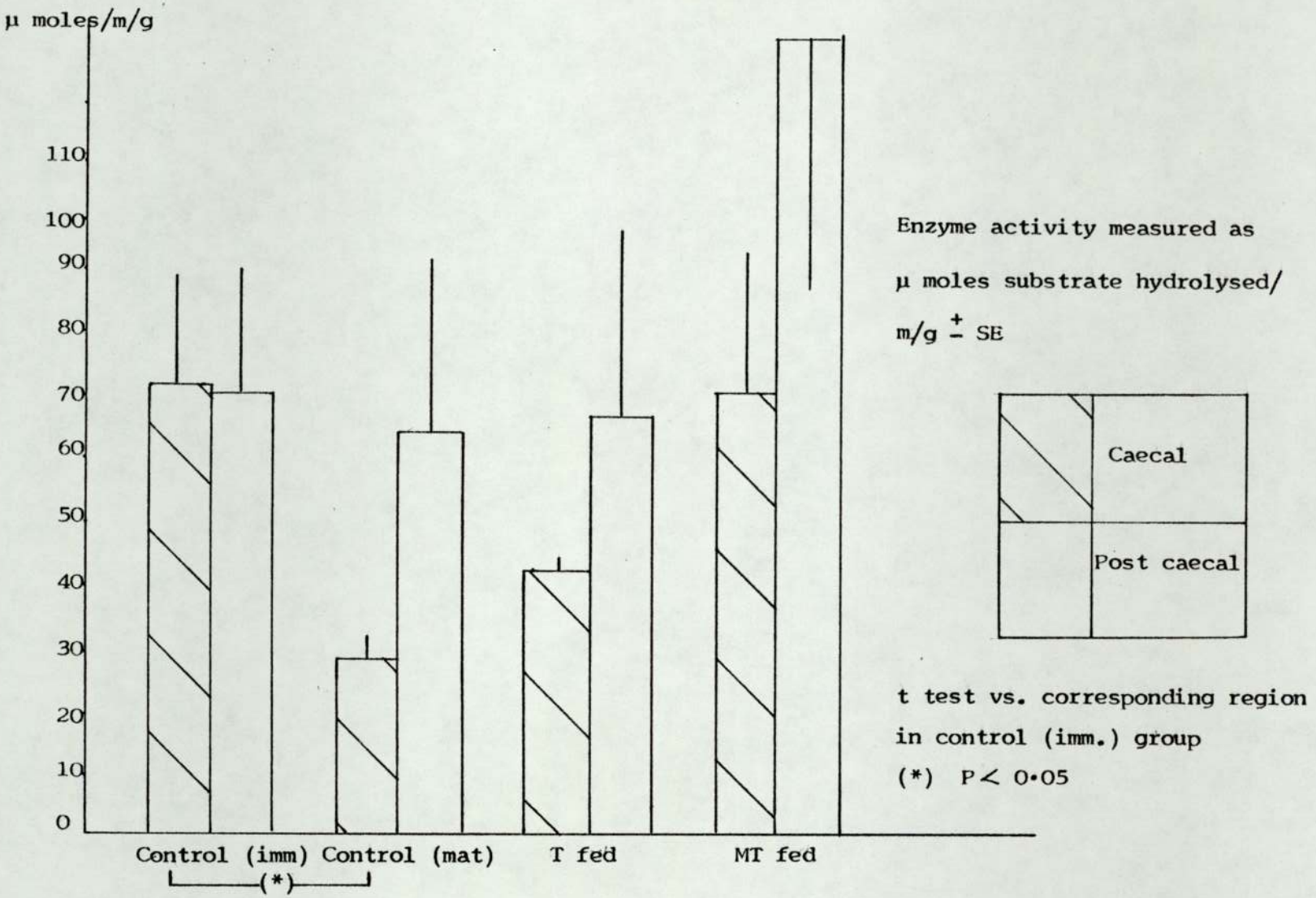
μ moles/m/g ± SE

Region	Control (immature)	Control (mature males)	Testosterone	Methyl-testosterone
	(*)			
Caecal	71•66 ± 17•59	28•00 ± 3•46 (-61%)	41•16 ± 11•26 (-41•1%)	69•66 ± 21•49 (-2•7%)
n	6)	4)	6)	6)
Post caecal	69•50 ± 15•75	64•26 ± 27•06 (-7•5%)	66•61 ± 27•19 (-4•1%)	126•10 ± 38•3 (+81•4%)
	6)	4)	6)	6)

t test (*) $P < 0.05$

(figures in brackets = % change relative to control value).

Fig. 4·3·1·7. Glycyl-leucine hydrolase activity in the caecal or post caecal regions of the intestine of control (mature or immature) or androgen treated (MT or T, 30 mg/kg diet) rainbow trout



The reported depression in digestibility associated with T feeding would seem to fit well with the reduction in peptidase activity in this group and the statistically significant reduction in activity in mature males may represent one mechanism through which reduced digestibility occurs in these fish. Surprisingly no depression of caecal mucosa enzyme activity was observed in MT treated fish, though a noted depression in digestibility was found in this group.

Within the present experiment evidence of the catabolic nature of high androgen levels was seen in the first phase of the experiment. Within this period a significant depression of muscle protein was observed in androgen fed fish. In the second phase of the experiment this catabolic effect was absent and, though androgen treated fish had lower mean levels of muscle protein, these were not significantly lower than those of control fish.

So the reduction in growth rate of androgen treated fish may arise in part from the depression of digestibility and catabolic effects of high androgens levels upon muscle composition. The relationship between these two factors may not be a simple one. Thus Lone and Matty (1980) reported an increase in skeletal muscle protein in carp fed 10 ppm MT, even though this group showed lower growth than control fish. Thus, it would appear that the sensitivity of the anabolic and food conversion effects may differ.

As in the mature male rainbow trout examined, a depression in VSI was observed in androgen treated fish in this experiment. This parallels the observation of Simpson (1976), who showed that the VSI decreased with MT feeding. However, Simpson explained his results as a discrepancy between the available food and the energy needs of his fish. In the present experiment, where fish were fed to a higher ration,

this seems an unlikely explanation. A more satisfactory explanation may be the mobilization of fat reserves stored in the viscera in response to androgen treatment. Androgens have been shown to be capable of exerting a depressive effect on visceral fat (Simpson, 1976; Fagerlund et al., 1978; Matty and Cheema, 1978). Mobilization and redeposition of visceral fat stores may explain the increases in muscle fat observed during the first phase of the experiment. Possibly the redeposition of fat in skeletal muscle reflects the absence of a developing gonad in these fish.

No effect of androgens upon the HSI of treated fish was observed in the present experiment. This contrasts with the work of Lone and Matty (1980) and may reflect a species difference in the relative roles of viscera and liver in rainbow trout and carp. Billard et al.(1981) reported no significant effect on the HSI of rainbow trout fed a range of steroids, including T and MT at a dose of 0·5 mg/kg; the only exception was estradiol-17β, which produced a significant increase in HSI.

Androgen treatment resulted in the appearance of male secondary sexual characteristics in fish of both sexes; an effect which was more pronounced with MT treatment. By the end of the experiment all fish in this group, irrespective of sex, resembled mature males. The development of male secondary sexual characteristics was less marked among T fed fish, with the appearance of slight skin darkening and kype development.

This influence of androgens upon the development and maintenance of teleost male secondary sexual characteristics is well recognized (Pickford and Atz, 1957; Dodd, 1960; Hoar, 1965; Lofts et al., 1966).

Evidence has been gathered by three main approaches:-

1) The administration of exogenous androgens, such as MT and 11-ketotestosterone, has been shown to be effective in inducing male secondary sexual characteristics, such as skin and colouration changes (Idler et al.,1961a,b; Lofts et al., 1966; Fagerlund and Donaldson, 1969; Yamazaki, 1972). Such androgenic effects have accompanied attempts to promote growth by androgen administration (Fagerlund and McBride, 1977).

2) Indirect evidence for the involvement of androgens in the development of male characteristics is the halting of these changes by castration (McBride and van Overbeeke, 1971).

3) As the appearance of male secondary sexual characteristics has been shown to coincide with naturally occurring high levels of androgens, androgens have been causally implicated in these processes (Sangalang and Freeman, 1974; Scott et al., 1980a).

MT has been shown to be a more potent androgen than testosterone in salmonids (Billard, 1976; Fagerlund and Dye, 1979; Yu et al., 1979; Billard et al., 1981). The approximate estimates of MT levels within the present experiment suggest blood MT levels were higher than testosterone levels, though both steroids were fed at the same dose rate. The appearance of male secondary sexual characteristics among female rainbow trout fed MT may be a reflection of the higher oral potency of this steroid. Although T levels are higher in mature female than in mature male salmonids, male secondary sexual characteristics do not appear. The absence of these changes in mature female trout may reflect the absence of the other natural teleost androgen, 11-ketotestosterone, perhaps coupled with differences in the degree of physiological inactivation of androgens by binding, as suggested by

Idler and Ng (1980). It may also reflect absence of suitable receptors for androgens.

Androgen treatment resulted in a depression of GSI in the males in both groups of treated fish, though no effect on female GSI was evident. As the control fish were of higher mean body weight, the depressive effect on GSI in androgen treated fish would be greater if this difference in body weight was not present. This effect was more marked in the group treated with MT. Although no detailed histological examination was carried out, the gonads examined from the MT treated males appeared abnormal in structure and contained some spermatogonial cysts, but were generally less well developed than those of control males. McBride and Fagerlund (1973) observed a decrease in spermatogonial numbers in MT treated coho salmon. Billard et al. (1981) report a depressive effect of MT and T on the testis of male rainbow trout. In this case the steroids were fed at a very low dose rate (0·5 mg/kg diet). Measurement of GTH levels suggests that this effect was due to a direct effect upon the gonad, rather than an indirect effect, mediated via the pituitary gland.

In view of the importance of the testis as a binding site for exogenous androgens (Fagerlund and Dye, 1979), it is hardly surprising that the administration of androgens can bring about changes in gonadal physiology and development. However, two reported effects of androgen treatment on the gonad of male teleosts are varied and reflect differences in species and age of fish and also the androgen used and its mode of administration and dose. Thus a positive response of androgens on male gonadal development and spermatogenesis has been reported (Sundararaj and Nayyar, 1967; Yamazaki and Donaldson, 1969; Billard,

1974; Yamazaki, 1976). Other studies demonstrate a negative or inhibitory response (Fagerlund and McBride, 1975; Yamazaki, 1976; Fagerlund et al., 1979a;Yu et al., 1979; Harbin et al., 1980). Some reports have shown no effect of exogenous androgens upon the teleost testis (Wiebe, 1969; Hyder, 1972).

It is generally agreed that MT is a more potent oral androgen than testosterone. In the present experiment high doses (30 mg/kg) of this androgen gave rise to a depression of GSI. Yamazaki (1976) showed MT (5 mg/kg) accelerated gonadal development of rainbow trout, while high doses (50 mg/kg) produced a depression of gonadal development in this species. However, Billard (1974) was only able to promote spermatogenesis in goldfish by treatment with high doses (100 - 200 µg/g body weight) of testosterone. The report that low doses (0·5 mg/kg diet) of MT are capable of completely suppressing spermatogenesis is somewhat surprising (Billard et al., 1981), as it is usually assumed that high doses exert this type of effect, while low doses usually promote gonadal development.

The serum levels of T measured during the two phases of the experiment demonstrate the importance of ration size and feeding practice in maintaining the blood steroid levels of treated fish. Following the reduction in diet ration on day 68 of the experiment, the serum levels of T in T treated fish fell to less than half the levels prevailing during the first phase of the experiment. The values of serum T, measured by RIA, show reasonable agreement with those calculated by Fagerlund and McBride (1978).

4•3•2 Effects of 11-ketotestosterone and testosterone upon secondary sexual characteristics, proximate composition, growth and gonadal development

The effects of feeding the androgens, T and MT, have been reported in the previous section. Both androgens produced changes in a number of parameters associated with maturation. As the most potent androgen used (MT) does not occur naturally, an experiment was carried out to compare the relative roles of the two natural androgens implicated in male sexual maturation in salmonids, i.e. testosterone and 11-ketotestosterone.

Materials and Methods

Immature rainbow trout, weighing approximately 75 g, were used in this experiment. A group of 45 fish were tagged and divided, at random, into the tanks of a three tank recirculating system. Fish were fed either a steroid treated or control diet prepared as described below.

Preparation of steroid treated diet

Steroid treated diets were prepared using B.P. Mainstream trout food (No. 4). Steroid was dissolved in ethanol and sprayed onto the diet. Steroids were added at the dose of 20 mg/kg diet. Treated diet was dried overnight and stored at $4^{o}C$ until use. Control diet was sprayed with ethanol only. These diets were fed throughout the experimental period, except for the 14 day period preceding faecal sampling, when similar steroid treated or control diets, but with added Cr_2O_3, prepared as described previously, were fed. This Cr_2O_3 diet was steroid treated in the manner just described. Fish were fed at the rate of 2% Bwt/d.

During the experiment fish were periodically weighed and examined

for the presence of male secondary sexual characteristics. On days 13 and 35 faecal samples were taken to allow determination of the dry matter digestibility. On day 35 five fish from each group were sacrificed for proximate analysis. Skin portions were removed and fixed in buffered neutral formalin and processed for histological examination. At the end of the experiment (day 83) further fish were sacrificed for proximate analysis.

Results

a) Growth

The growth of androgen treated and control fish is shown in Table 4•3•2•1 and Graph 4•3•2•1.

No significant differences were observed between the mean body weights of androgen treated or control fish at any time in the experiment. However, at all times T fed fish were smaller than either control or 11-KT fed fish. Fish fed 11-KT appeared to grow as well or better than controls.

b) Dry matter digestibility

Faecal samples for the measurement of dry matter digestibility were taken on days 13 and 35 and the results of these determinations are shown in Table 4•3•2•2 and Fig. 4•3•2•1. Changes were seen in the digestibility following steroid treatment, though the effect depended upon the steroid administered. Samples taken on day 13 showed no significant difference between androgen treated and control fish. However, the mean digestibility values of the T ($53•27 \pm 6•19\%$) and 11-KT ($71•32 \pm 1•72\%$) groups were significantly ($P < 0•05$) different. Although the mean digestibility of the T fed group was less than that

of controls, this effect was not statistically significant. In the 11-KT fed group the mean digestibility was slightly, though not significantly, higher than in the control group (69·25 $\pm$ 4·03%).

Later samples (day 35) showed a significant depression of mean digestibility resulting from T treatment (51·96 $\pm$ 5·42%) compared with a mean control value of 76·36 $\pm$ 4·88% ($P < 0·05$). Although the mean digestibility value of 11-KT fed fish was lower (58·59 $\pm$ 4·51%) than controls, this difference was not statistically significant ($P < 0·1$).

Thus the two androgens seemed to exert different effects upon digestibility, with T having a more marked effect upon this parameter.

c) Proximate analysis

The results of the proximate analyses carried out on fish sampled on days 35 and 83 are shown in Table 4·3·2·3 and Figs. 4·3·2·2, 4·3·2·3 and 4·3·2·4.

Viscerosomatic index (VSI). The analysis carried out on samples taken on days 35 and 83 revealed an effect of androgen treatment on VSI. Treatment caused a reduction in VSI among androgen fed fish. In the early samples (day 35) the mean VSI of control fish was 7·98 $\pm$ 0·57, while the corresponding values for androgen treated fish were 6·79 $\pm$ 1·63 (T) and 6·28 $\pm$ 0·53 (11-KT). However, due to the large standard errors, the mean VSI values were not significantly lower ($P < 0·1$).

Later samples showed a significant depression of mean VSI following treatment with both androgens. The mean control VSI was 7·58 $\pm$ 0·63, while that of T treated fish was 6·66 $\pm$ 0·46 ($P < 0·01$). In 11-KT fed fish the mean VSI was 5·38 $\pm$ 0·48 ($P < 0·001$). Thus a significant

Table 4·3·2·1

Mean body weights of androgen treated (11-KT or T, 20 mg/kg diet) and control rainbow trout

Mean body weight (g) ± SE

Day	0	6	13	20	28	35	54	67	83
Control	77·8	85·5 ± 4·1	104·2 ± 4·9	109·8 ± 5·0	122·0 ± 4·3	134·26 ±5·3	145·9 ± 3·8	173·1	203·0 ± 8·8
n	15)	13)	12)	12)	12)	12)	6)	7)	7)
Testosterone	76·9	83·5 ± 2·4	99·9 ± 2·9	106·1 ± 3·4	114·2 ± 3·5	124·8 ± 3·5	139·3 ± 5·2	161·2	188·4 ± 7·8
n	15)	15)	15)	15)	15)	15)	10)	10)	10)
11-ketotest-osterone	76·5	83·2 ± 3·7	101·9 ± 4·9	110·1 ± 5·0	120·3 ± 5·8	134·0 ± 7·4	160·4 ± 13·0	184·2	222·4 ± 17·6
n	15)	15)	15)	15)	14)	14)	8)	8)	8)

Table 4•3•2•2

Mean dry matter digestibility values of androgen treated (11-KT or T, 20 mg/kg diet) and control rainbow trout

Mean (%) ± SE

Day	13	35
Control	69•25 ± 4•03	76•36 ± 4•88
n	2)	3) (*)
Testosterone	53•27 ± 6•19	51•96 ± 5•42
n	3) (*)	3)
11-ketotestosterone	71•32 ± 1•72	58•59 ± 4•51
n	2)	2)

t test (*) $P < 0.05$ between treated/control for that day.

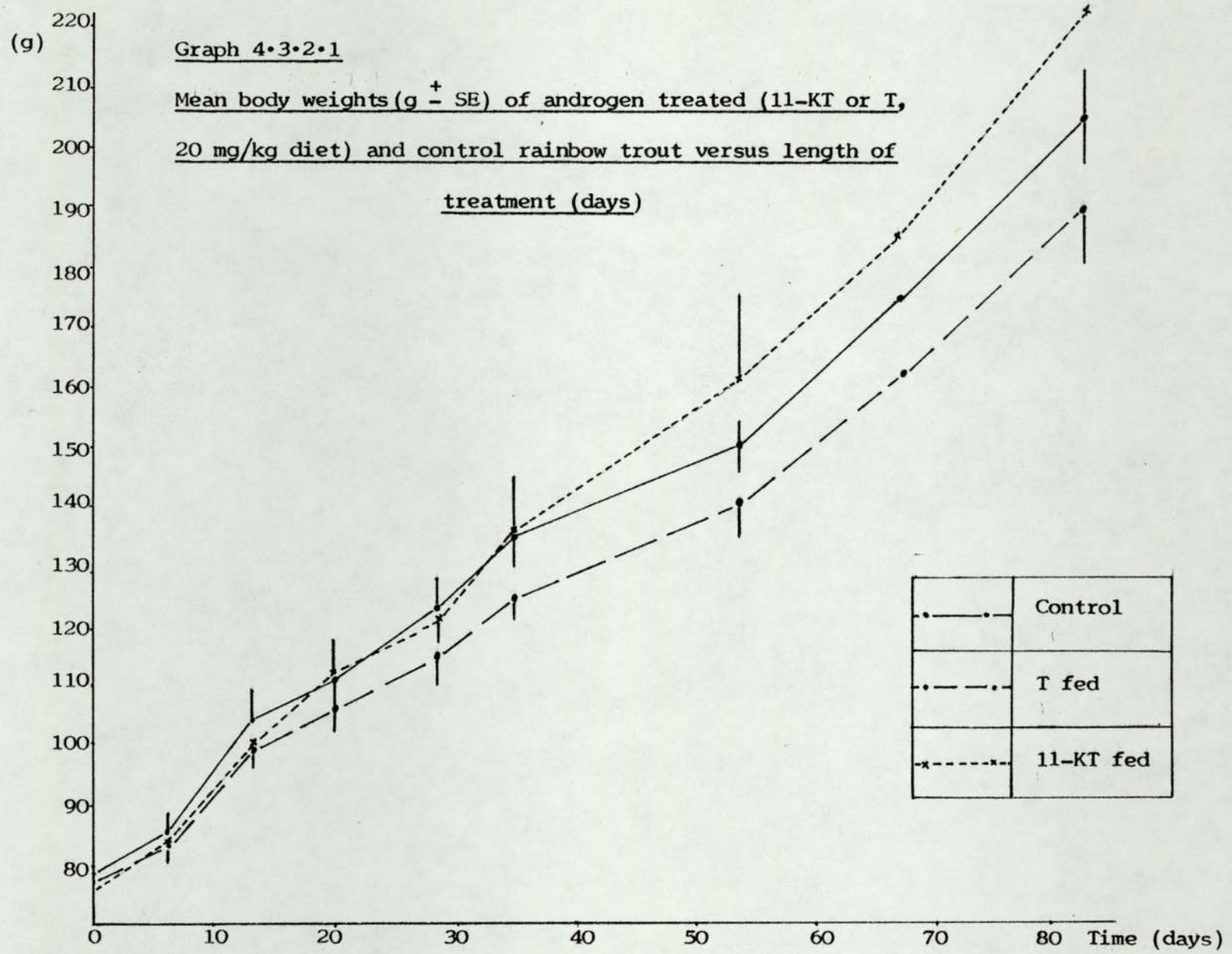
(n = number of pooled samples assayed - each sample represents the pooled faeces of sufficient fish to provide a reasonable sized sample.)

Table 4·3·2·3

Proximate analysis of androgen treated (11-KT or T, 20 mg/kg diet) and control rainbow trout after 35 and 83 days feeding

	Control	Testosterone	11-ketotestosterone
Day 35			
VSI	7·98 ± 0·57	6·79 ± 1·63	6·28 ± 0·53
n	5)	5)	5)
GSI male	0·09 ± 0·15	0·13 ± 0·03	0·08 ± 0·004
	4)	4)	2)
female	0·11 ± 0·0	0·58 ± 0·0	0·36 ± 0·37
	1)	1)	3)
% muscle water	77·54 ± 1·53	74·53 ± 2·36	76·93 ± 2·37
	5)	5)	5)
HSI	1·73 ± 0·16	1·88 ± 0·11	1·86 ± 0·03
	5)	5)	5)
% muscle fat	2·16 ± 0·58	3·29 ± 0·36	4·69 ± 0·32
	5)	5)	5) (**)
Day 83			
VSI	7·58 ± 0·63	6·66 ± 0·46	5·38 ± 0·48
	7)	8) (**)	8) (***)
GSI male	0·10 ± 0·12	1·06 ± 1·56	2·14 ± 1·48
	5)	4)	6) (*)
female	0·09 ± 0·001	0·13 ± 0·002	0·10 ± 0·01
	2)	5)	2)
% muscle water	73·19 ± 1·97	70·28 ± 8·8	75·24 ± 5·06
	7)	8)	8)
HSI	1·80 ± 0·12	1·71 ± 0·06	1·45 ± 0·10
	6)	7)	8)
% muscle fat	3·46 ± 1·00	4·65 ± 0·88	3·72 ± 0·38
	6)	8)	8)
% muscle protein	20·13 ± 0·39	18·08 ± 1·45	21·64 ± 2·19
	6)	8)	8)

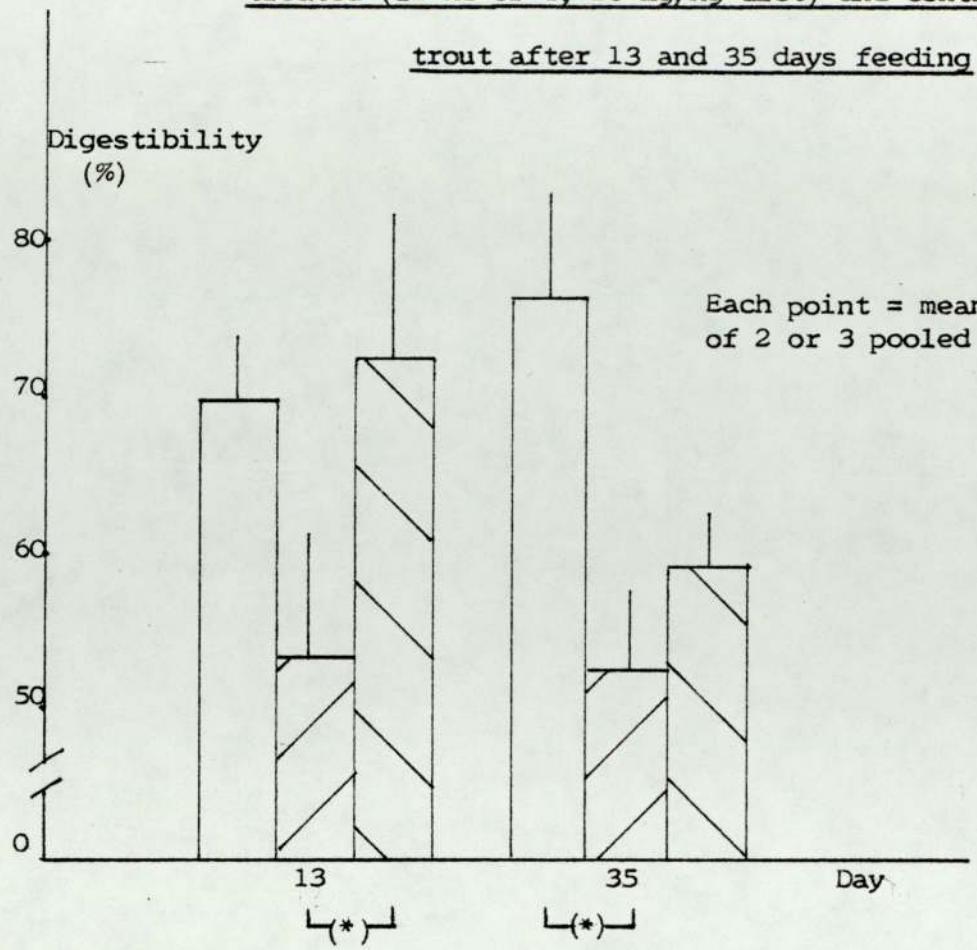
t test vs. controls (***) $P < 0·001$
(**) $P < 0·01$
(*) $P < 0·05$



Graph 4•3•2•1

Mean body weights (g ± SE) of androgen treated (11-KT or T, 20 mg/kg diet) and control rainbow trout versus length of treatment (days)

Fig. 4•3•2•1. Mean dry matter digestibility (% ± SE) of androgen treated (11-KT or T, 20 mg/kg diet) and control rainbow trout after 13 and 35 days feeding



t test comparing treated with controls and with each other
(*) $P < 0.05$

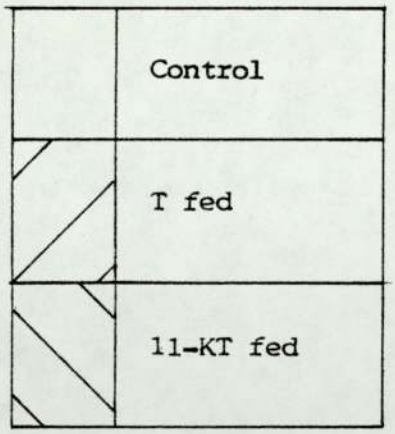
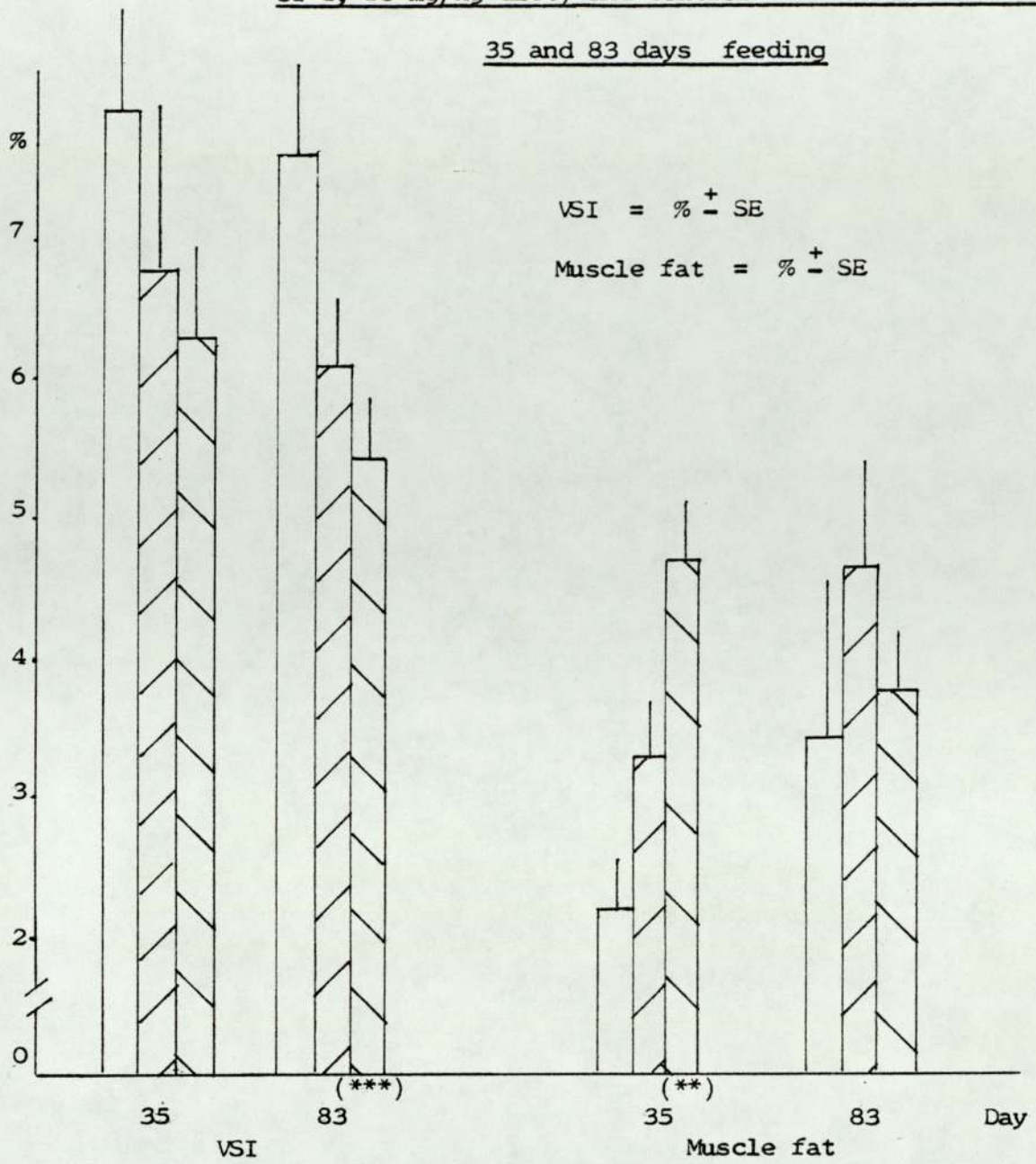
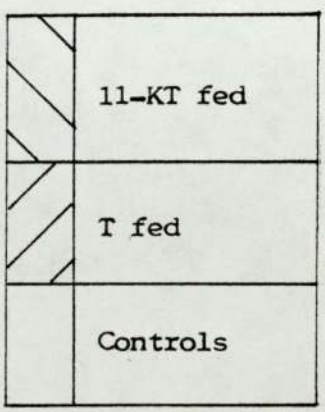


Fig. 4•3•2•2. Mean VSI and muscle fat of androgen treated (11-KT or T, 20 mg/kg diet) and control rainbow trout after 35 and 83 days feeding



t test vs. controls for that sample
(***) P < 0•001
(**) P < 0•01



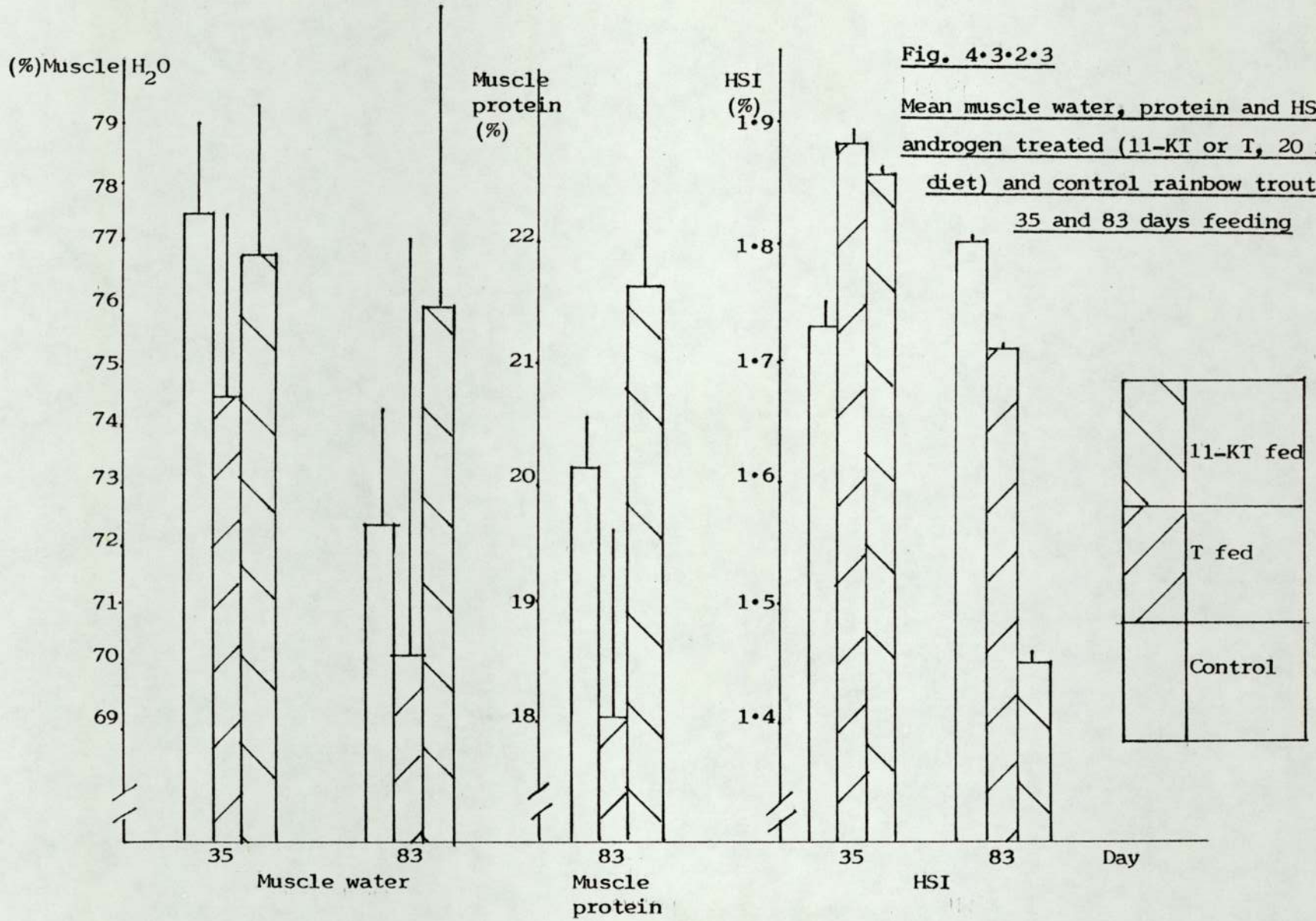
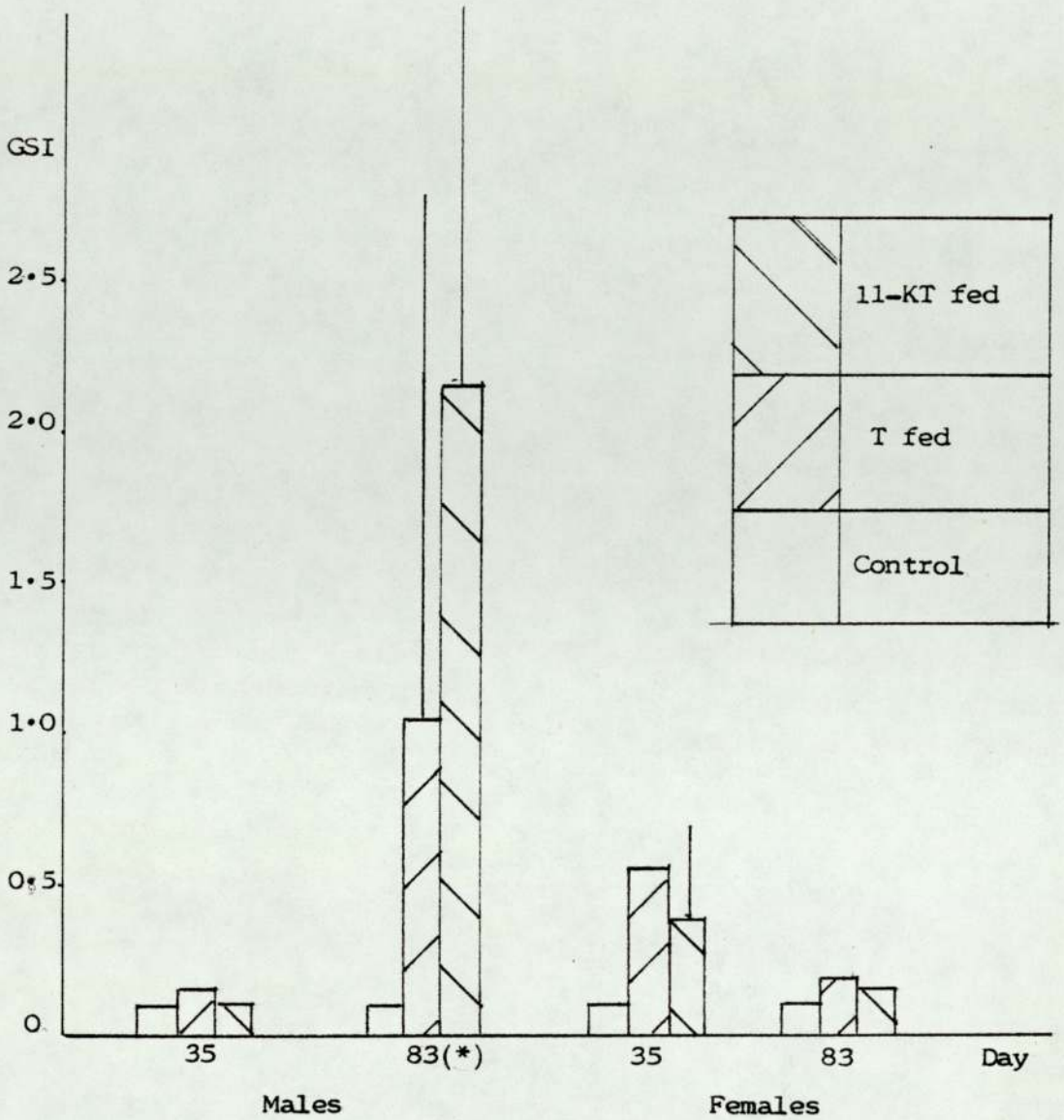


Fig. 4·3·2·3

Mean muscle water, protein and HSI of androgen treated (11-KT or T, 20 mg/kg diet) and control rainbow trout after 35 and 83 days feeding

Fig. 4•3•2•4

Mean GSI of androgen fed (11-KT or T, 20 mg/kg diet) and control rainbow trout after 35 and 83 days feeding



t test vs. control for that sample
(*) P 0•05

depression of mean VSI was observed following administration of androgens for 83 days, though not after the shorter period of 35 days.

Hepatosomatic index (HSI). No significant effects of androgen treatment upon HSI were observed, though on day 35 the mean HSI of T ($1{\cdot}88 \pm 0{\cdot}11$) and 11-KT treated ($1{\cdot}86 \pm 0{\cdot}03$) fish were both slightly higher than that of control fish ($1{\cdot}73 \pm 0{\cdot}16$). By day 83 the mean HSI values of both androgen treated groups, $1{\cdot}71 \pm 0{\cdot}06$ (T) and $1{\cdot}45 \pm 0{\cdot}1$ (11-KT), were both lower than that of control fish ($1{\cdot}80 \pm 0{\cdot}12$), though not significantly so.

% water. Androgen treatment did not result in significant differences in the mean muscle % water in treated fish, though T treated fish tended to have lower mean values than either control or 11-KT fed fish. Thus on day 35 the mean muscle % water was $77{\cdot}54 \pm 1{\cdot}53\%$ in control fish, and $74{\cdot}53 \pm 2{\cdot}36\%$ and $76{\cdot}93 \pm 2{\cdot}37\%$ in T treated and 11-KT treated fish respectively.

% fat and protein. A significant increase in muscle fat content was noted in 11-KT fed fish on day 35 of the experiment. At this time the mean muscle % fat was $2{\cdot}16 \pm 0{\cdot}58\%$ in controls and $4{\cdot}69 \pm 0{\cdot}32\%$ in 11-KT fed fish ($P < 0{\cdot}01$). Although muscle fat levels were elevated in T treated fish ($3{\cdot}29 \pm 0{\cdot}36\%$) this value did not differ significantly from that of control fish.

On day 83 of the experiment no significant differences were seen in either mean muscle fat or mean muscle protein levels. The mean muscle fat value for controls was $3{\cdot}46 \pm 1{\cdot}0\%$ and $4{\cdot}65 \pm 0{\cdot}88$ (T) and $3{\cdot}72 \pm 0{\cdot}38$ (11-KT) among treated fish. Thus though the mean fat content was slightly elevated by T treatment this difference was not significant.

The mean muscle protein content of control fish was slightly higher (20•13 $\pm$ 0•39%) than T treated (18•08 $\pm$ 1•45) fish, though lower than 11-KT treated fish (21•64 $\pm$ 2•19). Again these differences were not statistically significant.

Gonadosomatic index (GSI). Early samples (day 35) showed no difference between groups with regard to GSI. However, terminal samples (day 83) showed a statistically significant ($P < 0•05$) increase in mean male GSI among 11-KT fed fish (2•14 $\pm$ 1•48) compared with a mean GSI of control males of 0•10 $\pm$ 0•12. An increase in mean GSI was also seen in T treated fish (1•06 $\pm$ 1•56), though this did not differ statistically from that of controls. Thus 11-KT treatment at the dosage used (20 mg/kg) resulted in a significant increase in male GSI. Although an increase was also seen in T treated fish, this effect was far less marked. The stimulatory effect of 11-KT on gonadal development was apparent from the appearance of 'running' males in this group. By the end of the experimental period all males in this group had begun spermiation and could be easily stripped.

d) Secondary sexual characteristics

Androgen feeding resulted in the appearance of male secondary sexual characteristics among treated fish. The development of these was far more marked among fish fed 11-KT. By day 35 half the fish in this group showed the beginnings of kype development and skin darkening. The main effect seen in T treated fish was the appearance of skin darkening. However, kype development was far less noticeable in this group. The appearance of fish on day 35 of the experiment is shown in Plates 4•3•2•1 to 4•3•2•4. Within both groups the development of male secondary sexual characteristics became more advanced as the experiment progressed.

Table 4•3•2•4

Mean epidermal thickness of androgen treated (11-KT or T, 20 mg/kg diet) and control rainbow trout after 35 days feeding

Mean epidermal thickness (μm) ± SE

	Control	Testosterone	11-ketotestosterone
Mean epidermal thickness ± SE (um)	98•1 ± 6•7	119•9 ± 3•7 (*)	173•7 ± 5•6 (***)
n	4)	4)	4)
% increase relative to control		22	77

t test vs. control (***) $P < 0•001$
(*) $P < 0•05$

Table 4•3•2•5

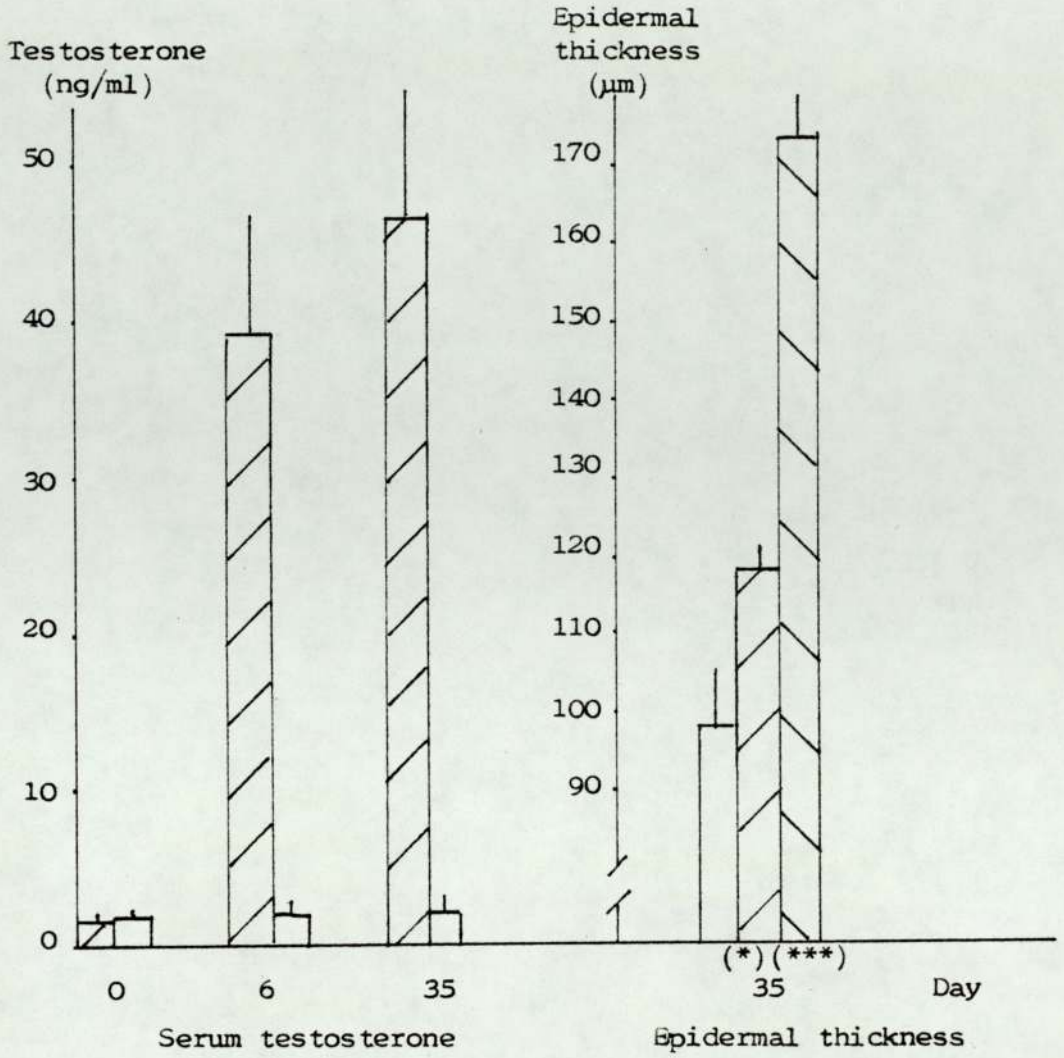
Mean serum testosterone levels of control and testosterone fed (20 mg/kg diet) rainbow trout

Testosterone (ng/ml) ± SE

Day	Testosterone fed	Control
0	1•20 ± 0•13	1•25 ± 0•25
n	5)	5)
6	39•21 ± 6•59	0•94 ± 0•30
n	5)	5)
35	46•05 ± 6•8	1•45 ± 0•19
n	5)	5)

Fig. 4·3·2·5

Mean serum testosterone levels (ng/ml ± SE) and epidermal thickness (µm ± SE) of androgen treated (11-KT or T, 20 mg/kg diet) and control rainbow trout



t test vs. controls
(***) P < 0·001
(*) P < 0·05

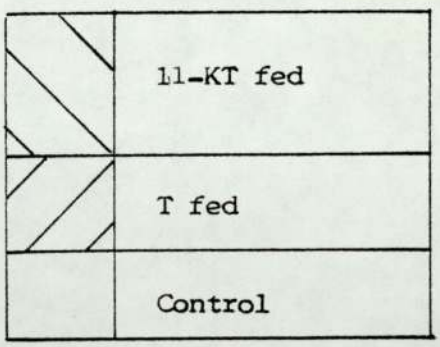


Plate 4•3•2•1 (overleaf)

Appearance of control (top), testosterone (middle) and 11-keto-testosterone (bottom) treated rainbow trout after 35 days feeding treated (20 mg/kg diet) or control diet

Plate 4•3•2•2 (overleaf)

Close up of control rainbow trout to show absence of secondary sexual features after 35 days feeding control diet

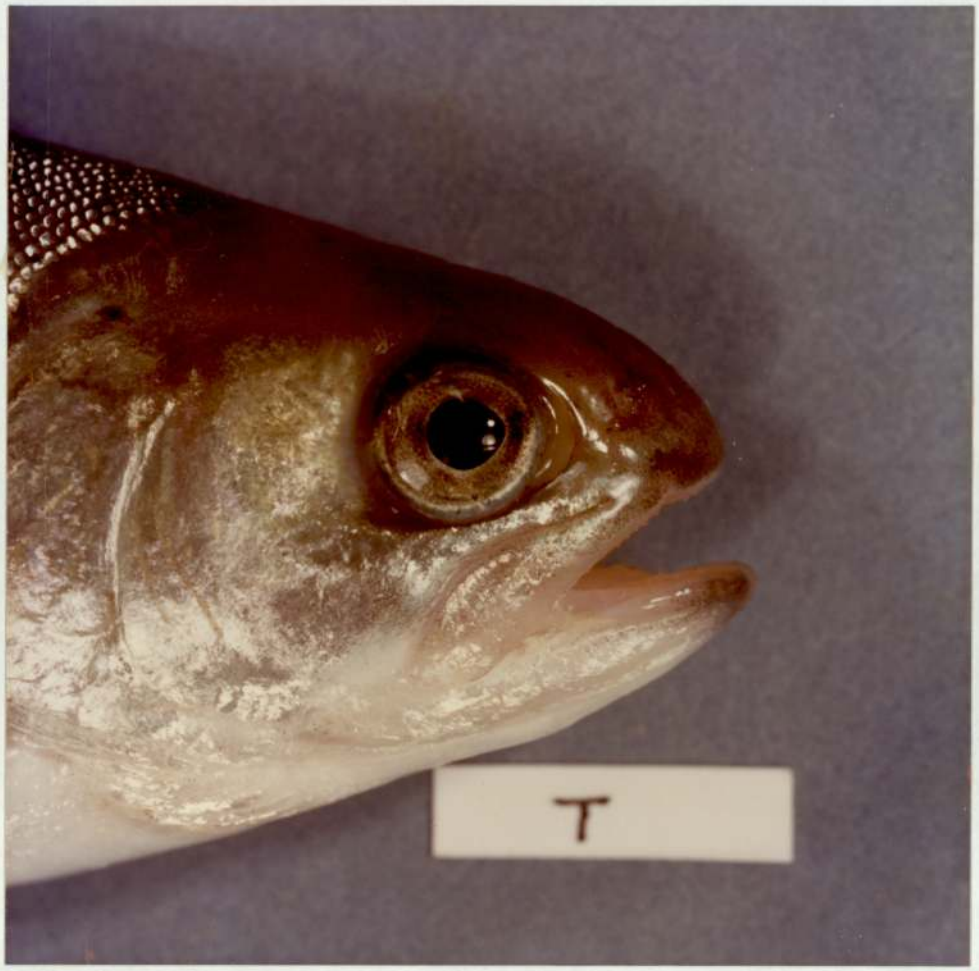

C

Plate 4•3•2•3 (overleaf)

Close up of testosterone treated rainbow trout (fed 20 mg T/kg diet) showing slight darkening after 35 days feeding treated diet

Plate 4•3•2•4 (overleaf)

Close up of 11-ketotestosterone treated rainbow trout (fed 20 mg 11-KT/kg diet) showing darkening and early kype development after 35 days feeding treated diet


T


11-KT

The effects of the two androgens upon epidermal thickness are shown in Table 4•3•2•4 and Fig. 4•3•2•5. Both androgens resulted in a significant increase in epidermal thickness. T treatment produced a 22% increase in thickness, from 98•1 $\pm$ 6•7 µm to 119•9 $\pm$ 3•7 µm. 11-KT feeding caused a 77% increase in epidermal thickness, from a control value of 98•1 $\pm$ 6•7 µm to 173•7 $\pm$ 5•6 µm. The effects of feeding T and 11-KT were both statistically significant at the $P < 0.05$ and $P < 0.001$ levels respectively, though 11-KT produced by far the greatest response for a given dose.

e) Hormone levels

Serum samples from control and T treated groups, taken on days 0, 6 and 35 were assayed for serum T. The results are shown in Table 4•3•2•5 and Fig. 4•3•2•5. T levels rose rapidly in hormone fed fish and by day 6 the mean T level of this group was 39•21 $\pm$ 6•59 ng/ml, compared with a mean control T value of 0•94 $\pm$ 0•03 ng/ml. By day 35 the mean level of serum T had risen slightly to 46•05 $\pm$ 6•8 ng/ml, while control levels remained low (1•45 $\pm$ 0•19 ng/ml).

Discussion

The two androgens, testosterone and 11-ketotestosterone, have been directly implicated in the sexual maturation of male salmonids and the cyclic changes of levels of these hormones have been reviewed earlier in this thesis.

Dietary incorporation of these steroids, at a dose of 20 mg/kg diet, produced a number of changes in proximate composition, gonadal development and secondary sexual characteristics in immature rainbow trout. The two androgens were found to differ in their potency in promoting these changes.

Thus 11-KT was more effective in bringing about the development of male secondary sexual characteristics in treated fish, and especially the development of a kype. 11-KT also had a more pronounced stimulatory effect upon the characteristic skin darkening and upon epidermal hyperplasia. It has been shown to be a potent androgen in teleost fish (Idler et al., 1961a, b; Arai, 1967; Fagerlund and Donaldson, 1969; Hishida and Kawamoto, 1970; McBride and van Overbeeke, 1971; Campbell et al., 1976) and the present study supports this observation. It is probable that 11-KT is the predominant androgen involved in the development of male secondary sexual characteristics, especially skin darkening, epidermal thickening and kype development.

Although T was able to induce some degree of skin darkening, it was less potent in promoting the appearance of male secondary sexual characteristics, especially kype development. Thus, while T treatment produced a 22% increase in epidermal thickness over the first 35 days of the experiment, 11-KT treatment produced a 77% increase over control values during the same period.

In contrast to the differences between T and 11-KT with regard to their effects on secondary sexual characteristics, T treatment was far more potent in causing a reduction in dry matter digestibility; an event which earlier experiments have shown to accompany male maturation, and which seems to be related to the growth depression observed among mature males. Although the growth of T treated fish was not significantly lower than that of control fish in the present experiment, at all times this group had a lower mean body weight than control fish. 11-KT treatment produced a slight, but not statistically significant ($P < 0.1$), depression in digestibility. As 11-KT was effective in

producing a depression of VSI, an effect also observed with T treatment, it would seem that the effect on digestibility is distinct from that exerted upon the viscera by both androgens.

The observation that the effects of T treatment on growth and digestibility can be, to a certain extent, divorced from each other suggests that this relationship is perhaps not as simple as has been postulated in previous experiments. However, it is possible that this variation may be in part dose related and that the two effects show a different steroid response threshold.

Thus it would seem that male secondary characteristics develop faster under the influence of 11-KT than T and that in the normally maturing male the appearance of these characteristics is more directly related to the serum concentration of 11-KT than of T. In addition, in the present experiment, 11-KT was shown to have a significant effect upon testicular development. Males receiving 11-KT treated diet had a significantly higher GSI than control males. This effect was especially noticeable by the end of the experiment and though no detailed histological examination was carried out, spermatogenesis was far more advanced in 11-KT treated fish and the majority of treated males were 'running' by the end of the experimental period.

Testosterone treatment had a stimulatory effect upon testicular development in some, though not all, treated males and in some fish, like the 11-KT treated males, spermatogenesis had begun. In control fish the testes showed an immature appearance, with little evidence of spermatogenesis. Similar observations were also made by Schreck and Fowler (1982), who noted that the % of mature male chinook salmon fed 10 and 15 ppm T were 30•3% and 83•3%, compared with an absence of male maturation in control fish.

Both the male secondary sexual characteristics and testicular development appear to be more sensitive to 11-KT administration. On the other hand changes in digestibility and perhaps growth may be more sensitive to T treatment. It may be that, within maturing males, a division exists between effects of the two androgens, with 11-KT influencing gonadal development and the appearance of secondary sexual characteristics and T causing a depression in digestibility and growth.

4·4 General Discussion and Conclusions

In addition to its role in the production of gametes, sexual maturation in salmonids results in profound changes in both physiology and morphology. These changes are especially pronounced in male salmonids. From the viewpoint of the commercial producer the most significant of these are the depressed growth (Nomura, 1963; Bye and Lincoln, 1979) and the appearance of unappealing secondary characteristics (Robertson and Wexler, 1960a;McBride *et al.*, 1963; Stoklosowa, 1970; Yamazaki, 1976) allied to increased succeptibility to disease and early mortality (Robertson *et al.*, 1961; McBride *et al.*, 1965; Roberts and Shepherd, 1974).

The reasons for this poor growth performance are likely to be complex, though a number of factors have been implicated including:-

i) Reduced food intake.

ii) Reduced food conversion efficiency.

iii) Diversion of energy and reserves towards gonadal growth.

iv) Catabolic effects of androgens.

v) Increased aggressive behaviour of mature males.

Many of these changes would appear to be directly related to serum

androgen levels; a connection which has been explored in the present series of experiments. The effects observed reflect the balance between the androgenic and anabolic effects of androgens. Such a balance is largely dose related. Thus Yamazaki (1976) was able to produce either a depression or a stimulation of growth, depending on the dose administered.

The anabolic effects of serum androgens may explain the good Spring growth observed among male salmon by Hunt et al. (1981). In the present series of experiments a depression of growth of male fish occurred, coincident with the time of high androgen levels, and at higher dose levels (30 mg/kg) the administration of exogenous androgens (T and MT) produced a similar depression in growth. Serum levels of T as measured by RIA were similar in normal mature males and in fish fed 30mg T/kg diet. This growth depression was not observed among fish fed androgens at doses of 20 mg/kg.

Appetite as estimated by ad libitum food intake was shown to fall in the mature male trout examined. This depression was apparent from both direct observation of ad libitum food intake and was also suggested indirectly by the reduction in the food index of mature males. This depression in food intake occurs as androgen levels are high. Yamazaki (1976) noted that high doses of exogenous androgens may cause a reduction in appetite. Conversely low doses of androgens have been shown to be capable of stimulating appetite in salmonids (Yamazaki, 1976; Fagerlund et al., 1978, 1979a, b, 1980).

Reduced food conversion was also noted in mature male rainbow trout and this would seem to be an obvious factor in the reduced growth rate of these fish. The present series of experiments suggests that included in the maturation induced changes are effects on total digestibility of

diet, which may represent an important component of this reduced food conversion.

These changes in digestibility were noted among male fish and also in fish administered exogenous androgens. Thus both T and MT at a dose of 30 mg/kg produced depressions in dry matter digestibility, which were unlikely to be size related (Windell et al., 1978b). 11-KT was less effective in promoting such an effect, though it did produce a fall in digestibility after more prolonged treatment.

The effect of androgens upon digestibility may represent a general inhibitory effect of androgens upon the alimentary canal. In support of this hypothesis all the androgens used in the experiments described produced significant depressions in VSI. A depressive effect of steroids upon VSI and visceral structure in salmonids has been recorded (Robertson and Wexler, 1960a;McBride and van Overbeeke, 1971; Simpson, 1976) and a reduction in visceral energy stores observed by Jensen (1980). However, 11-KT was equally effective in producing a depression of VSI, though it had less effect on digestibility.

Support for a more specific site of action of androgens are the observations that the mucosal peptidase activity of the caecal region of the intestine (thought to be the primary region responsible for protein digestion (Ash, 1980)), is reduced in mature males. A depression of activity was also observed in some of the androgen treated fish. In particular among T fed fish peptidase activity fell by approximately 42% compared to control levels. (Among mature males peptidase activity was depressed by 62% of control values). A relationship between androgen levels and intestinal enzyme activity has been shown in carp (Lone and Matty, 1981a) though in this case increased intestinal enzyme activity followed treatment with low doses of androgens.

However, as the administration of 20 mg T/kg diet gave a significant depression of digestibility, but only a slight reduction of growth rate, it appears that a reduction of growth does not always follow from depressed digestibility.

The overall effects of sexual maturation and androgen treatment are summarized in Table 4·4·1. Proximate analysis of mature male fish revealed a slight, but non-significant reduction in muscle protein and slight increases in muscle water, perhaps reflecting increased catabolic activity. The changes in carcass composition accompanying maturation were less than those reported by Davidson and Shostrom (1936), who recorded increases in muscle water and depressed muscle protein and fat levels in pink salmon. However, this is a migratory species with a pronounced migration linked fast. A highly significant depression in VSI was observed in all maturing fish, reflecting either a depressive effect of androgens on the alimentary canal and/or a utilization of visceral energy stores (Fagerlund et al., 1978; Matty and Cheema, 1978; Jensen, 1980).

Among precociously mature male fish no significant catabolic effects were observed and muscle water levels were slightly lower and fat levels slightly higher than in immature control fish. This difference may represent an age related variation in the maturation process, possibly related to low androgen levels of precociously mature salmonids (Dodd et al., 1978; Stuart-Kregor et al., 1981), though a depression of mean VSI was common to mature fish of both age groups. The reduced growth accompanying maturation in male salmon parr reported by Murphy (1980a) does, however, suggest that a significant increase in catabolic activities is part of this process.

Table 4•4•1

Effects of hormonal treatment, or natural maturation upon various maturation related parameters

Parameter	MT (30 mg/kg)	T (20 and 30 mg/kg)	11-KT (20 mg/kg)	Normal mature males (2 year)	Precociously mature
Skin thickening and darkening	***	*	***	***	**
Kype development	***	*	***	***	*
Growth depression	***	**(30 mg /kg * (20 mg /kg)	*	***	?
Depression of digestibility	***	***	*	***	?
GSI Male	↓**	↑* (20mg /kg) ↓* (30mg /kg)	↑**	***	***
VSI	↓***	↓***	↓***	↓***	↓***
Muscle H_2O	-	-	-	*	*
Muscle protein	↓**	↑** (30 mg/ kg)	↑*	↓*	-
Muscle fat	** (↑ early)	** (↑ early, 30mg/kg)	* (↑ early)	-	↑**

Assessment of extent of effect:
*** maximum
** intermediate
* slight
- no effect

Effect
↓ decrease
↑ increase

Of the androgens tested, 11-KT was shown to be the most potent in bringing about the development of male secondary sexual characteristics. It has already been shown to be a potent androgen in salmonids (Idler et al., 1961a, b; Arai, 1967; Hishida and Kawamoto, 1970). However, its effects on dry matter digestibility appear to be less than those of T; kype development, skin darkening and epidermal thickening were all observed following administration of 11-KT and T, though to a lesser extent following T treatment. It is perhaps paradoxical that, while 11-KT and MT were capable of bringing about male secondary sexual characteristics among both male and female rainbow trout, these features normally only occur in male fish. This is despite the fact that very high testosterone levels occur in mature female salmonids (Campbell et al., 1980; Scott et al., 1980b). Presumably this reflects the lack of 11-KT in female salmonids (Wright, 1976; Sangalang and Freeman, 1977; Scott et al., 1980b; Stuart-Kregor et al., 1981). The appearance of male characteristics among female trout treated with MT and 11-KT indicates that receptors for androgens are present.

Testosterone was less effective in promoting male secondary sexual characteristics, though skin darkening and very early kype development occurred. It did, however, have a greater effect upon the androgen induced depression of digestibility than did 11-KT. As these two steroids are the naturally occuring androgens in male salmonids, it may be that the growth depression associated with maturation is more directly related to T levels than to those of 11-KT.

The induction of male characteristics by anabolic doses of exogenous androgens has been a frequent problem in attempts to promote the increased growth of salmonids (Fagerlund and Donaldson, 1969; Fagerlund and McBride, 1975; Fagerlund et al., 1980).

The effects of the exogenous androgens administered varied in the two experiments reported. MT and to a lesser extent T were effective in suppressing gonadal development and spermatogenesis at a dose of 30 mg/kg. In the second experiment 11-KT and, again to a lesser extent T, were found to be capable of stimulating spermatogenesis and testicular growth at 20 mg/kg diet.

A depressive effect on gonad development with increasing MT doses has been noted (McBride and Fagerlund, 1973; Fagerlund et al., 1980) in Pacific salmon (Oncorhynchus kisutch), though the doses used were lower than those administered in the present experiments. Doses as low as 1·0 ppm MT were reported to be effective in depleting testicular germ cell numbers (Fagerlund et al., 1980). Interestingly this effect was more pronounced in fish treated in May than in February. Juvenile coho salmon also showed a similar depletion of testicular germ cells when treated with 2·5 ppm MT (Yu et al., 1979). In this latter study one of the experimental fish was found to show the opposite effect and had enlarged testes. Billard et al. (1981) were able to completely inhibit spermatogenesis in rainbow trout fed 0·5 mg/kg MT, and partially so in fish fed 0·5 mg/kg T, during the normal period of spermatogenesis. No depression of gonadotropin levels was observed, though the normal September increase was abolished, leading the authors to conclude that this inhibition is a primary effect on the testis, rather than an indirect one mediated via pituitary gonadotropins. (The claimed sterility of these fish must be treated with caution pending a long-term evaluation.)

Stimulatory effects of androgens on maturation and spermatogenesis have also been seen (McBride and Fagerlund, 1976; Schreck and Fowler, 1982). In the latter report an increased percentage of mature male chinook salmon followed treatment with T and MT at doses of 10 and

15 mg/kg, though again MT was the most potent. In the series of experiments reported here 11-KT was more potent than T in stimulating gonadal growth and spermatogenesis and of these two naturally occurring androgens 11-KT seems likely to have a greater role in stimulating these processes during the normal sexual development of male salmonids.

Owing to time limitations few samples were assayed for 11-KT. In view of the suggested importance of this androgen in the sexual development of salmonids, this is unfortunate. However, in the majority of cases, when it is necessary to follow the progress of sexual maturation, the monitoring of T levels is an acceptable alternative.

CHAPTER 5

ENVIRONMENTAL FACTORS INFLUENCING REPRODUCTION IN SALMONIDS

Contents

5•1 Introduction

Previous chapters have described the mechanisms responsible for the control of sexual maturation in male salmonids and in particular the vital role played by the endocrine system in regulating maturation. Integration and mediation of environmental factors via the nervous and endocrine system allow the synchronization of reproduction and enable it to take place at the most favourable time; an obvious adaptive feature.

A number of environmental 'cues' have been suggested to be important in such co-ordination among teleosts. These include light, temperature, nutrition, salinity, pheromones and social factors. The factors thought to be involved in reproduction have been reviewed (de Vlaming, 1972, 1974; Billard et al., 1978; Peter and Crim, 1979; Crim, 1982). The roles played by these factors and an assessment of each factor's importance in the maturation of male salmonids will be examined in this chapter.

5•2 Photoperiod

It is generally acknowledged that photoperiod is the major environmental variable responsible for synchronizing reproduction in salmonids. Thus photoperiodic variation has been shown to be capable of altering the timing of spawning in a variety of salmonid species (Hoover and Hubbard, 1937; Alison, 1951; Hazard and Eddy, 1951; Nomura, 1962; Henderson, 1963; Carlson and Hale, 1973; Kunesh et al., 1974).

However, other environmental variables were often not strictly controlled in these early studies. Using more rigorous methods, recent work has confirmed the role of photoperiod as a 'zeitgeber' or

synchronizer of reproductive development (MacQuarrie et al., 1978, 1979), and has also provided detailed information regarding the relationship between the endocrine dynamics of the reproductive process and photoperiod (Whitehead et al., 1978a, b, c, 1979, 1983).

With few exceptions (Whitehead et al., 1979; Lundqvist, 1980) studies have concentrated on the effects of photoperiod in regulating the reproductive process within female salmonids.

The effect of constant long, short and extended photoperiod cycles upon the sexual maturation of male rainbow trout

In view of the importance of photoperiod as an environmental cue influencing reproduction and the relative lack of data from male salmonids an experiment was carried out at Shearwater Fish Farming's Low Plains site. This experiment was designed to confirm the role of photoperiod in reproductive maturation in male salmonids and to provide information on the endocrine dynamics of the process. In addition, it was hoped to provide a rationale for attempts to modify or prevent male maturation among production salmonid stocks. The experiment formed part of a long-term study collecting data on the response of rainbow trout to changing photoperiod and possible mechanisms for such effects and was carried out in conjunction with Dr. Colin Whitehead.

Materials and Methods

Three year old rainbow trout from a stock reared at Low Plains were divided into four groups and housed in four 800 litre tanks within a light proof cabinet protected by black polythene (Diagram 5•2•1). Light was supplied by a 40 W. fluorescent tube controlled by an external time clock. Access to the tank was via a plywood window

Key to Diagram 5•2•1

a. Water inflow pipe

b. 400 L. fibreglass tank

c. Standpipe

d. Fluorescent tube (40 W.)

e. Inspection panel

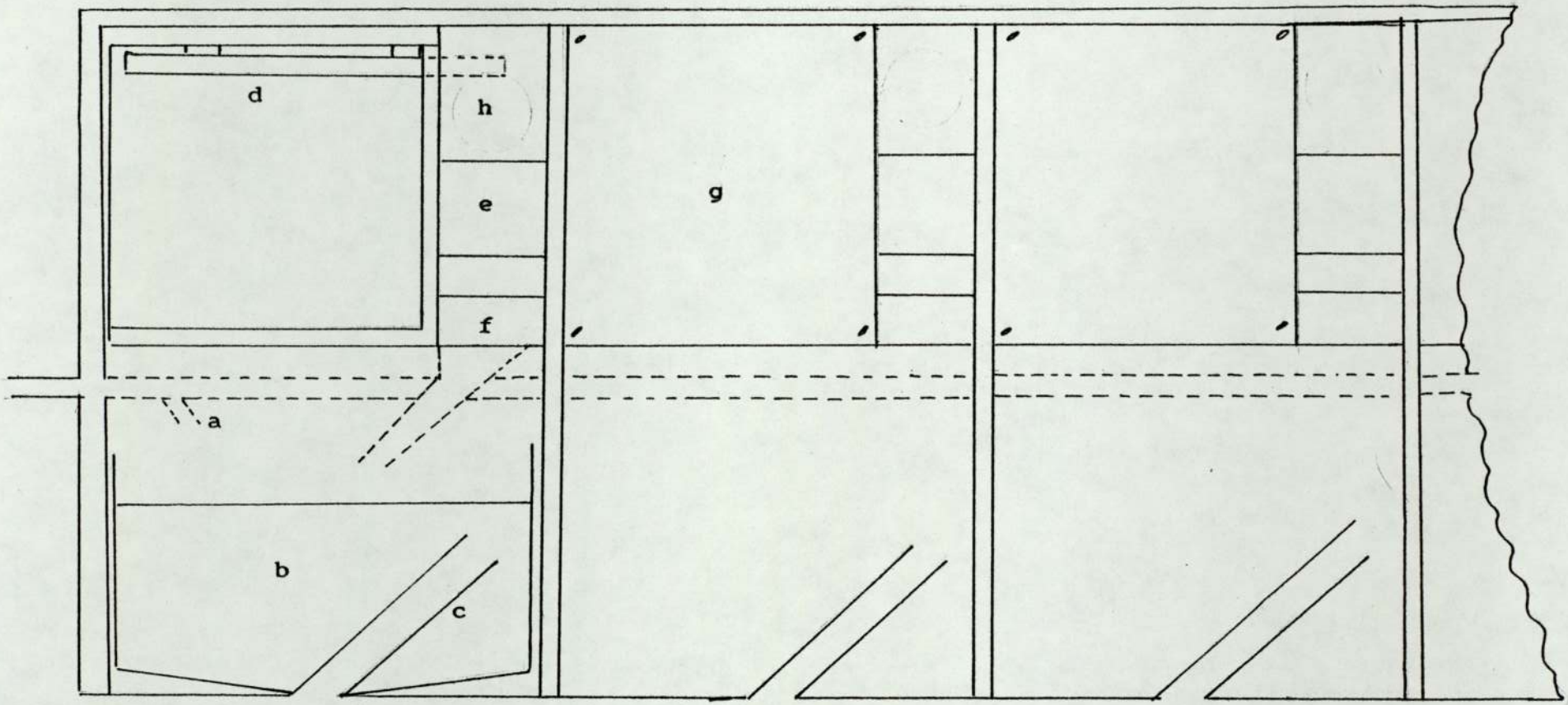
f. Feeding panel and chute

g. Cover allowing access

h. External time clock

Diagram 5•2•1

Photoperiod System showing details of one unit of the type used at Shearwater Fish Farming Ltd.



in the front of the case and a smaller window gave access to a tube from which the fish were fed. Water supply was by a single inlet pipe to each tank at the rate of 400 L./hour.

Water temperature was a constant 9°C and oxygenation maintained 100% saturation in effluent water. Fish were fed at a constant 0·5% body weight/day. The pH was a constant 6·6.

Fish were examined at monthly intervals for spermiation and secondary sex characteristics following benzocaine anaesthesia and blood samples taken for hormone assay.

Each tank was subjected to a different photoperiod regime regulated by the external time clock which was adjusted weekly.

a) Normal seasonal photoperiod.

b) 'Long day' photoperiod (16 hours light : 8 hours dark).

c) 'Short day' photoperiod (8 hours light : 16 hours dark).

d) Extended photoperiod (Normal cycle of photoperiodic change extended to 18 months - see Diagram 5·2·2).

The experiment was begun in February and carried on until June of the following year.

Results

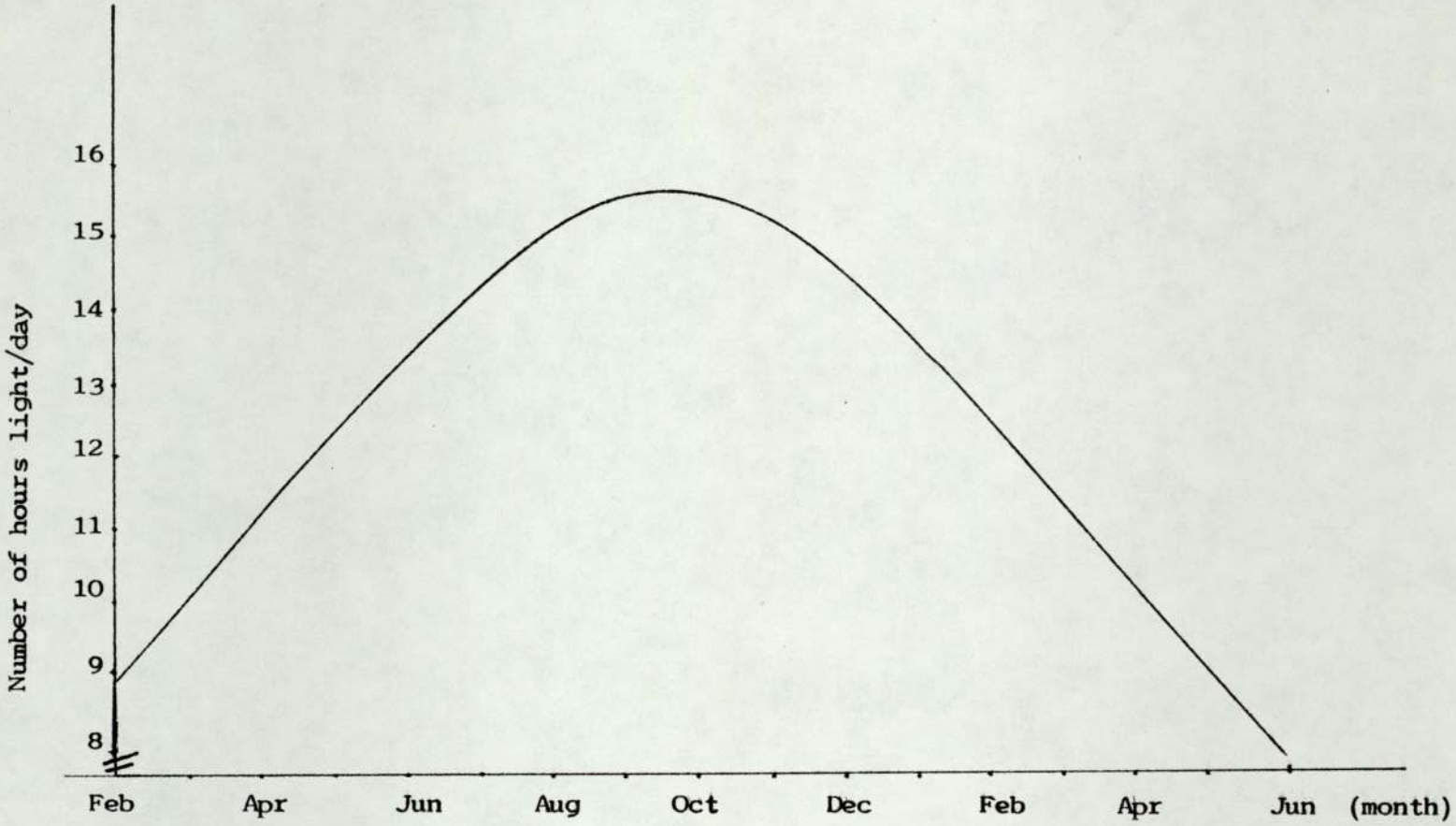
a) Timing of Spermiation

Under the normal seasonal photoperiod, spermiation was observed by December 1979 and all male fish were 'running' under slight hand pressure by January 1980. (Fig. 5·2·1).

Spermiation was advanced in males under the long day photoperiod. The majority of males in this group showed spermiation in October, two

Diagram 5•2•2

Photoperiod cycle of fish exposed to an artificially extended photoperiod regime consisting of the normal cycle extended into 18 months



months earlier than the control males (Fig. 5•2•2).

Within the extended and short day photoperiod groups a delay in male maturation occurred. Thus in the short day group spermiation was first observed among males in February 1980, but it was not until April/May that all the males were running; a delay of four months compared to controls (Fig. 5•2•3).

Under extended photoperiod conditions male fish began to run in February 1980, though spermiation was not demonstrated in all males of this group until March; representing a delay of two months compared to males in the control group (Fig. 5•2•4).

These results are summarized in Table 5•2•1.

b) Hormone levels

The serum testosterone levels of males under the various photoperiod regimes are shown in Table 5•2•2.

Male control photoperiod fish showed minimum levels of testosterone(T) in May 1979 (2•14 $\pm$ 0•66 ng/ml). Levels increased from July/August and reached a peak of 72•78 $\pm$ 15•93 ng/ml in January 1980 at the time of spermiation. Following this peak, levels fell rapidly to 5•79 $\pm$ 0•76 ng/ml in March (Graph 5•2•1).

Males under long day photoperiod showed minimum levels of T in June 1979 (2•69 $\pm$ 0•37 ng/ml) and levels rose rapidly from July to reach a peak in October (63•53 $\pm$ 16•67 ng/ml), again coincident with spermiation (Graph 5•2•2). Following a sharp fall in November, levels remained at 25 - 30 ng/ml until June 1980 when another fall was observed to 12•38 $\pm$ 21•03 ng/ml.

Within the short and extended photoperiod regimes minimum levels of T were observed in June 1979 (1·72 ± 0·35 and 1·94 ± 0·43 ng/ml respectively). Peak levels were observed in February 1980 within both groups (54·08 ± 10·14 and 72·52 ± 11·95 ng/ml); in the extended photoperiod group coincident with spermiation and slightly in advance of spermiation in the short day group. In the short day group a steady fall followed this peak and by May 1980 levels had fallen to 18·01 ± 6·59 ng/ml (Graph 5·2·3). A fall in T levels was also observed in fish on an extended photoperiod, though there was a considerable variation within this group, especially in March to April. By June 1980 T levels had fallen to 22·88 ± 8·99 ng/ml (Graph 5·2·4).

Table 5·2·1

Timing of Spermiation under varying photoperiods

	Control	Extended	Short	Long
Spermiation first observed	Dec. 79	Jan. 80	Feb. 80	Oct. 79
All males running	Jan. 80	Mar. 80	May 80	Nov. 79

Table 5·2·2

Mean Serum Testosterone Levels (ng/ml ± SE) of rainbow trout maintained under various photoperiodic regimes

Group	1979 Mar.	Apr.	May	Jun.	Jul.	Aug.	Oct.	Nov.	Dec.	1980 Jan.	Feb.	Mar.	May	Jun.
Control	24·16 ± 9·43 4	7·79 ± 1·58 5	2·14 ± 0·66 5	3·18 ± 0·70 4	6·80 ± 1·52 4	13·75 ± 4·65 4	30·34 ± 7·79 4	40·25 ± 7·79 4	54·19 ± 17·06 4	72·78 ± 15·93 4	30·61 ± 6·57 4	5·79 ± 0·76 4	6·10 ± 1·89 4	2·66 ± 1·75 4
Short	9·58 ± 5·38 8	5·26 ± 1·83 8	2·43 ± 0·35 7	1·72 ± 0·35 7	3·40 ± 0·52 7(*)	4·40 ± 0·79 7	5·54 ± 1·33 6(**)	14·48 ± 5·66 7(*)	23·26 ± 9·18 7	38·81 ± 7·34 4	54·08 ± 10·14 7	31·89 ± 6·71 6(****)	18·01 ± 6·59 6	20·44 ± 10·17 5
Long	22·17 ± 4·87 8	3·89 ± 1·14 9	3·41 ± 0·70 9	2·69 ± 0·37 8	11·95 ± 2·71 7	32·86 ± 6·51 7(*)	63·53 ± 16·67 6	31·05 ± 8·49 7	25·41 ± 4·89 7	25·27 ± 4·19 7(***)	36·70 ± 6·47 7	24·42 ± 6·31 6(***)	29·44 ± 2·78 7(****)	12·38 ± 21·03 5
Extended	6·67 ± 2·17 6	7·21 ± 1·39 6	2·43 ± 0·94 6	1·94 ± 0·43 5	3·36 ± 0·38 5	8·83 ± 1·65 5	11·55 ± 1·78 5(*)	19·58 ± 5·63 5	27·13 ± 5·24 5	42·42 ± 7·71 4	72·52 ± 11·95 5(***)	57·13 ± 16·12 5(***)	55·87 ± 20·81 5(*)	22·88 ± 8·99 5

P values, t test, mean value vs mean control value for each month. P less than 0·05 (*), 0·02 (**), 0·01 (***), 0·001 (****)

Fig. 5·2·1

Photoperiod Experiment (Shearwater Fish Farming)

Number and % of male fish spermiating (running) vs time

Control Photoperiod

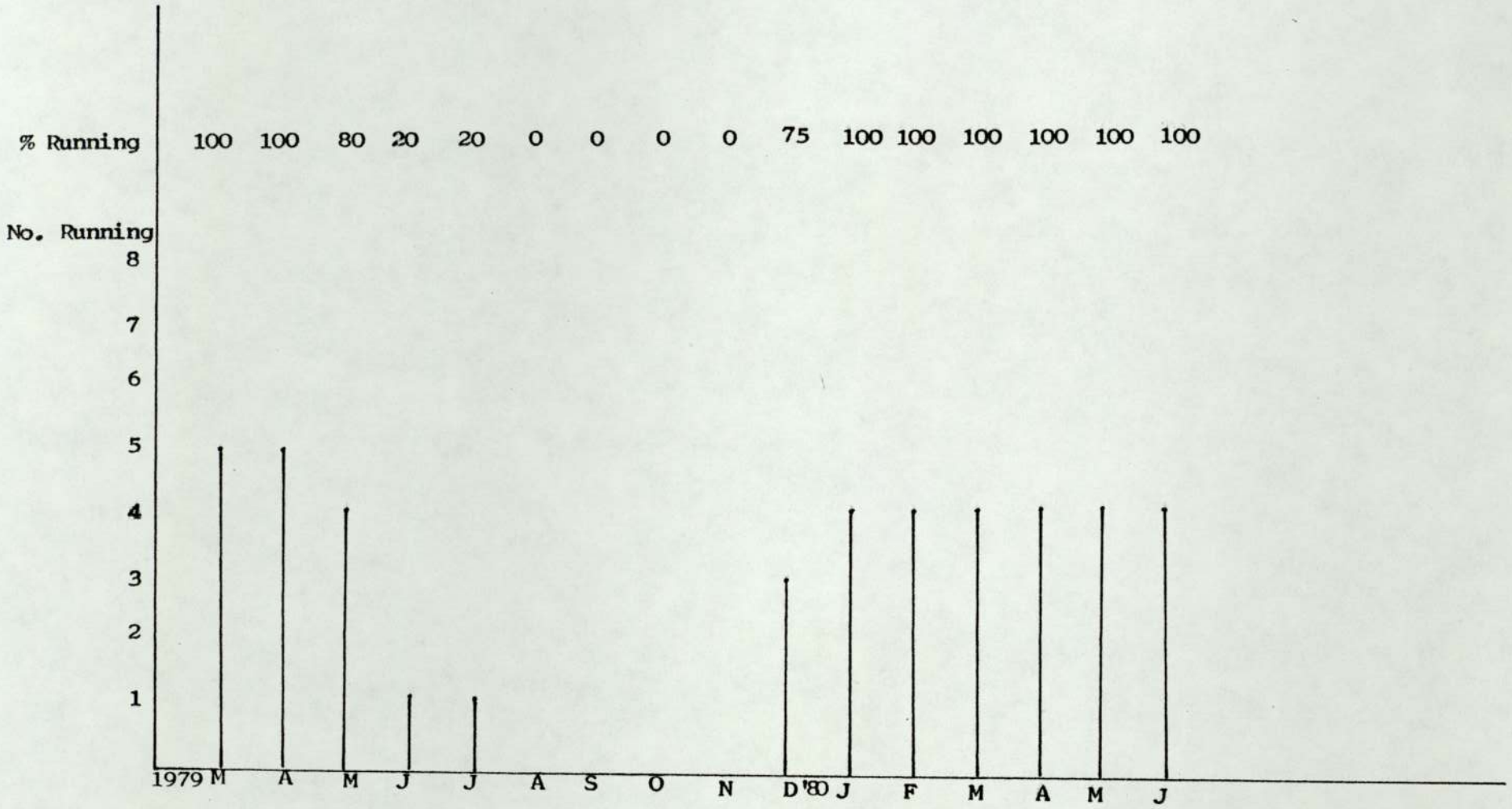


Fig. 5·2·2

Photoperiod Experiment(Shearwater Fish Farming)

Number and % of male fish spermiating (running) vs time

Long Day photoperiod regime (16 hours L : 8 hours D)

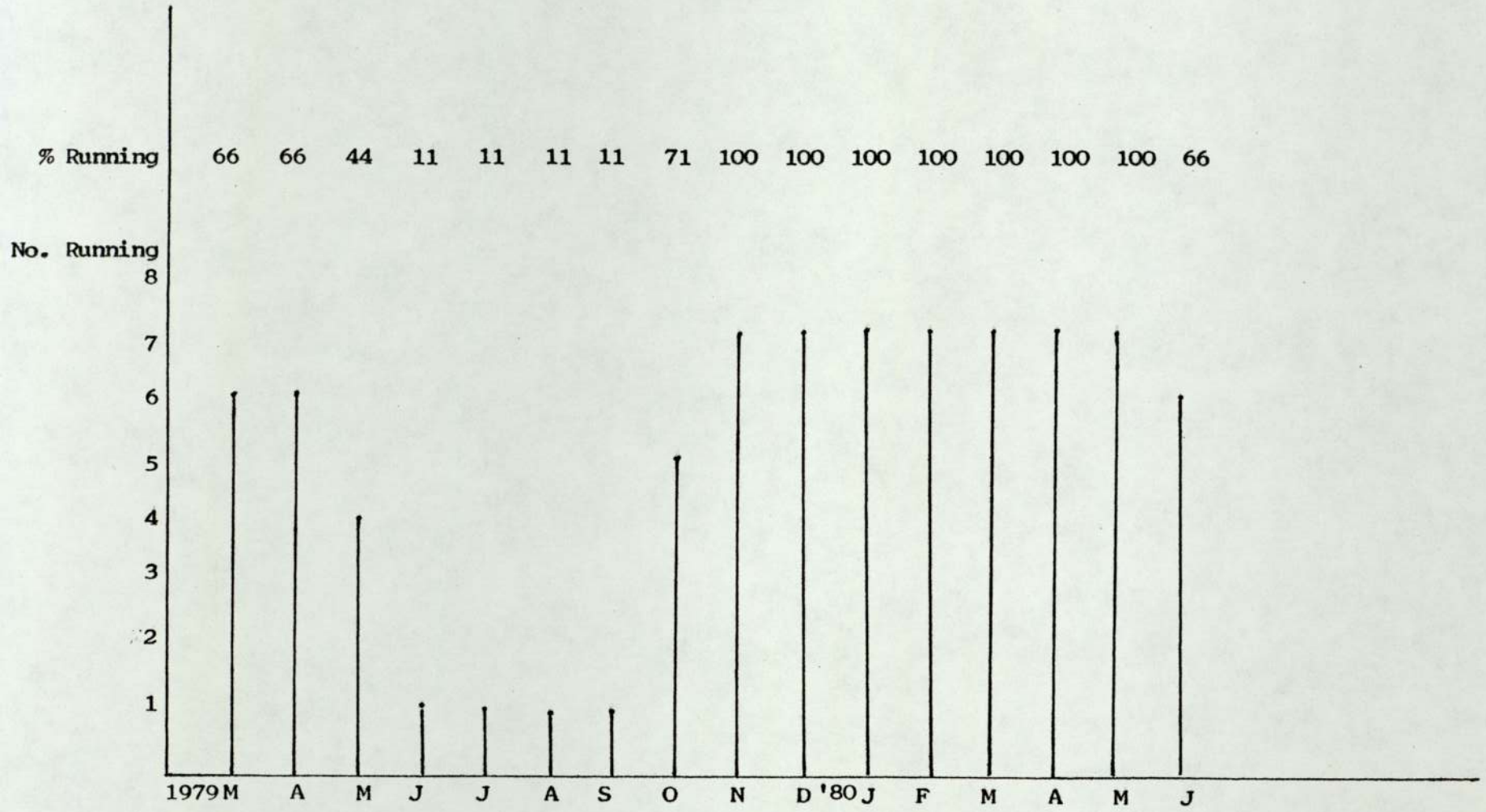


Fig. 5•2•3

Photoperiod Experiment (Shearwater Fish Farming)

Number and % of male fish spermiating (running) vs time

Short Day photoperiod regime (8 hours L : 16 hours D)

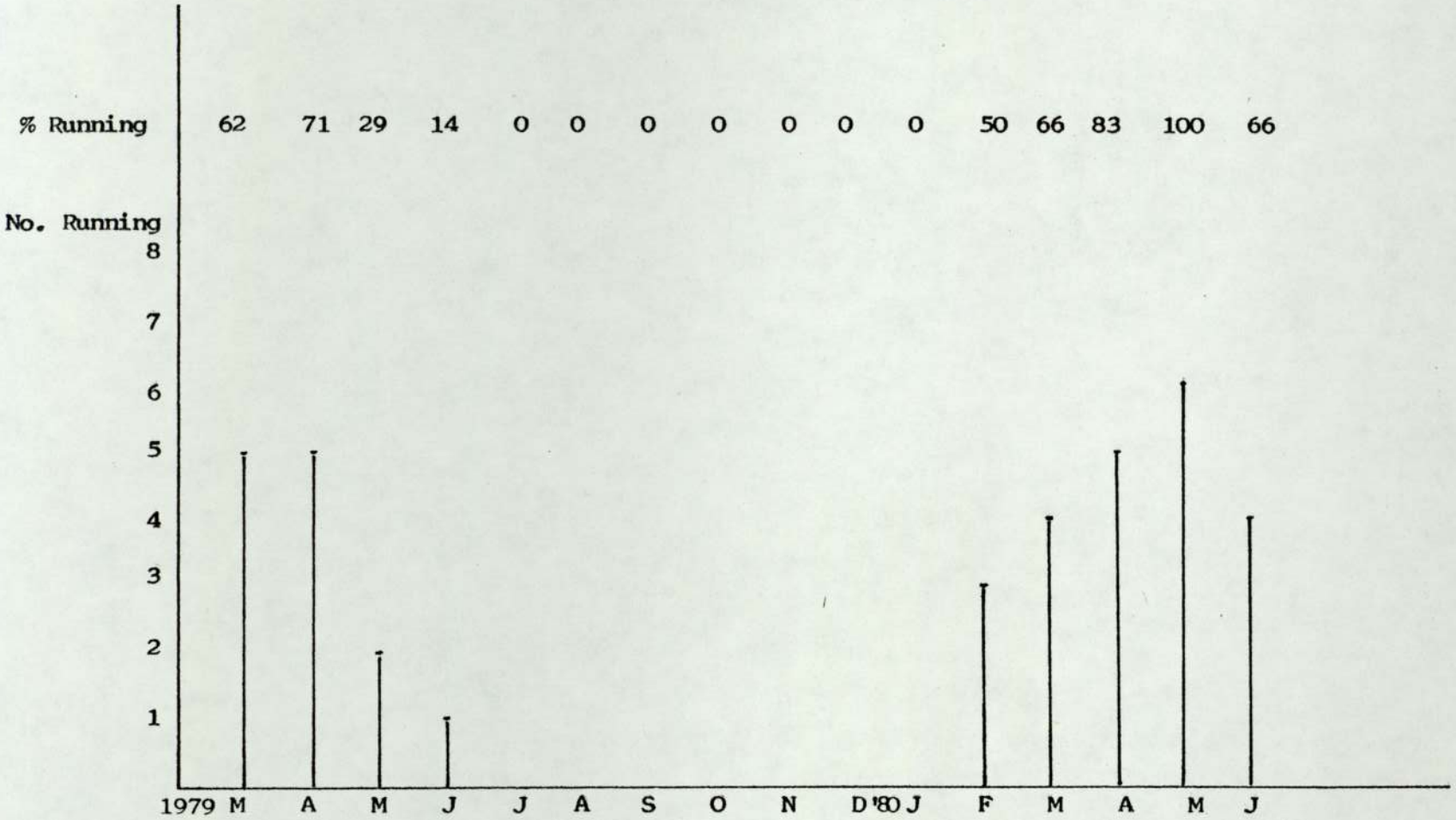
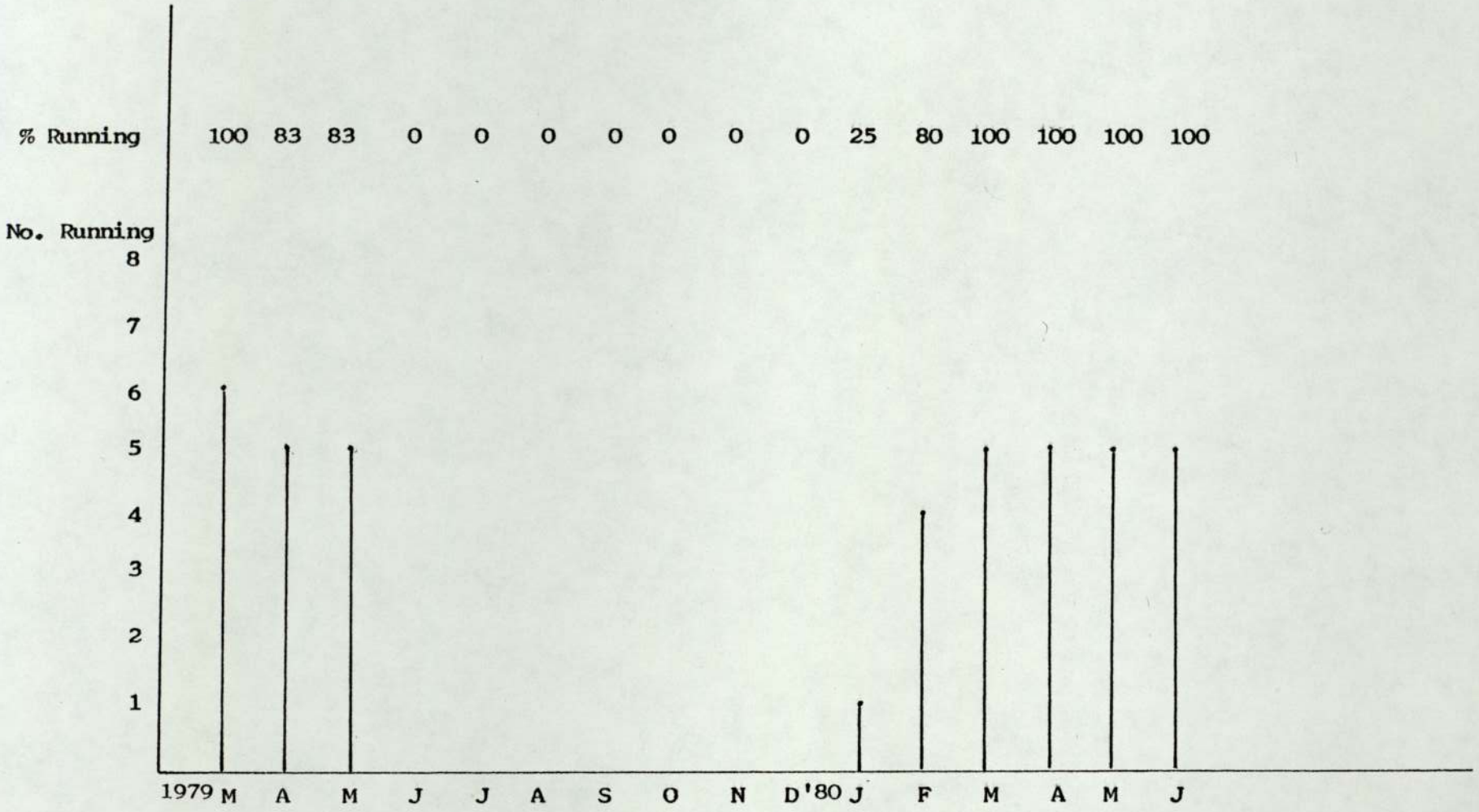


Fig. 5·2·4 Photoperiod Experiment (Shearwater Fish Farming)

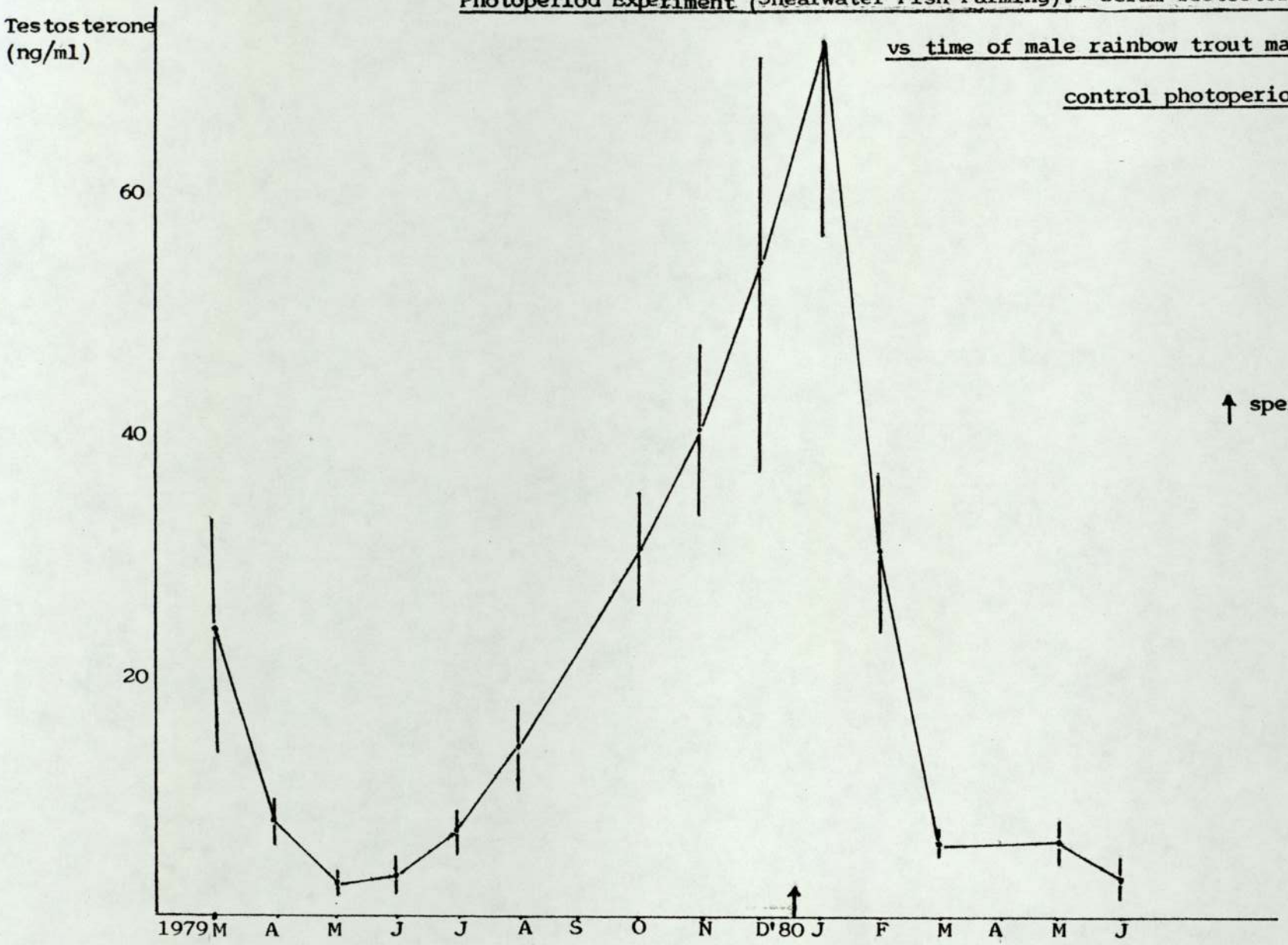
Number and % of male fish spermiating (running) vs time

Extended Photoperiod regime

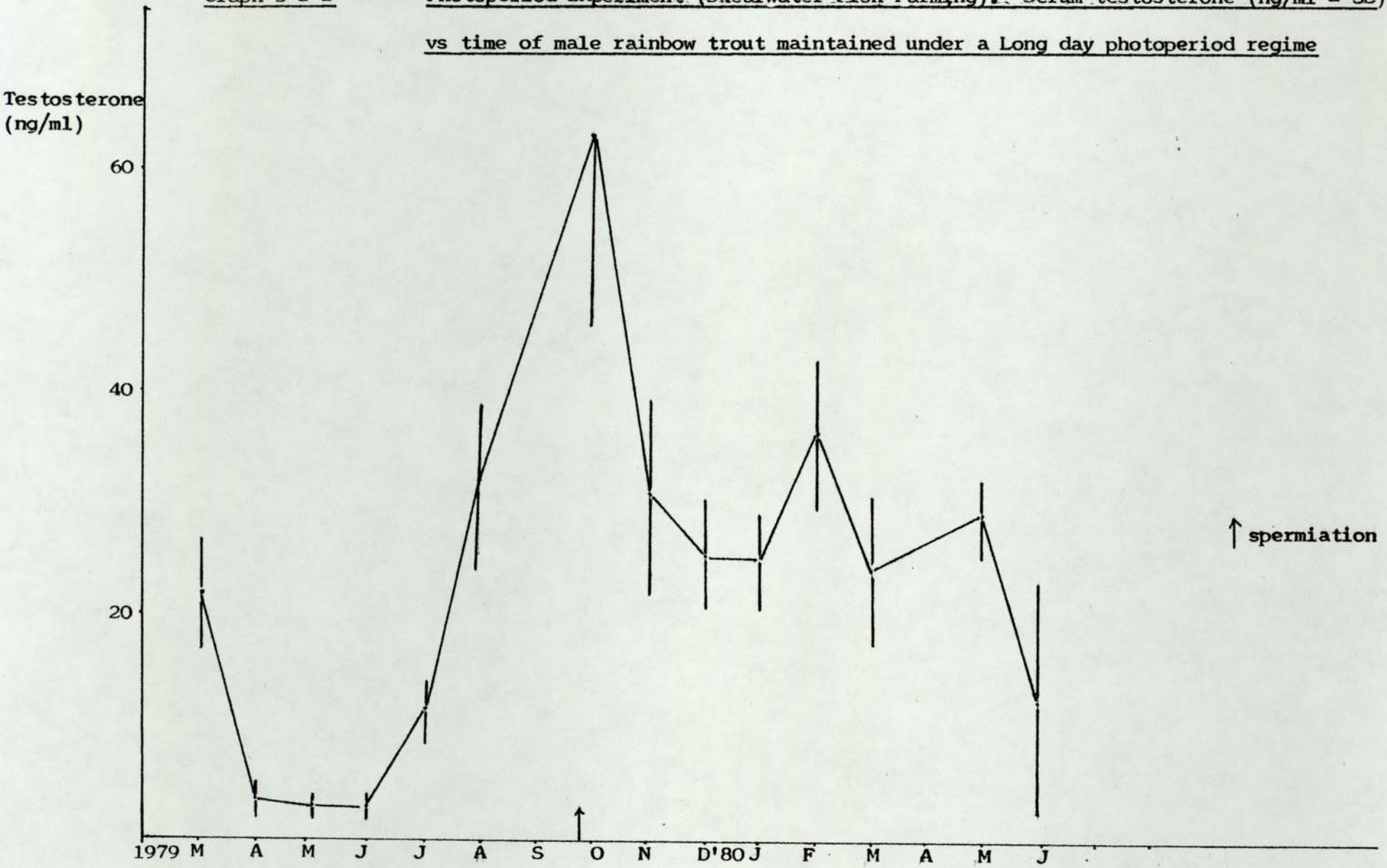


Graph 5•2•1

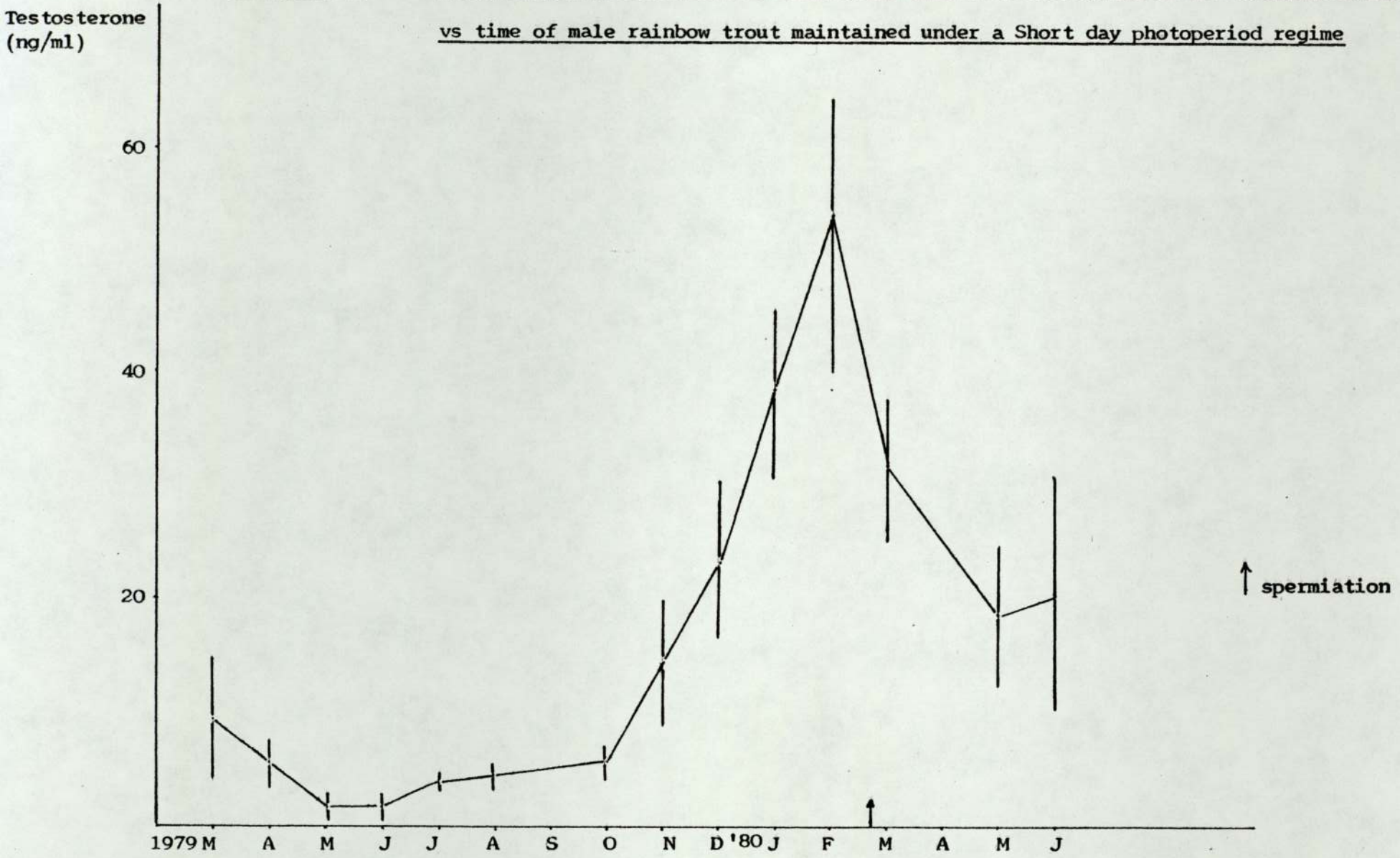
Photoperiod Experiment (Shearwater Fish Farming). Serum Testosterone (ng/ml ± SE) vs time of male rainbow trout maintained under a control photoperiod



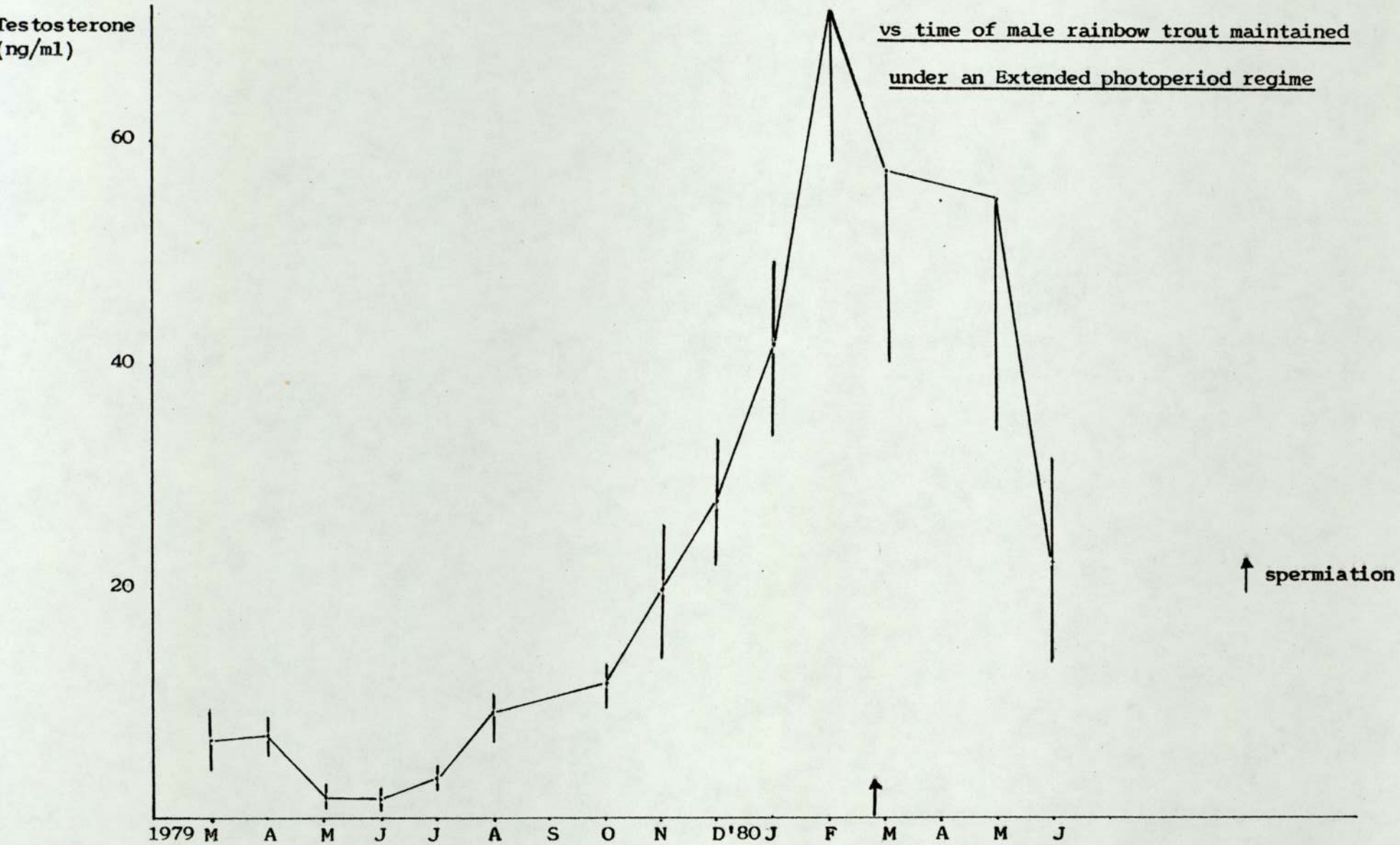
Graph 5·2·2 **Photoperiod Experiment (Shearwater Fish Farming). Serum testosterone (ng/ml ± SE) vs time of male rainbow trout maintained under a Long day photoperiod regime**



Graph 5·2·3 Photoperiod Experiment (Shearwater Fish Farming). Serum testosterone (ng/ml ± SE) vs time of male rainbow trout maintained under a Short day photoperiod regime



Graph 5·2·4 **Photoperiod Experiment (Shearwater Fish Farming). Serum testosterone (ng/ml ± SE)**



Discussion

The results clearly indicate the importance of photoperiodic cues in regulating sexual maturation of male rainbow trout. Under controlled experimental conditions where photoperiod was the only environmental variable, clear differences in the timing of spermiation and the associated cyclic changes of serum T were observed.

Thus advances in both the timing of spermiation and in the elevation of serum T levels were observed in male trout maintained on long day photoperiod.

The opposite effect was observed among males of the extended and short day photoperiod. Males within these groups failed to show spermiation until February/March compared with the normal timing of spermiation in December/January. Peak levels of serum T were delayed relative to the control group.

Maturation of both male and female salmonids may be either accelerated or delayed by compressing or extending the normal photoperiod cycle (MacQuarrie et al., 1978, 1979; Whitehead et al., 1978b, c, 1979) suggesting that the rate of change of photoperiod may be the important environmental cue regulating spawning. However, the demonstration that abrupt changes of photoperiod were as effective as a gradual change in regulating maturation implies that salmonids may be able to monitor absulute day length and that the rate of change of photoperiod is of less importance than its absolute value (Whitehead and Bromage, 1980).

The response to the constant photoperiod regimes used in the present experiment suggests this is indeed the case. It appears that different stages of the process of maturation may require different photoperiodic

stimuli. Thus long days early in the cycle give rise to earlier increases in serum androgens and earlier spermiation. In the absence of long days maturation is delayed by exposure to short days. The possibility exists that throughout the reproductive cycle an endogenous rhythm of sensitivity to photoperiod exists which is modified depending upon the photoperiodic information, previously supplied to the fish.

If such a rhythm of endogenous sensitivity to photoperiod exists, it may explain the variation in results obtained between different experimental designs. Lundqvist (1980) exposed Baltic salmon parr (Salmo salar L.) to long days and short days from August and found that, while short days had no observable effect on precocious male maturation, long days delayed maturation. However, as the experiment was of short duration, the previous photoperiod experienced prior to the experiment may be expected to influence the results. The results of Skarphedinsson et al. (1982), like the present experiment, suggest a stimulation of male maturation under long photoperiod.

Although gonadotropin levels were not measured in this experiment, it is reasonable to assume that a similar control mechanism is responsible for the changed secretion of androgens and timing of spermiation, as that described under compressed photoperiod cycles (Whitehead, 1979). So activation of the hypothalamic centres controlling the pituitary gonadotropins by photoperiodic inputs, and the resulting increased gonadotropin levels, gives rise to increased steroid levels and ultimately, spermiation.

5•3 Temperature

Temperature has been implicated as a prime environmental factor influencing reproduction in teleosts (de Vlaming, 1972, 1974). Environmental temperature is thought to be able to exert effects at a number of different levels within the reproductive system.

The environmental temperature may act directly upon the teleost gonad. Gametogenesis and gonadal cell division are especially susceptible to temperature effects. Thus temperature has been shown to be capable of controlling the duration and yield of spermatogenesis in the guppy, Poecilia reticulata (Billard, 1968) and the rice fish, Oryzias latipes, (Egami and Hyodo-Taguchi, 1967).

It has been recognized that different stages of gametogenesis may have different temperature requirements. Low temperatures have been shown to be necessary for stimulation of the early stages of spermatogenesis in Fundulus heteroclitus (Lofts et al., 1968) and Couesius plumbeus (Ahsan, 1966a). However, higher temperatures are necessary for the later stages of spermatogenesis in Couesius (Ahsan, 1966a) and for ovulation in the goldfish (Yamamoto et al., 1966).

Again, at the gonad level, temperature effects may be mediated via an action of temperature upon steroidogenesis. By influencing the enzyme systems responsible for steroidogenesis, temperature could exert a directional control of androgen biosynthesis. Kime (1979) has demonstrated an effect of temperature upon androgen biosynthesis in testicular homogenates of rainbow trout. Yields of 11-oxygenated androgens were maximal between 6° and 21°C, while steroid glucuronide levels increased with increasing temperature. Kime suggests a model in which β glucuronidase activity increases with temperature and removing free androgens

from the circulation. At lower temperatures lower β glucuronidase activity would preserve free androgens at the time most favourable for reproduction.

A similar temperature effect has been demonstrated in rainbow trout and goldfish liver homogenates, in which glucuronide production competes for free androgens more effectively at higher temperatures (Kime and Saksena, 1980).

Direct effects of temperature upon the hypothalamic-pituitary axis, as an initiating factor in reproduction, seem to be important especially among the cyprinid teleosts. Tench (Breton et al., 1975), goldfish (Gillet et al., 1977a, b) and carp (Weil et al., 1975) all demonstrate a direct effect of environmental temperature upon gonadotropin release. Further evidence is presented by Billard and Breton (1978).

A final aspect of the role of temperature is its influence upon growth and metabolism. This increased growth may affect reproduction, as has been demonstrated by Titarev (1975). He reported that male rainbow trout reared in a pond warmed by power station effluent matured at least one year earlier than they would normally do under the prevailing water temperatures of the region. This was apparently a non-specific effect of temperature acting via the increased growth rate.

Mackinnon and Donaldson (1972) have also reported the induction of early male maturation among one year old pink salmon (Oncorhynchus gorbuscha) reared under elevated temperatures. Again an increased growth rate was implicated in this response as the mature fish tended to be larger, though not significantly so.

Although a wide range of temperature related effects on teleost reproduction have been observed, it is generally agreed that in salmonids temperature is not the predominant environmental influence on reproduction. Thus Henderson (1963) found that the rate of gonadal development was the same among stocks of brook trout (Salvelinus fontinalis) at 16° and 8·5°C under the influence of natural photoperiod. However, within the cyprinids temperature does indeed seem to be the major factor responsible for the initiation of reproductive development (de Vlaming, 1972, 1974).

5·4 Nutrition

Nutrition plays a vital role in growth and metabolism, and in addition to these indirect effects, a direct influence of nutrition upon mammalian reproduction has been observed. Thus dietary deficiencies if vitamins A, B and E can give rise to a failure of spermatogenesis (Bell et al., 1972). Although little information is available regarding such possible effects in teleosts, it is normal for salmonid broodstock diets to contain increased amounts of vitamins.

The bulk of the work relating teleost reproduction and nutritional state has been directed towards the question of possible variations in female fecundity in response to different ration levels. Scott (1962) reported that female fecundity was directly related to feed levels in rainbow trout, with a decreased fecundity in response to low food levels. However, though Scott's experiment lasted for three years, very few (9%) fish actually matured.

Bagenal (1969) produced further evidence of a relationship between female fecundity and ration size in the brown trout (Salmo trutta). A reduction in female fecundity followed a reduction in ration size.

However, results in male trout appear more complex and while a reduction in male maturation at low feed levels was reported in the initial experiments, it was not observed in later experiments.

A nutritionally related variation of female fecundity has also been reported in the three-spined stickleback (Gasteosteus aculeatus L.) fed at three different ration levels. The percentage of females maturing and the number of eggs produced increased with increasing ration (Wootton, 1973).

However, though a reduction in feed rate gave rise to a fall in fecundity in the haddock (Melanogrammus aeglefinus), there was no evidence of a reduction in the percentage of females spawning. (Hislop et al., 1978).

Under practical farming conditions an increase in early male maturation has been observed, especially when salmonids are cultivated in sea pens (Edwards, 1978; Sutterlin et al., 1978). It has been suggested that increased feed rates are responsible for this phenomenon, as sea-cultured salmonids are fed to high levels (usually ad libitum).

A similar effect has been observed among stocks of rainbow trout at the Yorkshire Water Authority hatchery at High Costa Mill, Pickering. A batch of rainbow trout, reared in two separate ponds, were fed either once a day or four times a day. The incidence of precocious male maturation in the group fed four times a day was 12 - 15% of all fish examined, while in the group fed only once a day, only 2 - 3% of all fish examined were mature males. This difference in maturation was apparently correlated with growth rate (Harbin, unpublished).

However, when two groups of rainbow trout, maintained at Low Plains, were fed either to satiation or to table ration, very few mature male

fish were observed among the high-feed group, while the majority of the table-ration males matured (Smart, pers. comm.).

The effect of variation in ration size upon the sexual maturation of rainbow trout

In order to explore the relationship between feed rate and maturation in rainbow trout, an experiment was carried out at the University of Aston Fish Culture Unit.

Materials and Methods

Rainbow trout of approximately 90 g were obtained from Burwarton Fish Farms (Shrewsbury). Following quarantine, two groups of 20 fish were tagged and housed in two tanks of a recirculating system. Each group was fed a particular ration: either a reduced ration equivalent to three-quarters the recommended manufacturer's feed level (B.P. Nutrition) or twice the recommended feed level.

The experiment was begun in July and carried on until December, when it was terminated. Between these dates fish were either batch weighed at approximately fortnightly intervals or weighed individually at slightly longer intervals.

Fish were examined at these individual weighings for external secondary sexual characteristics and the presence of spermiation. Blood samples were also taken for hormone assay. At the end of the experiment fish were sacrificed and the gonads dissected free and weighed.

The Gonadosomatic Index (G.S.I.) was calculated from the formula

$$\text{G.S.I.} = \frac{\text{Gonad weight (g)}}{\text{Body weight (g)}} \times 100.$$

Results

a) Growth

The effects of the two feeding rates upon growth can be seen in Table 5•4•1 and Graph 5•4•1. As expected, a highly significant increase in growth was observed at the higher feeding level.

This was evident in individual weighings carried out on day 23 of the experiment when the three-quarter ration group's mean body weight of 129•7 $\pm$ 6.56 g was less than that of the high feed group (143•4 $\pm$ 4•2 g), though not significantly so ($P < 0•1$). All weighings after this date showed a highly significant ($P < 0•001$) effect of feed rate upon growth.

The growth results are analysed in greater detail in Tables 5•4•2 and 5•4•3, and Graphs 5•4•2 and 5•4•3, which show the mean body weight of fish of each sex and stage of maturity within groups.

Mature males showed the lowest growth rate in both groups. In the higher ration group, mature males grew at a slightly lower rate than immature fish until day 133. From this time, however, the growth rate fell drastically and at the time of the terminal sample, mature males were significantly lighter than both immature males ($P < 0•001$) and immature females ($P < 0•05$) (Table 5•4•2 and Graph 5•4•2).

Mature males within the three-quarter ration group were significantly lighter than immature males ($P < 0•05$) at the end of the experiment, though not significantly lighter than immature females. In this group the reduction in growth was more gradual than the high ration group (Table 5•4•3 and Graph 5•4•3).

Table 5·4·1

Mean body weights of rainbow trout maintained on either a high (x 2 manufacturer's recommended level) or a low ration (three-quarters manufacturer's recommended level)

Mean body weight ± SE (g)

	¾ ration	High ration
Day 0	95·9 ± 5·8	92·3 ± 3·0
12	106·4	120·7
23	129·7 ± 6·6	143·4 ± 4·2
40	139·8	177·4
54	151·3	206·1
68	168·3 ± 7·9 (***)	220·5 ± 6·7
80	175·0	272·3
91	186·6 ± 7·5 (***)	299·3 ± 7·9
104	200·3	320·6
120	215·0	368·5
133	240·5 ± 9·5 (***)	401·9 ± 11·0
146	252·2	440·2
161	267·8 ± 9·9 (***)	455·8 ± 19·6
174	276·5	487·0
183	301·0 ± 12·8 (***)	498·9 ± 27·9

t test between pairs (***) $P < 0.001$

Table 5·4·2

Mean body weights of rainbow trout fed a high ration (x 2 manufacturer's recommended level)

Body weight ± S.E. (g)

	0	23	68	91	133	161	183
Mature males (6)	91·8 ± 6·12	138·8 ± 9·1	228·2 ± 10·7	298·8 ± 14·3	385·4 ± 12·3	394·0 ± 13·6	396·2 ± 17·9
Immature males (3)	89·0 ± 9·06	158·6 ± 12·1	201·6 ± 20·0	291·3 ± 28·2	397·7 ± 45·4	493·0 ± 80·2	571·0 ± 95·8
Immature females (6)	92·0 ± 4·9	141·0 ± 6·5	216·3 ± 9·7	275·3 ± 10·9	403·0 ± 16·5	477·3 ± 27·5	545·5 ± 39·6
Mature females (2)	96·5 ± 7·1	148·0 ± 6·4	220 ± 13·3	306 ± 20·8	410·5 ± 18·8	435·5 ± 26·2	454 ± 25·9

(***) between mature males and immature males at 183 days

(*) between immature males and immature females at 183 days

t test between groups
(***) P less than 0·001
(*) P less than 0·05

Table 5·4·3

Mean body weights of rainbow trout fed a low ration (three-quarters manufacturer's recommended level)

Body weight ± S.E. (g)

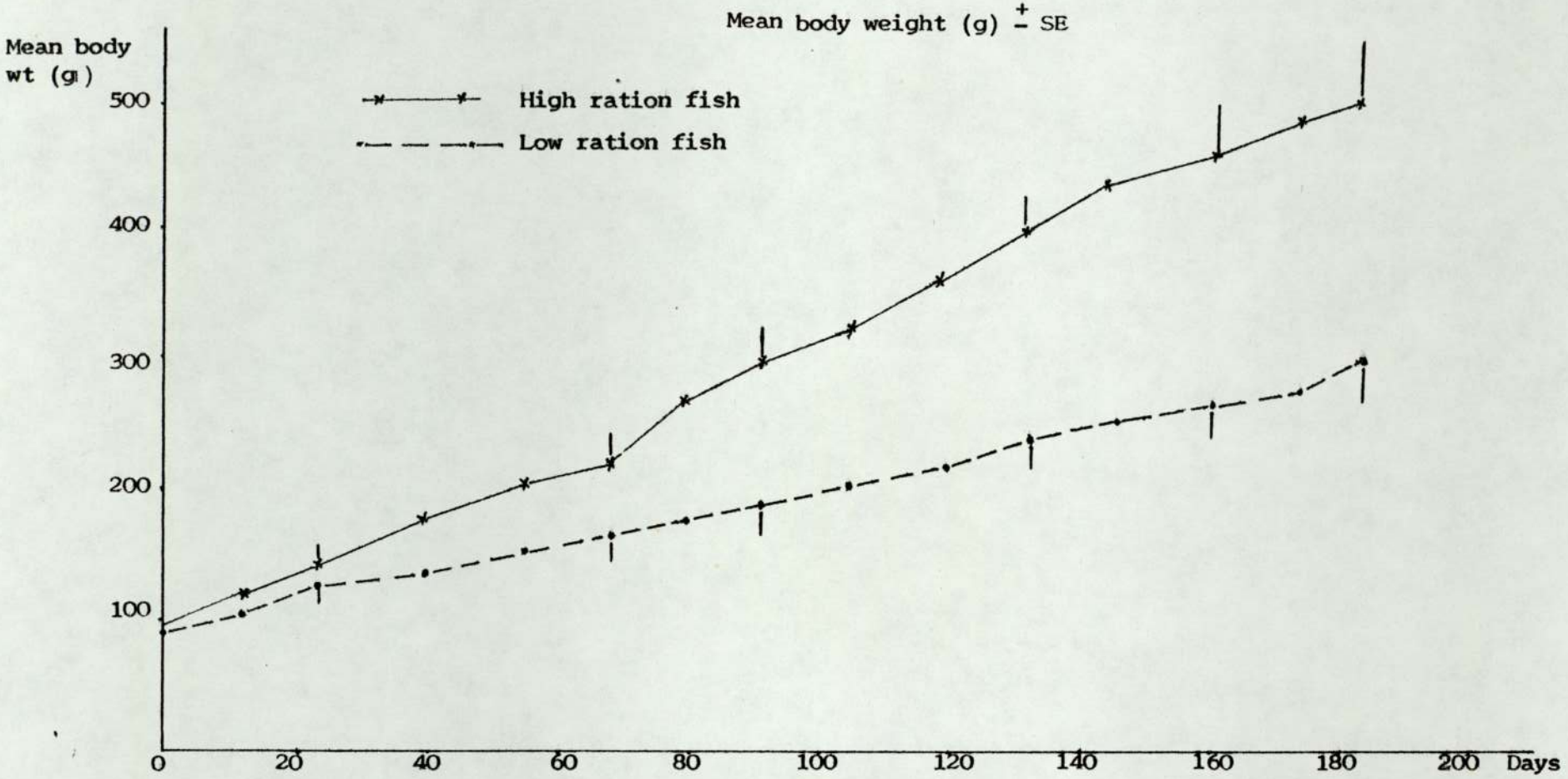
	0	23	68	91	133	161	183
Mature males (9)	94·3 ± 8·26	123·3 ± 9·0	160·9 ± 11·7	187·7 ± 13·0	227·5 ± 15·7	252·0 ± 17·8	271·4 ± 19·0
Immature males (2)	98·0 ± 8·4	129·0 ± 8·2	183 ± 9·9	206·0 ± 10·2	246·5 ± 13·4	291·5 ± 8·4	330·5 ± 9·8
Immature females (8)	93·0 ± 8·1	127·0 ± 8·5	161 ± 7·7	192·1 ± 6·3	242·7 ± 7·3	281·6 ± 8·6	297·1 ± 8·5

(*) between mature males and immature males at 183 days

t test between groups
(*) P less than 0·05

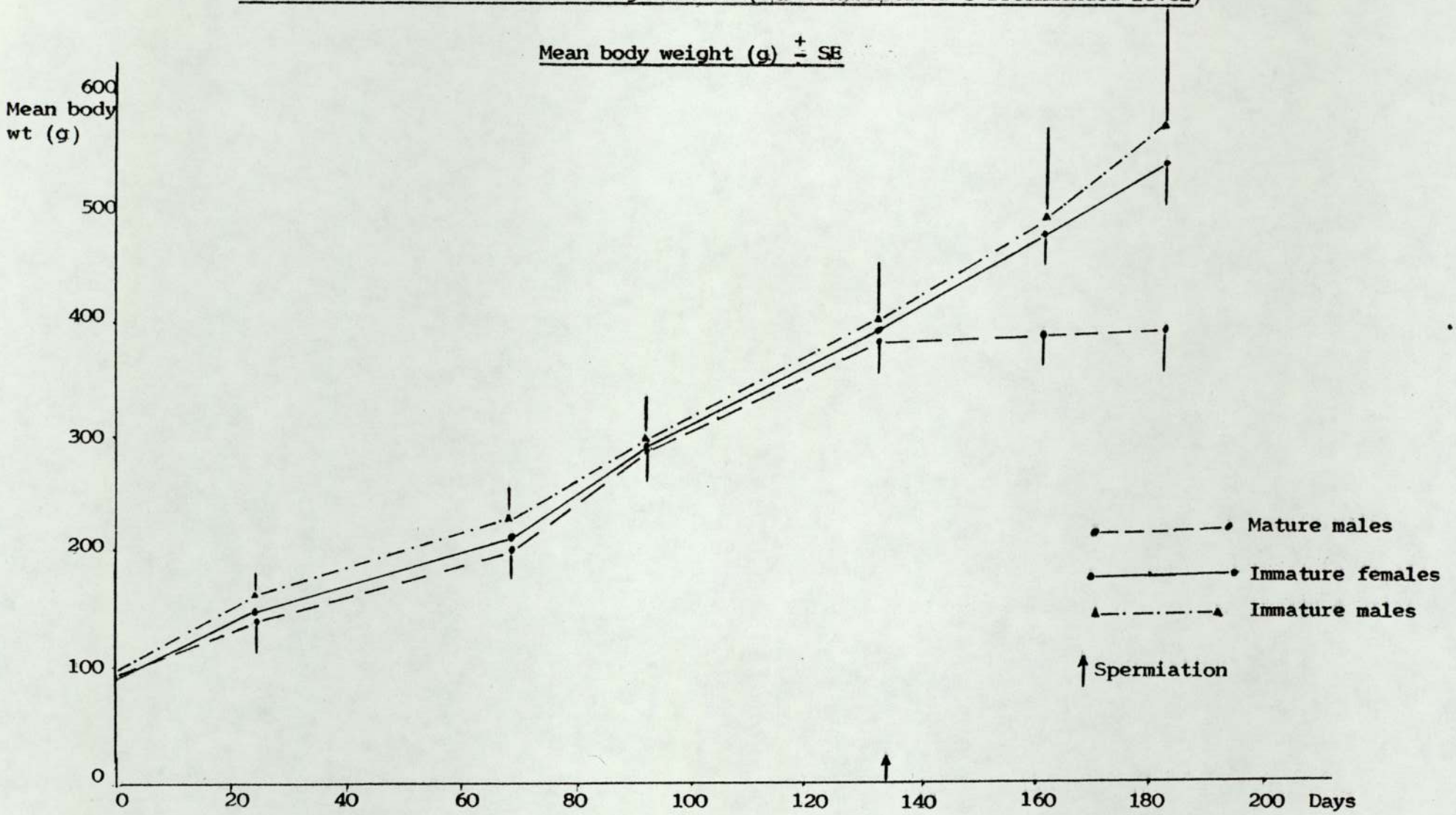
Graph 5•4•1

Growth of rainbow trout fed either a high (x 2 manufacturer's recommended level) or a low ration (three-quarters manufacturer's recommended level)



Graph 5·4·2

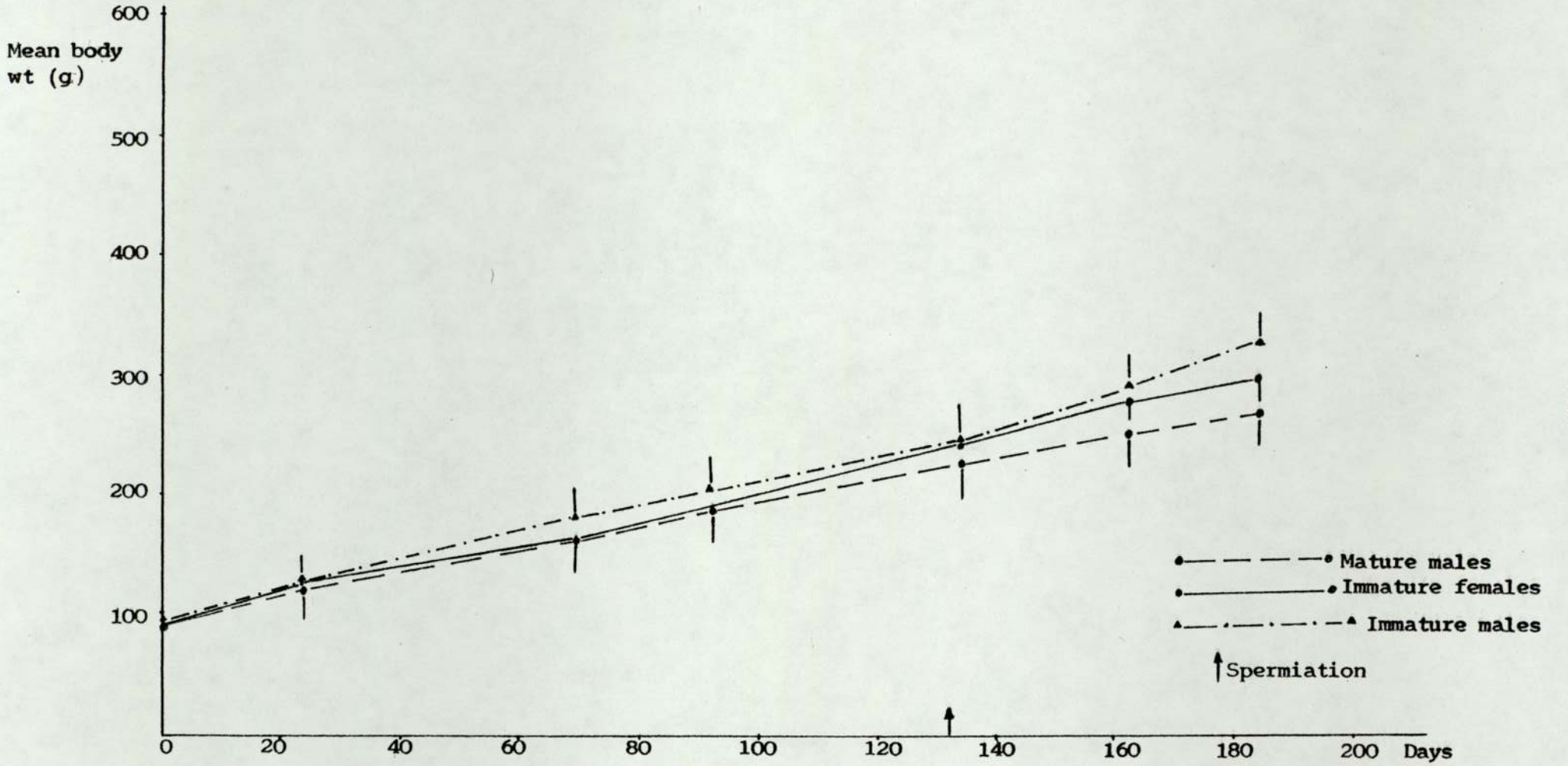
Growth of rainbow trout fed a high ration (x 2 manufacturer's recommended level)



Graph 5•4•3

Growth of rainbow trout fed a low ration (three-quarters manufacturer's recommended level)

Mean body weight (g) ± SE



b) Maturation

The percentage of mature fish within each group is shown in Table 5•4•4. No difference was evident between the two groups with respect to the incidence of male maturation. Within the high ration group 66% of all males were mature by the end of the experiment, while 82% of the males in the three-quarter ration group matured. However, due to the low number of male fish, this difference was not statistically significant.

Within the high feed group, two females matured, significantly earlier than would be expected, by the end of the experiment. No mature females were found in the three-quarter ration group.

c) Gonad weights

The mean gonad weights of each group are shown in Table 5•4•5. A significant difference was observed between the mean weights of testes from the two experimental groups. The high ration group had a mean testis weight of 20•5 $\pm$ 2•7 g, while the three-quarter ration group's mean testis weight was 11•05 $\pm$ 1•7 g ($P < 0•001$).

A significant ($P < 0•01$) difference in female gonad weight was also observed in females of the high ration group (0•58 $\pm$ 0•04 g) compared with the three-quarter ration group (0•43 $\pm$ 0•02 g).

d) Gonadosomatic Indices

The mean gonadosomatic indices of the two groups are shown in Table 5•4•6. While the mean G.S.I. of the mature males from the high ration group was higher than that of the three-quarter ration group (4•74 $\pm$ 0•50, high ration, and 3•78 $\pm$ 0•36, three-quarter ration) this

effect was not statistically significant. No significant differences were apparent between the G.S.I.'s of any particular sex and stage sub-group and its counterpart, suggesting that the differences in absolute gonad weight observed between ration groups reflect an increase in body size, as G.S.I. corrects for body weight differences it thus 'normalizes' the data.

e) Timing of maturation

Fish were briefly examined at weighing and the occurrence of spermiation checked. By day 133 spermiation was apparent in occasional fish of each group and was well pronounced by day 161, with four males in the three-quarter ration group and three males in the high ration group showing spermiation. By the end of the experiment five fish of the high ration group were 'running', while seven of the low feed males were in a similar condition.

f) Testosterone levels

Serum T levels were assayed only from male fish. Samples from five males were assayed for each sampling date examined. The results are shown in Table 5•4•7.

No significant differences were observed in mean T levels between the two groups on the days chosen for assay. In both cases an increase of serum T over the course of the experiment was observed, with maximum levels at day 161 of 60•18 $\pm$ 10•9 and 51•5 $\pm$ 8•8 ng/ml in the high feed and three-quarter ration groups.

Table 5•4•4

% maturation of rainbow trout fed either a high (x 2 manufacturer's recommended level) or a low ration (three-quarters manufacturer's recommended level)

	High ration	¾ ration
Males		
Total no.	9	11
No. mature	6	9
% mature	66%	82%
Females		
Total no.	8	8
No. mature	2	0
% mature	25%	0

Table 5•4•5

Mean gonad weights of rainbow trout fed either a high (x 2 manufacturer's recommended level) or a low ration (three-quarters manufacturer's recommended level) (Gonad weight $\pm$ SE (g))

	High ration	¾ ration
Mature males	20•5 $\pm$ 2•7 —(***)—	11•0 $\pm$ 1•7
n	6)	9)
Immature males	0•24 $\pm$ 0•06	0•13 $\pm$ 0•06
n	3)	2)
Immature females	0•58 $\pm$ 0•04 —(**)—	0•43 $\pm$ 0•02
n	6)	8)
Mature females	49•55 $\pm$ 7•05	-
n	2)	

t test high vs. low ration (***) ($P < 0•001$)
(**) ($P < 0•01$).

Table 5·4·6

Mean Gonadosomatic index (G.S.I.) of rainbow trout fed either a high (x 2 manufacturer's recommended level) or a low ration (three-quarters manufacturer's recommended level)

G.S.I. $\pm$ SE

	High ration	¾ ration
Mature males n	4·74 $\pm$ 0·50 6)	3·78 $\pm$ 0·36 9)
Immature males n	0·04 $\pm$ 0·005 3)	0·04 $\pm$ 0·017 2)
Immature females n	0·21 $\pm$ 0·09 6)	0·137 $\pm$ 0·005 8)
Mature females n	10·85 $\pm$ 0·45 2)	-

Table 5·4·7

Serum testosterone levels of male rainbow trout fed either a high (x 2 manufacturer's recommended level) or a low ration (three-quarters manufacturer's recommended level)

ng/ml $\pm$ SE

	High ration	¾ ration
Day 0 n	3·08 $\pm$ 0·6 9)	
91 n	35·82 $\pm$ 6·4 5)	30·9 $\pm$ 6·8 5)
161 n	60·18 $\pm$ 10·9 5)	51·5 $\pm$ 8·8 5)

Discussion

As expected, increased ration size gave rise to a significant increase in growth in the high feed group. Despite this increased growth the variation of ration size did not significantly influence either the timing or the number of males maturing. However, within the high feed group two females matured - well in advance of the expected time of female maturation. Although growth in the group fed three-quarter rations was reduced, again the percentage male maturation was not affected.

Within both groups the growth performance of mature males was significantly worse than that of immature fish. This effect was most apparent in the high ration group, where the overall growth rate of the group was high. In the three-quarter ration group the lower mean body weight was also statistically significant ($P < 0{\cdot}05$) by the end of the experiment, though the reduction in growth was less marked, reflecting the poorer growth of all fish within this group.

An increase in absolute gonad weight was evident between both the mature males and the immature females of each group. This difference appears to be related almost entirely to the increased somatic growth of the high ration group as no significant differences are apparent when the data are converted to G.S.I.'s, which correct for differences in body weight.

So at two very different ration sizes, no statistically significant effect of diet on the proportion of material deposited in the developing testis relative to body weight was seen.

The majority of work in this area has concentrated upon the relationship between fecundity and nutritional state in the female

teleost. Few reports have recorded the effects upon male maturation. Bagenal (1969) did observe depression of male and female maturation among brown trout fed at half table ration, but when experiments were repeated using a normal ration and a third ration group, no significant effect was seen.

In this experiment the two levels were chosen as having more commercial relevance. Very low feed levels giving rise to starvation/ stress effects on reproductive fecundity would be of obvious significance as a factor regulating wild populations. Thus very low food levels in the wild may play a part in regulating population levels. Under commercial conditions feed rates are normally at or close to the level of manufacturer's tables.

While nutrition has an obvious role in influencing growth rate, its effects upon reproduction are less certain. Generally in male teleosts it appears that if the dietary intake is above a minimum level it allows reproduction to take place. However, as Bagenal reports, maturation can take place among male brown trout fed only one third normal rations and the effects of reduced ration size seem to be very variable.

No increase in G.S.I. was observed through feeding an increased ration in these experiments,in contrast to the situation reported in the female teleost. Among female teleosts it seems increased ration size gives increased fecundity, though whether this is a valid effect or an effect of increased somatic growth remains unclear.

Differences between the sexes with respect to the effect of nutrition upon fecundity may well be explained by the relative role of the two sexes in spawning. Thus the female salmonid produces large yolky eggs and egg production must represent a major

energy/nutrient commitment. On the other hand, the material contribution of the male teleost is relatively low so the nutrient level needed to maintain this level of production may be less. Providing ration size does not fall below the level at which stress/starvation effects intervene, male maturation seems to be less dependent upon nutrition than that of the female.

Nutritional effects on reproduction may, perhaps more than other environmental factors, depend upon interactions with internal physiological factors, and thus their interpretation is likely to be difficult. As an example of this difficulty of interpretation, it was noted that in a series of experiemnts designed to study the effects of differing protein sources and levels on enzyme activities in yearling rainbow trout, there was a marked variation in the number of precocious males maturing in each group (D. J. Cooke, pers. comm.). Thus more males matured in the group fed high proportions of fish meal than those fed either casein or gluten diets (Table 5•4•8).

These changes did not appear to be related to changes in growth as diets were isocalorific and significant differences in growth were not observed between males in the fish meal, casein or gluten groups. However, when the experiment was repeated using similar sized fish, no effect of protein type or proportion was observed, illustrating the variable nature of nutrition effects on teleost reproduction.

Table 5•4•8

% maturation of male trout (expressed as a % of the total number of males examined) fed isocalorific diets containing a variety of proteins at different levels

Diet		% maturation
Fish meal	50%	58•0
	40%	45•0
	30%	18•0
Casein	50%	11•1
	40%	18•0
	30%	6•2
Gluten	50%	8•3
	40%	16•6
	30%	11•1

Data D. J. Cooke, unpublished.

5•5 Salinity

Changes in salinity have been suggested as a possible environmental variable having an influence on teleost sexual maturation. It has already been mentioned that mortalities among maturing male salmonids are high at increased salinities. Whether this arises from osmotic stress is uncertain, as is the extent to which the timing of maturation may be triggered by salinity changes.

An increase in the number of male rainbow trout reaching maturity in mixed salinity water has been reported (Smart, pers. comm.). However, the effect of salinity is complicated by its interactions with a number of other environmental variables, notably photoperiod, temperature and nutrition. In addition, a possible relationship between salinity and growth has been reported, which may be of significance in the timing of reproductive development. Increased salinity may increase growth rates of salmonids (Canagaratnam, 1959; Otto, 1971) or have either a negative or negligible effect (Kepshire and McNeil, 1972; Shaw et al., 1975; Clarke et al., 1981).

The occurrence of sexually mature salmon (Salmo salar) postsmolts has been reported under salt water conditions (Sanders and Henderson, 1965; Naevdal et al., 1975) and is thought to result from the accelerated rearing regime. In addition, sexually mature female postsmolts have been produced under salt water rearing conditions (Sutterlin et al., 1978). While the maturing females were the largest postsmolts, supporting the involvement of growth rate in female postsmolt maturation, the mature males were small. However, the relationship between growth rate and male maturation is undoubtedly complex and requires further work for its elucidation.

5•6 Pheromones and Social factors

Social interaction and in particular inter-fish communication has been suggested as a possible mechanism by which environmental factors may influence internal reproductive processes.

The occurrence of specific external chemical signals or pheromones (Karlson and Luscher, 1959) has been reported in a wide range of vertebrate and invertebrate species including teleosts (Bardach and Todd, 1970; Hara, 1971; Pfeiffer, 1974; Solomon, 1977).

Pheromones have been implicated in several areas which may be, either directly or indirectly, of reproductive significance. These include shoaling (Hoaglund and Astrand, 1973), homing (Solomon, 1973), alarm reactions (Pfeiffer, 1974), crowding factor (Rose, 1959a, b) and pair formation and spawning (Timms and Kleerekoper, 1972; Partridge et al., 1976).

Two factors of special interest are the crowding factors and the sex attractant factors. Crowding factor may be able to influence reproduction either directly or via an effect on growth and general metabolism. Rose (1959a) demonstrated a negative influence of crowding factor on growth in a variety of fish species. In addition, a density dependent-reduced fecundity of female guppies (Lebistes reticulatus) has been ascribed to crowding factor (Rose, 1959b). A similar density dependent inhibition was observed by Swingle (1953).

The role of pheromones in teleost pair formation and spawning behaviour is more widely recognized following the demonstration of their involvement in chemical communication during such behaviour. Timms and Kleerekoper (1972) showed a specific attraction of ripe male to ripe

female catfish (Ictalurus punctatus). Sex specific pheromones have also been demonstrated in rainbow trout (Newcombe and Hartman, 1973; Honda, 1980) and have been shown to be capable of attracting males to ovulating females. In the latter case the pheromone appeared to be specific to the gonad and the genital cavity fluid of mature female trout.

Although pheromones seem to have an involvement in courtship and later stages of spawning behaviour, there is little evidence that pheromones play a direct part in the initiation and control of male maturation. Anosmic Atlantic salmon, in which the olfactory mucosa was destroyed by cautery, showed similar gonadal development to intact control fish, indicating that olfaction is not mandatory to sexual development (Stabell and Refstie, 1980).

Solomon (1978) has made the point that due to fish culture operations where fish are maintained at artificially high densities, pheromones and other effects are likely to become more evident. Nevertheless, pheromones appear not to be a major environmental factor directly responsible for the initiation of reproduction, though they may influence later stages of the process, and as Liley (1982) points out, an understanding of their roles may allow improved control and manipulation of teleost breeding.

5·7 General Discussion and Conclusions

It is obviously of vital importance that reproduction is synchronized to allow it to take place at the most advantageous time. Thus the neuroendocrine and hormonal changes accompanying and giving rise to sexual maturation must be sensitive to environmental influences; these factors have been examined in this chapter.

There is general agreement that in salmonids and gasterosteids photoperiod is the main environmental cue for reproductive development (de Vlaming,1972; Crim, 1982), while in cyprinodontiformes, gobiids and clarids temperature becomes predominant. The importance of photoperiod in the maturation of male salmonids is confirmed by the present experiment, in which it was demonstrated that, under conditions of constant temperature, photoperiodic change is capable of influencing the timing of maturation. Thus when rainbow trout were exposed to short photoperiods (8 hours light : 16 hours dark) a delay of maturation was observed. This effect was apparent as a delay in spermiation and in the normal increase in serum androgen levels.

The opposite effect was seen when males were exposed to long photoperiods (16 hours light : 8 hours dark) and maturation was accelerated by this treatment. Under an extended photoperiod, in which the normal rate of photoperiodic change was extended to 18 months, male sexual maturation was delayed, though to a lesser extent than under short days.

So it appears that both absolute day length and changing day length provide environmental cues regulating the reproductive development of male salmonids. The results from this series of experiments using constant photoperiods and those of Whitehead (1979) suggest that it is unnecessary to use gradually changing photoperiod regimes to manipulate the reproductive development of male salmonids and that different stages of the reproductive cycle may respond to different photoperiodic inputs.

Although temperature seems to be less important in salmonids, it is dangerous to dismiss its possible influence. Breton and Billard (1977) showed that, while rainbow trout would not respond to constant long or

short photoperiods, spermatogenesis was stimulated by decreasing photoperiod. This effect of photoperiod upon serum GTH levels and testicular growth was modified by temperature. So fish maintained at higher temperatures (16°C) had higher serum GTH levels and larger testes than fish reared at 8°C. Although photoperiod may be the prime environmental cue in salmonids, temperature may act in a modifying capacity.

An indirect effect of environmental stimuli upon reproduction via an influence on metabolic processes and growth may also occur and nutrition might be expected to influence reproductive activity in such a manner. However, the evidence linking nutrition with male maturation, and especially of precocious maturation, has been largely based upon commercial work. Needham (1983) has collected some of the reports linking diet quality and quantity to the extent of grilse maturation of Atlantic salmon. In the present experiments no effect on reproduction was observed when rainbow trout were fed at two different ration levels. It has been suggested that if such a relationship exists, it is liable to be complex (Pyefinch, 1960) and while Thorpe (1975) found immature salmon smolts to be slightly larger than early maturing smolts, Sutterlin et al. (1978) noted the reverse effect.

The influences of salinity and pheromones upon salmonid reproduction are less certain, though it seems they are of minor importance.

The potential commercial use of environmental modification to regulate teleost reproductive development depends upon a number of factors, including effectiveness, cost and ease of manipulation.

Temperature appears to be of relatively minor importance in salmonids and is relatively expensive to control, though it has found application in controlling the incubation period of salmonid ova (Maddock, 1974).

Of the environmental factors examined, photoperiod control would appear to offer the greatest potential for the control of male maturation as it is relatively cheap and simple to manipulate and the commercial possibilities have already been recognised (Bye and Htun Han, 1978; Harvey, 1978; MacQuarrie et al., 1979; Whitehead, 1979), though in most cases these have concentrated on extending the season for egg production rather than on delaying male maturation.

Some larger scale preliminary experiments have been carried out at Shearwater Fish Farming, though with ambiguous results, perhaps in part due to the previous photoperiod to which the fish had been exposed. This may explain some of the variability in the reported effects of photoperiod. While Skarphedinsson et al. (1982) reported an acceleration of gonadal development of rainbow trout by long days, Lundqvist (1980) noted a delay of maturation under long photoperiod.

It is only relatively recently that the reproductive roles of isolated environmental factors have been classified and it seems likely that, in view of the case of manipulation of environmental cues such as photoperiod, their use will become an increasingly important aspect of commercial salmonid production.

CHAPTER 6

IMMUNOLOGICAL METHODS OF MANIPULATION OF SEXUAL MATURATION IN SALMONIDS

Contents

6.1 Immunity towards reproductive hormones

The fish's immune system provides a potential means through which the reproductive process may be influenced. Activation of the immune system towards one or more components of the reproductive system, and in particular the possibility of inducing an autoimmune state, directed either towards reproductive hormones or gonadal tissue, has an obvious potential as a control method by which the adverse effects of maturation may be prevented.

A considerable amount of work has been directed at the study of both passive and active immunity towards sex steroids in mammals (Edwards and Johnsone, 1976). Following immunological inactivation of sex steroids, their biological effects, including the induction and maintenance of the secondary sex characteristics, may be blocked. This procedure would in theory be capable of preventing the steroid-dependent aspects of maturation evident in male salmonids.

However, the endocrine control processes regulating sex steroid production will also be influenced by such treatment. That antibodies can react with and inactivate steroids is widely accepted. However, active immunization against sex steroids may cause a removal of negative steroid feedback at the CNS or pituitary level and thus increase gonadotropin production and release, with the consequent increase in gonadal steroid production and increased peripheral steroid levels (Edwards and Johnsone, 1976).

A more attractive site for immunological manipulation is the release of gonadotropin from the pituitary. This may be achieved either by the use of an antibody directed against gonadotropins

themselves or towards the releasing hormones responsible for gonadotropin release.

This area has been especially well explored in mammalian work. Mammalian gonadotropins have been shown to be directly succeptible to immunological activation by treatment with anti-GTH (Raghavan et al., 1977). Immunization with a synthetic releasing hormone, luteinizing hormone releasing hormone (LH-RH), has resulted in both long and short-term inhibition of gonadotropin release and accompanying gonadal atrophy in both male and female rats (Fraser et al., 1974, 1975). So autoimmunity towards releasing hormones may provide a selective alternative to hypophysectomy. However, although synthetic releasing hormone (LH-RH) has been shown to stimulate gonadal activity in salmonids (Crim and Cluett, 1974), the opposite effects of antibodies to releasing hormones on the gonadal development of salmonids has not been reported.

Among teleosts the use of such immunological methods has been largely limited to research applications by which details of interrelationships among reproductive hormones may be explored. Thus Ng and Idler(1980a) and Ng et al. (1980) used an antibody directed against maturational hormone (a gonadotropin fraction absorbable by Con-A-Sepharose) to block the hormone's normal actions and thus elucidate its role in the maturation of winter flounder (Pseudopleuronectes americanus), rainbow trout (Salmo gairdneri) and salmon (Salmo salar). Injection of an antibody to glycoprotein gonadotropin (GTH) has also proved effective in blocking the early ovarian growth of salmon (Salmo salar). (Wiegand and Idler, 1980).

Research use apart, it is by no means clear if the method could be effectively adapted to salmonid cultivation practice. It seems likely that the success of treatment would be critically dependent upon the dose of antibody administered and upon the degree of active immunity produced by an antigen-adjuvant complex. The teleost immune system is generally acknowledged to be less effective than that of mammals. Experimental work at the Department of Agriculture and Fisheries, Scotland (D.A.F.S.) showed antibody to hypothalmic releasing factor to be incapable of suppressing salmonid reproductive development (Johnstone, Pers. Comm.).

6.2 Testicular Autoimmunity

It has long been realised that the mammalian reproductive system is sensitive to immunological damage. In particular, immunisation of male animals with homologous testicular homogenates in conjunction with an immunological adjuvant resulted in the impairment of the spermatogenic cycle (Freund et al., 1955; Behrman, 1964; Johnson, 1970a). These results have been confirmed in a variety of species including guinea pig (Bishop and Carlson, 1965), rabbit (Yantorno et al., 1971), rat (Levine and Sowinski, 1970), mouse (Malkiel and Hargis, 1970), bull (Paronson et al., 1971), rhesus monkey (Andrada et al., 1969), Japanese quail (Wentworth and Mellen, 1964) and man (Mancini et al., 1965).

Similar autoimmune states may also arise as a result of physical damage or trauma (Broughton and Spector, 1963), heat (Eyquem and Krieg, 1965) or cold (Ablin and Soanes, 1972) or by injection of brain tissue (Katsh and Katsh, 1965).

The primary site for such immune lesions appears to be the spermatogenic tissue itself (Rumke and Hekman, 1975). However, in certain circumstances interstitial tissue and thus androgen metabolism may be affected (Becker et al., 1966), but results in this area are contradictory and have included Leydig cell hyperplasia (Yantorno et al., 1971). Normally interstitial cell damage only seems to follow extreme exfoliation of the germinal epithelium (Behrman, 1964).

The exact nature of the antigens responsible for experimentally-induced orchitis is still open to question and it appears that a variety of antigens associated with mature or maturing spermatozoa, are involved. Low molecular weight polysaccharide-polypeptide complexes associated with the outer surfaces of spermatozoa (Bishop and Carlson, 1965), and enzymes such as sorbitol dehydrogenase (Mancini and Andrada, 1971) are among the antigens implicated. At least three distinct antispermatogenic antigens have been located on the surface of guinea pig spermatozoa (Toullet et al., 1973).

Similarly, the immune mechanisms through which immune activation exerts its effect are uncertain. Some work has demonstrated the formation of antigen specific antibodies following injections of testicular extract plus adjuvant (Chutna, 1970), while others have observed evidence of cellular infiltration and hence cell mediated hypersensitivity (Levine and Sowinski, 1970). In certain cases transfer of sensitivity by leucocytes has been achieved (Laurence et al., 1965). More recent investigations suggest the need for a dual response of humoral and cell mediated components (Brown et al., 1972).

While the bulk of the research work in this area has utilized

common laboratory mammals, some recent work has suggested the potential of testicular autoimmunity as a method of preventing male sexual maturation among production stocks of salmonids (Laird et al., 1978). Injection of salmon parr with testicular extract, together with adjuvant, was reported to be capable of inducing an autoimmune state and to result in total destruction of testicular tissue. These claims were also reported at the 1978 Scottish Marine Biological Association/Highlands and Islands Development Board Conference on Fish Farming (Holiday, unpublished), and confirmation of the effects in male and female Atlantic salmon has since been published (Laird et al., 1980).

However, these initial results were based on examinations of a very limited sample of fish. In the initial report only three fish were examined, of which only two were males.

In view of the possible interest regarding the potential of this method of control, two experiments were carried out to test its effectiveness in preventing sexual maturation among stocks of rainbow trout.

6.2.1 The effect of administration of testicular extract on the reproductive development of rainbow trout

A small scale experiment was carried out at the University of Aston Fish Culture Unit to enable initial evaluation of the technique of autoimmune gonad rejection. Following injection it was planned to hold treated fish until the time of normal maturation, when the effectiveness of the method could be judged.

Materials and Methods

The experiment was begun in January. Rainbow trout (2 groups of 20)

(approximately 60 g weight) from a stock maintained in the Fish Culture Unit were tagged with numbered tags. Fish were selected at random, though any obviously mature male fish were rejected.

The fish were maintained in a recirculating system in the Fish Culture Unit for the duration of the experiment. Water temperature during the experiment was maintained at 12 $\pm$ 1°C. One week after tagging the fish were injected with either testis extract plus Complete Freund's adjuvant (C.F.A) or a control injection of Phosphate Buffered Saline (P.B.S.) plus C.F.A. Each fish was injected intraperitoneally with 200 µl of suspension via a 19G hypodermic needle and 1 ml syringe. Four weeks after the initial injection, a second injection was given to each group. The experimental group received an injection of sperm in P.B.S. while controls received P.B.S. only.

Injection Schedule

Group	Experimental	Control
Week 0	Injection 1	Injection 1
	200 µl Testis extract + Complete Freund's adjuvant.	200 µl P.B.S. + Complete Freund's adjuvant.
Week 4	Injection 2	Injection 2
	200 µl Sperm in P.B.S.	200 µl P.B.S.

Preparation of injections

Injection 1

Preparation of testis extract. A testis extract was prepared using the method described by Laird et al. (1978). Testes were removed from a freshly maturing male rainbow trout weighing 250 g. The total weight of gonadal tissue used was 10.6 g.

Testes were chopped with scissors and scalpel and then ground in a glass homogeniser with a small volume of P.B.S. They were then filtered through a fine gauze filter to remove any connective tissue or coarse material. The liquid obtained was centrifuged and the resulting pellet washed three times in P.B.S. and centrifuged (1000 rpm for 5 minutes) between each washing. The final pellet was suspended in 3 ml P.B.S. and mixed with 4 ml Complete Freund's adjuvant (C.F.A.) (Welcome Labs.) using an ultrasonic probe. The resulting emulsion was injected intraperitoneally to the experimental group (200 µl/fish).

Control injection. The initial control injection consisted of 200 µl of an emulsion formed from 3 ml of P.B.S. and 4 ml C.F.A. mixed with an ultrasonic probe.

Injection 2

Sperm in P.B.S. On the day before the second injection was given, milt was stripped from three mature male trout. This was mixed with an equal volume of P.B.S. and further mixed with the ultrasonic probe. This was stored overnight at 4°C prior to injection into the experimental group. Again 200 µl/fish was injected intraperitoneally.

Control injection. This consisted of P.B.S. only.

Nine weeks after the initial injection, five fish were selected at random from each group and a blood sample withdrawn via the Cuverian duct. The fish were sacrificed and the gonads removed, examined and fixed in Bouin's fixative for subsequent histology.

Twenty-seven weeks after the initial injection, a further five fish were sacrificed after blood sampling and the gonads removed, examined and fixed for histology.

The experiment was terminated earlier than planned in September, following a pump breakdown. However, the remaining fish were examined for gross gonadal appearance, though further histological and blood samples were not taken.

Blood samples were assayed for serum testosterone (T) and gonadal samples processed for histological examination; both as described in General Materials and Methods.

Results

a) Hormonal measurements

Serum T levels as measured by R.I.A. are shown in Table 6.2.1.1. Levels were low at all times during the experiment. Initial levels of experimental (1.49 ± 0.24 ng/ml) and control fish sampled (1.65 ± 0.42) were not significantly different when compared by a t test. In both groups there was a significant fall in T levels between day 0 and day 63, with levels of 0.70 ± 0.1 ng/ml (experimental) and 0.51 ± 0.1 ng/ml (control). ($P < 0.05$; Day 0 to Day 63). By the last sampling, serum T levels had risen to 2.96 ± 0.66 ng/ml (experimental) and 4.08 ± 1.10 ng/ml (control); in the case of the experimental group, a significant ($P < 0.05$) rise over both day 0 and day 63 samples. At no time was there a significant difference between the serum T levels of the two groups.

b) Histological examination

Histological examination of gonadal tissue from samples taken on days 63 and 190 of the experiment was carried out (Table 6.2.1.2). Initial examination of samples from early in the experiment (day 63) showed evidence of a lesion in the testes of one fish of the two male fish examined from the experimental group. In this fish local breakdown

Table 6.2.1.1

Serum testosterone levels of testis-extract injected and control rainbow trout sampled at 63 and 190 days after injection (ng/ml $\pm$ SE)

Time (days)	0	63	190
Testis extract injected n	1.49 $\pm$ 0.24 5) (*)	0.70 $\pm$ 0.1 5) (*)	2.96 $\pm$ 0.66 5)
Control injected n	1.65 $\pm$ 0.42 4) (*)	0.51 $\pm$ 0.1 5) (*)	4.08 $\pm$ 1.10 5)

t test vs. next sample, eg. day 63, day 0, (*) $P < 0.05$

Table 6.2.1.2

Histological examination of male rainbow trout treated with either testis extract or control injections

Time	Testis extract injected	Control injected
Day 63	1) Some lymphocyte infiltration and slight breakdown - peripheral only. Membrane thickening. Spermatogonial cysts. 2) Normal immature. Spermatogonia.	1) Normal immature. Spermatogonial cysts. 2) Normal immature. Spermatogonia. Some lymphocyte infiltration and thickening.
Day 190	1) Normal male. Spermatids - spermatogonia. 2) Normal male. Spermatogonia - spermatocytes. Some local thickening and lymphocyte infiltration. 3) Normal male. Spermatids - spermatogonia.	1) Normal male. Spermatogonia - spermatids. 2) Normal male. Spermatogonia - spermatocytes.

of testicular structure appeared to have taken place allied to infiltration of presumptive lymphocytes. Some thickening of the outer testicular membrane was also evident. However, this lesion was not seen in the other experimental male examined, and as possible evidence of limited lymphocyte infiltration and a local tissue reaction was also seen in an injected control male, the specificity of such a response may be questioned.

Again examination of samples at 190 days proved equivocal. Among experimental fish only one of the males examined showed any evidence of any type of lesion, and this consisted mainly of lymphocyte infiltration and membrane thickening. The two other males examined appeared normal with no evidence of germ cell damage or loss. Control fish examined appeared normal.

No effect of treatment was observed in the ovarian tissue examined.

c) Terminal samples

Following the early termination of the experiment in September, the remaining fish were examined to ascertain their gross gonadal state. Due to tag loss it was not possible to positively identify all remaining fish. However, the following observations were made. In the experimental group, the testes of 3 males, of the 4 male fish identifiable from this group, contained spermatogenic areas. Similarly, in the 3 males identified as controls, 2 contained spermatogenic testes.

Discussion

The effectiveness of a method of controlling maturation of male salmonids must ultimately be judged by its effectiveness in preventing

maturation. Within this preliminary experiment, a number of male experimental fish appeared to be approaching maturity at the termination of the experiment. It was not possible to hold these fish until December, when they would have been expected to be mature, but the presence of spermatozoa in the testes of some experimental fish indicated that they would probably have reached full maturity at the normal time.

In addition, the method of autoimmune gonad rejection appears less than fully effective by both histological and hormonal indices of maturation. No histological evidence of widespread gonadal breakdown and loss of germinal epithelum was observed. All male fish examined contained normal spermatogenic cells, which during the experiment appeared to undergo division to give rise to apparently normal spermatozoa, in at least some fish.

Lesions observed in the testes were not well developed and similar early lesions appeared to be present in both control and experimental fish. This suggests that the lesions seen may be due to the injection of adjuvant rather than a specific response to the injection of testis extract-adjuvant combinations. Laird et al. (1980) have reported a low level of adjuvant derived lesions within their control group, suggesting that Complete Freund's adjuvant can cause such lesions.

Hormonal assays showed no significant differences in serum T between groups at any sampling period in the experiment, though significant differences were seen within the groups with an initial fall after 63 days and an increase in T levels at 190 days. Again this temporary fall was seen in both groups and the cause remains

uncertain, though it cannot be a specific effect of testis extract injection.

6.2.2 The effect of administration of testis, ovary or combined extracts upon the subsequent reproductive development of rainbow trout

This experiment was an extension of the previous small scale experiment on autoimmune rejection. Its purpose was to evaluate the long-term effect of testis extract treatment on gonadal development, in fish kept to the time of normal maturation. In addition, an ovarian extract and a combined testicular/ovarian extract were administered in order to determine if an effect could be demonstrated on the female gonad.

Materials and Methods

The experiment was begun at Shearwater Fish Farming's Low Plains Farm in March. Rainbow trout of approximately 80 g weight were used. Fish were divided into four groups at random and injected according to the schedule outlined below. Thus testis extract (TE) injected fish received a first injection of testis extract plus C.F.A. with a follow-up injection of testis extract plus C.F.A. four weeks later. Ovary injected (O) fish received a similar treatment except that an ovarian extract plus C.F.A. was substituted. The third group were injected with a mixed extract of testis/ovary on both occasions. Control fish were injected with P.B.S. plus adjuvant only. Preparation of the individual extracts is also outlined below.

Group	Testis (TE)	Ovary (O)	Testis + Ovary (TE + O)	Control (C)
Injection 1 -	Testis extract (1) + C.F.A.	Ovarian extract (1) + C.F.A.	Testis/ovarian extract (1) + C.F.A.	P.B.S. + C.F.A.
Injection 2 -	Testis^{+CFA} extract (2)	Ovarian^{+CFA} extract (2)	Mixed extract (2) + C.F.A.	P.B.S. + C.F.A.

Preparation of injections

Injection 1

Testis extract (1). The testes were removed from a pair of mature rainbow trout and processed as described in the previous experiment. The pellet resulting from the final P.B.S. wash (16 g tissue) was divided and 8 g mixed with 15 ml of P.B.S. and 15 ml of C.F.A. with the ultrasonic probe. 200 µl of this emulsion was injected intraperitoneally into each fish.

Ovarian extract (1). The ovaries were removed from an immature trout and treated in the same way described for testis tissue. The final pellet, weighing 12 g, was split into two, and 6 g ovarian tissue mixed with 15 ml P.B.S. and C.F.A. using the ultrasonic probe. 200 µl was injected.

Testis/ovary extract (1). 6 g of ovarian material and 8 g testis material were pooled and mixed with 15 ml of P.B.S. and 15 ml of C.F.A. 200 µl of this mixed emulsion was injected.

Control injection. This consisted of 15 ml P.B.S. and 15 ml C.F.A.

Injection 2

The preparation of these injections was substantially the same as has been described. The only differences reflect the differing amounts of tissue pellet incorporated into each extract. Again 200 µl was injected per fish.

Testis extract (2). 10 g testis 'pellet' + 15 ml P.B.S. + 15 ml C.F.A.

Ovarian extract (2). 2 g ovarian 'pellet' + 15 ml P.B.S. + 15 ml C.F.A.

Testis/ovarian extract (2). 10 g testis 'pellet' + 2 g ovary 'pellet' + 15 ml P.B.S. + 15 ml C.F.A.

Control. 15 ml P.B.S. + 15 ml C.F.A. only.

This second injection was administered four weeks after the first.

Fish were maintained in 800 l fibreglass tanks at a constant temperature of 9°C and fed according to manufacturer's feed tables (B.P. Nutrition). They were maintained until November, by which time the male fish would be expected to be sexually mature and fish from each group were sampled at this time and on subsequent occasions.

Following weighing and blood sampling, fish were killed and the gonads removed and weighed to allow calculation of the gonadosomatic index (G.S.I.). Secondary sexual characteristics were assessed visually.

Following a routine fish movement in December, the female injected group was accidentally mixed with a second batch of fish. As fish were not individually marked, this group had to be discounted.

Results

a) Growth

The growth of fish during the course of the experiment is illustrated by Table 6.2.2.1 and Graph 6.2.2.1 showing the mean body weight of fish $\pm$ SE (g).

In both groups receiving testis extract, either alone or in combination with ovarian extract, a reduction in growth was observed. This was seen in the initial batch-weighing in July, though this was not statistically tested. However, in the November sampling, testis-injected fish weighed significantly less (C vs. TE, $P < 0.01$; C vs. (TE + O), $P < 0.05$). However, though testis-injected fish weighed less in December, this effect was not statistically significant.

b) Mortalities

Mortalities among all groups were similar, with the bulk of mortalities occurring in the month following the first injection. From November onwards, mortalities appeared to rise within all groups. Though no accurate mortality information was available, the bulk of the mortalities within this period appeared to be mature or maturing males.

c) Maturation within groups

Sexual maturation was assessed within experimental and control groups by the presence or absence of spermiation and male secondary sexual characteristics (i.e. darkening, kype development). The number of mature male fish as a percentage of the number of males examined at each sampling is shown in Table 6.2.2.2.

The number of mature males within each group was not significantly

Table 6.2.2.1

Mean Body weights of gonad extract and control injected rainbow trout sampled at various times after injection

Mean body weight (g) ± SE

Date	Control (C)	Testis extract (TE)	Ovary extract (O)	Testis extract + ovary extract (TE + O)
July 79 +	164.5	128.0	183.3	135.4
n	20)	20)	20)	20)
Nov. 79	485.6 ± 33.9	333.7 ± 14.6	514.9 ± 28.6	379.3 ± 20.6
n	20)	22)	21)	22)
Dec. 79	524.2 ± 44.2	452.0 ± 26.3	577.9 ± 35.6	471.2 ± 30.9
n	10)	10)	12)	9)
Mar. 80	820.1 ± 71.9	823.8 ± 26.3	-	738.8 ± 53.6
n	15)	21)		13)
May 80	1009.4 ± 71.4	911.3 ± 109.9	-	940.0 ± 55.3
n	9)	9)		10)

(**) (*)

+ weight of 20 fish weighed as batch.

t test vs. controls ** $P < 0.01$
* $P < 0.05$

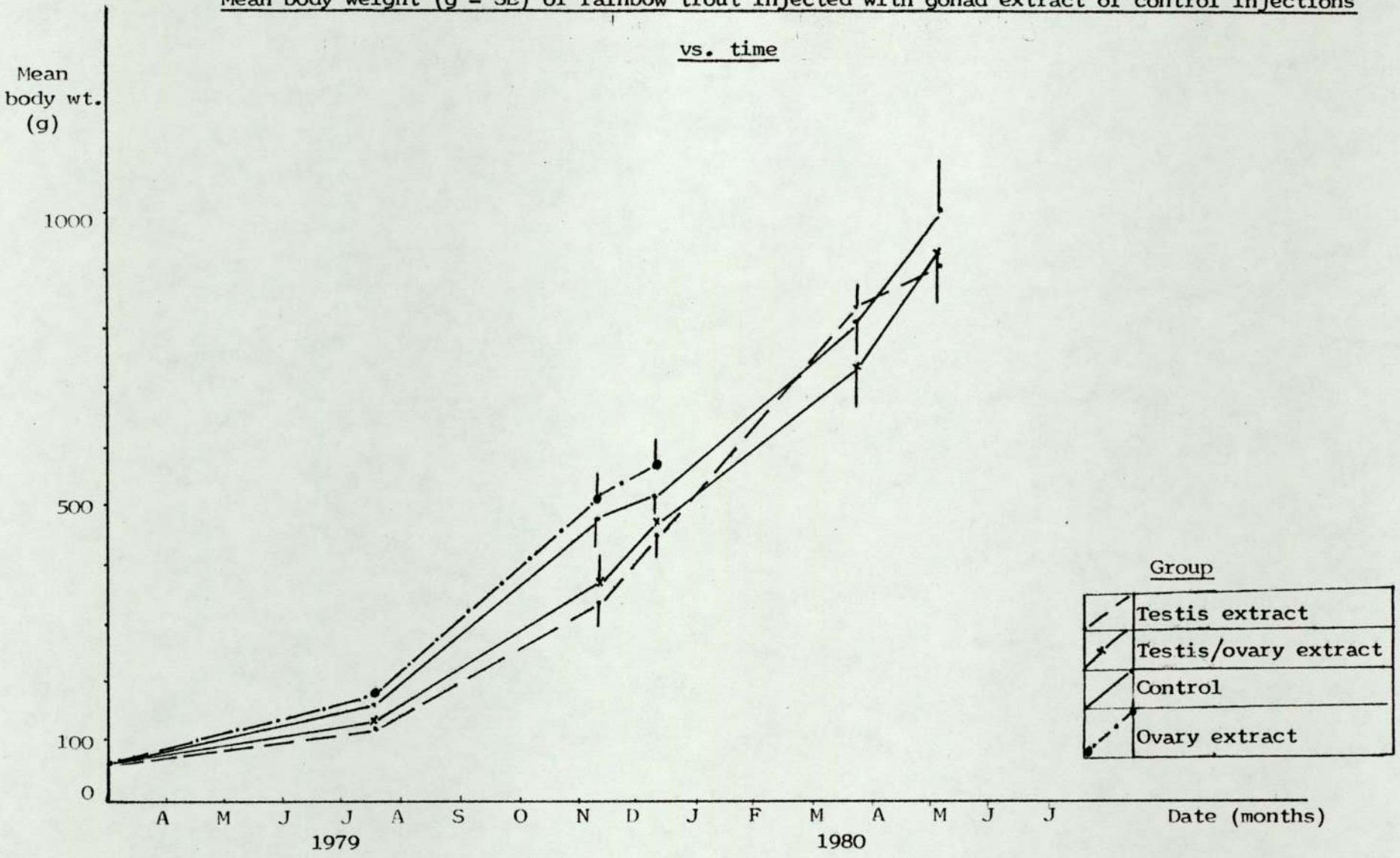
Table 6.2.2.2

% male maturation within gonad injected and control injected rainbow trout expressed as a % of the total number of males examined from each group

Group	C	TE	O	TE + O
Nov.	50	44	45	50
Dec.	60	50	50	50
Mar.	50	43	-	50

Graph 6•2•2•1

Mean body weight (g ± SE) of rainbow trout injected with gonad extract or control injections vs. time



different from that in the control group at any sampling date, when compared by a chi-squared test. A constant, though relatively low, level of male maturation was observed on all sampling dates. Approximately 50% of all males examined were mature.

d) Gonadosomatic Index

The G.S.I. (Gonadosomatic Index) was calculated for each group from the formula $G.S.I. = \frac{\text{Gonad wt.}}{\text{Body wt.}} \times 100$

The G.S.I. data reflect the generally low incidence of mature males all through the experimental group.

G.S.I. results are displayed in Tables 6.2.2.3, 6.2.2.4 and 6.2.2.5, which show the mean G.S.I. $\pm$ SE for mature males, immature males and females at each sampling period.

The G.S.I. of mature males from the control group was highest in November (3.56 ± 1.35) and fell to 1.27 ± 0.49 in March. A similar effect was noted in other groups with the exception of the (TE + 0) group where the maximum G.S.I. of mature males was observed in December (3.57 ± 2.12). No significant statistical difference was observed between the mean G.S.I.'s of experimental and control groups at any time during the experiment using Student's t test.

The G.S.I. of immature male experimental fish showed a similar pattern to that of immature control males, with a slight fall in G.S.I. from November to December, followed by an increase in March. Thus the control mean G.S.I. fell from 0.065 ± 0.006 in November to 0.04 ± 0.01 in December, with a rise to 0.07 ± 0.01 in March. Again, no statistically significant differences between mean G.S.I.'s within groups were observed.

Table 6.2.2.3

Mean gonadosomatic indices of mature male rainbow trout from gonad extract or control injected groups

G.S.I. ± SE

Group	Nov. 79	Dec. 79	March 80
Control	3.56 ± 1.35	1.84 ± 0.48	1.27 ± 0.49
n	5)	3)	3)
TE	5.04 ± 1.39	2.30 ± 0.61	1.13 ± 0.21
n	4)	3)	3)
0	3.04 ± 0.60	2.79 ± 0.30	-
n	5)	3)	
(TE + 0)	2.21 ± 0.92	3.51 ± 2.12	1.63 ± 0.36
n	3)	3)	3)

Table 6.2.2.4

Mean gonadosomatic indices of immature male rainbow trout from gonad extract or control injected groups

G.S.I. ± SE

Group	Nov. 79	Dec. 79	March 80
Control	0.065 ± 0.006	0.04 ± 0.01	0.07 ± 0.01
n	5)	2)	3)
TE	0.04 ± 0.006	0.03 ± 0.003	0.045 ± 0.01
n	5)	3)	4)
0	0.044 ± 0.009	0.04 ± 0.01	-
n	6)	3)	
(TE + 0)	0.052 ± 0.006	0.04 ± 0.01	0.06 ± 0.003
n	6)	3)	3)

Table 6.2.2.5

Mean gonadosomatic indices of female rainbow trout from gonad extract or control injected groups

G.S.I. ± SE

Group	Nov. 79	Dec. 79	March 80
Control	0.075 ± 0.006	0.098 ± 0.004	0.19 ± 0.03
n	10)	5)	10)
TE	0.09 ± 0.0002	0.10 ± 0.02	0.20 ± 0.01
n	12)	5)	13)
0	0.076 ± 0.008	0.090 ± 0.009	-
n	10)	6)	
(TE + 0)	0.085 ± 0.009	0.11 ± 0.02	0.17 ± 0.01
n	13)	4)	9)

Table 6.2.2.6

Mean Serum T levels (ng/ml ± SE) of gonad extract or control injected rainbow trout (Mean ± SE (ng/ml))

Sample date 12.11.79

	Mature Males	Immature Males	Females
Control	62.07 ± 20.46	5.1 ± 1.07	3.82 ± 0.86
n	4)	4)	4)
TE	58.06 ± 14.6	5.80 ± 1.25	-
n	3)	4)	
TE + C	58.80 ± 9.96	3.72 ± 0.97	4.94 ± 0.88
n	3)	4)	4)

In contrast, the G.S.I.'s of female fish of all groups showed a steady increase during the experiment. The control mean G.S.I. of 0.075 ± 0.006 (November) rose to 0.098 ± 0.004 (December) and to 0.19 ± 0.03 (March). A similar rise was observed in all groups. Thus the (TE + O) group's mean G.S.I. rose from 0.085 ± 0.009 in November to 0.17 ± 0.01 in March. The ovary extract injected group (O) also showed an increase in mean G.S.I. from 0.076 ± 0.008 (November) to 0.090 ± 0.009 in December. No statistically significant difference between the mean G.S.I.'s of groups were observed at any sampling date.

e) Hormone levels

The November serum samples were assayed for serum T to provide a second index of maturation and also to indicate any possible interstitial cell dysfunction. The results are shown in Table 6.2.2.6. Control mature males had levels of 62.07 ± 20.46 ng/ml in November. Slightly lower mean values were observed in the TE group (58.06 ± 14.6) and (TE + C) group (58.80 ± 9.96). These differences were not statistically significant. Immature males within each group had similar serum T levels. Immature males from the control group had a mean T level of 5.1 ± 1.07 ng/ml, while the mean T level of TE injected immature males was 5.8 ± 1.25 ng/ml. A slight but not statistically significant fall was observed in the (TE + C) group, with a mean level of 3.72 ± 0.97 ng/ml. None of these mean values was significantly different from control mean values.

f) Histological examination

Histological examination of gonads removed at the November sampling failed to reveal any significant germ cell destruction arising from gonad extract treatment. The testes of mature males from each group

appeared similar, with masses of spermatozoa packing the testicular tubules. Examination of immature fish from each group revealed a fundamentally similar picture, with the mass of the testes composed of spermatogonial cysts with some division to spermatocytes evident. The only evidence of any effect of treatment was the occasional presence of local thickening of the testicular membrane, and in two fish examined from the TE injected group, a slight increase in fibrous tissue. This thickening was also seen in control injected fish in addition to experimental fish. No evidence of any effect was observed in female fish in the control group, while in the ovary-injected group only one female of the five examined showed any unusual gonad structure, limited to a slight increase in fibrous tissue.

In summary, gonadal histology showed no evidence of destructive gonad lesions of the type which have been demonstrated im mammalian work and which are reported by Laird et al. (1978, 1980). Changes observed were limited to slight local lesions and increases in fibrous tissue and were observed within both control and experimental groups.

Discussion

In this experiment the injection of gonad extract plus adjuvant was ineffective as a specific method of preventing maturation of male trout. No statistically significant differences were observed between the number of mature male fish in the experimental and control groups. There was also no significant difference between the experimental and control groups with respect to maturation related parameters such as G.S.I. and serum T levels.

No difference was observed in gonad histology between groups, with no evidence of widespread germinal tissue damage. The only observable

histological effect of injection appeared to be a slight local thickening of the testis membrane and an increase in fibrous tissue within certain of the gonads examined. As this effect was seen in both control and gonad injected fish, it appears to be a non-specific effect of injection, possibly related to the Complete Freund's adjuvant administered.

One feature which was apparent in the experiment was the relatively low level of male maturation within male fish of all groups. Again this effect was a non-specific one and was seen in both control and gonad injected groups. No evidence of gonadal degeneration was found in the immature gonads examined histologically. The G.S.I. data obtained at sampling suggests a single peak of male maturation in November - December, while the immature males show no increase in G.S.I. over the course of the experiment. Thus suggesting that the immature fish were not merely late developing.

One factor which may have influenced this apparent high number of immature males is the selective higher mortality of mature males. However, this explanation fails to fully explain the observed results.

Due to lack of tank space, no uninjected control group was included in the design of the experiment, so it is not possible to comment further on the cause of this low level of male maturation. Again it may be related to a non-specific effect of C.F.A. injection, though not apparently acting directly at the gonad level.

Although it was not possible to continue the ovary injected group beyond December, no effect of injection was apparent up to this time. This was also the case in the group receiving a mixed injection of ovary and testis (TE + O) extracts. A steady increase in ovarian G.S.I. was observed over this period in females within all groups.

Histological examination revealed no major changes in ovarian structure.

Injection of gonadal material appeared to cause no increase in mortality as opposed to control injections. A statistically significant depression of growth was seen to result from the injection of testicular (TE) extract and combined (TE + O) extracts when fish were examined in November. This depression of growth was no longer evident by December.

6.3 General Discussion and Conclusions

Immunological approaches towards the control of maturation of salmonids have been examined in this chapter. While the technique of promoting immunity towards reproductive hormones has been, at least partially, effective in mammals (Fraser et al., 1974, 1975; Edwards and Johnsone, 1976; Raghavan et al., 1977), in teleosts it appears less so, though Wiegand and Idler (1980) report that the injection of antibody to glycoprotein gonadotropin blocked early ovarian growth in salmon.

Some encouraging results have been reported using the method of autoimmune gonad rejection (Laird et al., 1978, 1980), but in both experiments described in this chapter the attempted induction of an autoimmune state resulting in gonad rejection was unsuccessful. While the small scale experiment carried out at the University of Aston Fish Culture Unit provided some histological evidence of a slight testicular reaction, involving lymphocyte infiltration, this appeared to be a non-specific effect. The larger scale experiment at Shearwater Fish Farming Ltd. also produced no evidence of a significant anti-gonadal effect resulting from injection of gonad extract and adjuvant. No difference was seen between experimental and control groups in maturation and related parameters such as G.S.I. and serum T levels.

The failure of this method to achieve an effect upon maturation was also observed in a small scale trial run by Shearwater Fish Farming and our laboratory at Finnarts Bay. Rainbow trout of approximately 70 g weight were injected with testis extract - C.F.A. in February 1978, with a repeat injection in March 1978. The fish were maintained at Finnarts Bay until March 1980 when they were examined. All the three year old fish remaining were mature. No evidence of any antitesticular effect was observed.

The failure of autoimmune gonad rejection has also been reported in non-salmonid teleosts. It has proved ineffective in the prevention of maturation of turbot (Bye; 'Sexuality in Fish', Symposium of Fisheries Society, 1981, University of Aston in Birmingham).

These negative results contrast with the reports of Laird et al. (1978, 1980) and with the majority of the mammalian work (Freund et al., 1955; Behrman, 1964; Bishop and Carlson, 1965; Andrada et al., 1969; Levine and Sowinski, 1970; Malkiel and Hargis, 1970; Paronson et al., 1971; Yantorno et al., 1971). However, the difference may be that Laird et al. did not keep fish until the age of normal maturity and the success of the method was judged on histological evidence. The lesions observed may well be transient and not progressive as interpreted. A range of responses was observed ranging from a simple surface lesion to a deep lesion resulting in germinal tissue degeneration, suggesting that fish are not equally succeptible to treatment.

A number of additional reasons may be put forward to explain the variations in results obtained. Laird and co-workers have obtained their most promising results using Atlantic salmon (Salmo salar), which may be more succeptible to this technique.

A further variable is the environmental temperature. If the failure of the present series of experiments to cause widespread gonadal breakdown is indeed a failure of immunological activation, then temperature may be implicated in such a failure. While rainbow trout will produce antibodies at temperatures as low as 5°C, teleost immune responses generally improve with increasing temperature (Roberts, 1978). It seems the main effect of temperature on antibody production is at the primary phase of antibody induction (Avtalian et al., 1973).

Laird et al. suggest that the immune response would be speeded up by injecting fish when environmental temperatures were high, and certainly above 10°C. However, the pilot scale experiment at Aston was carried out at 12°C without success. So while temperature may play a part in the failure of this method at Low Plains (9°C) and Finnarts Bay (4 - 16°C), it should not be limiting in the Aston experiment.

A further factor suggested by Laird et al. is the age of fish at injection. They suggest injecting fish as early as possible. All rainbow trout used in our experiments were injected at approximately one year old. However, Laird et al. base this argument upon the appearance of a progressive lesion among injected fish and little evidence of such a progressive effect was observed in the experiments reported here.

As mentioned in the introduction, a number of problem areas still need to be resolved with respect to autoimmune gonad rejection in mammals. The exact nature of the antigen capable of provoking this state and the complete mechanisms by which it occurs require clarification

(Rumke and Hekman, 1975). In the mammalian testis an autoimmune effect is usually seen first at the area of the Rete testis, where it is suggested the blood-testis barrier is at its weakest (Johnson, 1970b).

Owing to the different structure of the teleost testis and the apparent absence of any such immunological 'weak-spot', any immune attack must proceed from the external surface of the gonad. So the external membrane of the gonad may act as a barrier to such attack. Laird et al. (1980) suggest that the lesions they observed were induced by means of macrophages from the peritoneal cavity. However, in the fish used in the Shearwater experiment, the number of peritoneal leucocytes harvested (mainly macrophages) was low. Using a technique widely used in amphibia, peritoneal leucocytes from immunised fish were collected. However, due to low yields the planned migration inhibition factor (M.I.F.) assay was not completed. In these injected fish it appears macrophages were not present in the peritoneal cavity in large numbers following immunisation. If indeed peritoneal macrophages are essential for the responses described by Laird et al., the failure of the method in the experiments described may reflect a failure of macrophage proliferation and activation.

In order for this technique to become commercially viable, the questions regarding its effectiveness must be clarified. In the two experiments reported in this chapter no significant effect of treatment was observed in rainbow trout. While it may be possible to improve results with variations in environmental parameters, it seems that the technique as it stands yields too variable a response to offer wide potential as a method of suppressing male maturation. If success necessitates very specific conditions, such conditions may be too specific and limit its commercial value. So although the technique

is theoretically attractive, especially if a purified antigen capable of administration by the osmotic immersion method of Amend and Fender (1976) could be produced, its effectiveness remains to be demonstrated.

So it would appear that the immunological techniques and in particular the autoimmune testicular rejection technique have not yet reached the stage at which they may be commercially utilized. Possibly a fundamental problem in the use of these techniques is that the immune system of teleosts is less responsive than that of mammals (Roberts, 1978), and the observed effects may be far more limited than in mammals.

CHAPTER 7

THE USE OF SURGICAL CASTRATION IN PREVENTING SEXUAL MATURATION IN SALMONIDS

Contents

7.1 Introduction

Surgical castration is obviously the most direct method for the prevention of sexual maturation. Removal of gonadal tissue prior to maturation will, in the absence of extra-gonadal sex steroid production, prevent the appearance of secondary sexual characteristics and the associated detrimental effects of maturation. As discussed in earlier chapters, extra-gonadal sex steroid production would seem to be of negligible importance in salmonids.

Surgical castration of teleosts has largely been used as a research technique to study the effects of a removal of natural sex steroids in a variety of species. These include Betta splendens, Tilapia sp., Poecilia sp. (Hoar and Randall, 1969), Lampetra fluviatilis (Pickering, 1976), Oncorhynchus nerka (McBride et al., 1963; McBride and van Overbeeke, 1969; Donaldson and Fagerlund, 1970) and Salmo gairdneri (Robertson, 1958; Schreck et al., 1972b; Billard et al., 1976, 1977, 1982; Wunder, 1977). Only Brown and Richards (1979) and Brown (1983) have reported the use of the technique as a possible routine measure aimed at avoiding the adverse effects of maturation.

Two main approaches have been adopted for the surgical castration of teleosts. The most frequently used is that of Robertson (1958). This operation necessitates a ventral incision of about 3 cm. An alternative incision site was used by Wunder (1977), who made use of two lateral incisions just below the lateral line and behind the pectoral girdle.

7.2 The short-term evaluation of alternative techniques for the surgical castration of salmonids

In order to evaluate the methods of surgical castration of salmonids and to provide information on the completeness of castration and short-term survival following operation, an experiment was carried out at the University of Aston Fish Culture Unit.

Materials and Methods

Rainbow trout of approximately 70 g weight, reared at the Aston Fish Culture Unit, were used in this experiment. Forty trout were split at random into two groups of 15 and one group of 10 fish, and were housed in three tanks of a recirculating system. After one week's acclimatization, the fish were removed and operated on, then returned to the recirculating system for the duration of the experiment. The operation schedules were as described below.

Group A	Group B	Group C
15 fish	15 fish	10 fish
Castrated by method of Robertson (1958).	Castrated by method of Wunder(1977).	Sham castrated, 5 by each method.

Group A (after Robinson)

Fish were anaesthetised using benzocaine and placed in a damp cloth on a dissecting board. A ventral incision was made beginning at the pectoral fins and running caudally for about 3 cm. A pair of shaped metal retractors was used to clear the field of view and expose the cephalic attachment of the gonad, which was then cut free. The gonad was then withdrawn via the incision and the caudal insertion

of the gonad located and freed. The gonad was then removed, and following the application of 5 - 10 mg of penicillin to the body cavity, the incision was closed with three lock sutures. The total time for the operation was around 3 - 4 minutes.

Group B (after Wunder)

This method requires a lateral incision just below the lateral line and behind the pectoral girdle. Following benzocaine anaesthesia the fish was wrapped in damp cloth and the incision made along the lateral flank as described. After retracting the lips of the wound, the gonad was removed via the incision. The incision was closed by a single lock suture and the fish turned over and the operation repeated on the other gonad. The time taken for this operation was very variable, depending upon the initial location of the gonad. Normally, operations were completed within 4 - 5 minutes.

Group C

This group were subjected to sham castration by either the method of Robertson or Wunder. Five fish were sham operated by each method.

It was originally planned to hold the fish for three or four months, but following the appearance of wound infections among a number of fish of all groups, it was decided to terminate the experiment after six weeks. At this time the fish remaining were sacrificed, carefully dissected and examined under a binocular microscope for the presence of any residual gonad tissue.

Results

a) Mortalities

Immediate post-operative survival of all fish was reasonable. Three fish in Group A failed to recover from anaesthesia, probably as they were the last fish to be anaesthetised and the anaesthetic may have become depleted of oxygen. Only one fish in Group B failed to recover from anaesthesia, as did one fish in the control sham operated group.

Post-operative mortalities increased after about 7 - 10 days and two and three fish in Groups A and B respectively died at this time. Due to the appearance of infected wounds among some fish, it was decided to terminate the experiment at 41 days post operation.

b) Autopsy of fish

Examination of the remaining fish following the termination of the experiment after six weeks revealed a relatively high proportion of fish still retaining some portion of gonadal tissue; in all cases at the anterior gonad insertion. Within Group A, five fish showed evidence of incomplete castration, while a further six fish of Group B contained gonadal fragments.

Discussion

Relatively high mortalities were observed in this experiment compared to previous reports giving details of mortalities (Schreck et al., 1972b; Brown and Richards, 1979). It seems likely this was due to the small size of fish used in these experiments. Most workers have used fish of 150 g plus, which may improve post-operative survival. Brown (1983) notes that the 250 - 300 g (22 - 25 cm) size range is the optimum for

successful castration of rainbow trout. Despite the addition of penicillin via the incision wound, infections were observed in a number of fish. This may reflect the maintenance of fish in a recirculating system where bacteria may build up in the faecal trap and filter gravel.

Residual gonad fragments were recorded in five fish in Group A and in six fish from Group B, showing that in a substantial number of cases incomplete castration resulted. This seems to be due to the anterior portion of the gonad splitting during removal and springing back into the body cavity. In all cases but one, the fragment of gonad remaining was in the anterior end of the gonad. Again this may reflect the small size of fish used relative to other workers, as reduced size means the anterior insertion of the gonad is difficult to locate.

If, however, the occurrence of residual gonadal tissue following surgical castration is widespread in larger fish, the effectiveness of the method may be limited by problems of regeneration. Compensatory hypertrophy of testis tissue has been demonstrated by Robertson (1958) and Wunder (1977). Even if a small fragment of testicular tissue remains, it would be sufficient to regenerate a testicular nodule capable of producing androgens and bringing about the deleterious secondary sexual characteristics. Work carried out at the University of Stirling suggests that this may indeed happen (Brown, 1983) and that gonad regeneration poses a problem even in larger fish.

At present surgical castration can only be performed by either a veterinary surgeon or an authorised Home Office licence holder, though in the latter case this may only be for experimental purposes. Thus it is likely that the cost per fish will be high, even if the

operation can be performed within ninety seconds, as claimed by Brown and Richards (1979).

If, as has been suggested (Brown, 1983), fish size is critical, surgical castration would not offer any potential for controlling precocious maturation of males. Its main value would seem to be in the production of relatively small numbers of very large fish, where the high cost per fish could be recovered. The main limitation would still be the effectiveness and freedom from regeneration which is still to be demonstrated. Indeed Brown (1983) recorded that the greatest disadvantage of the technique is in the regeneration of male testicular tissue.

CHAPTER 8

THE ADMINISTRATION OF EXOGENOUS STEROIDS DURING EARLY GONADAL DEVELOPMENT

Contents

8.1 General Introduction

The differentiation and development of the salmonid testis and its endocrine control have already been described. A considerable body of literature has detailed the effects of exogenous steroid treatment upon the subsequent reproductive development of both salmonid and non-salmonid teleost fish (Yamamoto, 1953, 1958, 1959; Ashby, 1957; Clemens and Inslee, 1968; Yamamoto et al., 1968; Yamamoto and Kajishima, 1968; Yamazaki, 1972, 1976; McBride and Fagerlund, 1973; Okada, 1973; Jalabert et al., 1974, 1975; Schreck, 1974; Fagerlund and McBride, 1975; Guerrero, 1975, 1976; Wenstrom, 1975; Simpson, 1976; Simpson et al., 1976, 1979; Johnstone et al., 1978; Bye and Lincoln, 1979, 1981; Goetz et al., 1979; Jensen and Shelton, 1979; Okada et al., 1979; Harbin et al., 1980; van den Hurk et al., 1980; Scott et al., 1980a; Donaldson and Hunter, 1982a).

A wide range of responses to steroid treatment have been observed depending upon the steroid used, its dose, method of administration and the stage of maturity at which fish are treated. The administration of exogenous steroids has suggested several possible ways in which the reproductive development of salmonid fish may be influenced for possible commercial advantage. This chapter will concentrate upon two main effects; that of sex reversal, in which steroid treatments result in a shift in phenotypic sex development and the possible sterilizing effects of high doses of androgens.

8.1.1 Sex reversal

The differentiation of the indifferent teleost gonad to either the male or female type is succeptible to the administration of

exogenous steroids during the critical period of differentiation, and early work in this field has been summarized by Yamamoto (1969).

a) Feminization

The use of estrogenic steroids such as 17B-estradiol (E_2) and estrone has been reported to shift gonadal development to the female type. An early attempt to influence the sexual development of salmonids by such means was that of Padoa (1937, 1939) in which immersion of juvenile rainbow trout in aqueous solutions of E_2 produced an increase in the percentage of females. Ashby (1957) showed 70% of the fish surviving immersion in 300 µg/L E_2 for 880°C days to be female, suggesting E_2 treatment shifted phenotypic sex differentiation to the female type. Estrone has also been used to attempt to feminize genetically male rainbow trout (Okada, 1973). This author fed estrone at 10, 50 and 100 mg/kg diet for 58 - 124 days and showed 79 - 94% of treated fish to be female at two months post-feed. However, if feeding was delayed until one month after hatching, estrone doses of 30 - 120 mg/kg diet fed for five months produced only 54% female fish at two years, though 30% of the fish examined were hermaphrodites.

Immersion of eyed eggs and alevins and dietary administration of E_2 at 20 mg/kg diet from early feeding produced feminization of salmon (Salmo salar) and rainbow trout (Simpson, 1976; Simpson et al., 1976). All salmon smolts examined were female following E_2 immersion prior to first feed and subsequent E_2 feeding for 120 days. All rainbow trout receiving dietary treatment for 30 and 56 days from first feeding were female, though only 70% of the trout examined were female if feeding was not continued beyond 15 days from first feeding. These results illustrate the importance of continuing treatment throughout the critical period of gonad development.

In contrast, contradictory results regarding the timing of treatment and method of administration of oestrogens were reported by Johnstone et al. (1978). Total feminization was achieved in salmon by a treatment regime consisting of either immersion of eyed eggs or alevins in E_2, followed by E_2 feeding (20 mg/kg diet) for 21 or 40 days following first feeding, or by dietary E_2 treatment alone for 80 days following first feeding. Rainbow trout were also successfully feminized by 30 days dietary treatment from first feeding, either with or without pre-immersion at the eyed egg/alevin stage. Thus in these species dietary treatment without pre-immersion in steroid appears to be successful in inducing feminization. However, some variability in the extent of feminization was noted in the above experiments (Johnstone et al., 1978), perhaps related to differences in feeding regimes and growth conditions at the two sites. Simpson et al. (1979) suggest that dietary E_2 treatment (20 mg/kg) for 60 days following first feeding should achieve a 100% feminization.

Direct evidence that E_2 produces feminization of genotypic male rainbow trout has been provided by Johnstone et al. (1979b). Some crosses of female fish resulting from E_2 treatment gave a male : female ratio of 2.2 : 1, rather than the 1 : 1 ratio one might expect from a normal cross, indicating the male genotype of some of these phenotypic females. The ratio achieved probably indicates that the YY genotypes have a low viability and also supports cytological evidence that in salmonids the male is the heterogametic sex (Thorgaard, 1977).

Immersion in aqueous solutions of progestogens such as progesterone has also been reported to feminize rainbow trout fry (van den Hurk et al., 1980). However, in contrast to these reports, Bye (quoted in Donaldson and Hunter, 1982a) reports a failure of E_2, administered at a dose of

20 mg/kg diet for 406°C days, to induce feminization of rainbow trout fry (though it did give rise to 5% hermaphrodites). This result may perhaps reflect differences in the strain of fish used or differences in feeding regimes between this trial and that of Simpson et al. (1976).

Successful feminization has been reported in other salmonid species. In lake trout (Salvelinus namaycush) 12 mg E_2/kg diet fed for 240 - 290°C days, from the time at which gonadal differentiation was normally complete, produced 80% females, perhaps surprisingly in view of the late timing of this treatment (Wenstrom, 1975). E_2 administered at 20 mg/kg diet to first feeding brook trout (Salvelinus fontinalis) resulted in 99% females when fed for 60 days (Johnstone et al., 1979a). Feeding for 40 days resulted in only 67% females with 12% hermaphrodites.

In Pacific salmon (Oncorhynchus kisutch) dietary E_2 (10 mg/kg) treatment for 90 days from first feeding resulted in only 54% females and 27.7% intersex fish (Goetz et al., 1979). In contrast, immersion in doses of E_2 ranging from 50 µg/L during the eyed egg and alevin stage together with feeding 10 mg/kg E_2 resulted in all female populations. In these trials 10 mg/kg E_2 was incapable of producing a significant feminization without pre-immersion in steroid solution. The apparent difference between the results in these trials and those obtained in Atlantic salmon and rainbow trout may be related to a species difference in the rate of gonad development and differentiation. Goetz et al. (1979) suggest that as the requirement for gonad differentiation in coho salmon is 840°C days, while that of Atlantic salmon is greater than 1050°C days (Persov, 1975), there is insufficient time for steroid treatment to achieve such a result without immersion prior to feeding. However, in the rainbow trout gonadal differentiation is

more rapid than in Pacific salmon and is usually complete by 600 - 640°C days (Okada, 1973; Laird et al., 1978; van den Hurk, 1980) and yet successful feminization has been reported in this species without pre-immersion (Simpson et al., 1976).

Direct evidence of the presence of feminized males in Pacific salmon is the occurrence of abnormal sex ratios when phenotypic females are crossed with normal males (Donaldson and Hunter, 1982a). Sex ratios close to 3 : 1 resulted, indicating the presence of XY females. The higher ratio of males compared to the results of Johnstone et al. (1979b) suggests that in Pacific salmon YY fish may have a higher viability than YY rainbow trout.

Donaldson and Hunter (1982a) have reported that, in Pacific salmon, immersion at the alevin stage, followed by doses of E_2 as low as 2 mg/kg can result in 100% feminization.

b) Masculinization

Similar modification of phenotypic sex development has been reported in salmonids following the administration of androgenic steroids, though in this case the pattern of development is switched to the male type (masculinization).

Jalabert et al. (1975) noted an increase in male and hermaphrodite rainbow trout after treating with a range of doses of methyltestosterone (MT) of 15 - 60 mg/kg diet. Yamazaki (1976) observed 87% male rainbow trout in a population fed MT (1 mg/kg diet) from one month after hatching to seven months post hatch. Treatment beginning at three months post hatch did not influence the sex ratio, presumably because differentiation of the gonad was too far advanced at the commencement of treatment.

Simpson et al. (1976) produced totally male groups of salmon and rainbow trout by dietary administration of 3 mg/kg diet MT for 90 and 120 days respectively. Immersion and feeding was also effective but immersion alone had no effect upon the subsequent sexual differentiation of treated eyed eggs or alevins. Immersion followed by a reduced dietary regime of 30 days feeding gave a partial masculinization only. MT treatment appeared to result in a reduction of germ cells within the gonad as well as influencing the differentiation of these germ cells. These results were confirmed by Johnstone et al. (1978).

When phenotypic male rainbow trout from a group fed MT during the critical period of gonad development were examined, only 16% of the males showing male secondary sex characteristics could be stripped of milt by gentle hand pressure. Occlusions and malformation of the sperm ducts seem to explain this inability to release milt. In nine out of ten crosses of these fish with normal females a 1 : 1 sex ratio was observed. In the remaining cross an all female group was produced, indicating the XX genotype of the reversed female (Johnstone et al., 1979b).

Similar results were produced by Okada et al. (1979), though a total masculinization was not achieved even at doses of 10 mg/kg, which gave rise to 88% male rainbow trout. Again the XX genotype was shown to be present in two out of eleven phenotypic males.

A further successful, though unpublished, masculinization of rainbow trout was seen in fry fed 3 mg MT/kg diet for 90 days from first feeding (Bye, 1980 - quoted in Donaldson and Hunter, 1982a). Of the phenotypic males resulting from this treatment 55% could be stripped of milt and gave a 1 : 1 sex ratio when crossed with normal

females. The remaining fish could not be stripped of milt due to gonad and duct abnormalities. When milt was surgically removed and used to fertilize normal ova, fourteen out of sixteen of such crosses gave all female progeny, indicating their XX chromosome constitution.

In other salmonids similar results have been obtained. 70% male populations of lake trout (Salvelinus namyacush) were produced by dietary treatment with testosterone propionate (700 mg/kg diet) (Wenstrom, 1975). In coho salmon (Oncorhynchus kisutch) 98% males resulted from feeding 3 mg MT/kg for 20 days following first feeding, while higher doses resulted in sterilization (Donaldson and Hunter, 1982a).

8.1.2 Production of XX males

The ability to manipulate teleost phenotypic sex differentiation through steroid treatment has been reviewed briefly in the previous section, and functional sex reversal, in both directions, has been recorded in salmonids.

Among salmonids the male is the heterogametic sex (Thorgaard, 1977; Johnstone et al., 1979b; Okada et al., 1979), thus the production of phenotypic males having the female genetic constitution (XX) presents a method of eliminating the male genotype. Genotypic females reversed to phenotypic males by early androgen treatment have been shown to produce all female progeny when crossed with normal females (Johnstone et al., 1979b; Okada et al., 1979; Lincoln and Bye, 1981).

The production of all female populations from XX males would allow the commercial producer to avoid the deleterious effects of male maturation (Bye and Lincoln, 1979, 1981; Simpson et al., 1979). Bye and

Lincoln (1981) report that such a technique produces all female populations, which may be masculinized by MT treatment to provide a stock of XX males for further production of all female fish.

Such a technique has the advantage that treatment would be restricted to brood stock fish and not fish destined for the table. The technique would appear to present an elegant approach to the problem of male maturation among salmonids. However, its use is not without problems. It appears that XX males fail to develop the correct duct structure through which milt may be stripped. Consequently these fish have to be surgically stripped of milt. This may provide difficulties in the assessment of the stage of maturity of the testis and lead to consequent problems with fertility. While Lincoln and Bye (1981) report no reduction in rates of fertilization using sperm stripped from such fish, in some cases very low levels of fertilization have occurred (J. A. Ralph; N. R. Bromage, Pers. comm.). Possibly such effects reflect a failure of spermatozoa to attain full maturity and viability prior to their use.

8.1.3 High dose androgen treatment

Treatment of salmonids, during or before the critical phase of gonadal differentiation, with high doses of steroid hormones and especially androgens, has been shown to suppress gonadal development and to lead to partial or complete sterilization.

Although his results were in part inconclusive, Ashby (1957) reported that immersion of brown trout fry in 50 - 60 ug/L solutions of testosterone propionate gave rise to inhibition of gonadal development. In a later study Jalabert et al. (1975) produced 12% sterile

two year old rainbow trout by feeding fry with 15 - 60 mg MT/kg diet for five months from first feeding. The effects of feeding 50 mg/kg diet MT upon the reproductive development of rainbow trout has also been reported by Yamazaki (1976). Feeding treated diet from first feeding resulted in sterility, which persisted to three years, though very few fish survived to this time.

Atlantic salmon and rainbow trout which appeared sterile resulted from the treatment of fry with 3 mg/kg diet MT for 90 and 120 days respectively, either with or without pre-immersion in steroid at the eyed egg/alevin stage (Simpson et al., 1976). However, only partial success was reported by Bye (quoted in Donaldson and Hunter, 1982a), who attempted to sterilize rainbow trout by the dietary administration of 25 mg MT/kg diet for 90 days following first feeding. Up to 60% sterility resulted, but the method appeared not to yield reproducible results and may have given rise to a paradoxical feminization.

A reduction in germ cell numbers was noted by Yamazaki (1972) following treatment of juvenile coho salmon (Oncorhynchus kisutch) with MT at the dose of 50 mg/kg diet. However, in this study feeding was not commenced until well after the time at which gonadal differentiation would be expected to be complete.

Goetz et al. (1979) and Donaldson and Hunter (1982a) have summarized research on steroid induced sterility in Pacific salmon species. It appears pre-immersion in 50 - 400 ug/L MT is necessary at the alevin stage, followed by the feeding of 10 - 20 mg MT/kg for a period of thirteen weeks. Either the omission of pre-immersion or a reduction of the length of dietary treatment resulted in a lower proportion of sterile fish (Donaldson and Hunter, 1982a). Sterility

appears to be complete in treated fish up to and beyond two years (Donaldson and Hunter, 1982a, 1982b).

Summary

Exogenous steroids administered during early gonadal development have been shown to be capable of exerting profound effects upon the subsequent reproductive development of salmonids. The effects of treatment have been shown to vary with the particular steroid used, the timing and method of its administration and the species of salmonid studied.

Three main effects offer potential for the elimination of problems of male maturation:-

i) Direct feminization with E_2 has been reported to be successful in rainbow trout and salmon (Simpson et al., 1976; Johnstone et al., 1978). Prior immersion in steroid appears to be necessary in Pacific salmon (Goetz et al., 1979; Donaldson and Hunter, 1982a).

ii) Masculinization of genetic females by low doses of androgens coupled with the selection of such reversed females and their use in crosses with normal females would yield all female progeny. This technique has some similarities with the technique of gynogenesis (in which the male chromosome contribution is eliminated by irradiation of sperm).

iii) The administration of high doses of steroid, especially androgens, may lead to sterilization of salmonids, thus eliminating both male and female maturation. In addition to avoiding maturation related problems, this technique would

allow the introduction of 'foreign' species into new environments with minimal risks of long-term competition with indigenous species. This option would be especially attractive in the cultivation of Pacific salmon, in which maturation is usually accompanied by death of spawners (Donaldson and Hunter, 1982a).

This chapter will examine the potential of both direct feminization and high dose androgen sterilization under commercial conditions. In addition ancillary experiments relevant to the method of steroid administration in salmonids will be described.

Due to lack of tank space no experiments were carried out on the production of XX males, although it was hoped to carry out some preliminary crosses with partially functional hermaphrodites, resulting from androgen treatment, and normal females. However, as no eggs were available at the time of maturation of these hermaphrodites, these crosses were not carried out.

8.2 Estrogen induced feminization

Introduction

Previous reports have suggested that the administration of exogenous androgens during the period of gonadal differentiation can lead to a shift in the pattern of gonadal development and a feminization of genetic male salmonids (Jalabert et al., 1975; Simpson, 1976; Simpson et al., 1976; Johnstone et al., 1978). Such feminization may be accomplished by direct dietary administration of steroid without pre-immersion of eyed eggs or alevins. Goetz et al. (1979) report

that such feminization in the Pacific salmon (Oncorhynchus kisutch) requires pre-immersion prior to steroid feeding.

In order to evaluate the possibility of the feminization of genetic male rainbow trout by estrogen administration, a series of experiments were carried out at Shearwater Fish Farming Ltd's Low Plains site. So it was possible to evaluate this technique under commercial farming conditions.

8.2.1 The effects of dietary administration of 17B-estradiol for the first 60 days from first feeding on the growth and gonadal differentiation of rainbow trout

In order to evaluate the possible feminizing effects of E_2 in rainbow trout fry, this steroid was fed at either 20 or 10 mg E_2/kg diet over a range of ration sizes and the effects of hormone treatment upon growth and sexual development monitored.

Materials and Methods

Groups of 5000 rainbow trout fry were fed either treated or control diet prepared as described below for a period of 60 days from first feeding (equivalent to 600°C days). Fish were fed every fifteen minutes between 08.30 - 17.30 using automatic feeders suspended above each tank.

		Dose E_2(mg/kg diet)	Ration (% of manufacturer's ration)
Group A	100% Dose/100% ration	20	100
B	100% Dose/75% ration	20	75
C	100% Dose/50% ration	20	50
D	50% Dose/100% ration	10	100
E	0 Dose/100% ration(control)	0	100

Diet preparation.

Treated diet was prepared using the method of Guerrero (1975) and Simpson et al. (1976).

A 10% portion of diet (B.P. 00 fry food or B.P. 01 fry food after day 21) was defatted by slow efflux of 3 volumes of ethanol/volume food in a glass column.

Steroid was added to the defatted portion dissolved in a small volume of ethanol in a round bottomed flask. Diet was then dried in a rotary evaporator using gentle warming (35°C). The diet was then air dried for 48 hours prior to use.

Defatted diet was mixed on site; one part of defatted, steroid diet was mixed with nine parts of untreated food, to give a final steroid concentration of 20 mg E_2/kg diet. Diet for Group D was prepared by mixing one part of defatted food with nineteen parts of normal food.

Control diet was prepared in a similar manner except that steroid was not added prior to evaporation on the rotary evaporator.

Mortalities were recorded throughout treatment, as was growth, which was measured by weighing batches of 100 fry at intervals throughout treatment. 120 days after the cessation of feeding, fish were sampled and weighed. The gonads were examined macroscopically using a binocular microscope and the fish sexed. A small portion of gonad was removed and fixed in Bouin's fixative to confirm the sexing.

Sex ratios were calculated and the number of fish of each sex within treatment groups tested by a Chi squared test.

Results

a) Mortalities

The % mortality in each group is shown in Fig. 8.2.1.1. The total mortalities of treated or control fish were similar over the first 60 days of the experiment. Only in group A (100D/100T) were mortalities higher than those of controls (7.4% vs. 4.6%). In the other groups, mortalities were similar to that of controls (5.2%, 4.2% and 5.4% in groups B, C and D respectively).

b) Growth

1) Early growth

Growth during the treatment period is shown in Table 8.2.1.1 and Graph 8.2.1.1. On day 18 of treatment all treated fish were slightly smaller than control fish. By day 49 the mean weights of the control group (0.81g) and the group receiving the lowest steroid dose (D) (0.72) were higher than all other groups (A, 0.56 g; B, 0.56 g; C, 0.53 g). By the end of treatment this effect was even more marked. Thus control fish (0.99 g) were heavier than group D fish (0.89 g), while the mean weight of groups A, B and C were 0.64 g, 0.71 g and 0.62 g respectively.

2) Later growth

i) 120 days after cessation of feeding (see Table 8.2.1.2)

The mean body weights of treated and control fish are shown in Table 8.2.1.2 and Fig. 8.2.1.2. The mean body weight of group A fish (100 D/100 T) was significantly different ($P < 0.001$) from that of controls (10.84 ± 0.56 g and 14.88 ± 0.68 g respectively). In addition the mean body weight of group B (11.21 ± 0.65 g) was significantly lower

($P < 0.01$) than that of controls. The mean body weights of groups C and D, 13.78 $\pm$ 0.64 g and 14.88 $\pm$ 0.68 g respectively, were not significantly different from that of control fish.

ii) 195 days after cessation of feeding (see Table 8.2.1.3)

The results of the sampling carried out 195 days after the end of feeding are shown in Table 8.2.1.3 and Fig. 8.2.1.2. On this date the mean body weight of control fish was 44.60 $\pm$ 3.12 g, while the mean weights of the other groups (A, B, C, D) were 39.78 $\pm$ 2.42; 43.61 $\pm$ 2.88; 41.94 $\pm$ 2.16 and 37.78 $\pm$ 2.34 g respectively. No significant differences were apparent between the mean body weights of the treated and control fish.

c) Sex ratios

The sex ratios of fish sampled 120 days after feeding are shown in Table 8.2.1.4. With the exception of group A, the sex ratios of all groups were close to those of control fish. In group A 68.5% of the fish examined were female compared with 52.9% of control fish. This increased number of females was statistically significant when compared by a Chi squared test ($P < 0.05$). No other group showed a difference from the control sex ratio. Thus in group B 52% of fish examined were female, while the corresponding values were 55.7% and 52.9% in groups C and D.

Discussion

The administration of dietary 17B-estradiol for a period of 60 days following first feeding produced a partial feminization of male rainbow trout. This effect was only evident at the highest dose

Table 8.2.1.1

Mean body weight (g) of E_2 treated and control rainbow trout fry during feeding treated or control diets at various ration levels

Group	Day 18	49	59
A 100 D- 100 T	0.38	0.56	0.64
B 100 D-75 T	0.35	0.56	0.71
C 100 D-50 T	0.33	0.53	0.62
D 50 D - 100 T	0.35	0.72	0.89
E 0 D - 100 T	0.40	0.81	0.99

D = % dose where 20 mg/kg = 100%
T = % ration where 100 = manufacturer's table level

Table 8.2.1.2

Mean body weights (g) $\pm$ SE of E_2 treated and control rainbow trout at 120 days after feeding treated or control diets at various ration levels

		n	
A 100 D - 100 T	10.84 $\pm$ 0.56	(57)	(***)
B 100 D - 75 T	11.21 $\pm$ 0.65	(50)	(**)
C 100 D - 50 T	13.78 $\pm$ 0.64	(56)	
D 50 D - 100 T	14.88 $\pm$ 0.68	(50)	
E 0 D - 100 T	14.28 $\pm$ 0.75	(50)	

t test vs. controls (***) $P < 0.001$
(**) $P < 0.01$

Table 8.2.1.3

Mean body weights (g) ± SE of E_2 treated and control rainbow trout at 195 days after feeding treated or control diets at various ration levels

Group	Body wt (g)	n
A 100 D - 100 T	39.78 ± 2.42	(50)
B 100 D - 75 T	43.61 ± 2.88	(49)
C 100 D - 50 T	41.74 ± 2.16	(58)
D 50 D - 100 T	37.78 ± 2.34	(41)
E 0 D - 100 T	44.60 ± 3.12	(50)

Table 8.2.1.4

Sex ratios of E_2 treated and control rainbow trout after feeding treated or control diets at various ration levels

	No. males	No. females	% males	Total
A 100 D - 100 T	37 (*)	17	68.5	54
B 100 D - 75 T	26	24	52.0	50
C 100 D - 50 T	29	23	55.7	52
D 50 D - 100 T	27	24	52.9	51
E 0 D - 100 T	27	24	52.9	51

Chi squared test vs. controls (*) $P < 0.05$

Fig. 8.2.1.1. Mortalities (%) among rainbow trout fry fed various combinations of dose and ration size of E_2 treated diet

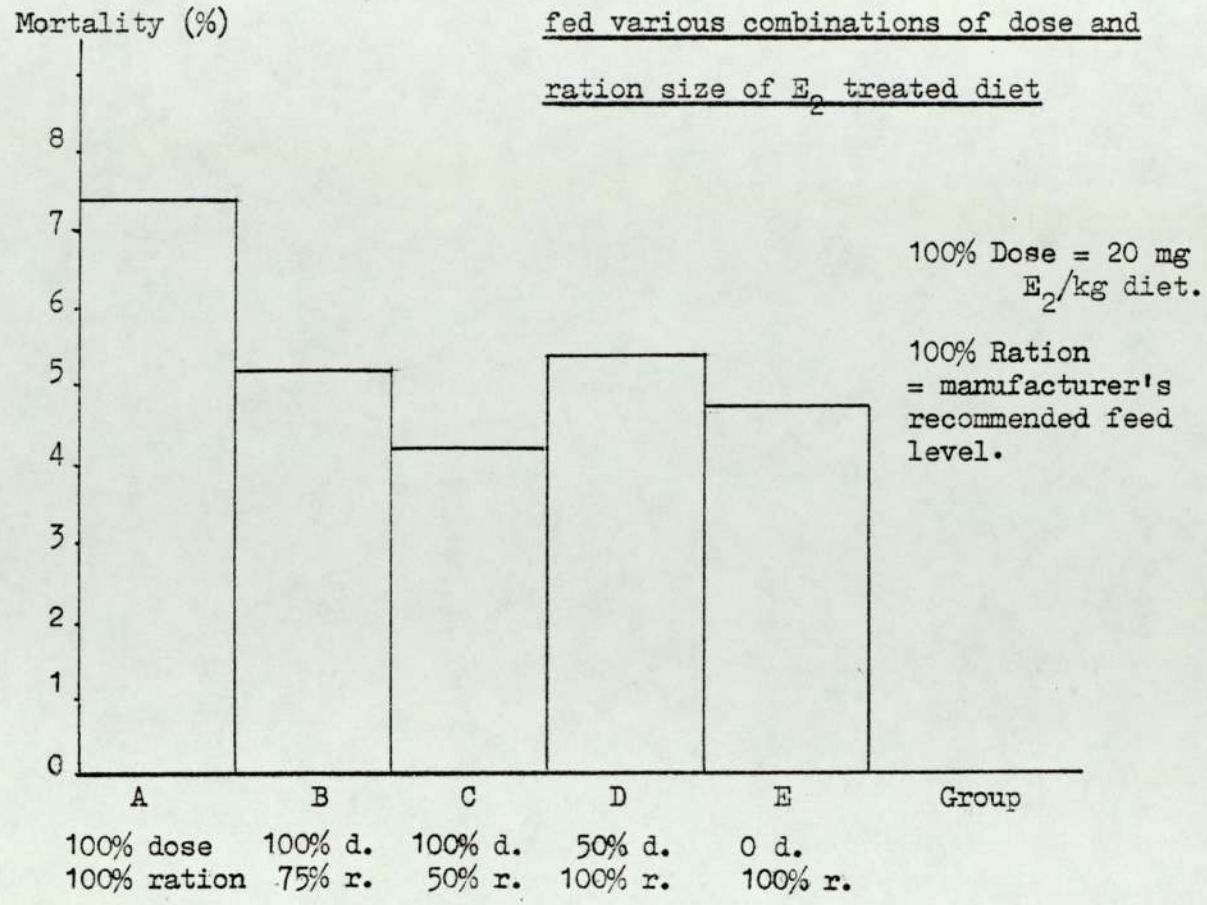
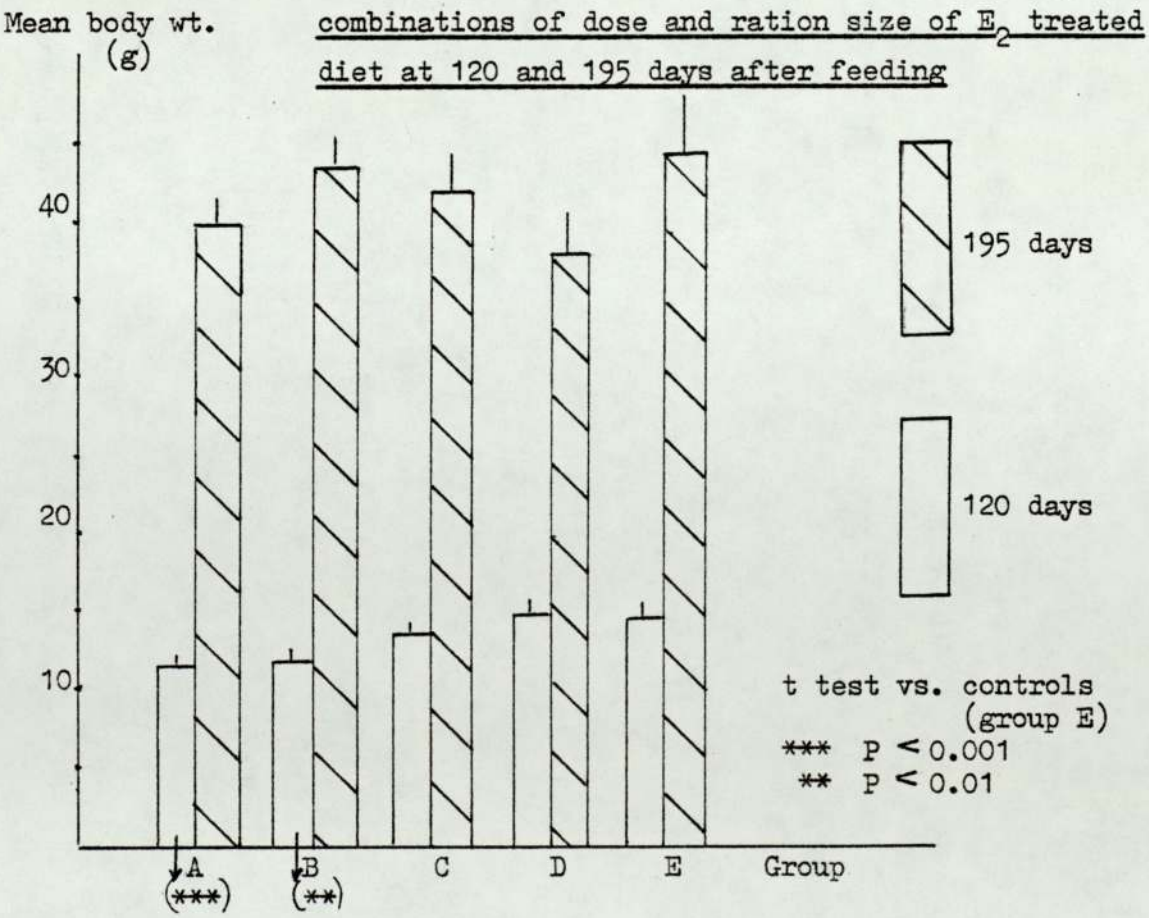
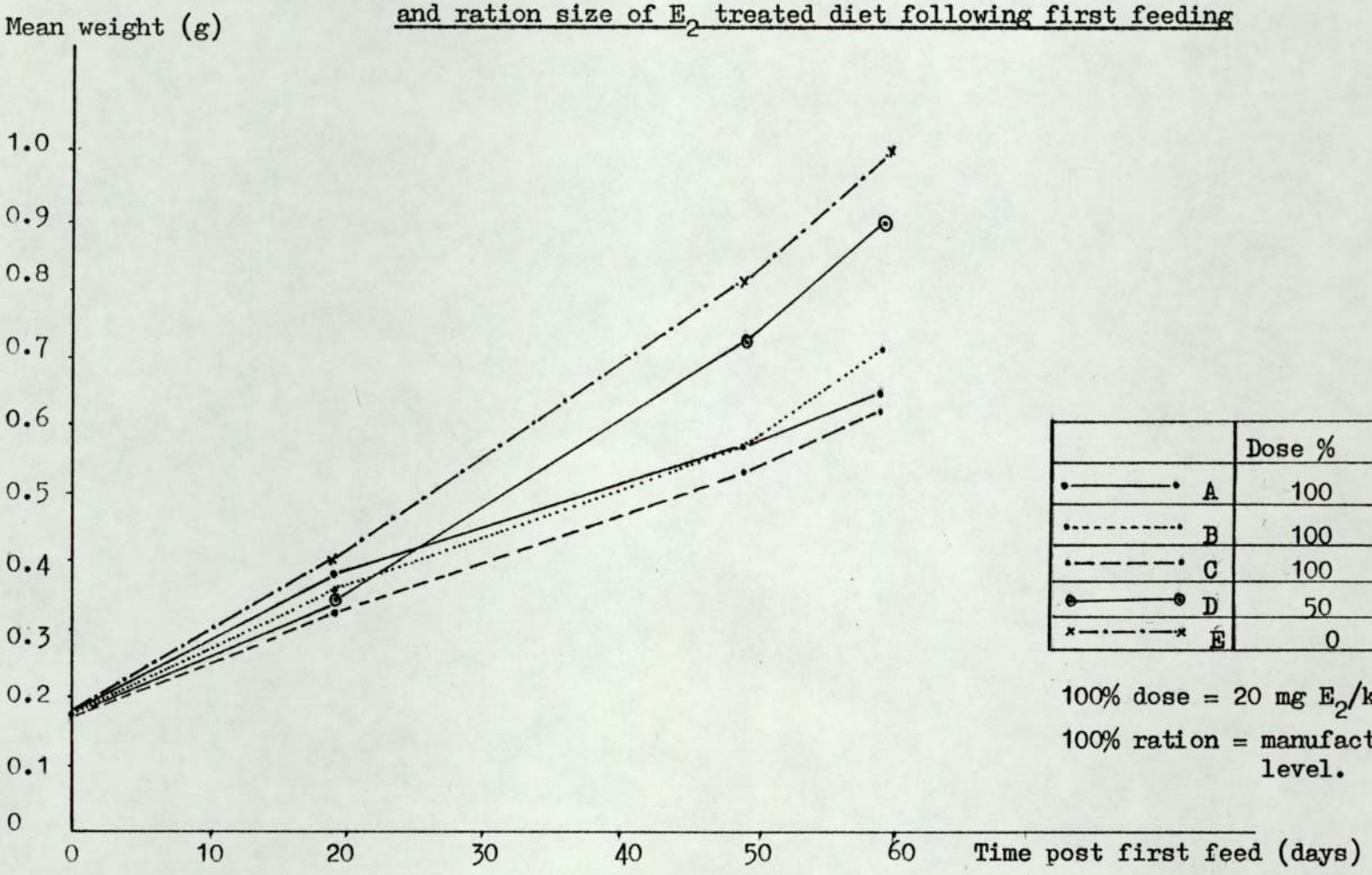


Fig. 8.2.1.2. Mean body weights of rainbow trout fed various combinations of dose and ration size of E_2 treated diet at 120 and 195 days after feeding



Graph 8.2.1.1. Mean body weights (g) of rainbow trout fry fed various combinations of dose and ration size of E_2 treated diet following first feeding



	Dose %	Ration %
A	100	100
B	100	75
C	100	50
D	50	100
E	0	100

100% dose = 20 mg E_2/kg diet.

100% ration = manufacturer's table level.

(20 mg E_2/kg diet, fed at 100% ration). If the steroid dose was reduced, either by reducing the steroid content of the diet or by reducing the feeding level, no feminization was observed; furthermore no graded response was observed.

However, a graded response was observed in the growth of treated fish with a depression in growth resulting from steroid treatment. The maximum growth depression was observed in fish receiving the highest steroid dose (groups A and B). In group D, receiving a reduced steroid dose, growth was depressed, though by a lesser amount. The low growth rate of group C probably reflects the reduced ration fed to this group.

A dose dependent depression of growth has previously been reported in rainbow trout (Simpson et al., 1976; Johnstone et al., 1978) and Pacific salmon (Goetz et al., 1979). Following cessation of treatment E_2 treated rainbow trout grew faster than control fish (Johnstone et al., 1978). In the present experiment it would appear that growth of treated fish was faster over the latter part of the experiment (to 195 days after feeding) as the groups fed higher doses of steroid were no longer significantly lighter than control fish by 195 days after feeding.

Only a slight increase in mortality was observed among treated fish. Thus mortalities were 7.4, 5.2, 4.2 and 5.4% in groups A, B, C and D respectively, compared with 4.6% among control fish. Simpson (1976) and Johnstone et al. (1978) report that E_2 treated fry appeared to be more sensitive to adverse environmental conditions and mortalities among treated fish may be higher than controls.

The absence of a significant feminization in the groups receiving reduced steroid doses suggests the dose threshold for feminization was

only reached in the highest dose group. The growth depression among treated fish may indicate that the thresholds for growth depression and feminization differ.

The results may be influenced by the interval between subsampling, during treatment and recalculation of ration size. Examination of the feeding records during the trial revealed that it is likely that, even in the high dose group, fish were fed below maximum level for some periods during treatment. In addition, following an outbreak of bacterial gill disease among some fish, all fish were treated with 'Betacide' on three consecutive days and would have received a reduced steroid dose on these days. Thus it seems likely that within this trial the steroid dose received may have been less than maximum.

8.2.2 The effects of dietary administration of 20 and 40 mg 17B-estradiol/kg diet and 20 mg ethinyl estradiol/kg diet for the first 60 days following first feeding on the subsequent reproductive development of rainbow trout

Introduction

To further evaluate the technique, a second experiment was carried out at Shearwater Fish Farming Ltd's Low Plains site. In this experiment E_2 was administered at 20 and 40 mg/kg diet. In addition, a synthetic 'long-lived' estrogen (ethinyl estradiol), was tested at a dose rate of 20 mg/kg diet.

Materials and Methods

Groups of 5000 rainbow trout fry were fed either treated or control diet prepared as previously described except that in addition to E_2 at 20 mg/kg diet, batches of E_2 at 40 mg/kg diet were prepared.

Ethinyl estradiol (EE) was also incorporated in batches of food at 20 mg/kg diet.

In view of the possible failure to maximise ration size in the previous trial, all fish were fed to excess using automatic feeders between the hours of 08.30 - 17.30. Fry were fed for 60 days following first feeding. The experimental groups are shown below.

Estradiol 17-B	20 mg/kg diet	E_2 (20)
" "	40 mg/kg diet	E_2 (40)
Ethinyl estradiol	20 mg/kg diet	EE (20)
Control	-	C

Fish were examined 168 days after the cessation of treatment, the gonads dissected free, examined macroscopically and fixed in Bouin's fixative for histological examination.

Results

a) Mortalities

Detailed mortality data were not available for this experiment. However, it was noted that mortalities were higher in the E_2 (40) and EE (20) groups. Within both these groups mortalities due to Costia and myxobacterial gill disease were diagnosed and treated.

b) Growth

At the end of the feeding period the mean body weight was depressed in all steroid fed groups compared to controls (0.64 g). The mean weights of the steroid fed groups E_2 (20), E_2 (40) and EE (20) were 0.59, 0.47 and 0.49 g respectively.

The mean body weights of fish sampled at 168 days following feeding are shown in Table 8.2.2.1 and Fig. 8.2.2.1. At this time fish from the E_2 (20) group were slightly, though not significantly, heavier than control fish (16.29 $\pm$ 0.88 and 16.08 $\pm$ 0.83 g). However, both EE (20) (12.91 $\pm$ 0.71 g) and E_2 (40) treated fish (9.01 $\pm$ 0.71 g) were significantly lighter than control fish ($P < 0.001$).

c) Sex ratios (day 168)

The sex ratios of treated and control fish are shown in Table 8.2.2.2 and Fig. 8.2.2.2. The greatest extent of feminization was observed among EE (20) fish in which 63.3% of the fish examined were female, 22.3% hermaphrodite and 13.3% male. In the E_2 (40) group the corresponding figures were 66.0%, 11.3% and 22.6%. No significant feminization was observed in the E_2 (20) group (52.9% females and 48.1% males). Sex ratios in the control group were close to unity (50.1% females and 49.9% males).

The sex ratios of the EE (20) and E_2 (40) groups were compared with that of control fish by a 3 x 2 Chi squared test and found to be significantly different from the control group ($P < 0.001$ and $P < 0.01$ respectively).

Discussion

A greater extent of feminization was observed in the group fed ethinyl estradiol at a dose of 20 mg steroid/kg diet. In this group 86% of fish examined were either completely female or contained some female gonadal elements. In the group fed E_2 (40 mg/kg) 77% of the fish examined contained female gonadal elements, though only 66% were completely female.

Table 8.2.2.1

Mean body weights of treated and control rainbow trout sampled on day 168 following cessation of feeding either estrogen treated or control diets

Mean weight (g) $\pm$ SE

	EE	E_2 (20)	E_2 (40)	Control
	12.91 $\pm$ 0.71 (***)	16.29 $\pm$ 0.88	9.01 $\pm$ 0.71 (***)	16.08 $\pm$ 0.83
n	51)	54)	51)	42)

t test vs. controls (***) $P < 0.001$

Table 8.2.2.2

Sex ratios of treated and control rainbow trout sampled on day 168 following cessation of feeding either estrogen treated or control diets

	EE	E_2 (40)	E_2 (20)	Control
Female	38 (63.3%)	35 (66.0%)	27 (52.9%)	26 (50.1%)
Male	8 (13.3%)	12 (22.6%)	25 (48.1%)	25 (49.9%)
Hermaphrodite	14 (22.3%)	6 (11.3%)	-	-
Total	60	53	52	51

No. in brackets = % of total examined.

Fig. 8.2.2.1. Mean body weights (g) ± SE of estrogen treated and control rainbow trout sampled 168 days after cessation of feeding treated diet

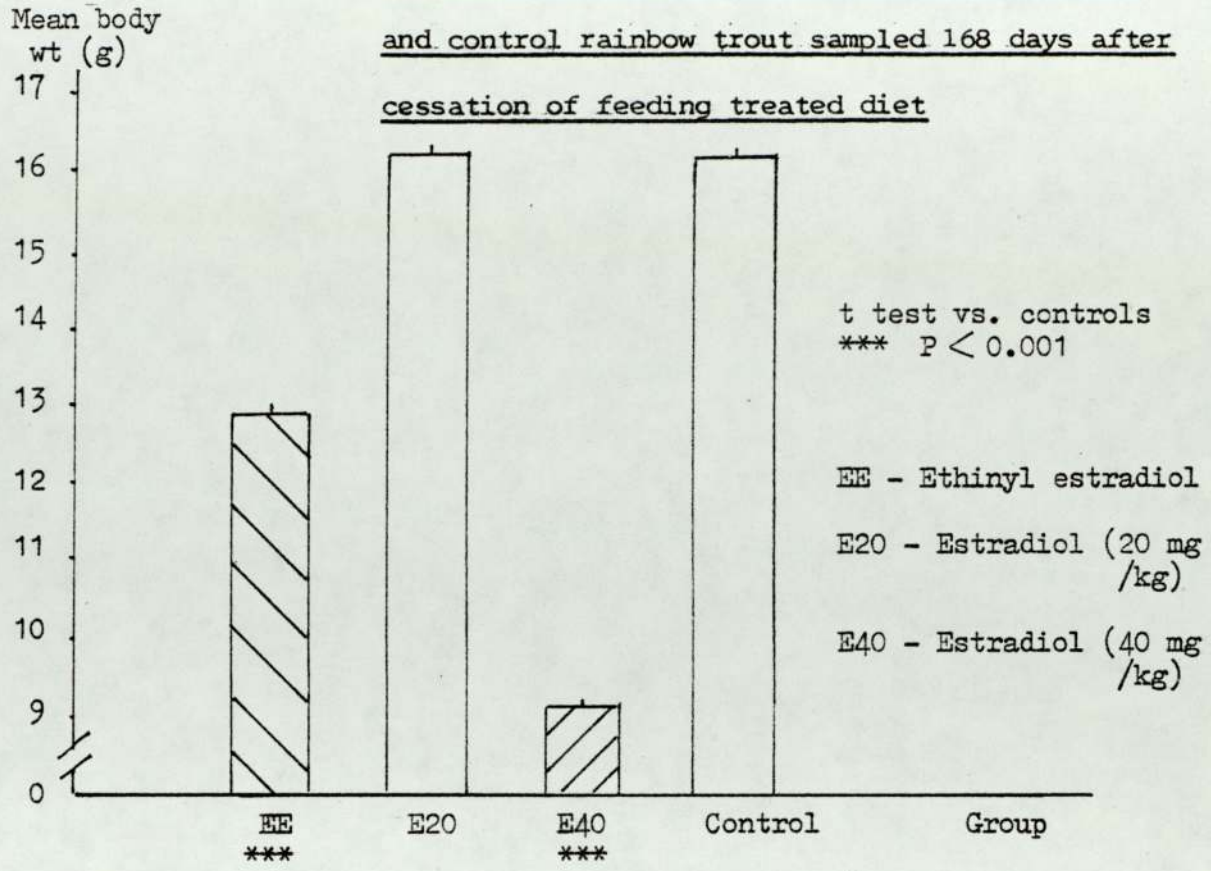
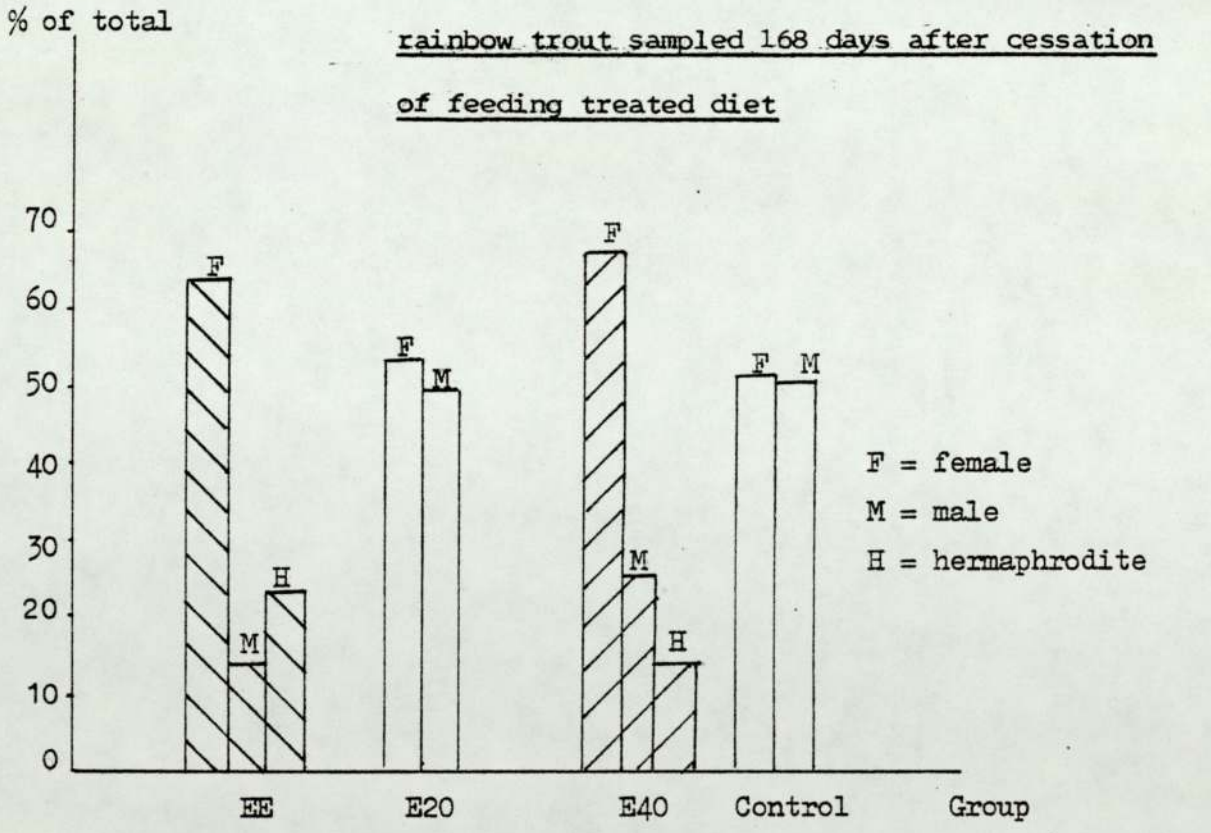


Fig. 8.2.2.2. Sex ratios of estrogen treated and control rainbow trout sampled 168 days after cessation of feeding treated diet



Surprisingly, in view of the partial feminization achieved by this dose in the previous experiment, the fish fed E_2 at a dose of 20 mg/kg showed no feminization and had a sex ratio very close to unity. The difference between the present experiment and that reported previously may reflect differences in feeding regime between the two experiments. In the present experiment fish were fed to satiation, using automatic feeders, while in the previous experiment they were fed to manufacturer's recommended level. It may be that using the treatment method in which only a ten percent portion of the food is steroid treated allows the fish to discriminate between non-treated and treated particles and thus reduce their steroid intake.

The growth results suggest that a greater steroid dose was received by the EE (20) and E_2 (40) treated fish than those of the E_2 (20) group. In addition the growth inhibition resulting from these two treatments seemed to be longer lasting.

In the experiment reported above, hermaphrodite fish were observed. Such fish were not observed in the previous experiment and again may reflect differences between the fish stocks or in the feeding conditions between each experiment. Alternatively it may reflect differences in methodology of assessing gonadal state in experimental fish. In this experiment the whole gonad was examined histologically while in the previous experiment only a portion was used to confirm the macroscopic examination. So it is possible that restricted areas of hermaphrodite tissue in the gonads of fish may not have been observed.

8.2.3 The effects of dietary administration of 25 - 35 mg 17B-estradiol for 77 days from first feeding, with or without pre-treatment in aqueous steroid solution, on the subsequent reproductive development of rainbow trout

Introduction

In view of the failure of a dose of 40 mg E_2/kg diet to achieve a complete feminization, a number of treatment parameters were varied in an attempt to maximize the steroid dose received and hence increase the extent of feminization.

As the previous experiments yielded the surprising result that feeding 20 mg/kg E_2 at a high ration level gave no feminization, while the same dose at a lower ration size produced 68% females, it was decided to modify the method of administration of steroid. In particular, in view of possible discrimination between treated and non-treated food particles, it seemed that treating only a 10% portion of the diet would favour such discrimination. So the method of steroid incorporation was modified to attempt to treat all particles.

In addition, the greater extent of feminization using the 'long-lived' estrogen, Ethinyl estradiol, suggested that an increase in feminization would result if blood steroid levels were maintained over twenty-four hours. (The effects of diet withdrawal on blood steroid levels is demonstrated in the ancillary experiments described in section 8.4.).

Although it has been reported that pre-immersion in E_2 is not necessary for complete feminization (Simpson 1976; Simpson et al., 1976), it was decided to treat a batch of eyed eggs/alevins as described by Goetz et al. (1979).

Materials and Methods

Eyed eggs from a batch of eggs held at Shearwater Fish Farming Ltd's Low Plains site were subdivided into three groups. The experimental design used is shown in Fig. 8.2.3.1.

Group 1. (Control)

This group was treated as group 2 but with a dipping regime consisting of ethanol only and a subsequent control diet sprayed with ethanol without E_2.

Group 2 (Dipped and fed)

Eyed eggs in this group were dipped in an aqueous solution of E_2 (100 µg/L) in hatchery troughs. The water level was run off to a predetermined level (100 L). E_2 was added at a dose of 10.0 mg in 40 ml ethanol and mixed well. After one hour's immersion the level of the trough was dropped and the inflow turned on to flush out steroid solution.

Eyed eggs were dipped in this manner at ten and five days pre-hatch.

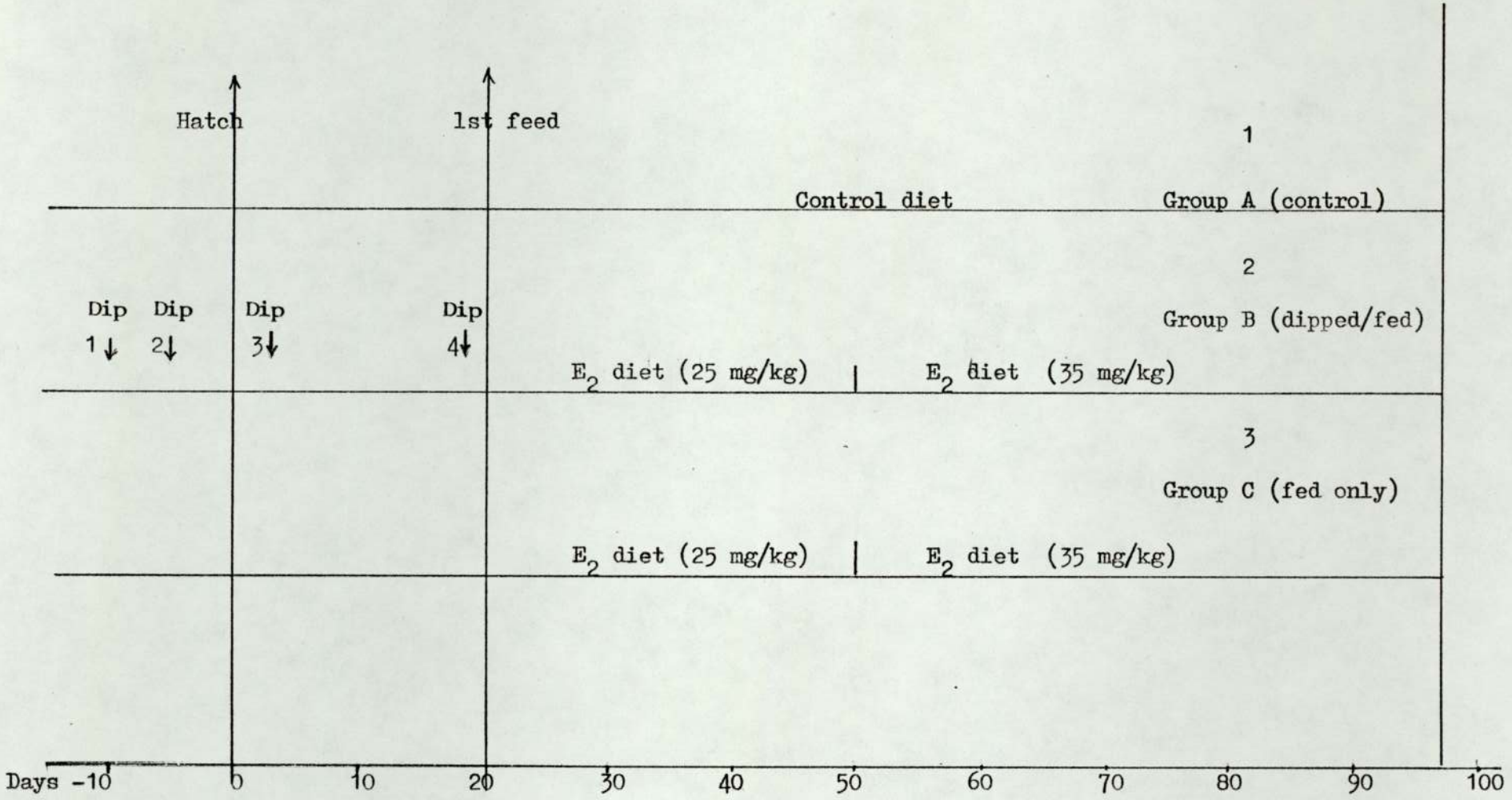
Alevins from these eggs were dipped at three and sixteen days post-hatch as described.

From first feeding (day 21 approximately) fry were fed an E_2 treated diet (25 mg/kg E_2). At day 51 the dose was increased to 35 mg/kg E_2.

Group 3 (Undipped, steroid fed)

Eyed eggs and alevins in this group were dipped in ethanolic solution without steroid at the same time and in the same way as group 2.

Fig. 8.2.3.1. Experimental design for comparing dipped and undipped E_2 treatments



From first feeding they were fed steroid treated diet (initially 25 mg/kg E_2 increasing to 35 mg/kg).

Diet preparation

BP Mainstream fry food (00 or 01) was sprayed with an ethanolic solution of E_2 (25 mg/kg diet in 50 ml ethanol) using an electric spray gun. After spraying it was air dried in a fume cupboard with a gentle airstream from a hairdrier for 48 hours. This diet was fed to groups 2 and 3 from day 20 - 21. At day 51 the steroid dose was increased to 35 mg/kg E_2.

Diet was stored at $4^{\circ}C$ prior to feeding.

Control diet was prepared by spraying fry food with ethanol only (50 ml/kg diet).

Fish were fed for the full twenty-four hours using automatic feeders and supplementary lighting.

Results

a) Mortalities

Treatment of eyed eggs and alevins by steroid dipping appeared to have no effect upon early mortalities and similar low mortalities were observed in groups dipped in ethanol and in steroid.

However, dietary administration of E_2 led to high mortalities in groups 2 and 3. In part this resulted from an increased incidence of Costia and myxobacterial gill disease, although histological examinations revealed evidence of damage to pancreatic, liver and gut mucosal cells, suggesting a direct effect of steroid.

In addition, the growth of fish was severely retarded, probably due to this damage, and although the fish fed well growth was reduced. In view of these high mortalities the experimental design was modified and the two steroid treated groups were pooled for analysis.

At the cessation of treatment mortalities fell to only slightly above normal, though the growth performance did not improve.

b) Sex ratios

As groups were pooled due to high mortalities, it was not possible to assess the relative effects of dipping and steroid feeding upon sexual differentiation. Examination of the fish at 120 days after treatment revealed them to be significantly smaller than control fish.

Macroscopic examination of the gonads of treated fish revealed no male fish. Of the 50 fish examined, all were either female, or had gonads consisting of a thin strip of connective tissue only.

Sex	%
Females	78
Reduced females	14
Sterile - gonads thin strip of connective tissue only.	8

Discussion

Changing the treatment method of steroid administration led to an increased effectiveness of feminization, though at the cost of steroid induced damage and high mortalities among treated fish. In view of the low mortalities resulting from feeding 40 mg/kg E_2 in experiment 2, it seems likely that this increased mortality resulted

from the increased dose of steroid received following modification of method of preparation and absence of an overnight non-feeding period.

As treated fish were pooled from dipped and undipped groups it is not possible to comment on the effect of dipping on feminization, although the results suggest that as no male fish were found, dipping may not be necessary for feminization in rainbow trout.

The occurrence of reduced female fish with very small ovaries and a low percentage (8%) of sterile fish is interesting as this effect is more commonly associated with high doses of androgens (see section 8.3) and again points to the high dose of steroid received by these fish.

8.2.4 General discussion of estrogen induced feminization

The reported experiments on E_2 induced feminization at Shearwater Fish Farming Ltd's Low Plains site yielded variable results. Unlike previous reports (Simpson, 1976; Simpson et al., 1976; and Johnstone et al., 1978) the dietary administration of E_2 at a dose of 20 ppm for 60 days did not produce complete feminization.

While in the first trial a partial feminization was observed, with 68% of the fish examined being female, doses lower than this failed to produce a significant effect on sex ratios.

Such variation in the extent of estrogen induced feminization has been previously reported. In the present series of experiments it has been suggested that variation may result from differences in feeding regimes between different sites. Johnstone et al. (1978) suggest such differences could reflect differences in growth density relationships and photoperiod conditions. Bye (quoted in Donaldson and Hunter, 1982a) reported the failure of E_2 at a dose of 20 mg/kg diet for 41 days from

first feeding to achieve a feminization of rainbow trout. He suggests differences in feeding practice together with strain differences in the timing of gonadal differentiation may explain this failure.

A greater extent of feminization was observed with increased doses of E_2 (40 mg/kg diet) and the use of a synthetic estrogen, Ethinyl estradiol. In the E_2 (40) and EE (20) groups 77% and 86% of all fish contained at least some female gonadal elements. However, the occurrence of hermaphrodite gonads in this trial suggests a shortfall in either dose or feeding regime from that necessary to achieve feminization. In addition the failure of E_2 (20) treatment to give a feminization effect in this trial suggests that the difference between the feeding regimes in the two experiments, and in particular feeding to satiation in the second trial, influenced the response to steroid treatment. This result raised the possibility that high feed levels may actually reduce the received steroid dose if the fish are able to discriminate between treated and non-treated particles. This would seem to be especially so in diets prepared using the method of Guerrero (1975) and Simpson (1976), where only a 10% portion of the diet is treated. Additional experiments (8.4.1) suggest the period between feeds on successive days would have been sufficient to allow blood steroid to fall to low, possibly non-effective, levels.

Attempts to increase the steroid dose received by fry by feeding throughout twenty-four hours using a treated diet in which all particles were treated, led to successful feminization; however, the higher steroid doses led to a reduction of growth and increased mortality. Similar high mortalities have been reported in steroid treated fish (Ashby, 1957; Jalabert et al., 1975; van den Hurk et al., 1980). It was also noted that in the final experiment 8% of the fish examined

were sterile, while the ovaries of a further 14%, though recognizably female, appeared to be grossly reduced.

Thus it would seem that while E_2 induced feminization may be achieved under commercial conditions, it requires careful evaluation of dose rate and feeding regime. It seems likely that in the first two experiments, while the absolute dose of E_2 was sufficient the long 'withdrawal' period overnight would allow sufficient fluctuation in blood levels to prevent a full feminization. (Further evidence on the relationship between blood levels of steroid and withdrawal is presented in section 8.4.1). It seems that a lower, though constant level of steroid would be more effective than erratic blood levels and would have less adverse effects upon growth and tissue structure. So a dose of 10 - 20 mg/kg E_2 fed for twenty-four hours using automatic feeders and prepared by spraying diet would merit further trial under the feeding and rearing conditions practised at Low Plains.

8.3 Effects of high dose androgen treatment

Introduction

The work previously reviewed has demonstrated that the administration of high doses of exogenous androgens to first feeding salmonid fry may produce sterile fish (Jalabert *et al.*, 1975; Yamazaki, 1976; Simpson *et al.*, 1976; Johnstone *et al.*, 1978). Jalabert *et al.* (1975) treated rainbow trout fry with doses of MT up to 60 mg/kg diet from one month post-hatch for a period of four weeks and reported the experimental fish to be sterile, though very few fish survived to the time of normal maturity. Yamazaki (1976) also reported sterilization to follow high dose androgen treatment at the fry stage.

Sterile fish would avoid the problems associated with both male and female maturation. In order to evaluate the potential of this method under commercial conditions two trials were carried out at Shearwater Fish Farming Ltd's Low Plains and Finnarts Bay sites. In both cases fish were maintained to at least the time of normal male maturation.

8.3.1 The effects of feeding 30 mg methyltestosterone (MT)/kg diet for 110 days from first feeding on the subsequent gonadal development of rainbow trout at Low Plains

Two groups of rainbow trout fry reared at the Shearwater Fish Farming Low Plains site were fed either a steroid treated (30 mg MT/kg diet) or control diet for 110 days from first feeding in order to evaluate the success of this method.

Materials and Methods

Two groups of 5000 first feeding rainbow trout fry were maintained in 400 L fry tanks and fed either a MT (30 mg/kg diet) or control diet, prepared as described below for a period of 110 days from first feeding. Fish were fed hourly between 08.30 - 17.30.

Diet preparation

Diet was prepared by the method of Guerrero (1975) and Simpson et al. (1976). Fry diet was defatted in a glass column by slow efflux of three volumes of ethanol per volume of diet. Diet was transferred to a round bottomed flask and a small volume of ethanol containing dissolved steroid added. Sufficient ethanol was then added to just cover the food. Ethanol was evaporated under vacuum using gentle warming (35°C). The diet was removed and air dried before use.

Steroid was added to the diet at the dose of 30 mg/100 g diet. Prior to feeding this was mixed with nine parts untreated fry food to give a final concentration of 30 mg/kg diet.

For the first two weeks of the feeding period BP 00 fry food was used to prepare diets. After this time the size of diet was increased to 01. Fry were fed for a period of 110 days (equivalent to 1100°C days).

The subsequent development of these fish was monitored over the next three years. Samples were taken to determine body weight, gonad weight and structure, hormone levels and secondary sexual characteristics. Serum T levels were determined both at Aberdeen (DAFS) and Aston University. 11-KT levels were measured at Aberdeen using the method of Simpson and Wright (1977). GTH levels were measured by Dr. B. Breton (INRA) using the method of Breton et al. (1971, 1976).

Both control and treated fish were maintained in individual tanks and sampled separately until 25 months, when the control group were lost following a routine fish movement. Treated fish were kept for a longer period.

Results

a) Early results

1) Mortalities and early growth

Mortalities throughout the feeding period were only slightly higher in the steroid fed fish (21%) than in the control fish (18%). No excessive increase in mortalities as a result of steroid treatment was noted.

MT treatment did, however, result in an initial depression of growth. At five months post-hatch (approximately two months after the cessation of steroid feeding) the mean body weight of MT treated fish (5.82 ± 0.15 g) was significantly lower ($P < 0.001$) than that of control fish (7.52 ± 0.19 g). However, this effect was transient and in subsequent samples MT treated fish were no lighter than controls.

2) Histological examination (five months post-hatch)

Gross examination showed the majority of MT treated fish to have filiform gonads. No fish examined showed a typical ovarian gonad.

Histological examination showed abnormal gonad structure among treated fish. Treated fish were largely devoid of germ cells and showed widespread connective tissue infiltration. If germ cells were present they were usually spermatogonia and no typical female germ cells were seen, though the abnormal appearance of these gonads made analysis difficult.

b) Twelve months post-hatch

1) Growth

At twelve months post-hatch MT treated fish were slightly heavier than control fish (107.72 ± 8.59 g and 90.25 ± 4.34 g). (This difference was not statistically significant ($P < 0.1$)).

2) Gonadosomatic index (GSI)

The mean GSI of treated and control fish are shown in Table 8.3.1.1 and Fig. 8.3.1.1. A depression of gonad weight was observed in MT treated fish. Within this group a number of fish (7 out of 27) contained reduced ovaries.

Table 8.3.1.1

Mean Gonadosomatic Index (G.S.I.) of MT treated and control fish at 12 months post-hatch following feeding 30 mg MT/kg diet or a control diet for 110 days from first feeding (Mean ± SE)

	Immature male	Mature male	Female	Sterile
Control	0.049 ± 0.009	4.22 ± 0.19	0.061 ± 0.01	-
n	14) (*)	2)	13) (***)	
MT treated	0.020 ± 0.006	-	0.006 ± 0.002	0.002 ± 0.0007
n	10)		7)	10)

t test MT vs. control (*) $P < 0.05$
(***) $P < 0.001$

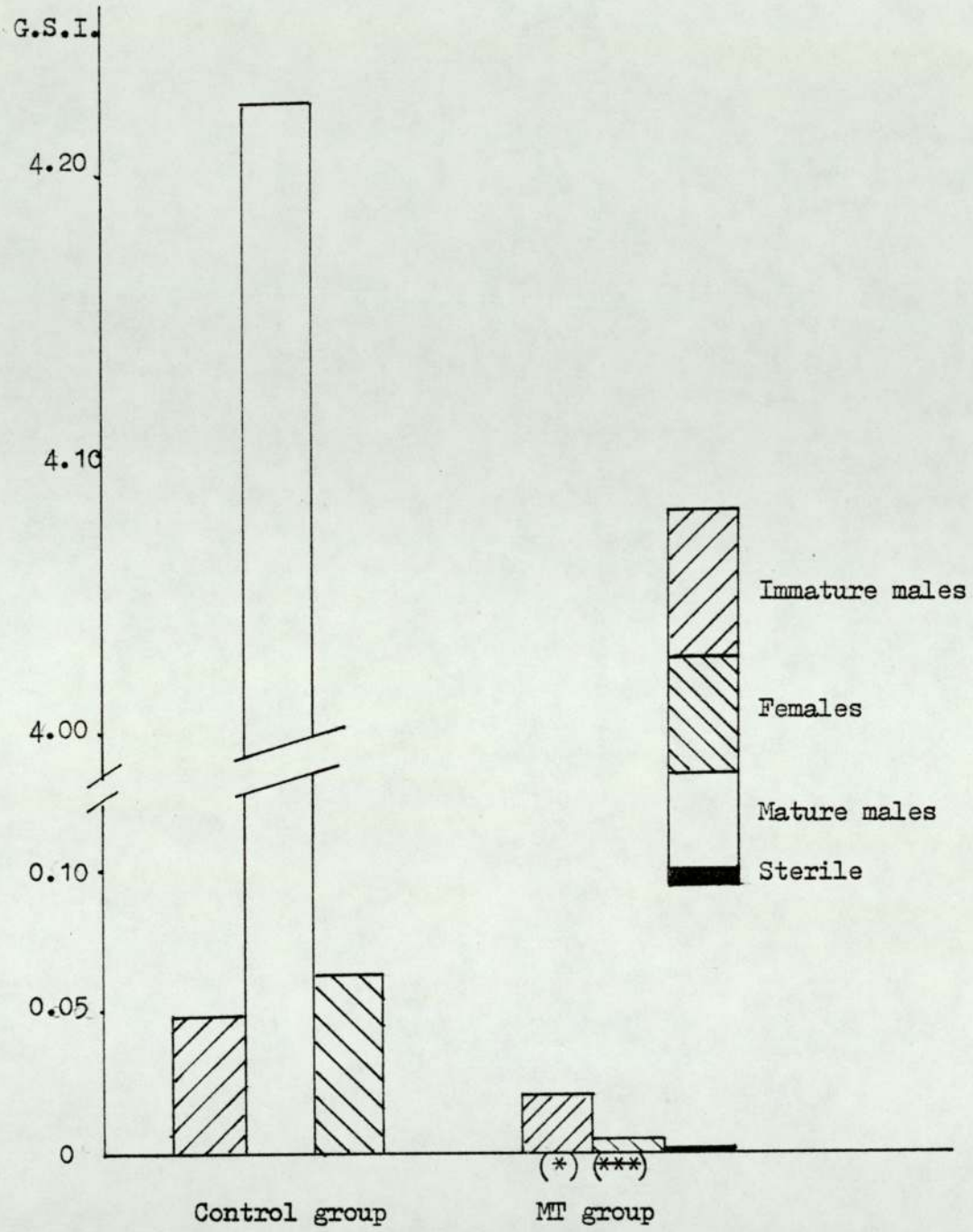
Table 8.3.1.2

Serum levels of testosterone and 11-KT in control and MT treated fish following feeding 30 mg MT/kg diet or a control diet for 110 days from first feeding (ng/ml ± SE)

	Immature male	Mature male	Female	Sterile
Control				
T	0.091 ± 0.034	10.33 ± 0.71	0.0	-
n	10)	2)	2)	
11-KT	2.24 ± 0.31	36.2 ± 2.45	0.85 ± 0.85	-
n	10)		2)	
MT treated				
T	0.23 ± 0.19	-	-	0.05 ± 0.05
n	7)			7)
11-KT	1.23 ± 0.25 (*)	-	-	1.89 ± 0.31
n	8)			6)

t test MT vs. control (*) $P < 0.05$

Fig. 8.3.1.1 Mean Gonadosomatic Index (G.S.I.) of MT treated and control fish at 12 months post-hatch following feeding 30 mg MT/kg diet as fry



t test vs. controls (***) $P < 0.001$
(*) $P < 0.05$

Identifiable germ cells were present in some of the gonads from treated fish, though 10 of the 27 fish examined appeared to be completely sterile. MT treatment depressed the GSI of both sexes. The mean GSI of treated male fish was 0.02 ± 0.06 compared with 0.049 ± 0.009 in male controls. The mean GSI of treated females was 0.006 ± 0.002 and that of control females 0.061 ± 0.01. These differences in GSI between treated and control males and females were significant at the $P < 0.05$ and $P < 0.001$ levels respectively.

Histological analysis showed a gross reduction in germ cell numbers and development compared with control fish. The majority of males in the treated group in which germ cells were visible were at the spermatogonia - spermatocyte stage, while control males showed germ cells at the spermatocyte - spermatid stage, and in two males mature spermatozoa were observed. A reduction in the number of oogonia and in their development was observed in the female gonads examined.

In those treated fish in which no gonadal development was seen the gonads consisted only of connective tissue.

3) Hormone levels

Both T and 11-KT levels were measured in samples taken at 12 months from treated and control fish. The results of these assays are shown in Table 8.3.1.2 and Figs. 8.3.1.2 and 8.3.1.3.

Hormone levels in all fish were low, with the exception of one mature male in the control group. No significant differences were noted between the T and 11-KT levels in treated and control fish with the exception of the mean 11-KT level of control and treated immature males (2.34 ± 0.31 ng/ml and 1.23 ± 0.25 ng/ml respectively) ($P < 0.05$).

Fig. 8.3.1.2

Serum testosterone levels (ng/ml) $\pm$ SE of control and MT treated rainbow trout sampled 12 months after feeding 30 mg MT/kg diet as fry

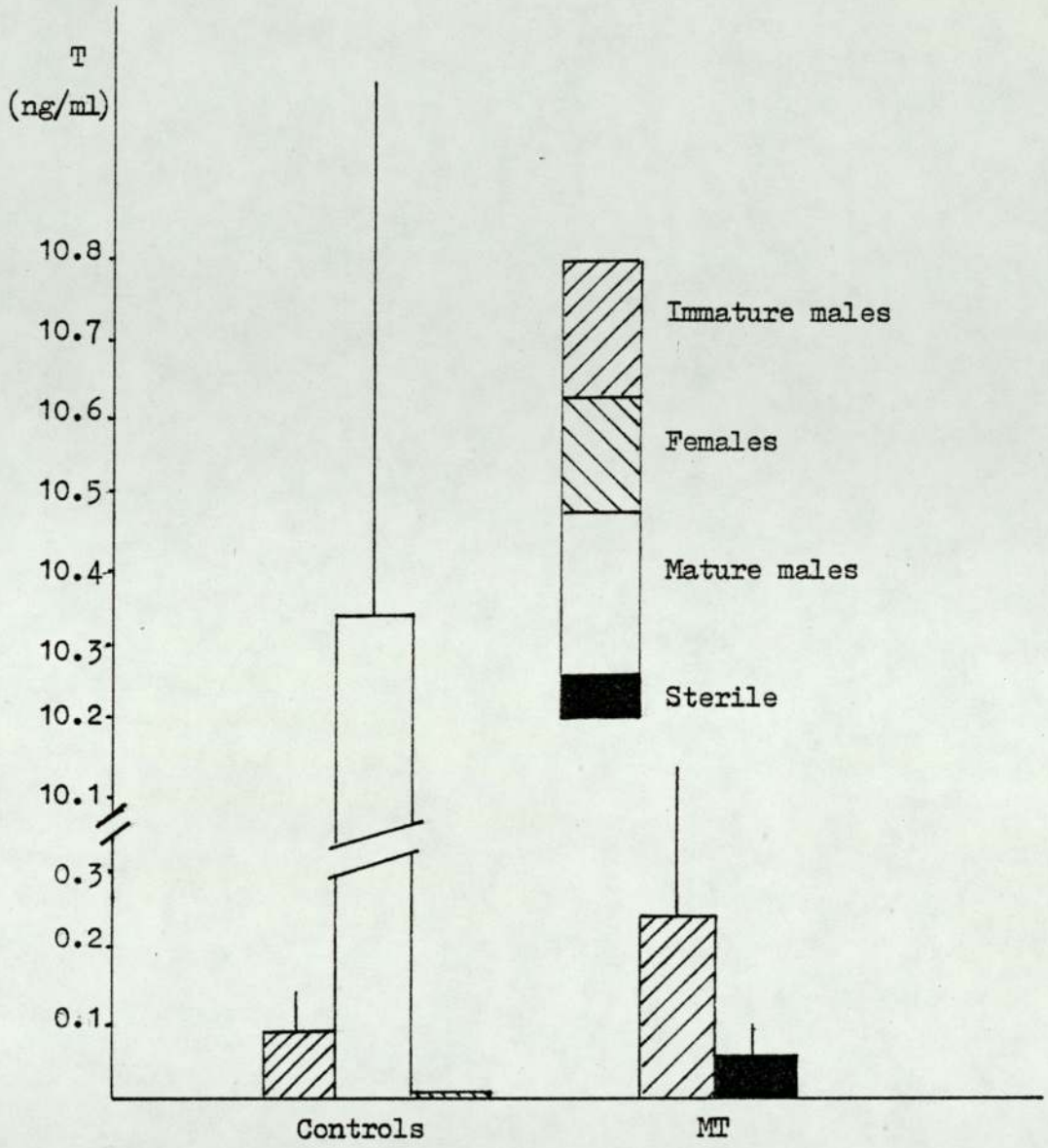
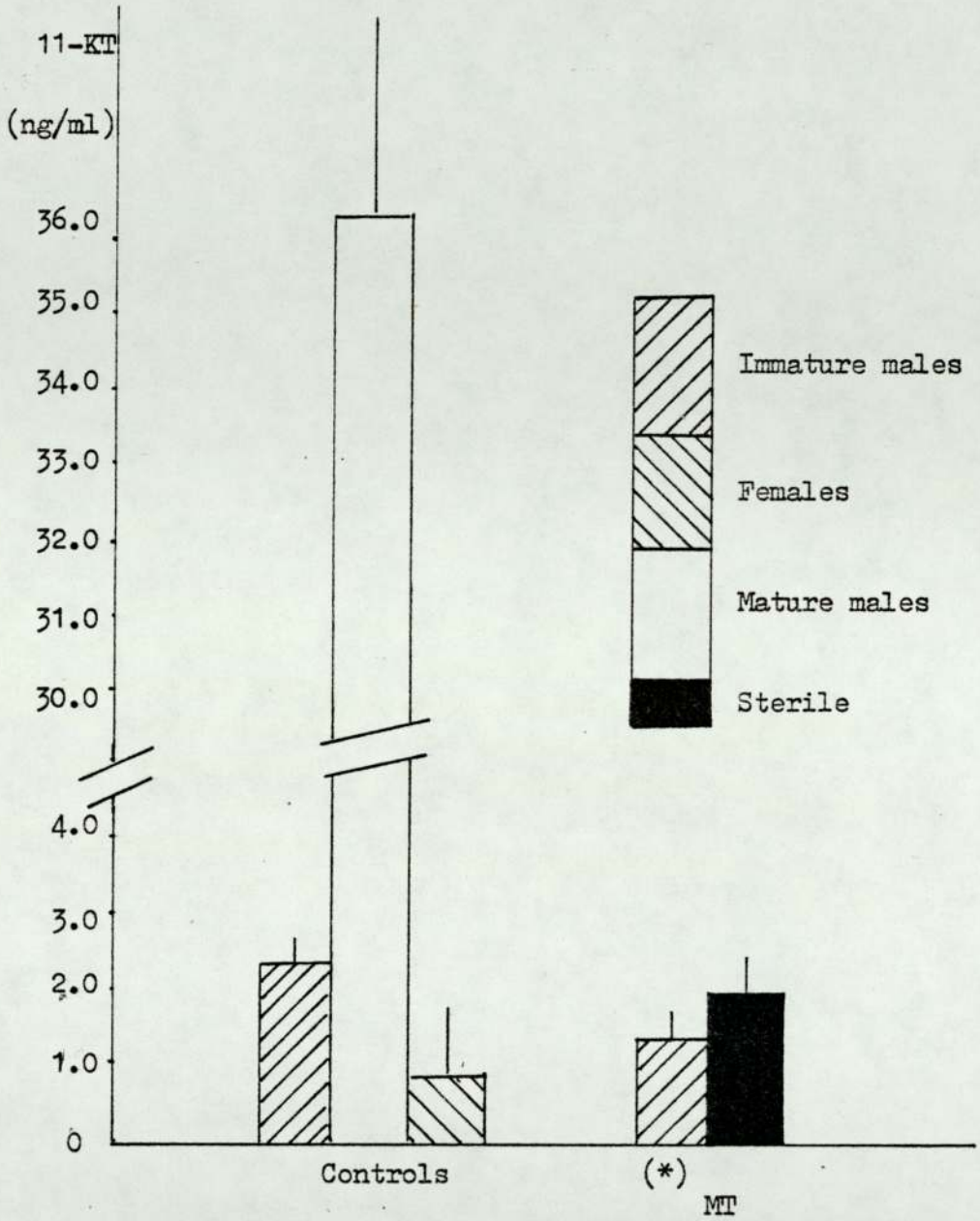


Fig. 8.3.1.3

Serum 11-ketotestosterone levels (ng/ml) ± SE of control and MT treated rainbow trout sampled 12 months after feeding 30 mg MT/kg diet as fry



t test vs. corresponding control group
(*) $P < 0.05$

Thus the 11-KT level of treated males was lower than that of control males.

c) Later Results (16 - 36 months)

1) Gonadosomatic index

The G.S.I.'s of fish sampled between 16 - 26 months post-hatch were calculated and the results are shown in Table 8.3.1.3 (controls) and 8.3.1.4 (MT treated).

The mean G.S.I. of control males increased rapidly from 16 - 19 months, from 0.25 ± 0.11 to 3.61 ± 1.07, by which time the fish were just prior to spermiation, which began at 20/21 months post-hatch. The mean G.S.I. of control males remained close to 1.9 in months 21 and 22 and subsequently fell to 1.05 ± 0.4 by month 26. The mean G.S.I.'s of control males are displayed in Graph 8.3.1.1. The mean G.S.I. of female control fish remained low at all times during this period.

Male secondary sexual characteristics began to appear in developing male control fish by month 16. By month 21 the majority of males examined showed full development of male secondary sexual characteristics and spermiation.

The mean G.S.I. of MT treated males remained low until 26 months post-hatch, when a slight increase in G.S.I. was observed to 0.19 ± 0.08. During all this period, with the exception of the 16 month sample, the G.S.I. of treated males was significantly lower ($P < 0.001$) than control males. From this time the mean G.S.I. increased steadily to month 28 (0.26 ± 0.11) with a sharp increase to 1.04 ± 0.22 at 30 months. A further increase to 1.72 ± 0.22 was seen by month 35, though the irregular sampling over this period makes the exact timing of increases

Table 8.3.1.3

Mean G.S.I. of male and female control fish sampled at 16 - 26 months post-hatch

Age (months post hatch)	% mature males	Male	Female
16	14	0.25 ± 0.11 (21)	0.09 ± 0.004 (28)
19	54	3.61 ± 1.07 (13)	0.43 ± 0.25 (9)
21	78	1.96 ± 0.48 (14)	0.07 ± 0.01 (8)
22	89	1.92 ± 0.30	0.21 ± 0.13 (5)
23	81	0.54 ± 0.29 (11)	0.13 ± 0.04 (9)
26	87	1.04 ± 0.40 (8)	0.13 ± 0.04 (11)

n = no. in brackets.

Table 8.3.1.4

Mean G.S.I. of MT treated fish sampled at 16 - 36 months post-hatch following feeding 30 mg MT/kg diet as fry (G.S.I. ± SE)

Age (mths post-hatch)	Males	% mature males	Females	Hermaphrodite	Sterile
16	0.09 ± 0.02 (17)	-	0.09 ± 0.004 (20)	-	0.002 ± 0.00 (5)
19	0.03 ± 0.006 (8) (***)	-	0.45 ± 0.29 (13)	0.03 ± 0.001 (2)	0.014 ± 0.014 (3)
21	0.02 ± 0.006 (8) (***)	-	0.01 ± 0.001 (13)	0.014 ± 0.004 (6)	0.005 ± 0.001 (3)
22	0.03 ± 0.001 (5) (***)	-	0.02 ± 0.003 (7)	0.014 ± 0.009 (3)	0.013 ± 0.006 (4)
23	0.034 ± 0.001 (7) (***)	-	0.02 ± 0.004 (10)	0.018 ± 0.004 (10)	-
26	0.19 ± 0.08 (11) (***)	27	0.01 ± 0.007 (5)	0.022 ± 0.022 (2)	0.0 (3)
28	0.26 ± 0.11 (7)	36	0.006 ± 0.02 (7)	0.38 ± 0.22 (4)	0.004 ± 0.001 (2)
30	1.04 ± 0.22 (9)	100	0.94 ± 0.27 (7)	0.47 ± 0.29 (2)	0.0 (3)
31	0.62 ± 0.59 (6)	83	0.23 ± 0.20 (2)	-	0.012 ± 0.012 (2)
35	1.72 ± 0.22 (23)	100	0.11 ± 0.26 (8)	-	0.0 (2)
36	0.75 ± 0.42 (6)	63	0.17 ± 0.05 (7)	1.11 ± 0.63 (4)	0.0 (2)
Totals	107		99	31	29
%	40.2		37.2	11.6	10.9

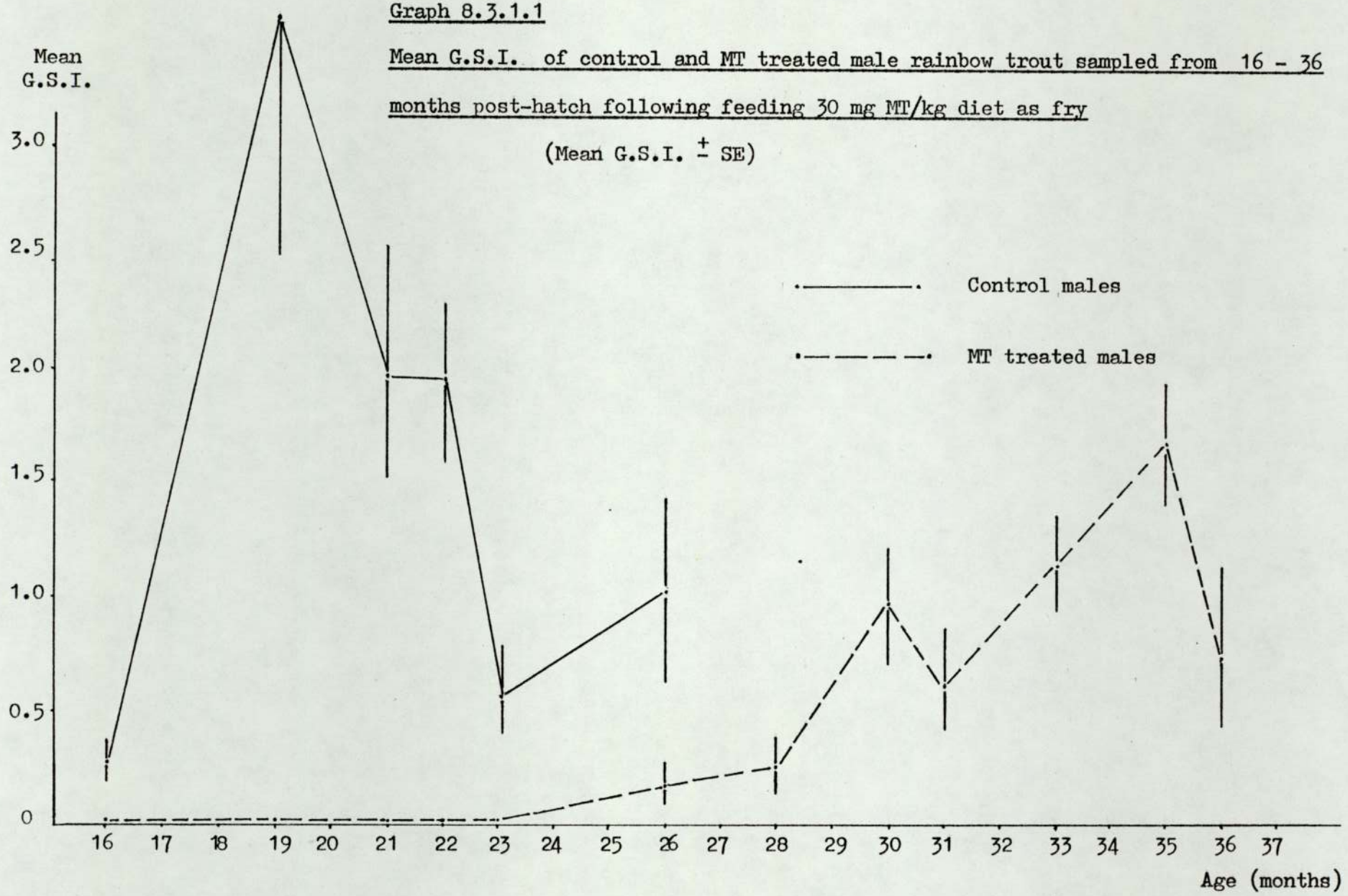
t test - significantly different from control males at these sample dates (***) $P < 0.001$.

n = no. in brackets.

Graph 8.3.1.1

Mean G.S.I. of control and MT treated male rainbow trout sampled from 16 - 36 months post-hatch following feeding 30 mg MT/kg diet as fry

(Mean G.S.I. $\pm$ SE)



in G.S.I. over this period difficult to determine with any certainty. The G.S.I. of treated male fish are displayed in Graph 8.3.1.1.

This increase in male G.S.I. was coincident with the appearance of male secondary sexual characteristics within treated males, albeit following a pronounced delay compared to control males. However, though the external secondary sexual characteristics of treated males were similar to those observed in normal males, gonadal development was abnormal in these fish. In the majority of fish examined the gonads appeared nodular and differed in appearance from normal maturing males. Only occasional fish could be hand stripped using gentle pressure, but in the majority of treated males which developed sexually, spermatozoa could not be stripped. On examination some of these maturing males were found to be hermaphrodites, having developing testis elements together with ovarian tissue.

The mean G.S.I. of female treated fish remained low throughout the experiment, with only an occasional female maturing at the later stages of the experiment.

At all times during the experiment the G.S.I. of fish assessed as sterile remained low. Histological examination revealed the gonads of these fish to consist largely of connective tissue with very few germ cells visible.

Like the treated male fish, the G.S.I. of hermaphrodite fish increased from month 28, largely due to an increase in size of the testicular component of the gonads. At month 28 the mean G.S.I. of hermaphrodites was 0.38 ± 0.22 and this subsequently increased to 1.11 ± 0.63 by month 36.

The cumulative % of each type of fish among treated fish was 40.2% male, 37.2% female, 11.6% hermaphrodite and 10.9% sterile.

2) Hormone levels

i) Testosterone

Serum samples from treated and control fish were assayed for T as described. The results are shown in Table 8.3.1.5 and Fig. 8.3.1.4.

At 19 and 21 months serum T levels of male control fish were significantly higher than those of male MT treated fish, reflecting the absence of male maturation among treated fish. At 19 months control males showed mean T levels of 20.31 $\pm$ 5.09 ng/ml compared with 1.95 $\pm$ 0.84 ng/ml in MT treated males ($P < 0.001$). The corresponding values for 21 months were 35.78 $\pm$ 6.59 ng/ml and 0.86 $\pm$ 0.22 ng/ml for control and MT treated males.

Serum T levels of control females were higher than those of treated females at 19 and 21 months, though not significantly so (2.72 $\pm$ 1.60 and 3.05 $\pm$ 1.80 ng/ml control, and 0.93 $\pm$ 0.29 and 0.76 $\pm$ 0.28 ng/ml treated at 19 and 21 months respectively).

ii) Serum gonadotropin (GTH)

Serum gonadotropin levels were assayed as described in General Materials and Methods by Dr. B. Breton (INRA). Serum levels as measured by assay are shown in Table 8.3.1.6 and Fig. 8.3.1.5.

On both occasions MT treated fish had higher mean serum GTH levels than control fish. At 19 months control males had mean GTH levels of 4.30 $\pm$ 0.42 ng/ml, while MT treated males had significantly higher mean serum levels of 7.72 $\pm$ 0.76 ng/ml ($P < 0.001$). At 21 months MT treated

Table 8.3.1.5

Mean serum testosterone levels in control and MT treated rainbow trout at 19 and 21 months post-hatch (ng/ml ± SE)

Age (months post-hatch)		Male	Female
19	Control	20.31 ± 5.09	2.72 ± 1.60
	n	9)	6)
	MT	1.95 ± 0.84	0.93 ± 0.29
	n	16) (***)	7)
21	Control	35.78 ± 6.59	3.05 ± 1.80
	n	10)	5)
	MT	0.86 ± 0.22	0.76 ± 0.28
	n	9) (***)	7)

t test Treated vs. control (***) $P < 0.001$

Table 8.3.1.6

Mean serum GTH levels in control and MT treated rainbow trout at 19 and 21 months post-hatch (ng/ml)

Age (months post-hatch)		Male	Female	Sterile
19	Control	4.30 ± 0.42	3.95 ± 0.87	-
	n	11)	7)	
	MT	7.72 ± 0.76 (***)	4.34 ± 1.28	16.78 ± 1.64
	n	16)	7)	2)
23	Control	3.24 ± 0.41	3.69 ± 0.37	-
	n	11)	9)	
	MT	7.42 ± 2.17	6.33 ± 0.63 (**)	34.28 ± 15.2
	n	11)	7)	2)

t test Control vs. treated for that month (***) $P < 0.001$
(**) $P < 0.01$

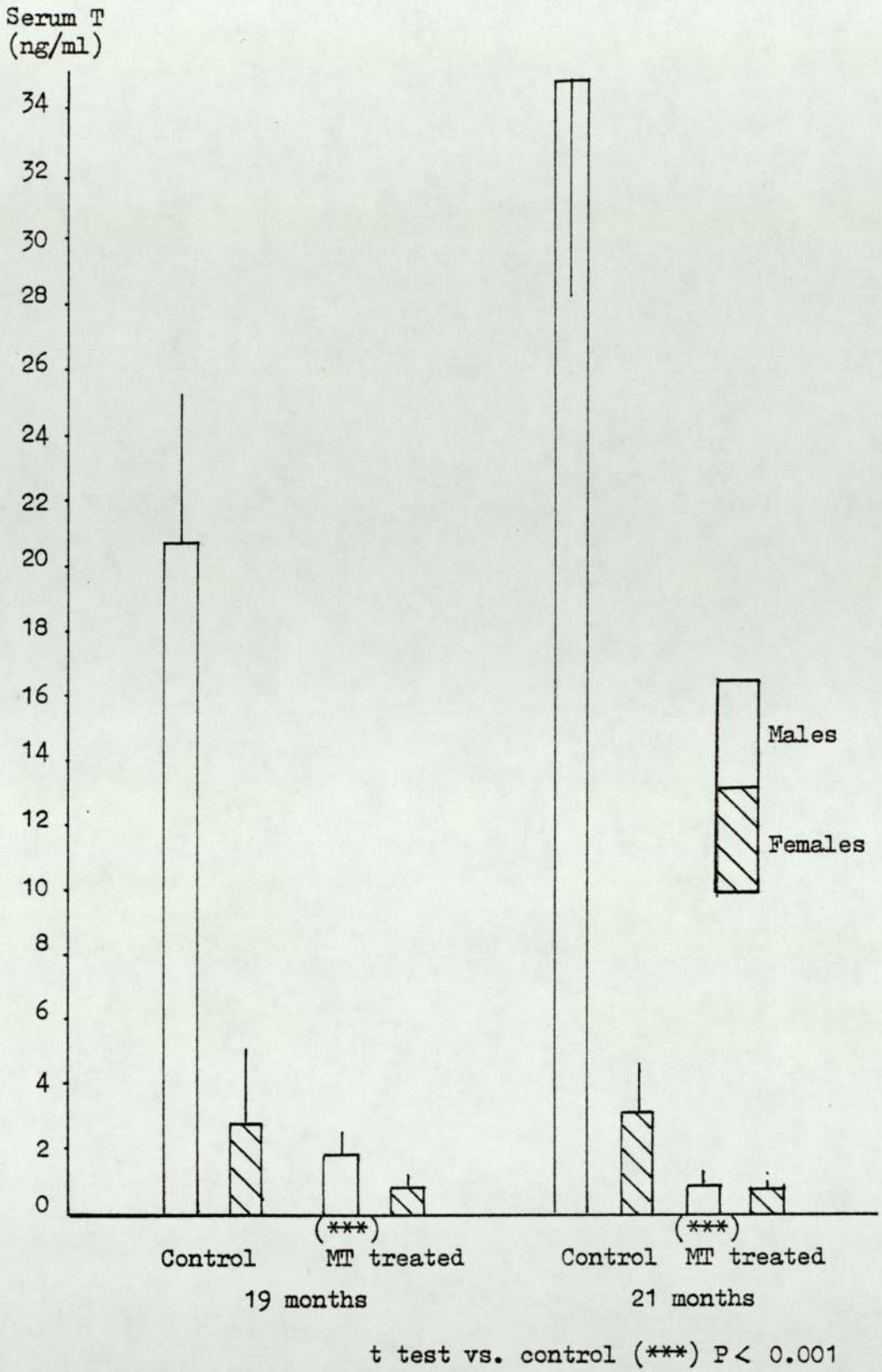
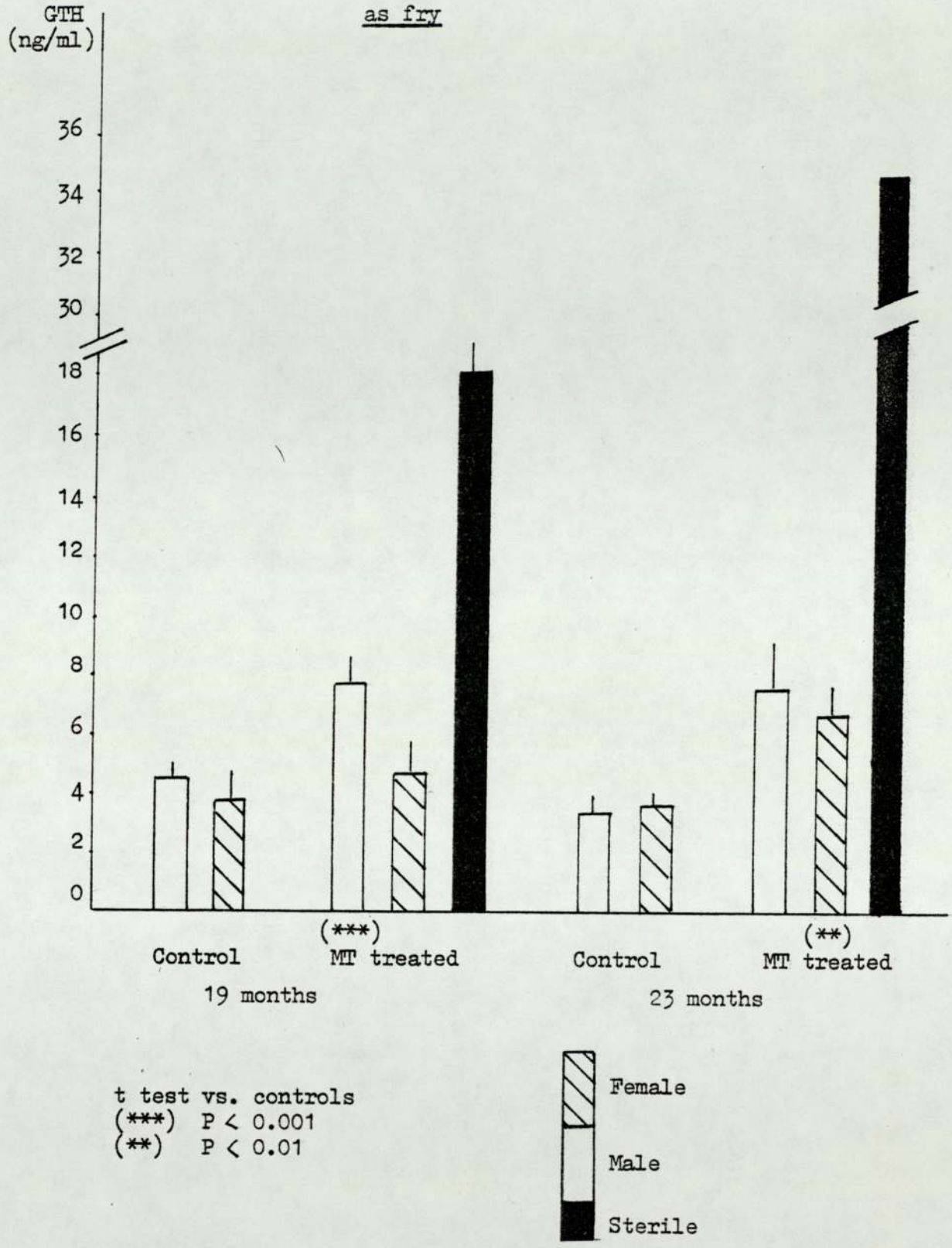


Fig. 8.3.1.4

Mean Serum testosterone levels (ng/ml) ± SE of MT treated and control rainbow trout sampled at 19 and 21 months following feeding 30 mg MT/kg diet as fry

Fig. 8.3.1.5

Mean serum GTH levels (ng/ml) ± SE of control and MT treated rainbow trout sampled at 19 and 23 months following feeding 30 mg MT/kg diet as fry



males had mean serum GTH levels of 7.42 ± 2.17 ng/ml, while the corresponding value for control males was 3.24 ± 0.41 ng/ml. This difference was not statistically significant. ($P < 0.1$).

Although the mean GTH value of control females was lower than that of treated females at 19 months (3.95 ± 0.87 and 4.34 ± 1.28 ng/ml respectively), these differences were not significant. However, at 23 months the mean GTH value of treated females (6.33 ± 0.63 ng/ml) was significantly higher than that of control females (3.69 ± 0.37 ng/ml) ($P < 0.001$).

GTH levels were determined in serum samples from a restricted sample of sterile fish (i.e. fish having gonads devoid of germ cells and consisting of connective tissue only). In both cases serum GTH levels were higher than in all other groups. At 19 months serum GTH levels were 16.78 ± 1.64 ng/ml and at 23 months 34.28 ± 15.2 ng/ml. However, these results were derived from only two fish sampled at each time and no further analysis of these results was attempted.

3) Body weight (16 - 25 months)

The mean body weights of control and MT treated fish are shown in Tables 8.3.1.7 and 8.3.1.8 and displayed in Graph 8.3.1.2.

The mean body weight of both male and female control fish increased steadily from month 16. A depression of growth was observed in male fish shortly after spermiation (month 20/21). The mean body weight of control males fell from 411.7 ± 34.2 g to 399.8 ± 21.7 g shortly after spermiation. In control males subsequent growth was slower following spermiation. Growth of females showed no such cessation of growth.

Table 8.3.1.7

Mean body weights of control fish fed on control diet as fry

(g ± SE)

Age (months post-hatch)	Male	Female
16	227.0 ± 8.0 (10)	220.7 ± 8.8 (12)
19	314.6 ± 18.2 (13)	331.7 ± 25.6 (9)
21	411.7 ± 34.2 (14)	423.1 ± 36.2 (7)
22	399.8 ± 21.7 (9)	471.0 ± 41.2 (6)
23	458.2 ± 29.8 (11)	546.2 ± 43.4 (9)
25	560.1 ± 46.8 (11)	612.2 ± 51.3 (8)

n = no. in brackets

Table 8.3.1.8

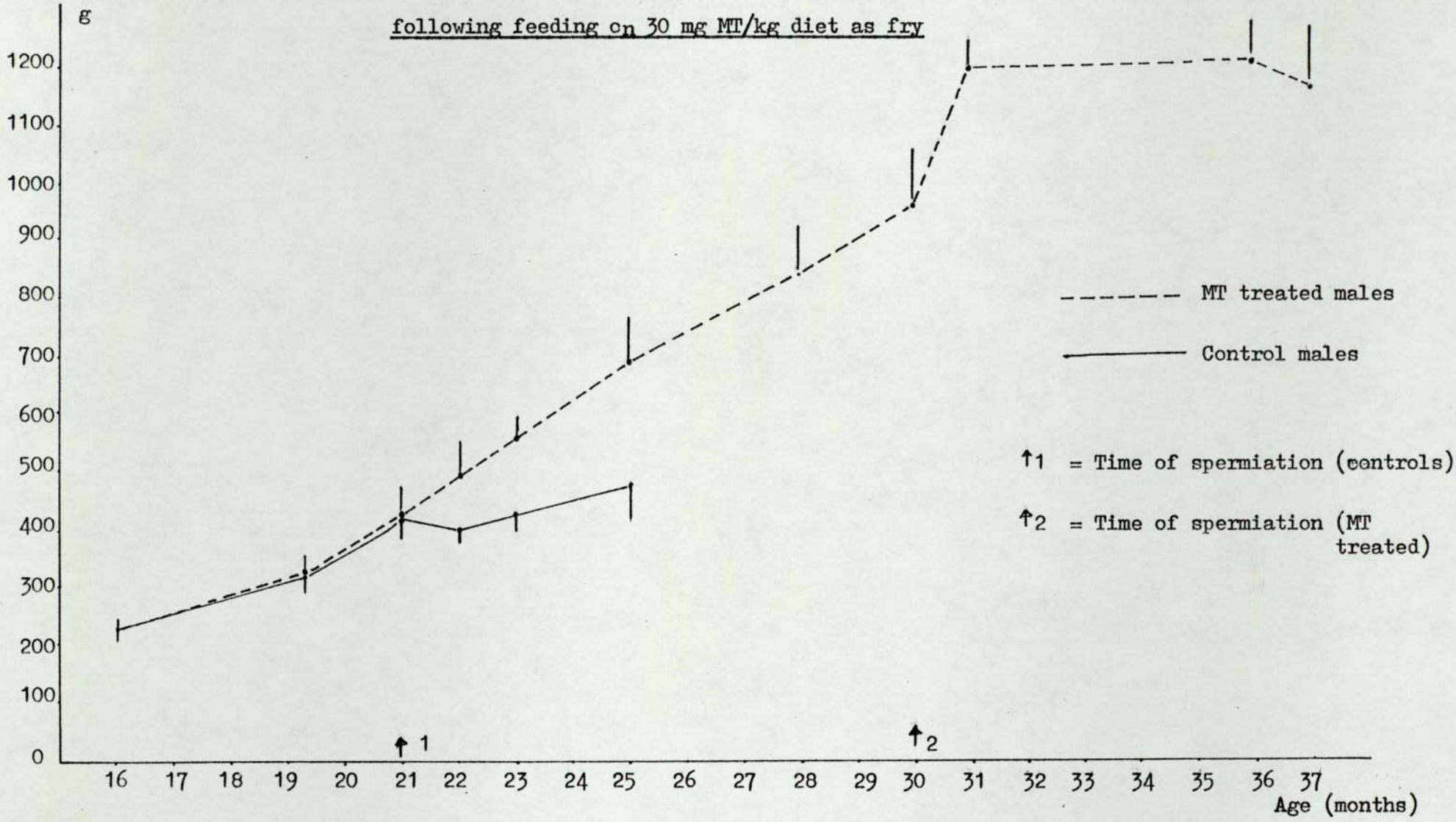
Mean body weights of MT treated rainbow trout at 16 - 36 months post-hatch following feeding 30 mg MT/kg diet for 110 days from first feeding

(g ± SE)

Age (months post-hatch)	Male	Female	Hermaphrodite	Sterile
16	218.0 ± 8.8 (8)	222.3 ± 9.0 (12)	-	-
19	304.4 ± 18.5 (18)	340.4 ± 27.9 (8)	378.5 ± 5.0 (2)	314.0 ± 14.0 (2)
21	422.8 ± 37.4 (10)	449.8 ± 19.5 (13)	531.0 ± 33.2 (6)	422.0 ± 40.7 (3)
22	494.0 ± 56.3 (5)	512.6 ± 13.1 (3)	439.7 ± 8.0 (3)	381.6 ± 55.5 (3)
23	559.9 ± 40.8 (10)	531.3 ± 47.9 (6)	-	455.5 ± 5.5 (2)
25	688.0 ± 57.1 (11)	637.4 ± 72.1 (5)	669.0 ± 54.0 (2)	495.0 ± 17.0 (2)
28	836.1 ± 63.8 (7)	1149.6 ± 133.5 (7)	1029.5 ± 87.5 (2)	975.0 ± 100.0 (2)
30	957.0 ± 85.5 (5)	970.0 ± 163.5 (3)	-	955.0 ± 30.0 (2)
31	1197.2 ± 32.6 (9)	1102.5 ± 148.3 (6)	1047.5 ± 97.5 (2)	975.0 ± 100.0 (2)
35	1203.0 ± 60.0 (23)	1350.4 ± 11.6 (11)	-	1450.0 ± 600 (2)
36	1165.0 ± 82.4 (16)	1368.9 ± 144.0 (9)	1437.5 ± 244.0 (4)	1095.0 ± 365.0 (2)

n = no. in brackets

Graph 8.3.1.2. Mean body weight (g) ± SE of MT treated and control males sampled from 16 - 36 months following feeding on 30 mg MT/kg diet as fry



The mean body weights of both male and female MT treated fish also increased steadily from month 16. However, no depression of growth was observed in treated male fish, associated with the absence of male maturation in these fish. The growth of male treated fish continued until shortly after sexual maturation in these fish (about 30 months) when the growth rate decreased abruptly.

The growth of female treated fish was similar to that of treated males, though no abrupt growth depression was noted after month 30. A slight reduction in growth rate did occur towards the end of the experiment (months 35 - 36).

The growth of sterile fish was not significantly better than that of either male or female treated fish. Due to the limited sample size and hence large standard errors, the differences in growth between sterile and male and female fish did not appear to be statistically significant. However, sterile fish appeared to grow slightly slower than either male or female treated fish.

Discussion

Treatment of first feeding rainbow trout fry with 30 mg MT/kg diet for 110 days from first feeding resulted in a depression of subsequent gonadal development. Early examination revealed abnormal gonad structure with a gross reduction of germ cell numbers and an apparent absence of female gonadal elements.

During the course of the experiment gonadal development was observed among treated male fish and the early results suggesting total sterility were not confirmed. However, the pattern of gonadal development observed

was abnormal, both in its timing and its nature. The testicular elements developing were abnormal in appearance and did not develop until after 28 months in treated male fish; some seven months after testicular development of normal control males. Widespread occurrence of spermatozoa among treated fish was not observed until about 30 months post-hatch.

Male secondary sexual characteristics and the depressed growth allied to normal male maturation were also delayed in their onset in the treated group and this presumably reflects the failure of these fish to develop elevated serum androgen levels at the normal time.

The testes of developing males previously treated with MT were abnormal in structure and consisted largely of nodules of testicular tissue, often with atypical and non-functional, duct systems. Thus the majority of developing males in the treated group could not be hand stripped. This nodular gonadal development is reflected in the maximum mean GSI of males from this group of 1.72 ± 0.22 compared with that of mature control males (3.61 ± 1.07). Of all the treated fish examined between months 16 - 36 11% were found to be sterile; in that their gonads consisted of connective tissue strands with very few detectable germ cells. Approximately 12% of the fish were found to be hermaphrodites, with gonads consisting of both testicular and ovarian components. In almost all cases the anterior portion of the gonad consisted of testis with posterior ovarian elements, which may reflect some gradient of gonadal morphogenesis and possibly a differing sensitivity to hormonal influence.

Control males showed normal sexual development from 19 months and elevated serum androgen levels were noted, associated with the appearance

of male secondary sexual characteristics. A reduction of growth rate was observed to follow shortly after the timing of spermiation of control males. This depression in growth was not observed in treated male fish until after 30 months.

Thus the main effect of high dose MT would seem to be a significant delay in the timing of sexual maturation of treated males until their third year. In addition this effect may also apply to females as only the occasional female treated fish was mature at three years.

The mechanisms through which steroid treatment exerts its effects seem likely to be a direct effect upon the developing gonad rather than an effect upon the pituitary-gonad axis. Certainly the preliminary assays of serum GTH have shown that this axis is functional in these fish and indeed among the few sterile fish examined GTH levels were higher than control fish. These high GTH levels in sterile fish may reflect a lack of negative steroid feedback upon the hypothalamus-pituitary normally operating in mature male salmonids (Billard, 1977a).

However, mean GTH levels of control males were lower than previously reported for male trout at spermiation (Sanchez-Rodriguez et al., 1978; Whitehead et al., 1979). Recently Fostier et al. (1982) have demonstrated that considerable variation in GTH levels may be observed, even within a relatively short period at and around the time of spermiation.

The reduction in germ cell numbers observed earlier in the experiment suggests an effect of high doses of MT upon either germ cell migration into or development within the presumptive gonad. Such a reduction in germ cell numbers was observed in treated fish two months after the cessation of steroid feeding. The gradual development of isolated groups of germ cells, perhaps under the influence of high

levels of GTH, may explain the appearance of nodular testicular elements. If such groups of germ cells were present and escaped detection in samples examined early in the experiment, they would explain the apparent decrease in the number of sterile fish observed. The appearance of female tissue among apparently all male or sterile MT treated fish has been reported (Simpson et al., 1976; Johnstone et al., 1978) and possibly reflects the presence of isolated groups of ovarian cells not observed in histological examination.

Throughout the later phase of the experiment (16 - 36 months) the sex ratio of control fish was close to unity. In treated fish the resulting sex distributions were 40% male, 37% female, 12% hermaphrodite and 11% sterile. These results are similar to Jalabert et al. (1975) who also reported the occurrence of appreciable numbers of females in rainbow trout receiving doses of MT ranging from 15 - 60 mg/kg diet. The observed sex distribution suggests that the steroid dose administered to the experimental fish was insufficient to achieve a masculinization of genetic females. However, though there are grounds for suggesting that the treatment regime did not optimize the effective dose of steroid received, it seems unlikely that this would be less than that achieved by feeding 3 mg/kg MT (the dose reportedly effective in producing complete masculinization in rainbow trout) (Simpson et al., 1976; Johnstone et al., 1978; Scott et al., 1980a).

An alternative explanation is the observation that in some teleosts, especially cichlids, the administration of high doses of exogenous androgens leads to a paradoxial effect in that, while low doses shift development to the male type, high doses can cause a feminization (Muller, 1969; Reinboth, 1969). Nakamura (1975) showed that in Tilapia mossembica, oral administration of androgen exerts a variable

influence on gonadal development and differentiation depending upon the dosage and duration of treatment. Long-term treatment with high doses could feminize the gonads of genetic males. It is thus possible that the females occurring in the present experiment represent a paradoxical feminizing effect, as has been reported to occur in rainbow trout by Bye (quoted in Donaldson and Hunter, 1982a).

It was intended to attempt to fertilize normal ova with spermatozoa from hermaphrodite treated fish. However, at the time no normal ova were available. The offspring of such a cross would have allowed the elucidation of the sex chromosome constitution of the hermaphrodite parent and possibly confirmed a paradoxical feminizing effect.

It is possible the high and low doses of androgens exert separate effects on gonadal development, with high doses acting directly upon germ cell numbers within the developing gonad, while low doses of androgens act only upon the differentiation of these germ cells and not their survival. This is suggested by the already reported observations that doses of up to 60 mg/kg MT were unable to prevent the appearance of ovarian tissue (Jalabert et al., 1975; Yamazaki, 1976), while doses as low as 3 mg/kg diet have produced complete functional sex reversal in a number of salmonid species, including rainbow trout (Simpson et al., 1976; Yamazaki, 1976; Johnstone et al., 1978; Okada et al., 1979) and Atlantic salmon (Johnstone et al., 1978).

8.3.2 The effects of feeding 30 mg MT/kg diet for 90 days from first feeding on the gonadal development of rainbow trout at Finnarts Bay

In order to further evaluate the effect of high androgen doses upon gonadal development and differentiation, a second experiment was carried out in which the effects of high doses of MT upon reproductive development were monitored. However, in this experiment sampling was confined to the expected time of normal male maturation. As this experiment took place at a different farm it provided a comparison between different sites.

Materials and Methods

The experiment was carried out at Shearwater Fish Farming Ltd's Finnarts Bay farm, Stranraer. Two groups of 1000 rainbow trout fry were fed steroid treated or control diet, prepared as previously described, for a period of 90 days from first feeding. During the feeding period the mean water temperature was 6°C and thus treatment covered a period equivalent to 540°C days.

Diet was prepared as described at Aston University, to be mixed on site with nine parts of untreated diet, giving a final concentration of 30 mg MT/kg diet. Fish were fed hourly between 08.30 and 18.00.

At 21 and 26 months post-hatch, fish from each group were sacrificed and samples taken for the determination of G.S.I., examination of gonad histology and assay of serum hormone analysis. However, due to high mortalities among control male fish following an outbreak of furunculosis, very few control males were available for examination at 26 months.

Results

a) Examination of 21 months

Ten fish from each group were examined at the expected time of normal maturation of male fish. The results of this examination are shown in Table 8.3.2.1.

In this sample all control males were sexually mature. Of the treated male fish only one of six examined was mature. In addition three of the fish examined in this group showed no evidence of gonadal development and their gonads consisted only of connective tissue. Only one of the treated fish was a female and its ovaries were very reduced.

b) Examination of 26 months

1) Sex ratios

Forty treated fish were examined at 26 months and the results are shown in Table 8.3.2.2. Of the twenty-three male fish the majority (sixteen) were males, having nodular gonads, showing a range of development. Only seven had testes containing spermatozoa, though of these only four could be hand stripped. Eleven of the remaining seventeen fish were sterile, with the gonadal tissue restricted to connective tissue only, while six fish were females with very reduced ovaries.

2) Hormone levels and secondary sex characteristics

The serum T levels for these fish are shown in Table 8.3.2.2 and Fig. 8.3.2.2.

The mean serum T levels (30.37 ± 6.09 ng/ml) and the mean G.S.I. (0.78 ± 0.13) of nodular males at 26 months suggested these fish were approaching sexual maturity. However, though at this stage darkening

Table 8.3.2.1

Mean G.S.I. ($\pm$ SE) of MT treated and control rainbow trout sampled at 21 months following feeding 30 mg MT/kg diet for 90 days from first feeding

Group	Running males	Immature males	Sterile	Female
Control	3.08 $\pm$ 0.42	-	-	0.11 $\pm$ 0.02
n	4)	0)	0)	6)
MT	3.43	0.017 $\pm$ 0.008	0.004 $\pm$ 0.000	0.001
n	1)	5)	3)	1)

Table 8.3.2.2

Mean G.S.I. ($\pm$ SE) and serum testosterone (ng/ml $\pm$ SE) of control and MT treated rainbow trout sampled at 26 months following feeding 30 mg MT/kg diet for 90 days from first feeding

	Sterile	Nodular male	Mature male	Female
Control				
G.S.I. $\pm$ SE	-	-	1.67 $\pm$ 0.5	0.20 $\pm$0.05
n			5)	4)
Testosterone (ng/ml)	-	-	9.62 $\pm$ 0.21	2.62 $\pm$0.58
n			3)	4)
MT				
G.S.I. $\pm$ SE	0.009 $\pm$ 0.008	0.78 $\pm$ 0.13	3.23 $\pm$ 0.22	0.026 $\pm$0.01 (**)
n	11)	16)	7)	6)
Testosterone (ng/ml)	2.02 $\pm$ 0.20	30.37 $\pm$ 6.09	12.76 $\pm$3.24	3.53 $\pm$ 1.51
n	11)	16)	7)	6)

t test vs. control (**) $P < 0.01$.

Fig. 8.3.2.1. Mean G.S.I. (± SE) of MT treated and control rainbow trout sampled at 26 months following feeding on 30 mg MT/kg diet as fry

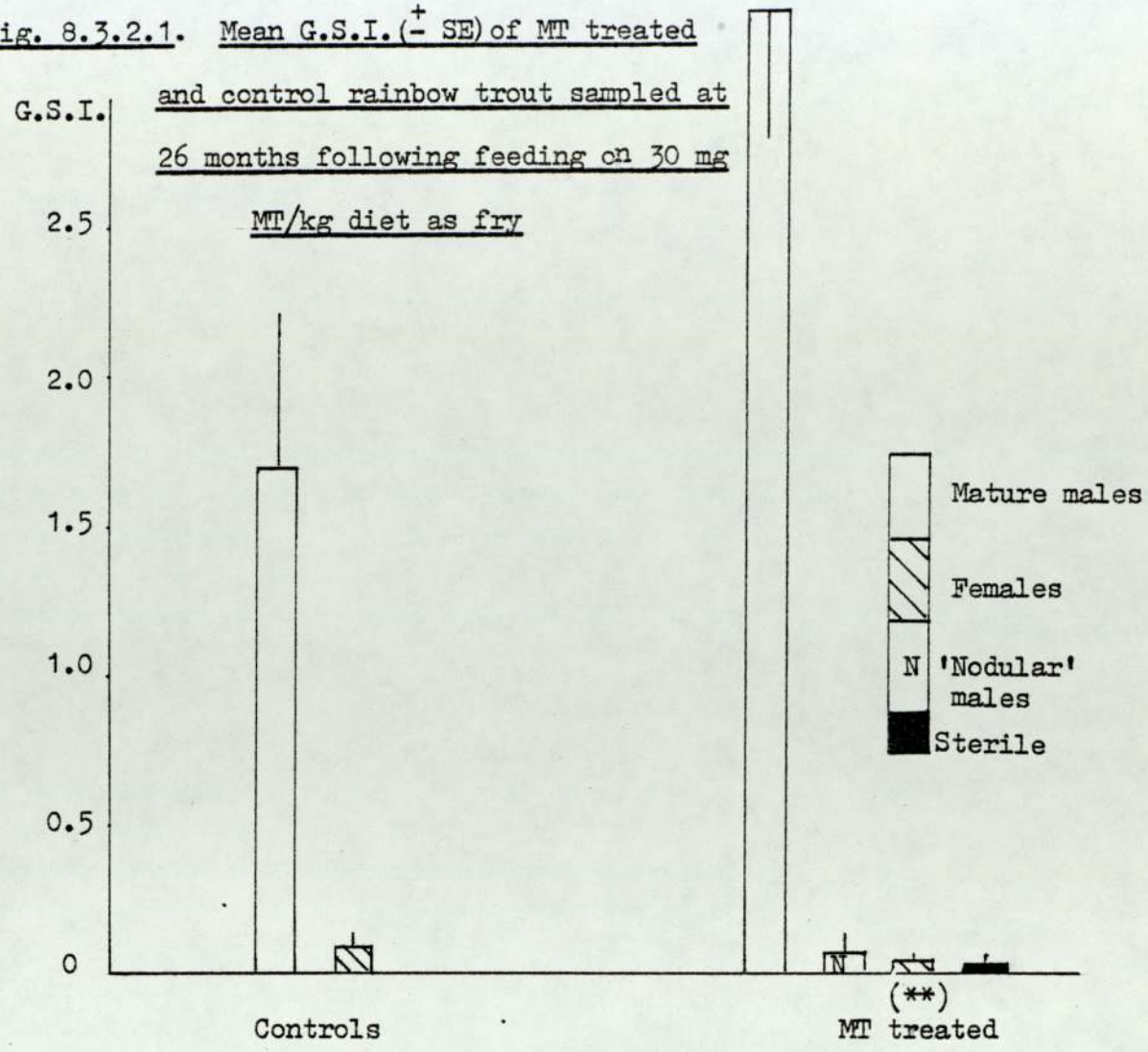
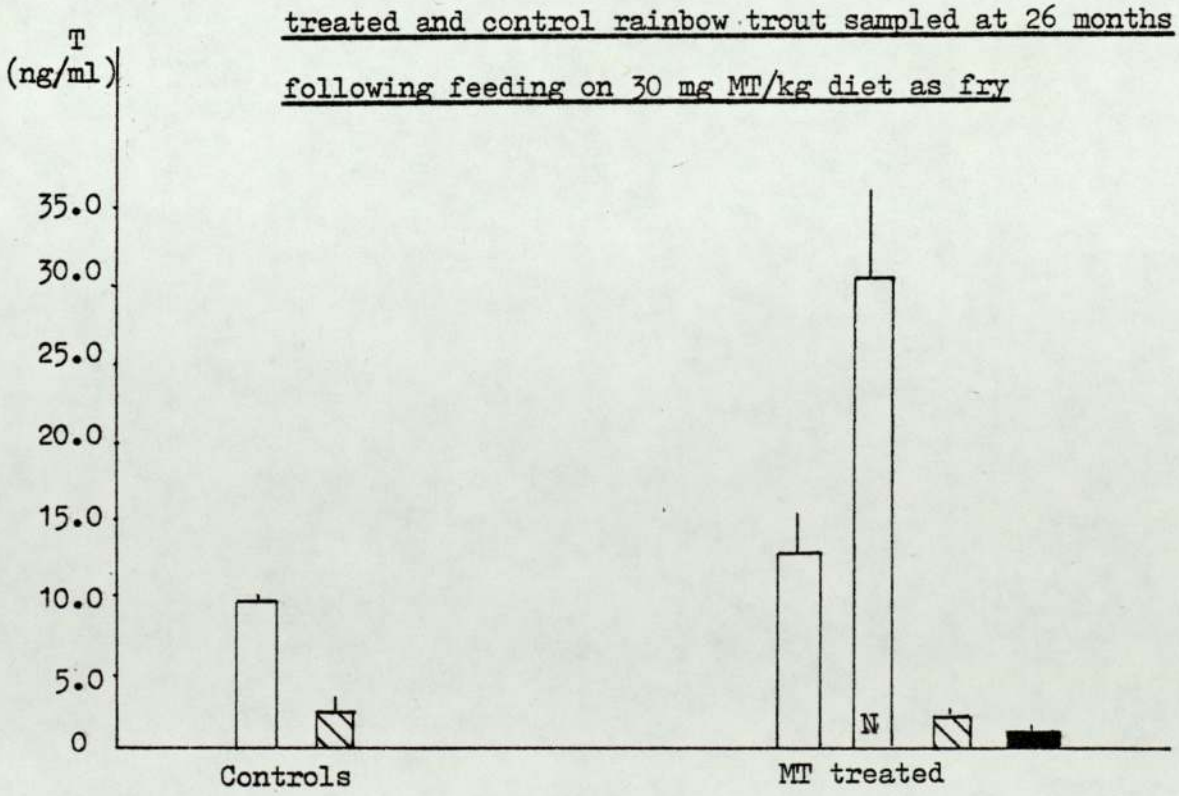


Fig. 8.3.2.2. Mean serum testosterone levels (ng/ml) ± SE of MT treated and control rainbow trout sampled at 26 months following feeding on 30 mg MT/kg diet as fry



and the beginnings of kype development were seen, male secondary sexual characteristics were not well developed in nodular males.

Among mature males secondary sexual characteristics were well developed. Serum T levels were, however, lower than in nodular males, suggesting that these fish had passed the time of peak steroid levels (12.76 ± 3.24 ng/ml, mature males, compared with 30.37 ± 6.09 ng/ml, nodular males). The mean G.S.I. of these mature males was 3.23 ± 0.22.

In reduced females and fish assessed as sterile serum T levels were low (3.53 ± 1.57 and 2.02 ± 0.2 ng/ml respectively). The mean G.S.I. of these two groups were 0.026 ± 0.01 and 0.009 ± 0.008.

Among the few control males examined the mean G.S.I. was lower in mature male controls (1.67 ± 0.5) (Table 8.3.2.2 and Fig. 8.3.2.1) than in MT treated mature males (3.23 ± 0.22), probably reflecting the earlier timing of maturation among control males. Serum T levels were also slightly, though not significantly, lower among control males than MT treated mature males (9.62 ± 2.1 vs. 12.76 ± 3.24). The mean T levels of control and MT treated females were not significantly different (2.62 ± 0.58 and 3.53 ± 1.51 ng/ml respectively), though the mean G.S.I. of control females (0.20 ± 0.05) was significantly ($P < 0.01$) higher than that of treated females (0.026 ± 0.01). The hormone and G.S.I. results for treated and control fish are shown in Table 8.3.2.2 and Figs. 8.3.2.1 and 8.3.2.2.

3) Body weights of treated fish

In view of the lower density of fish in the control tank following mortalities, the mean body weights of treated and control fish were not compared directly. However, the weights of fish within each sub-group of treated fish were compared (Table 8.3.2.3 and Fig. 8.3.2.3).

Table 8.3.2.3

Mean body weights (g ± SE) of treated fish (26 months)

fed 30 mg MT/kg diet as fry

Sterile	Male (nodular)	Male (mature)	Female
645.0 ± 74.4	856.6 ± 51.9	508.6 ± 45.6	872.5 ± 56.6
n 11)	16)	7)	6)
—(*)—	—(***)—	—(***)—	
—————(*)—————			

t test between groups (***) $P < 0.001$
(*) $P < 0.05$

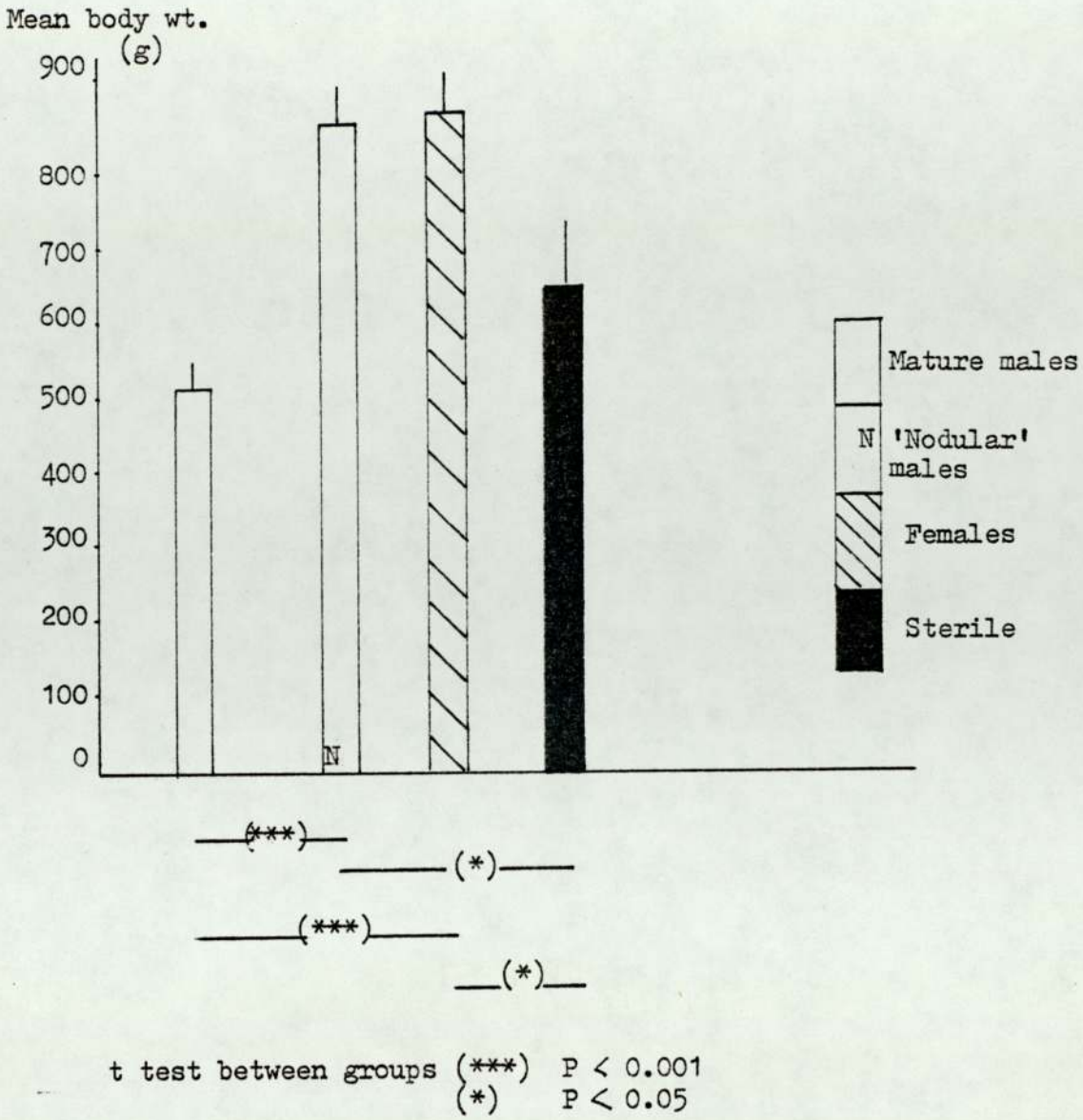


Fig. 8.3.2.3

Mean body weight of MT treated rainbow trout sampled at 26 months following feeding on 30 mg MT/kg diet as fry

Among treated fish mature males were significantly lighter (508.6 $\pm$ 45.6 g) than either nodular males (856.6 $\pm$ 51.9 g) or females (872.5 $\pm$ 56.6 g) ($P < 0.001$). However, mature males were not significantly lighter than sterile fish (645.0 $\pm$ 74.4 g). Sterile fish were significantly lighter than nodular males and females ($P < 0.05$). Thus both sterile fish and mature males showed poorer growth than either nodular males or immature females.

Discussion

As in the previous experiment a variable response to high dose MT treatment was observed. A higher percentage of treated fish (28%) were found to be sterile, having gonads consisting only of connective tissue with little germinal tissue. A shift to the male gonadal type was observed in treated fish. Of the fish examined at months 21 and 26 58% (29 of 50) contained male gonadal tissue, while only 14% (7 of 50) contained female elements.

Again MT treatment reduced gonadal development in both male and female fish, though some males matured earlier than in the previous experiment. At 26 months post-hatch (5 months after the maturation of control males) 71% of the treated males had not yet developed male secondary sexual characteristics. However, gonadal histology and assay of serum hormone levels suggested these fish would probably mature within one or two months.

This delay in male maturation was reflected in the mean body weights of fish at the termination of the experiment (month 26). Treated nodular males, containing gonadal elements which had not yet developed, and females were significantly heavier than those treated males which had

already matured. The low mean body weight of sterile fish again suggests a poor growth performance in these fish. Sterile fish were significantly ($P < 0.05$) lighter than either nodular males or females at 26 months, though they were slightly heavier than control males, but not significantly so (645.0 $\pm$ 74.4 g and 499.0 $\pm$ 79.4 g respectively).

Thus it appears that the growth of sterile fish is not as good as either female or late maturing male treated fish. The female treated and nodular male treated fish appear to have avoided the maturation associated depression of growth. Although sterile fish had not matured they did not show any increased growth compared to mature males. A lower growth rate of sterile fish compared to that of E_2 feminized females has been reported in coho salmon (Oncorhynchus kisutch) (Donaldson and Hunter, 1982b).

Hunt et al. (1982) suggest that good early spring growth in male salmonids may be due to the increases in serum androgens, especially 11-KT. In low doses androgens have been shown to exert anabolic, growth promoting effects in salmonids (Fagerlund and McBride, 1975, 1977). No significant differences were detected in the serum T levels of sterile and female treated fish in the present experiment, and in both groups serum T levels were low. In females 11-KT levels would be expected to remain low (Simpson and Wright, 1977) and it is difficult to explain the reduced growth of sterile fish relative to females by an anabolic effect of androgens. However, serum hormone levels differed between sterile and late maturing treated males and may explain the observed differences in growth rate.

The results reported in the present experiment showed several interesting differences to those previously reported from the Low Plains

site. In the Finnarts Bay experiment a higher percentage of sterile fish were observed following MT treatment (28%) than at Low Plains (11%), but no hermaphroditic gonads were observed among the fish examined at Finnarts Bay. This result does not exclude the possibility that some of the fish assessed as sterile may have contained occasional germ cells, which may have escaped detection. Gonadal development among treated fish, though delayed in the majority of males, took place earlier in the Finnarts Bay trial. Of the treated fish examined at month 26, 27% of the males were mature, while male maturation was not noted to a significant extent in the Low Plains trial until 30 months post-hatch.

These differences seem most likely to be explained by variation in the rearing conditions or the fish used in the experiments. The length of the treatment period and the differences in ambient temperature between the two sites are two important intersite variables. At Finnarts Bay fry were fed for the equivalent of 540°C days (90 days at a mean temperature of 6°C), while at Low Plains (at 10°C) they were fed for 1100°C days. As well as this variation in treatment period fry at Finnarts Bay experienced a variable temperature regime over the feeding period, while at Low Plains fish are maintained at a constant temperature of 10°C. As temperature is a prime determinant of feeding rate, then the amount of food taken by fry, the effective dosage received and the growth rate would be succeptible to variations in temperature. Temperature will also directly influence the rate of gonadal differentiation. In addition, some more subtle differences in feeding practice and growth rate may operate between the two sites.

8.3.3 High dose androgen treatment - general discussion

A variable response to high dose androgen treatment was observed in the two experiments reported in which rainbow trout fry were fed 30 mg MT/kg diet, with a partial sterilization and an inhibition of gonadal development in treated fish.

In the first experiment 11% of treated fish examined at 16 - 36 months were sterile, while in the second experiment 28% of the treated fish showed this condition. Earlier histological examination suggested a much higher degree of sterility. The discrepancy between the results at different ages possibly reflects the development of isolated groups of germ cells in otherwise sterile gonads. This would explain the appearance of nodular type gonads, with each nodule reflecting the proliferation of a group of germ or interstitial cells. Such a finding calls into question the validity of such early histological examination, unless it is sufficiently rigorous to completely exclude such isolated groups of cells.

MT treatment thus seems capable of reducing the germ cell numbers, either migrating into or surviving in the developing gonad. However, with the dosage and treatment regime used this effect did not result in permanent sterility. The development of such nodular gonads presumably indicates that the pituitary-hypothalamic axis is unimpaired by high dose MT treatment and this is confirmed by measurement of serum GTH levels in treated fish. Van den Hurk (1982) showed the pituitary gonadotropes of MT treated rainbow trout to be functional, though smaller than those of control fish at nine months post-hatch.

Although the remaining fish were not completely sterilized, the male gonad development that occurred was abnormal in timing and in form.

A very low extent of male maturation was observed among treated males at the time of normal control male maturation. The timing of such maturation that did occur varied between the two experiments, perhaps reflecting differences in the steroid dose received and feeding practice. At Low Plains little male gonad development was observed prior to 28 months, while in control males sexual development was observed at 20/21 months. Among treated male fish at Finnarts Bay only 17% contained spermatozoa at 26 months post-hatch. Thus at Low Plains treated fish were spared the adverse consequences of male maturation, including the development of male secondary sexual characteristics and the depressed growth associated with maturation, for a period of 6 - 8 months, while the delay was slightly less at Finnarts Bay.

Relatively few reports have considered the subsequent development of rainbow trout treated with high androgen doses from first feeding. Jalabert et al. (1975) reported that 11% of two year old rainbow trout fed 15 - 60 mg MT/kg diet as fry were sterile, though very high mortalities occurred in this trial. No mention was made of any delay in maturation of male and female treated fish. However, Bye and Lincoln (1979) reported that only 1.5% of fish treated with low doses of MT were mature at a time when 20% of control males were mature. Yamazaki (1976) noted that the apparent sterility resulting from feeding 50 mg MT/kg persisted beyond two years in rainbow trout. However, this finding was based on a very small sample of surviving fish. Sterility at 12 months in response to immersion in MT (30 to 300 ug MT per litre) was reported by van den Hurk et al. (1980), but fish were not maintained beyond this period and high mortalities (65%) were observed.

In other salmonid species a greater extent of sterility in response to high dose androgen treatment has been recorded. In Pacific salmon

(Oncorhynchus kisutch) treatment by immersion in MT (100 - 400 ug/L) followed by feeding 20 mg/kg diet MT resulted in apparently sterile fish at four months post-hatch (Goetz et al., 1979). This apparent sterility was confirmed at two years (Donaldson and Hunter, 1982a). Donaldson and Hunter also report similar results were achieved by immersion in 400 µg MT/L followed by feeding only 10 mg MT/kg diet. They report that 94% of treated fish did not develop secondary sexual characteristics at the time of normal maturation of control males. Interestingly their data suggest treatment at the eyed egg stage or the alevin stage (immersion in 400 µg MT/L) without subsequent steroid treatment can have at least a partial effect on gonadal development by inducing a low percentage of intersex fish. This finding is in contrast to the results of Simpson (1976) and Johnstone et al. (1978), who showed immersion prior to dietary steroid treatment was not necessary to achieve hormonal sex reversal in Atlantic salmon and rainbow trout. However, it is possible that the thresholds for sex reversal and sterilization effects differ.

In part these differences may reflect a species difference in the sensitivity of gonadal tissue to steroid treatment, perhaps related to the timing of gonad differentiation in these species. Persov (1975) reports the requirement for gonadal differentiation to be 840°C days in Pacific salmon and 1067°C days in Atlantic salmon. Estimates for the requirement for rainbow trout range from 600 - 640°C days (Okada, 1973; Laird et al., 1978; van den Hurk et al., 1980). Even within this period the sensitivity to steroid administration may vary depending on species. However, in the two experiments described the Low Plains experiment should have covered the full period of gonadal differentiation, while the Finnarts Bay experiment should have covered the majority of this period.

Bye (1982) has reported total sterility in turbot (Scophthalmus maximus) fed only 5 mg MT/kg diet; perhaps a further reflection of differing species' sensitivity to androgen treatment.

The results of the two experiments described are at variance with those reported for low dose androgen administration in rainbow trout. In rainbow trout doses of 1 - 10 mg MT/kg diet resulted in an overall shift to male gonad development (Simpson et al., 1976; Yamazaki, 1976; Johnstone et al., 1978 ; Okada et al., 1979; Scott et al., 1980a). Similarly low doses of androgen resulted in the masculinization of genetic female Atlantic salmon (Johnstone et al., 1978), Pacific salmon (Donaldson and Hunter, 1982a) and lake trout (Wenstrom, 1975). No masculinization was observed in the experiments described, though a higher percentage of males (58%) was observed among fish treated at Finnarts Bay. Although treatment and feeding regimes may not have maximized the dose of steroid received, it seems unlikely that this would have fallen lower than that supplied by feeding 1 - 3 mg MT/kg diet, a dose reportedly effective in masculinizing genetic females.

One possible explanation for this discrepancy is that androgen treatment exerts two different types of effect, with low doses influencing the differentiation of germ cells and high doses the survival of such cells. A further complicating factor is the possibility of paradoxical feminization, which could have been resolved by examining the sex ratio of the progeny of crosses between female treated fish and normal males. It was noted that the sex ratio was closest to unity among treated fish in the Low Plains trial, in which MT treatment was more prolonged.

The growth performance of those sterile fish examined was not impressive. Due to the lower level of sterility obtained in the Low Plains experiment, it was only possible to note that these fish appeared to grow no better than either late maturing males or immature females. However, in the second experiment, where a higher percentage of sterile fish was observed, the mean body weight of these fish was significantly lower than both late maturing treated males and treated immature females, though not of control males which had matured earlier. The reasons for this poor growth are uncertain, as these fish avoided the deleterious effects resulting from normal male maturation. It may be linked to a reduction in androgen levels in these fish, thus removing a possible anabolic effect of such steroids (Fagerlund and McBride, 1975, 1977; Hunt et al., 1982). However, such differences in serum androgens would not explain the difference in growth between sterile and immature female fish. Bye (1982) has reported that at two years hormonally produced sterile turbot(Scophthalmus maximus) were 27% heavier than control fish.

8.4 Steroid feeding - Ancillary experiments

In order to assess the effects of certain aspects of the steroid treatment regimes in causing the observed variation in results, treatment parameters were examined. The results of these, and other trials reported in the literature, suggest that variations in feeding regimes and treatment method may affect the dose of steroid received and hence the response observed.

Two ancillary experiments were carried out to evaluate aspects of this variation. Firstly, the effect of an alternative treatment method

(spraying) was compared with that previously used for the dietary administration of steroid (coating a 10% portion of the diet and mixing with nine parts of untreated diet). Secondly, alteration of feed rate and the effect of withdrawal of treated diet on blood steroid levels were studied. In addition an experiment was carried out to assess the rate at which treated diet may be eliminated from the digestive system of fry.

8.4.1 The effects of variation in feed rate and treatment method upon blood steroid levels in treated rainbow trout

In order to evaluate possible variation in the steroid dose received by treated fish, an experiment was carried out in which the effects of two alternative treatment methods and the effect of variation in feed rate were studied.

Differences in feeding regime between sites seems a likely cause of the reported variation. In addition different workers have used different methods of administering steroid by dietary feeding. Some workers (Guerrero, 1975; Simpson, 1976; Johnstone et al., 1978) treated one part of defatted diet. This was then mixed with nine parts normal diet to give the required concentration. Others (Goetz et al., 1979) sprayed the diet with steroid in ethanol.

Materials and Methods

Owing to the difficulty of sampling small fish, two year old rainbow trout were used. In order to avoid competition from endogenous androgens, immature fish were selected (largely immature females). Three groups of fish were maintained in separate tanks in a recirculating system at the University of Aston Fish Culture Unit. Each group

of fish were fed either a steroid treated diet, prepared as described below, or a control diet, based upon BP 'Mainstream' number 4 pellets.

Diet preparation

a) Defatted diet

Diet was prepared in the manner described by Guerrero (1975) and Simpson et al. (1976). A slight modification was that a 20% portion of diet was used rather than a 10% portion. This portion was defatted by ethanol efflux, placed in a rotavapor flask and steroid (10 mg T/ 100 g diet) added in ethanol. The ethanol was then evaporated off under vacuum, using gentle warming (35°C) and the diet air dried overnight. This portion of diet was stored at 4°C and mixed immediately before use with four parts normal diet to give a concentration of 20 mg T/kg diet.

b) Sprayed diet

Sprayed diet was prepared by applying steroid directly to BP number 4 diet. 1 kg batches were spread on a tray in a fume cupboard and 20 mg T dissolved in 50 ml ethanol and sprayed onto the food using a small electric spray gun. The diet was dried overnight. Again diet was stored at 4°C until required.

c) Control diet

Control diet was prepared by mixing one part of defatted food, without steroid added, to four parts of diet sprayed with ethanol, again without steroid added.

Feeding regimes

Fish were fed 1.5% body weight/day until day 49, on which day all groups were cut to 1% body weight/day. On day 61 steroid treatment was discontinued and both treated groups switched to control diet. On day 64 steroid treatment was recommenced in the sprayed group until day 72, when the experiment was terminated. The feeding regime is shown in Fig. 8.4.1.1.

Fish were regularly sampled throughout the experimental period. Normally blood samples were taken at 1600 hours, approximately four hours after the second of three daily feeds. Serum samples were collected and assayed for T as described.

Results

The results of serum T assay are shown in Table 8.4.1.1 and Graph 8.4.1.1. An initial difference was apparent in the mean serum levels of T treated fish. A more rapid increase was noted in serum T in fish fed sprayed diet compared to those fed defatted diet containing T. Twenty days after the start of feeding, fish fed sprayed diet had mean T levels of 96.46 $\pm$ 14.05 ng/ml, while the corresponding value for the other steroid treated group was significantly lower ($P < 0.05$) (54.42 $\pm$ 12.04 ng/ml). However, by day 34 of treatment no significant difference was apparent in the T levels of fish fed sprayed (91.72 $\pm$ 6.05 ng/ml) or defatted diet (92.25 $\pm$ 7.92 ng/ml).

A reduction in ration size to 1% body weight/day at day 49 resulted in a significant fall in T levels in both treated groups. Mean T levels of the defatted diet group fell from 93.64 $\pm$ 8.71 ng/ml to 67.38 $\pm$ 6.7 ng/ml (day 49 to day 61). ($P < 0.05$). Within the sprayed diet group

Table 8.4.1.1

Serum T levels of immature rainbow trout fed either steroid treated (20 mg T/kg diet) or control diets

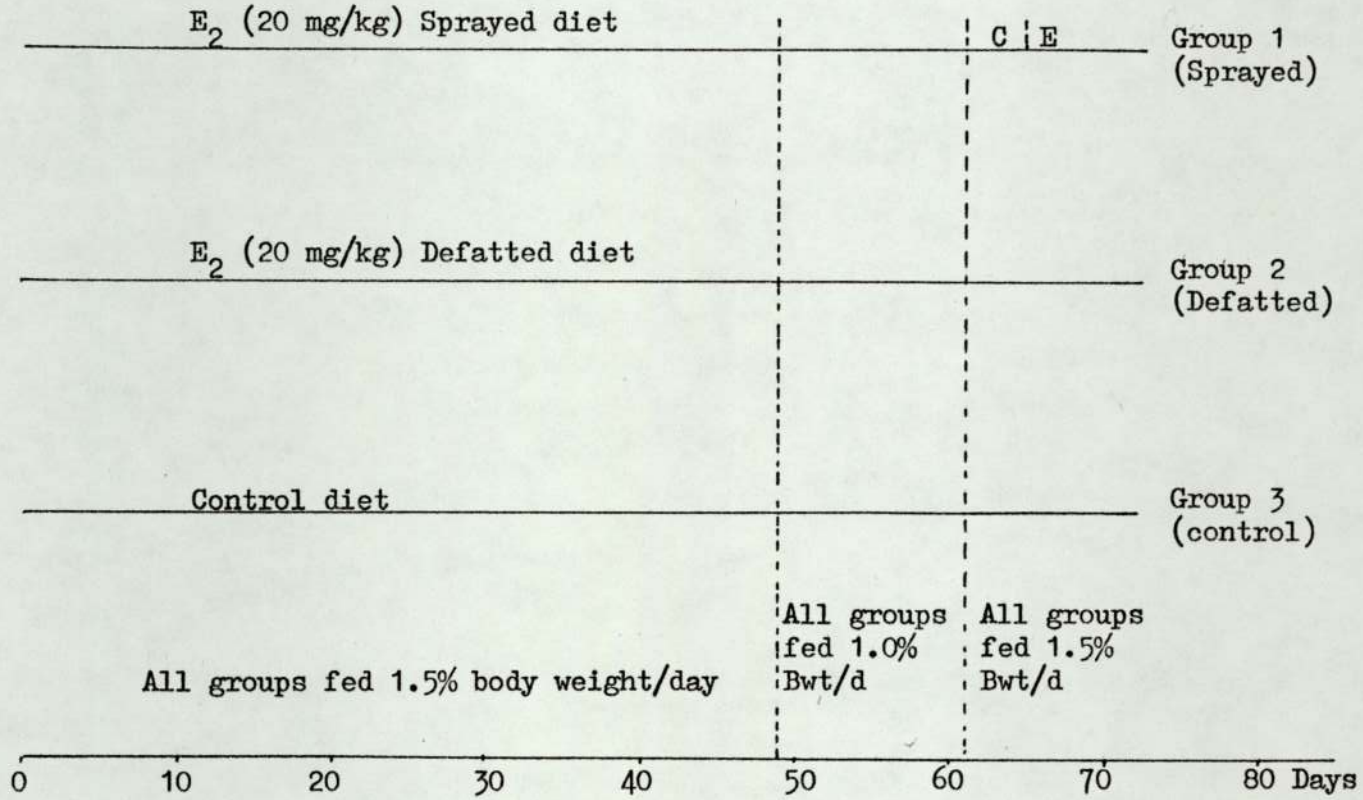
Day	20	34	49	61	62	64	72
Group							
Control	5.46 ± 0.52	4.55 ± 0.52	11.80 ± 0.70	6.20 ± 1.22	-	-	11.50 ± 4.25
n	6)	6)	6)	8)			5)
			(***)				
			(*)				
T treated (defatted)	54.42 ± 12.04	91.72 ± 6.05	93.64 ± 8.71	67.38 ± 6.90	-	-	10.68 ± 3.26
n	10)	8)	7)	9)		(***)	7)
	(*)						
				(***)		(***)	
T treated (sprayed)	96.46 ± 14.05	92.25 ± 7.92	89.48 ± 10.79	61.68 ± 5.86	12.26 ± 4.29	8.01 ± 4.45	60.67 ± 9.20
n	6)	9)	8)	9)	8)	8)	9)
				(*)			
			a	b		c	

a - Day 49. Switched from 1.5% to 1.0% body weight/day.
b - Day 61. Experimental groups switched to control diet.
c - Day 64. Sprayed group experimental diet restored.

t test between consecutive samples (*) $P < 0.05$, (***) $P < 0.001$.

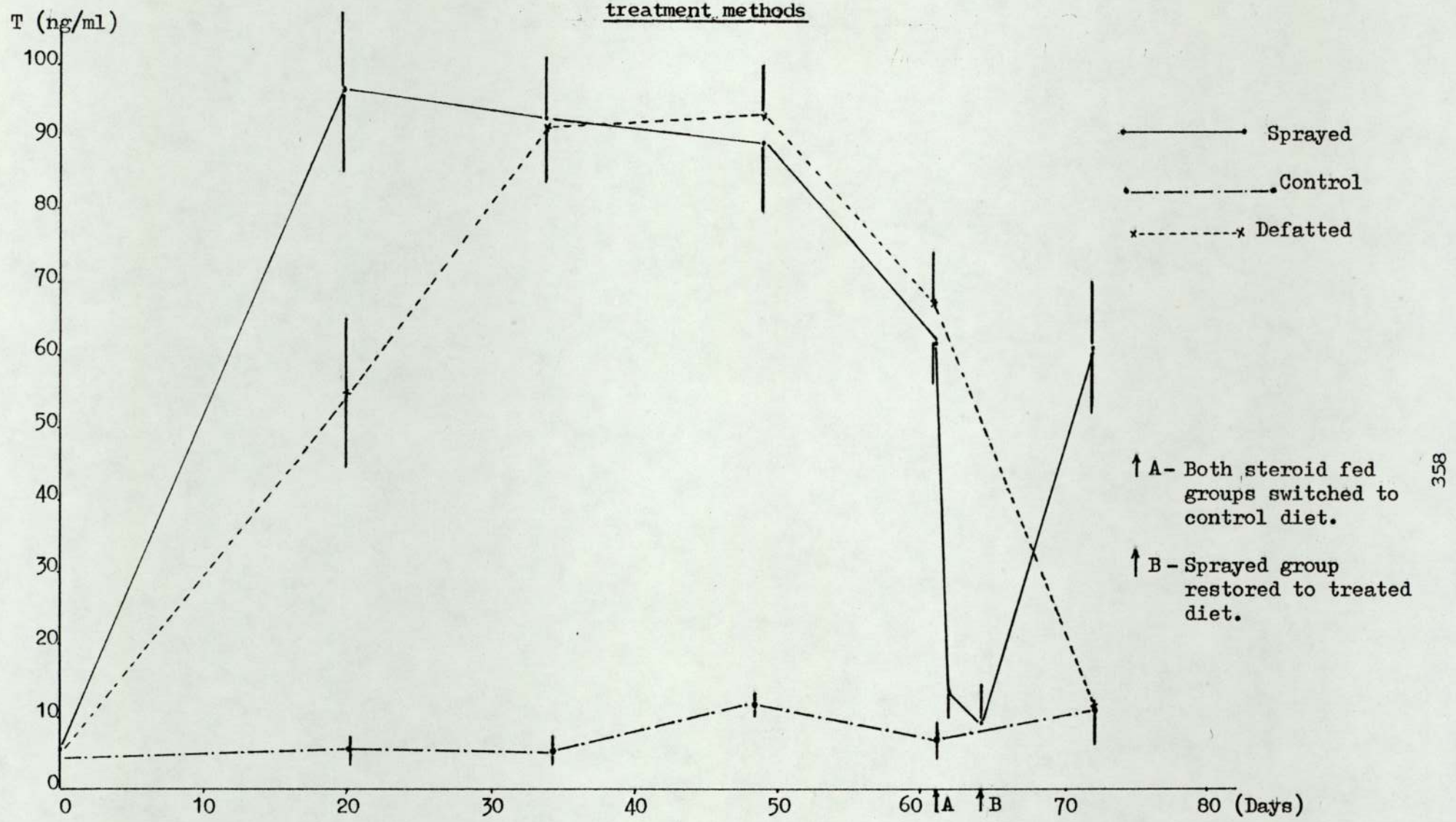
Fig. 8.4.1.1

Experimental design for comparing treatment method and steroid withdrawal upon blood levels of steroid



C Switched to control diet
E Switched to E_2 treated diet

Graph 8.4.1.1. Serum T levels (ng/ml) ± SE of control and T fed rainbow trout treated by two treatment methods



mean T levels fell from 89.48 ± 10.79 ng/ml to 61.68 ± 5.86 ng/ml over the same period ($P < 0.05$). Surprisingly a significant fall was also observed in control fish over the same period (11.80 ± 0.70 ng/ml to 6.20 ± 1.22 ng/ml).

Following the switch to control diet on day 61 of the experiment, mean T levels fell rapidly from 61.68 ± 5.86 ng/ml to 12.26 ± 4.29 ng/ml (day 62) in the sprayed group ($P < 0.001$). A further slight fall was observed to 8.01 ± 4.45 ng/ml by day 64. Steroid feeding was resumed in this group on day 64 and by day 72 mean T levels had risen to 60.67 ± 9.20 ng/ml.

In the group fed defatted steroid diet, mean T levels fell from 67.38 ± 6.90 ng/ml on day 61 to 10.68 ± 3.26 ng/ml by day 72 ($P < 0.001$). In the control group T levels had risen slightly, though not significantly between days 61 (6.20 ± 1.22 ng/ml) and 72 (11.50 ± 4.25 ng/ml).

Discussion

In order to allow easy measurement of blood steroid levels, T was administered, so the results obtained may not be typical of all steroids, though it is likely that similar profiles would be obtained for other steroids. MT may be expected to be taken up into the blood more rapidly than would T.

Significant differences were observed between the treatment regimes in the early stages of feeding, though due to the gap in sampling it is difficult to be precise about the exact shape of the steroid uptake curve over this early period. However, it is apparent that the rate of steroid uptake is higher from sprayed diet. Spraying resulted in a more rapid

increase in T levels. By day 20 a significant difference was observed between the sprayed and defatted steroid groups. This difference had vanished by day 34. The reason for this difference is uncertain, though one possible explanation is that the fish are able to discriminate between treated and non-treated particles in the defatted diet. In some steroid feeding trials it was noted that fish tended to reject food, possibly based on taste, and it is suggested these pellets are more likely to be steroid treated. Spraying would distribute the steroid evenly and avoid any such effect. Obviously such discrimination would be most marked at very high feed levels and this aspect perhaps deserves further study. It is possible that if discrimination takes place that higher feed levels, using defatted diet, would favour a lower blood steroid level.

Although the effect was not apparent beyond 39 days, in a treatment regime, for example sex reversal, where it is necessary to treat for the full extent of a critical period of gonadal differentiation, such a difference might be sufficient to cause steroid levels to drop below threshold levels.

A reduction in feed rate from 1.5% to 1.0% body weight/day at day 49 resulted in a significant ($P < 0.05$) fall in mean T levels in both treated groups. In the sprayed diet group the mean T levels fell from 89.48 ± 10.79 ng/ml (day 49) to 61.68 ± 5.86 ng/ml (day 61). In the defatted group mean levels fell from 93.64 ± 8.71 to 67.38 ± 6.70 ng/ml over the same period. Interestingly control levels also showed a significant fall ($P < 0.001$) over the same period. The reasons for this increase in control levels at day 49 and their subsequent fall are uncertain, though it is possible that some steroid may have circulated in the water.

Switching the sprayed diet group to control diet on day 61 of the experiment resulted in a rapid fall in serum T levels. By day 62 mean T levels had fallen to 12.26 $\pm$ 4.29 ng/ml ($P < 0.001$) with a further slight fall to 8.01 $\pm$ 4.45 ng/ml by day 64. At this point steroid treatment was resumed. This rapid fall in T levels suggests a short half life for blood borne T and confirms the results obtained by the administration of tritiated testosterone in Oncorhynchus kisutch (Fagerlund and McBride, 1978) and in Cyprinus carpio (Lone and Matty, 1981b). Similar work has studied the disappearance of ^{3}H MT in O. kisutch (Fagerlund and Dye, 1979) and its half life in the blood was shown to be short. Similarly Johnstone et al. (1978) have shown E_2 to have a relatively short half life in salmonids. However, these reports have recorded the disappearance of radioactivity, rather than immunoreactive steroid, and may reflect conversion of steroid to metabolites.

Thus it would appear that significant fluctuations in steroid levels may occur when feed levels are changed or a period with no fresh input of steroid treated diet intervenes. This may have some bearing on the situation at Low Plains and Finnarts Bay, where feeding was limited to the period from 08.30 to 17.00 hours. During the intervening period steroid levels could fall below the required threshold, as indeed seems to have occurred with the E_2 feminization trials. If feed rates are sub-optimum then the steroid dose received may be too low to achieve the desired effect. As a further complication attempts to avoid this by increasing feed levels may give rise to selection of untreated diet, when diet containing non-treated particles is used, and so cause a further reduction of the steroid reaching the fish.

The results of the final E_2 feminization trial suggest that this may indeed be the case, though in this experiment interpretation is

complicated as steroid dipping was also introduced. Certainly spraying seems to have increased the dose of steroid received, though in this case to levels high enough to induce mortalities and growth depression. This preliminary experiment has shown that feeding rate and farming practice can have an enormous impact on the steroid dose reaching treated fish and thus on the results obtained in steroid feeding trials. It is essential that all facets of the treatment regime used are considered in the light of the desired results and that as far as possible the steroid treatment method is designed to fit the particular rearing regime of each farm.

8.4.2 Gastric evacuation of rainbow trout fry

Under the feeding regime at Low Plains, fry were fed hourly between the period of 08.30 to 17.00 and a period of some 15 hours followed the last daily feed until the next day's first feed. As the half life of exogenous E_2 (Simpson and Wright, 1976), MT (Fagerlund and Dye, 1979) and T (Fagerlund and McBride, 1978) is short in salmonids, it is likely this period is sufficient for blood steroid levels to fall. The previous experiment demonstrates such a fall in steroid levels may occur following even relatively short-term withdrawal.

Steroid uptake from the gut depends upon a number of factors, a major one of which would be the rate at which steroid crosses the lining of the intestine to the blood. A second factor which would influence the uptake is the rate at which the alimentary canal empties. If the rate of emptying is faster than the rate of steroid uptake from the gut then this may limit the available uptake time.

Little information is available regarding the uptake of steroids from the teleost gut, though considerable data are available on the uptake of lipids (Cowey and Sargent, 1977). More information is available on the rate of gastric evacuation in salmonids, though not specifically in fry (Hopkins, 1966; Joblin and Davies, 1979). In order to evaluate the possible importance of gastric evacuation in influencing the blood steroid levels of treated fry, an experiment was carried out in which the rate of gastric evacuation of rainbow trout fry was estimated, using the direct method of removal of stomach and intestinal contents at set times following a single feed.

Materials and Methods

Rainbow trout fry (mean weight 0.42 g) were placed in a tank on a recirculating system and starved for 24 hours prior to the experiment. Following this period they were fed BP 00 fry food ad libitum for a 30 minute period. The fry were then removed to a clean tank to prevent consumption of any residual food. At intervals following feeding 20 fry were removed and the alimentary canal dissected free. The alimentary canal was split with a scalpel blade and the contents gently scraped out onto a clean, pre-weighed foil. The gut contents of all 20 fish were pooled at each sample time and then dried overnight at 100°C. They were then weighed and the mean weight of the gut contents calculated (dry weight). The square root of mean gut contents was plotted against time (Hopkins, 1966; Joblin and Davies, 1979).

Results

The mean gut contents weight (mg dry weight) at various intervals post-feeding are shown in Table 8.4.2.1 and the square roots of these

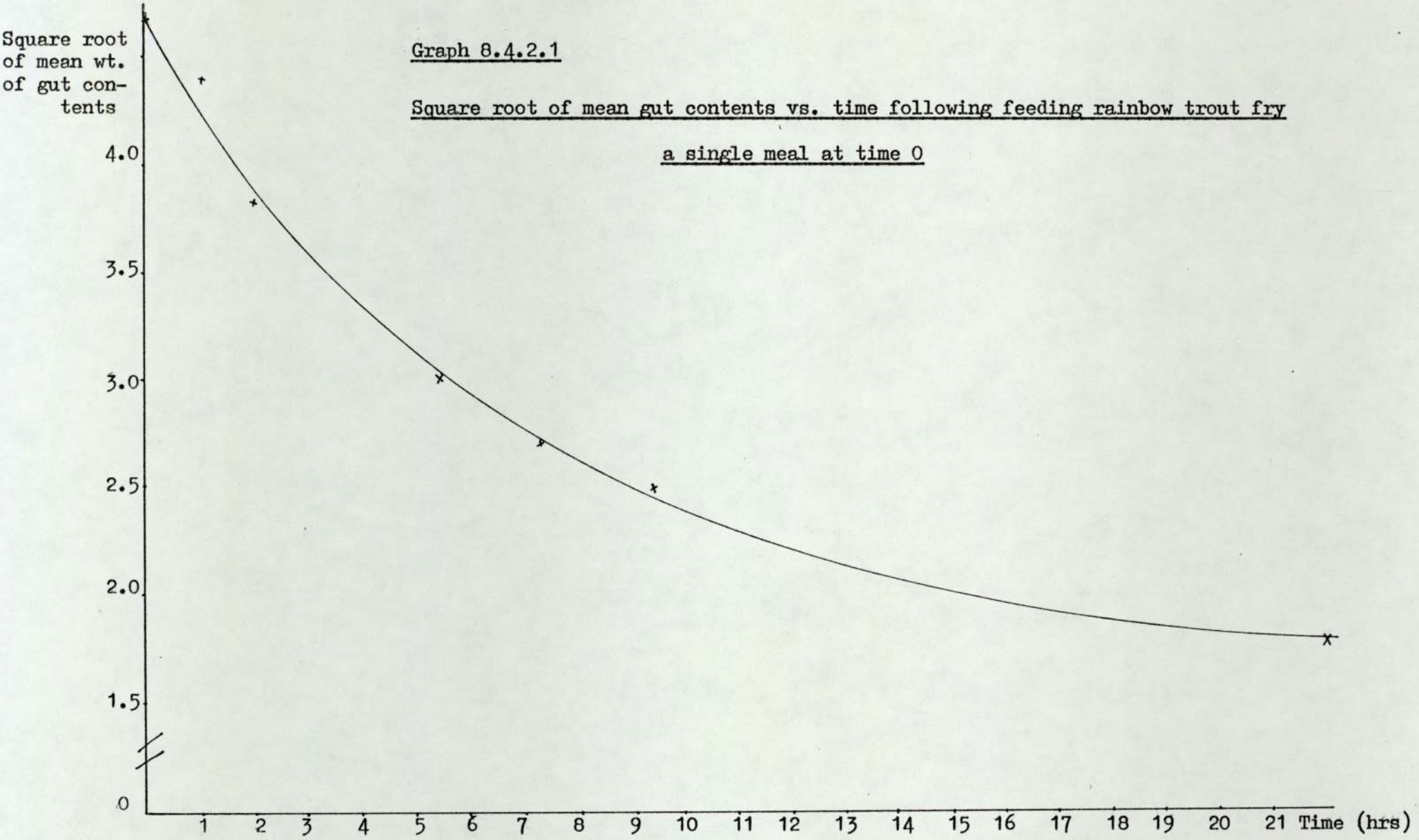
Table 8.4.2.1

Mean weight of gut contents (mg) and square root mean weight of gut contents vs time following feed in rainbow trout

Time (hours) after feeding	Mean gut contents (mg)	Square root of gut contents weight
0	22.3	4.72
1	20.1	4.48
2	14.8	3.85
3	12.1	3.51
$5\frac{1}{2}$	10.0	3.10
$7\frac{1}{4}$	7.4	2.72
$9\frac{1}{4}$	6.5	2.50
22	3.1	1.76

Graph 8.4.2.1

Square root of mean gut contents vs. time following feeding rainbow trout fry a single meal at time 0



values plotted against time in Graph 8.4.2.1. Over the first 3 - 5 hours of the experiment a rapid fall in gut contents was observed. However, from 5 hours the rate of loss of gut contents appeared to slow and a residual amount of material was present in the gut even 22 hours post-feed. The half life of food in the gut was calculated on the time taken for the mean weight of the gut contents to fall to half its initial value, and was approximately 6 hours.

Discussion

The gastric evacuation rate of salmonids has normally been determined in larger fish, either fingerlings or adults. Estimates of the time taken for complete stomach emptying range from 15 - 18 hours (Brett and Higgs, 1970; Grove et al., 1978). In the present experiment complete evacuation of the alimentary canal was not observed. This difference may reflect differences in methodology. Work with larger fish has concentrated on actual stomach emptying rather than the amount of material remaining in the alimentary canal (Elliott, 1972; Joblin et al., 1977; Grove et al., 1978; Fange and Grove, 1979). Elliott (1972), Brett and Higgs (1970) and Grove et al. (1978) all demonstrated an exponential fall in the weight of stomach contents with time in salmonids.

The results of the present experiment show an early exponential phase, probably representing loss of food by digestion and excretion, followed by a non-exponential phase, possibly representing slow excretion of a residual mass of material from the alimentary canal. This interpretation is supported by the observation that very few fish had material in the stomach (as opposed to the intestine) after 6 - 7 hours. Extrapolation of the early exponential phase, representing

the fall in stomach contents, suggests the complete emptying of the stomach would occur within 9 - 10 hours.

Thus in rainbow trout fry gastric evacuation would appear to take place faster than in adult salmonids.

The time required for steroid uptake from dietary sources is uncertain. If it is a rapid process the rate of gastric evacuation would not be expected to influence blood levels. Free fatty acids are taken up rapidly by direct uptake from the gut in only 1 - 2 hours. Thus following ^{14}C palmitic acid feeding Robinson and Mead (1973) noted a peak in blood radioactivity one hour post-feeding. However, lipid esters require times in excess of 30 hours needed for uptake in teleosts (Patton and Benson, 1975).

It seems likely that given the 15 hour period when steroid treated diet was not administered, a significant fall in blood steroid level could result. If steroid uptake is a direct process and takes place quickly, then for a period of 13 - 14 hours no fresh steroid will reach the bloodstream. If on the other hand steroid uptake is a slower process, then the time of gastric evacuation will limit the time during which steroid uptake is possible. In the final E_2 feeding trial in which automatic feeders and supplementary lights were used it seems maximum blood steroid levels were maintained over the treatment period.

8.5 The administration of exogenous steroids - General discussion and conclusions

The administration of exogenous steroids to teleost fry prior to the time of sexual development has been shown to yield a variety of responses, depending upon the steroid administered, the method by which treatment is applied and the species of fish used. The duration and timing of treatment are also important variables in determining the effect produced. The relevant literature has been reviewed.

Three main effects presenting commercial possibilities for the prevention of the adverse effects of male sexual maturation have been put forward:-

1) E_2 induced feminization of genetic males to give all female populations.

2) The production of all female fish by crossing XX males with normal females.

3) The production of sterile fish by treatment with high doses of androgens.

E_2 feminization has been claimed to offer a successful and cheap method of avoiding the problems of male maturation in salmonid cultivation (Simpson et al., 1976; Goetz et al., 1979). However, other authors have reported the failure of this method to achieve complete feminization in rainbow trout (Bye, in Donaldson and Hunter, 1982a). The results of a series of experiments carried out at Shearwater Fish Farming are reported. The treatment regime used by Simpson (1976) and Johnstone et al. (1978) proved ineffective in bringing about a complete feminization. Thus 68.5% of treated fish were shown to be female at 120 days post-feed.

Modification of the treatment regime, and in particular the use of automatic feeders, supplying food in more evenly spaced feeds within the treatment period (08.30 - 17.00), actually resulted in a reduced percentage of females among fish treated with 20 mg E_2/kg diet compared with the previous result. As suggested in the earlier discussion, this may be due to an effect of the method of diet preparation allied to the higher feed levels used. An increased extent of feminization was achieved by the use of the long lived synthetic estrogen, ethinyl estradiol. Feeding this steroid at 20 mg EE/kg diet gave 86% of fish containing female gonadal elements, though 28% of these were incompletely feminized and were hermaphrodites.

Further extensive modification of the treatment regime, including a higher dose rate of 40 mg E_2/kg diet, feeding for the full 24 hour period and attempting to treat all diet particles with sprayed steroid, increased the dose received by the experimental fish. However, this increase in steroid levels, though giving rise to all female fish, produced high mortalities and poor growth among treated fish. Of the fish examined at 120 days after feeding, no males were found and with the exception of 8% apparently sterile fish, all fish were female.

Thus it would appear that in order to achieve a complete feminization using E_2, careful consideration must be given to the possible sources of variation between different sites. One factor which seems of great importance is the feeding regime used and in particular the possibility of a reduction in effective steroid dose following an overnight 'withdrawal' period.

The possibility of producing XX males was not studied experimentally, though the literature on this topic is reviewed. This method offers an

elegant solution to the problem of male maturation. It is not completely problem free, however. A special broodstock must be set aside and gonadal tissue surgically removed, as these fish normally show abnormal duct development and cannot be hand 'stripped'. In addition, it has been reported that the fertilizing ability of spermatozoa from such XX males may be low (J. A. Ralph, Pers. comm.). This probably reflects the difficulties in assessing the state of maturity of fish which cannot be stripped. While it may be possible to increase the potency of these males by hormonal treatment this would introduce a further complication in the use of these fish.

The use of high doses of androgens has also been examined and the literature reviewed. In some reports sterilization has resulted from such treatment (Jalabert et al., 1975; Yamazaki, 1976; Goetz et al., 1979). However, the extent of sterilization appears to vary and the comments on feeding practice made in the discussion of E_2 feeding apply equally to this method. The results reported here suggest that the effects of high doses of MT (30 mg/kg diet) are by no means simple and that, though partial sterility was achieved, the most significant effect is a delay in gonadal development among treated fish. It is suggested this effect resulted from a reduction in cellular testicular elements and this is supported by the nodular nature of the gonads which did eventually develop. This delay may be sufficiently long to be of commercial significance in some situations. It was observed that the growth of sterile fish was relatively poor and that they were significantly smaller than the treated fish which matured after the normal time of maturation of male controls. If this is shown to be the case then it is possible that the control of the timing of maturation rather than its complete prevention may be a more attractive proposition for the commercial producer.

Measurement of blood levels of T have been reported as evidence of the influence of treatment method, feeding rate and steroid withdrawal upon the blood steroid profiles of treated fish. The rapid fall in blood levels following withdrawal, allied to the results of Simpson et al. (1976), Fagerlund and McBride (1978), Fagerlund and Dye (1979) and Lone and Matty (1981b) suggest that, provided the fish are grown to normal table size before consumption the relatively small amounts of steroid administered during early development would not pose a health risk. However, the question of hormonal treatment of fish destined for human consumption remains a thorny one and for this reason the production of all female stocks from XX males has a commercial advantage in that fish for the table are not directly exposed to steroid.

CHAPTER 9

MISCELLANEOUS METHODS FOR THE CONTROL OF MALE SEXUAL MATURATION IN SALMONIDS

Contents

9•1 Introduction

A variety of methods offering a potential application in the control of male maturation in salmonid stocks are considered in this chapter. These include a range of genetic techniques, the use of radiation and of antigonadal compounds like the antiandrogens and non-steroidal chemosterilants.

Although these methods are grouped together largely for convenience, some, such as radiation induced castration, have been tested in salmonids; others have not yet been examined with this application in mind. For instance, the use of the antigonadal agent methallibure has been reported in a wide variety of mammalian and teleost fish species, but few reports examine this compound's effects in salmonids.

As a result of the commercial impetus to develop methods of sex control in salmonids, the areas examined in this chapter are of increasing importance and the extent to which the research effort directed into these areas has been successful will be examined in this chapter.

9•2 Genetic methods

9•2•1 Introduction

A number of diverse approaches to the problems of male sex maturation may for convenience be grouped together under the heading genetic manipulation. Such techniques have been widely applied to almost every case of domestication of plants and animals to improve the quality and/or quantity of cultivated product and there is little doubt that as knowledge of the genetics of cultivated fish develops their importance will increase.

The techniques examined in this section have as their common feature the manipulation of the genetic constitution of fish in order to prevent sexual development. This manipulation includes the introduction of 'mismatched' chromosomes, as in intergenetic hybridization, and artificially induced increases in the normal diploid chromosome number (polyploidy). Similarly, the fertilization of normal ova with irradiated sperm coupled with thermal shocks can give rise to all female populations (gynogenesis). It would also include the variation in the individual genetic constitution of fish resulting from selection and breeding programmes.

9•2•2 Selective breeding

In contrast to domestic animals, relatively little work on the selective breeding of teleost fish has been reported (Purdom, 1972). Genetic improvement of salmonid stocks has been limited, largely due to the practice of selecting broodstock by appearance, rather than with any particular characteristics in mind. Despite this, improvements have been produced in teleost species by such 'rule of thumb' methods. This is especially so in two species of teleost, the common carp (Cyprinus carpio L.) and the rainbow trout (Salmo gairdneri R.) (Leintritz and Lewis, 1976; Moav, 1976; Moav et al., 1978).

The ability of selective breeding to produce a change in a particular characteristic depends upon the hereditability (h^2) of a characteristic, the selection pressure operating and the generation time between individuals. Hereditability of a characteristic is approximately equal to the ratio between the variance due to genetic factors (Vg) and the phenotypic variance (Vp). In order for selective breeding to produce an improvement in a particular characteristic, the

characteristic must show a reasonably high hereditability. Estimates of hereditability have been made for a number of important characteristics within salmonids (Aulstad et al., 1972; Gjedrem, 1975; Klup, 1979), though not for delayed maturation. Variable estimates of hereditability for growth rate have been obtained. In salmon parr hereditability for this characteristic was estimated at 0·08 - 0·15 (Refstie and Steine, 1978). Estimates have also been made of this characteristic in adult rainbow trout (0·20) (Gall, 1975), fingerlings (0·19) (Aulstad et al., 1972) and adult Atlantic salmon (0·31) (Gunnes and Gjedrem, 1978).

Reports have demonstrated selection induced changes in the timing of maturation, though such selection has aimed at producing earlier maturation, especially among female broodstocks (Lewis, 1944; Donaldson and Olson, 1957). However, these studies lacked adequate controls and the reported changes may have been due to environmental influences such as improved hatchery practices. This is a general problem in the development of strains of salmonids spawning at different times (Leintritz and Lewis, 1976). Again the importance of environmental factors has been inadequately explored. Movement of these stocks away from their 'home' area may result in a reversion to the original spawning time.

Any selection programme aimed at producing variation in the spawning time of broodstock would have to operate over an extended time scale. This is well illustrated by the time taken for the development of fall spawning rainbow trout strains from the original spring spawning strains. To achieve this, continuous selection from 1883 to 1933 was necessary (Leintritz and Lewis, 1976). At the Nikko hatchery, Japan, advancement of spawning time from late March to early December

was shown to require over 50 years of selection (Kato, 1973). Although such a time scale could be reduced by the use of advanced techniques such as polyploidy and gynogenesis, there is little doubt that a selection programme aimed at delaying maturation by a significant amount would be a time consuming project. In addition, Buss (1980) has commented on the resistance of spawning time to selection pressure in the brown trout (Salmo trutta) and brook trout (Salvelinus fontinalis).

In view of the possible genetic component in precocious maturation of salmonids, one aspect of breeding practice often suggested is avoiding the use of early maturing male salmonids as parents (Piggins, 1974; Schaffer and Elson, 1975; Thorpe, 1975). However, other workers (Glebe et al., 1980) have reported no influence of mature male parr parents upon the numbers of male progeny maturing early.

Perhaps the most important aspect of a selective breeding programme aimed at improving characteristics of salmonid stocks is a clear objective and some knowledge of the genetic basis of variation of the characters studied. Selective breeding is a long-term process, though techniques such as hybridization and gynogenesis may decrease the time needed for selection by increasing the coefficient of inbreeding of fish.

9·2·3 Hybridization

Compared with most animal groups teleost fish show a remarkable facility for hybridization (Purdom, 1972) and this capacity is well demonstrated in salmonids (Piggins, 1965, 1970; Susuki and Fukuda, 1971a, b, 1973a, b; Chevassus and Petit, 1975; Refstie and Gjedrem, 1975; Sutterlin et al., 1977; Blanc and Chevassus, 1979). As it is

commonly assumed that hybrids resulting from the crossing of two genetically distinct species are sterile, the hybridization of different salmonid species has been suggested as a method of avoiding the problems of male and female sexual maturation.

Early work in this field has been summarized by Alm (1955) and the now voluminous literature reviewed by Schwartz (1972), Dangel et al., (1973) and Chevassus (1979). The crossing of distinct salmonid species has yielded variable results for survival, growth rate and fertility, due in part to differences in methodology and intraspecific variability. One important variable is the actual criterion used for the assessment of the fertility of hybrids. Few workers have maintained such hybrids until sexual maturity and quantitatively assessed the production of viable gametes (Chevassus, 1979). Fewer still have obtained positive identification of hybrids based upon analysis of karyotypes (Nygren et al., 1972).

Of the hybrids so far examined, a number have been claimed to be sterile. Thus the crossing of brook trout males (Salvelinus fontinalis) with brown trout (Salmo trutta) yields so called 'tiger' trout, which are reportedly infertile (Harrison, 1961). The reciprocal cross was also claimed to produce a sterile hybrid (Susuki and Fukuda, 1973b; Chevassus, 1979), as did crosses between Salmo trutta and Salvelinus alpinus or S. malma (Refstie and Gjedrem, 1975).

Crosses between Oncorhynchus masou and S. fontinalis gave rise to sterile adults, with only 5 - 15% of adults maturing and very low egg or sperm production (Susuki and Fukuda, 1971, 1973a, b).

In other cases salmonid species have yielded progeny which show full fertility and may be backcrossed to either parent. Piggins (1965,

1970) obtained viable F_2, F_3 and backcross fish from a cross between Atlantic salmon (female) and sea trout (male). However, these results have been questioned by Nygren et al. (1975) who found low fertility and abnormal gamete production in this cross. Crosses within the genus Salvelinus and the genus Oncorhynchus (Chevassus, 1979) have been shown to be fertile.

The position regarding the fertility or sterility of hybrids appears to revolve around the respective chromosomal composition of the two parent species. In order for hybrids to be fertile the chromosome structure of the two parents must be virtually identical (Purdom, 1972). Thus while intrageneric hybrids are often fertile, sterile hybrids more often result from intergeneric crossing in which the chromosome structure of parents differs, giving rise to abnormalities of pairing at meiosis.

However, even within these 'sterile' hybrids, there is doubt regarding the extent to which sterility refers simply to the aspermatogenic condition or to the absence of secondary sexual characteristics. While a sterile hybrid in which the G.S.I. was low would largely avoid any diversion of energy and reserves towards the developing gonad, the possible presence of sex hormones could give rise to the androgen dependent deleterious changes examined in earlier chapters.

Thus, in summary, though hybridization may promote sterility, it is by no means proven and would be limited to intergeneric hybridization, which causes problems regarding the marketing of such progeny. By no means all hybrids yield viable progeny and poor survival may result (Susuki and Fukuda, 1971b). The hybrids chosen would have to be carefully tested for other characteristics such as growth rate in addition to their

lack of gamete production. Hybridization has yielded valuable results in growth improvement (Piggins, 1965) and disease resistance (Ord et al., 1976). Further long-term studies of promising hybrids, such as those described by Refstie and Gjedrem (1975) and Susuki and Fukuda (1971b) are necessary before hybridization may be seen to be a viable approach to the problem of the development of sexual maturation in salmonids.

9•2•4 Induced Polyploidy

It has been known for some time that while tetraploid (4n) plants are fertile, crosses between tetraploids and normal diploids (2n) give rise to sterile triploids (3n). (Wexelsen, 1964). It is generally assumed that such teleost triploids would be sterile and the production of triploid salmonids, either by crossing artificially produced tetraploids with normal diploids or by gynogenetic manipulation of salmonid eggs prior to fertilization with normal haploid sperm, would remove the problems associated with both male and female sexual development.

Naturally occurring triploids have been discovered among salmonid species, such as rainbow trout (Cuellar and Uyeno, 1972; Lincoln and Bye, 1980) and brook trout (Allen and Stanley, 1978), though the incidence of such triploids is low. The factors giving rise to such spontaneous polyploids are uncertain, but appeared in one case to be related to chromosomal disjunction, possibly brought about by low water temperatures during egg incubation (Allen and Stanley, 1978). Thorgaard and Gall (1979) reported finding six triploid rainbow trout among a stock of normal trout. Three males had partially developed gonads, while the three XXX females examined had abnormal gonads with unusual meiotic pairings.

Artificial polyploidy may readily be induced in a variety of teleost species by procedures such as thermal shock either before or after fertilization (Swarup, 1959a, b; Vesentskii, 1967; Purdom, 1972, 1976; Valenti, 1975). Treatment causes retention of the second polar body or interference with chromosomal separation. Such thermal shock originally proved less effective in inducing polyploidy in salmonid eggs (Lincoln et al., 1974) probably due to the larger egg size of salmonids. However, both heat (26 - 30°C for ten minutes) or cold shock (0 - 4°C for 6¾ hours) applied to eggs within one hour of fertilization with normal sperm were effective in promoting retention of the second polar body and giving rise to triploid rainbow trout embryos (Chourront, 1980). The discrepancy between this report and that of Lincoln et al. (1974) is probably related to the increased duration of cold shock used.

Thermal shocks have also been reported to be partially successful in inducing polyploidy among embryos of brook trout (Lemoine and Smith, 1980) and rainbow trout (Thorgaard et al., 1981). Recent work suggests that salmonids can give high yields of triploid embryos following heat shock treatments (Lincoln and Scott, 1983). However, their production characteristics are still undergoing evaluation. Although such triploid fish do not mature their growth appears to be not as good as the lead control fish. Analysis of the growth of sterile triploid plaice (Lincoln, 1981) showed that by the end of the experimental period the diploid control fish had recovered from maturation and were not significantly smaller than the non-maturing triploids.

An alternative approach is the use of cytokinetic inhibitors such as Cytochalasin B, which prevents cell division but not mitosis (Carter, 1967; Krishan, 1972). Treated cells become binucleate or multinucleate

and on transfer to fresh medium gave rise to tetraploids (Hoehn et al., 1973). Refstie et al.(1977) were able to induce polyploidy in Atlantic salmon, though with increased mortalities, by treating unfertilized eggs with Cytochalasin B at a dose of 10 µg/ml applied 34 hours prior to fertilization and stopping treatment before the four cell stage. However, Refstie (in Donaldson and Hunter, 1982a) reports a number of surviving fish to exhibit skeletal deformities.

Allen and Stanley (1979) were able to induce polyploid mosaic Atlantic salmon, without adverse effects on the percentage hatching, by treatment with 10 mg/L Cytochalasin B administered 35 to 110° hours after fertilization.

Brook trout eggs treated with 0·01% colchicine for 84 or 90 - 120° hours after fertilization resulted in polyploid mosaics in four of the six fish karyotyped (Smith and Lemoine, 1979).

One question which must be answered by this current work is the extent of triploid sterility. Although it is assumed that triploids are sterile, this question is not yet convincingly settled. The reports of Schultz and Kallman (1968), Purdom (1976), Allen and Stanley (1978) and Lincoln and Bye (1980) suggest triploids are sterile. Other work has reported fertile triploid populations, capable of reproduction by a gynogenetic mechanism (Schultz, 1967; Vrijenhoek and Schultz, 1974; Strommen et al., 1975). In addition, the results of Lincoln (1981) suggest that polyploidy may have some adverse effects upon somatic growth.

9•2•5 Gynogenesis

Gynogenesis is the process by which the embryo comes to contain exclusively female chromosomes as a result of fertilization of ova with inactivated sperm (Hertwig, 1911). If a thermal shock is administered following fertilization with such sperm, a doubling of maternal chromosomes results and a diploid embryo is produced. As all chromosomes of such an embryo derive from the female, the genetic constitution will be XX and all progeny female.

Gynogenesis may thus be considered to be a special case of polyploidy in which the spermatozoa makes no genetic contribution to the embryo and all the genetic material arises from the female parent following a suppression of the normal meiotic loss of chromosomes. Gynogenesis has been demonstrated in a number of teleost species (Romashov et al., 1963; Purdom, 1969, 1972; Nagy et al., 1978).

Fertilization of an egg by X or gamma irradiation inactivated spermatozoa lead to the production of haploid embryos with high mortalities, though 1 - 2% of the resulting embryos survived and were shown to be normal diploids (2n) (Purdom, 1972). If eggs are subjected to a thermal shock immediately following fertilization (preventing loss of the second polar body), the number of these diploid embryos is significantly increased (Chourront, 1980). As the female salmonid is the homogametic sex (Thorgaard, 1977; Okada et al., 1979), all resulting diploid embryos will have the female genotype and thus develop as females. So gynogenesis provides the means of producing all female populations (Stanley, 1976) and avoiding the detrimental aspects of male maturation.

As a means of producing all female populations gynogenesis is similar to the method of producing all female populations by the use of

XX males resulting from the masculinization of genetic females by androgen treatment (Bye and Lincoln, 1981). As this method involves genetic manipulation, though by endocrine means, the production of XX males is dealt with in the discussion of results in sex reversal and the administration of exogenous steroids.

It is interesting to note that Donaldson and Hunter (1982a) have suggested the use of these two methods in tandem. Thus if gynogenesis could produce an all female population, this population could be masculinized by androgen treatment to yield a stock of XX males for use as broodstock, giving all female progeny when crossed with normal females.

Although salmonids are less sensitive to the thermal shocks necessary in this technique, reasonable yields of diploids have been produced from thermally shocked rainbow trout eggs fertilized with irradiated spermatozoa (Chourront, 1980; Refstie et al., 1982).

Both polyploidy and gynogenesis seem to offer promise in the development of sterile, triploid (3n) or all female, gynogenetic populations. However, both techniques are at an early stage of development and are not yet viable commercially, though it is reasonable to expect that progress within these areas will see their use in aquaculture; not only to produce sterile or all female populations, but to give an impetus to breeding and selection experiments.

Further work is necessary before the remaining problem areas (such as the extent of triploid salmonid sterility) are resolved and the techniques in use must be shown to be effective, reliable and applicable on a large scale.

9•2•6 Discussion

The amount of genetic manipulation practised among teleost stocks is far less than that used in the majority of agricultural operations, due to the relatively late introduction of increasingly scientific methodologies in aquaculture.

Thus techniques such as selective breeding, hybridization, polyploidy and gynogenesis seem likely to contribute towards salmonid cultivation. The extent to which methods such as gynogenesis and the induction of triploidy provide solutions to male maturation problems will depend upon the progress of current research and the production of reliable techniques for the induction of such effects and analysis and identification of the required progeny. They must also be shown to be free of adverse effects.

Selective breeding and hybridization also require further long-term study before their potential can be assessed. Hybridization may yield useful crosses for aquaculture, but may give rise to problems in the marketing of such fish. As demonstrated, selective breeding is a long-term process, dependent upon the development of pure lines of parental stocks. Genetic methods of manipulation, such as gynogenesis, may be allied to other techniques including sex reversal, as has been suggested by Donaldson and Hunter (1982a). It seems these genetic techniques will become increasingly important as technical advances provide methods which increase the efficiency of such manipulations.

9·3 Radiation induced castration

Radiation induced damage to the germinal tissue of animals is a well documented phenomenon. Teleost fish have been shown to be sensitive to such manipulation and the use of radiation induced gonadal lesions suggested as a possible alternative to surgical castration. As in mammals it seems certain that the prime targets for radiation induced damage are the germinal cells of the gonad. Such a high sensitivity to radiation would be expected due to the succeptibility of meiotic chromosomes to damage (Mong and Berra, 1979). However, the interstitial tissue of the gonad may be less succeptible to such manipulation.

Three main approaches have been used in the administration of radiation and radioactive isotopes to teleosts, with the aim of modifying or preventing gonadal development, and the results from these studies illustrate this dichotomy of sensitivity of gonadal components.

The administration of X-rays to rice fish (Oryzias latipes) was shown to induce dose-dependent gonadal damage. After single doses of 100 - 200 R X-radiation, a depression of testis weight, largely resulting from germinal cell damage, was observed. When high doses of X-rays (8000 R) were administered, complete degeneration of testis structure was observed (Konno and Egami, 1966). Treatment of pink salmon with X-rays (0·35 Gy) suppressed gametogenesis for a period of up to seven months and apparently sterilized some males (Persov, 1975). An external radiation source (^{60}Co) has been used to irradiate rainbow trout with gamma rays, and was reportedly capable of inhibiting sexual development (Tashiro, 1972).

In order to avoid the high X-ray doses necessary for whole body irradiation, the direct administration of radioisotopes such as

Cobalt-60 (^{60}Co) has been studied. The administration of 10 μ Ci of ^{60}Co to female Indian catfish (Heteropneustes fossilis) reduced oocyte growth and induced oocyte degeneration (Srivanstana and Rathi, 1967). Similar results have been reported in the male of this species (Rathi, 1977) following intraperitoneal injection of 10 μ Ci of ^{60}Co. Although this report claims total sterilization resulted, the effects of injection upon interstitial cell structure are unreported and the observations described suggest only germinal tissue damage occurred.

Irradiation of eggs and alevins has been attempted (Donaldson and Bonham, 1964, 1970; Bonham and Donaldson, 1966, 1972; Erickson, 1971; Hershberger et al., 1978). Eggs and alevins of the Pacific salmon (Oncorhynchus tshawytscha) were irradiated by means of a ^{60}Co source at dosages ranging from 0·5 to 50 R/day during the period from fertilization to the start of feeding (approximately two and a half months). Examination of fish resulting from such treatments showed a retardation of gonadal development in smolts treated at 10 R/day (Bonham and Donaldson, 1972). The subsequent returns of fish from the sea were studied and returning males discovered to be sterile (Hershberger et al., 1978). However, as these fish showed normal male secondary sexual characteristics, it appears the interstitial cell sex steroid production was still proceeding and damage was limited to spermatogenic cells. However, in some fish the return to the home river was much delayed, indicating a substantial delay in maturation.

The use of radiation poses a number of problems as a routine treatment for the removal of male maturation. It has been shown that due to the relative lack of sensitivity of interstitial tissue, high radiation doses may be necessary to achieve full castration. Increased dose

levels lead to the possibility of further side effects. Bonham and Donaldson (1972) reported increasing mortalities and a depression of growth with increasing radiation levels in Oncorhynchus tshawytscha, as did White (1964) in Fundulus heteroclitus. As doses increase, dose-dependent side effects may be expected to become more severe.

The effectiveness of the techniques seems limited at lower dose levels, probably due to the dichotomy of sensitivity of gonadal tissue. A problem in terminology exists in that fish may be reported as 'sterile' when in fact they are aspermatogenic, as a result of irradiation of germinal cells. The interstitial tissue in these aspermatogenic fish may still be functional and capable of androgen production.

A further problem lies in the level of technology required to use radiation with safety. Radiation sources present obvious dangers and require a high level of technical expertise, and in the case of X-ray units, considerable capital investment. This requirement for specialised knowledge and expertise makes it unlikely that this technique will gain acceptance as a routine technique. Obviously direct administration of radioisotopes would be out of the question and the method seems likely to remain a research tool.

9•4 The Use of Antigonadal compounds - Chemosterilization

9•4•1 Introduction

A large number of non-hormonal compounds have been shown to be capable of exerting antigonadal effects in mammals (Neumann et al., 1975). Such compounds offer potential for the control of maturation of salmonids, though their early use has been restricted to investigation of the teleost endocrine system, rather than with commercial applications

in view. Investigation of their effects on maturation have been largely limited to two compounds: methallibure and cyproterone acetate.

a) Methallibure (ICI, 33,828) (1-α-methylallylthiocarbamoyl-2-methylthiocarbamoyl-hydrazine)

This compound, a dithiocarbamoyl hydrazine derivative, is perhaps the best known of the non-steroidal compounds capable of inhibition of gonadal function. Since Paget et al. (1961) reported this compound to be capable of selective interference with pituitary function in rats, its effects have been confirmed in a variety of mammalian species and the evidence suggests its main effect is to block the release and/or synthesis of pituitary gonadotropins (Schmidt-Elmendorff et al., 1962; Brown, 1963; Benson and Zagni, 1965; Harper, 1967). Methallibure has been administered to a variety of teleost species and its effects shown to include the suppression of spermatogenesis, inhibition of the appearance of secondary sexual characteristics and depression of steroidogenesis (Hoar et al., 1967; Wiebe, 1968; Martin and Bromage, 1970; Pandey, 1970; van den Hurk and Testrink, 1975). However, few studies (Donaldson, 1973; Flynn, 1973; Murphy, 1980a, b) have reported its effects upon salmonid sexual maturation. In a comprehensive report on the effect of this compound upon sexual development of salmon parr, Murphy (1980a) concludes that it offers potential as a chemosterilant but must be administered early in the process of gonad maturation.

b) Cyproterone Acetate (2α-methylene-6-chloro-pregnadiene-17α-ol-3,20-dione α acetate)

Cyproterone acetate is a synthetic progesterone-like steroid, which acts as a competitive inhibitor of androgens, and especially testosterone, at their receptor sites (Fang et al., 1969). There is also evidence that

in mammals it inhibits the biosynthesis of testicular C19 steroids (Hoffman and Breuer, 1968). Its effects on mammals include the blocking of the development of secondary sexual characteristics and reduction of plasma androgen levels (Neumann et al., 1967a, b).

The effects of cyproterone acetate in lower vertebrates has been the subject of a number of reports, though the majority have investigated the biochemical mode of action of this compound rather than its effects on sexual maturation. These reports have confirmed the antigonadal action observed in mammals.

Treatment of rainbow trout with cyproterone acetate (2·5 mg/fish) was shown to cause a reduction in the recovery of injected ^{3}H testosterone from the plasma and testes of immature males, though not the ovary and testes of mature fish (Schreck, 1973). A similar blocking of androgen-mediated effects, and presumably of androgen uptake, has been reported in the stickleback (Gasterosteus aculeatus) following the intraperitoneal implantation of cyproterone acetate in gelatin (Rouse et al., 1977). Cyproterone acetate has also been shown to block the androgen dependent hypersecretory activity of the seminal vesicles of castrated Indian catfish (Heteropneustes fossilis) (Sundararaj and Nayyar, 1969). On the other hand, in the swordtail (Xiphophorus) cyproterone acetate did not block the effects of exogenous testosterone propionate or 11-ketotestosterone upon the secondary sexual characteristics, and in particular the appearance of the characteristic male anal fin or 'sword' (Rastogi and Chieffi, 1975). Again few reports have dealt directly with the possibilities of using this compound as a chemosterilant in teleosts (Stanley, 1981) and fewer still have restricted such discussion to salmonids, though Murphy (1980a, b) is a notable

exception. Ambiguous results were obtained using this compound together with testosterone in chinook salmon (Schreck and Fowler, 1982).

A variety of other non-hormonal, antiandrogens have been shown to be effective in mammals. One which may offer some potential in the area of maturation control in salmonids is the non-steroidal antiandrogen flutamide (SCH 13521) (4'-nitro-3'-trifluromethylisobutylanilide). This compound has been shown to have antigonadal potency in mammals (Neri et al., 1972; Liao et al., 1974; Mainwearing et al., 1974; Peets et al., 1974), though its effects in fish have only recently been reported (Schreck and Fowler, 1982), when it was shown to be capable of slightly reducing the percentage male maturation of chinook salmon from 83% to 55% when fed in conjunction with 15 ppm testosterone.

9•4•2 The effects of feeding 40 - 50 mg methallibure and 50 mg flutamide/kg diet on the subsequent maturation of adult rainbow trout

In order to evaluate the commercial potential of this approach to the prevention of male maturation among salmonids, a pilot scale experiment was carried out using two of the compounds reviewed above: methallibure (ICI 33,828) and flutamide (SCH, 13521).

Materials and Methods

Immature rainbow trout weighing approximately 130 g were obtained from Burwarton Fish Farms. Three groups of 23 fish were tagged and housed in the tanks of a recirculating system. Fish were fed twice daily on either a treated or control diet for a period of eight months, from the commencement of the experiment (June) until well after the time of normal maturation (February).

Both compounds were applied to B.P. 'Mainstream' number 4 diet, dissolved in about 20 ml of ethanol and sprayed onto the diet using a small electric spray gun. Diet was then air dried overnight and stored at 4°C prior to use. Both compounds were initially administered at a dose of 50 mg/kg diet, but following early mortalities in the methallibure treated group this was reduced to 40 mg/kg in this group.

Fish were weighed, either individually or as a batch, at monthly intervals and examined for the appearance of secondary sexual characteristics. At the end of the experiment (February) the fish were sacrificed and the gonads removed to allow determination of the G.S.I. and the state of maturity.

Results

a) Growth

The mean body weights of control and treated fish are shown in Table 9•4•1. No significant differences were observed in the mean body weight of fish at any time during the experiment.

b) Mortalities

An increased number of mortalities were observed among treated fish, though this was no longer the case following a slight reduction in dose. During the first three weeks of treatment five mortalities occurred in the group receiving methallibure and two mortalities in the group receiving flutamide occurred over the first twelve weeks of the experiment.

c) Secondary sexual characteristics

These become apparent by the end of October. Within the control

group six out of twenty-three fish showed skin darkening and kype development, while the corresponding values for methallibure and flutamide treated groups were one out of eighteen and two out of twenty-one. By December these fish had begun to exude milt with gentle hand pressure.

d) Gonadosomatic Indices

The G.S.I. data from this experiment are shown in Table 9·4·2. No significant effect of treatment was observed on the G.S.I. of either treated group, though fewer males within the treated group had matured. No effect was seen on the G.S.I. of immature males or females.

e) Sex ratios

Table 9·4·3 shows the distribution of sex and developmental state among control and treated fish. The most striking aspect of the observed sex ratio was the preponderance of males among all groups. Almost 68% of all fish were found to be males. This effect was not treatment related. The possible reasons for this are examined in the discussion. In addition, a significant proportion of these males did not mature. Administration of both antigonadal compounds reduced the number of mature males in both groups. In the treated groups only 9% (methallibure) and 13% (flutamide) of males matured, while 37% of control males matured. However, when the numbers of mature and immature males were compared in a chi squared test the difference between numbers of mature males was found to be non-significant (chi squared = 4·03; $P > 0{\cdot}05$).

f) Serum T levels

Serum T levels were assayed in samples taken at the end of October, approximately four months after treatment had begun. The results are

Table 9•4•1

Mean body weights of control, methallibure (40 - 50 mg/kg diet) or flutamide (50 mg/kg diet) treated rainbow trout after 8 months feeding (February) (Mean wt. (g) ± SE)

Date	Control	Methallibure treated	Flutamide treated
7/6	124•3 ± 30•3	134•6 ± 29•4	143•2 ± 33•6
n	23)	23)	23)
12/8	198•9 ± 41•4	205•4 ± 42•6	219•4 ± 46•1
n	23)	18	22)
14/10	264•3 ± 53•16	278•1 ± 59•18	283•0 ± 60•2
n	23)	18)	21)
12/12	318•8 ± 71•6	335•4 ± 76•4	348•0 ± 79•3
n	23)	18)	21)
6/2	360•13 ± 83•6	380•6 ± 83•1	384•1 ± 83•0
n	23)	18)	21)

Table 9•4•2

Mean G.S.I.s of control, methallibure (40 - 50 mg/kg diet) or flutamide (50 mg/kg diet) treated rainbow trout after 8 months feeding (February) (G.S.I. ± SE)

Sex	Control	Methallibure treated	Flutamide treated
Mature males	3•00 ± 0•50	2•69	2•07 ± 0•06
n	6)	1)	2)
Immature males	0•04 ± 0•002	0•034 ± 0•007	0•035 ± 0•009
n	10)	10)	13)
Females	0•174 ± 0•012	0•165 ± 0•015	0•159 ± 0•03
n	7)	7)	6)

Table 9•4•3

Sex ratios of control, methallibure (40 - 50 mg/kg diet) or flutamide (50 mg/kg diet) treated rainbow trout after 8 months feeding (February)

	Control	Methallibure treated	Flutamide treated
Mature males	6 (37•5)	1 (9•1)	2 (13•3)
Immature males	10 (62•5)	10 (90•9)	13 (86•6)
Females	7	7	6
Total	23	18	21

figures in brackets = % of total number of males.

Table 9•4•4

Serum testosterone levels in control, methallibure (40 - 50 mg/kg diet) or flutamide (50 mg/kg diet) treated rainbow trout sampled 4 months after commencing treatment

(Mean T level (ng/ml) $\pm$ SE)

	Control	Methallibure treated	Flutamide treated
Mature males	26•22 $\pm$ 8•74	16•0	53•55 $\pm$ 5•73
n	6)	1)	2)
Immature males	1•53 $\pm$ 0•33	1•36 $\pm$ 0•26	1•82 $\pm$ 0•29
n	6)	6)	6)
Females	1•45 $\pm$ 0•05	1•74 $\pm$ 0•21	1•63 $\pm$ 0•25
n	6)	6)	6)

shown in Table 9•4•4. No differences were seen in serum androgen levels of immature male and female fish under treatment. The serum T levels of maturing males among each group were not compared statistically due to the small numbers of such fish, especially in the treated groups. However, no obvious differences were apparent.

Discussion

Interpretation of this experiment was complicated by the abnormal sex ratio among the experimental fish. Over 68% of all experimental and control fish were found to be male and the possible effect of treatment was largely masked by the large proportion of such fish which failed to mature at the expected time.

As the fish were obtained from a commercial fish farm it is not possible to account fully for this anomaly. One possible explanation is that some form of selection has taken place, removing females from the group prior to use. However, such selection would imply that males and females could be easily distinguished and would be possible only if the majority of males had matured prior to use, recovered their immature appearance and failed to spawn at the expected time.

It might be postulated that the low level of maturity among males in all groups could be due to the antigonadal compounds 'leaching' from the diet into the system's water. Indeed methallibure has been administered to teleosts in such a manner (Martin and Bromage, 1970; Pandey, 1970; Mackay, 1973), though as methallibure is relatively insoluble in water and is normally dispersed in solution using a wetting agent, such as Tween 80 (Martin and Bromage, 1970), this explanation may be discounted. Interestingly, in another report (Schreck and Fowler, 1982)

the sex ratios of fish treated with flutamide were significantly different from control fish.

The main conclusions seem to be that, at the dose used, both compounds are incapable of completely preventing male maturation in rainbow trout but apparently reduces such an occurrence. Among control males 37% matured while only 9% and 13% of methallibure and flutamide fish did so. Such a reduction would be sufficient to be of commercial importance to the producer. Murphy (1980a, b) also reported that methallibure was incapable of preventing male maturation when fed to salmon parr at a dose of 250 mg/kg diet for a period of approximately four months. Both methallibure and cyproterone acetate were, however, effective in lowering plasma androgen levels under these conditions.

Schreck and Fowler (1982) studied the effects of the antiandrogens cyproterone acetate and flutamide in chinook salmon. Both compounds were incapable of preventing the androgenic effects of exogenous testosterone (15 ppm) when administered at a dose of 10 ppm, though an inhibition of male maturation was noted from 83% of males (T alone) to 66•7% (T + cyproterone acetate) and 55•0% (T + flutamide).

In the present experiment the dose was chosen as equivalent to the minimum effective mammalian methallibure dose of 1 mg/kg body weight (Neumann et al., 1975) in order to reduce any toxic effects. Even at this dose mortalities were higher in this group. The methallibure dose used was one fifth that used by Murphy, though the compound was fed for a longer period. Flynn (1973) reports that the dose of methallibure necessary to produce an effect in Pacific salmon is higher than that necessary in warm water fishes. Both compounds did not reduce the growth rate of fish under treatment.

Even if such techniques can be refined until completely effective, doubts still remain about their potential under commercial conditions. Doubts have been expressed as to the safety of methallibure following its identification as a potential carcinogen. In addition, no information exists regarding the half life of such compounds in salmonids and it is likely that their use will produce considerable consumer resistance.

9.5 General Discussion and Conclusions

A number of diverse approaches, all with the common aim of producing either all female or sterile salmonids, have been examined in this chapter. The possibilities offered by such techniques are outlined in this discussion.

Selective breeding appears to be capable of producing changes in the timing of sexual maturation, especially in advancing the time of spawning of female salmonids. Thus selection has been used to try to produce earlier spawning female broodstock. Although such effects have been reported from hatcheries, they have yet to be fully and scientifically described. It is possible that changes reflect the interaction of local environmental factors with the fishes' genotype and would not be maintained in a different environment. Perhaps the greatest potential for the technique is when coupled to techniques such as gynogenesis, allowing the intensification of selection pressure.

Hybridization has yielded variable results. It is not always the case that hybrids are sterile. Sterility results from intergenetic rather than intragenetic hybridization and thus the progeny of intergenetic crosses may face marketing difficulties. There is some confusion over the use of the term sterile in this context. Some authors

use it to signify the inability to produce viable gametes, not the absence of developing gonads and the absence of secondary sexual characteristics. In addition, few authors have retained the progeny of crosses until the time that full evaluation may be carried out.

Of the genetic techniques, perhaps induced polyploidy and gynogenesis offer the greatest potential in the control of salmonid sexual development. Naturally occurring polyploids have been found in salmonid populations, though at low frequency. Techniques have been developed to produce such fish in respectable numbers, despite the fact that salmonid eggs (probably due to their large size) have proved resistant to such manipulation. However, one unanswered question concerns the possible growth performance of such fish. The growth of sterile triploid rainbow trout, though better than that of control males, was not better than that of the faster growing control fish (Lincoln, pers. comm.).

Gynogenesis, the production of progeny having all female chromosomes, is another promising technique. In common with hormonal feminization, all female populations result and male maturation is avoided. Like most of the genetic techniques, further long-term study and evaluation is necessary and it may be that such a result is more easily accomplished by hormonal means.

Radiation induced castration has been attempted in salmonids and has been proposed as a method for the prevention or delay of male maturation in species suffering high spawning mortalities, such as Pacific salmon. Some uncertainty is evident in the literature concerning the differential sensitivity of interstitial and germ cells to radiation. In some cases complete destruction of both interstitial

and germinal elements has been achieved, while other reports suggest a less complete response. In particular the hormone producing interstitial tissue seems more resistant to such an approach. The dangers associated with the direct administration of isotopes would appear to preclude this as a commercial technique for the production of fish for human consumption. Similarly the use of irradiation by external sources and X-rays imposes technical demands, which the commercial producer would be unable to satisfy. So this approach seems likely to remain a laboratory technique, with the exception of its possible environmental applications.

Although chemosterilization appears to offer a simple solution to the problems of male maturation in salmonids, its effectiveness remains to be demonstrated, though it has proved a useful experimental tool. The use of compounds such as methallibure, cyproterone acetate and flutamide has yielded inconclusive results. Problems of consumer acceptability and safety would require further work and data regarding the persistance of these compounds in salmonids is not yet available to answer these doubts.

Thus it would appear that though these techniques offer considerable potential, none have reached the stage of commercial viability. Further work will be necessary before the validity of these approaches is demonstrated and they are refined to the point of commercial use. In the absence of such work they must remain experimental tools by which the reproductive physiology of salmonids may be elucidated.

CHAPTER 10

GENERAL DISCUSSION AND CONCLUSIONS

The cultivation of any fish species involves the deliberate manipulation of its environment and the additional demands exerted by salmonid sexual maturation have made it necessary for the commercial producer to attempt to control this process in order to either prevent or delay such effects.

Fish farming within the U.K. is largely synonomous with trout and salmon farming, and the projected increase in such intensive farming from its present level of 5000 - 6000 tonnes/year to an annual level of 15000 - 20000 tonnes (Purdom, 1979) would be seriously curtailed by the absence of suitable control methods. These constraints will be more severe if the future development of the industry follows the pattern seen in Scandinavia (Edwards, 1978) and the U.S.A. (Donaldson and Hunter, 1982a), with a shift towards salt water cultivation of salmonids. Under such conditions male maturation and its adverse effects are especially pronounced (Drummond Sedgwick, 1973; Roberts and Shepherd, 1974).

These problems are compounded by the occurrence to a variable extent of early or precocious maturation, where predominantly male salmonids mature before the normal time of maturation. Among Atlantic salmon such precocious maturation is manifest, either among parr or grilse (Mills, 1971). The extent of such precocious maturation is extremely difficult to assess under commercial conditions (Forster, pers. comm.) due in part to its variability among stocks of different origin (Needham, 1983).

Commercially disadvantageous changes accompany sexual maturation, including a poor growth performance (Nomura, 1963; Bye and Lincoln,1979)

and deleterious changes in skin structure (Richards and Pickering, 1978). Despite the commercial importance of these factors, little attention has been directed towards the development of salmonid secondary sexual characteristics (Davidson and Shostrom, 1936; Sano, 1960; Robertson and Wexler, 1962; Nomura, 1963; Bye and Lincoln, 1979; Land, 1979; Murphy, 1980a). In particular the relative roles of the two natural androgens, 11-KT and T, in the induction of these changes has remained uncertain, though it is known that they develop under the influence of androgens, and that 11-KT appears to be an extremely potent androgen in teleosts (Idler et al., 1961a, b; Arai, 1967; Hishida and Kawamoto, 1970).

The role of androgens in the development and maintenance of the secondary sexual characteristics of rainbow trout has been examined in this work. Thus it has been shown that sexual maturation of male trout is associated with a reduction in growth rate and possible explanations for this phenomenon are examined. The depression of growth among mature male trout has been shown to occur when androgen (T) levels were high. A simultaneous depression of dry matter digestibility was also noted in mature males and is suggested to be an important component of the reduced food conversion efficiency occurring at this time. The dry matter digestibility was shown to be inversely related to serum T concentration during the period prior to and following the initiation of spermiation. Thus lower mean digestibility values were observed when steroid levels were at their highest. In immature female fish, maintained under similar conditions, digestibility values were not reduced and the effect is thus unlikely to be due to temperature (Windell et al., 1978b).

Although these changes have been shown to be related to androgen concentration, only T was directly measured by RIA and so the relative roles of the two natural androgens, 11-KT and T, were examined by the experimental administration of androgens to immature fish. Both MT and T (30 mg/kg diet) produced a depression of growth of immature rainbow trout and also resulted in reduced mean dietary digestibility. When 11-KT and T (20 mg/kg) were administered a depression of digestibility was seen in the T fed fish, though this effect was observed only after prolonged 11-KT treatment and this parameter seemed to be more influenced by T than by 11-KT. However, steroid administration at this dose level, though affecting digestibility, produced no significant depression of growth. Preliminary results suggest that this effect on digestibility may be mediated via an effect on interstitial peptidase enzyme activity.

All the androgens administered were able to induce male secondary characteristics, though the extent of these depended upon the particular steroid. Both MT and 11-KT were especially potent in promoting skin darkening and kype development and of the two natural androgens 11-KT was more effective in promoting skin thickening than T. In fish fed T slight development of sexual characteristics was noted, though this was less apparent than in those fed 11-KT or MT.

The effects of androgens upon gonadal development varied with the steroid and dose used. Thus both MT and T (30 mg/kg) depressed testicular development, though MT was more potent. At a lower dose (20 mg/kg) T and 11-KT stimulated testicular development and caused an increase in G.S.I., accelerated spermatogenesis and spermiation, though again T was the least effective of the two steroids. By the end of the experiment all 11-KT fed male fish were running.

Thus it is suggested that there may be a dichotomy of effect of the two natural androgens, T and 11-KT. While the initiation and maintenance of the male secondary sexual characteristics seem more succeptible to the influence of 11-KT, the observed depression of digestibility was more marked in fish fed T. It may be that the depression of digestibility occurring in mature males, and possibly also the reduced growth rate of such fish, results from an effect of T rather than 11-KT. The stimulatory influence of 11-KT upon testicular development, spermatogenesis and spermiation in male fish suggests that 11-KT is the predominant natural androgen influencing these processes during the normal maturation of male salmonids.

These steroid induced changes are manifestations of the action of the controlling hypothalamic-pituitary-gonad axis, which operates in a broadly similar manner to that of mammals. The control and interrelationships of this axis have been reviewed (Hoar, 1965; Donaldson, 1973; de Vlaming, 1974; Dodd, 1975; Fontaine, 1976; Billard et al., 1978, 1982; Dodd and Sumpter, 1982; Peter, 1982). This axis is succeptible to modification by either environmental factors or artificial internal manipulation and these two aspects represent methods by which the commercial producer may regulate the reproductive maturity of his stock.

Of the environmental factors implicated in teleost reproduction, photoperiodic change has been shown to be the predominant variable controlling the timing of normal reproduction (Kunesh et al., 1974; MacQuarrie et al., 1978, 1979; Whitehead et al., 1978a, b, c, 1979, 1983; Whitehead, 1979; Whitehead and Bromage, 1980), though few reports have examined the effect of photoperiod on the reproductive development of adult male salmonids (Whitehead et al., 1979). Work reported in

earlier chapters has confirmed the role of photoperiod in synchronizing the maturation of male salmonids and suggests a possible method by which environmental modification may be employed to delay the attainment of sexual maturation.

Thus maturation was delayed by a significant period by exposure of male rainbow trout to a constant 'short day' photoperiod (8 hours). In these males the appearance of male secondary sexual characteristics, spermiation and peak serum androgen levels were all delayed compared with control photoperiod males. An extended photoperiod, consisting of the normal seasonal photoperiod expanded into 18 months, also delayed maturation, though to a lesser extent. Constant long days (16 hours) accelerated maturation and the appearance of these maturation related parameters.

Photoperiodic manipulation may prove to be a useful method by which the adverse effects of maturation may be reduced, and thus would contribute to the flexibility of the producer's management strategy. However, in order to fully control maturation in the sense used by Donaldson and Hunter (1982a) it is necessary to consider more direct methods of intervention on the controlling hypothalamic-pituitary-gonad axis. As discussed in earlier chapters, a number of methods are available for such intervention. These methods are examined and evaluated in the rest of this chapter.

Some of the suggested commercial criteria by which a method for the prevention of male maturation will be judged are shown in Table 10·1. Such a list includes the main characteristics of an ideal method, including its cost-effectiveness, ease of use, lack of side effects and health dangers.

Table 10•1

Some commercial criteria by which the methods of maturation control in salmonids may be judged

1. Effectiveness - The technique must show a significant effect, though it need not be completely effective.

2. Cost effective - The cost per unit of production should be as low as possible.

3. Technically and operationally simple - Ideally the chosen method should be easily used in a farm situation, outside of the laboratory environment. It should require no specialised apparatus or expertise.

4. Free of detrimental side effects - It should show no adverse effects upon either growth or mortality.

5. Free of consumer resistance - It should not create consumer resistance or possible health dangers to either consumers or farm personnel.

6. Flexible - Ideally the method should be applicable to a range of fish stocks.

In the discussion that follows the techniques are grouped together depending upon their intended aim,ie. feminization or sterilization.

Feminization

Increasing the proportion of females in a population of salmonids is commercially advantageous for a number of reasons:-

1) As precocious maturation occurs among males, this will be avoided in all female populations.

2) Female salmonids normally mature a year later than males and so the growing period is extended.

3) The potential egg production of a population would increase with an increase in the number of females.

As already described, a number of techniques have been developed in order to increase the proportion of female salmonids in production stocks and ideally to eliminate the male phenotype (Bye and Lincoln, 1979).

a) Gynogenesis

Gynogenesis has been used to increase the proportion of females in teleost populations (Purdom, 1969, 1972, 1976; Chourront et al., 1980), though it is only recently that technical improvements have led to significant yields of such gynogenetically produced females (Refstie et al., 1982). This technique offers considerable potential in generating all female populations, though questions remain regarding the extent to which the sophisticated manipulations (including irradiation of sperm and precise temperature treatments of eggs) could be adapted to a farm situation. Doubts also exist regarding the viability of treated eggs as it has been noted that a reduced hatch may result

(Refstie et al., 1982). However, it seems likely that further technical advances will increase the success of this method and render its use more attractive.

One area in which it seems likely that gynogenesis will have a significant impact is in the acceleration of breeding programmes (Donaldson and Hunter, 1982a).

b) E_2 induced feminization

Direct feminization of salmonids has been reported following the administration of E_2 treated diet, early in gonadal development (Simpson, 1976; Simpson et al., 1976; Johnstone et al., 1978a). Prior immersion in steroid solution appears to be necessary in Pacific salmon (Goetz et al., 1979). In contrast with these successful reports, Bye (in Donaldson and Hunter, 1982a) recorded no increase in the proportion of females in populations of rainbow trout fed 20 mg E_2/kg diet from first feeding.

Results from this laboratory have shown an increase in the proportion of female rainbow trout after feeding E_2 treated diet, but the extent of such feminization was variable, probably due to aspects of the culture conditions such as the feeding regime. It appears that the method of incorporation and the feeding rate influenced the success of this method. Thus a reduced extent of feminization was seen at higher ration levels, suggesting fish may have been able to select non-treated food particles.

The use of a 'long lived' estrogen, ethinyl estradiol, produced an increased number of female and hermaphrodite fish. Total feminization was only achieved by drastic modification of the treatment regime.

However, this modified method gave rise to mortalities and a severe growth depression. It appears that this method requires careful monitoring and must be 'tailored' to the individual farm situation.

Since fish destined for human consumption are directly treated by this method concern has been expressed regarding the possibility of steroid residues reaching the consumer. Simpson et al. (1976) have shown that E_2 has a very short half-life in salmonids. In view of the low dose administered this suggests that the dose reaching the consumer would be minimal. It seems unlikely that the EEC will initiate legislation to limit the use of steroid hormones in livestock production, though at present the use of such compounds is governed by the Medicines Act and requires a veterinary surgeon's prescription.

c) The Production of all female eggs

As the male is the heterogametic sex in salmonids (Thorgaard, 1977) the progeny of crosses between genetic females, phenotypically sex reversed to functional males by treatment with androgens, and normal females will be female. Such an effect has been demonstrated in salmonids (Johnstone et al., 1979b; Okada et al., 1979; Lincoln and Bye, 1981) and grass carp (Shelton, 1982).

Commercial trials of this method have proved successful with nine of the twelve farms attempting the technique producing batches of all female eggs (Bye and Lincoln, 1981), though no adverse effects on fertility are reported. Some farms have experienced low fertilization (Ralph, pers. comm.), possibly related to the inexperience of farm staff in assessing the state of maturity or motility of spermatozoa.

Eggs produced by this technique have now become commercially available, though the supply is still rather limited, and it will be interesting to compare its efficiency at different farms. The technique is only marginally more complex if the farmer wishes to apply it to his own stock, as it requires him to hold special stocks of reversed females and to surgically remove sperm. It does have the great advantage that it avoids the need to directly treat table fish and so would bypass any possible legislation limiting the use of hormones.

Sterilization

Like feminization, the production of sterile salmonids would offer a number of commercial advantages:-

1) By preventing the diversion of energy and material towards gonadal growth, somatic growth would be maximized and the growing period increased.

2) The adverse secondary effects associated with both male and female maturation would be avoided.

3) The technique would allow the release of foreign or exotic species to new environments without the risk of harmful competition with indigenous species.

The techniques aimed at achieving sterilization have been described earlier in this work and are briefly evaluated below.

a) Surgical castration

Surgical castration is the most direct method of preventing sexual maturation of salmonids. However, the technique poses a number of problems in commercial use. It is technically difficult to achieve

complete castration of salmonids, especially if they are less than 250 - 300 g in weight (Brown, 1982).

In the brief experiment carried out in this laboratory it was found to be difficult to ensure that the gonads were completely removed. In particular the testes tended to split during removal and it appears that testicular regeneration would be a major limitation of this technique (Brown, 1982). Because of the restricted size range of fish in which this technique succeeds, it will not avoid precocious maturation. As it must be carried out by a veterinary surgeon and is therefore expensive, its main application would be in the production of small numbers of large fish.

b) Radiation induced castration

Radiation induced gonadal damage has been demonstrated by a number of workers (Donaldson and Bonham, 1964, 1970; Bonham and Donaldson, 1966, 1972; Hershberger et al., 1978). This method is especially suitable for the mass treatment of eggs or alevins but it requires considerable technical expertise and equipment which would not normally be available on farm sites.

The exact nature of radiation induced damage is uncertain. Hershberger et al. (1978) note that returning male Pacific salmon were sterile, though they showed normal secondary sexual characteristics, suggesting damage to the germinal rather than steroidogenic tissue. If this type of effect is widespread, radiation treatment would not prevent the appearance of androgen-dependent secondary characteristics. Possibly its main use will prove to be the large scale treatment of Pacific salmon eggs or alevins to reduce the incidence of precocious males (jacks) (Donaldson and Hunter, 1982a).

c) Genetic methods

1) Hybridization. The ability of salmonids to produce viable hybrids has been thoroughly studied (Piggins, 1965, 1970; Susuki and Fukuda, 1971a, 1973a, b; Chevassus and Petit, 1975; Refstie and Gjedrem, 1975; Sutterlin et al., 1977; Blanc and Chevassus, 1979; Chevassus, 1979). However, few workers have retained such hybrids to suitable age and commented on their state of sexual maturation. In some cases the hybrids have been reported to be sterile (Harrison, 1961; Susuki and Fukuda, 1971a, 1973a, b; Refstie and Gjedrem, 1975; Chevassus, 1979), in others this is not so (Piggins, 1965, 1970; Chevassus, 1979). Generally the offspring of intergeneric crosses are sterile, while those resulting from intragenetic crosses are usually not (Chevassus, 1979).

This approach is relatively simple, though a major problem is the marketing and description of resulting hybrids.

2) Polyploidy. The natural low frequency of spontaneous polyploid salmonids (Cuellar and Uyeno, 1972; Allen and Stanley, 1978; Lincoln and Bye, 1980) may be substantially increased by procedures such as thermal shock (Chourront, 1980; Lemoine and Smith, 1980; Thorgaard et al., 1981; Lincoln and Scott, 1983) or cytochalasin B treatment (Refstie et al., 1977; Allen and Stanley, 1979; Smith and Lemoine, 1979).

Technical improvements have developed this technique sufficiently that it yields high numbers of triploid (3n) offspring (Lincoln and Scott, 1983). These fish are now undergoing evaluation and it has been shown that they do not develop male sexual characteristics nor suffer the growth depression associated with male maturation. However, their

growth is not as good as the lead control fish and it seems that the growth performance of such triploids requires further careful evaluation.

d) Immune Castration

This is a technique, the effectiveness of which remains to be demonstrated. In contrast to the results of Laird et al. (1978, 1980) our work and that of Bye and co-workers at MAFF (Lowestoft) have failed to show a significant effect of such manipulation on the sexual development of male rainbow trout.

Due to doubt concerning the effectiveness of this technique, more positive evidence needs to be presented before it would be worth further commercial trials. The apparent failure of this technique is unfortunate as its relative simplicity inspired considerable interest throughout the fish farming industry.

e) Chemosterilization

A number of compounds have been tested as antigonadal agents in salmonids (Donaldson, 1973; Flynn, 1973; Schreck, 1973; Murphy, 1980a, b; Schreck and Fowler, 1982), though none has reached the stage of full commercial evaluation. The work of Murphy (1980a, b) suggests that methallibure may have some potential in the control of male maturation of salmonids, though in this trial both methallibure and cyproterone acetate were ineffective in suppressing precocious sexual development in salmon parr.

Work in this laboratory, using methallibure and the antigonadal compound flutamide has yielded ambiguous results. Both compounds reduced the percentage of mature males among treated fish, though the peculiar sex distribution of fish in this trial makes the results difficult to interpret.

As yet little information exists regarding the half life of such compounds in teleosts. In view of the possible health risks this gap must be filled before commercial use could be made of these.

f) High dose androgen treatment

A number of authors have noted the antigonadal effect of high doses of exogenous androgens (Ashby, 1957; Yamazaki, 1972; Jalabert et al., 1975; Goetz et al., 1979). However, this effect has not been observed in all trials. Bye (in Donaldson and Hunter, 1982a) reported that MT (25 mg/kg diet) produced up to 60% sterility, but the results were not reproducible. Interestingly a recent report has noted an inhibition of gonadal development in adult trout, by feeding 0·5 mg MT/kg diet (Billard et al., 1981).

Work in this laboratory has shown that high doses of androgens can inhibit gonadal development, though their effects appear complex. It appears the time of assessment may influence the interpretation of results and the apparent extent of sterility. Thus up to two years of age very few male fish matured and treated fish appeared to be totally sterile (Harbin et al., 1980). Continued monitoring showed a gradual development of testicular elements within treated fish. The males eventually reached spermiation approximately nine months after control males. However, gonadal development was abnormal and the majority of developing fish had nodular gonads. It is suggested this results from the persistence of small groups of cells, which survive steroid treatment and develop gradually under the influence of GTH.

The extent of total sterility varied between the two farm sites. In the Low Plains trial 11% of all fish examined were sterile, while in the Finnarts Bay trial 28% of all treated fish were sterile.

Although this provided a restricted sample for examination, some general comments on the growth performance of these sterile fish are possible. It was noted that the growth performance of these sterile fish was significantly worse than that of females and of those treated males which matured later than normal male controls.

So the efficiency of this technique in causing sterilization was relatively low; though in Pacific salmon it may be more promising (Goetz et al., 1979; Donaldson and Hunter, 1982a, b). The growth performance of sterile fish needs further monitoring. The most significant effect of treatment was the delay in male maturation and this, together with the prevention of precocious maturation, may be of commercial value, especially as the late maturing males appeared to grow faster than sterile fish. A further possible application of the technique might be to delay maturation in all female populations resulting from crossing sex reversed (XX) males with normal females, as it was noted in our trials that no females matured even up to three years.

Obviously the general comments on hormonal treatment made in the discussion of E_2 feminization apply equally here and in particular the use of hormonal techniques is dependent upon the attitude of bodies such as the EEC and upon current legislation.

Thus this work has examined the control of sexual maturation of male salmonids, the changes that accompany this process and the possible methods by which the fish farmer may avoid these adverse changes. The method chosen depends upon the particular problems faced by the producer and the production strategy in question. Precocious male maturation is prevented by feminization and so the producer facing

problems of precocious male maturation may adopt this approach. Alternatively, if a producer intends to produce large fish for a particular market, for example, smoking or restocking, then maturation of both males and females becomes a production constraint and sterilization would be the most desirable approach.

The evaluation of the available methods to satisfy these objectives is complicated by the task of attempting to compare reports from different laboratories and by the difficulties inherent in transposing a technique from the laboratory to the practical farm situation. However, some general conclusions may be reached.

The production of all female eggs by the use of sex reversed progeny has now reached the point of commercial use and appears to be an elegant answer to the problems posed by precocious male maturation. If it can be shown that fertilization is not adversely affected by difficulties in assessing the state of maturation of such reversed fish, it would seem to be problem free. It has the further advantage that direct hormonal treatment of 'table' fish is unnecessary. The results obtained using this technique appear less variable than those using direct E_2 feminization, which probably reflects intersite variation in culture conditions. The feeding regime, method of incorporation and length of treatment appear to be critical to the success or otherwise of this technique. In addition it is possible that gynogenesis may be utilized in producing all female populations following recent technical improvements.

Sterilization would remove both male and female maturation and prevent the diversion of energy and resources into gonad growth. So it would prolong the growing period.

High dose androgen treatment has proved effective in the sterilization of Pacific salmon (Goetz et al., 1979; Donaldson and Hunter, 1982a) and though Yamazaki (1976) reports it to be effective in rainbow trout, work in this laboratory has shown the effect to be incomplete, though with a significant delay. Bye and co-workers at MAFF (in Donaldson and Hunter, 1982a) also reports the failure of the method to yield total sterility.

The production of triploid (3n) rainbow trout is now a relatively simple technique and such fish avoid the growth depression associated with male maturation and appear to be sterile. However, they do not grow as fast as the lead control fish (Lincoln, pers. comm.). Before these techniques become more widely used it seems that the growth performance of sterile fish requires further evaluation and this is especially so in view of the comments on the hormonally produced sterile fish.

Although knowledge of the control of salmonid reproductive physiology has lagged behind that of mammalian species the considerable research interest, stimulated by the commercial importance of the topic, suggests that the technical expertise to fully control sexual development will soon be available. Possibly this will involve a combination of methods described and the greater flexibility such techniques will confer to the commercial producer should ensure that salmon and trout production fulfils its predicted potential.

APPENDICES

Appendix 1

Assay of mucosal glycyl-leucine hydrolase activity in rainbow trout

Technique

Fish were sacrificed and the intestine dissected clear and placed on a sheet of glass resting on an ice bath.

Fat and connective tissue were dissected clear and the intestine split by a scalpel blade.

The intestine was washed to remove any residual food using a wash bottle of chilled 0·9% NaCl.

The mucosa was scraped from the particular regions studied using a scalpel.

In the experiment reported two regions were examined:-

a) Caecal region. The mucosa was scraped from the intestine and as many of the caecae as could be easily split.

b) Post-caecal region. The mucosa was scraped from the region of the intestine approximately 2" below the caecae.

The mucosa was placed in a chilled LP3 tube packed in dry ice and was stored at -20°C until transport to Bradford for assay.

Assay

Glycyl-leucine hydrolase activity was assayed by Dr. Roger Ash at the University of Bradford using the 'two step' system of Nicholson and Kim (1975) as previously used by Ash (1980) using a substrate concentration of 10 mM and pH 8·0.

Results were expressed as μ mole substrate hydrolysed/min/g wet weight mucosa at 15°C.

Appendix 2

The use of implantable steroid delivery devices

Steroids and other potentially antigonad compounds may be administered by a variety of routes. Although such methods have been more widely explored in mammals, often with the intention of controlling reproduction, various methods have been used to administer steroids and other compounds to teleost fish.

Compounds may be administered in the environmental water (Ashby, 1957; Wiebe, 1968; Martin and Bromage, 1970; Pandey, 1970), by feeding treated diet (McBride and Fagerlund, 1976; Simpson, 1976; Yamazaki, 1976; Matty and Cheema, 1978; Fagerlund *et al.*, 1979; Yu *et al.*, 1979; Lone and Matty, 1980) or by injection (Hoar *et al.*, 1967; Nayyar and Sundararaj, 1970; Mackay, 1973, Hunt and Eales, 1979; Elliott *et al.*, 1980).

One problem common to these methods of administration is that they are influenced by variables such as feed rate, stress and the effect of handling. In particular the pattern of feeding can lead to fluctuating steroid levels. These variables could be eliminated by the use of implantable delivery devices, which could be left *in situ*.

Implantable devices have been used in the study of the teleost endocrine system. Higgs *et al.* (1975, 1976) used cholesterol based pellets to administer bovine growth hormone to coho salmon. Cocoa butter has been used as a matrix for antiestrogens (Billard and Peter, 1977) and testosterone (Crim and Peter, 1978). One problem with this type of delivery device is that the matrix may collapse, leading to irregular hormone release (Higgs *et al.*, 1976).

A more reliable method for the slow, controlled release of a variety of materials, such as hormones, vitamins, antibiotics, anti-coagulants, spermicidal agents and heart stimulants, is the use of a silastic implant. This uses a silastic matrix to give a slow release rate. Silastic (Dow Corning) is a medical grade elastomer, supplied as either a two component system, vulcanizing at room temperature after addition of the catalyst (stannous octoate), or as tubing.

This type of delivery device has been widely used in mammals (Dziuk and Cook, 1966; Prasad, 1973; Dubuc, 1974; Tanquary and Lacey, 1974; Frick et al., 1976; Gabelnick, 1976).

Few workers have attempted the possible use of such devices in teleosts, though Billard (1978) used an implantable silastic device of the reservoir type to administer T to rainbow trout. Similarly, MT has been administered to grass carp (Jensen et al., 1978; Shelton, 1982) using silastic devices.

Two main types of device have been used to administer steroids:-

i) Reservoir device - this consists of drug crystals, generally in a micronized form, enclosed in a silastic tube with both ends sealed with medical grade adhesive (Moghissi et al., 1976; Turek and Wolfson, 1978).

ii) Monolithic device - this type of system consists of a mixture of elastomer base and drug crystals, which is then catalysed and cast in a mould of the required size and shape. After vulcanization at room temperature, the device is then removed from the mould.

A) Preparation of Implants

Silastic 382 elastomer was chosen as the matrix for a monolithic delivery system as it is well tolerated, has a high permeability to steroids and permits their controlled delivery (Moghissi et al., 1976). Prior to use the elastomer was well stirred to ensure even distribution of filler. Elastomer was weighed out into the cut off barrel of a hypodermic syringe. A known weight of steroid was added (usually equivalent to 10 or 20% by weight). This was then mixed for five minutes and the correct amount of catalyst added (0·5% by weight). This was then stirred thoroughly for one minute. The mixture was then extruded into a polyethylene tube of 4 mm internal diameter.

After curing at room temperature the tubing was split and the silastic core removed, weighed and cut into sections, each having the required amount of steroid.

Prior to use implants were sterilized by soaking in Hibitane (Organon Laboratories).

Site of implantation

Three possible sites were examined:-

a) Subcutaneous (sc).

b) Intra muscular (im).

c) Intraperitoneal (ip).

Six fish were each implanted with a single placebo pellet consisting of silastic 382 only in one of the three sites. Immature rainbow trout (120 g weight) were used.

In the sc implants an incision was made in the skin slightly anterior to the dorsal fin and the skin lifted away. The pellet was

inserted and the incision closed by a pair of stitches.

Im implants were placed in a pocket, formed by cutting into the muscle layer at the same site. The pellet was inserted and the wound closed.

Ip implants were inserted into the peritoneal cavity through a single ventral incision of about 0·5 cm. The pellet was inserted and the incision closed by a single stitch.

Of the sites chosen the ip site was the most convenient due to the ease of wound closure. Subcutaneous implantation led to bruising and bursting of the skin and was far less satisfactory. When the fish were examined ten days later the ip site appeared to have healed well. This is in contrast with the results of Higgs et al. (1976) who reported high mortality when this site was used. The use of the ip site would have the advantage that the implants would be removed with the viscera when fish are gutted.

B) Hormone levels in implanted fish

Following implantation of immature rainbow trout with a single silastic pellet containing either 20 mg E_2, 20 mg T or a placebo pellet in the ip position, blood hormone levels were measured and survival monitored. In addition, five fish were implanted with a single solid pellet of either T or E_2, each of 20 mg (Organan Laboratories). Few mortalities were noted over the ten week experimental period, during which time blood samples were taken for hormone analysis.

Results

Of the 25 fish implanted all appeared to tolerate the operation well. Occasional early mortalities were noted, probably due to wound infection.

The results of the hormone measurements are shown in Table A•2•1. Serum calcium levels are also given as an indication of E_2 induced serum vitellogenin levels.

Discussion

The results demonstrate that the system described above is a relatively cheap and easily prepared delivery device for steroidal agents. In particular it appears able to give a relatively slow, controlled release of steroid. In both cases the silastic hormone implant appeared to release steroid at a much slower rate than did a solid hormone implant. One point of interest concerns the relatively low levels of E_2 released from both type of device into the blood. In the case of the silastic device this probably related to the reported lower release rate of E_2 from silastic devices (Lacey and Cowsar, 1974; Roseman, 1974), though other factors such as blood clearance rates can influence the blood levels observed.

C) The effects of steroid implantation upon maturation of rainbow trout

A silastic delivery system was used to test the possible anti-gonadal effects of a number of steroid hormones. Silastic implants were prepared containing 20 mg of T, E_2 and M.P.A. (Medroxyprogesterone Acetate). Placebo implants were prepared consisting of silastic only.

Table A•2•1

Mean serum T, E_2 (ng/ml ± SE) and calcium (mg % ± SE) of rainbow trout implanted with either crystalline or silastic steroid implants or placebo implants, sampled nine weeks after implantation

	T implanted (solid implant of crystalline T)	Silastic pellet + T implanted	E_2 implanted (solid implant of crystalline E_2)	Silastic pellet + E_2 implanted	Placebo silastic pellet
T (ng/ml ± SE)	58•02 ± 14•9	19•15 ± 4•5	1•76 ± 1•1	-	2•07 ± 1•52
n	5)	4)	4)		4)
E_2 (ng/ml ± SE)	-	-	3•70 ± 0•41	1•85 ± 0•06	0•39 ± 0•05
n			3)	4)	3)
Serum calcium (mg % ± SE)	-	-	43•74 ± 3•06	31•05 ± 12•7	9•29 ± 0•29
n			4)	4)	3)

Rainbow trout of approximately 140 g weight were obtained from Burwarton Fish Farm. Twenty fish were implanted with a single silastic pellet containing either one of the steroids or a placebo pellet as described. Implantation was carried out in July and the fish were maintained until December in a holding unit at the University of Aston, when following a pump failure the experiment was terminated.

Results

Due to high tag losses among experimental fish, and the fact that the experimental fish were not fully examined before disposal, only preliminary results are reported here. In no group did implantation completely suppress male maturation. Mature males occurred in all groups. The results are shown in Table A•2•2.

As the immature fish were not examined, it is not possible to comment on their sex or state of maturity. However, it is evident that at the dose used the implantation of the steroids T, E_2 and M.P.A. was incapable of suppressing male maturation.

Table A•2•2

Male maturation among rainbow trout implanted with a silastic pellet containing either T, E_2, M.P.A. or a placebo pellet sampled approximately six months after implantation

Steroid	Number of fish in group	Number of mature males	% mature males
Placebo	10	4	40
M.P.A.	6	2	33
T	10	3	30
E_2	6	2	33

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