The environmental control of reproduction in the female dace, *Leuciscus leuciscus*

Alison Joy Brook

Submitted for the degree of Doctorate of Philosophy.
Aston University

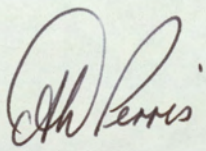
September 1989

DECLARATION

The work described in this thesis was carried out between 1985-1988 at Aston University in Birmingham. It has been done independently and has not been submitted for any other degree.

Signed: A J Brook.

Signature of internal supervisor:



The environmental control or reproduction in the female dace, *leuciscus leuciscus*

Alison Joy Brook PhD September 1989

Abstract

An investigation was made into the nature and control of the annual reproductive cycle of the dace, *Leuciscus leuciscus*. It includes 1) a study of the natural reproductive cycle, 2) the use of Carp Pituitary Extract (CPE) to induce final maturation and ovulation in captive fish, 3) the effect of artificial light treatments on ovarian development and 4)the measurement of serum melatonin levels under different photoperiod regimes. Ovarian development was monitored by endocrinological data, notably serum cycles of 17β-oestradiol (E2), testosterone (T), and calcium (as an index of vitellogenin), oocyte diameter, the gonadosomatic index and histological studies of the ovary.

Under natural conditions, ovarian development can broadly be divided into 4 stages: 1) oogenesis which occurs immediately after spawning; 2) a primary growth phase (previtellogenic growth) prevalent between spawning and June; 3) a secondary growth phase (yolk vesicle plus vitellogenic growth) occurring between June and December and 4) final maturation and ovulation which occurs in mid-March.

During the annual ovarian cycle, the sex steroids E2 and T showed two clear elevations. The first occurred occurred initially in April followed by a rise in serum calcium levels. This subsequently initiated the appearance of yolk granules in the oocytes in June. The second rise occurred in September and levels were maintained until December, after which there was a decline in serum E2 levels. It is proposed that in the dace, high serum E2 levels between September and December were required to maintain vitellogenin production and therefore its uptake into the developing oocytes which occurred during this time, albeit at a slower rate than in the summer months. After December, prior to final maturation, whereas serum E2 and calcium levels declined, serum T levels remained elevated.

In captivity, final maturation beyond the germinal vesicle migration stage failed to occur suggesting that the stimuli required for these events were absent. However ovulation could be induced by a single injection of CPE, which induced ovulation between 6 and 14 hours after treatment. Endocrine events associated with the artificial induction of spawning included a rise in serum levels of E2, T and the maturation inducing steroid 17α–hydroxy, 20β-dihydroxy progesterone.

Photoperiodic manipulation demonstrated that whereas short or increasing daylengths were stimulatory to ovarian development, long days delayed development. Changes from long to short and constant short daylengths early in the reproductive cycle advanced maturation (up to 5 months), suggesting that the stimulus for ovarian development and maturation was a short day. However, experiments conducted later in the reproductive cycle demonstrated that only a simulated ambient photoperiod could induce final maturation. It is proposed therefore that under natural conditions the environmental stimulus for ovarian development and final maturation are short and increasing daylengths respectively.

Further support that photoperiod is the dominant timing cue in this species was provided by the pattern of serum melatonin levels. Under different photoperiod treatments, serum melatonin, which is believed to be the chemical transducer of photoperiodic information (similar to other photoperiodic species) was elevated for the duration of the dark phase, indicating that the dace at least has the ability to 'measure' changes in daylength.

Keywords: Dace, reproduction, ovary, hormone, photoperiod.

Acknowledgements

This study was conducted at Aston University under the supervision of Dr Niall Bromage. During the final stages of study however, Dr Niall Bromage obtained a senior position at the Institute of Aquaculture, Stirling, Scotland. To enable me to complete my studies, using the purpose built photoperiod system and the pond system in Birmingham, Niall arranged for the maintenance of all the facilities required at Aston. Furthermore, through a kind invitation by Dr John Sumpter arrangements were made for me to complete my laboratory work at Brunel University. The completion of this study therefore required the help and co-operation of a number of people to whom I wish to express my gratitude.

Firstly, I would like to thank Charles Tyler for all his help and encouragement throughout this study with both his technical expertise and in reading the final manuscript.

I would like to express my gratitude to my supervisor Dr Niall Bromage for his assistance throughout this study and for his comments on reading the final manuscript.

Dr John Sumpter has given me considerable help during the final stages of this work in the provision of both laboratory facilities and general supervision at Brunel University. To him I offer great thanks. I would also like to thank John Carragher for his assistance in demonstration of practical techniques conducted at Brunel University.

I would also like to thank Dr Alan Perris for acting as my internal supervisor at Aston University after the departure of Niall Bromage to Stirling.

In addition, I am indebted to the following people for the provision of fish for my experiments: Keith Easton and Alan Churchward, Severn Trent Water Authority; Roger Sweeting, Thames water Authority and David Jordan, Anglian Water Authority.

My sincere thanks to all the technical staff at Aston University who were involved with fish maintenance and the provision of laboratory equiptment.

My final thanks go to friends and colleagues particularly the Fish Culture Unit who have continuously encouraged me and made my studies more enjoyable.

Contents

List of Figures, tables, plates and appendices.

Glossary

Anova	analysis of variance
Ca	calcium
CPE	carp pituitary extract
uCi	micro Curies
FSH	follicle stimulating hormone
GVM	germinal vesicle migration
GSI	gonadosomatic index
GTH	gonadotropin
GnRH	gonadotropin releasing hormone
GRIF	gonadotropin releasing inhibitor factor
17α20βP	17α-hydroxy,20β-dihydroxy progesterone
17αHP	17α-hydroxy progesterone
LH	luteinizing hormone
LHRH	luteinizing releasing hormone
LHRHa	luteinizing releasing hormone analog
E2	17β-oestradiol
RIA	radioimmunoassay
T	testosterone
VTG	vitellogenin

CHAPTER 1

General Introduction

1.1 General Introduction

Worldwide, there are over 20000 species of teleosts, representing a wide range of physiological and morphological adaptations. For such a large group of organisms, they have received very little scientific study. Research that has been conducted on fish is confined to a relatively small number of species, often those groups that have some commercial value, for example the salmonids.

The majority of British fish species belong to one or two orders, either the Clupeiformes, which include the salmonid family or the Cypriniformes, which includes the cyprinid family. Almost a third of the British freshwater fish belong to the cyprinid or carp-like family, and indeed on a worldwide basis, cyprinids include the majority of freshwater teleosts. Taxonomically, this group of bony fish have single anal and dorsal fins, the mouth is toothless, but the lower pharyngeal bones have strong teeth which crush the food against a horny plate (the 'carp-stone') in the roof of the gullet.

This study concerns the dace, *Leuciscus leuciscus*, a freshwater cyprinid which inhabits fast-flowing streams throughout Britain and Europe. In the British Isles, the importance of teleosts generally is assessed in terms of their value to sport angling or as food. Although the dace has some potential in angling, it has no food value and consequently little research has been directed towards this species.

However, in recent years, there has been a greater demand for the production of a more diverse range of freshwater fish, for the purposes of restocking inland water-courses, either to provide a 'new' fisheries for anglers, or to replace fish populations after pollution 'kills'. At present, there are a number of commercial hatcheries in Britain, which originally existed only to supply trout to anglers for stocking waters where natural production was insufficient to maintain adequate stocks. Now many of these are also supplying coarse fish. Furthermore, under the Salmon and Freshwater Fisheries Act (1969), the Water Authorities established in 1974 were required to "maintain, improve and develop fisheries", thus identifying a demand for coarse fish. Since 1974, the Severn-Trent, Thames, North-West and Yorkshire Water Authories have been developing culture techniques for coarse fish, mainly carp, (*Cyprinus carpio*), tench (*Tinca tinca*) and roach (*Rutilus rutilus*) and research efforts are being directed towards the culture of additional coarse fish species .

A detailed knowledge of the reproductive cycle and the factors controlling this cycle, are a prerequisite for the succesful culture of

any fish species. Thus simulation of the factors that naturally control reproduction ensures reproductive success in many captive species. It is therefore imperative that for each species cultured, detailed information concerning the natural reproductive cycle is first established.

In teleosts reproduction is cyclic, though the length of the cycle is extremely variable. Some fish e.g. Pacific salmonids and eels mature only once and then die, others only breed every 2 or 3 years (Billard and Breton, 1978). However the majority of fish breed once or several times each year. An annual reproductive cycle, with one batch of eggs produced each year is the most common strategy at mid and high latitudes, and includes most of the cyprinid family. While the majority of these fish show short and well defined breeding cycles, others appear to have extended breeding seasons lasting many months (Shackley and King, 1977). Less commonly, mature individuals may be found at any time of the year (Breder and Rosen, 1966). Clearly amongst teleosts, breeding cycles and strategies are diverse. Many are species specific, thus it is imperative that the factors regulating the reproductive cycle are examined for each species.

In the course of a typical reproductive cycle, the gonads develop, a process termed gametogenesis. In females this process which may take many months to complete culminates in final maturation and ovulation, two stages which by contrast are of limited duration. However to consider a reproductive cycle as consisting of but 2 stages, gametogenesis and final maturation is a misleading oversimplification. The morphology of a teleost ovary can be divided into a number of phases, namely oogenesis, specifically the transformation of oogonia into primary oocytes, cortical alveolus or yolk vesicle formation, exogenous vitellogenesis which contributes to the major growth phase of the ovary and lastly final maturation (germinal vesicle migration and ovulation). These in turn can also be divided into a number of more specific stages, such that 7 stages of ovarian development have been identified in the rainbow trout (Bromage and Cumarunatunga, 1988) and 8 in the herring (Polder, 1961). These stages of ovarian development are described in more detail in chapter 3.

Since most teleosts are seasonal breeders, these development differences occur at different times of the year, all of which require specific stimuli to control their periodicity. Based on similar environmental requirements for ovarian development, various annual reproductive cycles have been identified. These are usually classified according to when gametogenesis and/or spawning occurs.

Spawning is a loose term which describes the events shortly before and during oviposition (release of gametes into the external environment) and includes the final maturation stages as well as behavioural changes such as courtship and nest building. However like many authors, this thesis uses this term (spawning) simply to describe the release of oocytes into the surrounding water, i.e. the act of spawning.

According to when gametogenesis occurs, fish can be classified into three main groups (see Billard, 1982). The first group include the salmonids which all show the same characteristic annual reproductive cycle; gametogenesis beginning early in the year and vitellogenesis occuring during summer and autumn (under decreasing daylength and temperature) (table1.1). Other examples in this group include the pike, *Esox lucius* (Lofts and Marshall, 1957) and the female shiner perch, *Cymatogaster aggregata* (Weibe, 1968).

The second group is comprised of those species in which gametogenesis is arrested during winter (table 1.2).The roach, *Rutilus rutilus* is typical of this group (Escaffre and Billard, 1976); gametogenesis is inhibited in late summer and autumn until the following spring. Other cyprinids in this group include the minnow, *Phoxinus phoxinus* (Bullough, 1939), the bridle shiner, *Notropis bifrenatus* (Harrington, 1959) and the carp, *Cyprinus carpio* (Gupta, 1975). In addition the sticklebacks, *Apeltes quadracus* (Van den Ekoudt, 1946) and *Gasterosteus aculeatus* (Baggerman, 1972) show a similar cycle. There is strong evidence that dace may follow a similar gonadal cycle, as preliminary histological studies on the ovaries in October/November in this species (Brook and Bromage, unpub) suggests that the gonads are well developed by autumn.

The third and final group encompasses those species that undergo gametogenesis in late winter and spring (under both rising photoperiod and temperature) (table 1.3). A good example is the tench, *Tinca tinca* (Breton *et al*, 1980) which is also a cyprinid. Other species in this group include male *Cymatogaster aggregata* (Weibe, 1968) and the mackerel, *Scomber scomber* (Bara, 1960).

In the temperate zone, fish may also be classified according to when spawning occurs. Three main spawning seasons exist; these are late autumn/early winter (e.g. salmonids), early spring (e.g. pike) and late spring and early summer (e.g. cyprinids). However, species which belong to the same groups classified according to when gametogenesis occurs do not necessarily show the same spawning season. For example although all salmonids show a similar gametogenic cycle and generally spawn in autumn, *Thymallus* is a

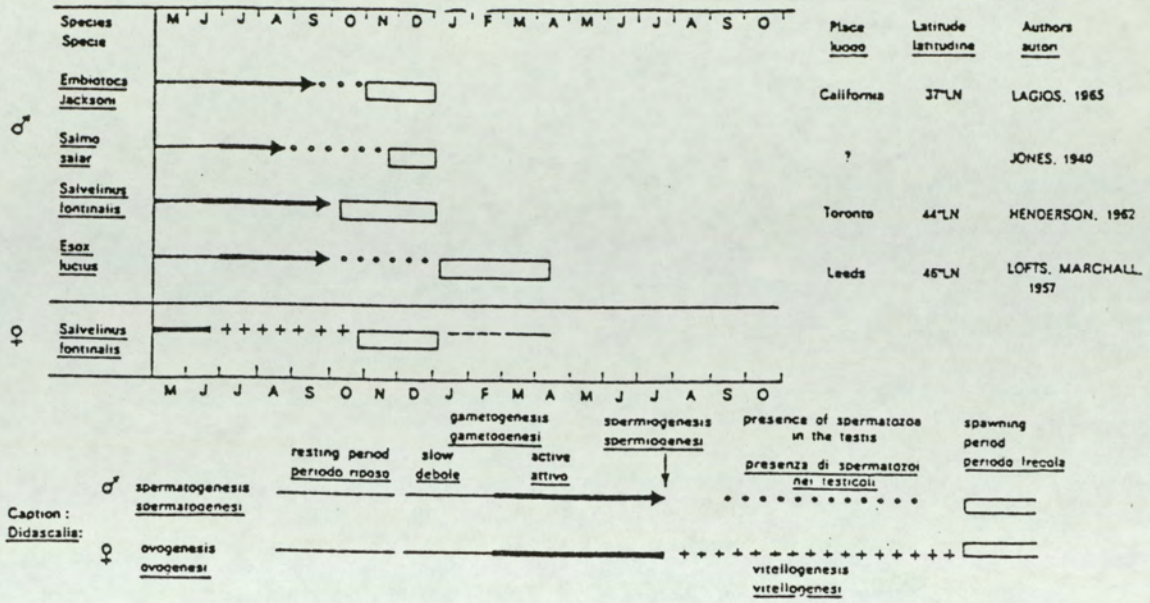


Table 11. Some examples of fish which undergo vitellogenesis under decreasing daylengths and temperatures. Taken from Billard 1982.

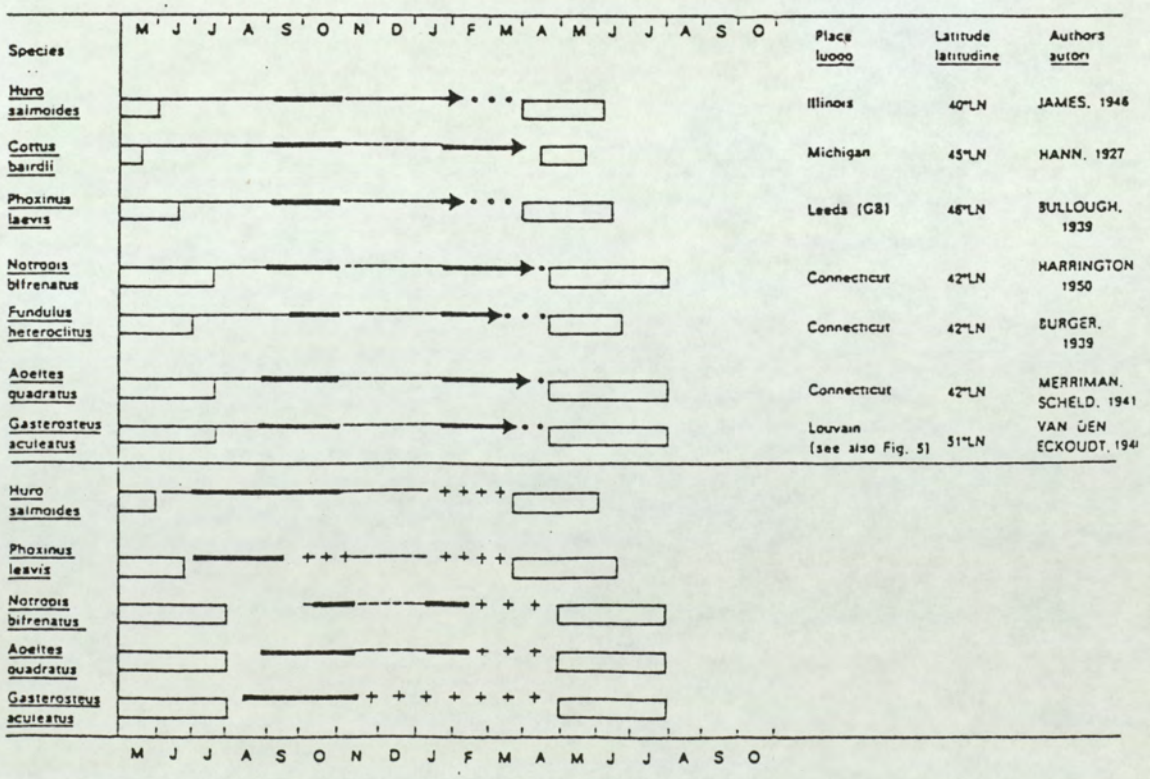


Table 12. Some examples of fish in which gametogenesis is arrested during the winter months. Taken from Billard, 1982).

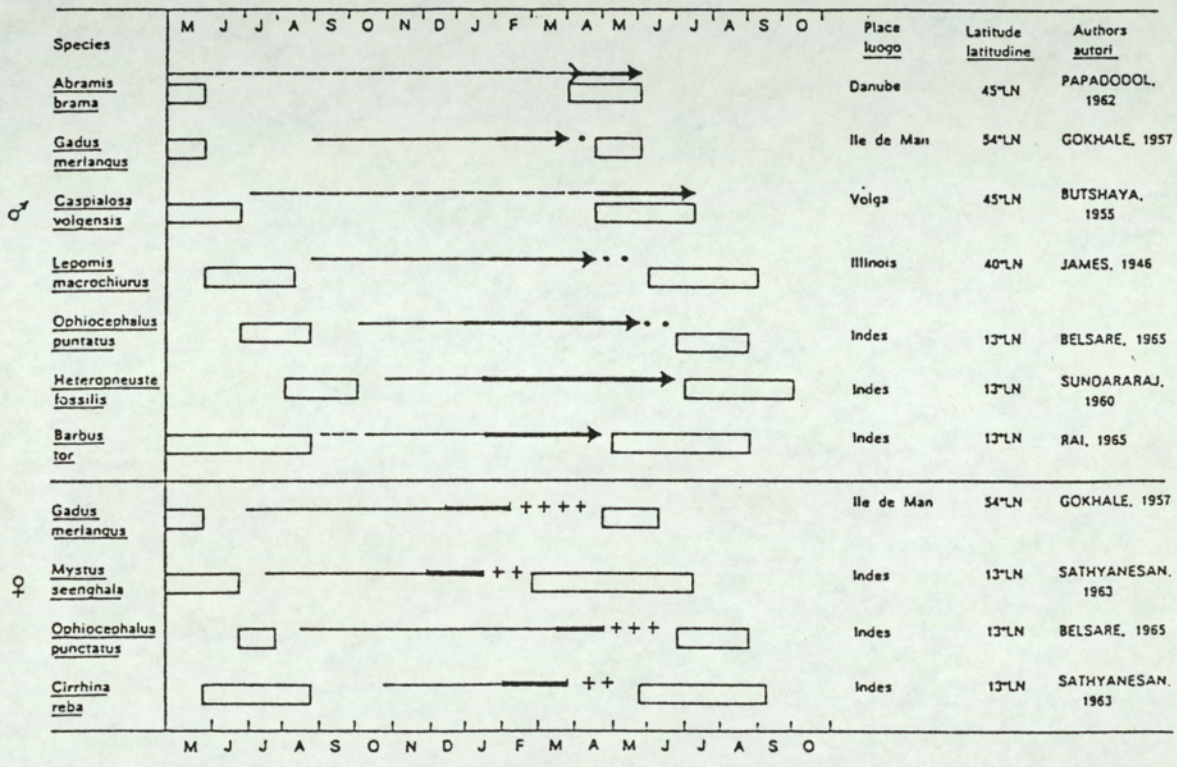


Table 1.3. Some examples of fish which undergo vitellogenesis under both rising photoperiod and temperature. Taken from Billard, 1982).

spring spawner.

Another important feature of these reproductive patterns, is that they are not characteristic of any one taxonomic group. For example cyprinids can be found in either Group II or III, classified according to when gametogenesis occurs, though spawning usually occurs in late spring/summer. By contrast the dace,although a cyprinid spawns earlier in the year, in March suggesting that ovarian development in this species may follow a different pattern to other cyprinids.

The preceeding few paragraphs have served to demonstrate that gametogenesis in different species may occur at different times of the year. It is reasonable to assume therefore that ovarian development in different species is timed by different cues. Although there are many cues which could potentially be involved in the timing of reproduction, they have broadly be divided into 2 types (Baker, 1938). Firstly, there are the 'ultimate factors' which determine the most advantageous time of year to reproduce. However, because such factors are not always reliable for determining the time of year and because they do not allow an organism to prepare in advance for the breeding season, there are also the so called 'proximate factors', i.e predictive cues which announce the approach of a changing environment. There are a wide range of both proximate and ultimate timing cues and each species depending upon its habitat will have evolved its own set of cues to ensure that the young are produced at the most appropriate time for their survival. Although much is known regarding the proximate factors involved in the long term control of breeding, little attention has been directed to the details of the final maturation and spawing (ultimate) requirements of fish. The difficulties encountered by fish culturists in obtaining oviposition in gravid fish (see Donaldson, 1975) indicates that for the final act, certain key (ultimate) stimuli are necessary.

In the temperate zone, the single most important ultimate factor governing the time of breeding appears to be the food supply (Nikolsky, 1969). Decreases in food availability can reduce gonadal development (Clemens and Reed, 1967) or delay the onset of sexual maturation (Bagenal, 1969), while an increase in food supply can hasten the onset of sexual maturation in a number of species (Cushing and Burd, 1956) and/or increase fecundity (Thibault and Schultz, 1978). This ensures that the offspring are produced when they have the greatest chance of survival, when food is abundant. It is likely that other ultimate factors are also important, but the nature of these factors, and to what extent they can regulate the timing of breeding appears to vary depending on the species. These stimuli

may include chemical parameters, for example pH, dissolved oxygen and salinity and physical factors such as the presence of appropriate vegetation (Scott, 1979). Although in most situations, climatic conditions would not be considered as ultimate factors, this is not without exception. For example, where rainfall is very seasonal, the fish living under these conditions have evolved a well defined breeding season that takes advantage of the flood period (Beumer, 1979). Furthermore the presence of a mate, or a member of the opposite sex, possibly at the correct stage of sexual maturity, maybe necessary for spawning. Finally exposure to other individuals of the same sex may also be important (Van Mullen, 1967) The specific stimuli required for spawning ensures reproductive synchronization between different individuals in a population.

In species with well defined breeding cycles, gametogenesis unlike final maturation, may take many months to complete and this is timed by the so called proximate factors. These can be either endogenous or environmental factors. The degree of importance of either factor varies. In some seasonally breeding animals, the environmental factors have the most influence, and largely govern the time of breeding, often to the extent that unless they become appropriate, the animal does not breed. In other animals, especially those living in constant environments, for example the deep ocean, it seems likely that the endogenous component of the timing mechanism will play the major role.

The environmental regulation of reproduction in teleosts has been reviewed in detail (e.g Billard *et al.*, 1978; Scott, 1979; Billard, 1982; Crim, 1982), and studies have shown that it differs according to the species under study. So far it has been suggested that environmental cues are restricted to either the initiation of gametogenesis or final maturation and spawning. However it is more likely that they coordinate all stages of gametogenesis. Wingfield (1984) has attempted to clarify this situation by classifying environmental variants into different groups (a) those factors which provide initial predictive information, (b) those factors which provide supplementary information, (c) those factors which operate close to or at ovulation or spermiation and (d) disruptive factors. However, in practice it is difficult to separate them as their precise nature is often unknown.

In the temperate zone where the climate varies from season to season, photoperiod and/or temperature changes are the most predictive and consequently more likely to be utilized to cue reproductive development. However, there is no reason why any

regularly occuring environmental factor should not act as a proximate timing cue, provided that it can be sensed by the animal, and that the animal can transduce this information to the reproductive system. The environmental cues controlling reproduction probably also interact with endogenous timing mechanisms, internal clocks which indicate when the reproductive season is imminent. The endogenous control of reproduction ensures even under constant environmental conditions, an animal will exhibit an approximately annual rhythm of gonadal maturation, i.e. they will come in and out of breeding repeatedly, each year, without any apparent environmental cues to trigger their breeding cycles. Under natural conditions however, environmental cues will synchronize this rhythm to the appropriate time of year. Most of the evidence for an endogenous control of reproduction has come from work on higher vertebrates (e.g.Gwinner,1973; Michael and Bonsall, 1977). However, more recently there has been some work on fish. Although the evidence for an endogenous control of fish reproduction is limited to only a few species (Baggerman, 1972; Sundararaj and Vasal, 1976; Duston and Bromage, 1986,1987), it has been suggested in many of the species studied to date (e.g. Scott, 1979; Hanyu *et al*, 1983).

An endogenous control of reproduction implies that environmental timing cues are generally not essential for gametogenesis: under constant environmental conditions gonad maturation is often completed though it maybe delayed (Pang, 1971) At the same time under suitable environmental conditions however, reproduction maybe advanced or occur more frequently than usual. Although environmental cues are not essential to gametogenesis, they are essential to successful reproduction, because individuals which mature out of phase with the environment, or each other will reduce their chances of producing offspring. Synchronization between individuals within a population is provided by accurate environmental (ultimate) information. However, such synchronization can only be effective if the follicles within each ovary are at a similar stage of development. In some species, for example the minnow, not all follicles in the ovary reach full maturity in spring (Scott, 1979) and late developing follicles remaining in the ovary after spawning would probably reach maturity completely out of phase with other follicles within the ovary. In nature this situation is avoided by the resorption of all vitellogenic follicles remaining in the ovary after spawning, reducing the ovary to a uniform datum on which the next year's cycle can be based. During this period the gonads are said to be refractory or insensitive to environmental factors. Thus, in the catfish, the ovaries of fish

collected during the post-spawning period were unresponsive to a long photoperiod, though the same photoperiod stimulated ovarian development a few months later (Sundararaj and Sehgal, 1970). Whereas in some species this ovarian regression is endogenously timed, e.g. in *Gasterosteus acculeatus* (Baggerman, 1988), i.e. it occurs after a predetermined time, even in the face of persisting suitable environmental conditions and regardless of whether spawning has occured or not, in others, breeding will only be terminated by unfavourable changes in the environment such as high temperatures (de Vlaming and Paquette, 1977).

So far it has been established that environmental conditions can influence ovarian development at different stages of the reproductive cycle. The next question that arises, is how is this information transduced to the developing ovary? Of the established environmental timing stimuli, only temperature by its action on enzyme systems has the potential to affect the gonad cells directly. Light and other less well established stimuli generally affect the gonad and other reproductive processes through a transducing system. Part of this transducing system, the hypothalamus-pituitary complex has been extensively investigated (e.g. Donaldson, 1975; Fontaine, 1976; Peter, 1982). Changes in the secretion of gonadotropin (GTH) from the pituitary gland and sex steroids from the developing gonad are thought to drive gonadal recrudescence and bring the fish to full maturity (e.g. Billard *et al*, 1978). With the improvement of techniques for measuring circulating levels of hormones, more detailed descriptions of the hormonal changes which mediate gonadal growth has become possible. In general, in fish showing well defined annual reproductive cycles, plasma GTH and sex steroid levels rise as the gonad matures, and falls after ovulation or spermiation. However, the positive identification of sex steroids in the plasma is still restricted to a relatively small number of species, and little is known of the functional importance of the different steroids. For example, testosterone, although it is a male androgen in most vertebrate groups, has been identified in the plasma of female teleosts, often attaining much higher levels in females just prior to ovulation, than in the corresponding males (Wingfield and Grimm, 1977; Whitehead *et al.* 1978; Scott *et al.* 1980). Its function in females however, still remains uncertain.

The release of GTH from the pituitary is under the control of releasing hormones secreted from neurosecretory cells in the hypothalamus. Major concentrations of these cells are located in the nucleus lateralis tuberis (NLT) and the nucleus preopticus (NPO)

regions of the hypothalamus (Peter and Nagahama, 1974). These neurosecretory cells are influenced by appropriate neural inputs from receptors for environmental stimuli.

In recent years, the pineal gland has been credited as a receptor for light, thus playing a major role in the control of reproduction (as an integral part of a pineal-hypothalamus-pituitary complex). Much of our information on pineal function is derived from studies on mammals (e.g. Reiter *et al*, 1976). Work that has been done on fish has been largely confined to a few species. From this work, both electronmicroscope studies (Oguri and Omura, 1973) and physiological data (Tabata *et al.* 1971) have confirmed that the teleost pineal gland is a photoreceptor and could potentially be involved in mediating the effects of photoperiod on sexual reproduction. In addition, the pineal gland has groups of cells that appear secretory (Hafeez, 1971) and therefore has also been described as endocrine. Further, indoleamine metabolism has clearly been demonstrated in the pineal of fish, which is governed by light/dark cycles (e.g. rainbow trout, Smith and Weber, 1976). The latter metabolism results in the synthesis and production of melatonin; melatonin is generally secreted during the dark or low light intensities, and it is believed to be the main chemical transducer of environmental, that is calendar information. Although the evidence is fragmentary, the most likely mechanisms by which environmental factors (via melatonin) influence gonadal function is by altering hypothalamic gonadotropin-releasing hormone and hence pituitary GTH and gonadal steroid secretion.

The preceeding few paragraphs have served to demonstrate that reproduction is a combination of many different complex processes. Clearly a basic understanding of these processes is necessary to begin to understand reproductive cycling and its control in the dace. This thesis investigates this further both under natural and artificial environmental conditions.

Previous studies on the dace have centred on the general biology and ecology of this fish (Kennedy, 1969; Hellawell, 1974; Mann, 1974; Wilkinson and Jones, 1977; Phillipart, 1981). Data on fecundity were presented by Kennedy (1969), Hellawell (1974) and Mann and Mills (1985), but little attention has been directed towards the annual reproductive cycle. A few researchers have investigated certain aspects of reproduction, including the attainment of sexual maturity and the timing of spawning. However, the available data were not consistent, and they do not consider all stages of the reproductive cycle. Reagan (1911) suggested that dace spawned at the same time as the chub, *Squalius cephalus*, in April to June. Jenkins (1958)

gives the spawning period of the dace as April and May. Hartley (1947) refers to the capture of a spent female dace as "early as 11th June" and records the capture of a "female full of developed eggs on 6th July".

Evidence that dace probably spawn earlier in the year than other cyprinids was advanced by Healy (1956), who found that a sample of dace caught in the Cork Blackwater, Ireland on 29th April, 1956 were all spent. Confirmation of early spawning was provided by Cragg-Hine (1963), who found that in a tributary of the River Nene, Northhamptonshire, the dace spawned in the last week of february in 1961; while in 1962, following a cold winter, they spawned in mid-March. Kennedy (1969) reported spent female dace on 1st April,1965, while roach, *Rutilus rutilus* in the same river showed no signs of imminent spawning. The most complete data on the spawning time of the dace are presented by Mann and Mills (1985). Each spring between 1974 and 1980, samples of mature female dace were collected from the River Frome, Dorset. These showed that dace in this river spawn over a 2 to 3 week period between late February and early April. These authors stated that the exact time was determined by the water temperature, although to date there has been no experimental evidence concerning the environmental control of reproduction in this species.

In other cyprinids, in which the environmental regulation of reproduction has been investigated, available data have shown that ovarian development and spawning are influenced by both temperature and photoperiod, although temperature is the predominant environmental influence in most species (e.g Billard *et al.*,1978). This is because most cyprinids spawn during the summer, when water temperatures are rising and very often spawning does not occur until a period of higher temperatures has been received. In addition elevated temperatures will advance final maturation in the goldfish, *Carassius auratus*, (Gillet and Billard, 1981). However, very few studies have been carried out to eliminate the role of photoperiod on the reproductive cycle. As the dace spawns earlier in the year than other cyprinids (early March), when water temperatures are still relatively low, it has been suggested that photoperiod rather than temperature may play the central role in controlling spawning in this species.

As the natural reproductive cycle of the dace has not been fully described, detailed knowledge of the natural gonadal pattern had first to be established. This is investigated in chapter 3; due to the large amount of literature concerning female reproductive cycles

alone, we decided to limit our study of the annual gonadal cycle of the dace to the female. A detailed examination of the annual female reproductive cycle was researched by both macroscopic and histological analysis of the ovary, and by measurement of the accompanying endocrine changes in serum sex steroid levels. In addition the annual cycle of serum calcium, as an index of vitellogenin levels (see section 2.5) was also determined.

During the course of this study, we found that female dace, like many other cyprinids would not spawn in captivity, and therefore we could not follow the endocrine changes associated with final maturation and ovulation. It was therefore necessary to artificially induce spawning in this species. In many captive species spawning can be induced by simulating natural environmental timing cues, for example a change in water temperature. However, although these techniques undoubtedly work for some, they cannot be applied to all species, for example cyprinids. However with an increasing knowledge of the endocrine mechanisms controlling reproduction in fish, it is possible to induce spawning using artificial exogenous hormone preparations. The most common endocrine method used to artificially induce spawning involves the injection of GTH of varying purity, usually a pituitary preparation. This technique of hypophysation has been used succesfully to artificially induce spawning in the cyprinids, carp (Yaron and Levavi-Zermonsky,1986), goldfish (Yamamoto *et al*, 1966) and roach (Worthington, 1983) as well as many other species (e.g. eel: Fontaine *et al*, 1964, pike: Anwand, 1963 and Grayling, *Thymallus thymallus*: Steffens, 1956). Chapter 4 therefore investigates the use of a carp pituitary extract to induce spawning in the dace, and specifically examines the endocrine changes associated with this induction.

Having established the natural pattern of ovarian development, further studies investigated the effects of photoperiod on the timing of gametogenesis. Artificial manipulations of photoperiod were imposed on the fish at different stages of the reproductive cycle, and the effects of these investigated by blood endocrinological and ovarian developmental (determined histologically) changes (chapter 5).

As a final effort into the investigation of the role of photoperiod on the reproductive cycle of the dace, chapter 6 investigates the mechanism whereby photoperiodic information could be transduced, notably a serum cycle of melatonin. If this proved successful, this would provide further evidence that photoperiod can be measured by the dace and be potentially involved in the control of

reproduction in this species.

CHAPTER 2

General materials and methods

2.1 Fish capture and husbandry

Maturing dace, *Leuciscus leuciscus*, of mixed sex were obtained from wild populations in streams using direct current (D.C.) electrofishing; the locations of which are described later. All electrofishing was conducted by the Water Authorities in which the water source were located. This method is based on the principle that fish under certain conditions respond to a direct current by swimming towards the positive pole (the anode). The current is produced by a portable generator and led through long rubber cables to a) the cathode or negative electrode which is a good conductor, for a example a cylinder of wire netting which is thrown into the stream and b) the anode (positive electrode) which is usually a ring of metal on an isolated shaft. To capture the fish, the anode is placed into the stream and fish within 3m radius will usually swim towards the anode, where they can be easily netted out. A direct current with a voltage of between 220 and 700 volts is sufficient to cause this reaction. At a continued use of the current or at still higher potentials, the fish either become paralysed or killed (electrocution). Due care is therefore taken at all times. The fish do not suffer any after effects provided they are netted out quite quickly and recover shortly after the current stops.

Once captured, the fish were transported to the laboratory in oxygenated plastic bags, and introduced into either a) recirculating water tank systems (experimental fish) (see appendix 1) or b) an outdoor pond approximately 144 square feet in area exposed to natural environmental conditions (control fish). The experimental fish were fed each day with commercial trout pellets (Mainstream, B.P. Nutrition), two hours after 'lights on', while the control fish, although their diet was supplemented periodically with trout pellets, obtained the major part of their sustenance from natural feed from the inflowing river.

All fish were individually identified by sub-cutaneous Panjet (Wright's Dental Products, Scotland) injections of Alcian blue dye applied at various combinations along the fish flank. The Panjet works by emitting a spray of dye solution which is able to penetrate beneath the skin; the mark lasting from 1 to 6 months.

2.2 Tissue sampling

In order to minimize handling stress, fish were anaesthetized using 2-phenoxyethanol (Sigma chemicals, Poole, Dorset) at a concentration of 1:1000. After sampling, the fish were maintained in a fast-flowing well aerated tank to aid recovery from the

anaesthesia. This procedure helped to minimise post-sampling casualties.

Blood samples were taken by cardiac puncture using 1.0 ml syringes (Richardsons of Leicester, Leicester) fitted with 21 gauge needles (Richardsons of Leicester, Leicester). Where possible 0.4ml of blood was removed and transferred to 0.4ml centrifuge reaction vials (L.I.P. Equiptment and Services Ltd). After clotting, the serum was separated from the blood cells after centrifugation using a Beckman microfuge at 15000 r.p.m. for 5 minutes. The resulting serum supernatant was stored at -20°C until assayed

The gonads were removed from sacrificed fish or those fish that had died prematurely during the course of an experiment. Left and right ovaries were individually weighed and fixed separately. One ovary was preserved in Gilsons fluid (section 2.5) to determine the number of oocytes and stage of development through size. The remaining ovary was fixed in formalin (section 2.6) for histological examination. After 24 hours, the fixed gonads were washed with and stored in 70% alcohol. The gonadosomatic index (GSI) was calculated as shown below:

$$GSI = \frac{\text{weight of left + right gonad}}{\text{body weight}} \times 100$$

2.3 Gonadal development

Adult male and female dace can only be distinguished from each other by external appearances during the breeding season. Sexually mature males can be identified by the presence of tubercles (the main secondary sexual characteristic) over the external surface, and adult females by their large swollen abdomens resulting from their large ovaries. Spermiation in males can be observed 2 weeks prior to ovulation by hand-stripping, a feature which enables the proximity of female ovulation to be determined. Unlike males in captivity the females are unable to complete maturation, unless induced using a pituitary extract (section, 2.3.2).

2.3.2. Assessment of the suitability of female dace for induction of spawning

a) materials

i.Stockards solution (clearing solution) containing:

glycerol	60ml
acetic acid	40ml
formaldehyde	50ml
distilled water	850ml

ii. 5ml syringe fitted with 11 gauge needle.

b) Method

Female dace were considered suitable for the induction of spawning when the germinal vesicle (nucleus) in the majority of oocytes was located in a peripheral position. An easy and rapid method for this determination developed by Bienarz and Epler, (1976) was used in this study. This procedure involves removing a sample of oocytes from the ovary by subcutaneous biopsy using a surgical needle (in this case, 11 gauge) attached to a syringe. The position of the oocyte nucleus was subsequently located after clearing the oocyte in Stockards solution for 10 minutes, and observing them under a binocular microscope (plate 2.1). Care was taken not to leave the oocytes too long in Stockards solution as this resulted in disintegration of the nucleus along with the rest of the oocyte.

2.3.3. Induction of spawning

Once selected for spawning induction, as described in section 2.3.2, the suitable fish were injected with carp piuitary extract (C.P.E.) (Stoller Fish Farms, USA). The acetone dried pituitary extract was solubilized as a 25 $mg.ml^{-1}$ suspension in 0.9% saline (Worthington, 1983). This solution was throughly mixed, centrifuged and the supernatant used for intramuscular injection. The injection volume was 0.1 $ml.100g^{-1}$ of body weight.

2.4. Serum analysis

2.4.1. Determination of calcium

Total serum calcium can be used as an indirect index for serum vitellogenin. Calcium is added during the post-translational modification of the vitellogenin molecule prior to its secretion from the liver into the blood (see Tata, 1978). It is well documented in teleosts that during the period of exogenous vitellogenesis production, serum calcium levels rise (Elliott *et al.*, 1979; de Vlaming *et al.*, 1980), an increase which has been shown to be directly correlated to blood vitellogenin levels (e.g Elliott, 1982). Calcium measurements were therefore used as a quick and reliable determination of blood vitellogenin.

Total serum calcium was measured using a Corning EL analyser (Corning Instruments, Medfield, Massachusettes, USA). This system operates on the following principle: a calcein dye forms an intense flourescent, non-dissociated complex with calcium ions in an alkaline medium. The technique is based on quenching of the florescence by

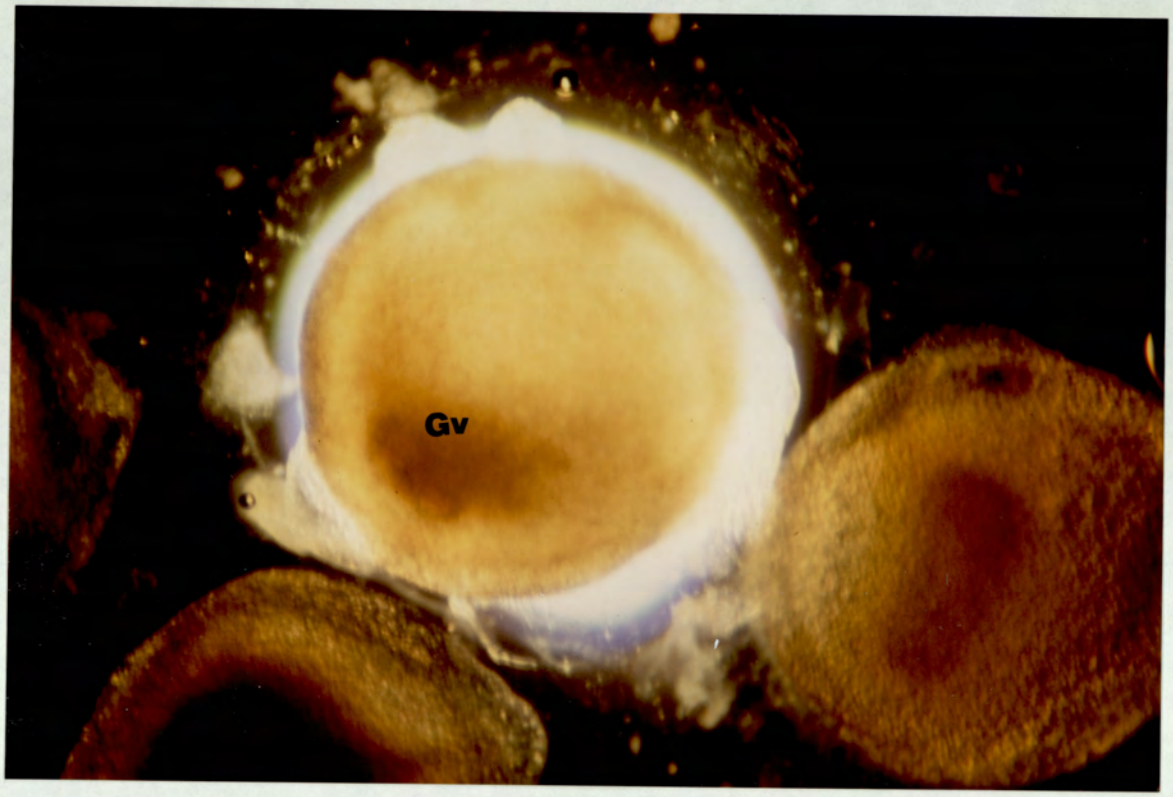


Plate 2.1. Whole oocyte showing germinal vesicle migration after clearing in Stockard's solution. This feature was an indication that the females were suitable for the artificial induction of spawning with C.P.E. GV = germinal vesicle. Magnification x 20.

chelating ions with EGTA, and detecting this with a colorimeter. Twenty ul serum samples were dispensed in duplicate. Precision and reproducibility were ensured by analysing trout standards each time. The inter and intra assay variations were 1.1 and 4.7% respectively.

Recently a vitellogenin radioimmunoassay (RIA) has been developed for cyprinids (Tyler and Sumpter, 1989). This assay was developed using labelled carp vitellogenin. Using this RIA, a dilution curve obtained from dace serum samples paralleled the carp vitellogenin standard curve (fig 2.1), which indicated that dace vitellogenin was immunologically similar to carp vitellogenin. In contrast rainbow trout vitellogenin did not cross react in this assay, demonstrating the specificity of this assay.

The correlation between total calcium used as an indirect assay of vitellogenin levels and vitellogenin measured directly using the above RIA, is shown in figure 2.2. This demonstrates that total calcium, as an index of vitellogenin used throughout this thesis is an accurate and specific determination of vitellogenin levels.

2.4.2 Determination of testosterone and 17β–oestradiol levels

RIA's for testosterone (T) and 17β-oestradiol (E2) were used in this study, according to the methods developed for rainbow trout serum by a) Whitehead (1979) and modified by Duston and Bromage (1987), further details of which are outlined below and b) T, Pottinger and Pickering (1985) and E2 , Bogomolynaya and Yaron(1984).

Two separate RIA procedures (a) and (b) were used in this thesis due to a change in laboratories during the project. Although the principles of the (a) and (b) RIA's are similar, separate validations were carried out due to differences in the source of antibody and label. The majority of the samples used in this thesis were analysed using the RIA developed by Duston and Bromage (1986) as outlined below:

2.4.2.1.Materials

a) assay buffer, phosphate buffered saline, pH-7, consisting of

Sodium chloride	4.5g
Gelatine	0.5g
Disodium hydrogenphosphate	8.88g
Sodium dihydrogen phosphate	5.82g
Sodium azide	0.03g

All the constituents were dissolved in 'Analar' water and made up to a final volume of 500ml.

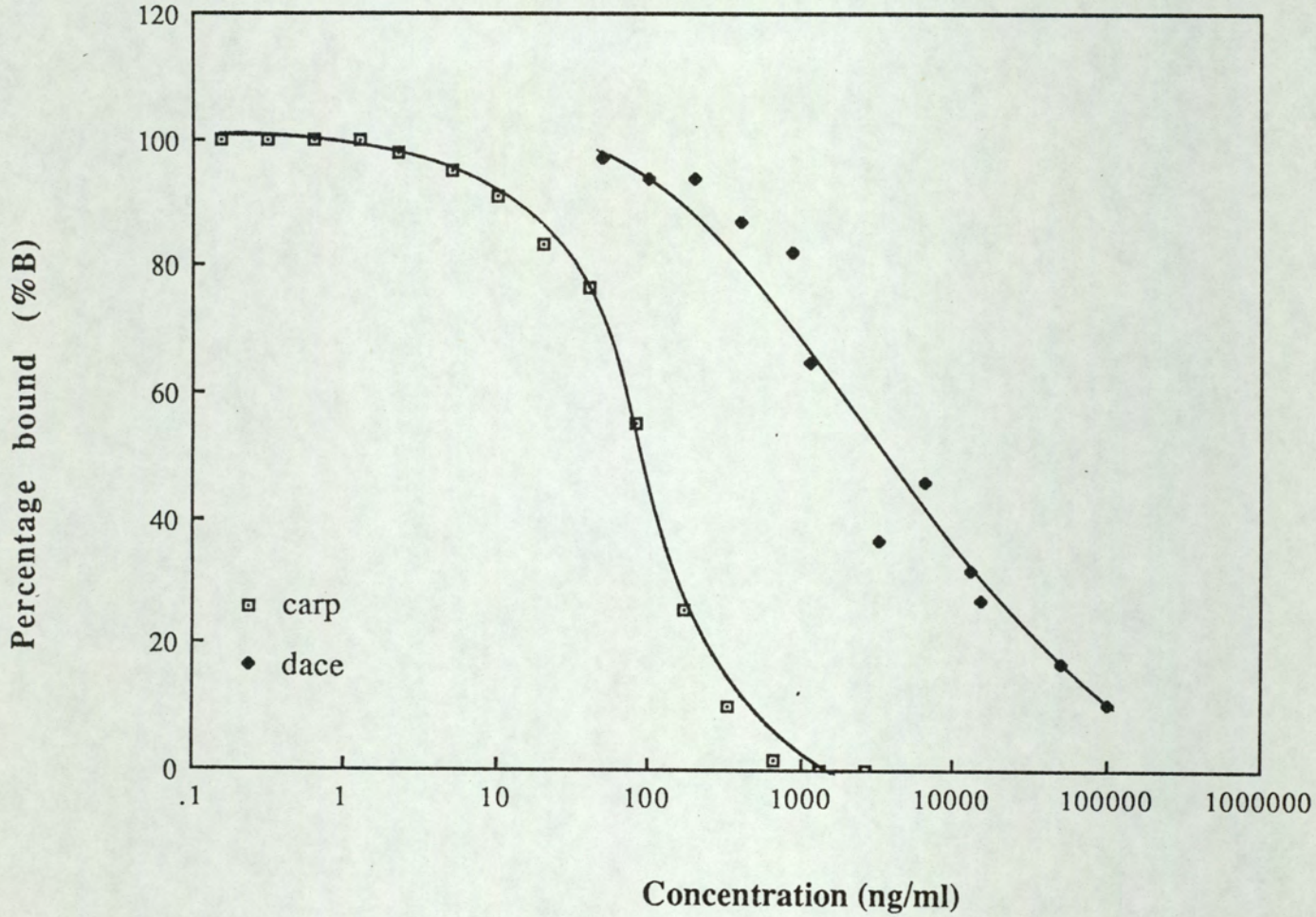


Figure 2.1 Parallelism of vitellogenic dace serum with carp vitellogenin using the carp radioimmunoassay. Redrawn from Tyler and Sumpter (1989).

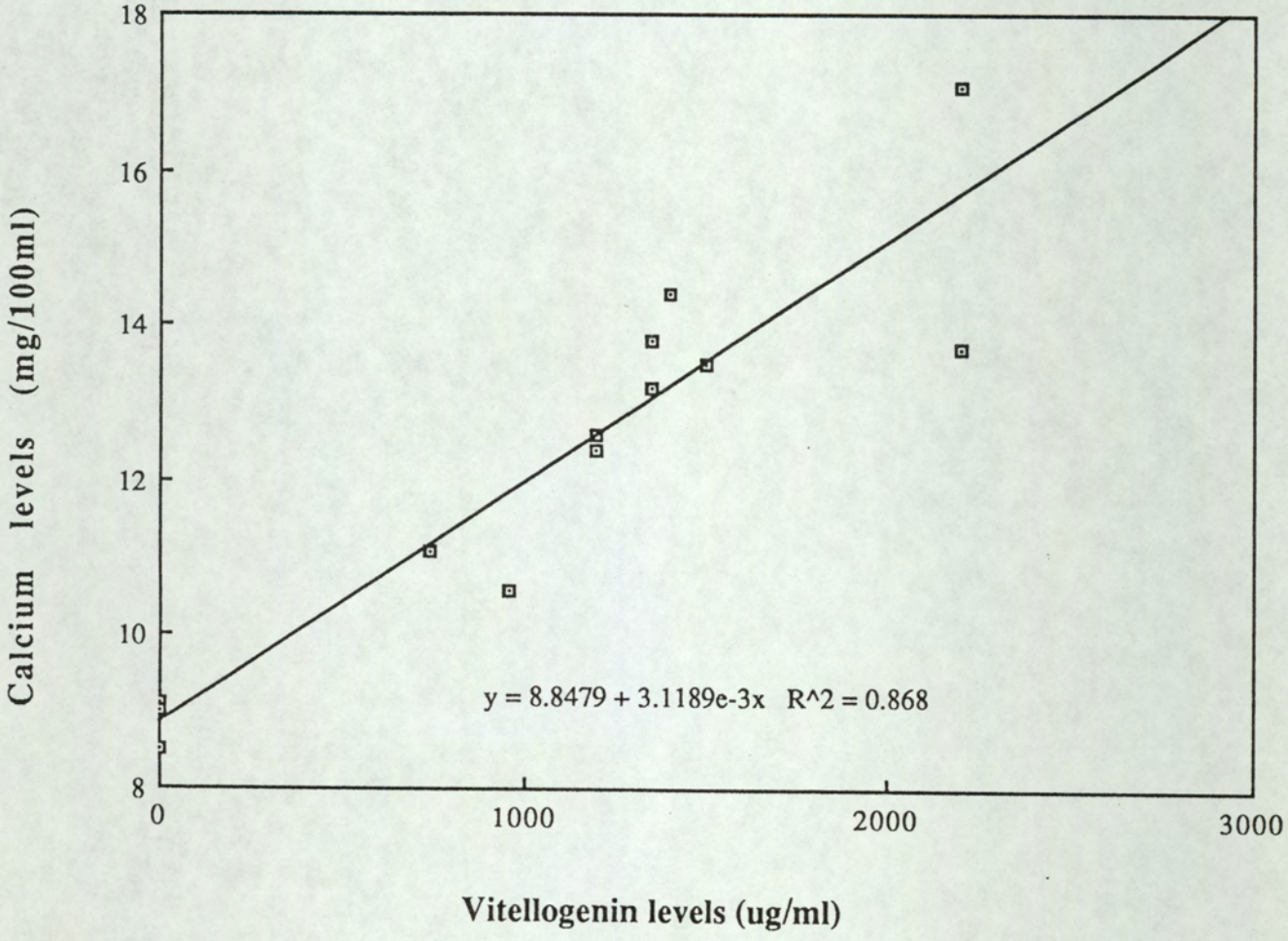


Figure 2.2 Correlation between vitellogenin measured as calcium (y-axis) and as vitellogenin using RIA (x-axis).

b) Scintillation fluid- Optiphase 'safe' (Pharmacia Ltd, Milton Keynes, Bucks)

c) Dextran coated charcoal

One 'Separex' dextran coated charcoal tablet (Steranti Research) was dissolved in 50ml RIA buffer. Each solution was made freshly for each assay.

d) Label

Tritiated testosterone and 17β-Oestradiol (Radiochemical centre, Amersham International, Aylesbury, Bucks) were stored at 10μCi.ml^{-1} of ethanol at -20°C. The working solution was prepared in the RIA buffer giving approximately 20000 d.p.m.100ul aliquot^{-1}.

e) Antisera

Testosterone and 17β-oestradiol (Steranti research) were reconstituted in assay buffer and stored at -20°C. The working solution was further diluted in RIA buffer. The specificity of the antisera was checked against a number of steroids in trout serum (Elliott, 1982) (see fig. 2.3).

2.4.2.2. Method

a) 50μl aliquots of serum were pipetted into polypropylene tubes for both the assay of testosterone and 17β- oestradiol.

b) 1.0ml of ethyl acetate was added to each tube, tightly stoppered and mixed on the rotary mixer for one hour.

c) The tubes were then centrifuged at 1500 r.p.m. for ten minutes.

d) 50μl and 100μl of supernatant were then transferred to rimless glass assay tubes for testosterone and 17β-oestradiol assays respectively. Smaller volumes of extractant were used for the testosterone assay due to the higher concentration of testosterone compared to 17β-oestradiol around maturation

e) A series of dilutions of standard hormone was prepared in duplicate in absolute ethanol. The range extended from 0-1000pg.

f) The extracts of standards and unknowns were then dried down (at less than 35°C) in a vacuum oven. This took 1-2 hours.

Steroid	% Cross-reaction	
17B-oestradiol	Taken as 100	5.8
Testosterone	1.6	Taken as 100
Oestrone	7.5	2.9
Oestriol	12.2	1.0
11-ketotestosterone	1.0	34.5
Androstenedione	1.8	1.0
17α -hydroxy-20B-dihydro progesterone	1.0	1.66
17 α -hydroxyprogesterone	1.0	1.0
Pregnenolene	1.0	3.3
Cortisol	1.0	1.0

Figure 2.3. Cross reactivity of 17β-oestradiol and testosterone antisera (used in the RIA'S at Aston univerity) with a number of structurally similar steroids. Taken from Duston and Bromage, 1987.

g) The dried extracts were cooled to 4°C before 100µl of antiserum and tritiated label were added to each tube. The tubes were vortex mixed for ten seconds before they were incubated at 4°C overnight.

h) A 0.5ml dextran coated charcoal suspension was added to each tube, vortex mixed and incubated for ten minutes at 4°C. The tubes were subsequently centrifuged at 2000r.p.m. for ten minutes at 4°C.

i) 0.4ml of supernatant was transferred from each tube into scintillation vials containing 0.9ml of scintillant, which were then stoppered.

j) Each vial was counted for five minutes in a scintillation counter (Packard Tri-carb 2660).

2.4.2.3. Calculations

a) percentage of bound hormone

Counts obtained for 100µl of labelled hormone (total counts) =**T**
Background counts were automatically subtracted from each scintillation vial as they were counted

.

Corrected counts obtained for standards

$$= \frac{\text{counts x 0.4 (fraction of supernatant counted)}}{\text{0.7 (total volume of supernatant, ml)}} = \mathbf{S}$$

Corrected counts for each unknown

$$= \text{counts x } \frac{0.4}{0.7} = \mathbf{U}$$

Therefore the percentage of bound hormone for standards and each unknown

$$= \frac{\mathbf{S} \text{ or } \mathbf{U}}{\mathbf{T}} \times 100$$

b) The percentage of hormone bound for each standard was plotted against the concentration of steroid in each standard ($pg.ml^{-1}$) to obtain a standard curve (fig. 2.4).

c) The concentration of steroid ($pg.ml^{-1}$) present in the unknown samples or the controls (x) could then be read directly from the

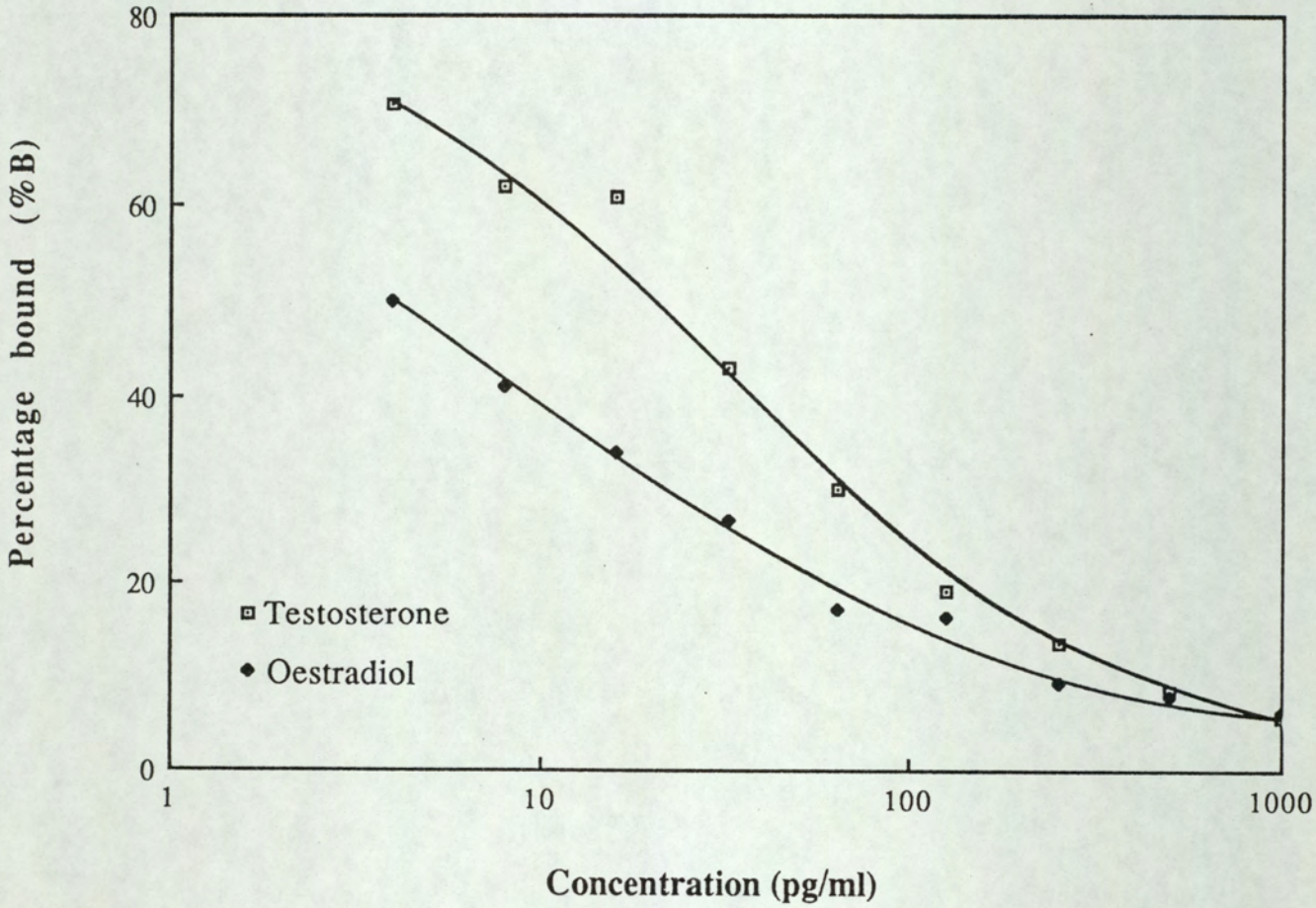


Figure 2.4 An example of the Standard curve obtained for testosterone and 17β-oestradiol, using the RIA's developed by Duston and Bromage (1987).

standard curve. The concentration of steroid ($pg.ml^{-1}$) in the original serum could then be obtained by multiplying x by 20 for 17β – oestradiol and 40 for testosterone. Due to the high levels of steroid in some samples, in particular testosterone, the concentrations were expressed in $ng.ml^{-1}$

2.4.2.4. Validation

a) During each assay, a standard trout serum sample (line standard) was used at the beginning and end of each assay so the inter and intra assay variations could be determined. For the testosterone assay, the inter assay variation was 5.5% and the intra assay variation 6.6% (see Appendix 2); the 17β-oestradiol inter assay was 6.2% and the intra assay variation was 9.5% .

b) This RIA was validated for use with dace serum by i) spiking equal volumes of serum with known concentrations of steroid; the accuracy of the assay was determined by correlating known amounts of testosterone or 17β-oestradiol added to the serum and the amounts detected by the RIA. (Fig. 2.5).
ii) comparing a series dilution of dace serum with the assay standard curve for both testosterone and 17β-oestradiol. The curves for both assays were parallel (fig. 2.6) indicating that the steroid measured in dace serum was immunologically similar to the standard hormone.

2.4.2.5. RIA procedure conducted at Brunel University

The RIA protocol for testosterone and 17β-oestradiol was similar to the method outlined above except for the following:

a) The assay buffer (phosphate buffered saline) was supplied as a tablet (Oxoid,) containing 0.1% sodium azide and 0.1% gelatine.

b) The standards were prepared in RIA buffer and not dried down with the unknowns. The dried extracts of the unknowns were consequently redissolved in 100ul of buffer.

c) The charcoal solution (made up fresh) was prepared as a 0.5% solution plus dextran. 500µl of charcoal suspension was transferred to each tube, incubated for ten minutes at 4°C and centrifuged at 4°C for 15 minutes. All the supernatant was transferred to the scintillation vials.

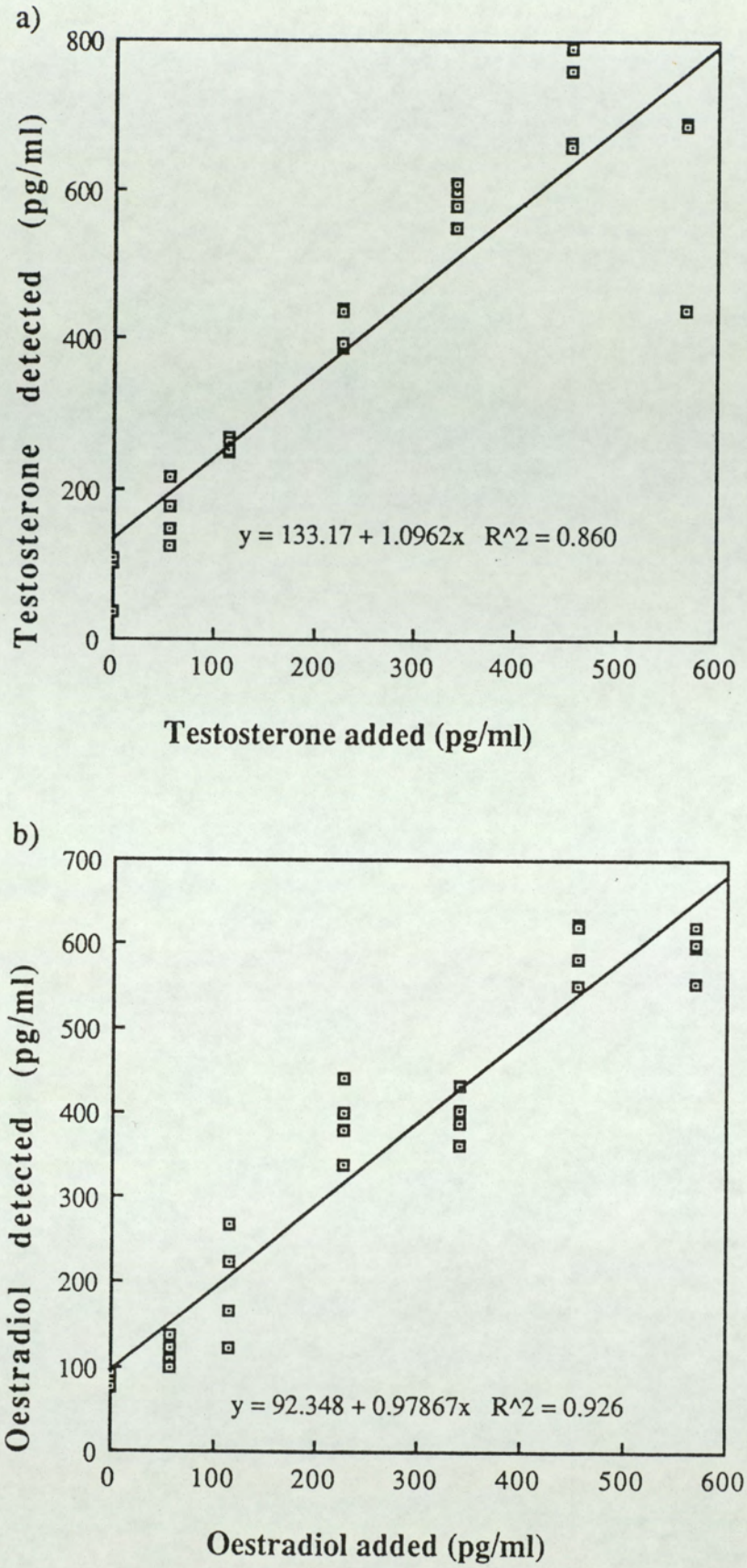


Figure 2.5 a and b Correlation between the amount of a) testosterone and b) 17β-oestradiol added to the serum and the corresponding amounts detected by RIA

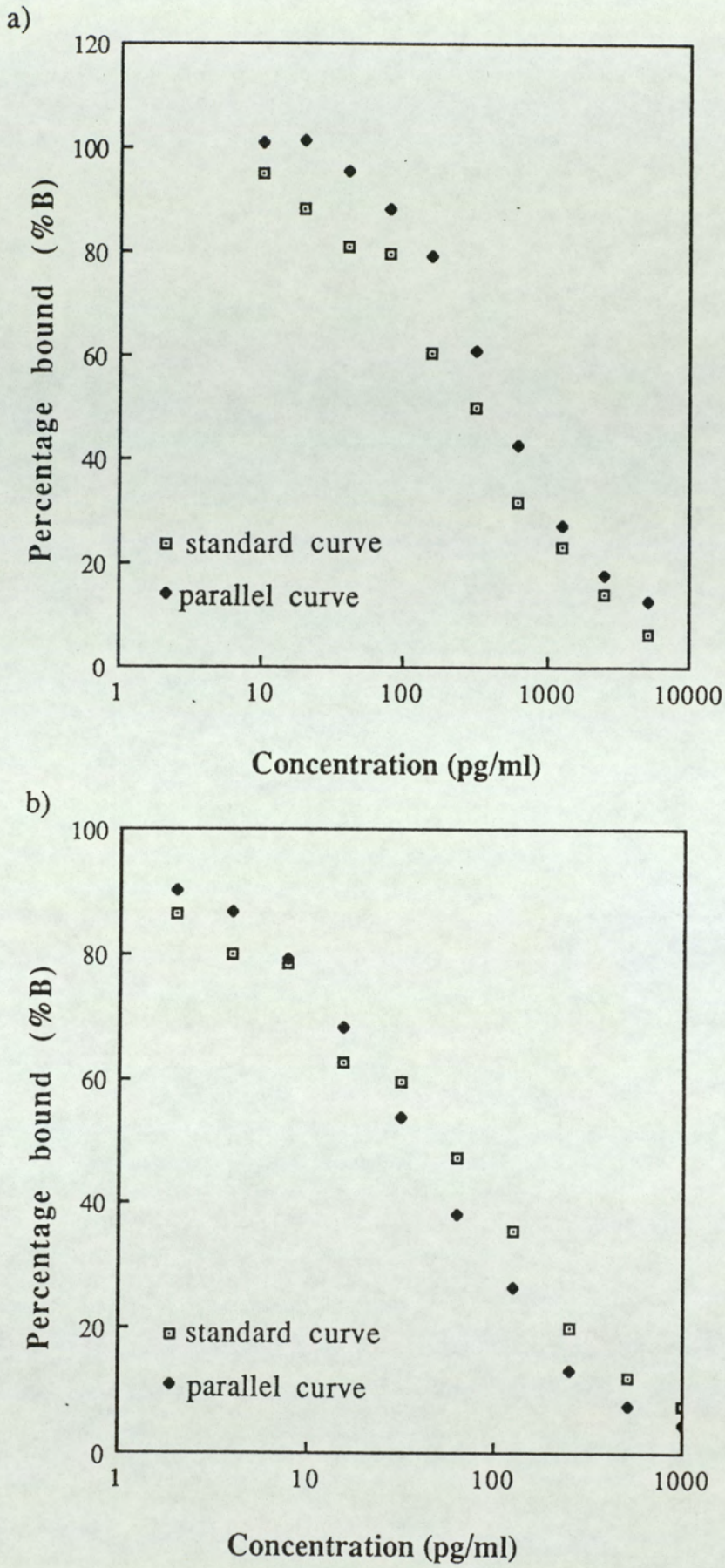


Figure 2.6. A comparison of the amount of hormone detected in a series dilution of dace serum with the standard curves of a) testosterone and b) 17β-oestradiol

d) The scintillation fluid used (5ml) was Liquiscint (National Diagnostics, Aylesbury, Bucks).

e) The antisera used in the testosterone assay was that employed by Pickering *et al* (1987). The dilution used was 1:25000.

f) The antisera employed in the 17β-oestradiol assay was provided by Dr Z. Yaron (Tel Aviv, Israel) and used at a dilution of 1: 150000. The detection limit of the assay was $8.6 \pm 1.7 pg.ml^{-1}$. Ethyl acetate extractions of dace serum diluted, parallel to the standard. The specificity of the testosterone antisera (Scott *et al*, 1984) and the 17β-oestradiol antisera against other steroids are shown in figure 2.7.

The correlation between testosterone and 17β-oestradiol levels recorded in this assay with those recorded in the assay developed by Duston and Bromage (1986) are shown in figure 2.8. These data confirm that both assay procedures are specific for detecting testosterone and 17β-oestradiol in the dace.

2.5 Oocyte counting/sizing

2.5.1. Materials

a) Gilsons fluid

Mercuric chloride	20grams
60% ethanol	100ml
80% nitric acid	15ml
Glacial acetic acid	18ml
Distilled water	850ml

b) 2.5ml plunger sampling pipette and a 250ml dilution bottle (Hydro-bios, Apparotebalk, Gmbtt, Kiel-Hottenau, Fed Rep. of Germany) (Appendix 3),

c) S-shaped counting chamber especially designed for the present study.

d) Plastic storage jars with plastic lids. This avoids the corrosive problems caused by contact of Gilsons fluid with metallic lids.

2.5.2 Method

Preservation of ovaries in Gilson's fluid for up to 3 months obtained

a)

Steroid	% Cross-reaction
Testosterone	100
17B-oestradiol	0.05
11-ketotestosterone	2.4
17 hydroxy progesterone	<0.01
17 hydroxy-20B dihydro-progesterone	<0.01
Androstenedione	0.4
Progesterone	<0.01
20B hydroxy progesterone	<0.01
17 hydroxy pregnenolene	<0.01
DHEA	0.05
5 dihydroxy testosterone	31
Androsterone	0.04
11B-hydroxy testosterone	8.9
Oestrone	0.01
Cortisol	<0.01
Cortisone	-
Corticosterone	-
o	<0.01
11 deoxycortisol	<.0.01

b)

Steroid	% Cross-reaction
Oestradiol	100
Oestrone	0.344
Oestriol	0.426
16 -hydroxyoestrone	0.067
Androsterone	<0.0001
Dehydroisoandosterone	<0.0001
Testosterone	<0.0031
11B-hydroxy-testosterone	<0.0001
11-ketotestosterone	<0.0001
Cortisol	<0.0001
Cortisone	<0.0001
Corticosterone	<0.0001
11-deoxycortisol	<0.0031
Deoxycorticosterone	<0.0001
Dehydrocorticosterone	<0.0001
Pregnenolene	<0.0001
Progesterone	<0.0001

Figure 2.7 a and b. Cross reactivity of a) testosterone (from Scott et al., 1984) and b) oestradiol (from Bogmolnaya and Yaron, 1984) antisera (used in the RIA's at Brunel University) with other steroids.

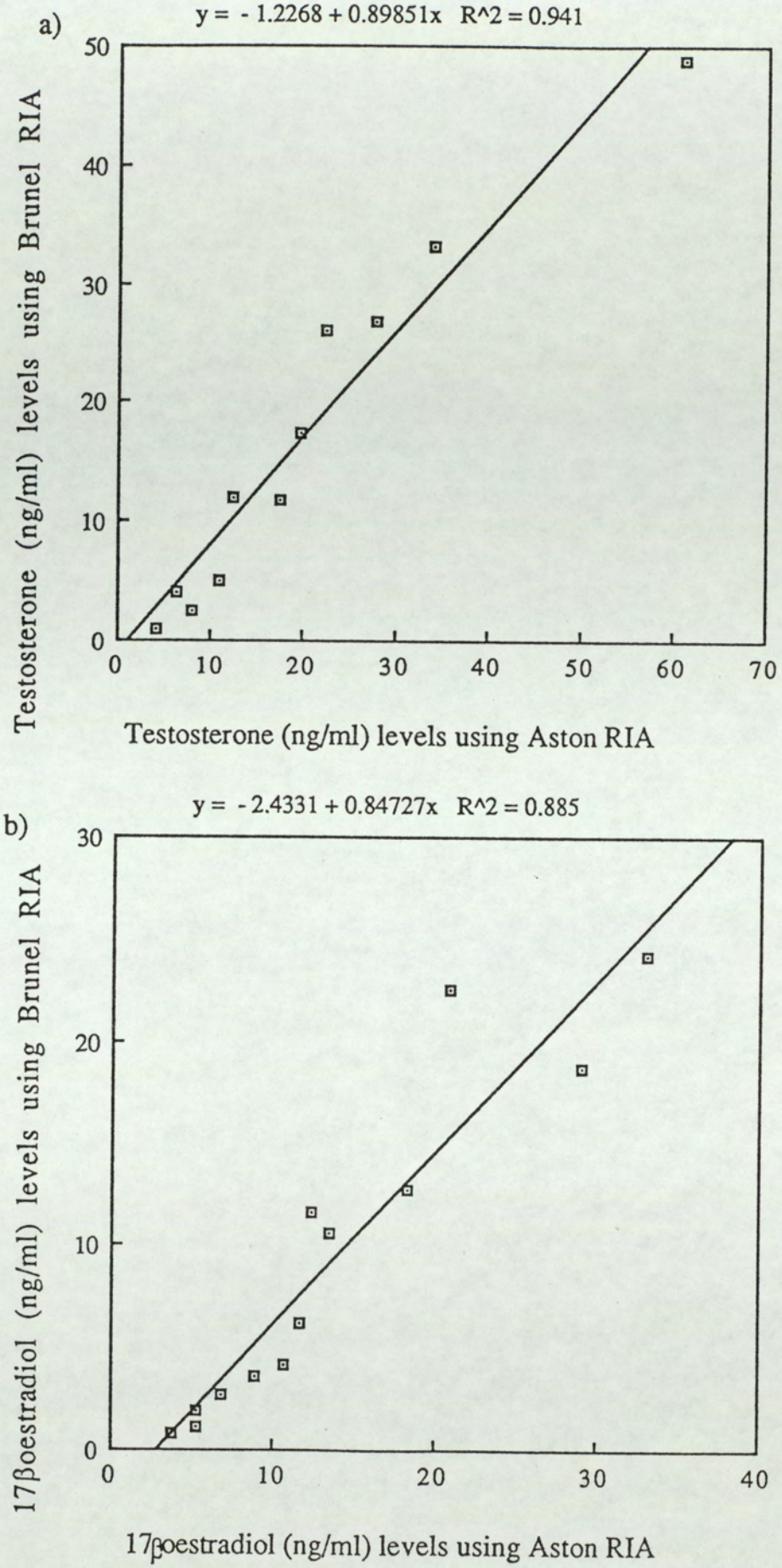


Figure 2.8. Correlation between the concentration of a)testosterone and b)17β-oestradiol levels detected in the two assays.

the best results; all of the oocytes at this stage had been completely separated from the follicle layers and connective tissue.

After completing separation, each ovary was placed in the 250ml dilution bottle after having been thoroughly washed in distilled water. The sample was then made upto 250ml with distilled water and the pipette put in place. Vigorous shaking at this point helped to distribute the oocytes within the bottle evenly. A sub-sample of 2.5ml was then removed and transferred to the S-shaped counting chamber.

All the oocytes were counted and their diameters measured using a binocular microscope fitted with a micrometer eye piece graticule. They were then sorted into the different developmental stages on the basis of their diameter. From each sample, at least 3 sub-samples were used for counting. The intra-sample coefficient of variation was 6.16$\pm$3.68% (see Appendix 2).

2.5.3. Calculations

Total gonad weight = **Gt**grams
Weight of right ovary = **Gr**grams
Weight of left ovary = **Gl**grams

$$Gr + Gl = Gt$$

Volume of total sample = 250ml
Volume of sub-sample = 2.5ml

Total number of oocytes in sub-sample =**St**
Total number of vitellogenic oocytes in sub-sample =**Sv**
Total number of previtellogenic oocytes in sub-sample =**Sp**
Total number of atretic oocytes in sub-sample =**Sa**

$$St = Sv + Sp + Sa$$

Total number of oocytes in left gonad $Sl = \frac{St}{2.5} \times 250$

Total number of oocytes in both gonads $St = \frac{Sl}{Gl} \times Gt$

or $\left(\frac{St}{2.5} \times 250\right) \times \frac{Gt}{Gl}$

Total number of vitellogenic oocytes in both gonads = **Sv**

$$= \frac{Sv}{2.5} \times 250 \times \frac{Gt}{Gl}$$

In the same way the number of oocytes of each development stage could be calculated.

2.6. Histological methods

a) Formalin
b) 70% ethanol
c) LKB Historesin kit (Pharmacia Ltd, Milton Keynes, Bucks) consisting of i) basic resin made up of
Glycomethacrylate (GMA)
Hydroquinone
Polyethyleneglycol 400 (PEG 400)
Water 5-8%

Glycomethacrylate is a water soluble ester of methacrylic acid. Hydroquinone prevents the self-polymerization of the plastin monomer. The softner PEG 400 is used to improve the sectioning properties of the final resin.

ii) Activator made up of benzoyl peroxide and a plasticizer. Benzoyl peroxide, a strong oxidising agent reactivates the glycomethacrylate. The plasticizer secured the benzoyl peroxide which is explosive as a dry powder.

iii) Hardener, made up of a derivative of barbituric acid and dimethyl sulfoxide (D.M.S.O). The derivative of barbituric acid initiates the polymerization reaction when added to the infiltration medium.

iv) Historesin mounting medium consisting of 2 component methacrylate polymers, namely polymethacrylate (PMMA) ,a benzoyl peroxide with 50% stabilized plasticizer and a liquid component made up of methlmethacrylate (MMA) and dimethyl paratoluidin.

v) Polyethylene histomoulds
vi) Wooden mounting blocks
Vii) Slides and coverslips
viii) D.P.X. mountant
ix) Staining materials, Ehrlich's Haematoxylin and Eosin and Mallory's Triple stain.

2.6.2 Procedure

a) Fixation

Whole ovaries or individual oocytes were fixed in formalin for 24 hours, then washed and stored in 70% alcohol.

b) Infiltration

The infiltration solution was prepared by mixing 50ml basic resin + 0.5g activator. The oocytes were transferred from 70% ethanol into a 1:1 mixture of infiltration solution and 70% ethanol in

glass bottles and left overnight at 4°C, before being transferred into 100% infiltration medium. Three changes of infiltration solution were carried out in total, over a period of 6-10 days. The temperature was maintained at 4°C in order to slow down the rate of polymerization. Several changes of infiltration solution ensured complete penetration of the oocyte. Infiltration is completed when the specimen appears slightly translucent and sinks to the bottom of the infiltration vessel; the oocytes were then ready for embedding.

c) Embedding

The embedding medium was prepared by mixing 15ml of infiltration solution with 1ml of hardener. At room temperature the embedding medium begins to polymerize almost immediately, becoming too thick too quickly. Therefore all embedding procedures were carried out on ice to slow down the polymerization process and extend the working time.

The lower depression of the histomould was filled with embedding medium before the specimen was immersed. The mixture was left to polymerize for upto 4 hours at room temperature.

d) Mounting

The mounting medium was prepared by mixing 2 parts powder to 1 part liquid by weight, and used immediately. The upper depression of the histomould was filled with a thin layer of mounting medium and the wooden block pressed firmly down onto the mould. The block is ready for sectioning after 10-15 minutes.

e) Microtomy

Two um thick sections were cut using a retracting rotary microtome (Rotary one, Pharmacia Ltd, Milton Keynes, Bucks). Sections were floated on a beaker of distilled water to remove any folding of the section, before they were mounted onto glass slides. The preparations were then placed on a hotplate at 40-45°C to dry and adhere the sections to the slide.

f) Staining

Sections were stained with either Ehrlich's haematoxylin and eosin (Pantin, 1960) or Mallory's triple stain and mounted in DPX. Stages of ovarian development were assessed by reference to results obtained in the rainbow trout (Bromage and Cumarunatunga, 1988).

g) Photography

Stained sections were photographed using a Zeiss Photomicroscope II (West Germany).

2.7 Statistics

2.7.1. Estimation of the mean

The arithmetic mean of a sample represents an estimate of the true population mean (u) and is calculated by summing all the individual observations of items in the sample and dividing the sum by the number of items in the sample. An estimate of the population mean throughout this thesis is written as the arithmetic mean $\pm$ one standard deviation ($Y \pm SD$).

$$\text{Arithmetic mean} = Y = \frac{x}{n}$$

where n= the total number of ampling times, x = independent variables, x = sum of independent variables and $(x-Y)^2$ = variance of a sample.

The standard deviation (SD) =

$$\frac{(x-Y)2}{n}$$

2.7.2. Comparison of more than two samples

One way analysis of variance was used wherever 3 or more sets of replicated readings were compared. 'Anova' calculations were carried out using a 'Minitab' statistical package (Ryan *et al*, 1981) on a Harris mainframe computer. The test compares the mean values of the different treated groups and determines whether there is a difference between them. Sample sizes of unequal numbers of items were analysed in this way.

If the null hypothesis were true (that there is no difference between the sample groups is correct), we would expect the ratio of the comparisons to exceed unity. In practice we calculate this ratio as the statistic F and determine the level of probability of obtaining such a ratio (or one larger) if the null hypothesis were true. The F ratio is determined by dividing the mean squares of the item to be tested by the error mean square.

The significance of the F ratio can then be determined by referring to the F tables at the appropriate degrees of freedom. Significance is determined by the probability of the result occuring by chance. Probability is generally accepted as significant if $P< 0.05$, that is a 5% chance that the results would occur by chance alone rather than the differences in treatments.

The levels of probability are $P<0.05$ significant *, $P<0.01$ highly significant** and $P<0.001$ very highly significant ***.

However, the analysis of variance only determines whether there is a difference between different groups. In order to determine which of the groups are different, the least significant difference (LSD) is calculated, whereby,

$$LSD = t\ (5\%\ \text{degrees of freedom of error}) \times \sqrt{\frac{2 \times \text{error MS}}{\text{number of replicates}}}$$

The mean of each group is then subtracted from the grand mean. If this value is larger than the LSD value, then the effect of the treatment is significant. If the value is less than the LSD value, the effect of the treatment is not significant.

CHAPTER 3

The annual reproductive cycle of the female dace

3.1 Introduction

Reproduction in most temperate zone teleost species is controlled by regular, predictable changes in the environment, such as photoperiod and temperature. This ensures that the young are hatched each year under the most favourable conditions for their survival, that is when food is most abundant. The available data on the environmental control of reproduction have, however, largely been obtained from only a few species, generally those which have an econonic importance. There has therefore been a tendency to generalize from the few species studied to the remainder of the teleost groups. However, this is unjustified, as each species will have evolved its own set of reproductive timing cues. To understand the environmental timing mechanisms involved in reproductive cycling, an awareness of the gonadal and endocrine cycles accompanying reproduction must first be established.

In teleosts, egg production is a complex process, involving considerable changes in both gonad morphology and hormone changes in the blood. The complex cycles accompanying reproduction include a number of individual stages and events which are all essential for the succesful development of the gonad. Considerable information is available on the histological changes in the ovaries and endocrine changes that occur during the seasonal pattern of sexual development in a number of teleost species. Together these features are essential for our understanding of how changes in the environment are perceived and relayed to their effector organs. An awareness of the histological cycles of ovarian development and endocrine events accompanying reproduction in other teleosts is central to this study examining the natural reproductive cycle of the dace. The following paragraphs therefore give a brief account of the general changes that occur in the ovary and the endocrine glands and secretions that accompany the changes in reproductive development in the fish species studied to date. With this information, comparisons can be made to the ensuing results from this study in the dace.

Teleost ovaries develop in the dorso-lateral lining of the peritoneal cavity, one to each side of the dorsal mesentery. The ovaries develop as hollow organs, and consist of many tissue septa which project into the lumen; it is on these that the oocytes develop. In species which show an annual reproductive cycle, one batch of eggs is produced each year, for several years. The annual ovarian cycle can broadly be divided into several stages of oocyte growth. Recognised by their

structural and functional differences, these are oogonial proliferation, oogenesis, folliculogenesis, cortical alveolar formation, vitellogenesis and final maturation proceeded by ovulation (Khoo, 1979; Kagawa *et al.*, 1981; Wallace and Selman, 1981). These stages of development are demonstrated more clearly in figure 3.1. It should be stressed however, that although this figure demonstrates that each phase of development sequentially replaces each other, this is not always the case, and often 2 or 3 phases may occur simultaneously in different oocytes (Wallace and Selman, 1981).

The first phases of the ovarian cycle, including oogonial proliferation, oogenesis and folliculogenesis constitute the primary growth phase (PGP). This phase of development is initiated when oogonia, distinguished by their small size and scant cytoplasm, undergo mitotic division, namely oogonial proliferation. It is unclear exactly when this period of ovary development occurs, though in the majority of teleosts with well defined annual cycles, oogonial proliferation and subsequent formation of new oocytes has been observed just before, during or immediately after the spawning period (e.g. Franchi *et al.*, 1962).

The transformation of oogonia into primary oocytes constitutes the first phase of meiosis; the chromosomes replicate and proceed through leptotene, zygotene and pachytene stages of prophase (see figure 3.1). From the completion of pachytene, until a few days before ovulation, the meiotic process is arrested at diplotene (Wallace and Selman, 1981). Growth during the meiotic prophase can further be divided into 3 sub-phases; chromatin nucleolus, early perinuclear and late perinuclear stages. At the start of the meiotic division, the oocytes at the chromatin nucleolus stage are very similar to the oogonia, that is they contain scant cytoplasm and a large centrally located nucleus. These oocytes are observed to go through the leptotene and zygotene stages of chromosome development (Tokarz, 1978). During the perinuclear stage, oocytes are easily identified by the large number of nucleoli close to the nuclear membrane (e.g. Khoo 1979). Oocyte become larger than the chromatin nuclolar stage mostly due to the growth of the cytoplasm and also due to the growth of the nucleus (Lambert, 1970; Wallace and Selman, 1981; Foreberg, 1982). In the early perinucleolar stage the cytoplasm loses its basophilic nature due to the presence of aggregates of ribonucleoprotein particles (Wallace and Selman, 1981). The aggregates become surrounded by less densely stained cytoplasmic material to form the complexes known as Balbiani bodies (Wallace and Selman, 1981). Their function is unclear. Towards the end of the early perinuclear stage, the Balbiani bodies have migrated

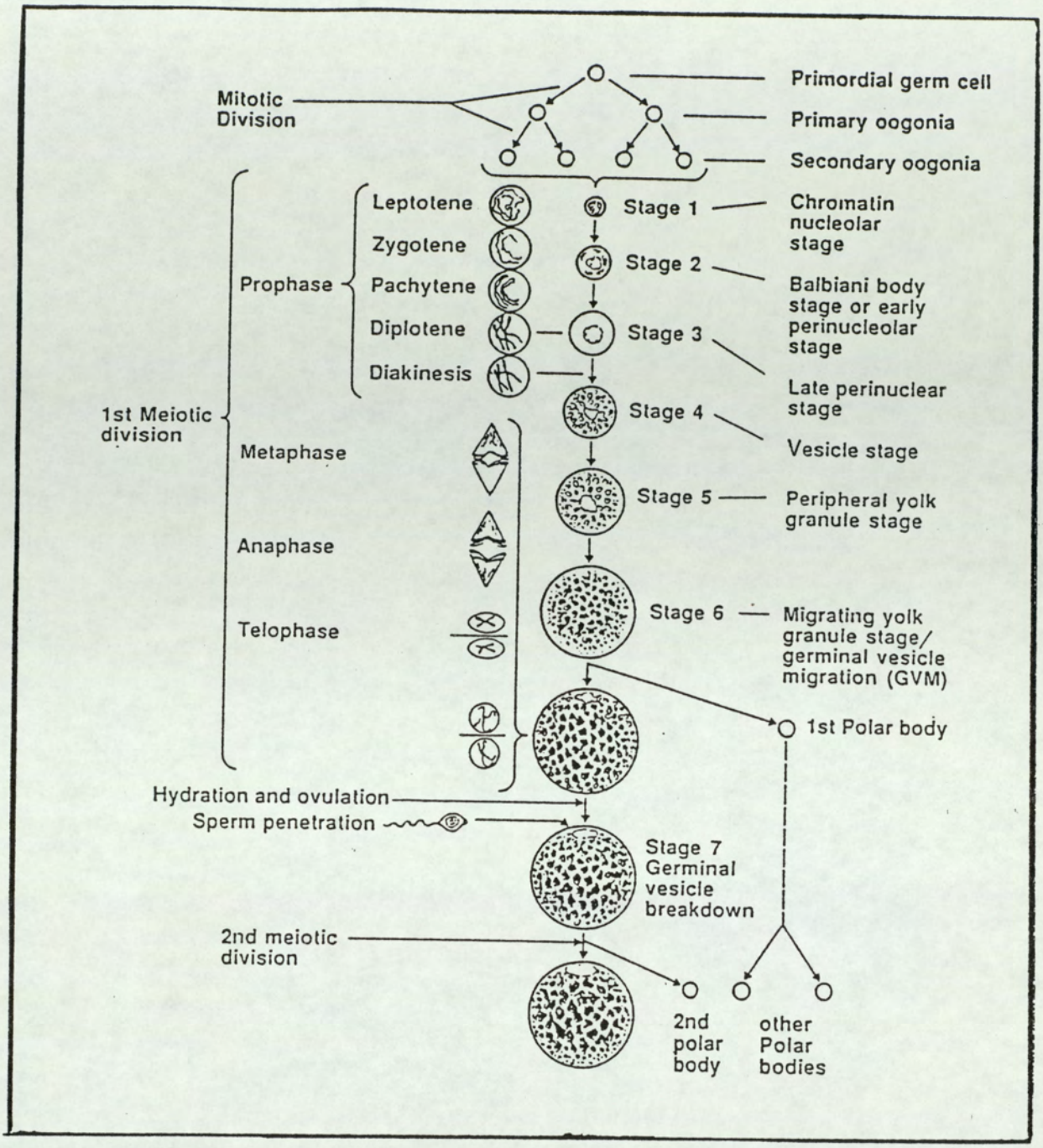


Figure 3.1 Ovarian developmental stages in the rainbow trout. (from Bromage and Cumarunatunga, 1988).

towards the periphery of the cytoplasm (e.g. Brusle, 1980; Wallace and Selman, 1981). By the late perinuclear stage, concomittant with an increased size of the oocyte, the Balbiani bodies dissolve and the cytoplasm becomes clear again (e.g Wallace and Selman, 1981; Bromage and Cumarunatunga , 1988). By the end of this growth phase, in most teleosts the oocyte has grown from a diameter of 10-20 µm at leptotene to a diameter ranging from 100-200µm (e.g. Wallace and Selman, 1981; Bromage and Cumarunatunga, 1988). Thus approximately a thousandfold increase in volume has taken place, primarily due to the elaboration of cytoplasmic organelles and the accumulation of cytoplasmic proteins. It is important to emphasize, however that ovaries containing oocytes only in primary growth phase stages are still relatively small and generally perceived as immature.

Coincident with the above changes within the oocyte, folliculogenesis also occurs; this results in the formation of a follicle outside the oocyte. The meiotic transformation of oogonia into oocytes is accompanied by the movement of primary oocytes away from the oogonial nests and their association with prefollicular cells, initiating folliculogenesis (Moser, 1967). The teleost follicle comprises an oocyte surrounded by cellular epithelial cells or the granulosa. External to the granulosa is a layer of connective tissue cells, the theca. By the end of the primary growth phase, the follicular layers have become separated from the oocyte by a non-cellular layer, the zona radiata (also known as zona pellucida, chorion and vitelline membrane) (e.g. Wallace and Selman, 1981; Dodd and Sumpter, 1982; Bromage and Cumarunatunga, 1988).

The granulosa is usually a single layer of cells (e.g. Guraya *et al.*, 1977). Initially the granulosa cells are in close contact with the oocyte, contact being maintained by interdigitating cytoplasmic processes (Anderson, 1967). With the development of the oocyte, intercellular spaces develop between the oocyte and the granulosa (Flugel, 1967) which may play an important role in vitellogenesis (Anderson, 1967). Electron microscopical studies have revealed a full complement of secretory granules indicative of protein synthesis (Kagawa *et al.*, 1981). In addition, some of the granulosa cells may help to produce the zona radiata (Wourms and Sheldon, 1977).

The theca consists of a layer of flattened fibroblast cells which first appear at the end of the perinucleolar stage of the primary growth phase (Forberg, 1982). The first layer of thecal cells is closely associated by a rich capillary plexus to the granulosa. The theca can further be divided into the theca interna and theca externa; both

these layers have a smooth muscle-like appearance similarly containing fibroblasts and collagen fibres which gives strength to the growing follicles. In addition recent cytochemical studies have indicated that some of the thecal cells (special thecal cells) by virtue of their high enzyme activity, are probably involved in steroid synthesis (Hurk and Peute, 1979; Kagawa *et al.*, 1981). In trout, these special thecal cells, belonging to the theca interna, are seen just before and after spawning (see Dodd and Sumpter, 1982). However special thecal cells have not been identified in all teleost species, and while they are found throughout the cycle in some fish (e.g mackerel), in others like char and trout, they are observed in mature and post-ovulatory follicles (Hurk and Peute, 1979; Kagawa *et al.*, 1981).

The zona radiata first appears at the end of the primary growth phase. It is secreted between the oocyte and the granulosa, and shows many variations in its development and structure in the growing oocyte of different teleost species. In oviparous teleosts, the zona radiata usually consists of 3 distinct layers, differing in structure and possibly origin, into which villi from both the oocyte and granulosa cells project (see Dodd and Sumpter, 1982). These layers have been shown to change markedly histochemically and in appearance during follicular development, though the functional significance of the changes is unknown.

The formation of the follicular envelope marks a transition in the manner whereby the oocyte reacts with its somatic environment. A cellular layer is now formed which can receive and transmit signals, most likely via hormones, and also regulate nutrient access. This marks the beginning of the secondary growth phase, whish includes cortical alveolar formation, vitellogenesis and final maturation. Depending upon a number of factors, including hormonal and environmental stimuli, a number of the larger primary growth phase oocytes undergo recruitment into the secondry growth phase.

During the cortical alveolar stage, a number of vesicles termed cortical alveoli, cortical vesicles, yolk vesicles or intravesicular yolk (see Selman *et al.*, 1986) appear in the cytoplasm, initially at the periphery of the oocyte. The structures contain an endogenously synthesized glycoprotein called intravesicular yolk (Korfsmeier, 1966). These vesicles gradually increase in size and number until they fill the oocyte cytoplasm (Wallace *et al.*, 1987). In subsequent stages, displaced by yolk granules, these vesicles migrate to the periphery of the cytoplasm and lie adjacent to the oolemma (Khoo, 1979; Wallace and Selman, 1981). Towards the end of vitellogenesis, as the oocyte enters final maturation, the cortical alveoli fuse with

the vitelline membrane, and at the time of fertilization, release their glycoprotein contents into the perivitelline space (Wallace and Selman, 1981; Wallace *et al.*, 1987). The significance of these cortical alveoli with their 'putative' yolk in ovarian development is still poorly understood and it is questionable whether the contents of these vesicles should be called 'yolk' because it probably bears no relationship to true yolk, which provides nutritive substances to the developing embryo.

A second event, that is initiated during the cortical alveolar stage is the formation of lipid inclusions or droplets consisting predominantly of phospholipid (Guraya, 1965), which occur in the cytoplasm in addition to vesicles and protein yolk granules. Lipid accumulation, usually oil droplets, occurs in some species well before the yolk vesicle stage (e.g. Beams and Kessel, 1973). In other species, these oil droplets have been observed to disappear prior to or during subsequent vitellogenesis (Guraya, 1965) but may persist in others (Shackley and King, 1977). Much more variability in this process occurs among fish species, than for previously mentioned events, it is therefore worthwhile questioning the importance of oil droplet accumulation in oocyte development.

Vitellogenesis comprises the major portion of oocyte growth. For most nonmammalian vertebrates, including teleosts, yolk proteins contribute more than 80-90% of the dry weight of the egg. During this growth phase, a large amount of an extraovarian, hepatically derived proteinaceous material, termed vitellogenin (VTG) is sequestered by the developing oocytes (Wallace, 1978; Tyler *et al.*, 1987a). Vitellogenin and egg yolk proteins have been characterized in some detail in a number of teleost species (Wiegand, 1982). Vitellogenin is a lipoglycophosphoprotein with a high molecular weight (300000-600000). It is selectively taken up by the oocytes by receptor mediated endocytosis (Wallace, 1985; Tyler *et al.*, 1988b). On uptake by the oocytes, it is enzymatically cleaved to form the yolk proteins lipovitellin (a lipid rich protein) and phosvitin (a phosphate rich protein)(Tyler *et al.*, 1988a). Histologically the onset of vitellogenesis is marked by the appearance of yolk granules in the periphery of the ooplasm, and as more VTG is sequestered, they increase in number and size until they completely fill the ooplasm (Bromage and Cumarunatunga , 1988). In the majority of teleosts, the yolk accumulates in fluid-filled spheres which migrate centripetally and fuse to form a continuous yolk mass (Wallace and Selman, 1981), conferring the characteristic transparency to the ovulated egg. As a consequence of yolk fusion, the yolk vesicles or cortical alveoli are

displaced towards the periphery of the oocyte.

After completion of yolk deposition, and with the appropriate hormonal stimulation, oocytes undergo enlargement by hydration to reach their full size (Wallace and Selman, 1978). The final egg size can vary considerably among teleosts, ranging from less than 0.33mm diameter for the sea perch *Cymatogaster aggregata* (Eigenmann, 1892) up to 30mm for the marine catfish, *Bagre marinus* (Gudger, 1918). However, the majority of British freshwater teleosts show egg sizes between 0.5 and 5mm in diameter. In a fully grown vitellogenic oocyte, the nucleus (usually referred to as the germinal vesicle) with its chromosomes still arrested at the diplotene stage of meiotic prophase, lies at the centre of the nucleus. During this process of maturation, the germinal vesicle migrates to the periphery at the animal pole or micropyle and breaks down (germinal vesicle breakdown (GVBD)); the chromosomes condense and proceed to first meiotic metaphase followed by the elimination of the first polar body; the remaining chromosomes enter second meiotic metaphase and meiosis is arrested once again (see fig. 3.1). In teleosts as in other vertebrates, once this second arrest occurs, the oocyte has become mature and fertilizeable, i.e, it is an egg. Ovulation generally follows oocyte maturation (usually accomplished within 24 hours of maturation)(see Wallace *et al.*, 1987). Ovulation is the process whereby mature eggs are released from their follicles into the body cavity; they are now ready for oviposition or expulsion into the external environment.

Oocytes during any stage of their development can undergo degeneration or atresia (Ryan, 1981) and this commonly occurs among all vertebrate groups (e.g. Byskov, 1978; Bromage and Cumarunatunga , 1988). The function of atresia and the factors involved in controlling its level are not well understood. The incidence of atresia is believed to be influenced by age, the stage of the reproductive cycle, nutrition and the individuals hormonal status (Bromage and Cumarunatunga , 1988). However, it is probable that atresia is an uncommon event in physiologically healthy females Tyler and Sumpter, 1989b). Where it does occur, particularly at the end of the breeding season, atresia may come into operation as a 'mopping-up' process, especially in those species which have relatively asynchronous ovaries or multiple batches of eggs (e.g Barr, 1963).

The reproductive phases of ovarian development detailed above are synchronized by internal and environmental rhythms. The significant energy costs associated with reproduction emphasize the importance of the physiological mechanisms that ensure these costs

are expended at the 'right' times. It is now well recognised that the reproductive cycle of teleost species is controlled by modifications in levels of hormones from the hypothalamo-pituitary gonadal axis timed by cues from the external environment (reviews: see Fontaine, 1976: Dodd and Sumpter, 1982; Scott, 1988). Studying hormonal changes throughout annual reproductive cycles helps to determine the most important factors mediating gonadal growth. Most of the data available on the hormonal control of reproduction have focused on pituitary and blood gonadotropin (GTH) or circulating sex steroids. In general, plasma GTH and sex steroid levels rise as the gonad matures and falls after ovulation (e.g. Wingfield and Grim, 1977; Billard *et al.*, 1978).

In teleosts, a wide variety of steroids have been identified in ovarian extracts, *in-vitro* incubates of ovaries and in the plasma of maturing females. Both oestrogens, notably 17β-oestradiol and oestrone and androgens, for example testosterone and androstenedione are major excretory products of teleost ovaries. The androgens produced by the ovary, are the obligatory precursors of 17β-oestradiol and oestrone respectively (see figure 3.2) (Scott, 1988). The conversion of the androgens to oestrogens is effected by an enzyme complex termed aromatase (Scott *et al.*, 1982). Ovarian aromatase is at its most active in females undergoing vitellogenesis (Scott *et al.*, 1982). Other explanations for the role of female androgens include stimulation (Scott *et al.*, 1980) or inhibition (Brommalaer *et al.*, 1981) of GTH secretion and stimulation of behavioural responses (Lambert *et al.*, 1986).

At the time of oocyte maturation, in all fishes studied so far, androgen and more so oestrogen production declines, and the major products of steroidogenesis appear to be the C21 steroids: the progestagens 17α-hydroxy 20β-dihydroprogesterone and 17α hydroxyprogesterone produced by salmonid and cyprinid ovaries (e.g. Jalabert, 1976), while the corticosteroids, 11-deoxycortisol and 11-deoxycortisone are produced by the ovaries of a number of marine teleosts (Colombo *et al.*, 1978) (see chapter 4). The formation of steroid glucuronides may also increase at this stage (Lambert, 1978; Lambert and Van den Hurk, 1982).

Ovarian steroidogenesis has been identified in the follicle layers, specifically the granulosa and thecal layers (Kagawa *et al.*, 1981,1982). Available data suggest that the theca layer synthesizes androgens, which are then transferred to the granulosa layer where they are aromatized to oestrogens (see Scott, 1988).

Most of the important phases in gonadal development are

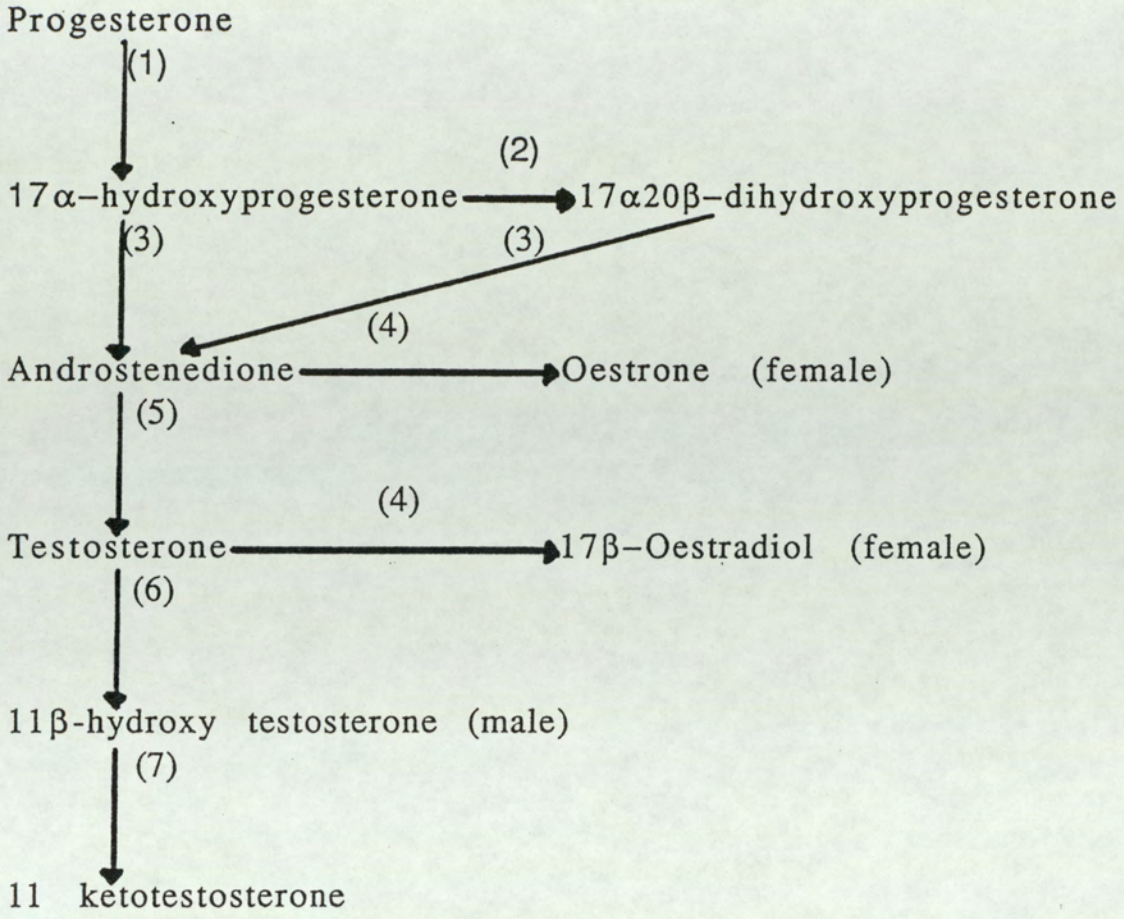


Figure 3.2 The major biosyntheyic pathways of gonadal produced sex steroids. The numbers in brackets represent the enzymes involved at each step; (1)- 17α hydroxylase, (2)- 20-β-hydroxysteroid dehydrogenase, (3)- C21-C19 desmolase, (4)-aromatase, (5)- 17β-hydroxysteroid dehydrogenase, (6)- 11b-hydroxylase, (7) 11β–hydroxysteroid dehydrogenase.

controlled by GTH(s) produced by the gonadotroph cells, lying in the adenohypophysis of the pituitary gland (e.g Campbell and Idler, 1976; Peute *et al.*, 1978). The release of GTH from the pituitary is in turn under the control of secretions from the hypothalamus. It has been established that the teleost brain contains a peptide similar in its structure and actions to mammalian luteinizing-hormone releasing hormone (LHRH) (see Peter, 1982) which is referred to as gonadotropin releasing hormone (GnRH). Brain lesion studies have been used in several teleosts to identify sites which may be involved in GnRH production. Because a GnRH RIA was not readily available, the effect of these lesions were measured indirectly, either by RIA for GTH or by determining changes in the gonadosomatic index (see section 2.2). The available data suggests that the nucleus lateralis tuberis (NLT) of the hypothalamus is the site of GnRH production (see Peter, 1982).

In addition to providing evidence for a GnRH in teleosts, similar brain lesioning studies have also shown that, at least in the goldfish, the brain produces a gonadotropin release-inhibitory factor (GRIF), located in the nucleus preopticus region (NPO) of the hypothalamus (Peter *et al.*, 1982; Ball, 1981). Subsequent work strongly indicates that dopamine may function as GRIF in goldfish (Peter, 1980,1982) and trout (Crim, 1982). Injections of pimozide, a dopamine antagonist followed by LHRH injections, 12 hours later induce virtually 100% ovulation and very high plasma GTH levels (Peter, 1980).

GTH has been shown to induce oestrogen production by the ovary which is in turn responsible for the stimulation of hepatic vitellogenin production (Crim and Idler, 1978; Fostier *et al.*, 1979). It also acts on the ovarian follicle to stimulate uptake of vitellogenin (Campbell, 1978; Upahayday *et al.*,1978; Abraham *et al.*, 1984) and subsequently to induce the synthesis of maturation stimulating hormones (see Nagahama,1987). Up until recently there has been considerable controversy with regards to the number and nature of GTH's in fish. One hypothesis maintains that a single GTH , rich in carbohydrayte is responsible for inducing both maturation and ovulation (Burzawa-Gerard, 1974) and the second hypothesis suggests that 2 distinct GTH'S exist, each with separate functions; one inducing vitellogenin uptake (carbohydrate poor) and the second (carbohydrate rich) inducing ovulation (Campbell and Idler, 1976; Ng *et al.*, 1980). The controversy that exists is largely due to different purification procedures and through the use of heterologous bioassay's. However now, it is generally accepted that there are 2 types of GTH. Itoh *et al* (1988) have recently purified 2 chemically

distinct types of GTH's from chum salmon, homologous to mammalian leutenizing hormone (LH) and follicle stimulating hormone (FSH),one which is elevated during vitellogenesis and the other prior to maturation (Dickhoff and Swanson, 1989). Using these GTH preparations work both *in-vitro* and *in-vivo* in the rainbow trout have clearly shown separate functions for these 2 GTH's : GTH I which stimulates ovarian growth and GTH II which is responsible for maturation (Tyler *et al.*, 1989c).

In summary therefore, GnRH and GRIF (dopamine) respectively, serve to promote or inhibit GTH release by direct innervation of the gonadotroph cells of the pituitary. GTH I in turn promotes ovary growth by stimulating steroidogenesis and GTH II final maturation and ovulation. It would appear therefore that the primary growth phase is probably GTH independant and the secondary growth phase, distinguished by the formation of several follicle layers and therefore capable of responding to hormonal stimuli, being GTH dependant.

During vitellogenic growth, follicle cells respond to circulating GTH I by producing oestrogens, mainly 17β-oestradiol, which promote the production of VTG in the liver and its subsequent release into the bloodstream and uptake by the developing oocytes (Wallace *et al.*, 1987). The elevated levels of VTG during this phase of development can be monitored in the plasma indirectly, by measuring calcium levels (see section 2.4.1). In addition to high levels of serum 17β-oestradiol during vitellogenesis, testosterone levels have also been shown to be elevated in many species (e.g Wingfield and Grim, 1977; Whitehead *et al.*, 1978). The most plausible explanation for the presence of androgens in the plasma is that they are synthesized by the ovary, released into the plasma and converted into oestrogens when they reach the brain and pituitary. Both of these organs have been shown to contain large amounts of aromatase (Callard, 1982).

Although oestrogens and aromatizable androgens are capable of inducing GTH synthesis and accumulation in the pituitary both by positive (Crim *et al.*, 1981) and negative (Billard, 1978) feedback mechanisms, they do not appear to promote its release. Indeed there is evidence that ovarian steroids inhibit GTH release during vitellogenesis, for example castration of female rainbow trout at this stage results in a dramatic increase in plasma GTH levels (Brommalaer *et al.*,1981).

Following the completion of vitellogenesis, oocytes are then maintained in the ovary for a variable (species specific) period, until a series of endocrine events stimulates their final maturation.

.Follicular maturation is ultimately under the control of GTH II in response to hypothalamic signals (e.g. Goetz, 1983; Nagahama, 1987a, Dickhoff and Swanson, 1989)). GTH is carried to the ovary where it stimulates the production of maturation inducing steroids by the follicular cells (Nagahama, 1987b; Goetz, 1983) (see chapter 4). These steroids trigger oocyte maturation; ovulation follows.

Ovulation which involves the active expulsion of oocytes from the follicles, is controlled by prostaglandins which appear to act directly on the smooth muscle fibres which are found in the theca layers of the follicles and the ovarian lamellae (see Scott, 1988). However different species would appear to respond to different prostaglandins (Goetz *et al.*, 1982).

Oviposition (the shedding of ovulated eggs) generally requires the presence of a male, a suitable spawning substrate (e.g vegetation for goldfish) and appropriate environmental conditions (e.g warm temperatures for goldfish and carp) (see Bye, 1984; chapter 4). It is usually accompanied by elaborate courtship behaviour, involving chemical, visual and tactile signalling. Studies of at least 19 oviparous species have shown that sexually mature females, which are ready to spawn, emit pheromones. These serve to attract males and stimulate their sexual activity (Liley, 1982). The significant event in the onset of pheromone production, appears to be the occurence of ovulation (Liley, 1982).

The ovarian and hormonal cycles detailed above are important for the following interpretation of events accompanying the natural reproductive cycle of the dace. In this study, seasonal changes in the ovary were assessed using both the increase in weight of the gonad, relative to body weight (GSI)(see section 2.2) and by using histological analyses (Section 2.6). Although GSI's provide good indications of the rate and state of gonadal development, they do not allow the stage of oocyte development to be defined. Histological data on the other hand allow the stages of oocyte development to be clearly defined. To understand the hormonal events accompanying ovarian development, serum cycles of testosterone, 17β–oestradiol and calcium (index of VTG) were also measured.

3.2 Materials and methods.

Dace of mixed sex were electrofished from the R. Ray, Reading (1986) and the R. Worfe, Wolverhampton (1987 and 1988) just prior to ovulation. The females were induced to ovulate using carp pituitary extract (see chapter 4) to ensure that all females were at the same stage of ovarian development, at the beginning of the new gonadal cycle. Both sexes were stripped and tagged (section 2.1), before being introduced into an outdoor earthern pond, subjected to natural photoperiod and temperature fluctuations. The approximate size of the pond was 144 square feet, with a water depth of 3 feet. Although commercial trout pellets (Mainstream B.P. Nutrition) were occasionally fed to the dace, the major part of their diet was provided by natural feed from the inflowing river.

Each month, throughout this study (3 years in total), 10-20 dace, depending on the numbers of fish caught, were blood sampled, and the resulting supernatant analysed for testosterone and 17β-oestradiol (see section 2.4.2) and vitellogenin (measured as calcium: section 2.4.1) levels. In addition during the first year of this study, 5 female fish were sacrificed at each sampling date and their ovaries retained for GSI determinations (section 2.2) and histological study (section 2.6).

3.3 Results

3.3.1 Ovarian development

a) Oogonia

At the start of a new gonadal cycle (March/April), the ovary consisited largely of oogonia. These cells were characterized by their small size (0.01mm), large nucleus, prominant nucleolus and a thin band of cytoplasm (plate 3.1). Although predominating in the early stages of development, oogonia were observed throughout the annual cycle.

b) Primary growth phase oocytes

i) chromatin nucleolar stage

Chromatin nucleolar stage oocytes were observed at the beginning of the primary growth phase. These oocytes were very similar in appearance to oogonia; i.e the nucleus contains contains a large nucleolus and is enveloped by a thin band of cytoplasm. However, these primary oocytes were slightly larger (0.06mm) than oogonia and surrounded by a layer of follicle cells (plate 3.1). Oocytes at this

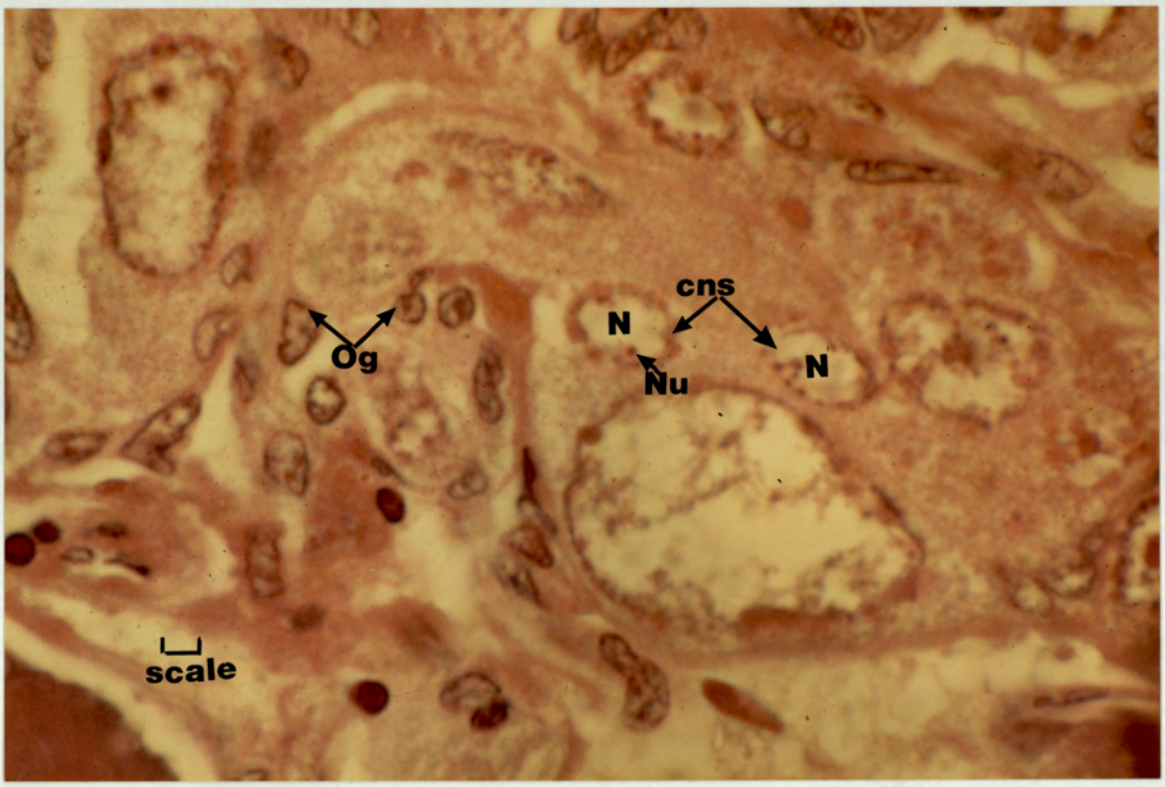


Plate 3.1. Section of an ovary showing oogonia (og) and chromatin nucleolar stage oocytes (cns). These oocytes were predominant during the primary growth phase. N = nucleus, Nu = nucleolus. Stained with Ehrlich's Haematoxylin and Eosin. Scale: 0.01mm.

stage of development were also present throughout the annual cycle.

ii) Early perinucleolar stage

In the course of oocyte development, the nuclei increased in size and multiple nucleoli appeared. The band of cytoplasm surrounding the nucleus also become enlarged. These oocytes, slightly larger (0.13mm) than the chromatin nucleolar stage oocytes were characterized by the presence of Balbiani bodies within the cytoplasm. Initially the Balbiani bodies were located close to the periphery of the cell (plate 3.2)(size 0.19mm).

iii) Late perinucleolar stage

Towards the end of the primary growth phase, the Balbiani bodies disappeared and the cytoplasm becomes clear again (plate 3.3). The oocytes at this stage measured 0.23-0.26mm in diameter. The follicle cells at this stage of development consisited of a single layer of granulosa cells surrounded by a layer of thecal cells. Oocytes in the primary growth phase were observed throughout the annual cycle, but predominated during early development, that is from the end of March (after the previous spawning period) until June.

c) Secondary growth phase oocytes

i) Cortical alveolar stage

These oocytes were readily identified by the presence of yolk vesicles, initially found at the periphery of the cell (plate 3.4) and later spread throughout the cytoplasm (plate 3.5). Follicle diameter at this stage of development ranged from 0.3-0.5mm. The follicle layers at this stage consisted of a narrow zona radiata immediately surrounding the oocyte, granulosa, thecal and epithelial layer. Oocytes in the cortical alveolar stage first appeared in the ovary in April, were most common in May, but became less frequent as the cycle progressed. No cortical alveolar stage follicles were observed from December onwards.

ii) Vitellogenic oocytes

These oocytes were readily identified by the presence of yolk granules (early stages)(plate 3.6) or a central yolk mass (later stages)(plate 3.7). During the later stages of vitellogenesis, the yolk vesicles were gradually forced towards the periphery of the oocyte by the increasing central yolk mass. The follicular layers at this stage had increased in size to take up a larger proportion of the total follicle, particularly the zona radiata. Irregularity between granulosa

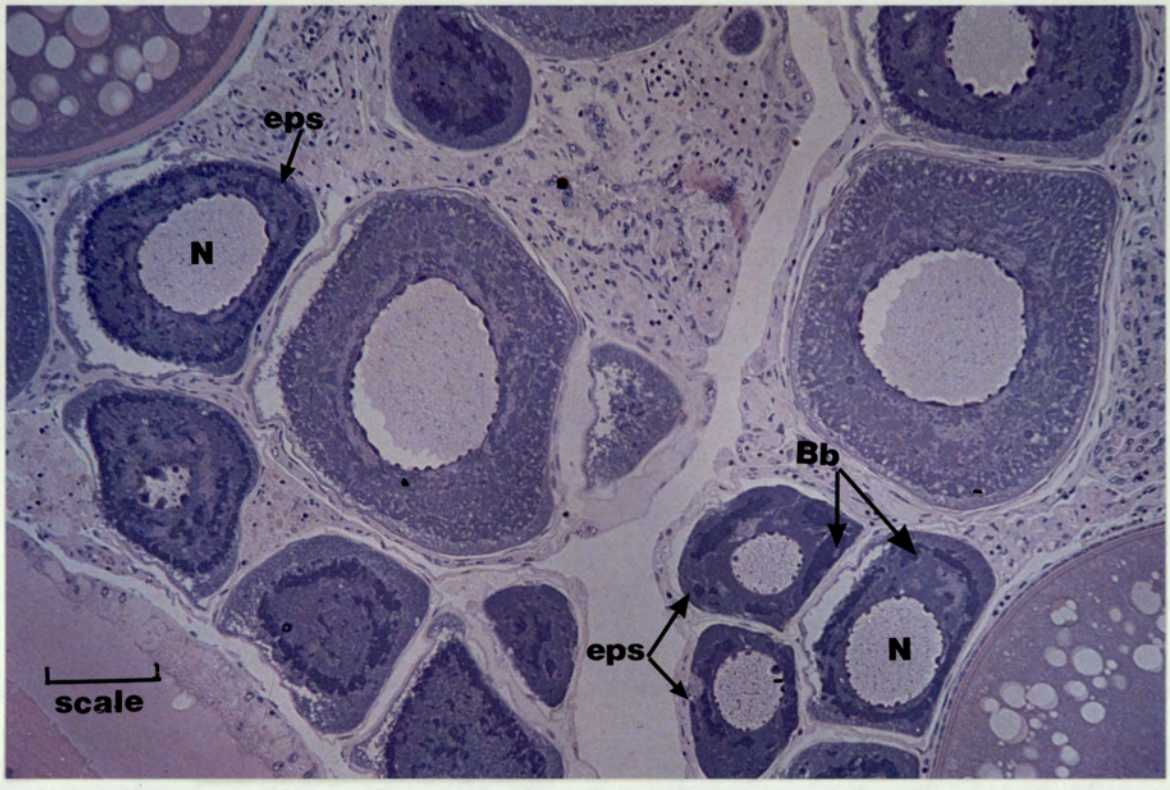


Plate 3.2. Section of an ovary showing early perinuclear stage (eps) oocytes. These are characterized by the presence of Balbiani bodies (bb) within the cytoplasm. N = nucleus. Stained with Ehrlich's Haematoxylin and Eosin. Scale: 0.1mm.

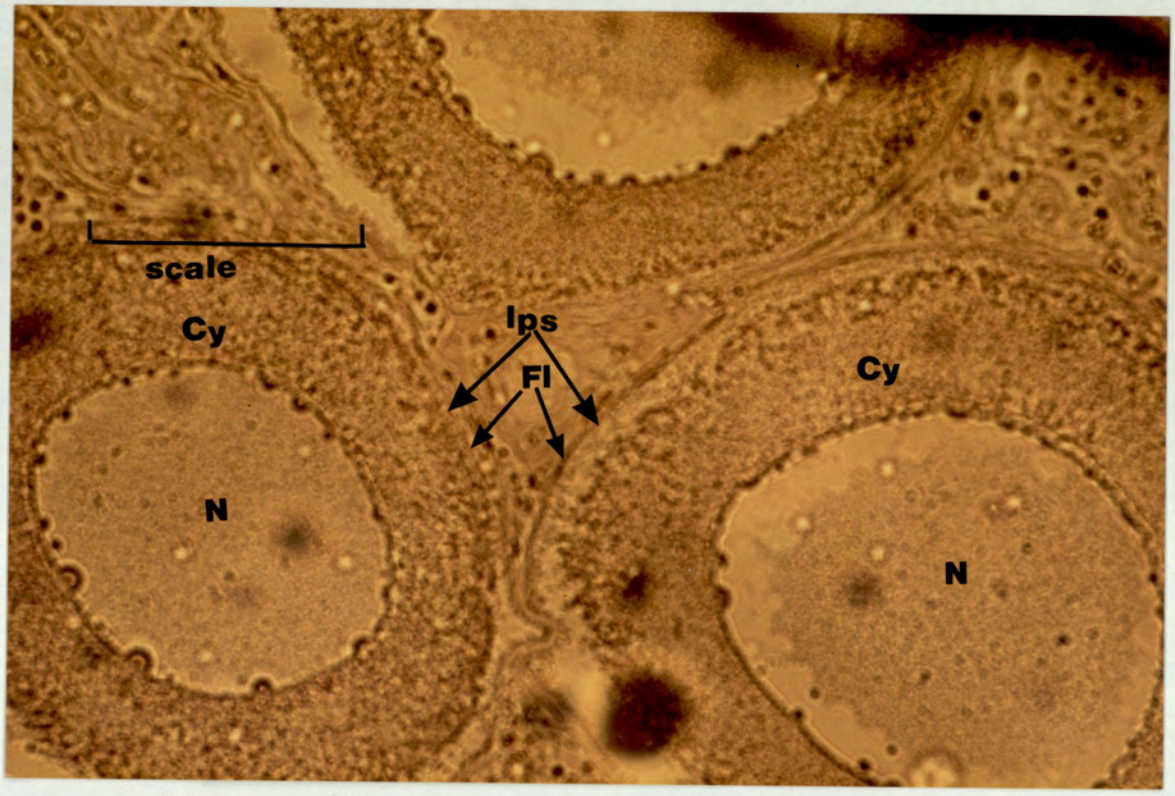
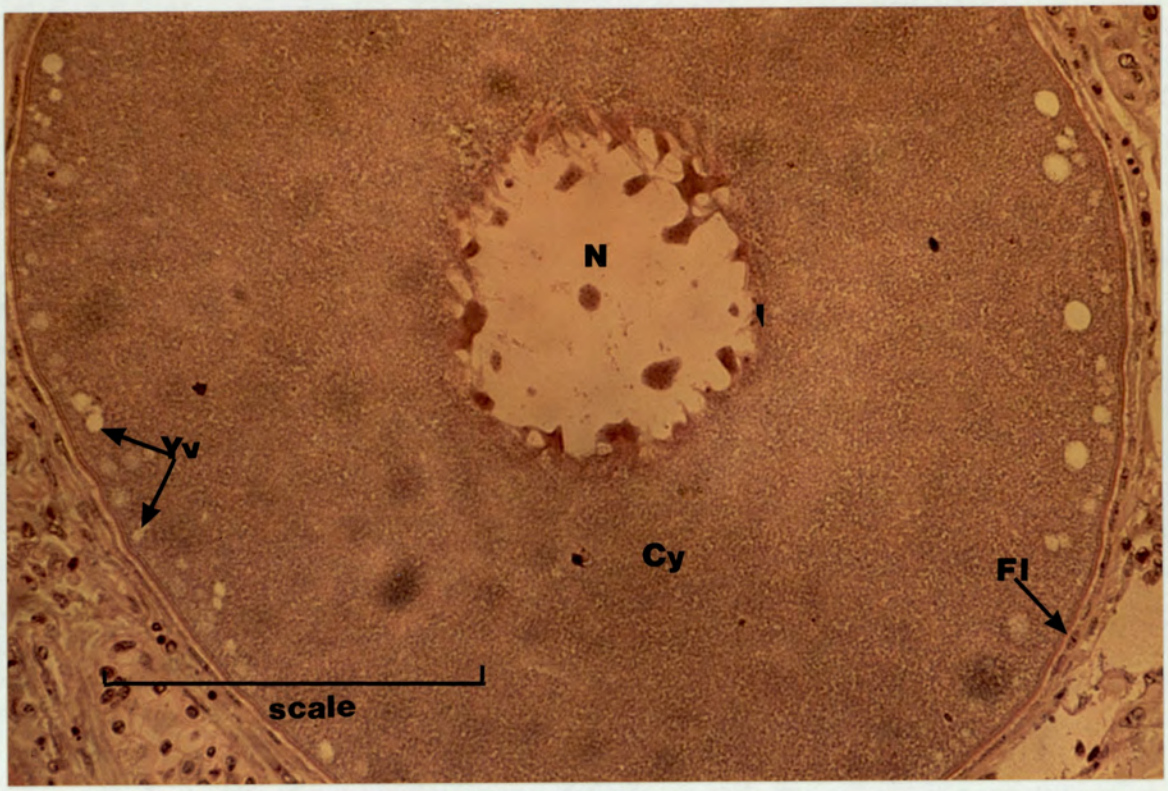


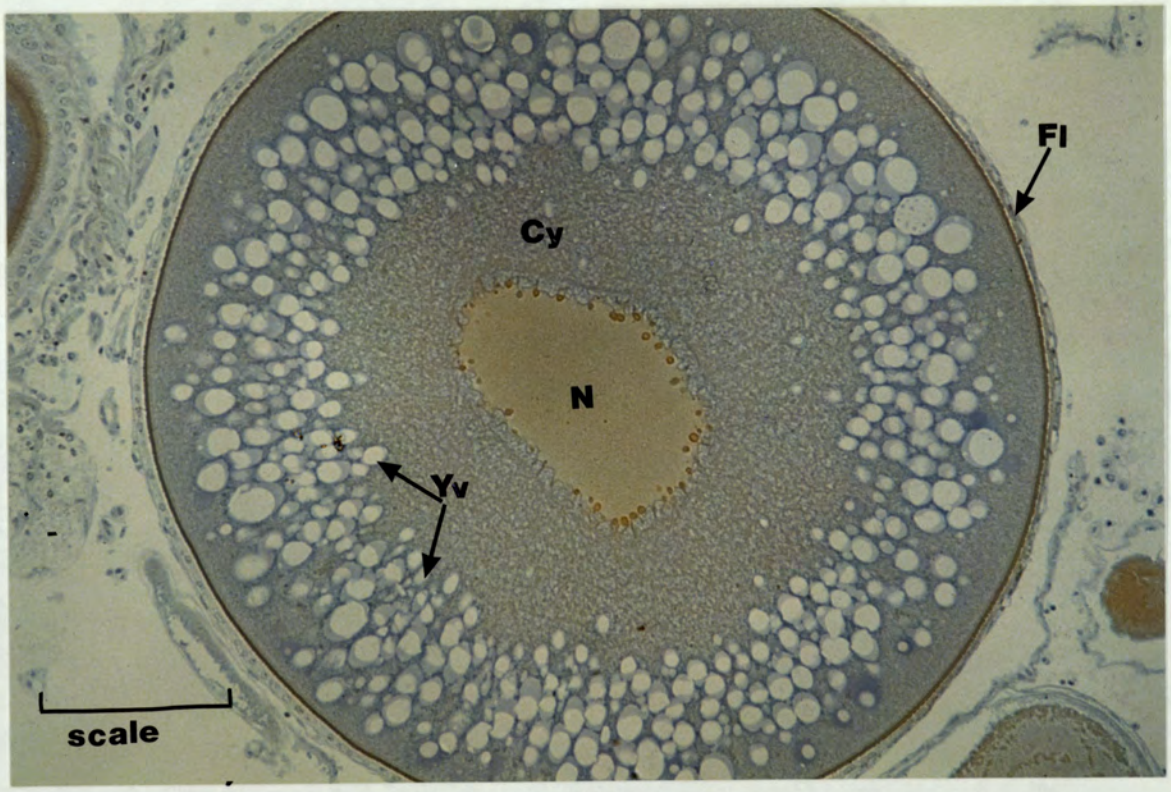
Plate 3.3. Section of an ovary showing late perinuclear stage oocytes (lps). At this stage of development, the Balbiani bodies have dissapeared and the cytoplasm has become clear again. N = nucleus, Cy = cytoplasm, Fl = follicle layers. Stained with Ehrlich's Haematoxylin and Eosin. Scale: 0.1mm.

Plates 3.4 and 3.5. Sections of oocytes showing the cortical alveolar stage of the secondary growth phase.

Plate 3.4. Section of an oocyte at the beginning of the cortical alveolus stage. This was characterized by the presence of yolk vesicles at the periphery of the cell. N = nucleus, Yv = yolk vesicle, Cy = cytoplasm, Fl = follicle layers. Stained with Ehrlich's Haematoxylin and Eosin. Scale: 0.1mm.

Plate 3.5. Section of an oocyte showing the cortical alveolar stage at a later stage of development. Oocytes at this stage are characterized by the presence of yolk vesicles throughout the cytoplasm. N= nucleus, Yv = yolk vesicle, Cy = cytoplasm, Fl = follicle layers. Stained with Mallory's Triple stain. Scale: 0.1mm.

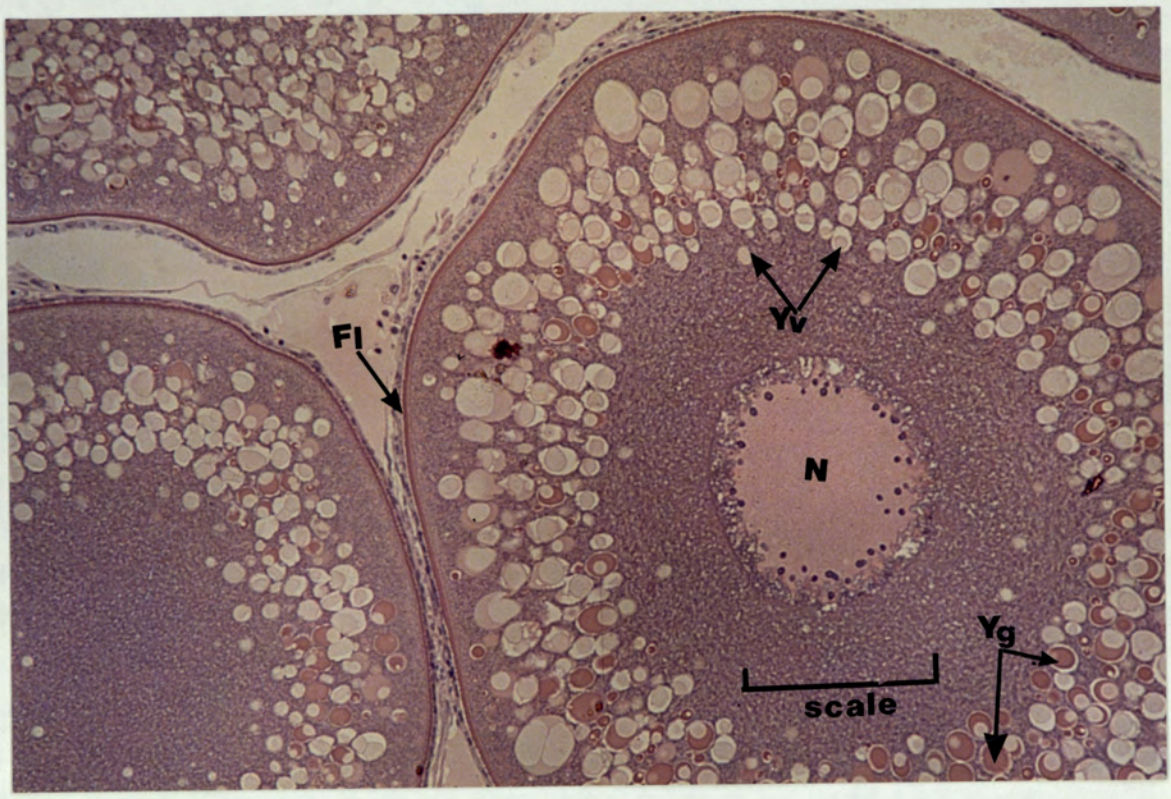

N
Yv
Cy
Fl
scale

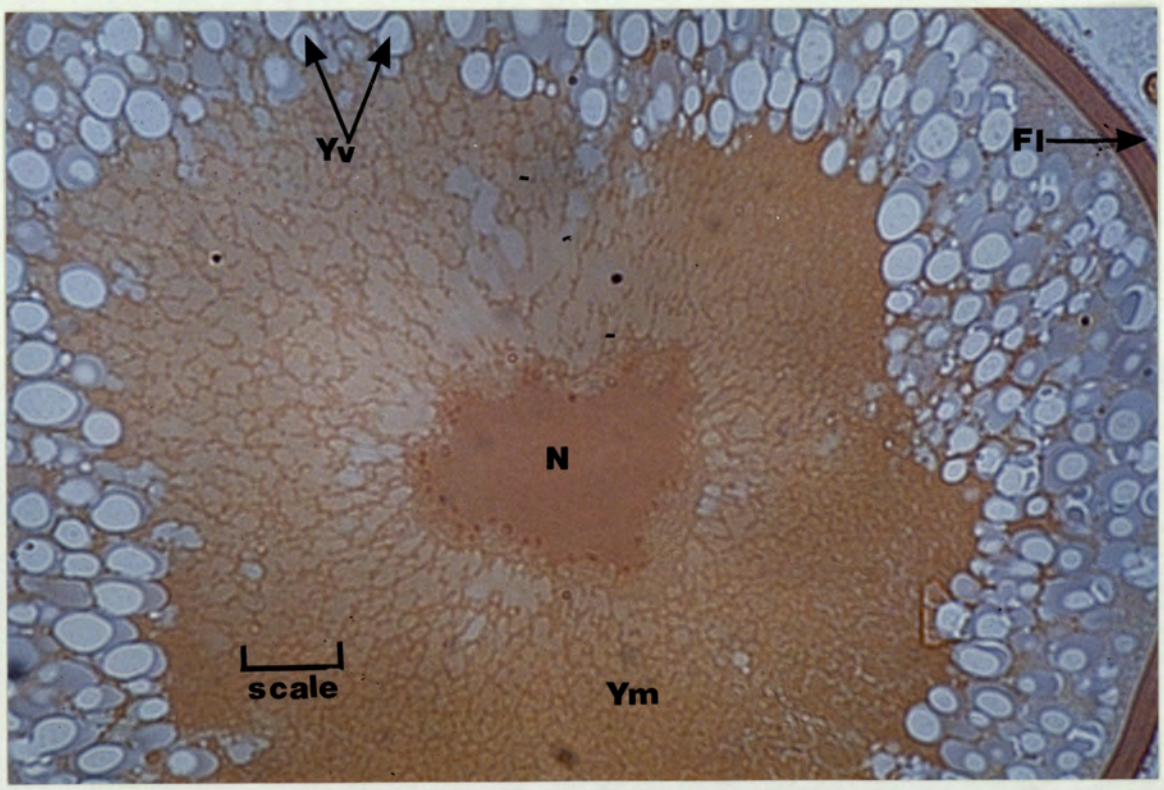

Fl
Cy
N
Yv
scale

Plates 3.6 and 3.7. Sections of oocytes showing the vitellogenic stage of development.

Plate 3.6. Section of an oocyte during early vitellogenesis. These were readily identified by the presence of yolk granules towards the periphery of the cell. N = nucleus, Yv = yolk vesicle, Yg = yolk granule, Cy = cytoplasm, Fl = follicle layers. Stained with Ehrlich's Haematoxylin and Eosin. Scale: 0.1mm.

Plate 3.7. Section of an oocyte during late vitellogenesis. At this time there is a central yolk mass. N = nucleus, Yv = yolk vesicle, Ym = yolk mass. Fl = follicle layers. Stained with Mallory's Triple stain. Scale: 0.1mm.


Fl
Yv
N
Yg
scale


Yv
Fl
N
scale
Ym

cells was caused by the presence of nodular protrusions extending from the zona radiata. These nodular extensions of the zona radiata, and the rest of the follicle layers are clearly represented in plates 3.8 and 3.9).

Vitellogenic follicles ranging from 0.4-1.4mm in size were present from June through to spawning the following March, that is throughout the annual ovarian cycle.

iii) Migrating nuclear stage

This stage of oocyte development was characterized by the migration of the nucleus (germinal vesicle) from a central position in the oocyte to the periphery of the cell, towards the micropyle (plate 3.10 and 3.11). Follicle diameter during this stage ranged from 1.4-1.7mm. Oocytes only underwent this stage of development in ovaries that were close to spawning (within a few days). During this stage, the micropyle was clearly visible.

iv) Germinal vesicle breakdown (GVBD) stage

This stage closely followed the stage of nuclear migration. On migration towards the periphery of the oocyte, the nuclear membrane broke down releasing the nuclear contents into the cytoplasm. This stage of follicle development was observed only hours prior to ovulation. Ovulated eggs ranged from 1.7-1.85mm. They still had the nodular extensions of the zona radiata, suggesting that they might be involved in the attachment of the egg to the substrate.

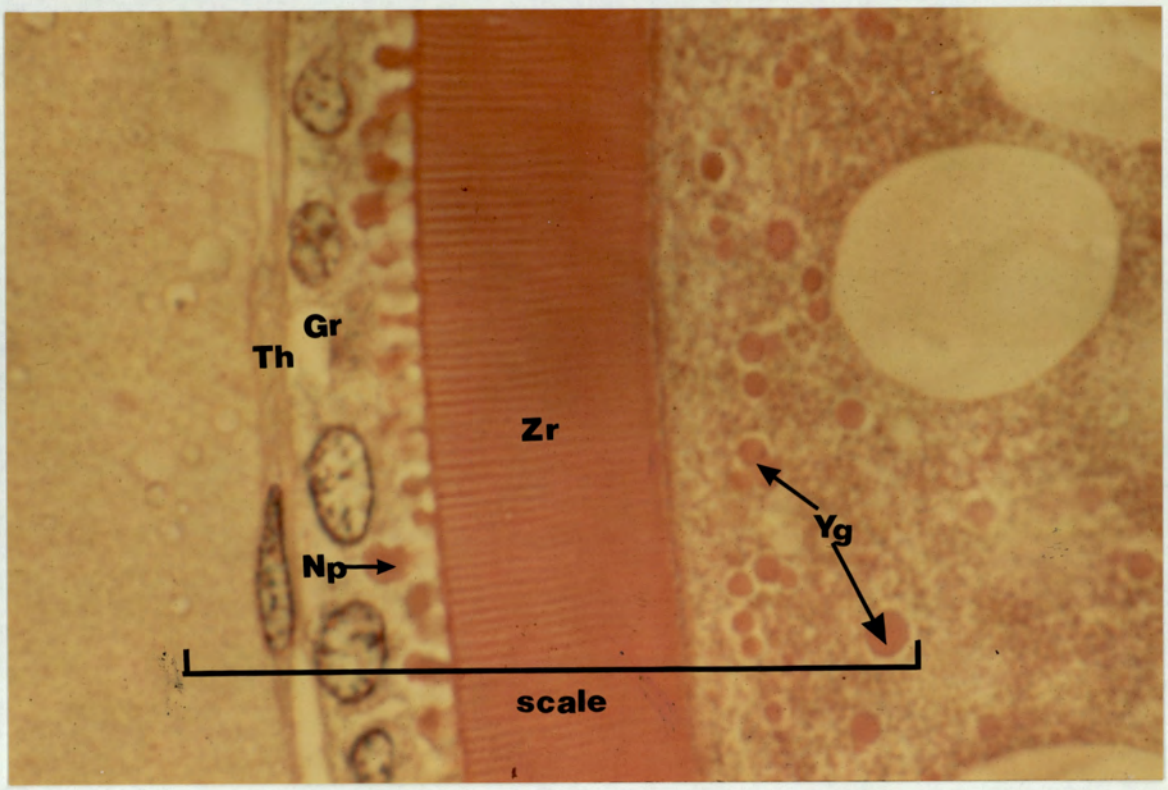
3.3.2. Seasonal cycle of ovarian growth, measured by the GSI

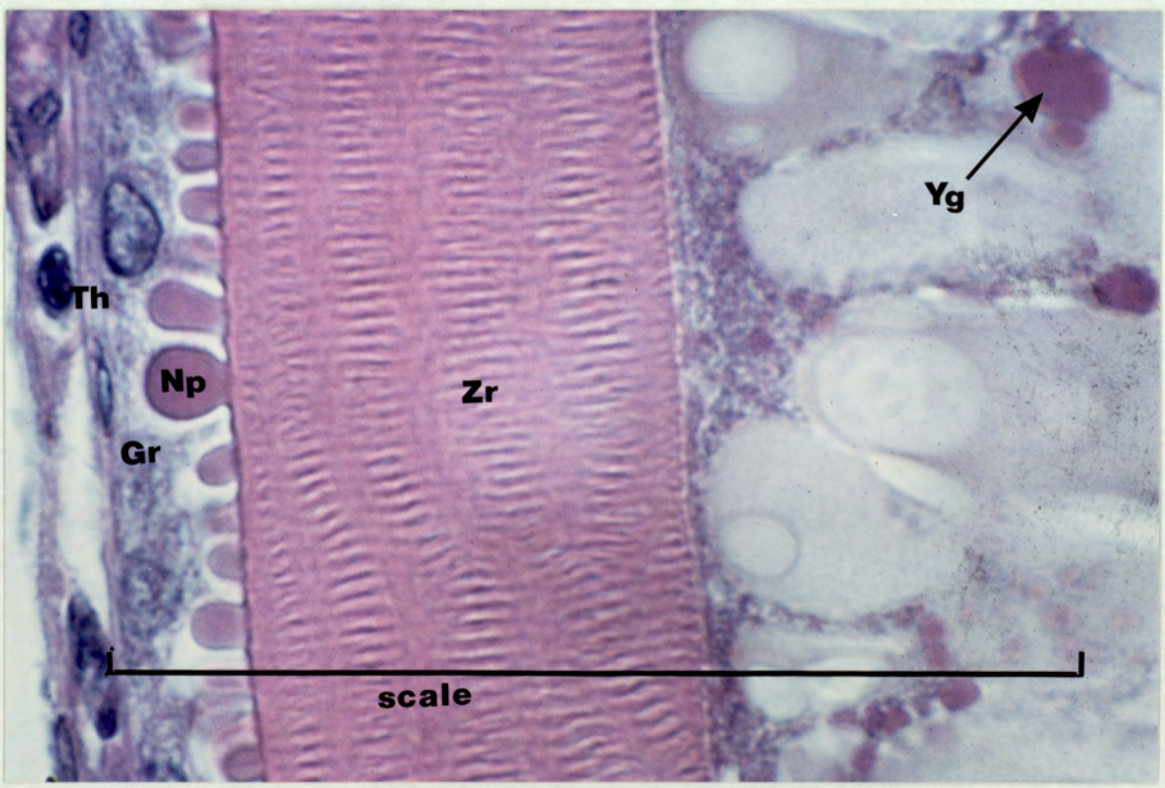
Figure 3.3 shows the increase in the gonadosomatic index (GSI) of the dace as the ovaries progress through the annual reproductive cycle. This figure demonstrates that after spawning, the ovary recovers almost immediately. An increase in GSI could be detected as early as May. Subsequently the GSI increased rapidly until October. During the winter months, the GSI still increased (although it would appear at a reduced rate). From December until spawning (mid-March) the GSI increased rapidly to reach a maximum of 20% prior to spawning.

3.3.3. Annual cycles of serum testosterone (T), 17β-oestradiol (E2) and calcium (Ca)

Figure 3.4 illustrates the seasonal pattern of T, E2 and Ca in the blood. Initially (at the start of a new gonadal cycle, in April), an increase in serum T and E2 was observed to 2-3ng.ml^{-1} and 4-

Plates 3.8 and 3.9. Sections of oocytes showing the follicle layers during late vitellogenesis. Zr = zona radiata, Gr = granulosa, Th = theca, Np = nodular protrusions, Yg = yolk granule. Stained with Ehrlich's Haematoxylin and Eosin. Scale: 0.1mm

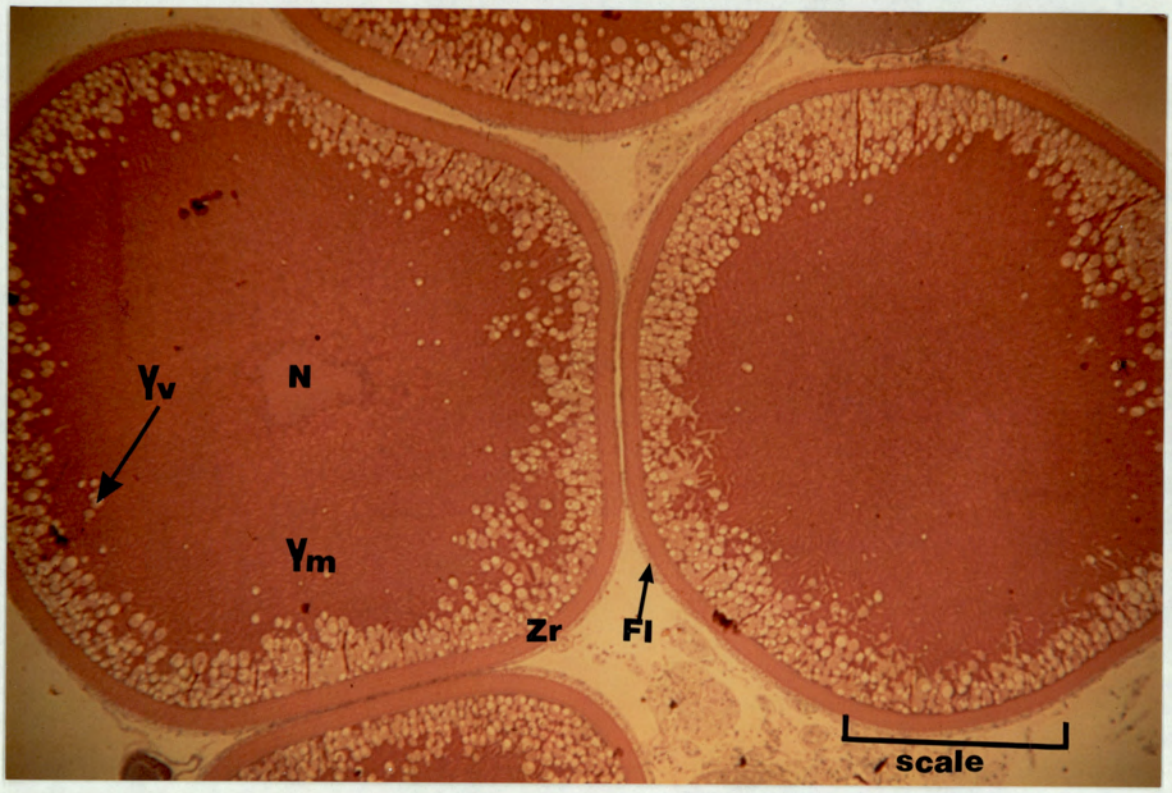

Gr
Th
Zr
Yg
Np
scale

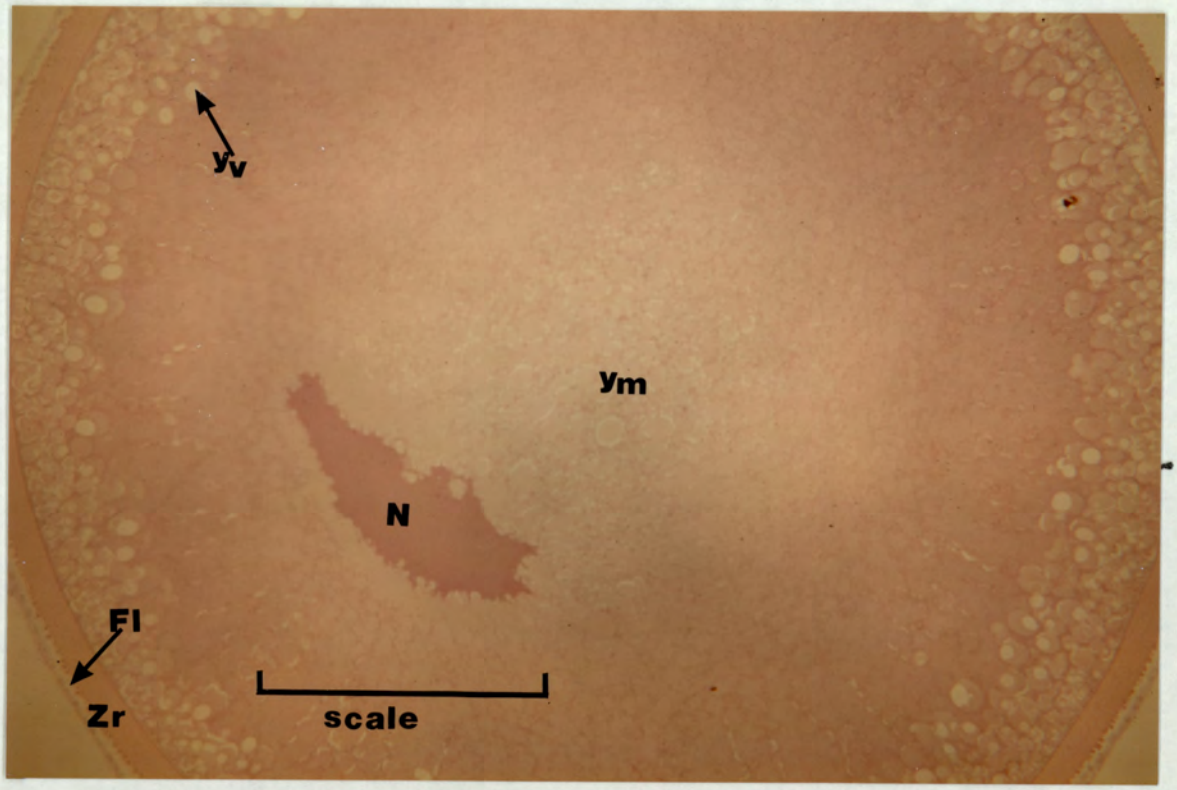

Yg
Th
Np
Zr
Gr
scale

Plates 3.10 and 3.11. Section of oocytes showing the migrating nuclear stage.

Plate 3.10. Section of an oocyte showing the nucleus (germinal vesicle) just starting to migrate. N = nucleus, Yv = yolk vesicles, Ym = yolk mass, Fl = follicle layers, Zr = zona radiata. Stained with Ehrlich's Haematoxylin and Eosin. Scale: 0.5mm.

Plate 3.11. Section of an oocyte showing the nucleus (germinal vesicle) almost at the periphery of the cell. N = nucleus, Yv = yolk vesicle, Ym = yolk mass, Fl = follicle layers, Zr = zona radiata, Stained with Ehrlich's Haematoxylin and Eosin. Scale 0.5mm.


Yv
N
Ym
Zr
Fl
scale


yv
ym
N
Fl
Zr
scale

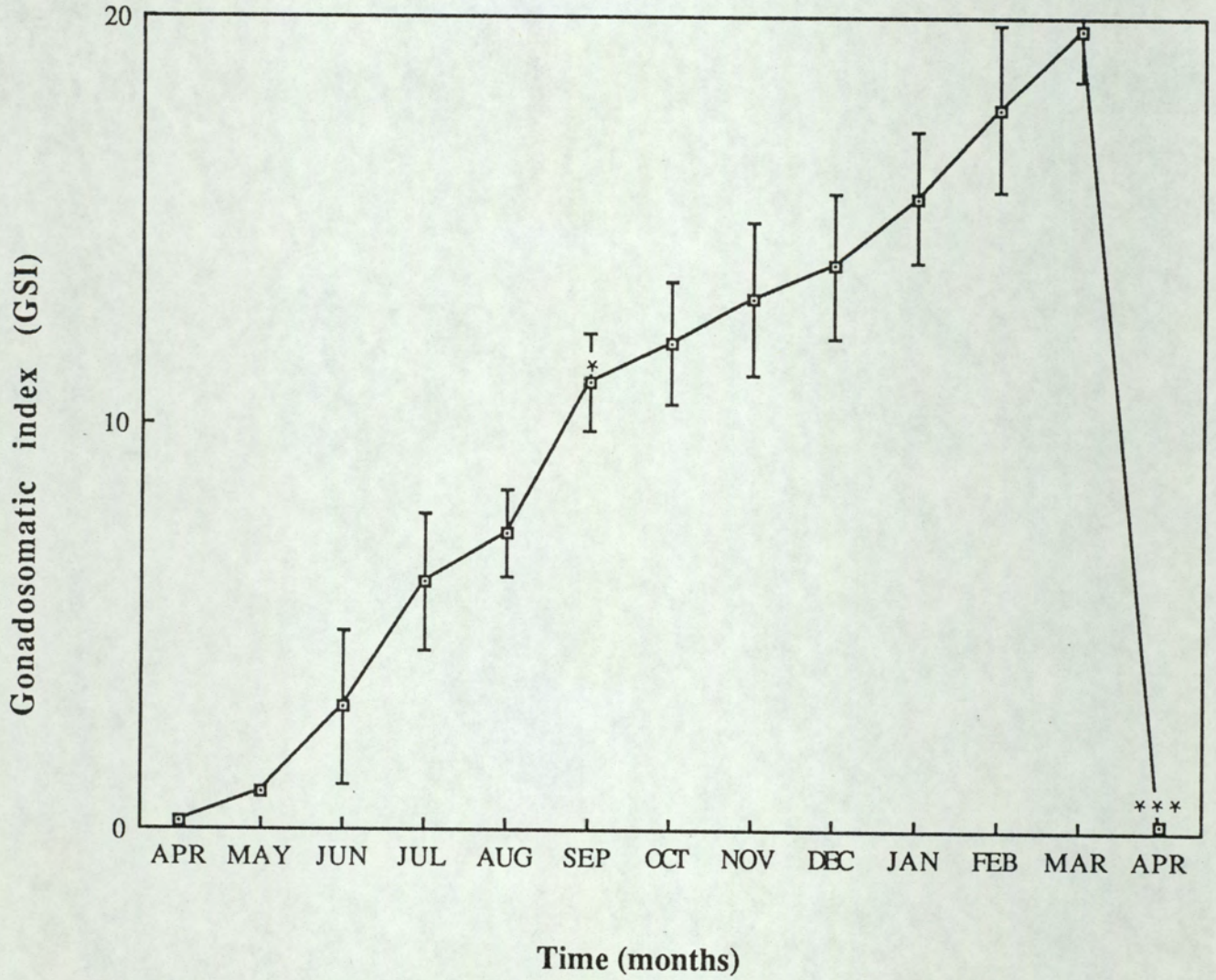


Figure 3.3. The annual cycle of ovary growth measured as the gonadosomatic index (GSI)(ovary weight/body weight x 100). Vertical bars denote standard deviation.

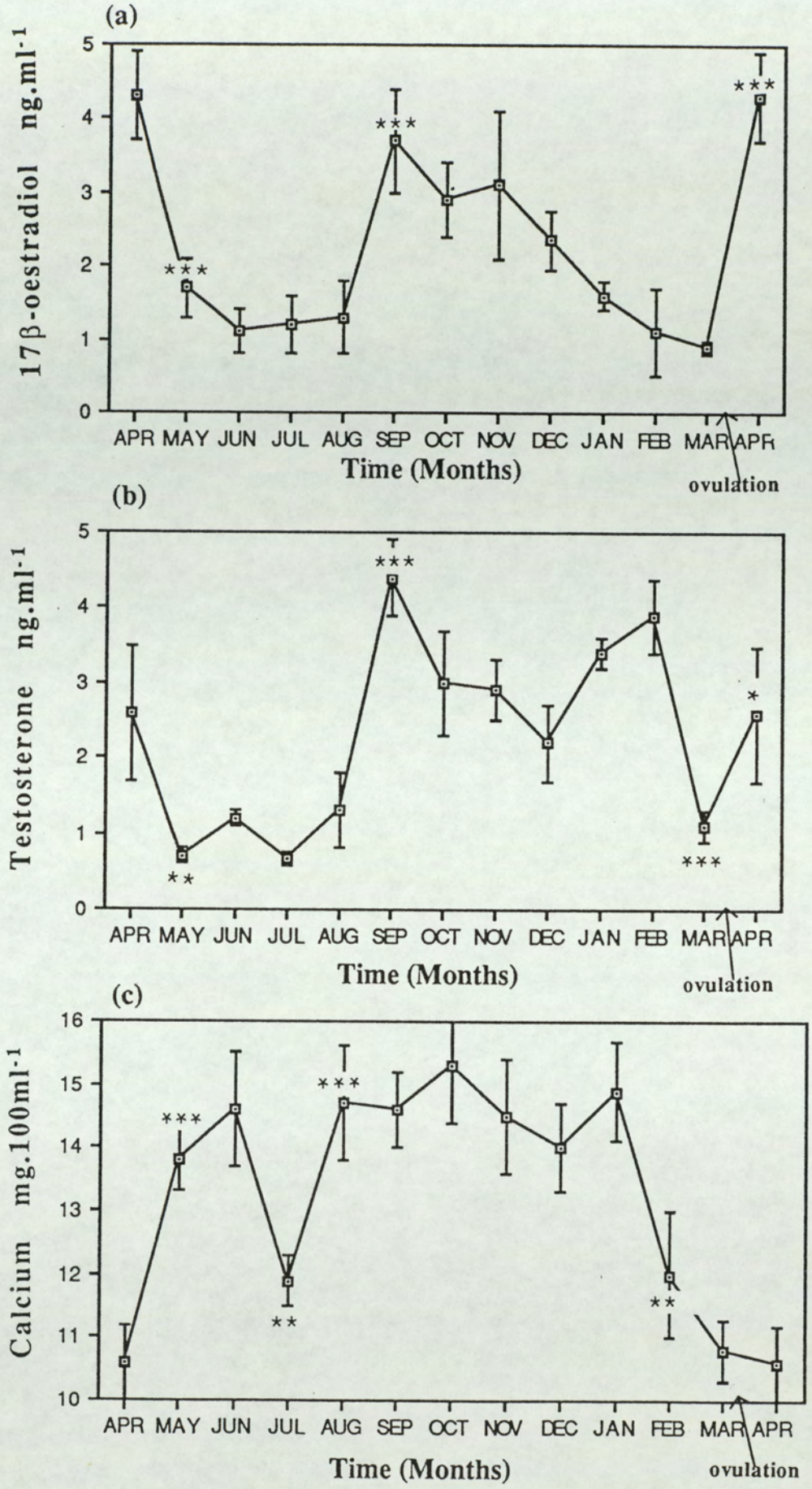


Figure 3.4. Serum cycles of (a)17β-oestradiol, (b) testosterone and (c) calcium measured throughout the annual reproductive cycle. Vertical bars represent standard deviation.

5ng.ml^{-1} respectively. Following the rise in steroid production in April, serum Ca levels started to rise in May (approx. 14mg.100ml^{-1}), just prior to the recruitment of the first vitellogenic follicles in the ovary in June. Serum E2 and T levels declined after the initial rise in April. Ca levels, however, remained high throughout the summer and winter months, though there was a significant ($P<0.01$) drop in July (from 14.5 to 12mg.100ml^{-1}). Ca levels subsequently declined in January and remained low through to ovulation.

A second peak of serum T (4-5ng.ml^{-1}) and E2 (3-4ng.ml^{-1}) was observed in September. These levels then declined slowly during the winter months. Whereas serum E2 levels had returned to basal (approx. 1ng.ml^{-1}) by February, serum T increased in January and remained high (4-4.5ng.ml^{-1}) until ovulation.

3.4 Discussion

Histological examination of dace ovaries revealed a similar pattern of ovarian development to other teleosts which produce yolky eggs, for example the rainbow trout (see figure 3.1; Bromage and Cumarunatunga, 1988).

At the start of the annual reproductive cycle, immediately after the previous spawning period, the ovary contained large numbers of oogonia and primary growth phase oocytes (that is chromatin nucleolar, early perinuclear and late perinuclear stages). Although not the predominant stages of ovarian development in later histological sections, primary growth phase oocytes could be observed throughout the annual reproductive cycle. However, it would appear that their recruitment into secondary growth phase oocytes, initially cortical alveolus stages, occurs between the end of the previous spawning (in March) and August. This was confirmed by the presence of cortical alveolus stage oocytes throughout this period. At any time between March and August, a mixture of primary growth phase, cortical alveolus and vitellogenic (different sized) oocytes could be observed within a single ovary (although they were found in different proportions at different times). That is, although this chain of events sequentially replace each other in a single oocyte, within an ovary, oocyte development is asynchronous. Asynchronous oocyte development during early development of the annual ovarian cycle has also been observed in the rainbow trout; in this species vitellogenic oocytes ranging in size from between 1-2.7mm could be observed within a single ovary (Tyler, pers. comm.). These observations in the dace and the rainbow trout raise important questions as to how the hormonal control of oocyte development is brought about. For species which have oocytes at different stages of development, one must conclude that either sequential hormonal events do not occur, or each oocyte has an influence on the manner by which the overlying follicle cells respond to hormonal stimuli, notably gonadotropins. Thus to a certain extent each oocyte must be considered as a seperate entity, each controlling its own growth rate in response to hormonal stimuli. Further evidence in support of oocytes controlling their own growth rate has largely come from work on amphibian oocytes (Wallace and Misulovin, 1978). In amphibian oocytes, vitellogenesis and the development of stage specific events can occur *in-vitro* in the absence of external hormones, provided the appropriate nutrients are present (Wallace and Misulovin, 1978). Indeed even ' full grown' oocytes can resume

growth when placed in culture conditions (Wallace *et al.*, 1981). Similar work on cultured rainbow trout follicles also support the contention that ovarian follicles can grow in the absence of hormonal stimuli (Tyler *et al.*, 1987a).

It is well documented that recruitment of oocytes in the primary growth phase into cortical alveolar and vitellogenic stage is under the control of gonadotropin (e.g. Campbell and Idler, 1976; Ng and Idler, 1983). This in turn promotes the production of sex steroids, notably oestrogens, which contribute further to the production of vitellogenin and in turn the growth of the oocyte (e.g. de Vlamming *et al.*, 1977; Elliott *et al.*, 1979). Therefore in the dace, it is likely that the rise in serum 17β-oestradiol produced by the follicle cells (presumably in response to GTH) in April, was the stimulus for vitellogenin production. The production of vitellogenin in response to high levels of 17β-oestradiol (4-5ng.ml^{-1}) in this study was supported by an increase in serum vitellogenin levels (measured as calcium; up to 14.5mg.100ml^{-1}) one month later, in May. This in turn was followed by the appearance of the first yolk granules in the ovary in June. In addition to a rise in serum 17β-oestradiol levels prior to the presence of yolk granules within the ovary, a rise in serum T levels (2-3.5ng.ml^{-1}) was also observed. A similar rise in serum levels of E2 and T prior to yolk granule appearance in the majority of developing oocytes has also been observed in the carp, *Cyprinus carpio* (Yaron and Levavi-Zermonsky,1986). Although a role for E2 in vitellogenesis is well documented, that is the stimulation of vitellogenin production, a function of T in female fish is unknown. As mentioned in the introduction to this chapter, T is usually described as a 'male' hormone. However, in many teleosts, large amounts of testosterone have been measured in females, and usually at higher levels (up to 150ng.ml^{-1}) than in the corresponding males (for example in the winter flounder: Campbell *et al.*, 1976; and the rainbow trout: Whtiehead *et al.*, 1978). Though it seems likely that these elevated androgen levels during the reproductive cycle in female fish, are involved in the control of reproduction, at present their function(s) is/are unknown. A likely role for these androgens however is the immediate precursors for the production of oestrogens.

Although serum E2 and T levels had declined by May, histological evidence (demonstrated by an increase in oocyte size) and high serum Ca levels (as an index of vitellogenin levels) during the ensuing summer months (June-August) confirmed that the production and uptake of vitellogenin into the developing oocytes was continuing to occur, even though serum E2 levels had returned

to basal (1-1.5ng.ml^{-1}). Indeed, in the rainbow trout, once initiated by E2, vitellogenin production and its subsequent release into the bloodstream has been shown to continue for upto 5 months, even though blood E2 levels had returned to basal within a few days of its production (Elliott *et al.*, 1979). Although there was a significant ($p<0.01$) drop in serum calcium levels in the dace in July (down to approx. 12mg.100ml^{-1}), these levels are still reasonably high indicating that vitellogenin was still present in the bloodstream. However this short-term decline (July only) in calcium levels suggests that perhaps the sequestration of vitellogenin from the blood by the developing oocytes was occurring faster than its production in the liver and subsequent release into the bloodstream. A faster rate of oocyte growth during this period is suggested by the gonadosomatic index (GSI)(see figure 3.3).

Growth of the ovary as demonstrated histologically (by an increase in oocyte size) and the GSI (see figure 3.3) continued throughout the winter months, albeit at a slower rate than that in the summer months. Further evidence that vitellogenesis remained active during the winter months was provided by serum peaks of E2 (3-4 ng.ml^{-1}) and T (4-5ng.ml^{-1}) in September. Possibly this boost in E2 levels, was required to maintain vitellogenin production throughout the winter months. Unlike the dace, in the carp, *Cyprinus carpio*, oocyte growth is halted at the beginning of winter due to cold water temperatures and does not resume until water temperatures are considerably higher (e.g. Billard *et al.*, 1978). Although it is likely that vitellogenin production and its subsequent uptake into the ovary is much reduced at cold temperatures, Tyler *et al.* (1987a) have demonstrated that rainbow trout oocytes cultured *in-vitro* sequester vitellogenin at temperatures of 5°C and below, although at a slower rate than higher temperatures. This provides supporting evidence that vitellogenin uptake can occur in dace ovaries during the winter, albeit at reduced rates. For as in the trout, this species is adapted to fast-flowing streams which are generally subjected to cold temperatures.

A further increase in the rate of ovary growth (as indicated by the GSI; see figure 3.3) was observed from the end of December until the time of spawning. However, an important feature concerning ovary growth during this period, is that unlike the earlier development stages when there was a considerable 'asynchrony' in oocyte size, by December/January, individual oocytes had 'caught-up' in size with each other. This suggests that individual oocytes may be able to communicate with each other; that is some factor(s) maybe

released by the cells which could either stimulate faster growth rates in the smaller oocytes or inhibit growth in larger oocytes. This would enable smaller oocytes to 'catch-up' in size with larger ones. Although growth factors have not been identified in lower vertebrates, they have been characterized in mammals and known to play an important role in cell to cell communication (Sporn and Roberts, 1988).

During the period of ovary growth observed between December and spawning, calcium levels declined suggesting that this growth may not have been due to vitellogenin uptake. Indeed, in the rainbow trout, recent evidence suggests that although maximum rates of vitellogenin incorporation into oocytes occurs during the mid-phase of vitellogenic development, vitellogenesis is largely completed 1-2 months before ovulation; the further growth which occurs in oocytes from this time up until ovulation is due largely to water uptake (Riazi and Fremont, 1988). Furthermore reports by Wallace and Selman (1981) on *Fundulus heteroclitus* have shown that during final maturation of the egg, hydration contributes far more to the follicle enlargement, than vitellogenin uptake. Indeed 80% of the normal enlargement of follicles undergoing maturation can be achieved *in-vitro* in the absence of added nutrients (Wallace and Selman, 1981) indicating that vitellogenin sequestration accounts for less than 20% of the normal follicle development during the final maturation process (Selman and Wallace, 1983). In the dace, there is no evidence that the final growth of the follicle is due to vitellogenin uptake (that is low E2 and calcium levels), and it is possible that a large proportion of this growth may also be due to water uptake.

In contrast to the decline in E2 levels prior the final maturation stages, serum T levels remained high (3.5-4.5 $ng.ml^{-1}$).The reason for high T levels during this period is unknown. Similar increases in T levels during this period have also been observed in the rainbow trout (Scott *et al.*, 1980). A possible role for T during this period could be for the production of T glucoronides (e.g. Lambert *et al.*, 1986: see chapter 4), which have recently been shown to have pheromonal influences (Lambert, 1978; Stacey, 1984; Lambert *et al.*, 1986); pheromones are required to induce behavioural responses between sexes within a population, for example courtship, nest building and synchronized spawning. Thus in the dace, high serum T levels may be associated with the production of steroid glucoronides which allow communication regarding maturation between opposite sexes. Alternatively high levels of T may also provide large quantities of precursors for the production of T, E2 and maturation-inducing steroids that occur over the very short period

(few days) prior to ovulation.

Final maturation, as demonstrated histologically (by GVM migration and the presence of the micropyle) occurred a few days prior to ovulation. The hormonal events associated with final maturation and ovulation are described in detail in the following chapter (chapter 4). Therefore they will not be discussed here, other than to say that a further a peak of T and E2 (not shown in figure 3.4) and a peak of the maturation-inducing steroid, 17α20β - dihydroxy progesterone were detected just hours prior to ovulation.

Ovulation was highly synchronous, and occurred at approximately the same time each year, in mid-March, in fact within a week of each other for the 3 separate years over which the study was conducted. This suggests that water temperatures which play an important part in the timing of reproduction in other cyprinids (see Billard *et al.*,1978) do not control the timing of final maturation and ovulation in the dace. As water temperatures over the 3 year study period, at spawning varied between 5-10°C, it does seem far more likely however, that daylength is a critical factor required to induce final maturation, for this parameter does not vary from year to year and is therefore more predictable. The role of daylength as an important environmental cue during the reproductive cycle of the dace is further investigated in Chapter 5.

Ovulated eggs ranged in diameter from between 1.5-1.8mm depending on the size of the fish. A peculiar feature of the eggs, unlike other fish species, was the presence of nodular like protrusions extending from the zona radiata (see plates 3.8 and 3.9). These were first visible in cortical alveolus stage oocytes coincident with the formation of the zona radiata. Their precise function is as yet unknown. However, these nodules, which confer upon the zona radiata a convoluted appearance, may function by increasing the total surface area of the developing oocyte, and therefore aid vitellogenin uptake. This would be beneficial for the developing embryo, as its gestation period is longer than that of most other cyprinids. As dace embryos require more nutrients or egg yolk for their development, a mechanism which increased the amount of available yolk would be advantageous. Alternatively as these protrusions from the zona radiata were still present on the ovulated egg, this suggests that they may be required for a process that occurs after ovulation. As dace spawn in fast flowing streams they have to adhere their eggs to a substrate suggesting that perhaps these small structures may function to provide the adhesive substance which would help to attach the fertilized egg to its substrate. Obviously further examination of these structures is

necessary to understand their precise role in the development of the egg.

In summary, these results demonstrate that histologically, oocyte development shows a similar pattern of development to other fish species, for example the rainbow trout (Bromage and Cumarunatunga, 1988). A major difference however, unlike other teleosts, is the presence of small nodular protrusions which extend from the zona radiata. Oocyte development during the early stages of ovary growth in the dace is 'asynchronous', that is different sized oocytes (including different stages) could be observed at any time between March and August. However, by December, individual oocytes were of a similar size.

The annual cycle of ovarian development began around May, when the GSI started to rise. This was preceeded by a peak in serum E2 and T levels, followed by a rise in serum calcium levels and the subsequent appearance of yolk granules in the oocytes in June. Ovary growth continued throughout the summer and winter (albeit to a lesser degree) months largely due to the uptake of vitellogenin (as indicated by serum calcium levels). After serum calcium and E2 levels had declined in January however, follicle diameter still increased. This may have been due to some other factor (rather than vitellogenin) for example water uptake, In contrast to the decline in serum E2 and calcium levels, T levels remained high until ovulation; high serum T levels during this period may have been involved with the production of steroid glucoronides and/or maturation-inducing steroids. Ovulation occurred in mid-March around the same time each year, suggesting that the main stimulus for this process is daylength and not water temperature.

CHAPTER 4

The hormonal changes accompanying the artificial induction of spawning using C.P.E.

4.1 Introduction

Often fish maintained in captivity are unable to complete reproduction. In these fish, the final maturation phases of ovarian development, namely germinal vesicle breakdown (GVBD), ovulation and oviposition fail to occur. Similarly in this study, mature dace maintained in captivity failed to undergo these developmental stages. This clearly limited our studies to investigate the endocrine changes associated with reproduction in this species, occluding the hormonal changes during final maturation. Furthermore before initiating a number of the photoperiodic experiments in the subsequent chapter, male and female dace were required at an identical phases of reproductive development, ideally immediately after spawning. To obtain females in this condition (unlike males which spermiate in captivity) a methodology was required to artificially induce ovulation.

The inability of the final stages of ovarian development to be completed in captivity, is not unique to the dace. In aquaculture, it is common practice amongst many species, including other cyprinids. Artificial final maturation and spawning inducing techniques are now widely used in aquaculture. Among the advantages of this capability include the ability to control the timing of spawning and hence the production of fry and to synchronize spawning between different individuals. In some species this can be achieved by simulating the natural environmental cues that control these processes.

The natural control mechanisms have evolved to ensure that spawning occurs when environmental conditions are most suitable for the development of their young. These mechanisms are complex; in many species ovulation is not simply the consequence of completed vitellogenesis, but rather is a rapid response to a variety of specific factors such as temperature change, pheromones and spawning substrate. In captivity, by simulating these natural cues, the spawning of captive species can often be acheived. Thus, by exposing captive goldfish, *Carassius auratus* to a change in water temperature from 12-20°C induces ovulation (Kobayashi *et al.*, 1988) within a few days, though oviposition will not occur spontanously without the presence of aquatic vegetation (the natural substrate for oviposition) (Stacey *et al.*, 1979b)). Although the induction of ovulation by simulating natural cues undoubtedly works for some, these methods cannot be applied to the majority of species maintained in captivity, as the precise cues required for ovulation in different species are unkown. Because environmental cues are

mediated through the endocrine system, in recent years, treatments of exogenous substances, usually hormones or hormonal substitutes have been commonly used to artificially induce ovulation and spawning, a process known as hypophysation.

The majority of induced spawning practises involves the injection of hormone preparations (hypophysation). To succesfully induce a species to spawn using these methods, it is necessary to have detailed information of the endocrine events that accompany final maturation under natural conditions and how they might interact not only with each other but also with the agent chosen to induce ovulation. The endocrine control of final maturation and ovulation has been reviewed several times (e.g. Fontaine, 1976; Peter, 1982; Scott, 1987) and are summarized in figure 4.1. However a brief account will be given here.

The hypothalamo-hypophysial unit is at the centre of control of this process. This unit releases specific hormones, namely hypothalamic gonadotropin releasing hormone (GnRH), gonadotropin release-inhibitory factor (GRIF), and pituitary gonadotropin (GTH) which in turn mediates the ovarian hormones involved in the final maturation process (see chapter 3).

In fish a pronounced surge in GTH is seen prior to ovulation in goldfish (Stacey *et al.* 1979), carp (Stantos *et al.* 1986; Weil *et al.* 1980) and a number of salmonids (Fostier *et al* 1983; Breton *et al.* 1983). This feature and the observation in a number teleost species that mature females can be induced to ovulate by injection of a variety of GTH preparations, for example carp pituitary extract (C.P.E) (e.g. Bienarz *et al.*,1980; Kime and Dolben, 1985; Yaron and Levevi-Zermonsky, 1986) strongly implicates this hormone as a prerequisite for ovulation. Indeed, in many captive species, it is generally accepted that it is one or more of the specific factors necessary for the GTH rise and consequently the final phases of reproductive development which is/are absent.

There is evidence to suggest that the GTH rise that accompanies ovulation is mediated through the hypothalamus (e.g. Peter, 1982). Thus synthetic GnRH (Lam *et al.*, 1976; Chan, 1977) and superactive GnRH analogues (Co-op Team for Hormonal Application in Pisciculture, 1977) are effective in artificially inducing ovulation (see fig.4.1). Similarly, mammalian LHRH and its analogues have been used with some success to artificially induce ovulation in several species (see Donaldson, 1981), though higher doses are usually necessary. Further evidence suggests that both gonadotropin releasing hormone (GnRH) and gonadotropin inhibitory factor (GRIF) respectively, serve to promote and inhibit GTH release from the

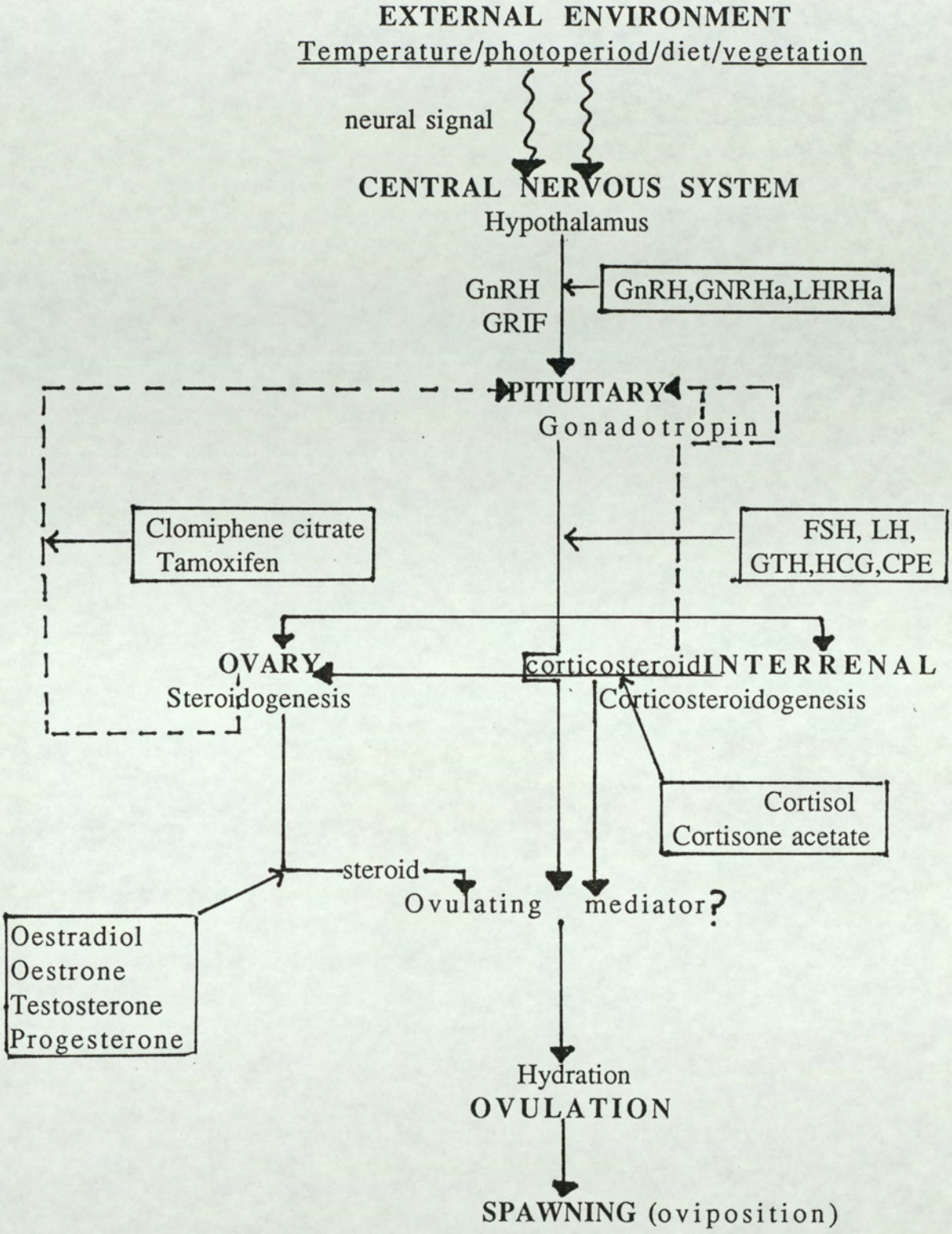


Figure 4.1 The endocrine regulation of final maturation and spawning. The boxed text demonstrates where man could intervene to artificially induce spawning.

gonadotroph cells of the pituitary (Peter, 1982; Ball, 1981) (see chapter 3). It is not clear, however, how the apparent dual regulation of GTH release by GnRH and GRIF might normally participate in the control of teleost ovulation. In the goldfish, preovulatory GTH release may be due at least in part to temporary withdrawl of GRIF which normally inhibits GTH release (Peter, 1982). Another possible mechanism for preovulatory GTH release maybe the increased pituitary sensitivity to GnRH. Alternatively, there is the possibility in some species (e.g. salmonids) preovulatory GTH release is not triggered by altered neuroendocrine activity, but is a pituitary response to some other factor such as changing steroid levels.

Considerable research effort has focused on the mechanisms of GTH induction of oocyte final maturation and ovulation in teleosts, as an understanding of these processes may lead to other techniques which do not require the use of GTH or LHRH. Both steroids and prostaglandins have been demonstrated to mediate the ovulatory action of GTH. Preovulatory levels of GTH trigger 2 distinct processes: both of which can be dissociated experimentally, final maturation of the oocyte which is stimulated by ovarian steroids (Jalabert, 1976) and follicular rupture which is thought to be stimulated by prostaglandins (Goetz, 1983). Studies in a variety of teleosts showing that GTH induces final maturation indirectly by stimulating follicular steroidogenesis (Jalabert, 1976) have led to investigations of the nature of the steroid(s) mediating these effects of GTH. In the rainbow trout, goldfish and northern pike, *Esox lucius* (Jalabert, 1976), yellow perch, *Perca flavescens* (Goetz and Theofan, 1979), brook trout, *Salvelinus fontinalis* (Duffey and Goetz, 1980) and amago salmon, *Oncorhynchus nerka*, (Nagahama *et al.*, 1980), the steroid most potent in inducing final maturation is 17α,hydroxy-20-β-dihydroxy progesterone (17α20βP). In support of these findings 17α20βP has been shown to increase in the blood just prior to spontaneous ovulation in a variety of teleost species (Scott and Baynes, 1982; Kagawa *et al*, 1982; Fostier *et al*, 1983; Scott *et al*, 1984; Jalabert *et al*, 1977) and to be synthesized *in-vitro* by GTH stimulated ovaries of ayu, *Plecoglossus altevilus* (Suzuki *et al.*, 1981).

In more recent studies however, 17α-hydroxy progesterone (17αHP), a precursor of 17α20βP, which reaches serum levels of 2-13 $ng.ml^{-1}$, has been suggested as the maturational hormone in the carp (Kime and Dolben, 1985). Although 17αHP has been measured in the plasma of other species (Nagahama *et al*, 1982; Duffey and Goetz, 1980), it has generally been at significantly lower levels than

17α20βP. Whereas in salmonid species, the maturational hormone is most definitely 17α20βP, there appears to be rather less specificity in a number of non-salmonid species. In the carp for example, significant maturation can also be induced by androstenedione, progesterone and deoxycortisone acetate (Epler and Bienarz, 1983) (see fig. 4.1). However in this study, 17αHP was not measured. Similarly 17αHP has not been measured or used to induce final maturation in many other species. In another species the zebrafish, *Brachydanio rerio*, deoxycortisone is more effective than 17α20βP in inducing maturation (Van Ree *et al.*, 1977). In the catfish, *Heteropneustes fossilis*,there is also evidence that GTH induces ovulation by stimulating synthesis of interrenal corticosteroids, which in turn act on the ovary (Sundararaj and Goswami, 1977; Truscott *et al*, 1978).

The gonadal steroids, for example testosterone and 17β-oestradiol produced by the ovary, are also important for the final maturation process (Jalabert, 1976; Scott *et al.*, 1980). Thus, in some species several of these have been shown to successfully induce ovulation artificially (eg. Kobayashi *et al.*, 1988) (fig. 4.1). However, the natural progression of steroid production during the final maturation process varies between species (e.g. see Kime and Dolben, 1985; Scott *et al.*, 1982) making the precise role of these steroids during final maturation difficult to understand. For example in the rainbow trout, 17β-oestradiol (E2) levels which are high during exogenous vitellogenesis, decrease before maturation (Fostier *et al*, 1976; Scott *et al*, 1980) while testosterone (T) levels remain high (Scott *et al*, 1980) and show a further rise prior to ovulation. In contrast, both T and E2 rise prior to ovulation in the carp (Jalabert, 1976). The apparent differences in steroid production prior to ovulation is possibly related to their biosynthetic pathways (Fig. 4.2). It is assumed that the activity of the enzymes involved in the production of gonadal steroids are the major influences on their corresponding serum levels. Thus switching 'on' or 'off' a different set of enzymes in different species could account for the apparent discrepancies in steroid production.

In addition to being local ovarian mediators of the ovulatory action of GTH, ovarian steroids may also act at the level of the brain on the pituitary to regulate GTH release by both positive and negative feedback mechanisms. In the goldfish (Kobayashi *et al*, 1987) and rainbow trout (Fostier *et al*, 1983), E2 exerts both a positive and negative feedback on GTH release and ovulation. In conjunction with this, anti-oestrogens such as clomiphene citrate and tamoxifen, have been shown to block the effects of E2 on the

pituitary, and hence induce ovulation (Pandey *et al*, 1973) (5). Moreover, by using an anti-oestrogen drug, such as clomiphene citrate, ovulation has been induced in the goldfish at 13-14°C, a temperature below which this species would not normally ovulate (Pandey and Hoar, 1972).

Although in some species, ovulation normally follows *in-vitro* steroid stimulated final maturation (Goswami and sundararaj, 1971; Goetz and Theofan, 1979), in most cases *in-vitro* steroid treatment induces ovulation only infrequently (Jalabert, 1976; Goetz and Bergman, 1978; Wallace and Selman, 1978). Considerable evidence (see review by Stacey and Goetz, 1982) indicates that ovulation in teleosts is stimulated by prostaglandins. Thus the prostaglandin synthesis inhibitor, indomethacin completely blocked *in-vivo* follicular rupture in goldfish and in yellow perch oocytes injected with human chorionic gonadotropin (Stacey and Pandey, 1975) or 17α20BP (Goetz and Theofan, 1979) respectively; in both studies, several prostaglandins were effective in restoring indomethacin blocked follicular rupture.

Although many different procedures for artificially inducing final maturation and ovulation are available, the practice of injecting GTH of varying purity, usually a pituitary preparation, is the most common method (e.g. Kime and Dolben, 1985; Yaron and Levavi-Zermonsky, 1986). Despite their effectiveness, there are several disadvantages to the use of pituitary extracts. Firstly, it is considered that such a harsh method of spawning induction may provide levels of GTH far higher than those that would under natural conditions. Secondly, they normally contain large numbers of other hormones besides GTH which are not always beneficial to the physiology of the fish and especially to the quality of the genital products. Therefore, an increasing effort has been made to isolate the gonadotropic hormones from the pituitary. Both fish and mammalian gonadotropins are used, e.g. human chorionic gonadotropin (HCG) either alone or in combination with piscine hormones (Liao *et al*, 1971) to achieve spawning success. Fish pituitary gonadotropin has been purified from salmon, carp and mullet. Much smaller doses are required to elicit ovulation with purified GTH than with the more commonly used less refined spawning induction methods. Another disadvantage of using pituitary extracts, is that it is impossible to standardize dosages without performing an assay of pituitary GTH content beforehand. This is particularly important since the GTH content of pituitaries varies seasonally (Peute *et al*, 1976) and diurnally (de Vlaming and Vodicnik, 1977). In addition, correct

dosing is rendered difficult due to species specificity (Fontaine *et al*, 1972) and deterioration of the potency of pituitaries during storage (Yaron *et al*, 1982). Thus, synthetic compounds have been investigated increasingly e.g. LHRH, LHRHa and dopamine citrate, corticosteroids and progestogens (review: Lam, 1982). Despite all the disadvantages of the less refined spawning induction methods, pituitary extracts, in particular C.P.E. injections are still frequently practised.

The objective of the present study, as mentioned earlier was to artificially induce ovulation and spawning in the dace. A carp pituitary extract (C.P.E) was used, as this was readily available. This enabled the two main objectives of this chapter; that of determining the final endocrine changes related to final maturation and ovulation, and also the synchronization of groups of animals for the subsequent photoperiod experiments. As the hormonal content of the pituitary extract used in this procedure was unknown, by recording hormonal levels upto 12 days after the C.P.E. injection enabled us to ascertain whether or not the injection of such a crude extract had any long term effects on the reproductive cycle of the dace and consequently the fish used in the following photoperiod experiments (see chapter 5).

4.2 Aim of experimemt

The aim of this experiment was to artificially induce dace to spawn using a carp pituitary extract (C.P.E.) and to monitor the pattern of testosterone (T), 17β-oestradiol (E2) and 17α20β-dihydroxyprogesterone (17α20βP) before and after C.P.E. treatment.

4.3. Protocol

Mature dace of mixed sex prior to ovulation were obtained from the River Worfe, Wolverhampton and immediately introduced into a water recirculatory tank system (see appendix 1). The fish were divided into 8 groups, each consisting of 5 females and 5 males, easily distinguished at this stage of the annual reproductive cycle, by their external appearance (see section 2.3). Each group was placed into different tanks. The males at this time could be induced to spermiate by a slight pressure to their abdomen (section 2.3). The photoperiod in each tank was maintained as close to the ambient photoperiod as possible (11.75L:12.25D) and the water temperature at 11±0.5°C.

Prior to being induced to ovulate, the females were ovarian biopsied to ascertain their suitability for spawning induction (section 2.3.2.). The results from this procedure demonstrated that the majority of oocytes had begun germinal vesicle migration (GVM) and the females were therefore ready for spawning induction. On the same day 25 female and 25 male fish were blood sampled.

The following day at 10,00am (day 1), females from 4 of the tanks were injected with carp pituitary extract (C.P.E.) as described in section 2.3.3., and from the other 4 tanks with isotonic saline solution, using the same injection volume. Fish were blood sampled 6, 22, 32,48 hours and 4,5 and 12 days post-injection. To reduce stress from handling, only fish from two tanks were sampled, at each sampling time, one in which the females had been treated with C.P.E. and one in which the females had been treated with isotonic saline; in addition as far as was practical different tanks were sampled at each sampling time.

All blood samples were allowed to clot, centrifuged and deep frozen. Subsequently the resulting supernatant was analysed for T and E2 using the RIA procedure described in section 2.4b.

17α20βP was analysed using an RIA based upon that described by Scott *et al.* (1982) and similar to the procedure for T and E2 described earlier, with minor modifications which are given below.

a) Label

As the radiolabelled steroid was not available commercially, it was prepared in the laboratory by the method described by Scott *et al.* (1982).

b) Antisera

The antibody which was a gift from Dr A.P. Scott (MAFF) to Dr J. Sumpter, was used at an initial dilution of 1:20000. The cross reactivity of this antisera with other steroids are presented in Scott *et al* (1982); these results indicate that of the steroids tested, only 17α20βP significantly cross reacted with the antisera.

c) Standard Hormone

The standard which was purchased from Sigma (P, 625) was diluted in assay buffer to obtain a $1ng.ml^{-1}$ solution. The standard solution was serially diluted in each assay to cover a range of zero to 500 $pg.ml^{-1}$. An example of a typical standard curve is shown in figure 4.2.

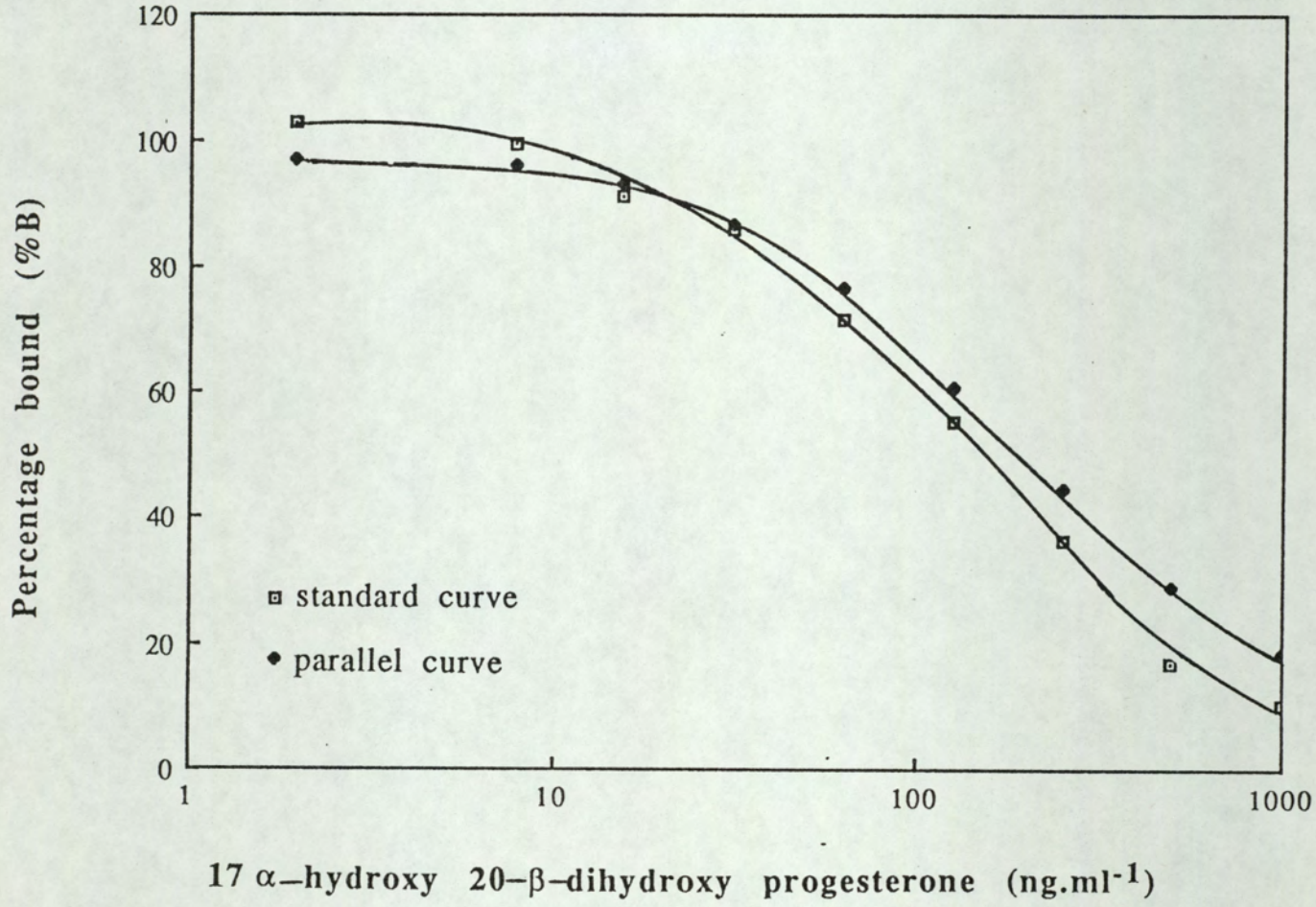


Figure 4.2. An example of the 17α –hydroxy,20β–dihydroxy progesterone RIA standard curve.

4.4 Results

All female fish, apart from one, injected with C.P.E., spawned (released oocytes into the external environment) between 6 and 14 hours post-injection, that is during the night between day 1 and 2. The one fish that had not spawned spontaneously, had however ovulated (rupture of oocyte from follicle) and could therefore be stripped (day 2). In contrast, all females injected with saline failed to ovulate, and consequently could not be stripped.

Throughout the experiment, all males could be induced to spermiate by applying gentle pressure to the abdomen. However, they did not spawn spontaneously and consequently they all had to be stripped in preparation for the following photoperiod experiments. Therefore, the ovulated eggs were not fertilized and we could not determine the viability of eggs oviposited using the C.P.E. procedure.

Figures 4.3, 4.4 and 4.5 a and b illustrate the changes in serum E2, T and 17α,20βP respectively, between day 0 and day 12 of the experiment, in saline and C.P.E. treated fish. Prior to the C.P.E. or saline injections, serum T, E2 and 17α20βP levels were 19.4, 15.2 and 2.2 $ng.ml^{-1}$ respectively. After C.P.E. treatment (day 1), serum levels of all 3 steroids increased significantly ($p<0.001$) with serum T reaching the highest levels (65.7 ± 20.4 $ng.ml^{-1}$). In the saline treated fish on day 1 (6 hours post-injection), only E2 levels were elevated (23.6 ± 9.4 $ng.ml^{-1}$)($p<0.05$); both serum T and 17α20βP had declined.

On day 2 (22 hours post-injection), E2 levels in the C.P.E. treated fish had increased further (47.2 ± 12.2 $ng.ml^{-1}$)($p<0.01$), T and 17α20βP levels had however decreased. By 32 hours post-injection (day 2), all steroid levels in these fish had returned to levels similar to those observed prior to treatment. In the saline injected fish, on day 3, serum T and 17α20βP remained at low levels; however serum E2 levels followed a similar pattern to those recorded in the C.P.E treated fish, that is a further increase to 40.2 ± 11.3 $ng.ml^{-1}$ at 22 hours post-injection, followed by a decrease to 13.1 $ng.ml^{-1}$($p<0.001$) 32 hours post-injection.

By day 3 post C.P.E. and saline treatment, all steroid levels had returned to low levels and remained low for the remainder of the experiment.

In males, in tanks where the females had been treated with C.P.E., serum T initially at 26.2 $ng.ml^{-1}$ (day 0) prior to the females being treated, remained elevated until day 3 (fig 4.4a). In contrast serum T in males maintained with saline treated females were significantly lower ($p<0.001$).

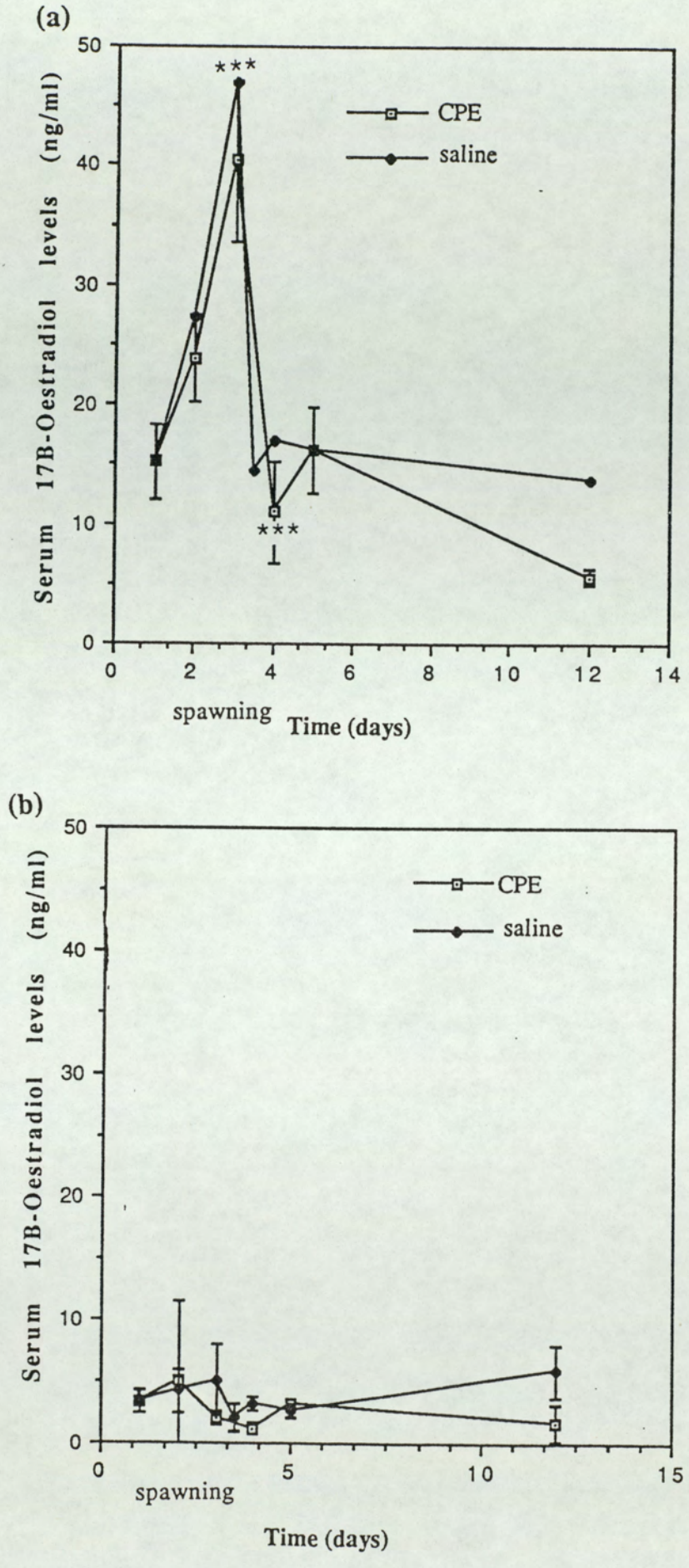


Figure 4.3 Serum 17β–oestradiol levels ($ng.ml^{-1}$) in fish injected with saline and CPE: (a) females and (b) the associated males.

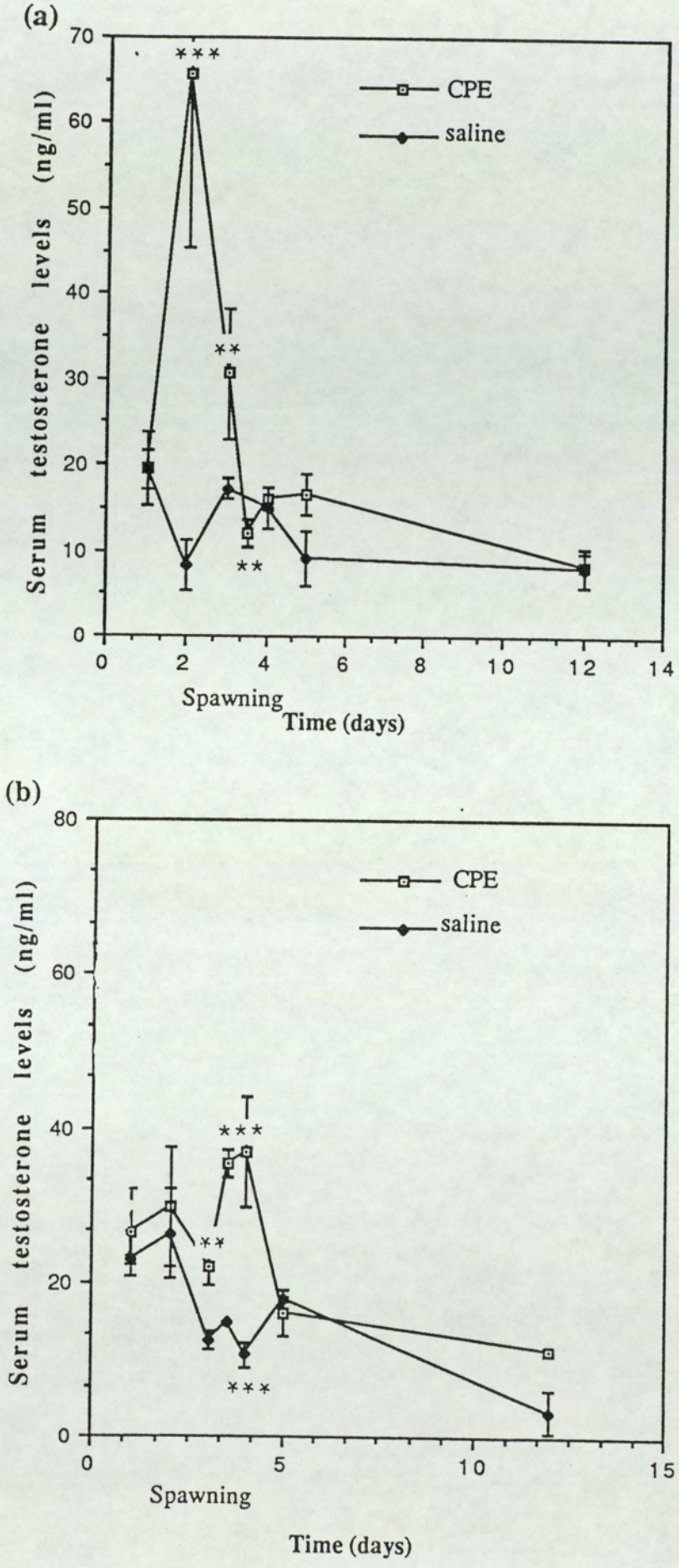


Figure 4.4 Serum testosterone levels ($ng.ml^{-1}$) in fish injected with saline and CPE: (a) females and (b) the associated males.

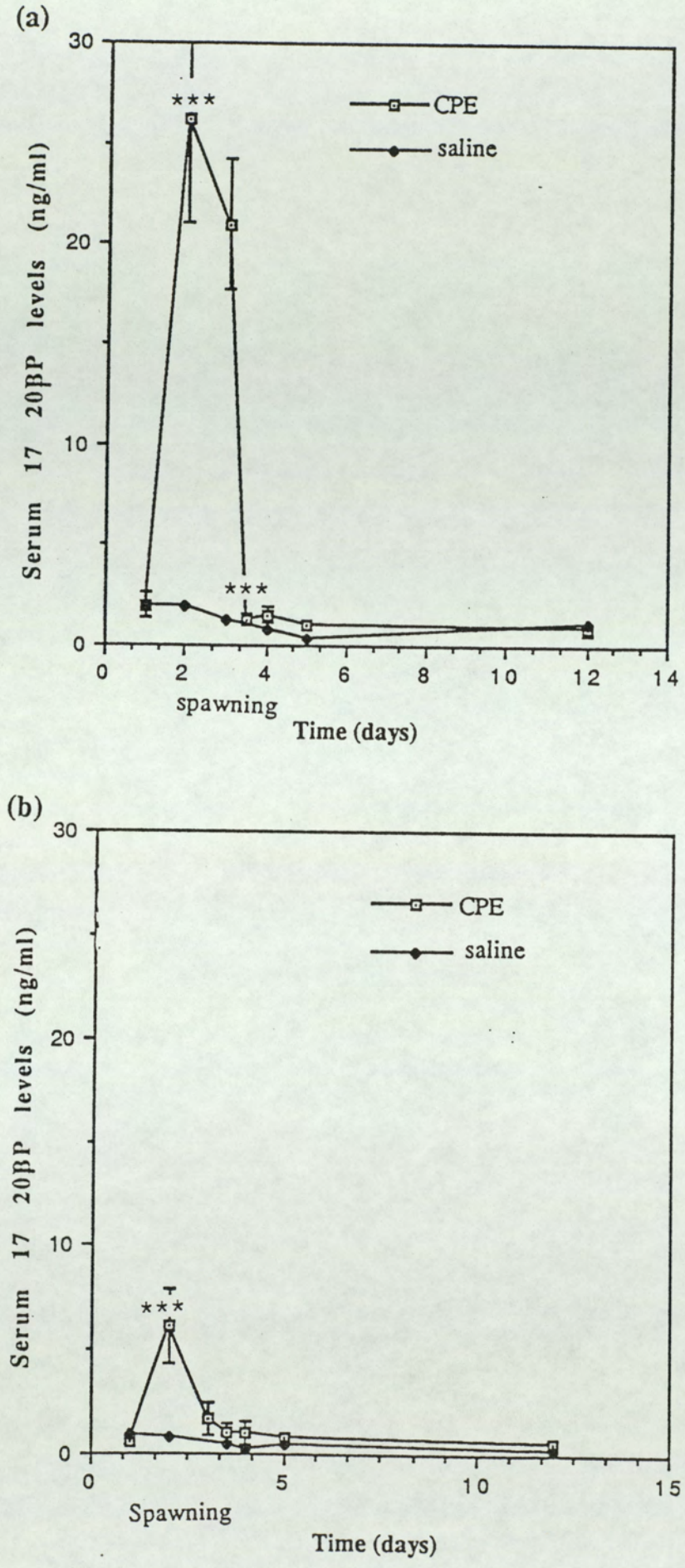


Figure 4.5 Serum 17α20β levels ($ng.ml^{-1}$) in fish injected with both saline and CPE :(a) females and (b)the associated males.

Unlike serum T in males, serum 17α20βP showed a slight peak on day 1 in tanks where both C.P.E. or saline treated females were present. Indeed, serum 17α20βP levels reached higher concentrations in the male fish when maintained with the saline treated females than they did in the C.P.E. treated females (Fig. 4.5b).

In contrast serum E2 levels remained low in all the male fish, throughout the experiment.

4.5. Discussion

The results in this chapter demonstrate that C.P.E, as a source of GTH, successfully induced ovulation in the dace, similarly to that shown in other species. Thus as final maturation is ultimately under the control of GTH release (Jalabert, 1976), the absence of this phase of reproductive development in captive fish appears to be related to the lack of a GTH surge. As the ovulatory surge in GTH only occurs when both environmental and endocrine conditions are optimum for spawning, this suggests in the dace, that the specific environmental factors required to induce final maturation and spawning naturally, were absent under captive conditions. In other cyprinids, for example the goldfish (Stacey *et al.*,1979b) and roach (Worthington *et al.*, 1982) water temperature, and the presence of a spawning substrate were required to induce final maturation and ovulation (Stacey *et al*, 1979b). However, the natural spawning stimulus for dace is at present unknown; neither the presence of males or gravel (used to mimic the natural spawning substrate) was sufficient to stimulate final maturation.

Successful induction of spawning in cultured fish depends mainly on two factors; the quality and the dose of the administered agent and the suitability of the recipient fish to respond to this agent. A number of procedures exist for administering C.P.E, but in general a 'priming' dose followed by a 'resolving' dose appears to be the most effective (reviews: Donaldson, 1975; Fontaine, 1976; Lam, 1982). In this study however, only a single injection of C.P.E. was required to successfully induce ovulation and oviposition, which occurred between 6 and 14 hours after treatment. Successful ovulation using this method also depends on the stage of follicle development when the agent is administered. *In-vitro* experiments have shown that GVBD is attained faster and in larger proportions after stimulation with C.P.E., if the percentage of follicles with migratory or peripheral GV is initially higher (Epler, 1981). Thus, in this study a routine biopsy procedure was used to ensure that only the most suitable females were selected for induction. This routine prevented treatment too early in the development process, which normally results in deleterious effects on further spawning of the fish in the same season (abnormal hydration and/or atresia) (Yaron and Levavi-Zermonsky, 1986). The fact that the dace ovulated and oviposited between 6 and 14 hours after the C.P.E. injection demonstrates the effectiveness of the biopsy procedure used combined with a suitable concentration of C.P.E. In previous years (data not shown) the same dose of C.P.E. induced ovulation but not

oviposition and in this case all the females had to be stripped, as did one female from this years experiment. This suggests that the C.P.E. was not administered at the optimum time in these fish. In this study, as one female had to be stripped, this suggests that the follicles in this fish were slightly out of phase to the other females, possibly indicating some asynchrony between the fish. Alternatively, if this fish was the last to be treated with C.P.E., final maturation may just have been delayed, and ovulation and oviposition might have occurred within the next hour or so.

The use of C.P.E. as an artificial agent for spawning induction in the dace has also been demonstrated elsewhere (Brighty*et al.*,87). In addition this author was able to induce spawning in this species with des Gly^{10}, [D-Ala^{6}]-leutenizing releasing hormone (LHRH-A)(0.1ug. g^{-1}) coupled with an injection of either chlorpromazine (0.1 or 1ug. g^{-1}) or droleptan (1µg.g^{-1}), both dopamine antagonists. Together these data suggest that the control of final maturation and ovulation in the dace, as in many other species, is ultimately under the control of the hypothalamo- pituitary axis via GTH. In other species, there is evidence to suggest that the pattern of GTH release and the subsequent steroid production associated with final maturation were similar both in C.P.E. treated fish and in fish under natural conditions (Yaron and Levavi-Zermonsky,1986; Fostier *et al.*, 1973; Jalabert *et al.*,1976). Thus it is likely that the steroids associated with final maturation, produced in this study as a result of C.P.E treatment are similar to those which occur under natural conditions.

In the dace, as a result of C.P.E. injection, increases of serum T, E2 and 17α20βP levels were observed in conjunction with GVBD and ovulation. In other species, there is a natural succession in the production of steroids prior to final maturation and development. In this study , because blood sampling was limited to only once or twice a day and the fact that spawning occurred quite rapidly after the C.P.E. injection, the natural succession in production of T, E2 and 17α20βP could not be fully determined. In a number of other species that have been studied, there are some major differences in the production of gonadal steroids, especially between salmonid and cyprinid groups. In the carp, for example, a priming injection of C.P.E. stimulates increased T after 3-6 hours, followed by an increase in E2 (Kime and Dolben, 1985). Following the second injection another peak of T accompanied by a peak of T glucuronide was observed and finally a peak of 17α−HP (Kime and Dolben, 1985). By contrast in other cyprinids, the final maturation hormone detected was 17α20βP (Stacey *et al*, 1983; Yaron and Levavi-Zermonsky, 1986). In salmonid species both T and E2 decrease significantly prior to

ovulation, though like cyprinids 17α20βP rises (Scott *et al.*,1982; Fostier *et al.*, 1981). As previously mentioned, the apparent discrepancies in steroid production prior to final maturation in these species can probably be related to their biosynthetic pathways (Fig, 4.2). By switching 'on' or 'off" a particular set of enzymes, depending on the species, could account for the apparent differences in steroid production.

It is assumed that the activity of the enzymes involved in the production of gonadal steroids are the major influences on their corresponding serum levels. In the rainbow trout (salmonidae), Scott and Baynes (1982) postulated a gonadotropin-induced switching 'off' of the C21-C19 desmolase and an induction of the 20β-hydroxysteroid dehydrogenase so that T production is replaced by a significant rise in 17α20βP (see fig. 4.2). In addition aromatase activity has been shown to decline (Van den Hurk, 1974), which probably accounts for the drop in E2 observed prior to ovulation.

In the carp or goldfish (Family Cyprinidae), a similar pattern of enzyme activity does not appear to occur. In these fish the rise in T followed by a peak in E2 is consistent with a precursor-product relationship (see fig 4.2). After a second injection of C.P.E. however, there is a shift in steroidogenic pattern similar to that observed in the rainbow trout, i.e. a switching 'on' of the 20β-hydroxysteroid dehydrogenase (2) and the production of 17α20βP. Thus T declines and instead its glucuronide (T glucuronide) is produced by conjugation. Conjugation may in fact be a means of deactivating T (Kime, 1982).

As in the goldfish and the carp (Cyprinidae), E2 and T peaked prior to ovulation in the dace suggesting a similar enzyme activity pattern as described above. In the dace treated with C.P.E. E2 levels increased to 27.3 $ng.ml^{-1}$ prior to ovulation; however they did not peak until a few hours after ovulation (47 $ng.ml^{-1}$, 22 hours post-injection) (Fig 4.4a). Thereafter E2 levels returned to lower levels (10-15 $ngml^{-1}$), but still remained higher than basal (2-5 $ngml^{-1}$). The functional significance of rise in E2 prior to ovulation in the dace is at present unknown. It is possible that E2 may be involved with oogonial proliferation and the next gonadal cycle which follows almost immediately. This would also explain why the E2 levels in the saline treated fish (i.e. unovulated) followed a similar pattern to those seen in the C.P.E. treated fish (fig 4.4b). In addition this suggests that a rise in E2 at this time is not under the control of GTH but rather pre-programmed (i.e. endogenous). This would ensure the development of the next gonadal cycle, if for any reason, one or more

of the specific factors required for final maturation were absent and ovulation failed to occur.

In mammals high levels of E2 prior to ovulation are thought to act as a positive feedback for the release or the LH surge prior to ovulation (Karla and Karla, 1985). However, in the rainbow trout, a decrease in E2, has been suggested to trigger the GTH surge prior to ovulation, functioning as a negative feedback mechanism (Fostier *et al*, 1983; Scott *et al*, 1983). This is not the case in the goldfish, however, since administration of E2 does not inhibit the occurence of the GTH surge and ovulation (Pankhurst and Stacey,1985; Kobayashi *et al.*, 1987). In another study on the goldfish, the implantation of ovariectomized goldfish with silastic capsules containing either T or E2 induced the GTH surge, though to a lesser extent in fish implanted with E2 (Kobayashi *et al.*, 1988). Thus in cyprinids under natural conditions, E2 maybe involved at least in part in the release of GTH. This does not explain, however, a role for E2 after the GTH surge observed in the dace and other studies, where the fish were artificially induced to spawn. Perhaps this increase in E2 may serve to maintain GTH levels by positive feedback for the period immediately before ovulation or alternatively they may be the result of high T levels, E2 being the product of a precursor-product relationship.

Testosterone levels in the dace prior to ovulation reached a maximum of 65.7 $ng.ml^{-1}$, significantly higer ($p<0.001$) than levels obtained after ovulation. Preovulatory peaks in T as well as in the carp and goldfish, have also been shown in the Indian catfish, *Heteropneustes fossilis* (Lamba *et al.*, 1983) and the white sucker, *Catostomus commersoni* (Scott *et al*, 1984). In the goldfish T is thought to be more important than E2 for the induction of the GTH surge (Kobayashi *et al* ,1988). Other roles for T prior to ovulation may include accumulation of GTH within the pituitary (Fostier *et al.*, 1983) which may enhance the action of GTH on follicle maturation (Jalabert, 1976) or acting as a precursor for the synthesis of 5b reduced androgens which often function as teleost pheromones (Colombo *et al*, 1982). Concommitant with the production of T, T glucuronide is also produced in the carp, African catfish, *Clarius lazeras* (Lambert and Van den Hurk, 1982) and zebra fish, *Brachydanio rerio* (Lambert, 1978) in association with ovulation. Further evidence for the role of pheromones during the final phases of reproductive development has been presented by Lambert *et al.* (1986). In their studies on the zebrafish, courtship behavior will only occur when the female has ovulated, and a female will only ovulate

when post-vitellogenic follicles are present in the ovaries and when a male is close. It is reasonable to assume therefore that the female receives a signal from the male, and that the male can perceive whether a female has ovulated. In addition water in which males had been held has been shown to induce ovulation in female zebrafish (Van den Hurk and Lambert,1983). There are strong indications that these signals are pheromones produced by the testes and ovaries. Indeed following treatment with the enzyme b-glucuronidase, to eliminate possible steroid glucuronide synthesis, the ovulation inducing capacity of the water fraction was virtually eliminated (Schoonen and Lambert, 1986). Together these data point to steroid glucuronides acting as pheromones to synchronize male and female final maturation. Both T and E2 (although to a lesser extent) glucuronides have been shown to function as pheromones (Lambert *et al.*, 1986).

As a result of the above evidence, it is possible that the inability of females to undergo ovulation in captivity may be due to the absence of steroid glucuronide production. Indeed the enzymes responsible for the glucuronidation show a much stronger action in nature than in aquarium fish (Lambert *et al.*, 1986). This may at least in part, account for the inability of captive dace in this study to undergo ovulation. However, a peak of T observed in the male fish associated with the ovulated females (no peak was seen in males associated with unovulated females) suggests that there maybe pheromonal communication between males and females, particularly as the T peak observed in males occurred only as a result of the females ovulating. On the other hand, however, the T peak observed in the male fish associated with the ovulated females may have occurred as a result of C.P.E. leakage from the female into the surrounding water, thus providing the hormonal stimulus for T production in males. As the holding water was not analysed for GTH or other hormones associated with the extract, caution should be taken in the interpretation of these results.

The peak of preovulatory 17α20βP observed in female dace (fig, 4.6a) is seen in nearly all species tested to date, both under natural conditions and as a result of the artificial induction of spawning. 17α20BP was first isolated from the plasma of male and female Pacific salmon over 20 years ago (Schmidt and Idler, 1962). There is now much evidence to suggest that 17α20BP is the mediator of gonadotropin-induced follicle maturation, as this hormone has succesfully induced premature ovulation in the carp and salmon (Jalabert, 1976) and has the same effects *in-vitro* (Fostier *et al.*, 1973).

Although 17α20βP is the main maturation inducing steroid in most species studied to date, the pattern of its production is quite different between salmonid and cyprinid groups. In salmonid species, 17α20βP begins to increase upto 8 days prior to ovulation (Bry, 1981); this encompasses the period in which follicle maturation is known to occur. In contrast, in cyprinids, there is usually only a short peak just hours before ovulation. Likewise after ovulation in salmonids, 17α20βP took 2-3 weeks to return to basal, whereas in the carp (Cyprinidae), levels fall within a few hours. According to Brighty (1987), the pattern of 17α20βP in the dace follows a similar pattern to that observed in salmonid species (i.e. an extended period of 17α20βP production), though in this case being elevated from December to March suggesting that final maturation is a protracted event in the dace. Although in this study 17α20βP was not recorded during this preovulatory period, levels prior to C.P.E. induction were only 2.2 $ng.ml^{-1}$ indicating that final maturation is restricted to only a few hours, as in other cyprinid species. As Brighty *et al.*, (1987) failed to mention the levels of 17α20βP he recorded, it is difficult to compare and understand his contradictory findings.

Serum 17α20βP levels have also been recorded in spermiating male fish, though at lower levels than those recorded in the females, in both the rainbow trout (Scott and Baynes, 1982) and in this study in the dace. In the male rainbow trout, 17α20βP production is not associated with final maturation, but with potassium ion concentration of the seminal fluid; K^+ ions serving to inhibit sperm motility (Baynes *et al.*, 1981). As a small increase of 17α20βP in male dace was observed in fish maintained with both ovulated and unovulated females, and the fact that all the males could be induced to spermiate by applying slight pressure to the abdomen, suggests that in the male dace 17α20βP may also be correlated with K^+ levels in the seminal fluid.

In the rainbow trout (Scott *et al.*, 1982) and the carp (Yaron and Levavi-Zermonsky, 1986), 17α20βP prior to ovulation reached levels of 400-800 $ng.ml^{-1}$ and 111 $ng.ml^{-1}$ respectively. As 17α 20βP levels in dace only reached 26 $ng.ml^{-1}$, this indicates that the fish were not necessarily sampled at the optimum time of 17α20βP production. If this was the case, concommitant with the production of 17α20βP occurring after androgen and oestrogen synthesis in other species, it is possible that in the dace a switch in enzyme activity from desmolase to dehydrogenase may also occur (see fig. 4.2). Thus in the dace, like many other species 17α20βP is produced after T and E2 production.

In summary, C.P.E. successfully induced ovulation and oviposition in the dace, and induced temporary elevations in plasma steroid levels. Both T and E2 increased as a result of C.P.E. (GTH) treatment. Their precise role in final maturation is at present unknown, though available data suggests they may have a pheromonal role. High levels of E2 after ovulation maybe associated with or induce the next gonadal cycle, which commences almost immediately. Like in other species, 17α20βP could be the final maturation-inducing steroid, stimulating GVBD and the seperation of the oocyte from the follicle, resulting in ovulation. Although these data do not enable us to convey precisely how this process is controlled in the dace, they do explain, at least in part how final maturation and ovulation may occur in this species.

CHAPTER 5

The influence of photoperiod in the timing of reproduction in the female dace

5.1 Introduction

The previous chapters show that the annual reproductive cycle of the dace can be divided into a number of stages, namely oogonial proliferation, oogenesis, cortical alveolus formation, exogenous vitellogenesis and final maturation and spawning. Under natural conditions, each stage of ovarian development is accurately timed to occur at a specific period in the year. In this chain of events, cortical alveolus formation occurs soon after spawning, predominantly between April and June, exogenous vitellogenesis occurs between June and January and final maturation is timed to occur in the following March. These stages are repeated annually, until the fish eventually dies; in some cases this maybe 10 or 12 years. This chapter investigates how the dace maintains this precise synchrony of gonad development.

Reproduction is ultimately timed so that the young hatch at the most appropriate season, for example when food is abundant, to ensure maximal survival. Clearly reproduction therefore, has to be closely coordinated to the environment. The cues which coordinate reproductive and environmental cycles are those which provide the most reliable 'calendars' and are most appropriate to the animal in its own niche. Daylength, temperature and food availability predominate in temperate regions.

In addition to an environmental regulation of reproduction, there is evidence to suggest that this interacts with an underlying endogenous rhythm (see reviews: Bye, 1984: Stacey, 1984). The endogenous control of reproductive development in seasonally breeding teleosts ensures that even under constant environmental conditions, fish will exhibit a rhythm of gonadal maturation, that approximates to an annual cycle, though under natural conditions, this rhythm is entrained by changes in photoperiod and temperature; this ensures that the gonads are mature at the appropriate seasonal period. Due to the length of time experiments have to be conducted, the evidence for an endogenous control of reproduction in fish is restricted to only a few species, for example, the rainbow trout *Salmo gairdneri* (Whitehead *et al.*, 1978), and the catfish, *H. fossilis* (Sundararaj *et al.*, 1973)) which were maintained under experimental conditions for 1 and 3 years respectively. However, from shorter term experiments there is evidence in other fish that certain stages of the reproductive cycle are endogenously timed. One such stage is refractoriness when the gonads are insensitive to environmental factors following the breeding season. A refractory period is presumed to be a mechanism which prevents the fish

breeding, even though environmental conditions are favourable at a sub-optimal time of year. Although a refractory period is thought to exist in many species, one has been unequivocally demonstrated only in the stickleback, *Gasterosteus aculeatus* (Baggerman, 1972), a centrarchid, *Lepomis cyanellus* (Kaya, 1973), the medaka, *Oryzias latipes* (Egami and Hosokawa, 1973) and the catfish, *Heteropneustes fosilis* (Sundararaj and Vasal, 1976). Other species, however, do not show a refractory period; de Vlaming (1975) found that regardless of the time of year when the experiment was initiated, the correct environmental conditions could stimulate gonadal development in the cyprinid, *Notemigonus crysoleucas.*

It is has already been mentioned that photoperiod and temperature are probably the dominant environmental factors involved in reproductive cycling, however ovulation and spawning usually require further specific stimuli. This is discussed in more detail in chapter 4. Briefly, these stimuli may include both chemical parameters, for example pH and dissolved oxygen content and physical factors such as the presence of appropriate vegetation (Scott, 1979). As these are sometimes difficult to identify, it is not easy to mimic the precise conditions required for final maturation in the laboratory with the result that G.V.B.D., ovulation and spawning often fail to occur (see review; Donaldson, 1975). Under natural conditions the specific stimuli required for spawning further ensures reproductive synchronization between individuals within a population.

With regard to photoperiod and temperature, considerable variation is seen in the reproductive timing mechanisms among teleosts, making it difficult to propose any unifying hypothesis. As de Vlaming (1972) has remarked 'because teleosts occupy such a diverse range of habitats, and the fact that species of a family or even different races of the same species occupy an extended geographical range, few generalizations can be made with regard to phylogenetic, geographical or ecological relationships of the fishes studied to date'.

Datum available from work on cyprinids indicate that in this group of fish, temperature is the most important factor involved in reproductive timing (de Vlaming, 1972; Billard *et al.*, 1978). Many experiments show the strong influence of temperature in cyprinid fish: in carp, *Cyprinus carpio*, reproduction only occurs in warm conditions and under a temperature of 20-22°C, continuous gametogenesis has been reported (Kossman, 1975); temperature plays a role in the spring time induction of vitellogenesis in the tench, *Tinca tinca* (Breton *et al.*, 1980; Morawska, 1984) and the

Japanese bitterling, *Rhodeus O. ocellatus* (Hanyu *et al.*,1983); furthermore heated water (+6°C above ambient) increases the number of tench spawning and the total duration of the spawning period (7 spawnings over 3 months, compared to only 2 in the controls (Billard and Breton, 1977).

In some species, low temperatures have also been suggested as being important for stimulating the early stages of gametogenesis (Ahsan, 1966; Breton *et al.*,1980; Gillet and Billard, 1981), and since in many late spring and summer spawners, gametogenesis is initiaited during the winter, this may be more important, than has been previously realized. The importance of warm temperature as an environmental timing cue in cyprinid reproductive cycles is largely associated with those species which spawn in late spring/summer, when water temperatures are high. Because the dace spawns earlier in the year (in March) than the afore-mentioned species, when water temperatures are still relatively low, it has been sugested that photoperiod may be more important than temperature in the timing of reproduction in this cyprinid (Bromage, pers. comm.). This chapter therefore investigates the role of photoperiod on reproductive cycling in the dace.

Of all the environmental factors which influence reproduction in fish, the effect of light has received the most extensive study. Most of our understanding concerning the role of photoperiod has been obtained from research on salmonids, where significant progress has been made in the use of photoperiod to control reproductive development. From work on the rainbow trout, *Salmo gairdneri*, it is now clear that both long and short daylengths influence subsequent ovarian processes in autumn and winter breeding populations (Elliott *et al.*, 1984; Sumpter *et al.*, 1984; Scott, 1988). In general long photoperiods advance spawning (compared to normal time) if initiated before June/July, but delay spawning if initiated after it (Bromage *et al.*, 1984).

In contrast to rainbow trout maintained under continuous long days from the time of transfer, those which were subsequently transferred to short (<12L:12D) photoperiods show accelerated (by 8 weeks on average)(Bromage *et al.*, 1984; Scott, 1988) and more synchronized (Duston and Bromage, 1986) spawnings. However, it is unclear whether this is the result of accelerating vitellogenesis and/or the subsequent processes of final maturation and ovulation. Recently Randall *et al* (1987) suggested that the direction of change of photoperiod rather than the absolute daylength involved maybe of importance in experimental situations. Thus 10 or 12 hours of

light per day can be perceived as long or short depending on the previous photoperiod.

Recently, as previously mentioned, it has been shown that rainbow trout maintained under constant environmental conditions exhibit an endogenous circannual rhythm of maturation (Duston and Bromage, 1986). Under natural conditions this rhythm is entrained by the seasonal changes in daylength, such that an abrupt reduction in photoperiod from 18L:6D to 6L:18D during the early stages of maturation results in a phase advance of the timing of spawning (Duston and Bromage, 1987). Furthermore, in the rainbow trout, it has been proposed that the circannual mechanism responsible for the control of the timing of maturation is an autonomous clock (Duston and Bromage, 1987). This proposal is based on an experiment in which 2+ year old virgin females were maintained on 18L:6D in mid-January until May, when they underwent an abrupt reduction to 6L:18D. In this experiment the timing of spawning was phase advanced. However only some of the fish in the first year spawned, though during the second year all the fish spawned (between July and September). Since all the fish spawned at the same time during the second year, Duston and Bromage (1987) proposed that the clock of not only those fish which spawned in the first year, but also those which did not, must have been phase advanced. They concluded that maturation in virgin fish will only occur if they have reached a certain stage of development when the clock is at its specific phase of its circannual cycle.

Research on the role of photoperiod in non-salmonid species has largely been confined, for practical purposes, to small species of fish, often of no aquacultural significance, for example the minnow, *Phoxinus phoxinus* (Cyprinidae)(Scott, 1979) and the stickleback, *Gasterosteus aculeatus* (Gasteroidae)(Baggerman, 1980). Both of these species have proved good experimental models in establishing the manner in which fish normally time their reproductive cycles.

In *G. aculeatus*, under natural environmental conditions, both photoperiod and temperature are important in regulating the reproductive cycle; the lengthening of photoperiod and increasing temperatures of spring induce sexual maturation, subsequently the long photoperiods and warm temperatures of summer maintain an extended breeding season. The number of spawnings within a single season however is a function of food availability (Wooton, 1977; Baggerman, 1980).

As with salmonids, there is evidence for an underlying circannual rhythm in *G. aculeatus*, which has a free-running period of 6-7 months under constant 16L:8D at 20°C. This response to

continuous long days has also been recorded for the brook trout (Henderson, 1963), the dab (Htun-Han, 1975) and the rainbow trout (Bromage *et al.*, 1984; Scott *et al.*, 1984) though the underlying mechanism cannot yet be explained. Bromage *et al.* (1984) suggest that it is a 6 month phase advance of the endogenous clock.

There is also some evidence for the presence of a diel (24 hour) rhythm of sensitivity to light in *G. aculeatus* (Baggerman, 1980). It is this rhythm which is likely to be the basis of a teleosts ability to assess changes in daylength. Using skeleton photoperiod experiments consisiting of 2 light pulses per day (a 6 hour +2 hour light pulse), Baggerman (1980) has demonstrated that it is not the duration of the daily photoperiod that is important in inducing sexual maturation, but its relationship to the underlying cycle of photosensitivity. Photoperiodic induction of gonadal development occurs only when light coincides with the photosensitive phase.

In fish, the possession of a photosensitve phase has only been demonstrated in two other species, the Asian catfish, *H. fossilis* (Sundararaj and Vasal, 1976) and the rainbow trout (Duston and Bromage, 1976). In the catfish, single hour light periods around midnight were sufficient to initiate gonadal development; any additional light periods served only to attenuate the response. In some animals, for example the hamster, as little as one minute of light falling in the photosensitve phase period of a hamsters circadian rhythm can initiate gonadal recrudescence (Hoffman, 1979). Therefore it is important when conducting photoperiod experiments to maintain precise experimental conditions; even brief light exposures during the dark may influence the gonadal development of fish.

In most cyprinids, although temperature is the predominant environmental influence, in others, sexual development has been shown to be modulated by photoperiod albeit to varying degrees. However, caution should be taken in the interpretation of experiments involving photoperiod as Scott (1979) has emphasized, this can give a false impression of the natural response of an animal, although demonstrating its physiological potential. A clear example of this difficulty is seen in the studies conducted on the minnow, *Phoxinus phoxinus*. The earlier studies using varying photoperiod and constant temperature regimes in this species, concluded that photoperiod was the dominant timing cue (Bullough,1939, 1942; Scott, 1979). However further work on fish maintained in their natural environment established that although this species can use photoperiodic cues, temperature was the main factor regulating the

reproductive cycle (Scott, 1979); changes in water temperature produce behavioural changes which places the minnow in a position in the environment where it can respond to the appropriate photoperiod for reproductive development.

Similarly the early studies on the Asian catfish, *H. fossilis* using constant temperature and varying photperiod, supported the thesis that the catfish reproductive cycle was primarily timed by photoperiodic responses (Sundararaj and Sehgal, 1970). Further work with changing temperatures indicated that under natural environmental conditions, the main factor regulating the reproductive cycle is water temperature (Sundararaj and Vasal, 1976).

In the cyprinid species mentioned so far (for example, carp, minnow and tench), it is apparent that photoperiodic influences are subtle serving to diminish or enhance responses which are more directly controlled by temperature. The importance of temperature or photoperiod in reproductive cycling is probably a direct result of the nature of these cycles. That is, depending on when vitellogenesis or spawning occurs in different species, these events will be regulated by different environmental cues. For example most cyprinids spawn in late spring/summer, whem water temperatures are relatively high; thus it is not surprising that temperature has an important influence in these species. In contrast, in species which spawn in the winter months, when water temperatures are low, for example salmonids, photoperiod would appear to be the dominant regulatory factor. Similarly photoperiod is the dominant regulatory factor in autumn and winter spawning cyprinids. In the case of the Japanese bitterling, *Pseudoterilampus typus*, only photoperiods decreasing below the threshold (13L:11D) initiated vitellogenesis; this occurred regardless of the temperature (Hanyu *et al.*, 1983). Similarly in *Mirogrex terrae-sanctae*, although oogonial proliferation and vitellogenesis may be stimulated by high and low temperatures respectively, short daylengths were more effective in each case (Yaron *et al.*, 1980). The evidence from the latter two species is indicative that photoperiod is probably the dominant regulatory factor in other winter and early spawning cyprinids. It is proposed therefore that photoperiod is the dominant regulatory factor in the dace as this species spawns earlier in the year than most other temperate cyprinids, in March, before water temperature have started to rise. It is unlikely therefore that temperature in this species is an important cue.

This chapter therefore investigates the role of photoperiod on the annual reproductive cycle of the dace. In the following

experiments, both long and short photoperiod manipulations were used to determine the importance of light in this species at different stages of the reproductive cycle. Changes in blood hormones, notably testosterone and 17β-oestradiol and gonadal development (determined by GSI and histological analyses) were used to monitor the effect of artificial photoperiod treatments on reproductive development.

5.2 General methods

All fish used in the following experiments were obtained from natural streams by electrofishing and introduced into light-proof water recirculatory tank systems (plate 5.1). The tanks were circular, 0.7m in diameter with a water depth of 0.5m. Illumination was provided by 1m fluorescent tubes positioned 0.7m above the tanks and controlled by electric time clocks. The light intensity at the water surface was approximately 30 lux. Gravel was placed at the bottom of each tank to provide a spawning substrate. The fish were fed daily with a commercial trout pellet (B.P.Nutrition, U.K. Ltd,) approximately 2 hours after 'lights-on'. Water temperatures were maintained constant at $11 \pm 0.5°C$ in all experimental tanks.

After an acclimatization period of 2 weeks under an ambient photoperiod, all fish were weighed and exposed to their appropriate experimental photoperiod regimes. In some experiments, fish were required at the beginning of a new gonadal cycle. These were obtained using fish that were close to spawning and inducing the act of ovulation using CPE (Carp Pituitary extract)(Section 4.2). These treatments ensured all the fish within a treatment group were at the same stage of development. Individual fish were identified by a series of blue dye marks which had been applied with a panjet (see section 2.1) positioned at various places along the fish's flank.

For each experiment, blood samples were taked from fish at the beginning of the study and subsequently at monthly intervals (section 2.2). The serum obtained was analysed for those hormones known to undergo cyclic changes during reproduction (see chapter 3), namely testosterone (T) and17β-oestradiol (E2) using RIA (section 2.4.2). Levels of vitellogenin in the serum were also estimated by measurement of changes in blood calcium (Ca) (section 2. 4.1).

At the termination of each experiment, the body weight and gonadosomatic index (GSI) (gonadal weight/body weight x 100) of individual fish were determined. Both ovaries were weighed and fixed seperately, the left in Gilson's fluid to determine the numbers and sizes of developing oocytes (section 2.5) and the right ovary in formalin for histological sectioning (section 2.6). All results were analysed using a one-way analysis of variance (section 2.7).

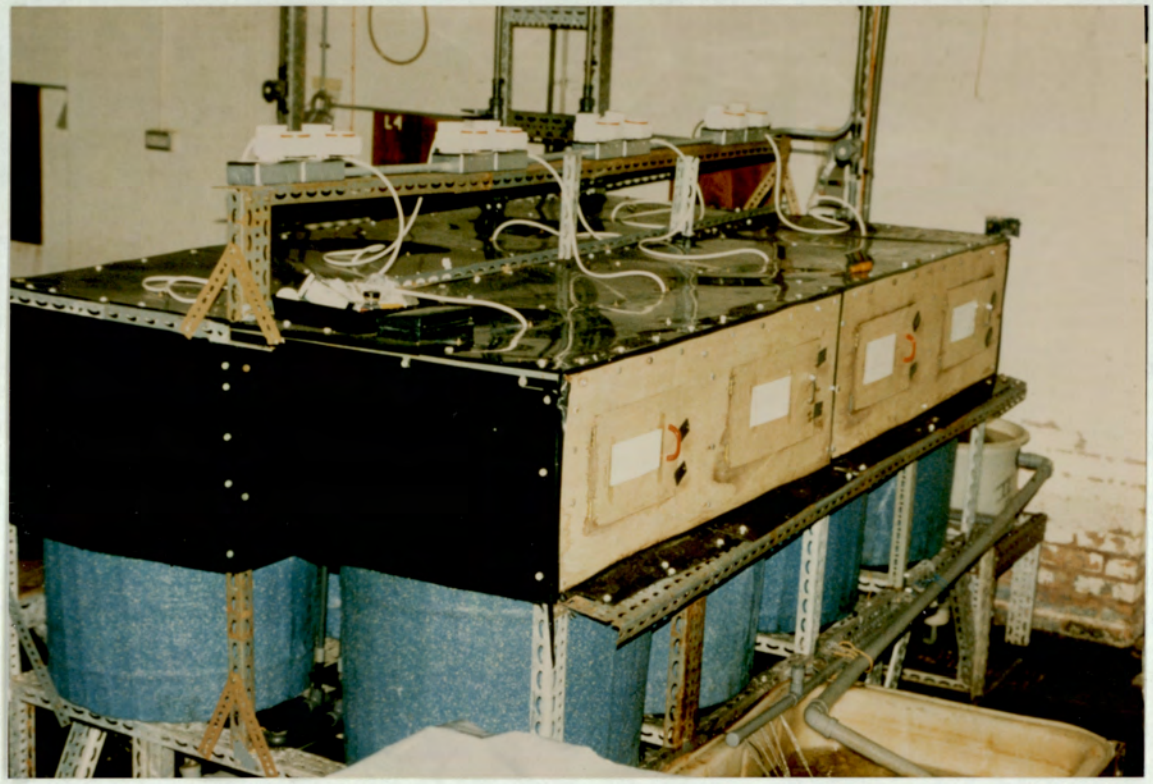


Plate 5.1. The light-proof recirculatory water tank system used for housing fish in all the photoperiod experiments.

5.3 Experimemt 1: The effect of a long, short and increasing photoperiod on the final maturation stages of gonadal development.

The experiment was designed to investigate the role of photoperiod on the completion of vitellogenesis, final maturation and spawning, which under natural conditions occur in the spring, just prior to spawning. As water temperatures are low in early spring (the time of natural spawning in the dace), it was proposed that the increasing daylengths of spring are probably the most likely factors controlling the completion of gonadal maturation in the dace.

5.3.1 Protocol

After an acclimatization period of 2 weeks, 30 dace of mixed sex were divided equally (with regards to sex and weight) and maintained under the following photoperiod treatments from the 31st December: Group A-18L:6D, Group B-6L:18D and Group C-ambient photoperiod and a constant temperature. Temperatures were maintained constant at $11\pm0.5^{0}C$ in all three Groups. In addition a further Group of fish (Group D) were maintained under natural conditions of photoperiod and temperature (controls).

This experiment took place in January after a reduced period of ovarian growth during the winter months. As a result, the oocytes at the start of the experiment were at the same stage of development as they were in September, in the exogenous vitellogenic phase.

The experiment was terminated in May, after natural ovulation and spawning failed to occur in any of the experimental groups.

Figure 5.1 provides an outline of the treatments to which the different groups of fish were subjected.

5.3.2. Results

Serum levels of T, Ca and E2 are shown in figures 5.2, 5.3 and 5.4 respectively. In the control fish (Group D), a rise in serum levels of T and a decline in serum levels of Ca and E2 were observed prior to final maturation and ovulation, which occurred in the second and third week of March (see Chapter 3). After this time, in association with a new gonadal cycle, rises in serum E2 and T (April) were observed. A similar endocrine pattern to that seen in the controls was only observed in fish maintained on an ambient photoperiod and constant temperature regime (Group C). Unlike in Groups C and

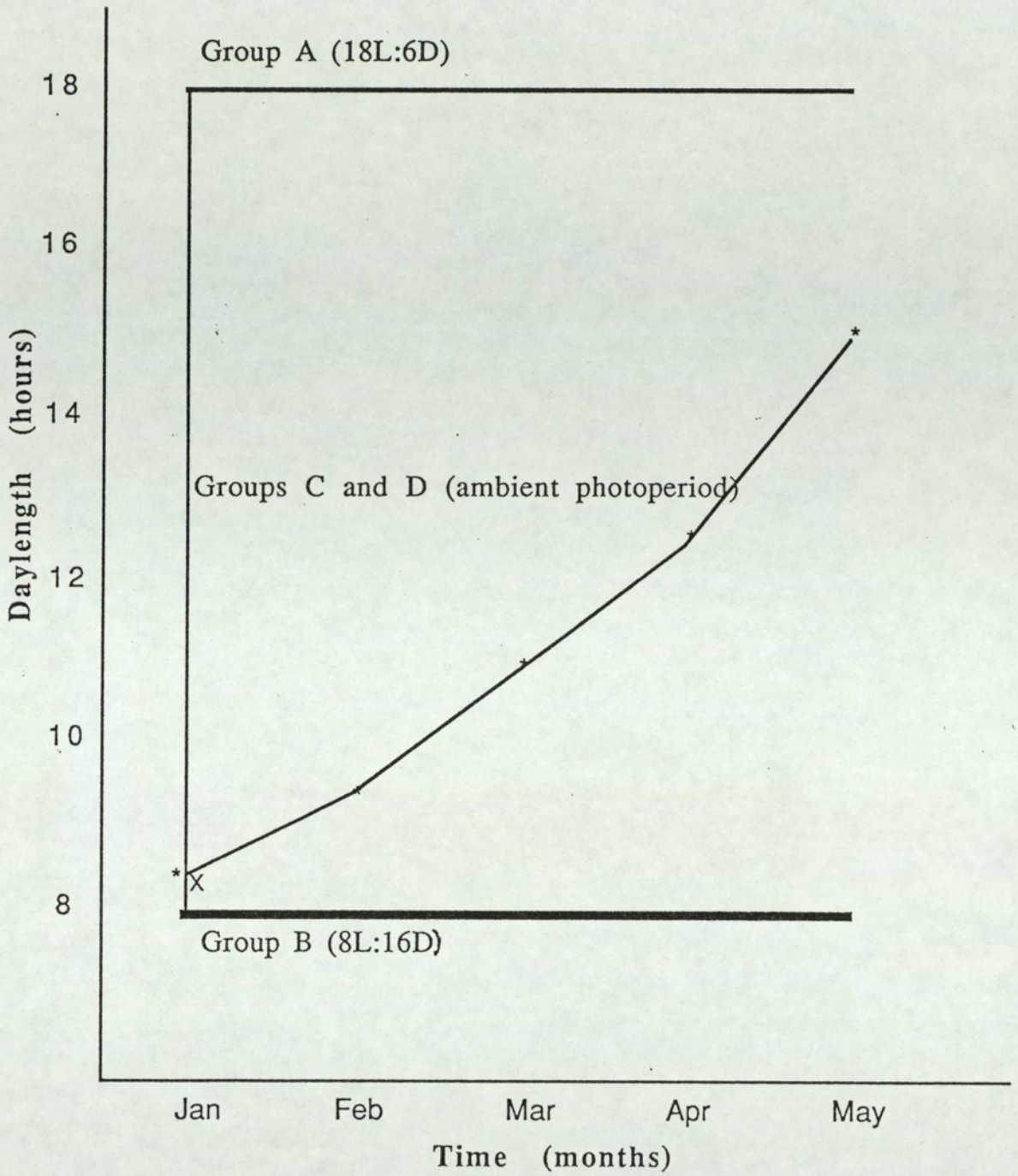


Figure 5.1 Experimental photoperiod regimes in fish maintained, between January and May. X marks the daylength at the start of the experiment. Temperatures were maintained constant at 11±0.5°C in Groups A-C. Group D (controls) were maintained under naturally fluctuating temperatures.

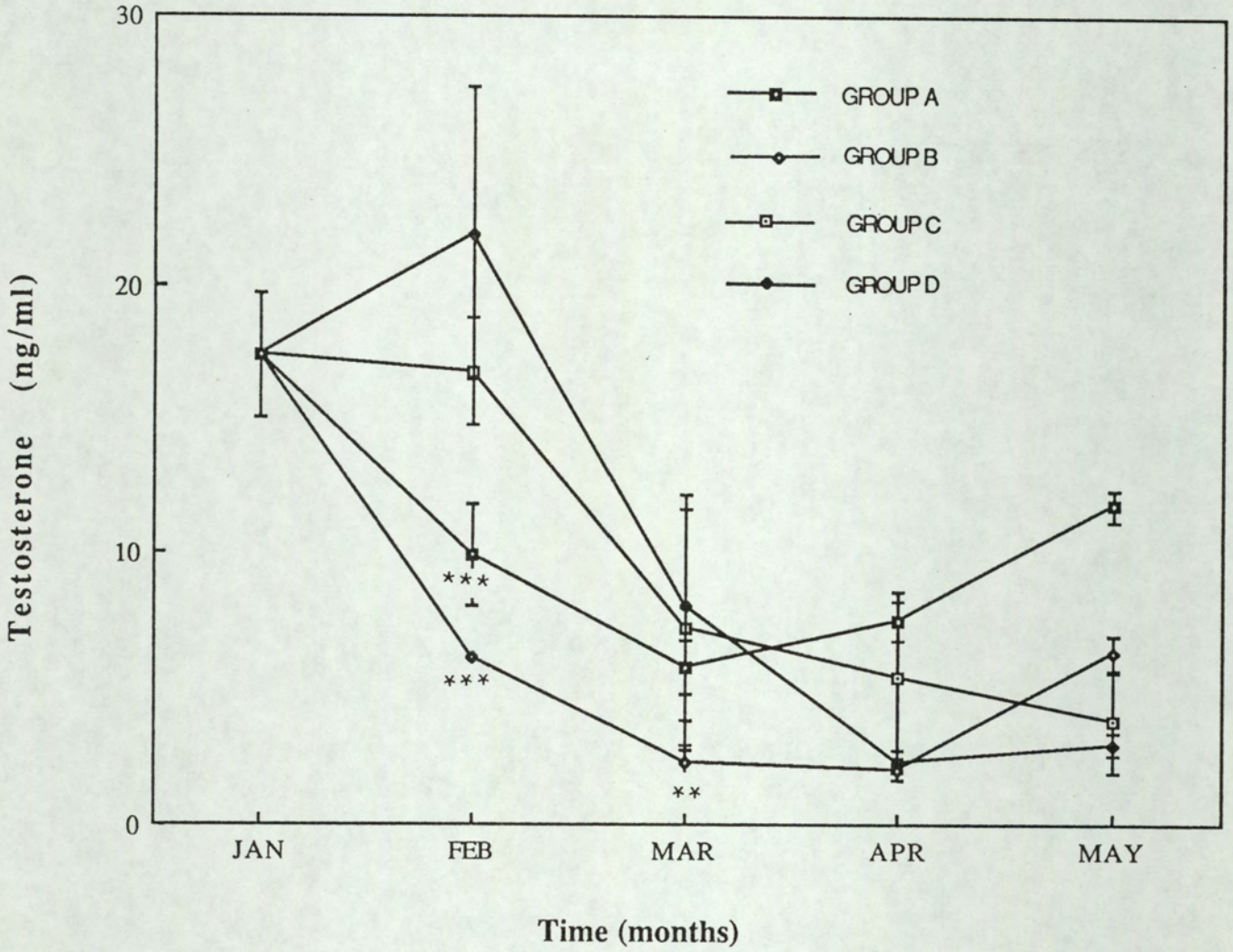


Figure 5.2 Serum testosterone levels ($ng.ml^{-1}$) in Groups A (18L:6D), B (8L:18D), C (ambient photoperiod / constant temperature) and D (controls, maintained under natural conditions of photoperiod and temperature) in experiment 1. Vertical bars denote standard deviations.

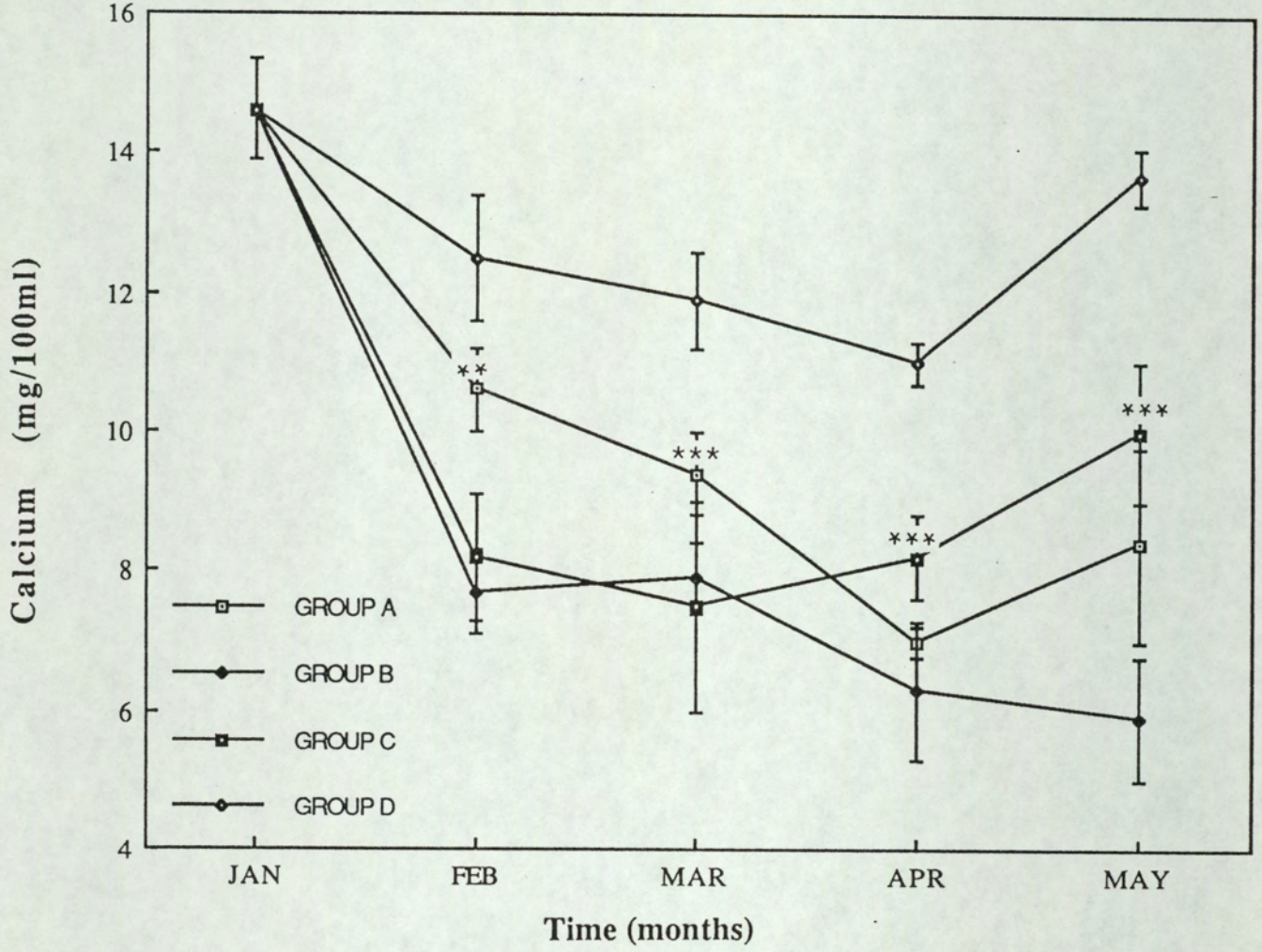


Figure 5.3 Serum calcium levels ($mg.100ml^{-1}$)(as an index of vitellogenin levels) in Groups A (18L:6D), B (6L:18D), C (ambient photoperiod / constant temperature) and D (controls, natural conditions of photoperiod and temperature) in experiment 1. Vertical bars denote standard deviations.

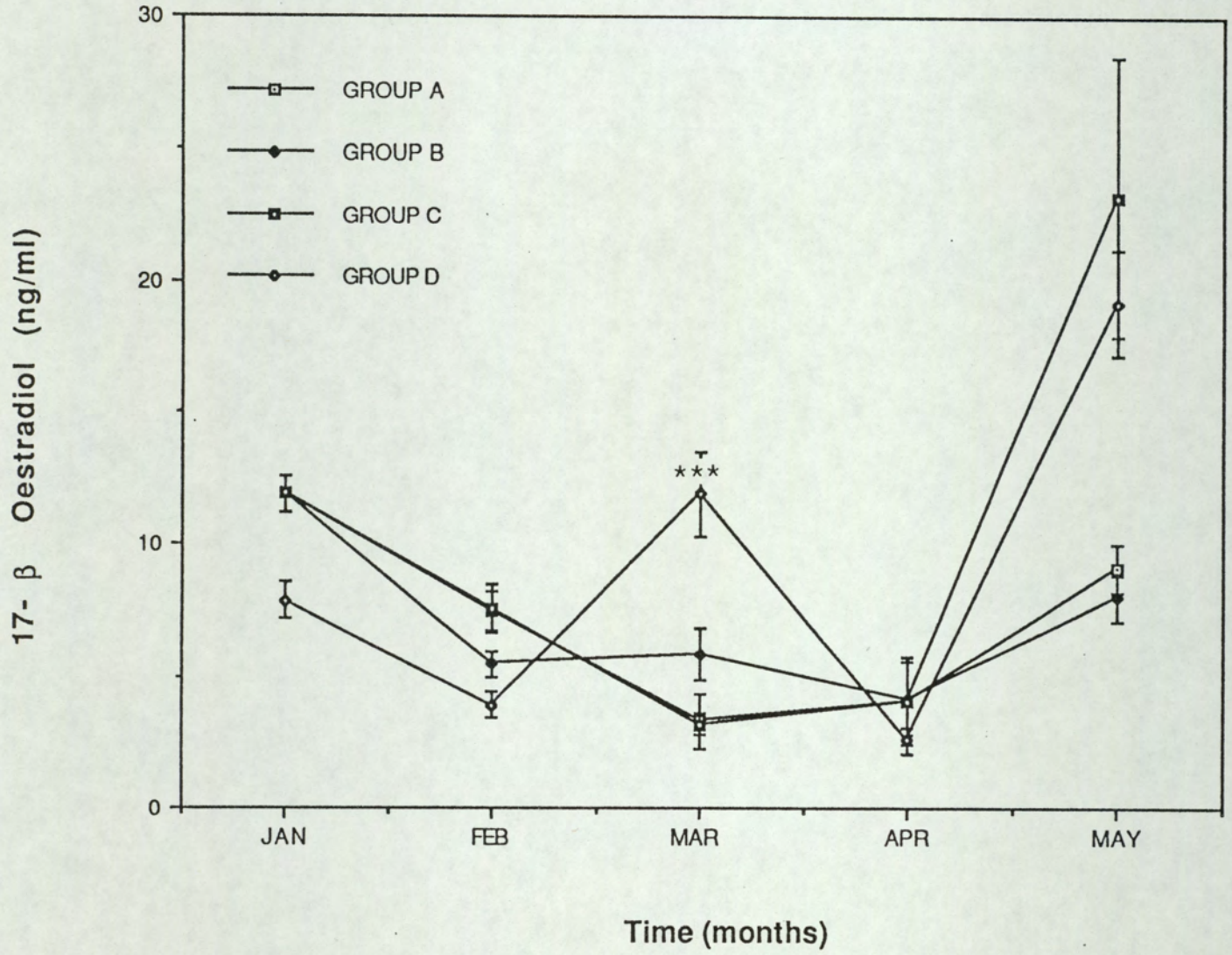


Figure 5.4 Serum 17β-oestradiol levels (ng.ml-1) in Groups A (18L:6D), B (6L:18D), C (ambient photoperiod / constant temperature) and D (controls, natural conditions of photoperiod and temperature) in experiment 1. Vertical bars denote standard deviations.

D, serum levels of T, E2 and Ca in Groups A and B remained at low levels throughout the experimental period. These results demonstrated that only fish maintained on an increasing daylength (Group C) had undergone final maturation similar to the controls. This was confirmed by the stage of ovarian development at the end of March, that is germinal vesicle migration (GVM) stage which was observed in the majority of oocytes (section 2.3.2). Although final maturation (up to GVM) had occurred in Group C fish, no further development was observed and consequently they failed to spawn. Because none of these fish had spawned by May, the experiment was terminated. Final maturation in Group C fish was further supported by histological and GSI changes in the ovaries at this time. A histological examination of the ovary in May demonstrated that GVM was still visible in Group C fish (plate 5.2). In contrast, the nucleus was still in a central position in oocytes (plate 5.3) from Groups A and B fish. In addition a number of oocytes in these fish had started to undergo atresia (plate 5.4)(see section 3.1). Similarly the GSI values in Group C fish were significantly ($P< 0.01$) higher (8.5%) than Groups A (3.2%) and B (3.6%)(figure 5.6). However GSI values in Group C were still significantly lower than those recorded in the controls (Group D)(18.5%).

5.3.3 Summary of results

1) Only fish maintained from the end of December on a simulated ambient (increasing) photoperiod/constant temperature regime (Group C). underwent final maturation similar to the control fish (Group D). This was supported by serum levels of T, E2 and Ca, the GSI and also the position of the germinal vesicle in March; neither fish maintained on constant long (Group A) or short (Group B) daylengths showed any further development.

2) Although final maturation was observed in Group C fish, development beyond the germinal vesicle migration stage, that is G.V.B.D. and ovulation failed to occur.

5.4 Experiment 2: The effect of different photoperiod regimes on the early stages of ovarian development

Under natural conditions, a major proportion of ovary growth occurs during the summer between June and September, under decreasing daylengths. The importance of photoperiod during this time is investigated in this experiment.

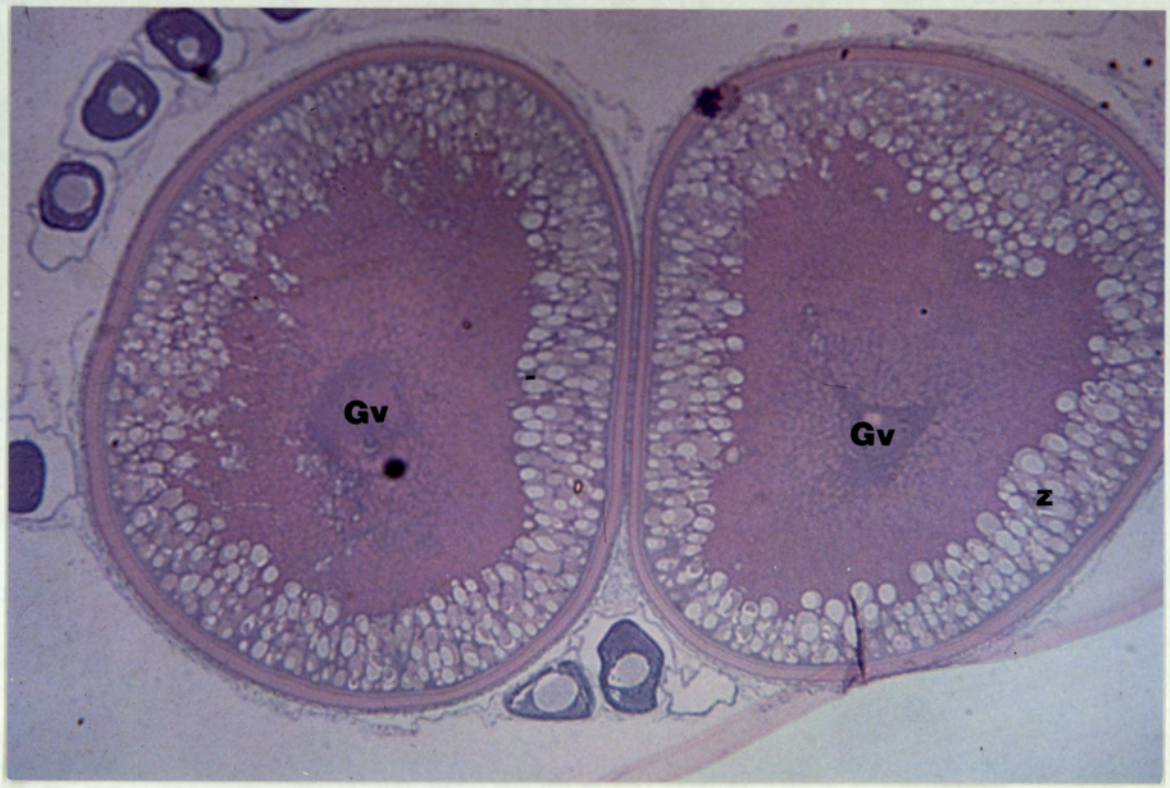
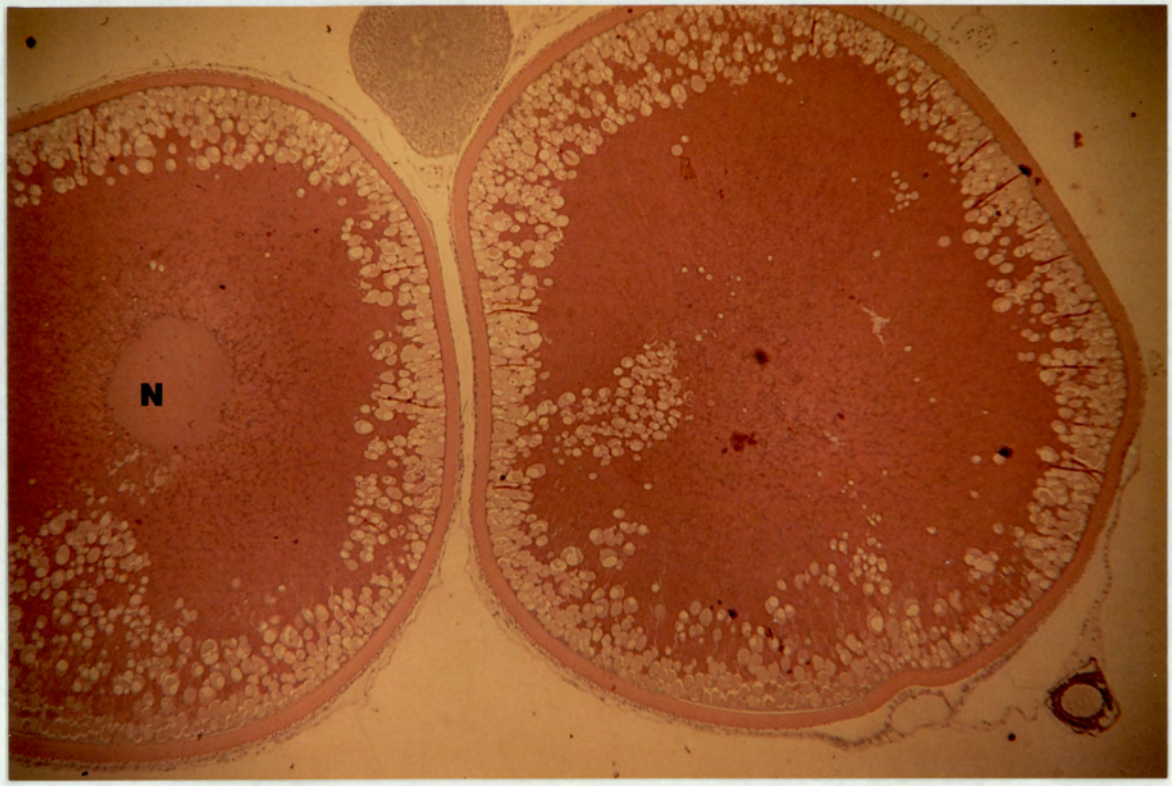


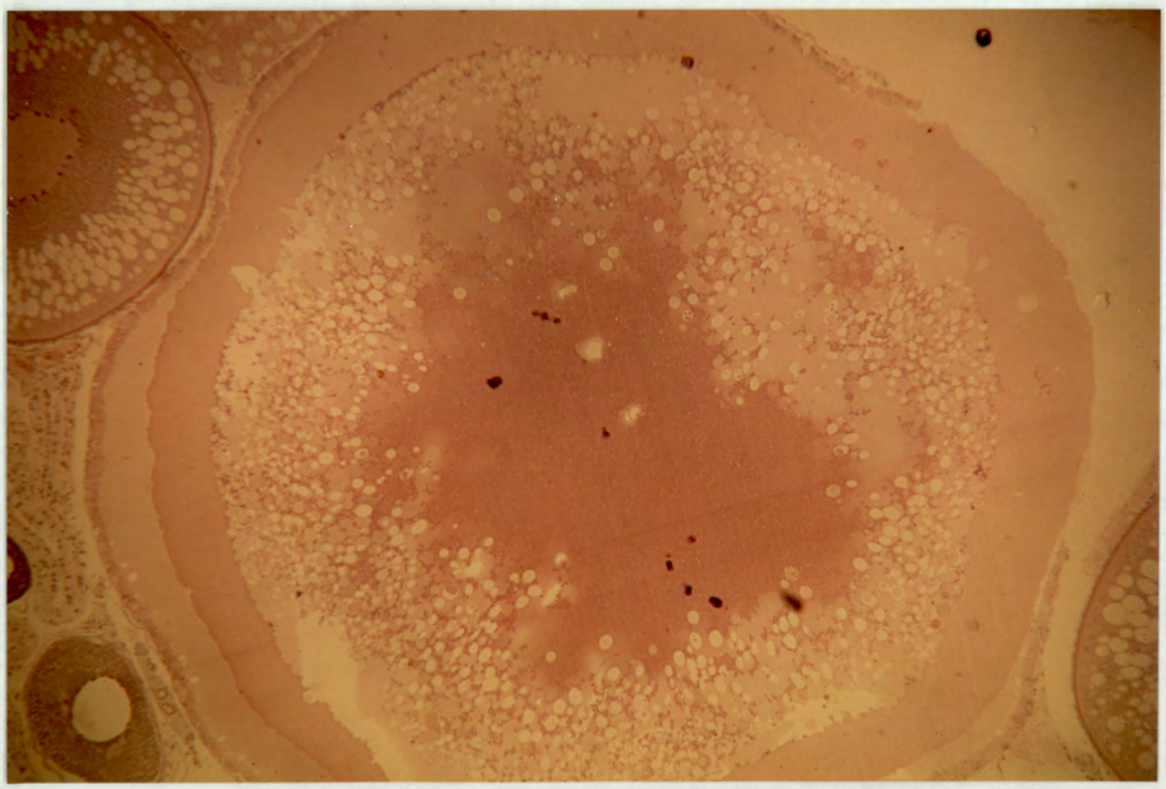
Plate 5.2. Section of an ovary showing germinal vesicle migration (GVM). This stage of oocyte development was only visible in Group C fish maintained under an ambient photoperiod and constant temperature and the controls maintained under naturally fluctuating conditions of temperature and daylength. GV = germinal vesicle (nucleus). Stained with Ehrlich's Haematoxylin and Eosin. Scale : 0.1mm.

Plates 5.3 and 5.4. Sections of oocytes from fish maintained under constant long (Group A) and short (Group B) photoperiods.

Plate 5.3. A section of an oocyte from fish in Groups A and B, demonstrating that the nucleus (germinal vesicle) was still in a central position. N = nucleus. Stained with Ehrlich's Haematoxylin and Eosin. Scale 0.1mm.

Plate 5.4. Section of an atretic oocyte from fish maintained in Groups A and B.


N



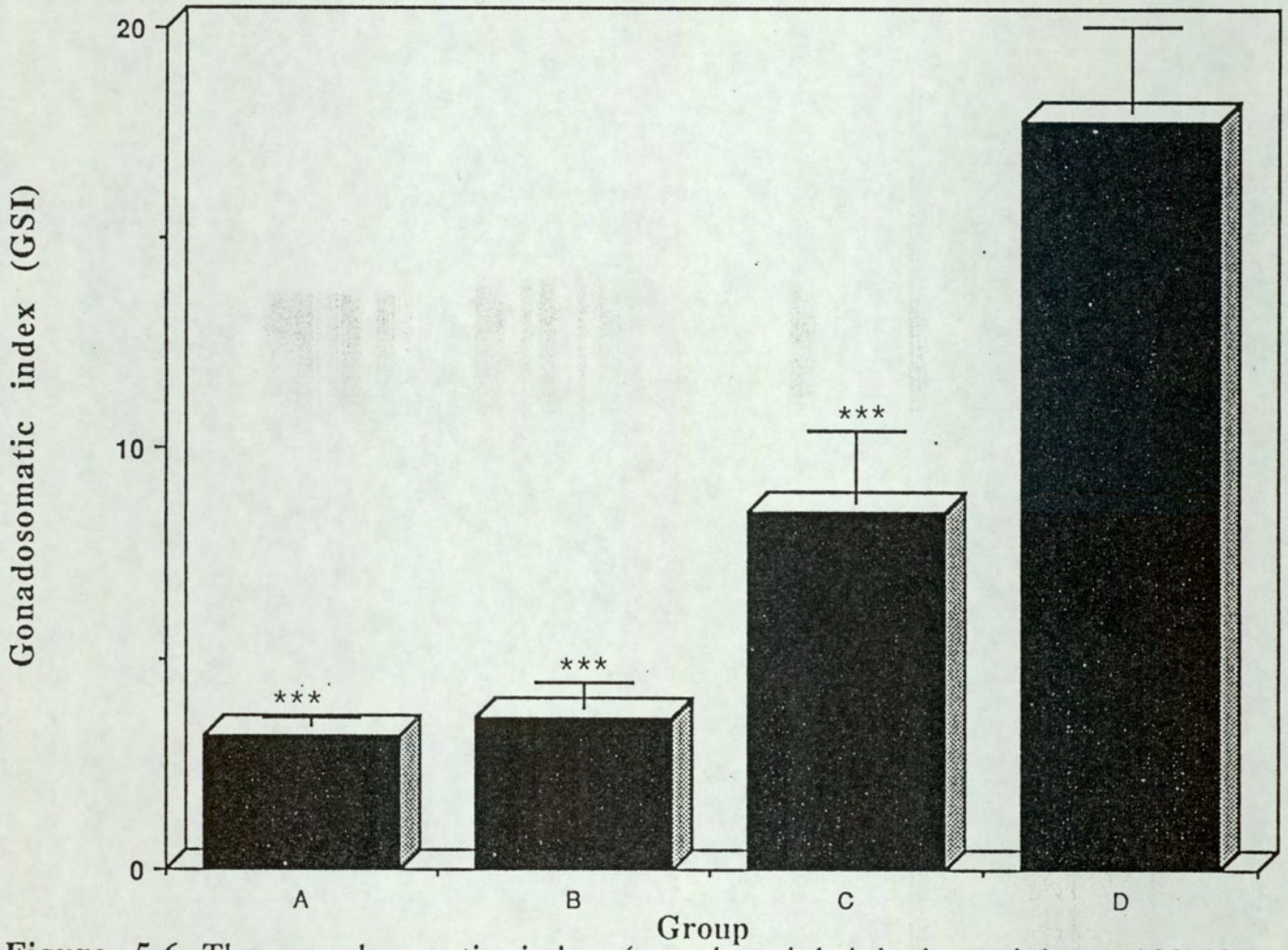


Figure 5.6 The gonadosomatic index (gonad weight/ body weight x 100)(GSI) recorded at the end of experiment 1, in fish maintained under 18L:6D (Group A) and 6L:18D (Group B) photoperiods, ambient photoperiod/ constant tempetature (Group C) and under natural conditions of both photoperiod and temperature (Controls; Group D). Vetrical bars denote standard deviations.

5.4.1. **Protocol**

Fifty dace of mixed sex, previously exposed to natural environmental conditions were equally divided (with regards to sex and weight) and maintained from June 1st (when the ambient daylength was 16L:8D) until the end of September under the following photoperiod regimes:- Group A-8L:16D, Group B- 12L:12D, Group C-14L:10D ,Group D-18L:6D, Group E- ambient photoperiod /constant temperature. Temperature was maintained constant at $11 \pm 0.5°C$ throughout the experimental period. In addition a further group of fish (controls)(Group F) were maintained under natural conditions of photoperiod and temperature.

Figure 5.7 outlines the photoperiods to which the different groups were treated.

5.4.2. **Results**

Figure 5.8 illustrates the change in fish weight from the beginning to the end of the experiment. At the end of the experiment, all the females had increased in weight. Differences between group weights were larger at the end of the experiment presumeably due to different lengths of daylength and therefore feeding time and also to differential growth of the ovary.

Vitellogenic oocytes were found in all experimental groups at the termination of the experiment. (see plate 3.6). However, oocyte size, the number of vitellogenic oocytes and the GSI indicies varied with the different photoperiods (figs. 5.9, 5.10 and 5.11).

The results demonstrated that ovarian development in fish exposed to an abrupt reduction in photoperiod at the start of the experiment, from 16L:8D (ambient) to 8L:16D (Group A) had been advanced with respect to the other Groups and the controls (Group F). Thus at the termination of the experiment in late September, Group A fish were close to spawning; GVM, a feature associated with final maturation and ovulation was clearly visible in this group (plate 5.5). In addition GSI values in this group were significantly higher ($P<0.001$) than Groups B,C,D and E, though similar to the controls (Group F)(fig. 5.11). Furthermore oocyte sizes in Group A were significantly greater than Groups B and C ($P<0.05$) and D ($P<0.001$) though again similar to the controls (Group F) (fig. 5.9).

In contrast to Group A, fish exposed to an increase in photoperiod from 16L:8D (ambient) to 18L:6D (Group D) had been delayed with respect to Groups A,B,C,E and the controls (Group F). Both the number of vitellogenic oocytes, oocyte size and GSI indices were significantly lower ($P<0.001$) in this Group.

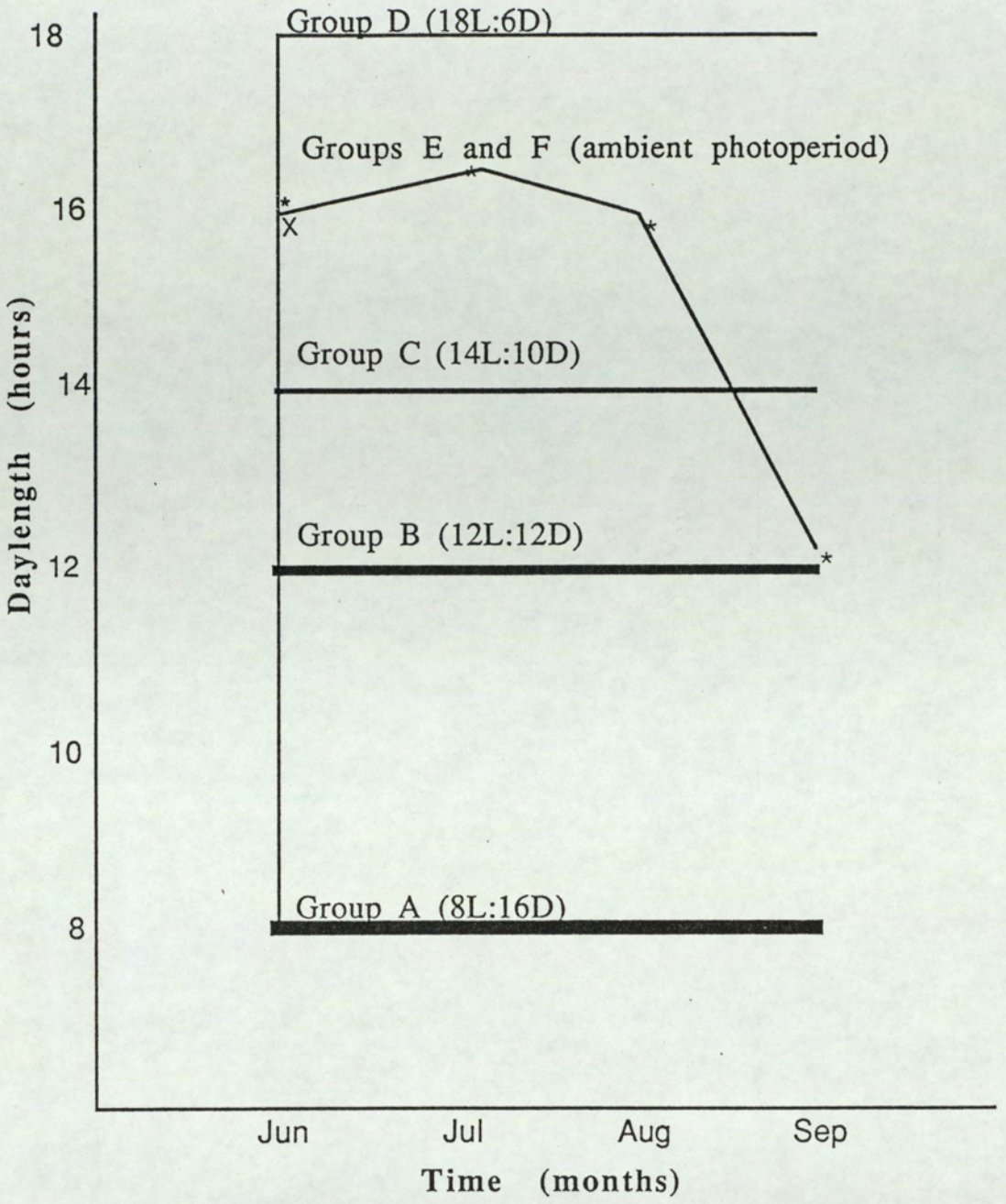


Figure 5.7 Experimental photoperiods in fish maintained in experiment 2 between June and September. X marks the natural photoperiod at the start of the experiment. Temperatures were maintained constant at 11+0.5°C in Groups A-E. Group F (controls) were maintained under naturally fluctuating photoperiod and temperature.

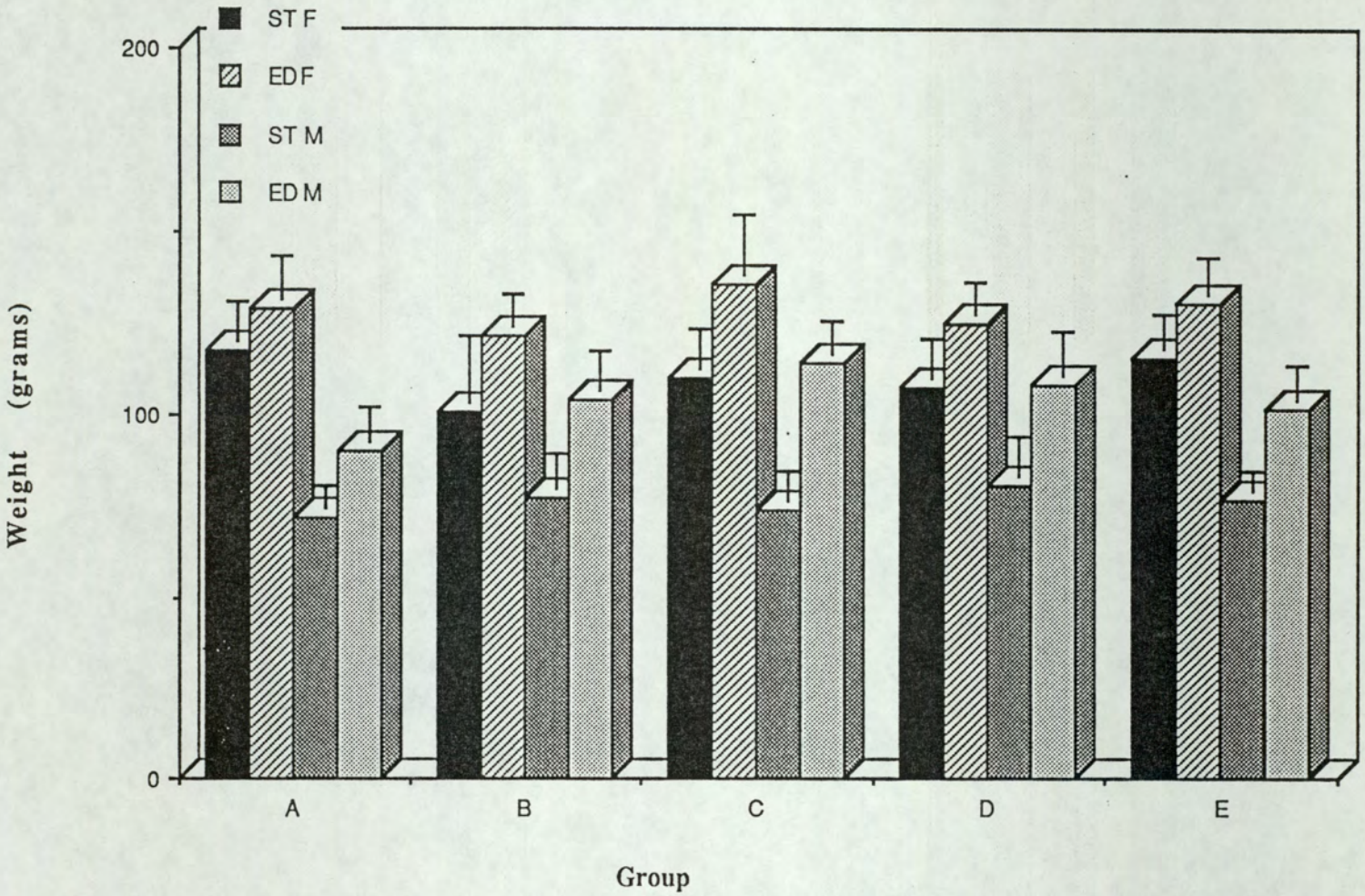
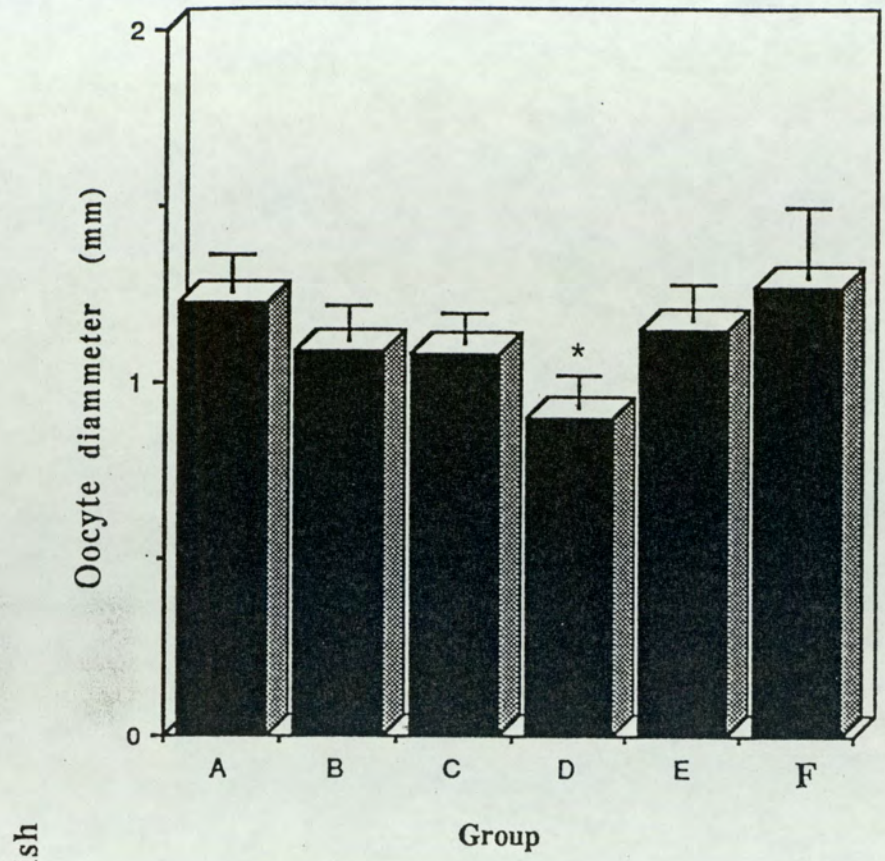
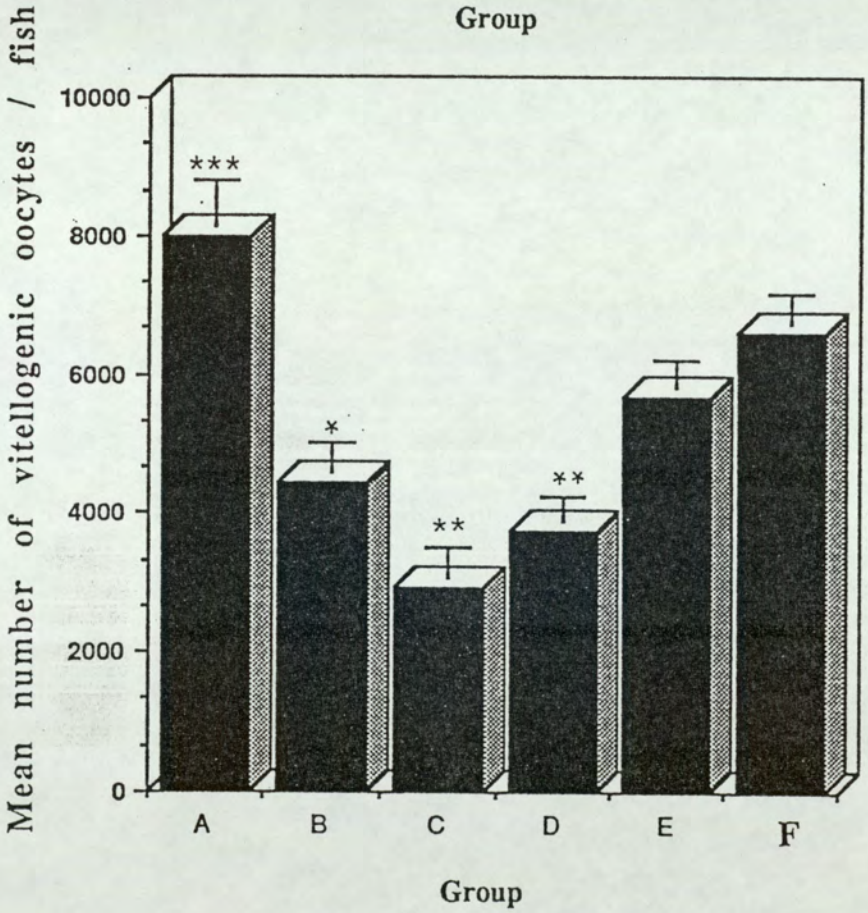


Figure 5.8. Growth of fish maintained in Groups A (8L:16D), B (12L:12D), C (14L:10D), Group D (18L:6D) and Group E (ambient photoperiod/constant temperature in experiment 2. Fish were weighed at the start (ST) and end (ED) of the experiment. F= female and M= male. Vertical bars denote standard deviations.





Figures 5.9 and 5.10 Oocyte diammeter (Fig. 5.9) and the mean total number of vitellogenic follicles / fish (Fig. 5.10) in fish maintained in experiment 2. Groups A, B, C, D, E and F (controls) represent fish maintained under 8L:16D,12L:12D,14L:10D,18L:6D, ambient photoperiods (constant temperature) and natural conditions of photoperiod and temperature respectively. Vertical bars denote standard deviations.

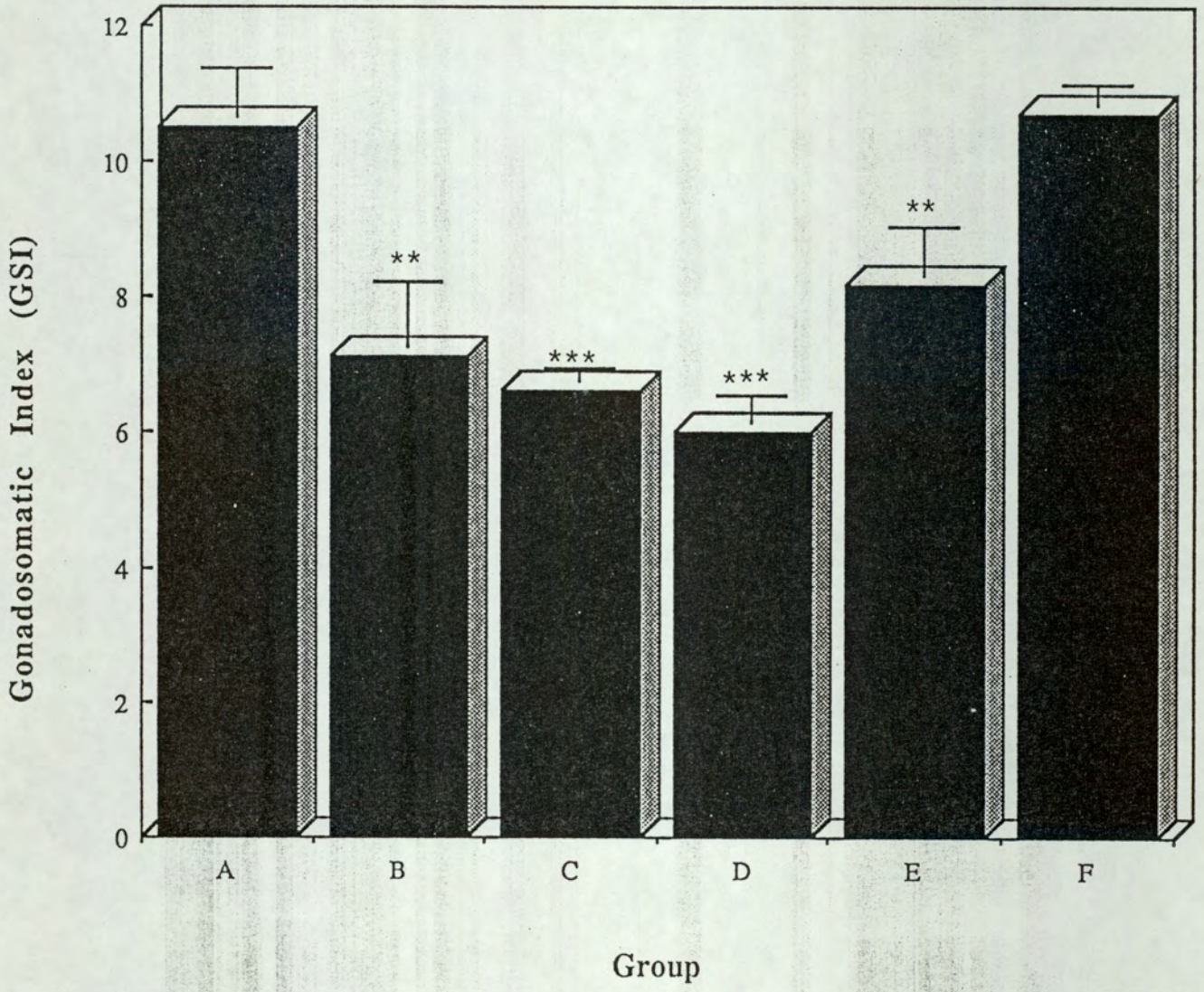


Figure 5.11 The gonadosomatic index (gonad weight/ body weight x 100)(GSI) of fish maintained in experiment 2. Groups A, B, C, D, E and F (controls) represent 8L;16D, 12L:12D, 14L:10D, 18L:6D, ambient photoperiod(constant temperature) and natural conditions of photoperiod and temperature respectively. Vertical bars denote standard deviations.

Intermediate rates of ovarian development were observed in fish exposed to lesser reductions in photoperiod, that is from 16L:8D (ambient) to 12L:12D (Group B) and 14L:10D (Group C). This was confirmed by both oocyte size (figure 5.9) and GSI (figure 5.11) data. Only fish maintained under an ambient photoperiod/constant temperature regime (Group E) showed similar gonadal development to the control fish (Group F) exposed to naturally fluctuating photoperiods and temperatures.

The photoperiod induced changes in ovarian development were paralleled by the associated changes in blood testosterone and calcium (index of vitellogenin) levels (figures 5.12 and 5.13). Similar patterns of serum T and Ca levels were observed in both fish maintained on an ambient photoperiod/constant temperature regime (Group E) and those maintained under natural conditions of photoperiod and temperature (Group F). In these Groups, a rise in serum levels of T and Ca occurred in August/ September (15-20 $ng.ml^{-1}$) and June (11-12 $mg.100ml^{-1}$) respectively. Whereas only a single peak of serum T, which occurred in August/September, was observed in the control fish (Group F), serum Ca levels remained elevated throughout the experiment (12-16$mg.100ml^{-1}$). Compared to the control fish (Group F), an elevation of serum T levels in fish exposed to shorter daylengths from the start of the experiment (Groups A, B and C) was advanced Thus an initial increase in T was observed in June in Group A (15$ng.ml^{-1}$) (8L:16D) and July (10-12$ng.ml^{-1}$) in Groups B and C. Interestingly serum T levels remained higher in Group A Fish than in Groups B and C. In contrast to fish maintained on shorter daylengths, in fish exposed to an initial increase in photoperiod (Group D), a rise in serum T did not occur until August/September (15$ng.ml^{-1}$), at the same time as the controls.

In contrast to the pattern of serum T levels in fish exposed to a shorter daylength at the start of the experiment (Groups A, B and C), serum Ca levels remained high (that is between 10-14$mg.100ml^{-1}$) throughout the experiment, similar to the controls (Group F). However the actual levels varied between the experimental Groups. Serum Ca levels in Groups B (10$mg.100ml^{-1}$) and C (10.5 $mg.100ml^{-1}$) were significantly lower ($P<0.01$) than those observed in Group A (14 $mg.100ml^{-1}$) maintained on the shortest day and the controls (Group F)(14.5$mg.100ml^{-1}$). Compared to fish maintained on short daylengths (Groups A,B and C), serum levels of Ca in fish maintained on long daylengths (Group D) did not rise above 8.5 $mg.100ml^{-1}$(basal) until September.

Serum E2 levels were initially high in April (up to 16$ng.ml^{-1}$) one

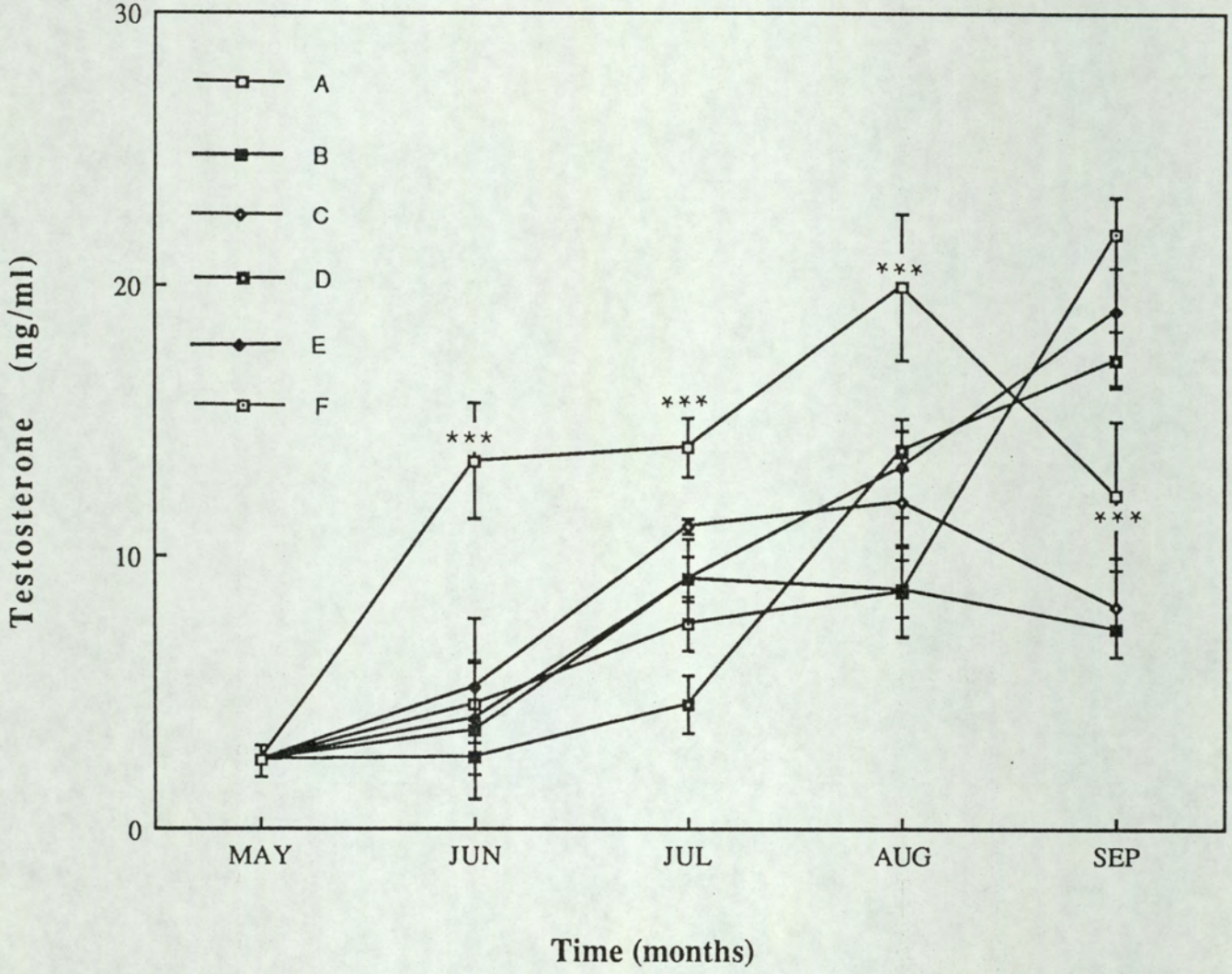


Figure 5.12 Serum testosterone levels ($ng.ml^{-1}$) in fish maintained in Groups A (8L:16D), B (12L:12D), C (14L:10D), D (18L:6D), E (ambient photoperiod / constant temperature) and F (controls maintained under natural conditions of photoperiod and temperature) in experiment 2. Vertical bars denote standard deviations.

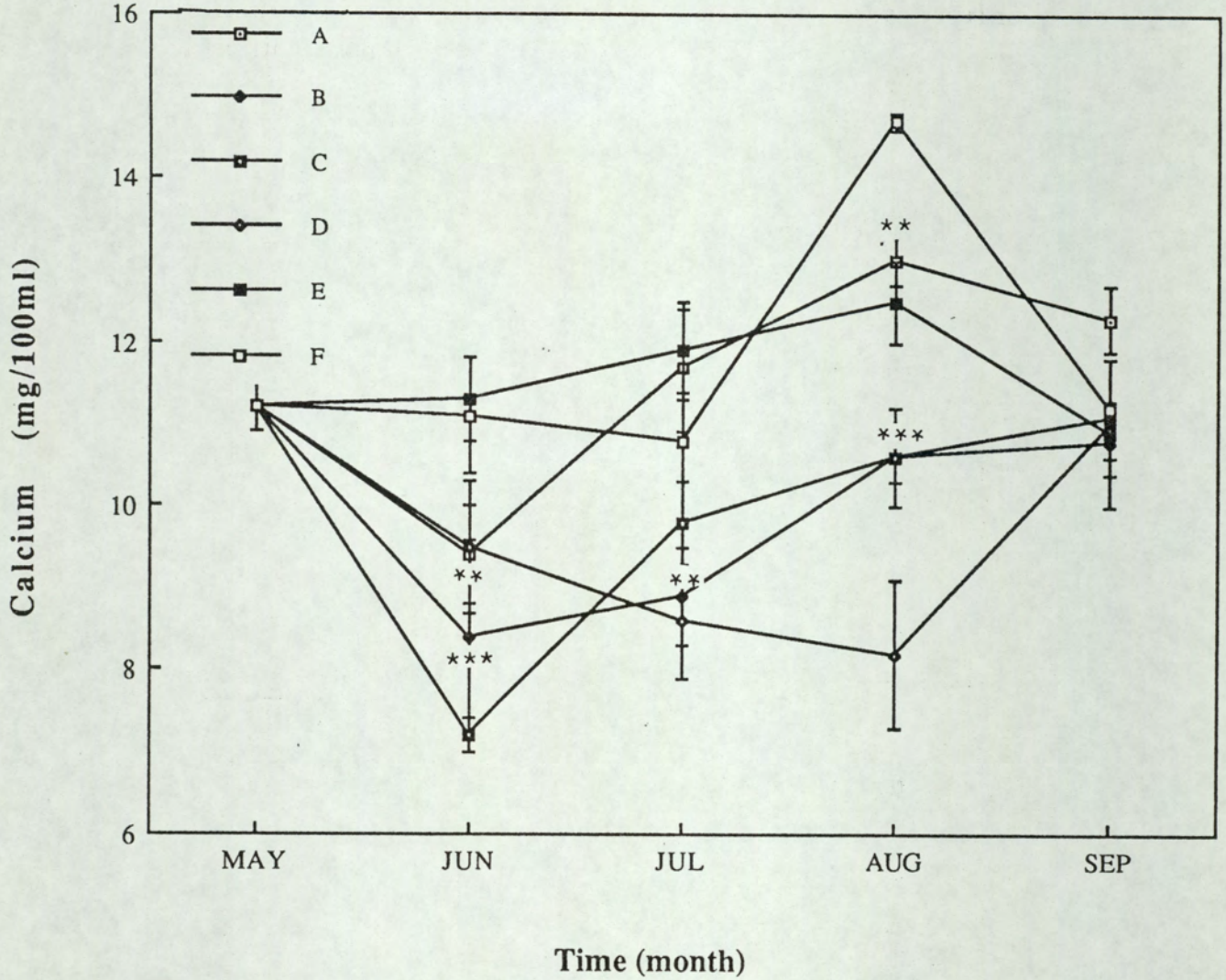


Figure 5.13 Serum calcium levels (mg.100ml^{-1}) in fish maintained in Groups A (8L:16D), B (12L:12D), C (14L:10D), D (18L:6D), E (ambient photoperiod / constant temperature) and F (controls maintained under natural conditions of photoperiod and temperature) in experiment 2. Vertical bars denote standard deviations.

month prior to the experiment, after which they declined to basal (upto 8ng.ml^{-1})(fig. 5.14). Therfore, at the start of the experiment E2 levels were basal. A further increase in serum E2 levels only occurred in Group E maintained on a simulated ambient photoperiod and the controls (Group F), which occurred in September (16ng.ml^{-1}); no elevations were observed in any of the other experimental Groups.

5.4.3. **Summary of results**

1) Ovarian development was advanced, with respect to the control fish, in fish exposed to a reduction in photoperiod at the start of the experiment (June). Observations on the number of vitellogenic oocytes, oocyte size, the GSI at the end of the experiment and serum hormone levels during the experiment indicate that Group A fish exposed to a reduction in photoperiod from 16L:8D (ambient) to 8L:16D were more advanced than Groups B and C exposed to less pronounced reductions in photoperiod. Furthermore only germinal vesicle migration could be oberved in Group A fish. In contrast fish exposed to an increase in photoperiod, that is from 16L:8D (ambient) to 18L:6D were delayed with respect to the controls (Group F).

2) A similar pattern of ovarian development was observed in fish maintained on an ambient photoperiod/constant temperature regime (Group E) to the controls maintained under natural conditions of photoperiod and temperature (Group F).

3) The photoperiod induced changes of ovarian development in Groups A-D were paralleled by the associated changes in serum T levels.

4) Serum levels of Ca, after an initial increase remained elevated throughout the experiment in all Groups except those maintained on long photoperiods (Group D). In addition serum Ca levels were significantly higher in Groups A (8L:16D) exposed to the shortest reduction in photoperiod (8L:16D) and F (controls) than Groups B (12L:12D), C (14L:10D), and D (18L:6D).

5) Serum E2 levels were only elevated in the controls (Group F) and fish maintained on an ambient photoperiod/constant temperature (group E) in September; basal levels were maintained in Groups A-D throughout the experimental period.

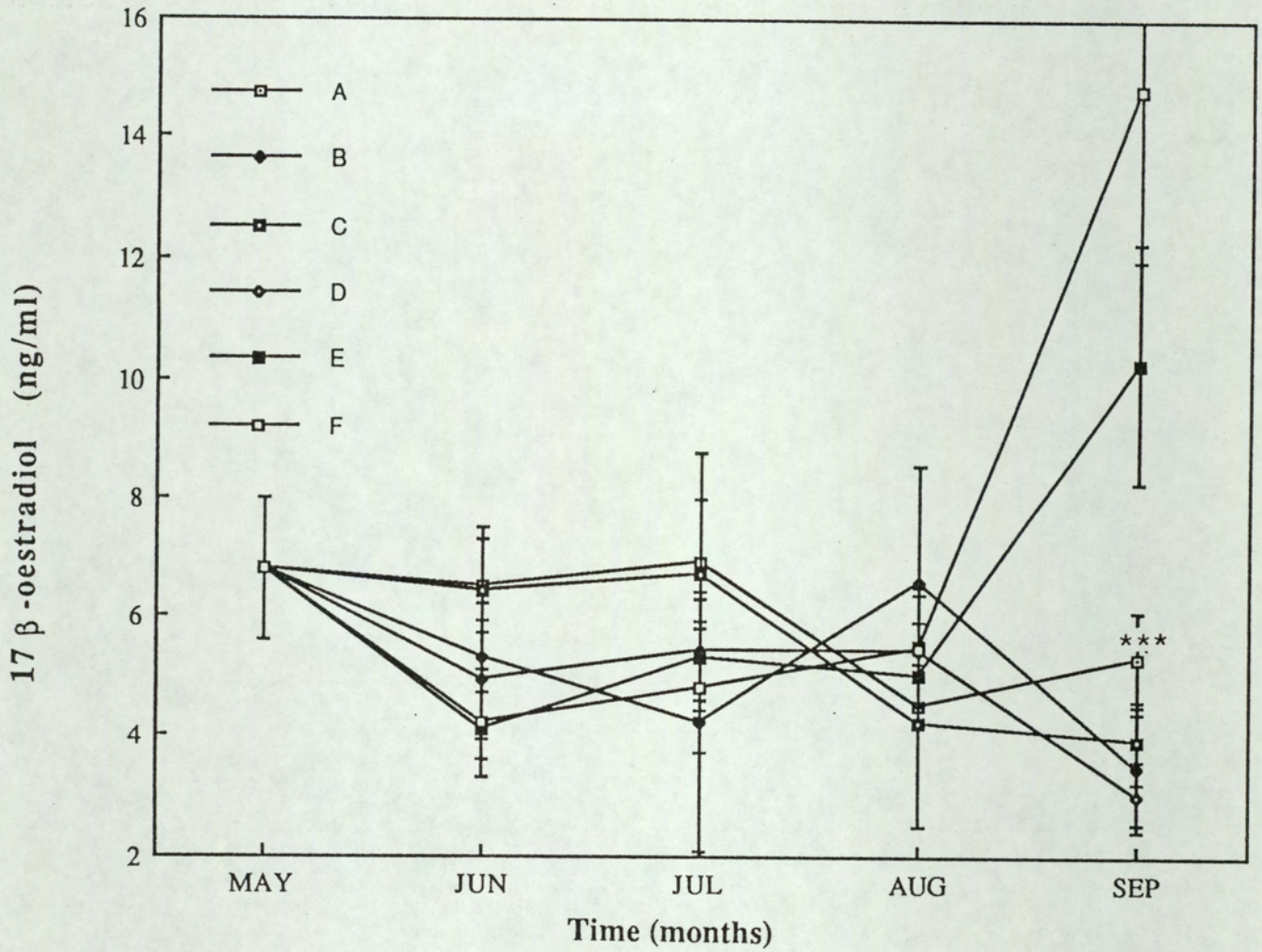


Figure 5.14. Serum 17β-oestradiol levels ($ng.ml^{-1}$) in fish maintained in Groups A (8L:16D), B (12L:12D), C (14L:10D), D (18L:6D), E (ambient photoperiod / constant temperature) and F (controls maintained under natural conditions of photoperiod and temperature) in experiment 2. Vertical bars denote standard deviations.

5.5 Experiment 3: The effect of constant long days prior to a reduction in photoperiod to short days during the early stages of ovarian development.

This experiment was run in parallel with the previous experiment. However, this study was initiated immediately after the previous spawning of the experimental fish, at the end of March.

5.5.1 Protocol

Thirty two dace of mixed sex, previously exposed to natural conditions were divided into 2 groups of 16 fish equally matched by weight and sex and transferred into 2 tanks (Groups A and B). The ovaries at this time were very small consisting only of previtellogenic follicles. In both groups, the fish were initially exposed to an increase in photoperiod from ambient (12.5L:11.5D) to a long daylength (18L:6D). This photoperiod regime was maintained until June, after which the fish were exposed to an abrupt reduction in daylength to 18L:6D. The photoperiod was then maintained on short daylengths for the duration of the experiment in Group A but changed to a long day in September in Group B. The temperature was maintained at $11\pm0.5°C$. The control fish (Group C) were the same as the previous experiment.

Figure 5.15 further illustrates the photoperiods both Groups of fish were exposed to.

5.5.2 Results

The results from ovarian biopsies of fish in both Groups A and B in September demonstrated that final maturation (upto GVM) had occurred, producing oocytes close to ovulation and spawning. Concomittant with Group A fish in the previous experiment, final maturation had therefore been advanced by approximately 5 months with respect to the controls (Group C).

The advancement of gonadal maturation presumably induced by the sharp reduction in photoperiod in June (to 8L:16D), was accompanied by the associated changes in serum T (Figs. 5.16). Serum T levels once elevated (9-10ng.ml^{-1}) then increased gradually until September to reach a maximum of 20ng.ml^{-1}. Therefore the highest levels of serum T occurred just prior to final maturation. In contrast an elevation of serum T levels did not occur in the control fish (Group C) until August/September.

Serum Ca levels are illustrated in Fig. 5.17. This figure

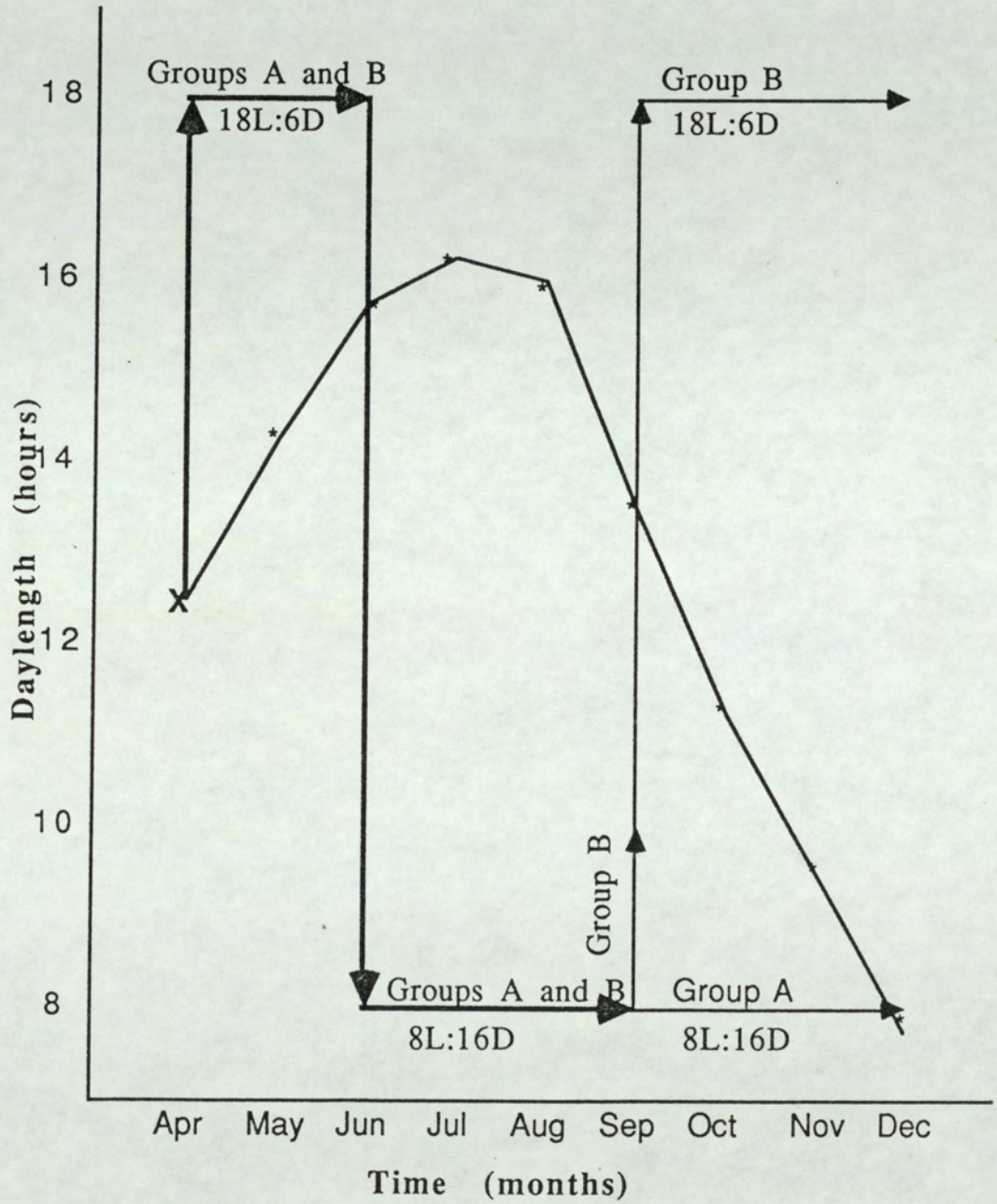


Figure 5.15 Experimental photoperiod regimes used in experiment 3 between April and December. X marks the photoperiod at the start of the experiment. Temperatures were maintained constant at 11+0.5°C in Groups A and B. Group C were maintained under naturally fluctuating conditions of photoperiod and temperature.

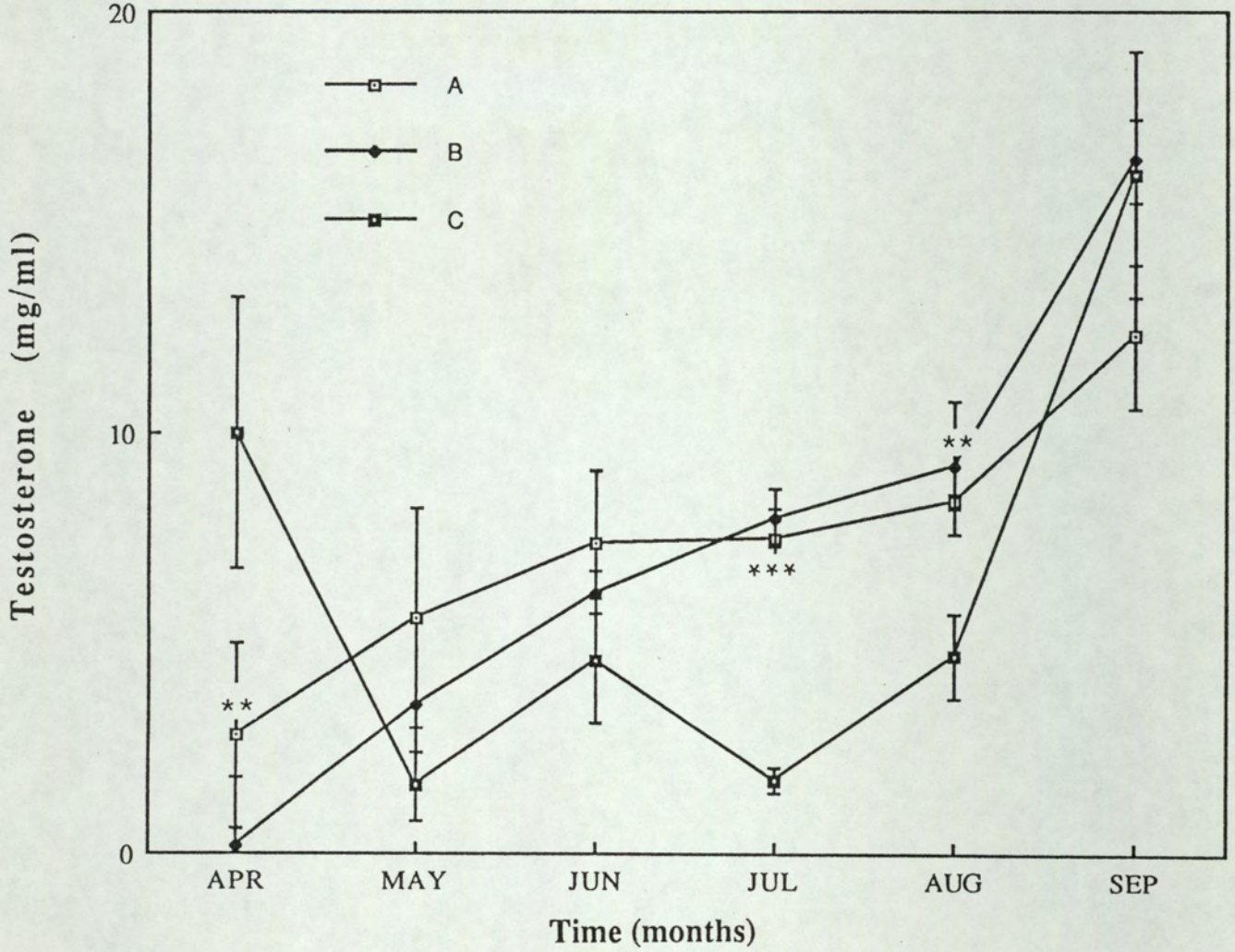


Figure 5.16 . Serum testosterone levels ($ng.ml^{-1}$) in fish maintained in experiment 3. Groups A and B were maintained under the same photoperiod (18L:6D from April to June and 8L:18D from June to September, after which Group B was rexposed to 18L:6D). The controls (Group C) were maintained under natural conditions of photoperiod and temperature. Vertical bars denote standard deviations.

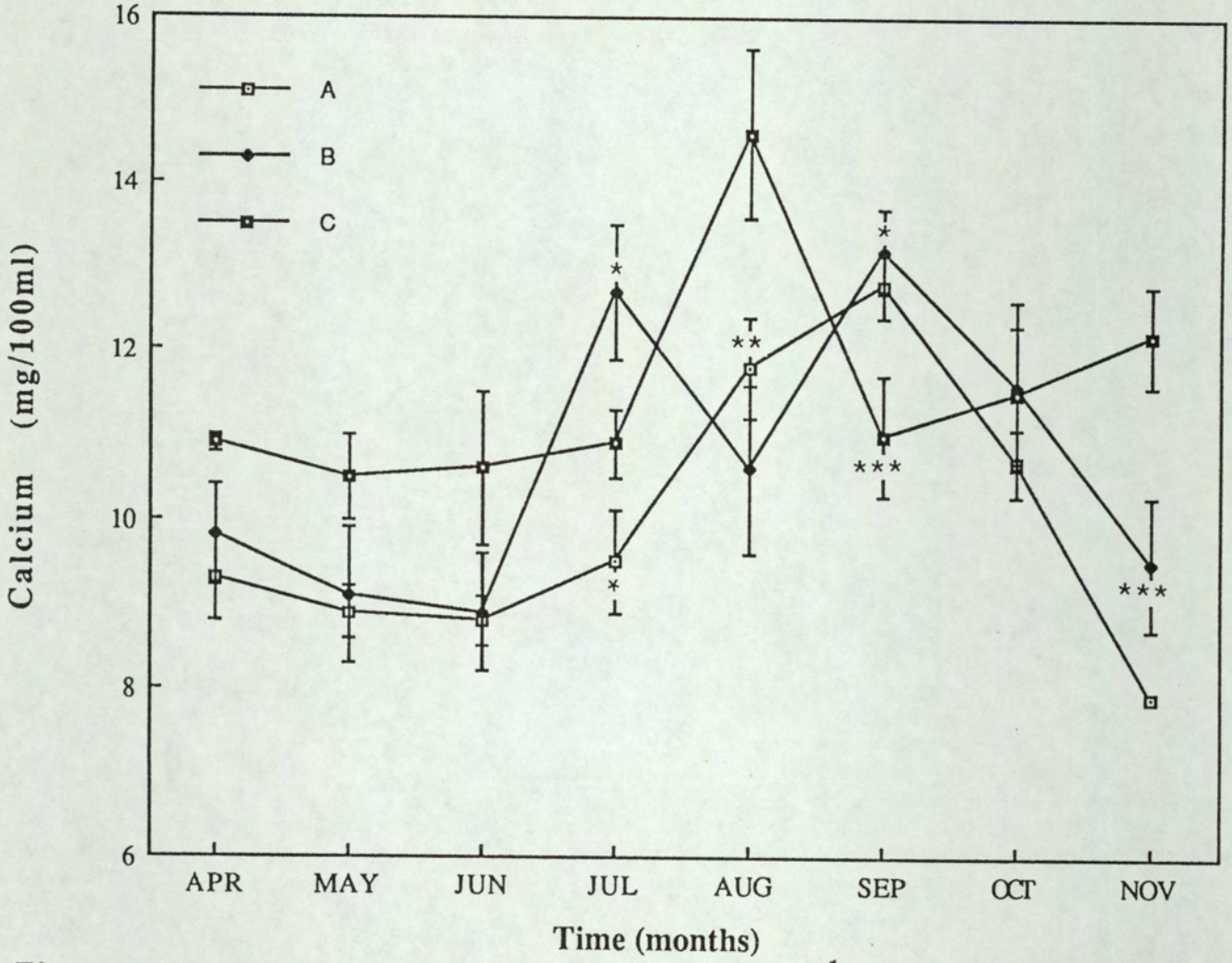


Figure 5.17 . Serum calcium levels ($mg.100ml^{-1}$) in fish maintained in experiment 3. Groups A and B were maintained under the same photoperiod (18L:6D from April to June and 8L:16D from June to September after which Group B was rexposed to 18L:6D). The controls (Group C) were maintained under natural conditions of photoperiod and temperature. Vertical bars denote standard deviations.

demonstrates that serum Ca levels in the controls (Group C) were significantly ($P<0.01$) higher (up to 11mg.100ml^{-1}) than Groups A and B (up to 9mg.100ml^{-1}) until June. After this time when Group A and B fish had been exposed to a reduction in photoperiod from 18L:6D to 8L:16D, elevations in serum Ca levels were observed in both Groups A and B up to 12.4 and 12mg.100ml^{-1} respectively. High levels were then maintained until September (the time that final maturation occurred) in both Groups A and B and the controls (15-16mg.100ml^{-1}), after which they returned to basal.

In contrast to the pattern of serum T and Ca levels, serum E2 levels after an initial rise (which occurred one month after the controls) at the beginning of the experiment (in May) remained at low levels throughout the experiment in both Groups A and B(fig. 5.18). A further elevation of serum E2 levels (15ng.ml^{-1}) was only observed in the control fish (Group C). This occurred in September.

Although the experiment was not terminated in September, 2 of the females were sacrificed from each tank. As spawning had not occurred in the remaining fish they were also sacrified in December. GSI values in this experiment were highest in September, around 10% at the time of final maturation. In fish sacrificed in December, histological examination of the ovary revealed massive levels of atresia (section 3.1) and the mean GSI had fallen from 10% to 1-2%.

Similar levels of gonadal development and the associated changes in hormone levels were observed in both experimental groups, irrespective of the change in photoperiod to long daylengths in Group B at the end of September.

5.5.3 Summary of results

1) A similar pattern of ovarian development was observed in Groups A and B maintained initially on the same photoperiod regime and later on different ones (from September- Group A-maintained on a 8L:16D photoperiod and Group B-changed to a 18L:6D photoperiod regime). In these Groups final maturation occurred in September 5 months in advance of the controls (Group C). This was demonstrated by G.V.M. in the majority of oocytes, the GSI and serum levels of T.

2) After September, because the fish were not artificially induced to ovulate, the ovaries irrespective of the photoperiod regime in the different Groups regressed. In association with this, serum levels of T, E2 and Ca had all returned to basal and the GSI had declined.

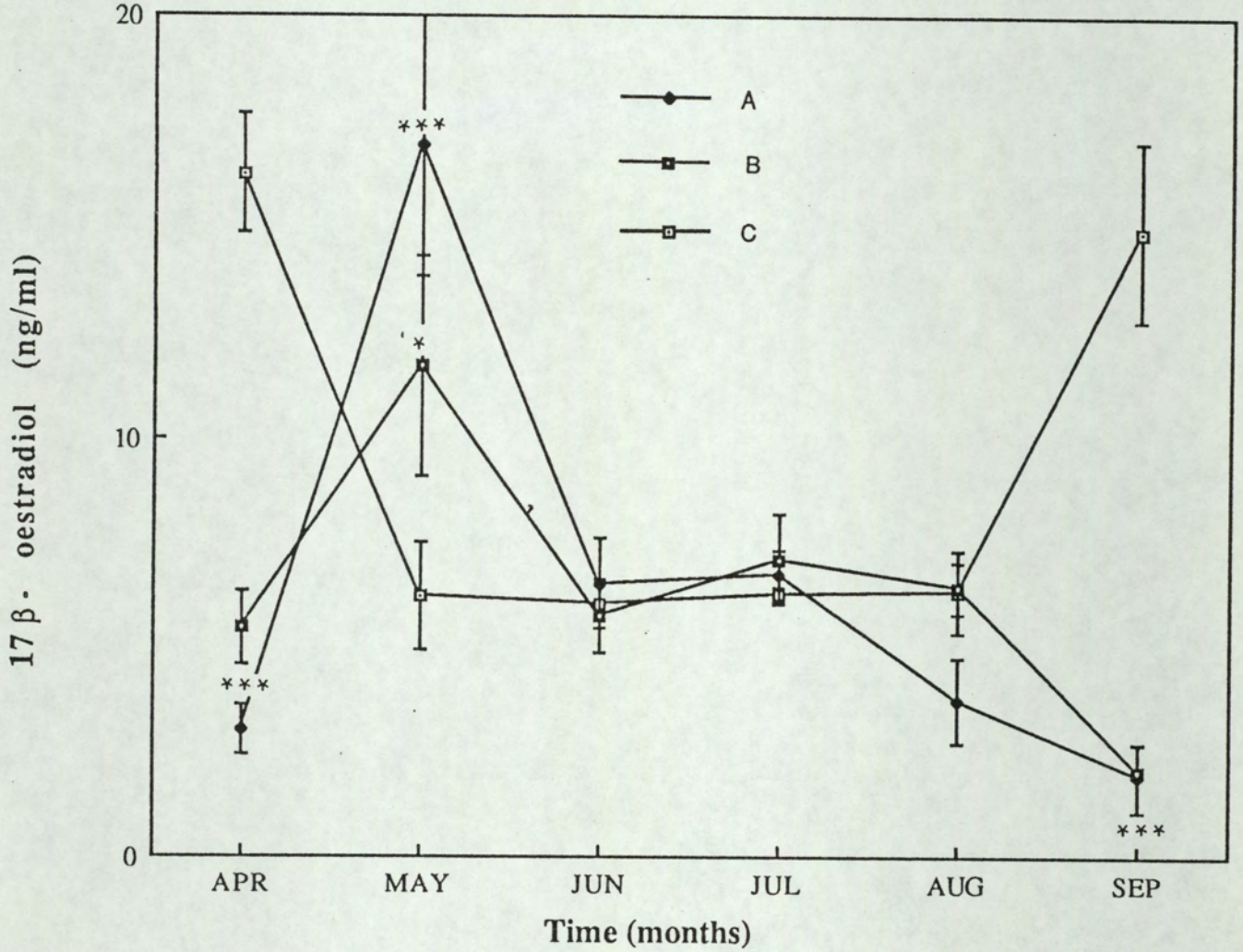


Figure 5.18 Serum 17β-oestradiol levels ($ng.ml^{-1}$) in fish maintained in experiment 3. Groups A and B were maintained under the same photoperiod (18L:6D from April to June and 8L:16D from June to September, after which Group B was rexposed to 18L:6D). The controls (Group C) were maintained under natural conditions of photoperiod and temperature. Vertical bars denote standard deviatons.

5.6 Experiment 4: The effect of abrupt changes in photoperiod from long to short days at various time intervals between April and September during the early stages of ovarian development.

This experiment was designed firstly to investigate whether the presence of constant long daylengths before a transfer to short daylengths, were required to advance final maturation, and if so for what period of exposure to long daylengths, and secondly to investigate whether constant short days from the start of the experiment have the same effect on gonadal maturation, as an abrupt change from long to short days. As this experiment involved an initial change in photoperiod at the start of the experiment, (that is from ambient to either a long or short days which may have influenced the results), one group of fish were also maintained on the ambient photoperiod at the start of the experiment (12.5L:11.5D).

5.6.1. Protocol

Eighty dace of mixed sex matched by sex and weight were equally divided and maintained under the following photoperiod regimes from the end of March, when the ambient photoperiod was 12.5L:11.5D:- Group A- maintained on the ambient photoperiod at the start of the experiment (12.5L:11.5D), Group B- constant short daylengths (8L:16D), Group C- constant long daylengths (18L:6D), Group D- long daylengths (18L:6D) for one month followed by a reduction to short daylengths (8L:16D), Group E- long daylengths for two months followed by a reduction to short daylengths, Group F- long daylengths for 3 months followed by a reduction to short days, Group G- long daylengths for 4 months followed by a reduction to short daylengths and Group H long daylengths for 3 months followed by a reduction to short daylengths for one month. This was followed by a further increase to long daylengths. The temperature was maintained constant at 11 ± 0.5°C in all the Groups.

In addition a control group of fish were maintained under natural conditions of photoperiod and temperature (Group I)

Figure 5.19 further outlines the experimental photoperiod regimes used in this experiment.

The experiment was terminated at the end of September.

In this experiment a different RIA procedure was used to analyse

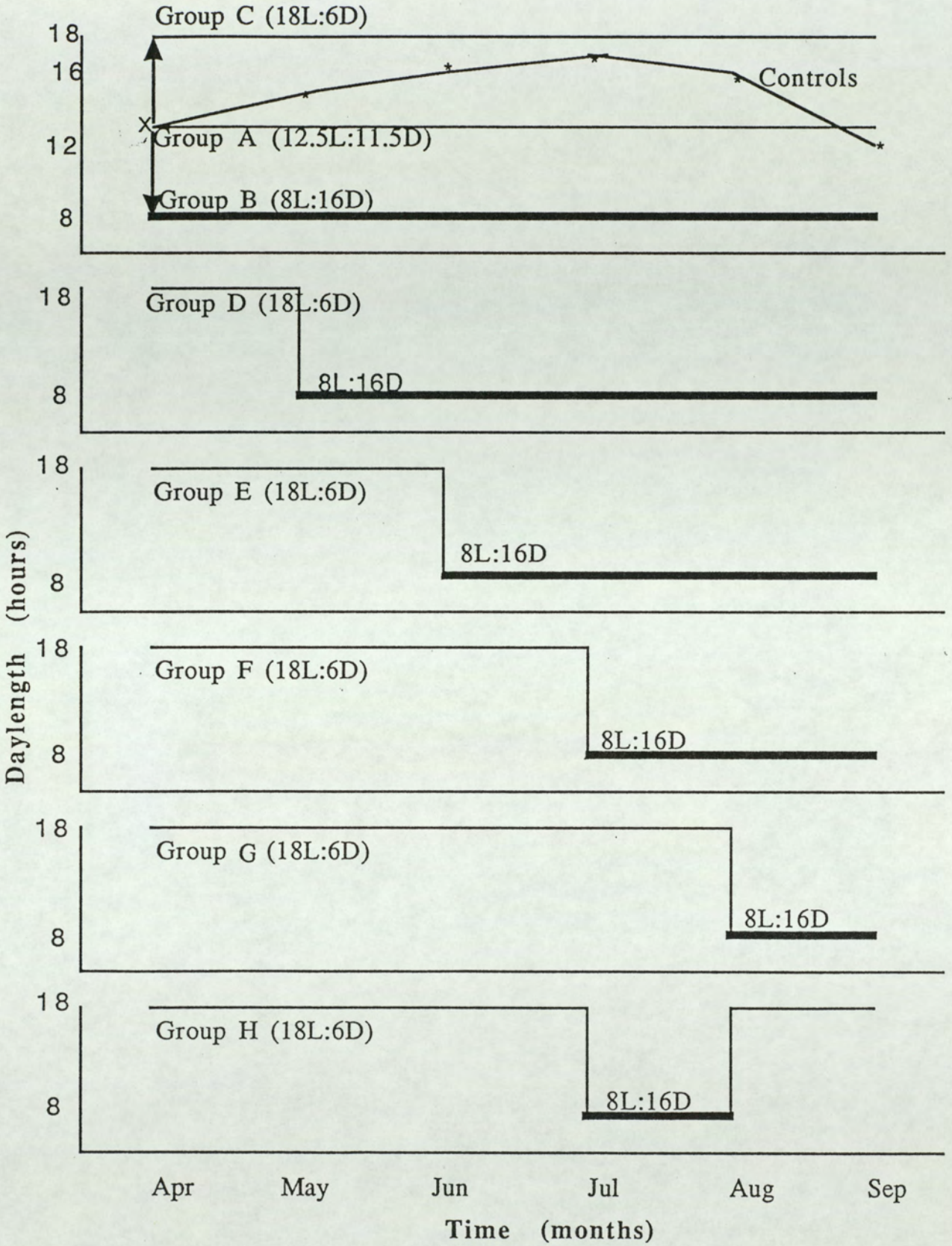


Figure 5.19 Experimental photoperiod regimes used in experiment 4. Fish were exposed to different daylengths between the previous spawning (March) and September. In all Groups, except Group I (controls), the temperature was maintained constant at 11±0.5°C. In the controls (Group I), fish were exposed to naturally fluctuating photoperiod and temperature.

serum levels of T and E2 compared to the previous experiments i.e different label and antisera were employed (see section 2.4.2.4.). Although higher hormonal levels were comparable using the different procedures, basal levels were generally lower with the method used in this experiment.

5.6.2. **Results**

The mean weights of fish at the beginning and end of the experiment are shown in figure 5.20. All experimental Groups showed an overall increase in body weight, indicating that the fish had acclimatized to captive conditions.

The results demonstrate that gonadal maturation was advanced (compared to the controls, Group I) in all Groups exposed to a short daylength during the experiment (Groups A,B,D,E,F,G and H). In contrast, in fish exposed to continuous long days (Group C), gonadal development had been delayed with respect to the control fish. This datum was indicated by both serum levels of T, Ca and E2 during the experiment and histological analyses of the ovary at the end of the experiment.

The levels of serum T, E2 and Ca are shown in figures 5.21, 5.22 and 5.23 a and b respectively. The different patterns of ovarian growth which occurred in the experimental Groups, are indicated by serum T levels. In all cases on transfer of fish to a short daylength, high serum T levels were detected one month later. In fish maintained on constant ambient daylengths (12.5L:11.5D) from the start of the experiment (Group A) and those exposed to a reduction in photoperiod from ambient to short daylengths (Group B), serum T levels began to rise in July and June respectively in advance of the control fish (Group I)(fig. 5.21). Peak serum T levels were observed in both these Groups in August (Group A- 10-11.5 $ng.ml^{-1}$ and Group B- 8-10$ng.ml^{-1}$). In fish changed from constant long days (18L:6D) to short days (8L:16D), after various periods of time, serum levels of T increased first in Group D (June)(5-6 $ng.ml^{-1}$) exposed to a reduction in daylength in May and Group E (July)(3.5- 5$ng.ml^{-1}$) exposed to a reduction in photoperiod in June. Similarly serum T levels increased in Groups F (10$ng.ml^{-1}$) and H (4$ng.ml^{-1}$) in August, and Group G(8-10$ng.ml^{-1}$) in September. In contrast, in fish maintained on constant long days serum T levels did not show an increase until September similar to the controls (Group I). In Group H fish after an initial rise in serum T levels on exposure to short days (8L:16D), levels declined on transfer to long days (18L:6D) by the end of August .

Similar to the pattern of serum T levels, serum Ca levels were also

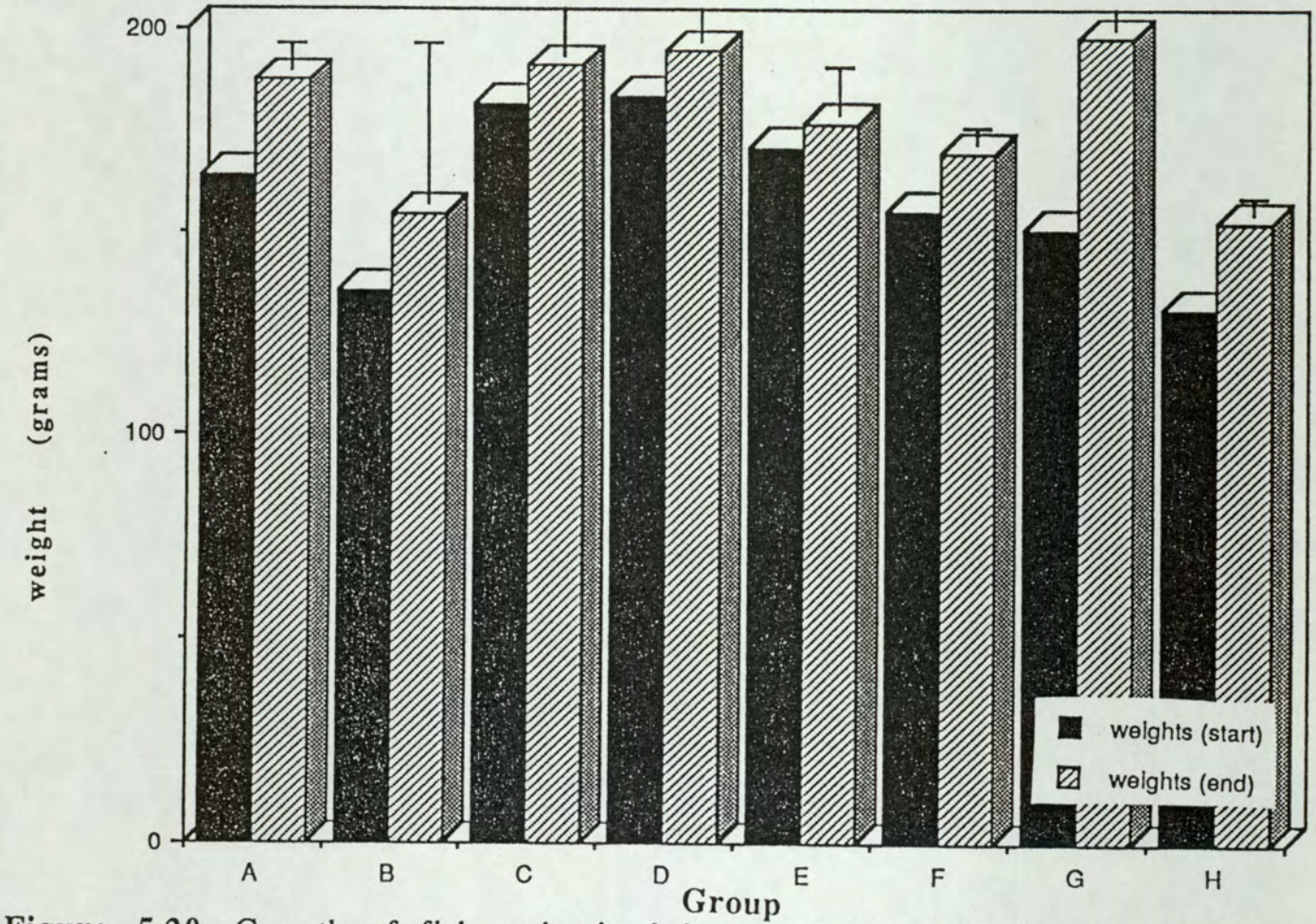


Figure 5.20. Growth of fish maintained in experiment 4. Each Group represents fish maintained under different photoperiod treatments. These were Groups A (12.5L:11.5D), B (8L:16D), D, E, F and G (18L:6D for 1, 2, 3 and 4 months respectively followed by a decrease to 8L:16D), H (18L:6D for 3 months, 8L:16D for 1 month followed by rexposure to 18L:6D) and I (controls maintained under natural conditions of photoperiod and temperature). Each fish was weighed at the start and end of the experiment. Vertical bars denote standard deviations.

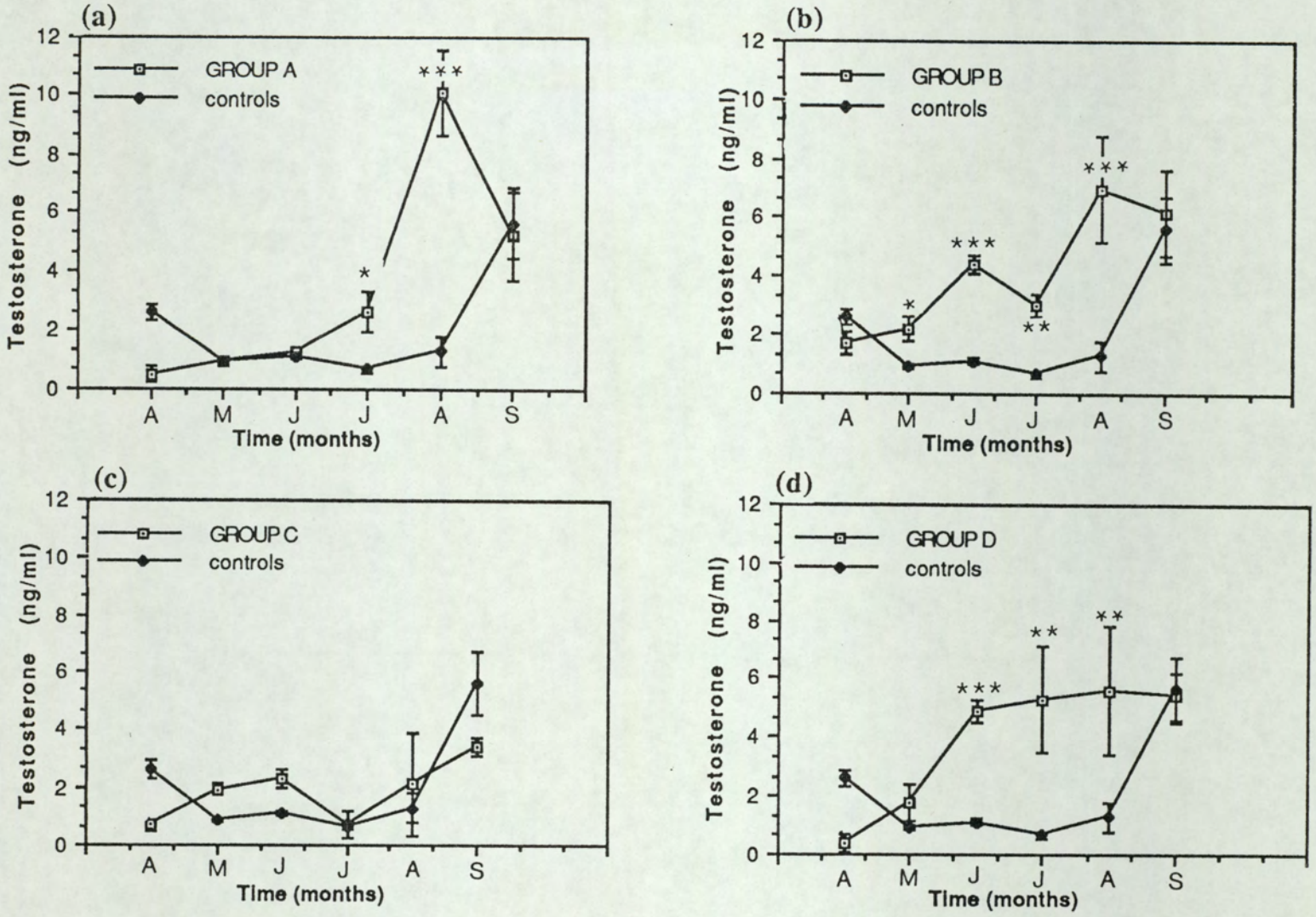


Figure 5.21 a-d. Serum testosterone levels ($ng.ml^{-1}$) in (a) Group A (12.5L:11.5D), (b) Group B (8L:16D), (c) Group C (18L:6D) and (d) Group D (18L:6D for 1 month followed by 8L:16D) in experiment 4. Control fish (Group I) were maintained under natural conditions of photoperiod and temperature. Vertical bars denote standard deviations.

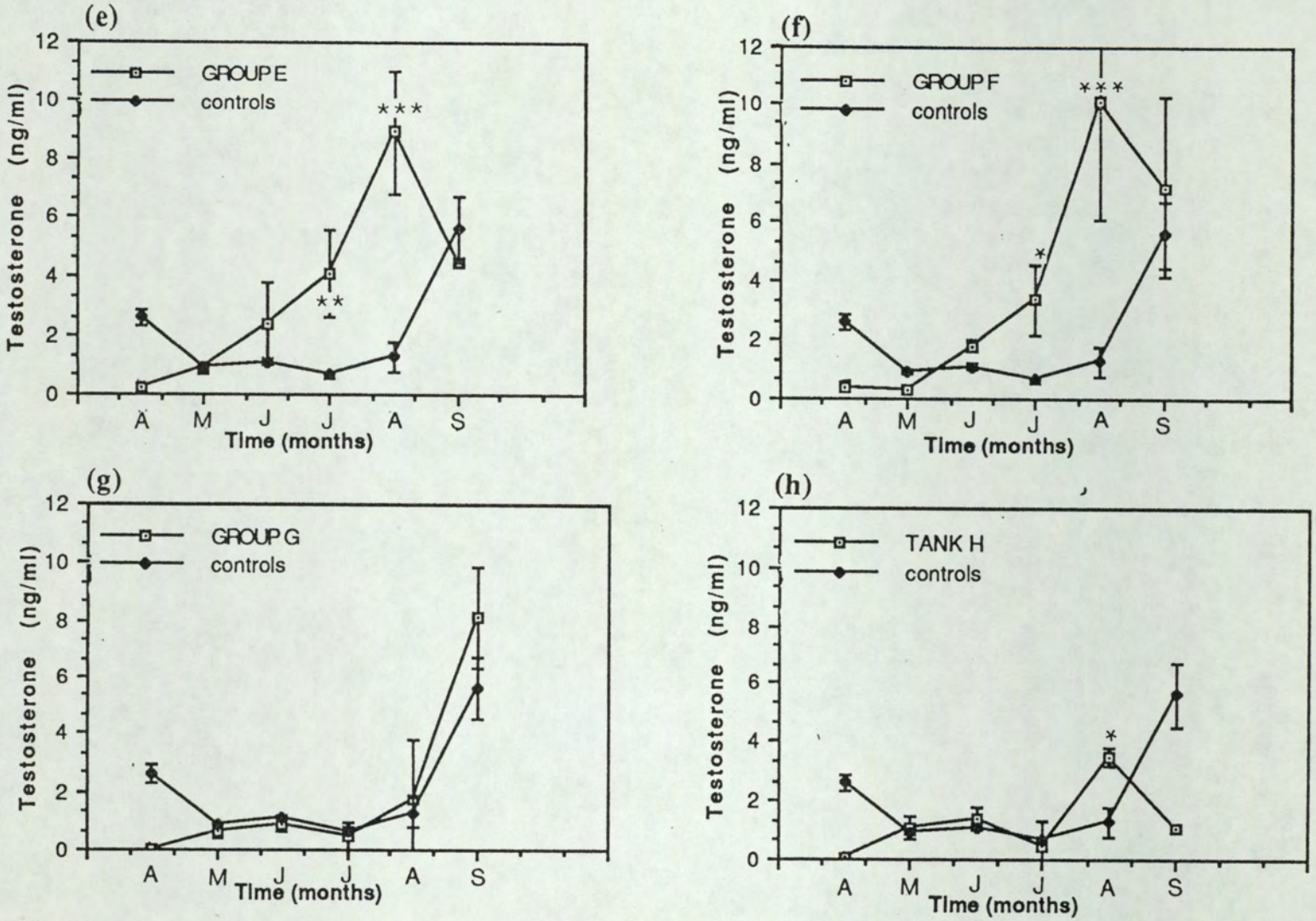


Figure 5.21 e-h. Serum testosterone levels ($ng.ml^{-1}$) in (e) Group E (18L:6D for 2 months followed by 8L:16D), (f) Group F (18L:6D for 3 months followed by 8L:16D), (g) Group G (18L:6D for 4 months followed by 8L:16D) and (h) Group H (18L:6D for 3 months, 8L:16D for 1 month followed by 18L:6D) in experiment 4. Control fish (Group I) were maintained under natural conditions of photoperiod and temperature. Vertical bars denote standard deviations.

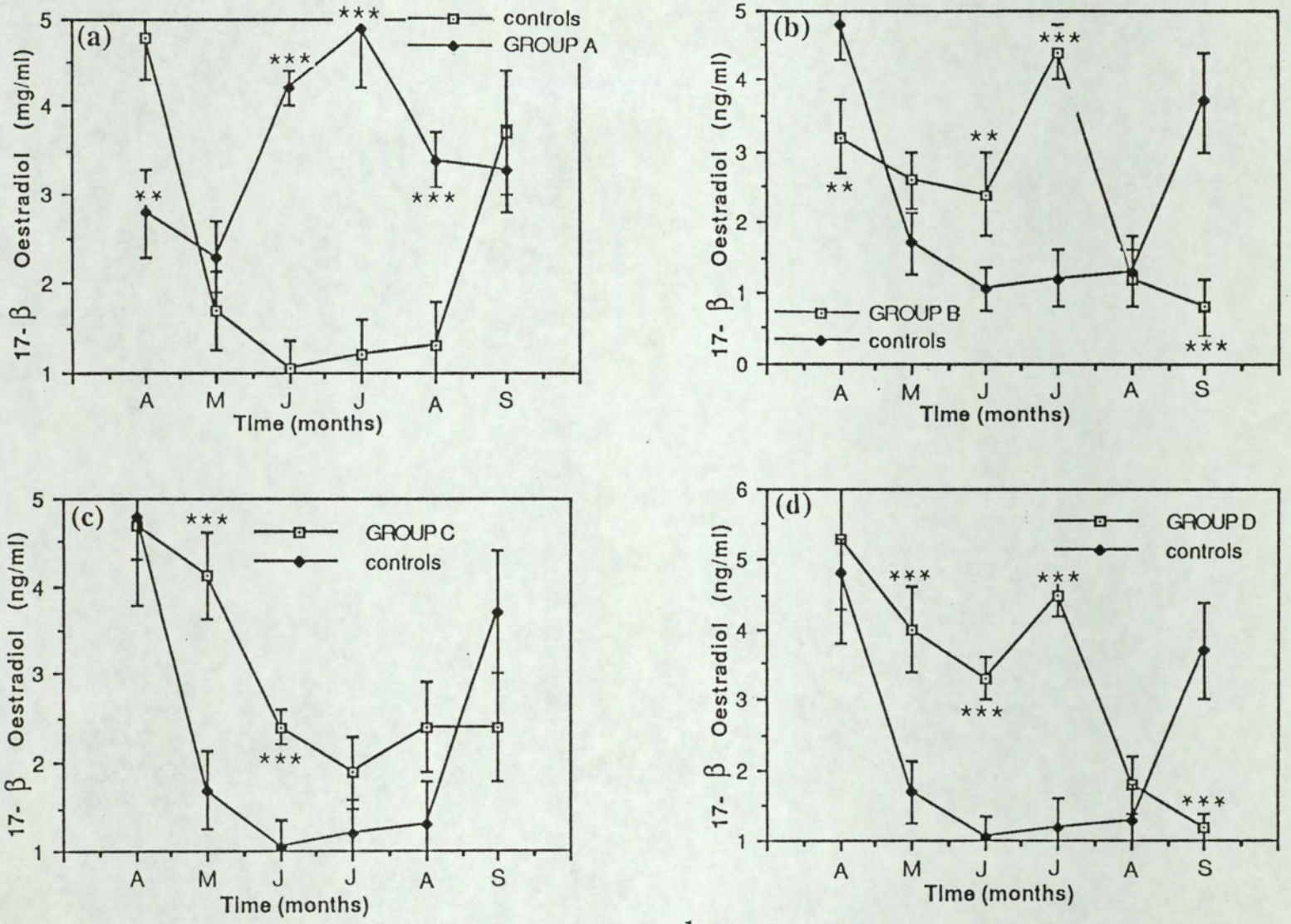


Figure 5.22 a-d. Serum 17β-oestradiol levels ($ng.ml^{-1}$) in (a) Group A (12.5L:11.5D), (b) Group B (8L:16D), (c) Group C (18L:6D) and (d) Group D (18L:6D for 1 month followed by 8L:16D) in experiment 4. Control fish (Group I) were maintained under natural conditions of photoperiod and temperature. Vertical bars denote standard deviations.

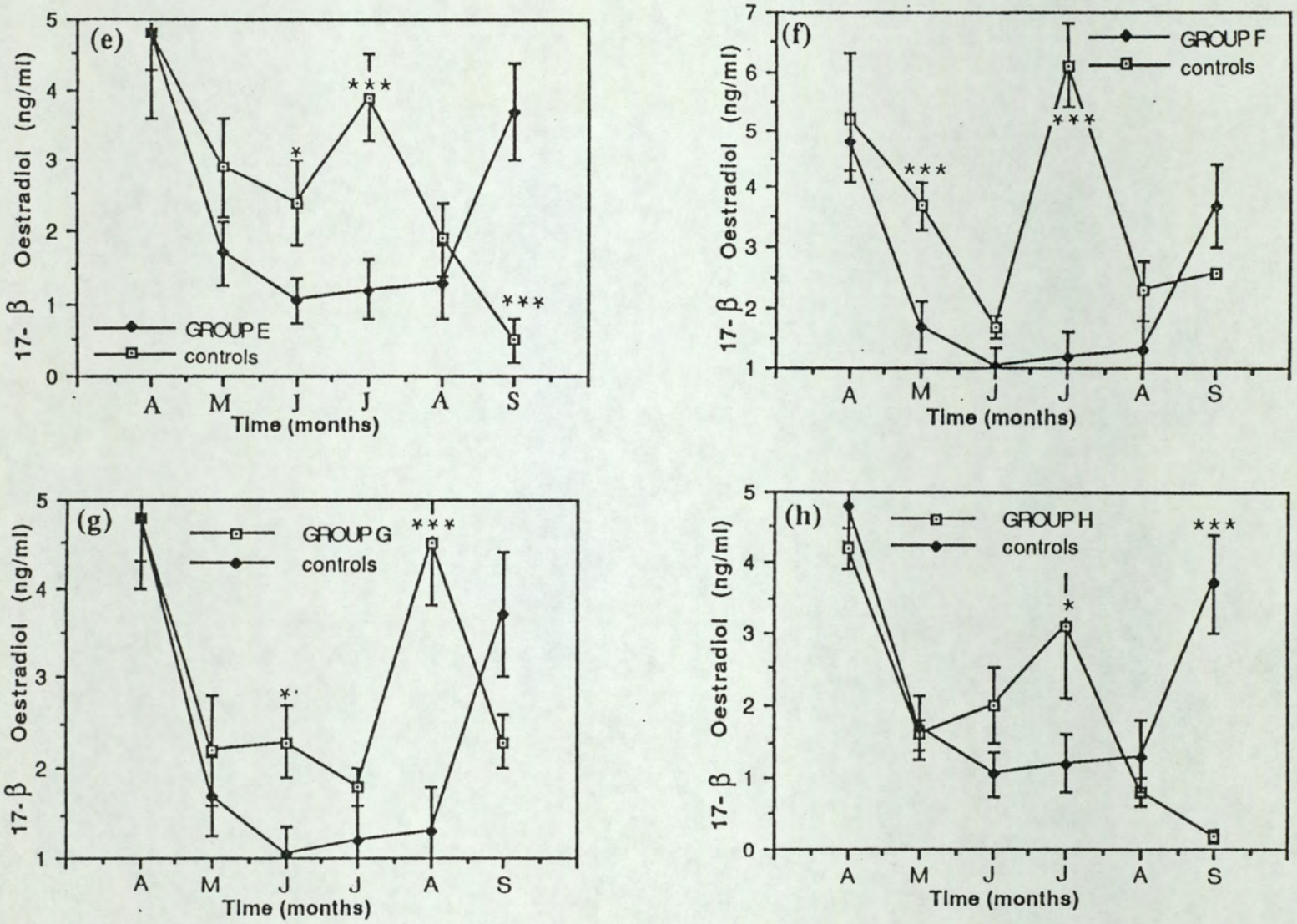


Figure 5.22 e-h. Serum 17β-oestradiol levels ($ng.ml^{-1}$) in (e) Group E (18L:6D for 2 months followed by 8L:16D), (f) Group F (18L:6D for 3 months followed by 8L:16D), (g) Group G (18L:6D for 4 months followed by 8L:16D) and (h) Group H (18L:6D for 3 months, 8L:16D for 1 month followed by 18L:6D) in experiment 4. The control fish (Group I) were maintained under natural conditions of photoperiod and temperature. Vertical bars denote standard deviations.

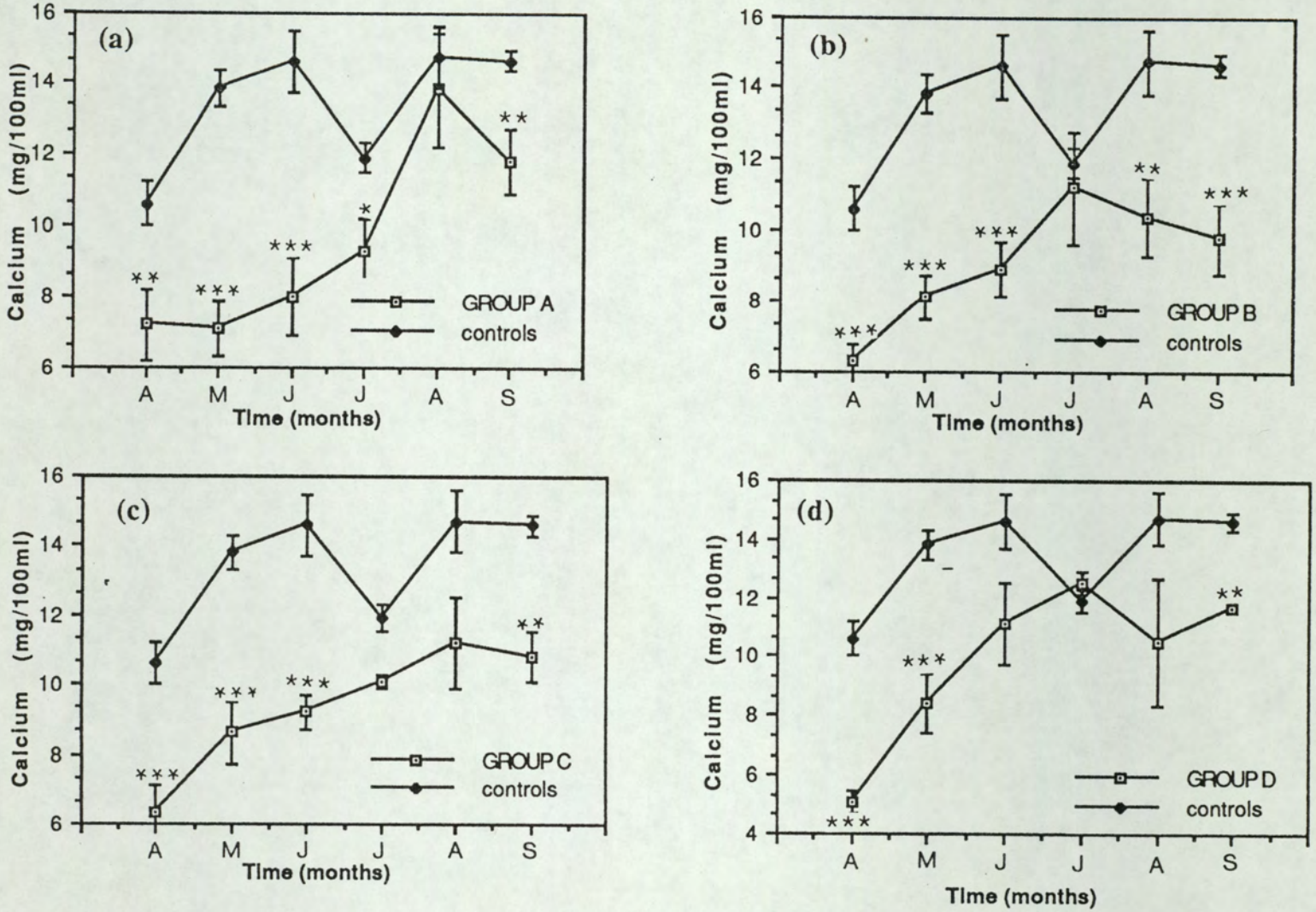


Figure 5.23 a-d. Serum calcium levels ($mg.100ml^{-1}$) (as an index of vitellogenin levels) in (a) Group A (12.5L:11.5D), (b) Group B (8L:16D), (c) Group C (18L:6D) and (d) Group D (18L:6D for 1 month followed by 8L:16D) in experiment 4. The controls (Group I) were maintained under natural conditions of photoperiod and temperature. Vertical bars denote standard deviations.

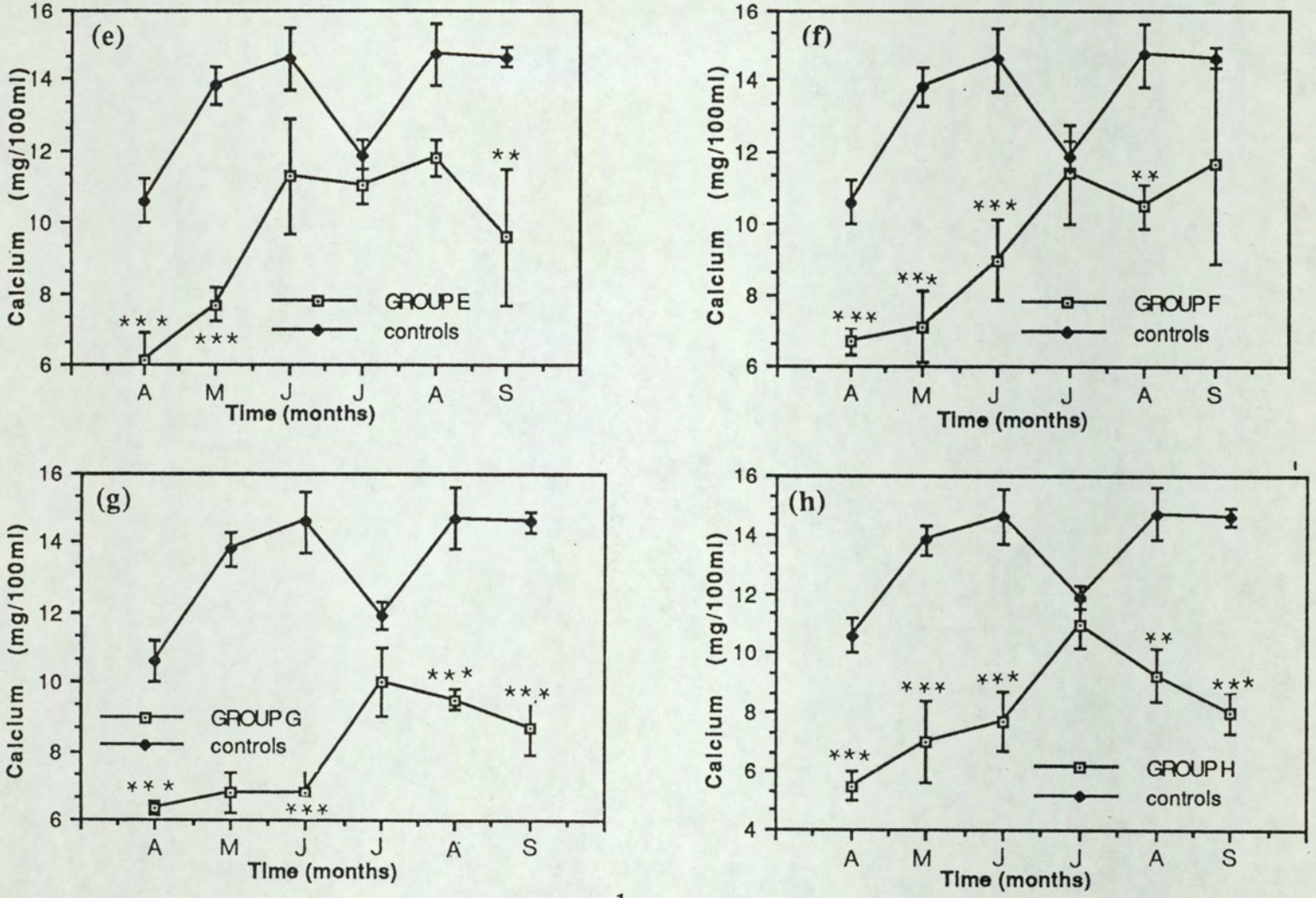


Figure 5.23 e-h. Serum calcium levels ($mg.100ml^{-1}$) (as an index of vitellogenin levels) in (e) Group E (18L:6D for 2 months followed by 8L:16D), (f) Group F (18L:6D for 3 months followed by 8L:16D), (g) Group G (18L:6D for 4 months followed by 8L:16D) and (h) Group H (18L:6D for 3 months, 8L:16D for 1 month followed by 18L:6D) in experiment 4. Th controls (Group I) were maintained under natural conditions of photoperiod and temperature. Vertical bars denote standard deviations.

influenced by a transfer of fish from long to short days (figs. 5.23a and b), that is they were elevated. However, unlike serum T levels, Ca levels regardless of the photoperiodic conditions and similar to the controls (though serum Ca levels in the controls showed a slight drop in July; see Chapter 3, which did not occur in any of the experimental Groups) had already been increasing slowly from the start of the experiment in all experimental Groups. In the experimental Groups, a transfer to short days only served to elevate the levels further. Therefore, in Groups D,E,F and G exposed to short days in May, June, July and August respectively, Ca levels although they had been steadily rising from the start of the experiment showed a further increase one month after transfer to short days. That is in June, July, August and September respectively. In fish exposed to constant short days, that is 12.5L:11.5D (Group A) and 8L:16D (Group B), Ca levels increased gradually, with peak levels being observed in August (12-14 $mg.100ml^{-1}$), one month prior to final maturation (fig. 5.23a) In contrast, in fish maintained on constant long days (Group C), Ca levels were still rising in September. With respect to Group H , that is, fish exposed to one month of short days (August) in an otherwise long day photoperiod, serum Ca levels rose sharply on transfer to short days ($12mg.100ml^{-1}$) and declined on further exposure to long days (levels were between $7-8mg.100ml^{-1}$). Regardless of the pattern of serum Ca levels, in nearly all cases, levels were significantly lower ($P<0.001$) than the controls (Group I).

Irrespective of the photoperiodic conditions, serum E2 unlike levels of T and Ca, were initially high (between $3-5ng.ml^{-1}$) in all the experimental Groups in April, similar to the control fish (figs. 5.22a and b). Furthermore in all Groups, E2 levels subsequently declined. However a second peak in E2 levels was observed in July in Groups A,B,D and E and in August in Groups F,G and H. This was in advance of the control fish (Group I) which did not show a rise in E2 levels until September. This strongly indicates that gonadal maturation was advanced in the afore-mentioned experimental Groups. Unlike serum E2 levels in fish transferred to a shorter daylength, in Group C fish maintained on long daylengths, E2 levels did not show a second peak.

Histological analysis of the ovary confirmed that gonadal maturation had been advanced in Groups A,B,D,E,F,G and H with respect to the controls. GVM, which is normally associated with final maturation was clearly visible in these Groups (see plates 5.2 and 5.5). However in Group H fish which were further exposed to long days after a period of short days, although GVM was visible in some of the oocytes, the majority had started to undergo atresia (see plate

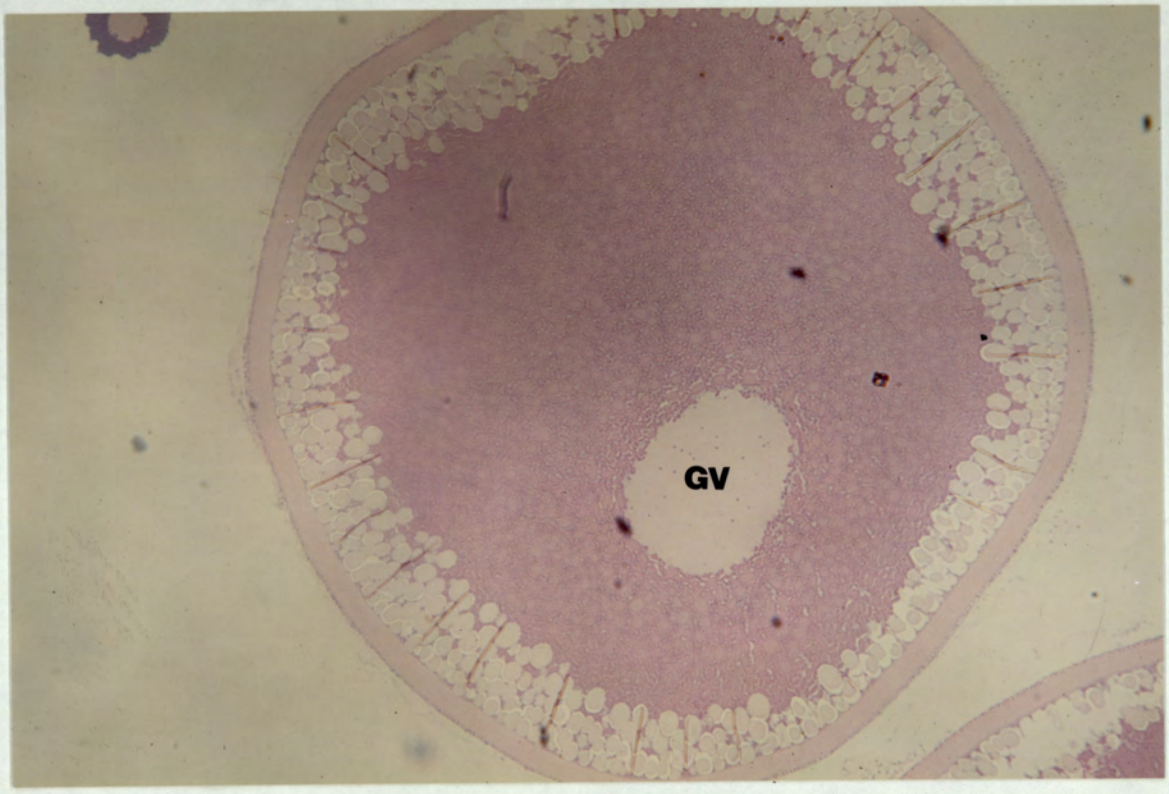


Plate 5.5. A typical example of an oocyte undergoing advanced germinal vesicle migration (GVM).

5.4 and section 3.1). In addition GSI values in these Groups, with the exception of Group H exposed to one month of short days (August) in an otherwise long daylength regime, were significantly higher ($P<0.00.$) than values obtained for Group C fish maintained on constant long days (fig. 5.24). Similarly oocyte diameters were significantly higher ($P<0.001$) in fish which showed advanced gonadal maturation compared to Group C fish maintained on constant long days (fig. 5.25). Furthermore, the number of vitellogenic oocytes in fish which showed advanced development, with the exception of Group H were significantly higher ($P<0.001$) than numbers obtained in Group C fish (fig. 5.26). Interestingly the GSI values and the numbers and sizes of vitellogenic oocytes in the experimental Groups close to spawning were very similar to the controls in September. However, the control fish were some 5 months away from spawning.

5.6.3. **Summary of results**

1) Final maturation was advanced in fish maintained on both the ambient photoperiod at the start of the experiment (Group A) and those exposed to a reduction in photoperiod between April and August (Groups B,D,E,F,G and H). Interestingly final maturation occurred at the same time, in September in each Group irrespective of when they were transferred to short daylengths. This was confirmed by the position of the G.V.M in September. In contrast, ovarian development in fish maintained on long daylengths was delayed.

2) Although advanced ovarian development occurred in Group H in 2 of the fish (i.e G.V.M. was visible), in the other 3 massive levels of atresia were observed, presumably due to the exposure of these fish to long daylengths in August.

3) The GSI, oocyte diameter and number of vitellogenic oocytes were significantly higher in fish exposed to a reduction in photoperiod than fish exposed to constant long daylengths and Group H fish rexposed to long daylengths in August.

4) G.V.B.D. and ovulation did not occur in any of the experimental fish.

5)The effects of different photoperiod conditions were clearly seen through the associated changes in serum levels of T, E2 and Ca. Serum levels of T and Ca in particular were affected directly by a change in photoperiod.

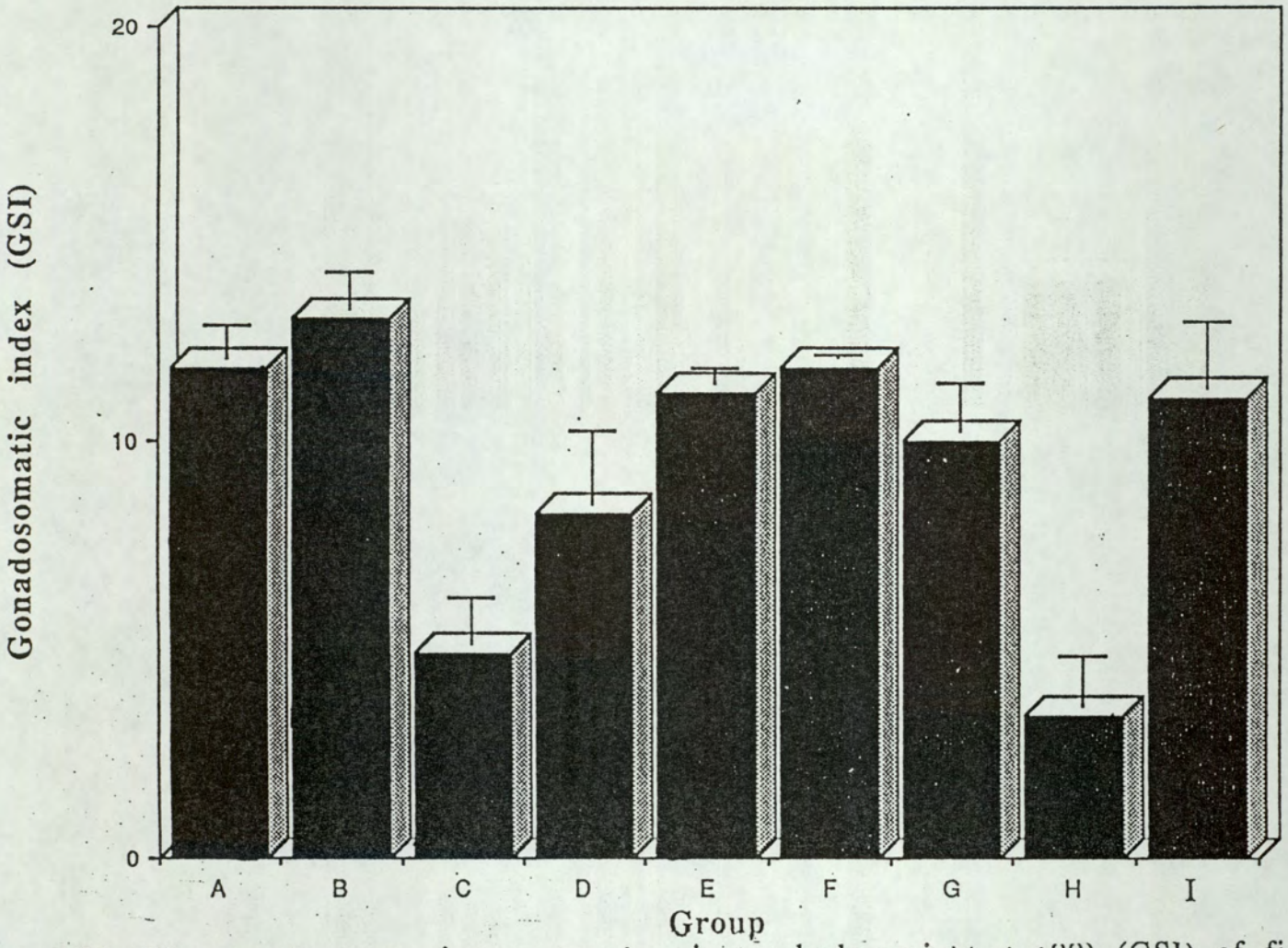
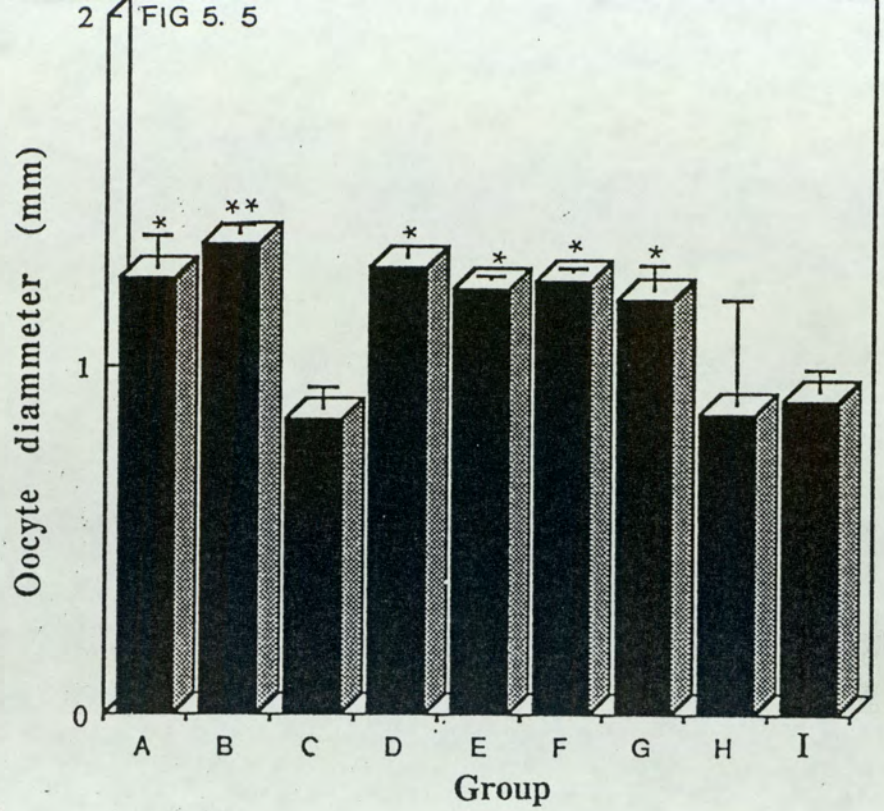
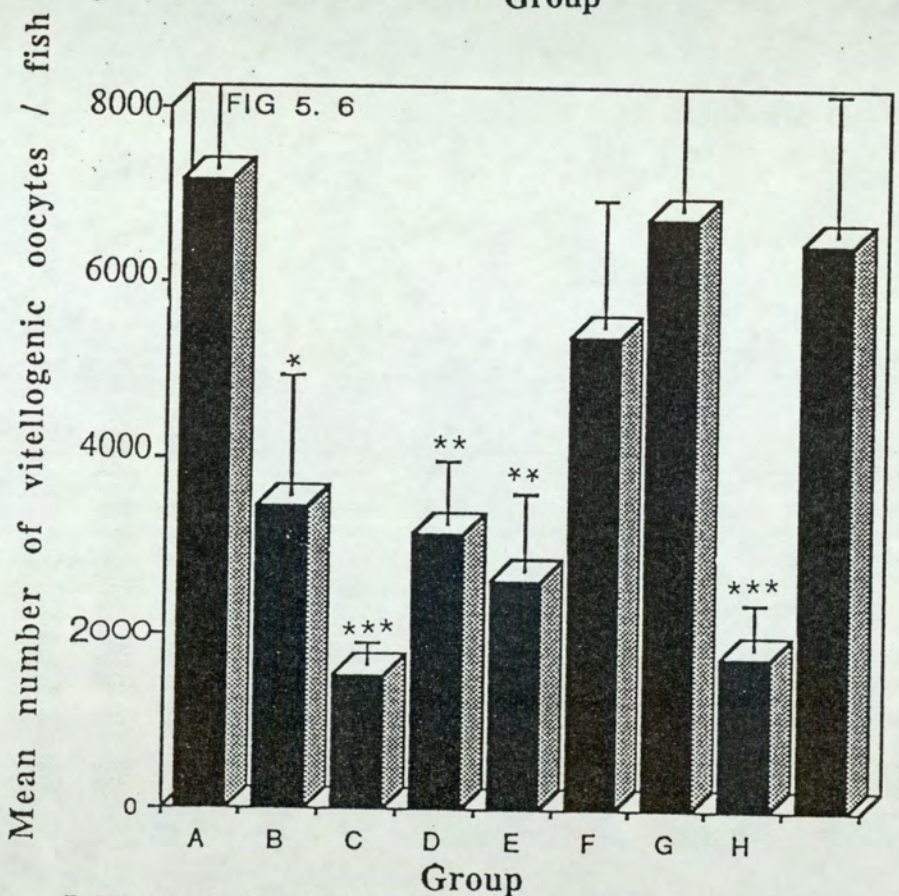


Figure 5.24. The gonadosomatic index (gonad weight / body weight x 100) (GSI) of fish recorded at the end of experiment 4. Groups A-H represent different photoperiod regimes. These were Group A (12.5L:11.5D), Group B (8L:16D), Group C (18L:6D), Groups D,E,F and G (18L:6D for 1,2,3 and 4 months respectively followed by 8L:16D) , Group H (18L:6D for 3 month, 8L:16D for 1 month followed by 18L:6D) and Group I (controls maintainded under natural conditions of photoperiod and temperature). Vertical bars denote standard deviations.





Figures 5.25 and 5.26. Oocyte size (Fig.5.25) and the mean total number of vitellogenic oocytes / fish (Fig, 5.26) in experiment 4. Groups A-H represent different photoperiod regimes. These were Group A (12.5L:11.5D), Group B (8L:16D), Group C (18L:6D), Groups D, E, F, G (18L:6D for 1,2,3 and 4 months respectively followed by 8L:16D), Group H (18L:6D for 3 months, 8L:16D for 1 month followed by 8L:16D) and Group I (controls maintained under natural conditions of photoperiod and temperature). Vertical bars denote standard deviations.

5.7 Summary of Chapter 5

1. Short (experiments 2,3 and 4) or increasing daylengths (experiment 1) are stimulatory to gonadal development. In experiment 1, only increasing daylengths, during the period in which gonadal development occurs under natural conditions (January-March) stimulated final maturation (upto GVM); neither constant long (18L:6D) or short (6L:18D) photoperiods stimulated this process. In experiments 2,3 and 4, short days (8L:16D) exposed to fish at various intervals between March and August (the period in which under natural conditions, the major portion of gametogenesis occurs), advanced final maturation with respect to the controls. In addition fish maintained on the ambient photoperiod (12.5L:11.5D) from the start of the experiment (beginning of April) also showed advanced maturation
2. In contrast, long daylengths (18L:6D) during late spring/summer (experiments 2,3 and 4) and late winter/early spring (experiment 1) delayed or were inhibitory to gonadal development.
3. A reduction in photoperiod from 16L:8D to 12L:12D and 14L:10D (experiment 2) in June also advanced maturation, but to a lesser degree than a reduction in photoperiod from 16L:8D to 8L:16D.
4. Similar patterns of ovarian development were observed in fish maintained on an ambient photoperiod/constant temperature regime and the controls maintained under naturally fluctuating conditions of temperature and photoperiod.
5. Advanced or delayed gonadal development was indicated by serum levels of T, and Ca though serum E2 levels did not seem to be affected as much. In addition, GVM in oocytes at the end of an experiment confirmed whether final maturation had been advanced or not.
6. Additional information such as GSI values, oocyte size and number data at the end of each experiment confirmed that ovarian development in fish maintained under short days were advanced compared to fish on long photoperiods, that is they were greater.
7. In all experimental groups in which final maturation had occurred, only oocytes up to the GVM stage could be observed. Therefore, spawning did not occur in any of the experimental animals.

5.8 Discussion

The results demonstrated that photoperiod has an important influence on reproductive timing in the dace. Whereas short or increasing daylengths were stimulatory to gonadal development, long daylengths delayed maturation. However, in fish exposed to a simulated ambient photoperiod/constant temperature regime between December and March, final maturation occurred at the same time as the controls, maintained under natural conditions of photoperiod and temperature. Similarly fish exposed to a simulated ambient photoperiod/constant temperature between April and December showed a similar pattern of ovarian development to fish maintained under natural conditions. These data would suggest therefore, that temperature changes are not important for ovarian development in the dace and support the proposal that photoperiod is the main requirement for reproductive development.

Although progressively-changing photoperiods are probably the cues for ovarian development under natural conditions, under experimental conditions, photoperiods of constant length also induced final maturation. Thus, it would appear that a seasonally changing photoperiod is not required for the timing of ovarian development. Similar findings have also been demonstrated in the rainbow trout, *Salmo gairdneri* (Bromage *et al.*, 1982,1984), the catfish, *Heteropneustes fossilis* (Sundararaj and Vasal, 1976) and the stickleback, *Gasterosteus aculeatus* (Baggerman, 1980). Furthermore as constant short and long photoperiods advanced and delayed maturation respectively, these results would suggest that as in other photoperiodic fish so far investigated (e.g. Baggerman, 1972; Sundararj and Vasal, 1976; Duston and Bromage, 1986), it is the amount of light received per day and where it appears in the 24 hour light cycle which is important and not its seasonal rate of change.

The importance of increasing or short photoperiods as a requirement for gametogenesis and/or final maturation has also been demonstrated in other fish, for example *Fundulus heteroclitus* (Day and Taylor, 1984), *Blennius pavo* (Patzner, 1983) and *Gasterosteus aculeatus* (Baggerman, 1972). In these species full gonadal development in response to long photoperiods and warm temperatures will not occur unless previously exposed to a period of short daylengths and cold temperatures. Similarly short photoperiods are important for the winter spawning *Mirogrex terrae-sanctae* (Yaron *et al.*, 1980) and the bitterling, *Pseudoperilampus typus* (Hanyu *et al.*, 1982). In contrast to the stimulatory effect of short

daylengths on ovarian development in the dace, long daylengths delayed maturation. This occurred both in fish maintained on long daylengths from the start of a new gonadal cycle and after re-exposure to long daylengths after a period of short (stimulatory) daylengths. In the latter situation, in response to long daylengths, the GSI and follicle size at the end of the experiment were significantly smaller than those obtained in fish maintained under short daylengths and massive levels of atresia were visible, indicating an inhibitory effect of long daylengths. Furthermore ovarian development could be achieved completely in the absence of long daylengths which would suggest that long daylengths are not a requirement for ovarian development.

The effects of changes in photoperiod on ovarian development were clearly seen through changes in serum T and Ca. That is the changes in serum levels of T and Ca that accompany gonadal maturation under natural conditions were either advanced or delayed to coincide with the photoperiod induced changes in ovarian development. These results support similar findings in other species where environmental manipulations have been used to modify gonadal development (e.g. *Salmo gairdneri*, Breton and Billard, 1977; Whitehead *et al.*, 1978: *Carrasius auratus*, Gillet and Billard, 1977). In the dace, whereas serum levels of T and Ca increased on exposure to short photoperiods, they declined or remained at low levels on long photoperiods. Although serum E2 levels were similarly affected by exposure of fish to short and long photoperiods in some fish, the changes were not as marked or as consistent as serum levels of T and Ca, making E2 levels less reliable as indicators of the pattern of gonadal development.

The findings that a change in photoperiod from long to short and a short to long advanced and delayed maturation respectively strongly support the proposals by Randall *et al.*, (1987). Working on *Salmo gairdneri*, these authors demonstrated that not only a change in photoperiod is important for gonadal development but also the direction of change. In addition, in dace, results from fish exposed to various degrees of changes in daylength, that is from 16L:8D (ambient) to 14L:10D, 12L:12D or 8L:16D would suggest that not only the direction of change in photoperiod is important but also the amount of change. Thus a reduction in photoperiod from 16L:8D to 8L:16D was more effective in advancing maturation than less pronounced reductions in photoperiod.

Advances in maturation induced by alterations in artificial environmental conditions has also been demonstrated in several other species. Using similar experimental conditions to those used to

advance maturation in the dace, that is a change from long to short daylengths, maturation has also been advanced in the rainbow trout, *Salmo gairdneri* (Whitehead and Bromage, 1980; Randall *et al.*, 1987). In addition accelerated light cycles (12 month natural cycles compressed into shorter cycles) have also advanced maturation in rainbow trout(e.g. Billard and Breton, 1977; Whitehead *et al.*, 1978), roach, *Rutilus rutilus* (Worthington *et al.*, 1982) and barbel, *Barbus barbus* (Poncin and Phillipart, 1986). The response of fish to stimulatory daylengths earlier in the year raises important questions regarding the mechanisms involved in time measurement. In the rainbow trout, it is proposed that the advancement of spawning exhibited by fish exposed from long to short daylengths or accelerated light cycles is due to the phase advance of an endogenous clock (Randall *et al.*, 1987; Duston and Bromage, 1987). Although there is no evidence for an endogenous control of reproduction in the dace, it is possible that a similar mechanism also operates. Thus, short daylengths earlier in the year would be perceived by the fish as 'running behind' time, and result in a compensating advance of maturation. An interesting feature regarding the exposure of fish from long to short days is that regardless of when the fish were transferred to short days between the previous spawning in March and August, final maturation occurred at approximately the same time, in September (as indicated by the presence of G.V.M.). Why this should happen is at present unclear. However, it is suggested that each follicle may require a certain minimum period of time to develop before they can mature. Thus, fish exposed to stimulatory (short) daylengths at a later stage of follicle development would be in a position to undergo maturation faster than fish exposed at an earlier stage of development. As none of the fish matured before September this would suggest that the minimum period reqired for follicle development in dace is 6 months.

In addition to fish exposed to a reduction in photoperiod, fish maintained on a constant photoperiod (that is the ambient photoperiod from the start of the experiment in April which was 12.5L:11.5D) also showed advanced maturation. This would suggest therefore that a reduction in photoperiod as suggested earlier in the text, although successful is not essential to advance maturation. Because gonadal maturation in other experimental Groups in the dace was only advanced under short photoperiods, this would suggest that a photoperiod of 12.5L:11D is probably also perceived as a short daylength. Further evidence that a photoperiod of 12.5L:11.5D is probably a short daylength is demonstrated by experiments in other

cyprinids, for example the goldfish (Razani *et al.*, 1987) and the bitterling (Hanyu *et al.*, 1983). In these species, daylengths between 13 and 14 hours of light per day (that is the critical daylength) were found to be the transition point between short and long daylengths. Thus in these species, daylengths below 13 hours of light per day are considered as short. Although the transition point between what is perceived as short and long days in dace is unknown, the results from other species provide further evidence that a photoperiod of 12.5L:11.5D is a short daylength. Thus the requirement for advanced maturation in dace is not necessarily a change in photoperiod from longer to short daylengths as previously suggested, but may be just a short daylength.

Under experimental conditions, both the initiation of ovarian development and final maturation occurred under short daylengths suggesting that under natural conditions, ovarian development is probably similarly controlled. As daylengths, although increasing are still 'short' after the previous spawning in March, approximately 12.5L:11.5D, it is proposed that gametogenesis is initiated at this time. Evidence that gametogenesis is initiated at this time is supported by high serum levels of E2 and T almost immediately after spawning (see chapter 3). The stimulation of ovarian development immediately after the previous spawning would strongly suggest that there is no refractory period in the dace, i.e. a period of gonadal insensitivity to environmental factors following the breeding season. Similar findings have also been observed in the cyprinids, *Notemigonus crysoleucas* (de Vlaming, 1975a) and *R. ocellatus* (Hanyu et al., 1982). In *Notemigonus crysoleucas,* de Vlaming (1975) demonstrated that regardless of the time of year the experiment was initiated, the correct environmental conditions would stimulate gonadal development.

Although constant short daylengths earlier in the year than they occur naturally stimulated final maturation under experimental conditions, it is proposed that under natural conditions the final stages of development are stimulated by increasing daylengths which occur from December onwards. This proposal is supported by the experimental results during this period where only a simulated ambient photoperiod stimulated final maturation; neither constant short or long daylengths had any affect on this process. The inability of dace at this time of year to respond to daylengths of constant length compared to earlier in the year would suggest that like the stickleback (Baggerman, 1980), there is a seasonal variation in photosensitivity. A seasonal variation in photosensitivity would also explain why short daylengths under natural conditions prior to

December do not induce final maturation, that is the fish are probably insensitive to short daylengths at this time. Alternatively low water temperatures during this period may be more important than the effects of short daylenghts which would prevent fry from being produced at a sub-optimal time of year.

Although final maturation could be obtained under laboratory conditions in fish maintained under increasing or short daylengths, the maximum GSI and oocyte size at the termination of an experiment (when oocytes were undergoing G.V.M.) were significantly less than those observed in the controls at a similar stage of development. A lower GSI and oocyte diameter in oocytes that had been advanced is presumably due to the fact that they had less time (5 months) to develop. However, in experimental fish which developed at a similar time to the controls the GSI and oocyte size were still significantly lower in the fish maintained in the laboratory. This would suggest that the full complement of environmental stimuli required for final maturation under natural conditions were absent under experimental conditions. Thus the highest GSI value recorded in fish maintained in the laboratory was only 11%, very much lower than the GSI (20%) recorded under natural conditions. Similar results have also been observed in the minnow, *Phoxinus phoxinus* (Scott, 1979). In this species a simulated increasing daylength stimulated a GSI of only 10.5% in laboratory fish compared to 12-14% in nature. In the minnow, GSI values similar to those observed in nature could only be acheived under experimental conditions by exactly mimicking the conditions experienced in nature, that is , by exposing the fish to a sudden increase in daylength (see Scott, 1979). Similarly *Blennius pavo* (Patzner, 1983), *Fundulus heteroclitus* (Day and Taylor, 1983) and *Gasterosteus aculeatus* (Baggerman, 1980) do not exhibit full gonadal development in response to warm long daylengths , unless these conditions are preceeded by short daylengths and cold temperatures, similar to the situation in nature. Therefore, in dace undergoing final maturation under experimental conditions, it is likely that one or more environmental trigger(s) were still missing, preventing full ovarian maturation similar to the controls.

In association with the above, although final maturation as far as the G.V.M. stage occurred under experimental conditions, G.V.B.D. and ovulation failed to occur. This is similar to the findings in many other species maintained in captivity (see Chapter 4). Therefore, it is reasonable to assume that the stimuli required for these processes were absent under experimental conditions. In the goldfish,

Carrasius auratus (Stacey *et al.*, 1979) and the roach, *Rutilus rutilus* (Worthington *et al.*, 1982), the presence of appropriate nesting material, a spawning substrate or members of the opposite sex were required to induce final maturation beyond the G.V.M. stage. In the dace as yet, it is unclear what conditions are necessary to induce G.V.B.D. and ovulation; neither the presence of gravel as a spawning substrate or males were sufficient to induce these processes. However, there is evidence to suggest that the absence of G.V.B.D. and ovulation under experimental conditions may not be due to the lack of cues, but instead to the presence of inhibitory stimuli. In other species exposed to noxious stimuli, the fish produce a stress response, a symptom of which is gonad degeneration (Scott, 1979). A similar response is also seen in the dace exposed to long daylengths, after a period of short (stimulatory) daylengths; that is massive levels of atresia. Stress produced by conditions, particularly handling, captivity and confinement are also known to block several steps of the reproductive cycle including gametogenesis, oocyte maturation, spawning and spawning behaviour (Billard *et al.*, 1981). Therefore, it seems likely that the inhibitory effect of captive conditions may have blocked G.V.B.D. and ovulation in the dace. Collectively these results would indicate that stress caused by noxious stimuli may be more important than has previously been realized. It is interesting to note however, that the stressful environment created by man when handling and breeding fish in captivity in some species can be overcome by domestication. After several generations, cultivated barbel, *Barbus barbus* become adapted to captive conditions and reach sexual maturity at an earlier age and size than wild populations, and are able to undergo repeated spawnings throughout the year (Poncin *et al.*, 1985).

In summary the results suggest that temperature does not play a major role in reproductive timing in the dace and support the proposal that photoperiod is the main environmental cue for ovarian development. Under experimental conditions ovarian development could be advanced and delayed by short and long days respectively. These were most effective during the early stages of follicle development. In contrast to the effect of long and short daylengths at the beginning of the annual cycle, towards the end of the cycle, they had no effect; only a simulated ambient photoperiod could induce final maturation (upto G.V.M.). The experimental results suggest that ovarian development under natural environmental conditions may be similarly timed by short or increasing photoperiods. Thus gametogenesis is probably timed by the short daylengths that occur after the previous spawning in March and final maturation by

increasing daylengths from December onwards. Similar to many other species maintained in captivity, G.V.B.D. and ovulation failed to occur in any of the experimental fish, suggesting that either the trigger(s) for these events were missing or that the imposed conditions were detremental to development.

CHAPTER 6

An investigation into the perception of daylength

6.1. Introduction

The previous chapter (chapter 5), clearly demonstrated that photoperiod plays an important role in the timing of reproduction in the dace. The next question that arises is how is this photoperiodic information relayed to the target organs, in this case the reproductive system. Based on research from a wide variety of vertebrates, this chapter investigates how the dace perceives light, how this photoperiodic information interacts (if any) with circadian rhythms, and how this is transduced into endocrine information and gonadal development.

Available data indicate that there are several sites capable of perceiving the daily photoperiod. In mammals, as may be expected, daylength is perceived via the eyes (Karsch *et al.*, 1984), and animals cannot respond to the photoperiod if blinded. However, even mammals appear to be able to use visual receptors other than the eye, at least to some degree (Karsch *et al.*, 1984). Neither birds nor reptiles use the eye to perceive photoperiodic information (Oliver and Bayle,1982; Underwood, 1979). In the case of birds, a light sensitive area of the hypothalamus is the site of photic imput which controls the reproductive cycle. The case for photoperiodic perception in most other animals has been the subject of much research, but as yet is not fully resolved. In reptiles light may be perceived by more than one pathway. Like birds, some reptiles possess extra-retinal, non-pineal encephalic photoreceptors (Underwood,1973; Kavaliers and Ralph, 1980). In addition some reptiles for example, *Scheopordacus occindentalis*, possess an extra-cranial third or parietal eye that bears resemblance to the mammalian retina (Eakin and Westfall,1960), and is sensitive to light/dark cycles. Finally reptiles may also use their pineal gland to perceive light (Meissl and Ueck,1980; Okshe and Hartwig,1975).

The amphibian pineal organ has also been shown to have photoreceptive properties. In *Rana esculenta* and the clawed toad, *Xenopus laevis,* it has been demonstrated electrophysiologically and morphologically that the pineal organ has photoreceptive capabilities (Cadasseau *et al.*,1979; Koo *et al.*,1981).

In fish, the situation regarding the perception of daylength is much less clear. This, at least in part, is due to the fact that so few species have been investigated and/or those that have been investigated may not use photoperiod as a primary environmental cue. Studies on fish, similar to those for other vertebrate groups,

reported above, have shown that in several teleost species, the pineal gland is directly photosensitive. For example, histological examination of the pineal organ in *Phoxinus phoxinus* revealed photoreceptor cells similar to those in the retina (Okshe and Hartwig,1975). However, in other species of fish, the pineal gland does not appear to be required in the perception of photoperiodic information. In the lake chub, *Couesius plumbeus* (Kavaliers,1979) for example and *Fundulus heteroclitus* (Day and Taylor,1983), an extra-retinal, non-pineal photoreceptor is suggested. Indeed, photosensitive areas have been shown in regions of the diencephalon, the paraphysis and the third ventricle (Okshe and Hartwig,1975; Hartwig and Van Veen,1979). These encephalic receptors have been suggested to be involved in determining the light responses of blinded and pinealectomized minnows, *Phoxinus phoxinus* (Cyprinidae) and the eel, *Anguilla anguilla* (Von Frisch,1911 and Van Veen *et al.*,1976).

The pineal organ and eyes of many teleosts have been examined in an attempt to to determine how the photoperiodic control of reproduction is brought about, however the results suggest that no specific trends exist within or between genera. The eyes do not appear to be required for appropriate reproductive responses in some photoperiodic fish (for example *Gasterosteus aculeatus* Borg, 1982; *Fundulus heteroclitus* Day and Taylor,1983), although other species may depend on their eyes (for example, *Carrasius auratus* Delahunty *et al.*,1979).

Although the above data indicate that there are various pathways by which light is perceived, there is no indication of how light/dark cycles of different lengths maybe distinguished, that is how does an animal know what time of year it is? Several theories have been proposed to explain how an animal measures time. One of these, initially proposed by Bunning,(1973), states that animals are able to measure the length of the photoperiod by means of a daily (circadian) rhythm of sensitivity to light. Circadian rhythms have undergone considerable investigation in higher vertebrates and the findings summarized in a number of comprehensive reviews (Bunning,1973; Pittendrigh,1974). Generally the evidence available indicates that circadian rhythms are inherent, genetically determined functions of the organism, that have several specific characteristics, which are outlined below:

1) They are endogenous , that is they will" free-run" under constant environmental conditions, with a periodicity of approximately 24 hours, either a little longer or shorter than 24 hours, which is why they are called circadian meaning" about a

day".

2) Under natural environmental conditions, the rhythms show a period of exactly 24 hours. This is because they are entrained by light/dark cycles.

3) They are temperature compensated, that is they do not speed up or slow down as the temperature fluctuates.

Circadian rhythms coordinate the activity of the internal environment of an organism with its external environment, and in order to understand the basic characteristics of these rhythms, it is necessary to understand some of the theories proposed to explain how an organism" measures" time. Returning to Bunning's theory, also known as the external coincidence model, light must be present during the photoinducible phase of the photosensitivity rhythm which is entrained by the daily photoperiod. The position of the photosensitive phase relative to the 24-hour clock is thought to be species dependant but in long-day animals, it is imagined to occur during the subjective night, i.e. between 12 and 24 hours after dawn.

A more complicated theory, the internal coincidence model relies on light entraining two or more internal rhythms that underlie the photoperiodic response. This model suggests that since the phase relationship between internal rhythms may vary under different photoperiodic conditions, an inductive reponse between internal oscillators mediating photoperiodic time measurement will only occur under certain light-dark cycles (Pittendrigh, 1981). Thus in a non-inductive photoperiod, the rhythms will not overlap, but on transferring to an inductive photoperiod, the internal rhythms coincide resulting in gonadal maturation. In both models, a notable feature is that it is not the amount of light or dark/day that is important, but when the light or dark falls in relation to the underlying rhythm of photosensitivity.

Although these two models are not the only theories used to explain how an organism "measures time', most experimental evidence would suggest that they are the most important. In fish, as briefly mentioned, few experiments have been conducted on reproductive responses to alterations in photoperiod. However, in those species that have been examined, for example, the rainbow trout (Duston and Bromage, 1987), the stickleback (Baggerman, 1972), the catfish (Sundararaj and Vasal, 1976) and the medaka (Chan ,1976) the results support the hypothesis that circadian rhythms are involved in controlling reproduction.

The next question that arises is where are these rhythms

located? In mammals, although many different structures are capable of generating their own circadian rhythms, those generated in the suprachiasmatic nucleus (SCN) appear to control the onset of breeding (Turek *et al.*,1984). In fish, considerably less is known about the control of circadian rhythms. The role of the SCN in circadian organization of fish has not been investigated, however the pineal gland is capable of generating circadian rhythms (Kavaliers,1979; Underwood, 1982). Furthermore, in a number of birds, lizards and amphibians (Menaker and Zimmerman, 1976; Underwood, 1979; Demain and Taylor, 1977), there is substantial evidence to support the theory that the pineal organ plays an important role in the control of circadian rhythms.

The teleost pineal is a well vascularized organ lying on the dorsal surface of the posterior diencephalic region of the brain (see Takahashi,1969; Omura and Oguri, 1969; Owman and Rudeberg, 1970; Rizkalla,1970; Hafeez,1971). Frequently the pineal body lies beneath a partially depigmented and translucent area of the cranial roof (Hafeez, 1971) which may allow for the passage of light (Rivas,1953). Thorough morphological descriptions of teleost pineal organs are beyond the scope of this study but are reviewed in detail by de Vlaming and Olcese (1981), Fenwick, (1969) and Owman and Rudeberg (1970). Briefly the teleost pineal organ consists of a well vascularized end vesicle (epiphysis cerebri) connected by an epiphseal stalk to the posterior diencephalon. A smaller parapineal organ, which degenerates in most adult forms, if present, is connected to the stalk (Fenwick,1969). Hafeez and Zerihum (1974), demonstrated the presence of pinealofugal fibres extending to the lateral labenular nucleus, several thalmic nuclei and various portions of the mesencephalon and diencephalon. There is also a close anatomical relationship with, and neural connections to the ependymal regions of the third ventricle and epithalmic diverticulum (Okshe and Hartwig,1975). This suggests that communications and interactions between the pineal and other brain regions are likely.

From an evolutionary point of view, there is evidence that the pineal gland originated from a single cell type, the photosensory pinealocyte (Collins, 1981), and that there has been a gradual shift from a direct photosensory function of these cells in the more primitive vertebrates to a neuroendocrine role in the advanced forms (Kappers, 1971). Indeed some of the cells of the teleostean pineal gland also appear secretory (Takahashi, 1969; Hafeez, 1970). Available evidence indicates that these cells are not only photoreceptive but also the site of an active biosynthetic pathway

with melatonin being the major secreted product (Collins and Okshe, 1981; Vivien-Roels *et al.*, 1981; Falcon *et al.*, 1985 and Mcnulty, 1986). In addition to being a major site of melatonin biosynthesis, the photoreceptor cells of the pineal also display electrical activity in responses to changes in illumination (Meissl, 1986). Thus, it appears that photoreception and melatonin formation are coupled processes in the photoreceptive cells of non-mammalian vertebrate groups.

The biosynthesis of melatonin in the pineal gland is illustrated in figure 6.1. Initially tryptophan is converted into serotonin with 5-hydroxy tryptophan being an intermediate product. The conversion of serotonin to melatonin is a two step sequence involving the enzymes N-acetyl transferase (NAT) which converts serotonin to N-acetyl serotonin and hydroxy indole-o-methyl transferase (HIOMT) which converts N-acetyl serotonin to melatonin.

In teleosts, melatonin secretion has been measured directly from isolated pineal organs (Herwig, 1981; Falcon *et al.*, 1985) or indirectly as melatonin circulating in the plasma (Gern *et al.*,1978). From these studies, it is clear that melatonin levels are high during the night and low during the day. This is the case in all animals studied so far. Like melatonin, NAT also reaches peak levels during the dark; HIOMT activity remains constant however, indicating that NAT regulates rhythms in melatonin synthesis. The melatonin rhythm is entrained to the light/dark cycle, so that its secretory pattern changes with the photoperiod, and it is this rise during the night, that is important in determining the reproductive resonse to daylength, the duration being directly proportional to the length of the night.

The fact that pinealectomy in trout does not completely abolish the melatonin rhythm, suggests that unlike the situation in mammals, where the pineal organ is the only source of melatonin, melatonin in fish is also synthesized elsewhere (Gern *et al.*, 1978). In the fish, *Hemigrammus* (Herwig *et al.*, 1981) and the rainbow trout (Gern and Ralph,1979), the eyes also synthesize melatonin, with strong indications that the retina of several other species may also synthesize this molecule.

Although the full range of actions of the pineal or circulating melatonin in fish has probably not been defined, evidence indicates that the fish pineal does effect many metabolic and endocrine events. Melatonin has potent reproductive (Vodicnik *et al.*, 1978; de Vlaming *et al.*, 1974) and metabolic (Delahunty *et*

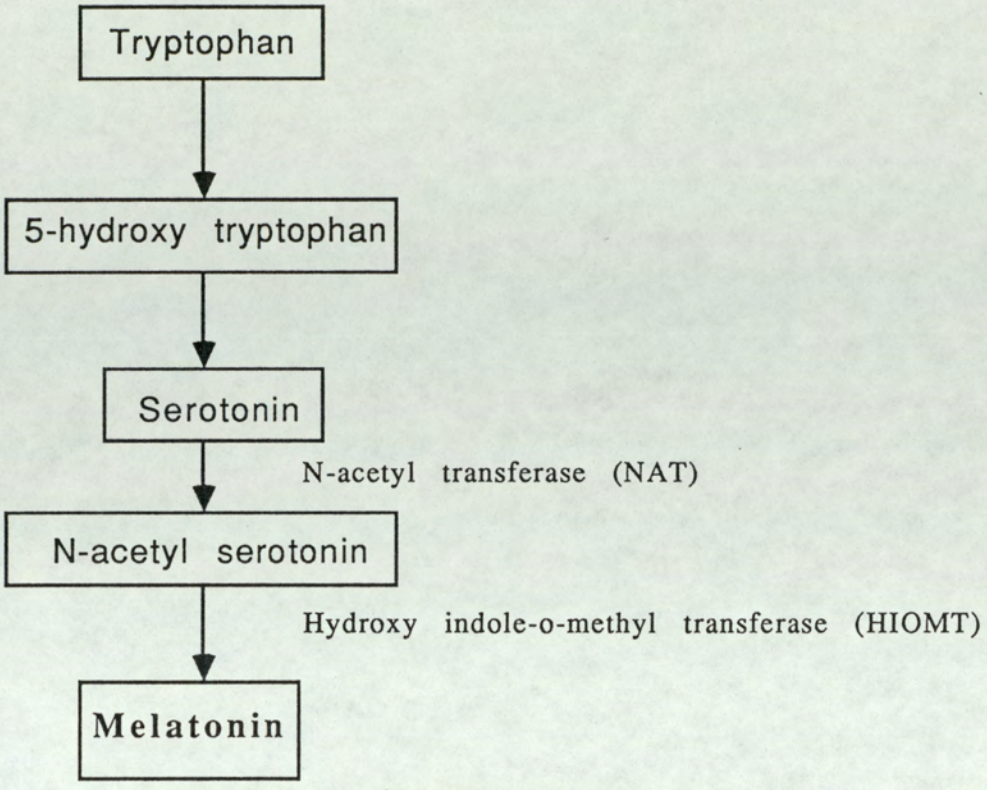


Figure 6.1. The melatonin biosynthetic pathway

al., 1978) effects on fish and can alter plasma cortisol levels (Delahunty *et al.*, 1978). Until recently (Kavaliers,1979), there was very little evidence that the pineal organ had a definite role in determining the circadian rhythmicity of fish. The pineal body of fish has been shown to affect the diel arrangement of locomotor activity rhythms (Kavaliers, 1979,1980), but there is only a vague understanding of melatonin's contribution to these rhythms. The studies of Kavaliers (1979,1980) also indicate that the pineal organ is not the only source of circadian organization, but it may serve as a sychronizer or coupler of endogenous, self-sustained oscillators.

It has been suggested in birds, that the pineal may transfer circadian modulation to subordinate systems (Turek *et al.*, 1984; Gwinner and Benzinger, 1978). In fish, melatonin injections have been shown to decrease the locomotor activity of trout (Hafeez,1970) and juvenile sockeye salmon (Fujii and Miyashita,1978), so a similar mechanism of action does not appear unlikely. Furthermore, melatonin is extremely effective in inducing melanosome aggregation in both dermal and epidermal melanophores of the siluroid catfish, *Parasilur asotus* (Fujii and Miyashita, 1978). The thorough study of these workers, provide substantial evidence that melatonin possesses peripheral actions in several teleosts.

There is also considerable evidence to support the contention that the pineal organ and melatonin, have a role in the control of gonadal maturation, although the mode of action remains unclear (Reviews; Ralph, 1978,1983; de Vlaming and Olcese,1981). The data suggest that the pineal can be anti- or pro- gonadal depending on the time of year the experiments are performed. Pinealectomy of goldfish, *Carrasius auratus* (Fenwick, 1970; Vodicnik *et al.*,1978; de Vlaming and Vodicnik, 1978) and Japanese killifish, *Oryzias latipes* (Urasaki,1972,1973) when they are commencing gonadal recrudescence under long days of spring or artificial long photoperiods resulted in a decline in the gonadosomatic index (GSI). In contrast pinealectomy of cyprinids maintained on artificially short photoperiods in the spring resulted in an increase in the GSI compared to controls (Fenwick,1970; Vodicnik *et al.*, 1978; de Vlaming and Vodicnik,1978; Sagi and Abraham, 1984). During the period of reproductive quiescence in goldfish (between late summer and autumn) pinealectomy had no effect on gonadal development (de Vlaming and Vodicnik,1978) or pituitary, or plasma levels of gonadotropin (Vodicnik *et al.*, 1978). From the results presented here, it is difficult to assess the role of the pineal in gonadal maturation. These discrepancies maybe explained by

the fact that retinal produced melatonin was not taken into account, so that melatonin levels were not completely abolished by pinealectomy. However, whatever the role, there is evidence to suggest that pinealectomy affects gonadal development in fish by altering secretions from the hypothalamo-pituitary axis (de Vlaming and Vodicnik,1978; Vodicnik *et al.*, 1978; Hontela and Peter,1980).

In general intra- peritoneal injections of melatonin cause gonadal regression. Daily injections of 20ug melatonin into goldfish, (*Carrasius auratus*) prevented an increase in the GSI and the activity of pineal gonadotrophs, normally observed when the fish are maintained under 16L:8D (Fenwick,1970). Daily injections of melatonin (< 250mg) into fish undergoing gonadal recrudescence have also reduced the GSI in a number of species, including *Oryzias latipes* (Urasaki, 1972), *Fundulus similis* (de Vlaming *et al.*, 1974), *Gasterosteus aculeatus* (Borg and Ekstrom, 1981), *Mystus tengara* (Saxena and Anand, 1977) and *Heteropneustes fossilis* (Sundararaj and Keshavanth, 1976). It is interesting to note that in the catfish (*Heteropneustes fossilis*) although melatonin administration inhibited vitellogenesis and caused gonadal atrophy, cessation of the treatment resulted in the restoration of activity, indicating that the effects are not permanent (Sundararaj and Keshavanth, 1976). As a diurnal rhythm of melatonin has been demonstrated in at least one teleost, the rainbow trout, it was suggested that possibly the time of day that melatonin was administered maybe important (Gern *et al.*, 1978). In contrast, de Vlaming *et al.*,(1974) concluded that the effect of melatonin treatment on *Fundulus similis* was independent of the time of day it was administered. Furthermore, it is also difficult to assess the significance of melatonin treatment, when the site and action of endogenous melatonin is unknown.

These results, and others which monitor other (non-reproductive) physiological or endocrine parameters, demonstrate that the effects of pinealectomy or melatonin treatment among teleosts are generally photoperiod dependent (Delahunty *et al.*, 1980,1978; de Vlaming et al., 1980). In summary these data provide good evidence that the pineal organ functions as a component in the pathway(s) by which seasonal daylength controls and synchronizes annual physiological cycles.

For this reason, it has been suggested that the pineal and its hormone melatonin maybe involved in the control of gonadal development in the dace. If melatonin could be demonstrated in

this species, particularly a rhythm which is dependant on light/dark cycles, it would provide evidence that the dace has the ability to measure changes in photoperiod, and therefore a mechanism which measures time. This was investigated in the following experiment:

6.2 Serum melatonin profiles in dace maintained under a short, 6L:18D and a long, 18L:6D photoperiod.

The aim of this experiment was to investigate whether a diel rhythm in melatonin production occurred under different photoperiod regimes.

6.2.1 Materials and methods

Two groups of 20 fish were maintained in light-proof tanks under a 6L:18D and 18L:6D photoperiod regime. The experiment commenced on 1st July, 1988, using fish that had previously been exposed to ambient conditions. Sixty watt bulbs were used which provided 30 lux at the water surface and the period of illumination was controlled by an electronic time switch. Water temperature was maintained at 11 ± 0.5°C. The fish were fed daily, 2 hours after the lights were switched on. After an acclimation period of 20 days, fish in both groups were blood sampled every 2-4 hours; sampling times were designed to coincide within half an hour of lights on or off in both experimental groups. During the night, blood sampling was conducted under an infra-red lamp. Although the photosensitivity of the dace to different wavelengths has not been established , in the rainbow trout, which is the only fish species in which work has been carried out,the fish are relatively insensitive to this wavelength (Douglas, 1980). However, as a precautionary measure, a light-proof damp cloth was placed over the eyes and pineal area of the brain during sampling. At each sampling time, 3 fish were blood sampled under each of the experimental photoperiod regimes. The blood samples from each fish were allowed to clot at 4°C, and after centrifugation, the serum was removed and stored at -20°C until assayed. The serum was assayed for melatonin as described below:

6.2.2 Melatonin RIA

A direct radioimmunoassay originally developed by Arendt (1983) and modified by Randall (personal communication) was used to measure melatonin. This RIA has proved successful in determining melatonin levels in the rainbow trout (Duston and Bromage, 1987) and preliminary recordings in the dace (own observation, data not shown).

6.2.2.1 Assay materials

All chemicals were Analar unless otherwise stated. Freshly made deionized water was used throughout.

a) Buffer

Tricene	2.688g
Sodium chloride	1.35g
Gelatine	0.15g

The buffer constituents were made up to 150ml with deionized water and dissolved by placing the buffer + container into boiling water. The assay buffer was made immediately before each assay.

b) Label

The 3-H melatonin was obtained in 0.25 mCi aliquots from Amersham International (Aylesbury, Bucks), and diluted 1 in 100 with ethanol, before storage at 20°C. The working solution was subsequently prepared before each assay, was made by a further dilution in assay buffer to give a final concentration of 4000 dpm.100 μl^{-1}.

c) Antiserum

The antiserum to melatonin was supplied freeze dried from Guildhay Antisera (University Surrey, Guilford, Surrey, U.K.). This was reconstituted with 2ml deionized water before storage at -20°C. The working solution was subsequently prepared before each assay, was made by a further dilution in assay buffer to give a final concentration of 1 in 4000.

d) Standard

The melatonin standard obtained from Sigma (Poole, Dorset), code No. M5250 was prepared by dissolving 10mg melatonin in 10ml absolute ethanol. This solution proved stable for at least 3 months at 4°C. The working solution, prepared before each assay, was obtained by a further dilution in assay buffer to give a final concentration of 1000pg.ml^{-1}.

e) Activated charcoal solution

One tablet of dextran coated charcoal (Steranti Research, St. Albans, Herts) was dissolved in 50ml buffer. This was stirred continuously on ice 30 minutes before use.

f) General

All glassware used in this assay was thoroughly cleaned by soaking in chromic acid for 24 hours.

6.2.2.2 **Method**

1. Duplicate plastic LP3 tubes (Luckham Ltd, Burgess Hill, Sussex) were used for all samples, standards, totals and non-specific bindings (NSB)
2. 20 tubes were set aside for the standard curve. 500µl of each dilution was prepared ranging from 0- 500pg melatonin.tube^{-1}.
3. 100µl of serum + 400µl of buffer were added to the assay tubes.
4. 200µl of antisera was added to each tube, vortexed and incubated at room temperature for 30 minutes.
5. 100µl of 3-H melatonin was added to each tube and incubated overnight at 4°C.
6. The antibody bound melatonin was seperated from free melatonin by incubating with 500ul of the activated charcoal solution for 15 minutes at 4°C. Immediately after adding the dextran coated charcoal, the tubes were vortexed and centrifuged for 15 minutes at 4°C.
7. 1ml of the supernatant was transferred into vials containing 9mls of the scintillant.
8. Each vial was counted for 5 minutes in a scintillation counter (Packard Tri-carb 2660), and the concentration of melatonin in each of the unknowns (pg.ml^{-1}) determined by the procedure outlined below:

6.2.2.3 Calculations

1. The percentage of bound melatonin in each standard

Counts obtained for buffer + label + antisera= maximum bound melatonin (MB)
Counts obtained for label + buffer = non-specific binding (NSB)

The percentage bound for each standard=

$$\frac{\text{counts for standard - NSB}}{\text{(Total - NSB)}}$$

2. The percentage bound melatonin for each standard was plotted against the concentration of melatonin ($pg.ml^{-1}$) in each tube, to obtain a standard curve (see figure 6.2).
3. The concentration of melatonin in each of the unknown samples could then be read directly from the standard curve.

6.3 Results

The results presented in figures 6.3 and 6.4 clearly demonstrate that melatonin levels were significantly higher ($P<0.001$) during the night than during the day in fish maintained under both the long and short photoperiods.

In fish maintained under the short photoperiod, 6L:18D (fig. 6.3), melatonin levels were increased from around $1.8pg.ml^{-1}$ during the day to a maximum of 1230 $pg.ml^{-1}$ during the night. Interestingly, during the dark, 2 peaks of melatonin were observed. However the slight drop in levels recorded during the middle of the night were still significantly higher ($P<0.001$) than daytime levels.

Similar to the fish on short days, dace maintained on a long photoperiod, that is a short night (18L:6D) showed daytime levels of less than $5pg.ml^{-1}$. Although nighttime levels were lower than those observed in fish maintained on a short day, that is $820pg.ml^{-1}$, they were still significantly higher ($P<0.001$) than daytime levels.

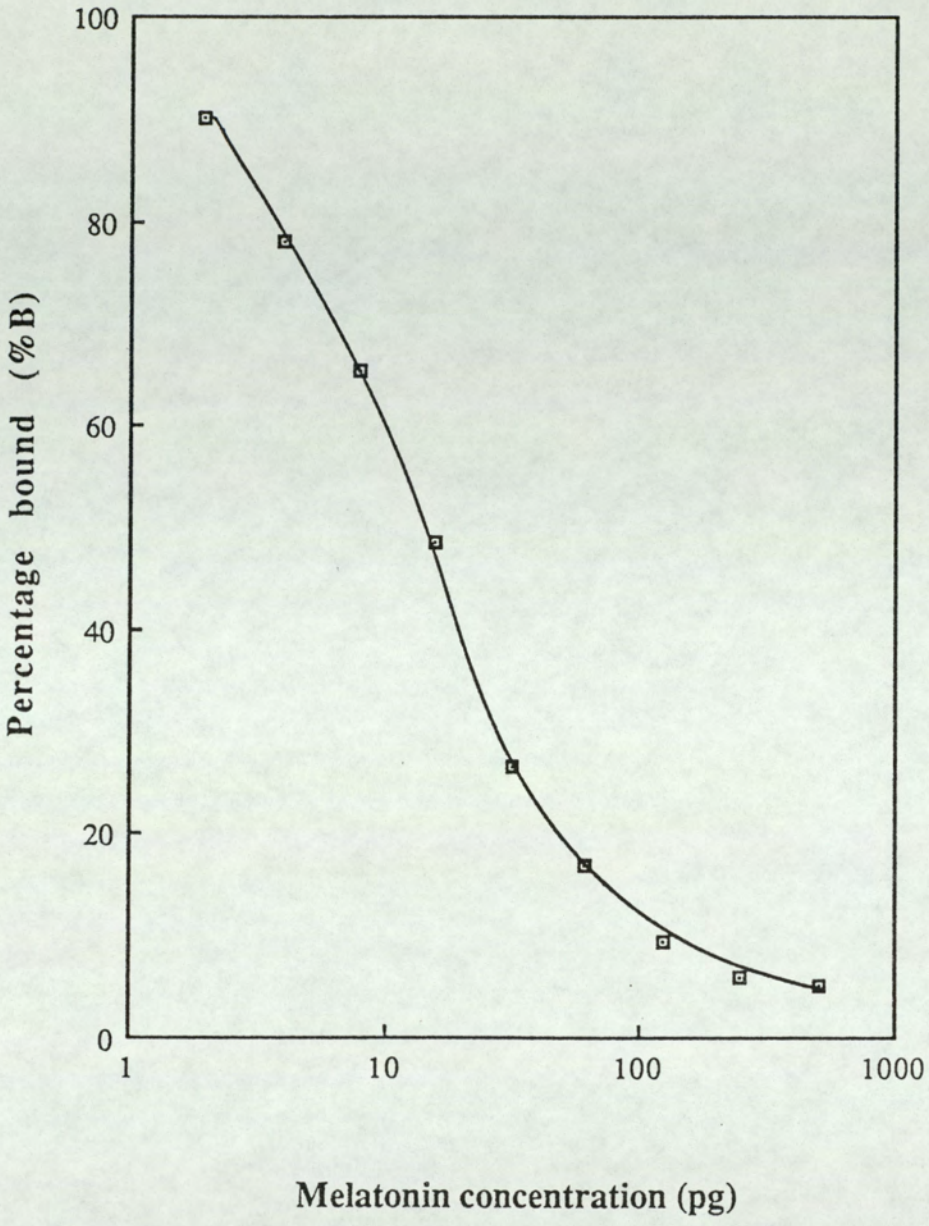


Figure 6.2. An example of the melatonin RIA standard curve.

Figure 6.3

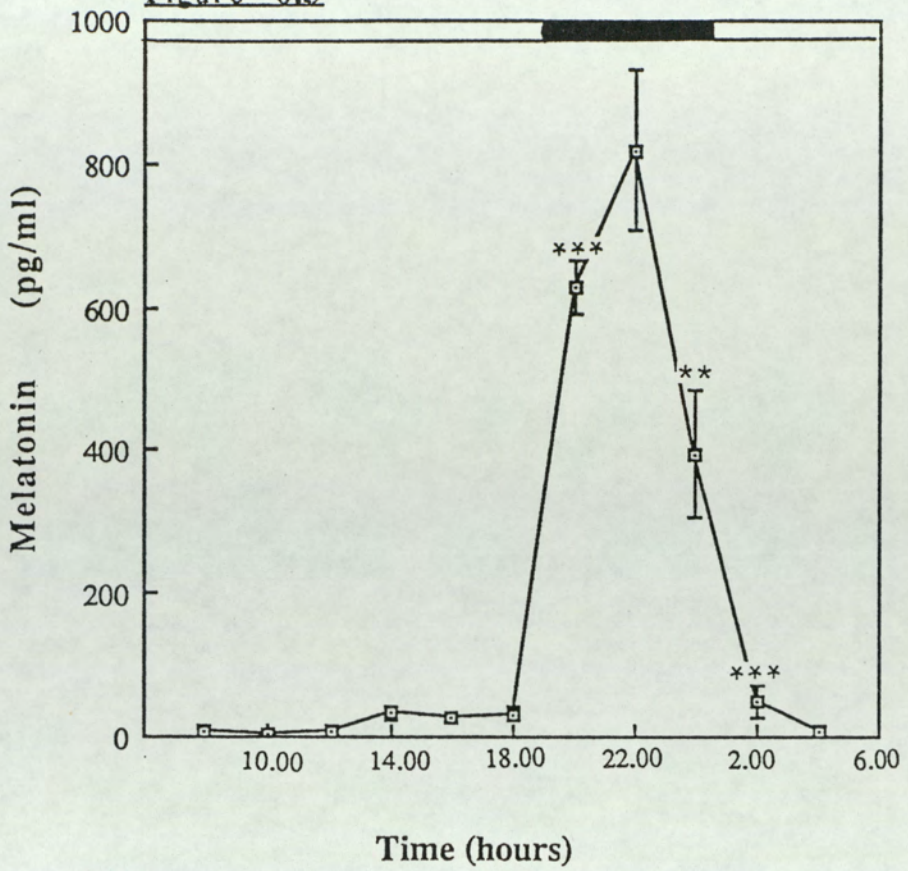
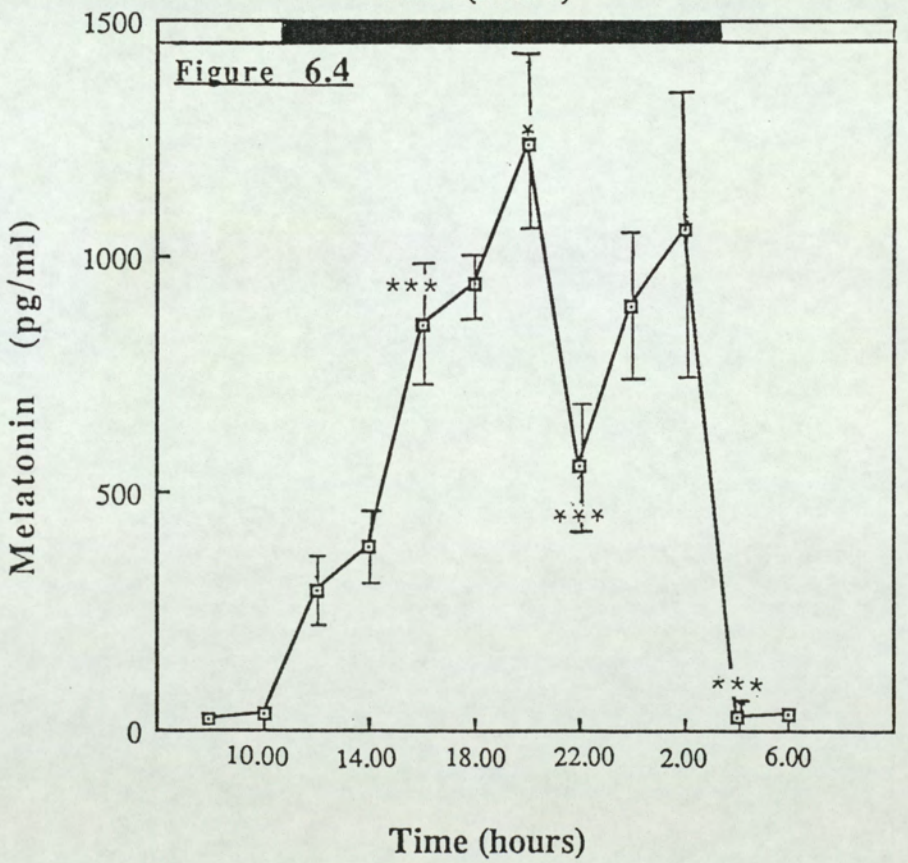


Figure 6.4



Figures 6.3 and 6.4 Serum melatonin levels of dace maintained under a long (18L:6D) and short photoperiod (6L:18D) respectively. The vertical bars represent the standard error of the mean. The shaded areas represent the dark period.

6.4 Discussion

The nocturnal rise in melatonin levels shown in the dace is a characteristic feature of all species investigated to date: these include lizards (Kennaway *et al.*, 1977), birds (Ralph, 1976) mammals (Lynch *et al.*, 1975; Rollag and Niswender, 1976) and other fish (for example the rainbow trout: Gern *et al.*, 1978). Although the magnitude of the rise varies among and between phyla, generally nightime levels are between 2-10 times greater than daytime levels. In this experiment however, nightime melatonin levels were up to 50 times greater than that measured in the daytime in fish maintained on a short day (fig.6.3). and 20 times greater than daytime levels in fish maintained on a long day (fig.6.4). Why nocturnal melatonin levels in the dace should be considerably higher than most other species, is at present unknown. However, as the fish in this experiment were blood sampled on more than one occasion, the overall higher melatonin levels recorded may have been due, at least in part, to a corticosteroid-induced response to stress. Stress has been shown to cause a general imbalance in fish physiology (Strange *et al.*, 1977; Leach and Taylor, 1980) and more specifically increase melatonin output in rats (Lynch *et al.*, 1977). If fish melatonin secretion responds to stress in a similar fashion to that seen in rats, this may account for the extremely high melatonin levels recorded in the dace. The similarity in response in melatonin levels in the dace to those found in the rainbow trout (Duston and Bromage, 1987), although higher, that is a nocturnal rise in melatonin which is dependent on the photoperiod strongly suggests that the heterologous RIA adopted in this study was specific in detecting melatonin. It should be stressed however, that the results in this study be treated with caution as the RIA was not validated in these studies. However, melatonin levels recorded in the trout using a different heterologous but validated assay (Gern *et al.*, 1978) provided levels very similar to those reported by Duston and Bromage (1987) in the same species, using the same assay procedure adopted in this study on the dace. This provides strong evidence that the melatonin levels recorded in the dace are accurate.

The observations in this study showing that nightime melatonin levels recorded in fish maintained on a short day rose to higher levels than those in fish maintained on a long day is in contrast to a number of other species, for example the rainbow trout (Duston and Bromage, 1987; Gern *et al.*, 1978); quail (Cockrem and Follett, 1984). and sheep (Lincoln *et al.* 1985) where the amplitude of the nocturnal rise was greater in animals maintained on a long

photoperiod. The reasons for this observation are at present unknown. However, because this experiment was only performed once, and only a small number of fish were used, individual variation in melatonin levels may have accounted for the differences observed in this study compared to those found in other species. In addition, it is not known in fish, whether the photoperiodic history of the animals before an experiment is undertaken, affects the melatonin response. Therefore, this may also account for the differences in the nocturnal melatonin pattern under different photoperiods found in this study.

Despite the differences in the magnitude of the melatonin response, the rhythm of melatonin secretion into the blood, being high during the night and low during the day, appears to be a consistent feature in all the species from the different vertebrate classes studied to date. However, in mammals the pattern of the increase in melatonin during the dark phase seems to be specific for a given species. Three different patterns or types of nocturnal rhythms have been identified, designated Type i, ii and iii (see Reiter, 1987)(fig.6.5). In a type i response, seen for example in the Syrian hamster, *Mesocricetus auratus* (Panke *et al.*, 1979; Tamarkin *et al.*,1979), the onset of darkness is not associated with immediate stimulation of melatonin production, instead melatonin concentrations remain similar to daytime levels for the first few hours, before a sharp increase and subsequent peak in melatonin production later in the night. In this pattern, the levels decline before the onset of light.

A type ii melatonin rhythm is typical of the rat (Johnston *et al.*, 1982) and the human (Waldhauser *et al.*, 1984). In these species melatonin production begins to increase at or shortly after the onset of darkness, the levels continue to rise gradually to reach a peak near the middle of the dark phase, melatonin levels then drop to reach daytime concentrations at about the initiation of dawn. In the type iii pattern of melatonin secretion, melatonin values increase soon after "lights off" to reach a plateau which is maintained for the duration of the dark phase; either shortly before, or at the time of "lights on", the melatonin levels drop to the low daytime levels. This type of rhythm is typical of that produced by the djungarian hamster (Goldman *et al.*, 1982) and sheep (Lincoln *et al.*, 1985).

The physiological significance of these different types of rhythms in melatonin secretion into the blood is unknown. However several studies have shown that the type of melatonin rhythm in a particular species may relate to a number of factors, for example the genetic

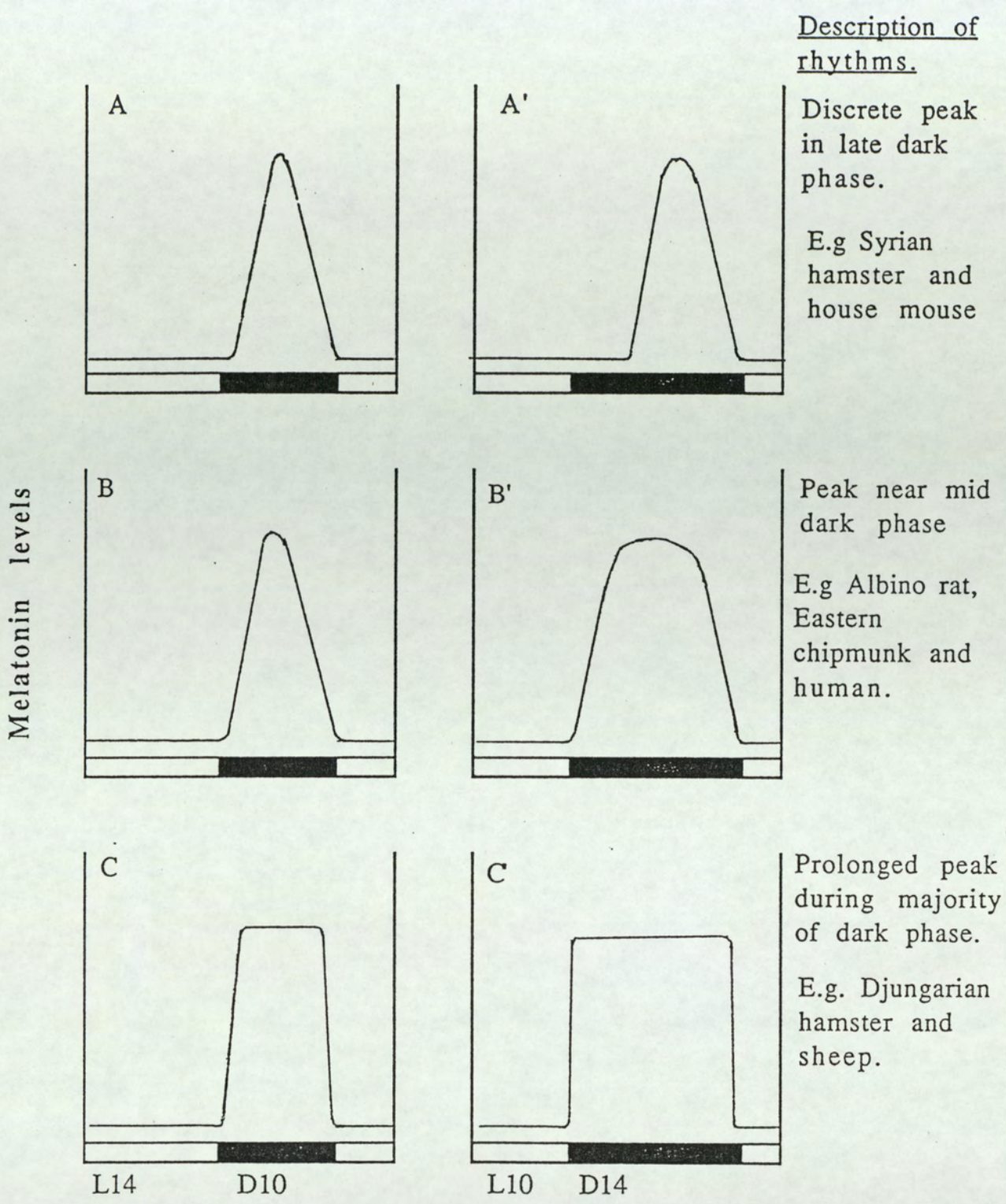


Figure 6.5. Types of melatonin rhythms found in mammals. A-C corresponds to Type i, ii and iii rhythms respectively. Irrespective of the nature of the melatonin rhythm, increasing the duration of the dark phase of the daily light:dark cycle causes a prolongation of the daily melatonin peak (A'-C'). The solid black bars represent the daily dark periods. Redrawn from Reiter (1987).

make-up of the animal (Ebihara *et al*, 1986), photoperiod to which the animal has been exposed to previously (Reiter *et al.*, 1983), the light intensity during the daytime (Lynch *et al.*, 1981) and the endocrine state of the animal (Reiter, 1986).

In fish, because so few species have been investigated, different types of melatonin rhythms, indeed, if they occur, have yet to be established. In the trout (Duston and Bromage, 1987) and the pike (Falcon *et al.*, 1987), a type iii rhythm of melatonin production has been found, that is the levels of melatonin gradually increase at the onset of nightime to reach peak levels near the middle of the dark period. In the dace, although the rhythm of nightime melatonin in fish maintained on a long day would appear to be that of a type ii response, the rhythm in fish maintained on a short day is more like that of a type iii rhythm, that is elevated for the duration of the night. Another unusual feature of the melatonin rhythm in dace maintained on short days is that during the night, melatonin levels were not maintained at a constant levels, but appeared to be episodic. However, the lowest value recorded during the night was still significantly higher($P<0.001$) than daytime levels. Although this may have been due to individual variation, Gern and Greenhouse (1988) working on superfused pineal glands of the rainbow trout demonstrated that melatonin was also released from the pineal in a pulsatile manner. The cause and significance of rapid fluctuations of melatonin levels are unknown. Furthermore, it is not known whether they result from episodic secretions, alterations in clearance from the blood, or changes in pineal blood supply. It is worthwhile emphasising that regular oscillations like this are characteristic of other neurohormones, such as oxytocin or GnRH.

The fact that melatonin in dace maintained under a short day appears to be secreted in a pulsatile manner, and that nocturnal elevations in melatonin begin to decrease before the onset of light suggests that melatonin rhythms in this species are not merely due to the turning "on" or "off" of the light, but due to a more complex mechanism. Cockrem and Follett (1984), cited the fall in pineal levels of melatonin before "lights on" in the Japenese quail as evidence of a circadian involvement. However, one of the features of circadian rhythms, introduced earlier in the chapter, is that they persist under constant conditions, though they may deviate from 24 hours. However, Gern and Greenhouse (1988) working on superfused trout pineals failed to demonstrate a rhythm of melatonin secretion under constant darkness (DD). However, a change in the photoperiod to 4L:10D did alter the pattern of melatonin secretion, demonstrating that the organ was still capable of responding to changes in

illumination. Several reasons may account for the lack of melatonin rhythmicity observed under DD in these *in-vitro* experiments. For example an essential component of the medium may have been lacking (Takahashi *et al.*, 1980) or it may have been due to necrosis of the organ while in culture. Further it may have been inherent to the particular strain of rainbow trout they used as genetic make-up is also important (Ebihara *et al.*, 1986). However this lack of endogenous rhythmicity in melatonin secretion in trout pineal organs maintained under DD is supported by data on NAT (the rate limiting enzyme of melatonin biosynthesis) rhythmicity in the pike (Falcon *et al.*, 1987). No rhythmicity in NAT was found in organs maintained under DD, though a low significant peak was maintained under LL. This is in contrast to mammals, where NAT activity continues to oscillate *in-vivo* being entrained by an endogenous or exogenous oscillator (Binkley, 1983; Klein *et al.*, 1981; Reiter, 1982). In poikilotherms, however, temperature conditions have been shown to influence melatonin rhythms (Vivien-Roels and Arendt, 1983). For example the amplitude of the rhythm is reduced when temperatures are reduced from 37 to 22°C in the lizard, *Anolis* (Underwood, 1983), and completely abolished in the turtle at +5°C. In a similar fashion, in the pike, the damping of the NAT activity rhythm in DD might have resulted from previous adaptation of short daylenghths and cold temperatures. It is of interest to note here, that both the rainbow trout and the pike, in which the NAT and melatonin rhythms were removed under DD breed under winter conditions. As the dace also breeds under relatively cold temperatures, it would be interesting to investigate what happens to the melatonin rhythm under DD in this species, as the lack of circadian rhythmicity may be characteristic of animals breeding under cold temperatures.

It appears that in the dace, as in other teleosts and higher vertebrates, melatonin is secreted in a rhythmic manner, with pattern of secretion synchronized to the external environment. Because daily variations of melatonin in the plasma parallel those in the pineal organ (Gern and Greenhouse, 1988; Falcon *et al.*, 1987), it is suggested that newly synthesized melatonin is released into the bloodstream. This finding is corroborated in fish where previous data on rainbow trout demonstrated a lowering of plasma melatonin levels after pinealectomy (Gern *et al.*, 1978). Melatonin and its pattern of production would therefore seem to have all the properties of a time-keeping hormone: its secretion is light sensitive and changes under different photoperiods; it originates from a structure known to mediate the photoperiodic response of an

organism, and generally it has the characteristics of a circadian rhythm. Furthermore, with respect to the latter property, there is now compelling evidence that circadian rhythms are used to measure the length of the day in a wide variety of birds and mammals (Follett and Sharp, 1969; Almeida and Lincoln, 1982; Turek *et al.*, 1984).

One important question that arises from these data, is how do circadian rhythms measure daylength? Several models have been proposed (see introduction to this chapter), but as yet none provide the definite answer. The first hypothesis, the duration hypothesis, initially proposed by Bunning (1973), involves an endogenous timer. If we apply the endogenous timer theory to the pineal gland, then a product (melatonin) is produced during darkness and the length of the dark period is measured by the duration of elevated melatonin levels. Irrespective of the nocturnal pattern of melatonin production, increasing the duration of the daily dark period, within limits, also causes an increase in the duration of elevated melatonin (Arendt, 1983; Goldman *et al.*, 1982). Another feature of this melatonin rhythm is that a rapid adjustment in the melatonin level occurs in response to change in daylength (Bittman *et al.*, 1983). In addition, in the sheep (Bittman et al., 1983; Bittman and Karsch, 1984) and the djungarian hamster (Carter and Goldman, 1983), studies have demonstrated that the timing of the melatonin infusion relative to the light/ dark cycle was unimportant in determining the inhibitory effect of melatonin on the reproductive sysytem. On the basis of these findings, these phenomena have led to the idea that the duration of the elevated melatonin levels is the signal which provides the message from the pineal gland to the effector organs of the organism. Thus the increasing duration of nightime melatonin levels with increasing night length in many species provide further evidence that the pineal gland perceives and transduces the photic environment, providing the organism with a means to measure the time of year.

The duration of nightime melatonin alone however, does not explain all of the physiological responses to melatonin. In one experiment conducted by Hoffman *et al.* (1985), Djungarian hamsters were maintained on either a 16L:8D or 6L:18D photoperiod regime. After 8 weeks the hamsters on long days had large gonads while those maintained on short days had small atrophic reproductive organs. On transferring both groups of hamsters on to a 14L:10D photoperiod regime, the original group maintained on long days interpreted this new regime as a short day (their gonads became involuted) while those originally maintained on a short day interpreted 14L:10D as a long day (their gonads regenerated).

However, the nighttime melatonin levels in the hamsters maintained on 14L:10D were the same, indicating that the melatonin rhythm was not providing the same response in both groups of animals. Thus not only the duration of the melatonin levels are important, but also the direction of change may be essential in determining the ability of melatonin to influence a given system.

The external and internal coincidence models described briefly in the introduction to this chapter explain how light/dark cycles are integrated with the internal environment. These theories if applied to the pineal organ and its product, melatonin, describe how melatonin exerts its endocrine actions in the whole organism. In the external coincidence model, which relies on a circadian rhythm of sensitivity (Pittendrigh, 1976), the photosensitive phase of the underlying rhythm of sensitivity coincides with the experimental dark phase and in the case of the pineal gland with the product melatonin only under short day conditions. Under these conditions, the interaction of melatonin with an appropriate sensitive target organ(s) leads to the appropriate response(s).

According to the internal coincidence model, there are at least two rhythms operative in determining the action of melatonin. One is the already described melatonin rhythm; the second is the rhythm of organ sensitivity to melatonin. According to this hypothesis, the product (melatonin) is only capable of acting when melatonin production and secretion coincides with a "window" of maximal organ sensitivity. Thus under long day conditions, for example, Syrian hamsters have large functional reproductive organs despite the fact that there is a melatonin peak every night. According to the coincidence model, the neuroendocrine axis is not sensitive to the melatonin signal at this particular time. However, the animal could be sensitive to melatonin at some other time during the 24 hour period.

The coincidence models, in particular the internal coincidence model has greater flexibility than the duration hypothesis. It is possible that each organ system could have its own window of sensitivity to melatonin (Reiter, 1985). Thus at any given photoperiod, the sensitivity of one organ system would be coincident with peak melatonin levels while the sensitivity rhythms of other organ systems may be out of phase with the melatonin rhythm. This would explain why when melatonin is administered, it may influence the function of some organs but not others, and also why exogenously administered melatonin's ability to influence the function of some organs depends on the time of day or night that it is

given. Also important is the phasing of the melatonin rhythm with the organ sensitivity rhythm. This sensitivity rhythm of the target organ, for example, the reproductive system can be greatly changed by factors such as quantity and quality of food consumed (Sorrentino *et al.*, 1971); olfactory information (Reiter *et al.*, 1980) and changes in ambient temperature (Desjardins and Lopez, 1980).

To summarize the results from this chapter, we have demonstrated that like in many other species, melatonin in the dace is elevated during the subjective night, and that the pattern of melatonin secretion is determined by the photoperiod regime to which the animals are subjected. The possibility that melatonin in the dace is secreted in a pulsatile manner and that levels in dace maintained under short days decrease before "lights on" is suggestive of more complex circadian rhythms, as discussed earlier. Although in mammals, there is compelling evidence that circadian rhythms are used to measure the length of day (Follett and Sharp, 1969; Lincoln *et al.*, 1981; Almeida and Lincoln, 1982; Karsch *et al.*, 1984), in fish only work on locomotor rhythms (Kavaliers, 1979) has indicated that the pineal gland has an important role in the organization of circadian and possibly circannual rhythms. However, numerous studies involving pinealectomy and melatonin infusions, have shown that the reproductive system is dramatically affected by this gland and its product melatonin. Overall, although not conclusive, the results support the hypothesis that dace can measure changes in the photic environment by means of the production of a rhythm of melatonin, which in turn drives the reproductive response.

CHAPTER 7

Conclusions

7.1 Conclusions

The results presented clearly show that under natural conditions the dace exhibits an annual cycle of ovarian development which culminates in spawning in mid-March.

The cycle of ovarian development can broadly be divided into four phases according to the morphological and histological state of the oocytes: a) oogenesis; this occurs in the period immediately after spawning under natural conditions largely between March and April, b) a primary growth phase (largely that determined by previtellogenic growth) occuring from April up until June, c) a secondary growth phase (vitellogenic growth) which largely occurs between June and December and finally d) maturation which occurs during a short period between early and mid-March leading to ovulation and spawning.

Data collected from both histological studies and determined by the numbers and sizes of oocytes in the developing ovary show that the dace may be considered as a 'group synchronous' spawner, in that in the ovary at any one time, there are two populations of oocytes, primary oocytes from which the previtellogenic and vitellogenic stages are recruited and a group of 'synchronously' developing secondary oocytes which form the season's batch of eggs. Although the seasons' batch of secondary oocytes develop simultaneously, it should be stressed that within an ovary during early vitellogenic development, there is a clear variation in the size of vitellogenic oocytes. A 'tight' synchrony in oocyte sizes did not occur until the end of December, two months prior to spawning.

The variation in size of secondary oocytes during the vitellogenic phase would suggest that ovarian development is not regulated solely by blood hormones because if so, all the oocytes in the secondary growth phase should respond to a systemic hormonal signal and subsequently develop at equal rates and therefore be of equal size, however, this is clearly not the case. One possible explanation is that individual follicles regulate or at least 'fine-tune' their own growth rates through more local elements such as growth factors secreted from the follicles themselves. To date however, growth factors have not been identified or isolated in teleosts.

Analyses of the blood steroids, testosterone and 17β-oestradiol during the natural reproductive cycle illustrated elevated levels in April. These elevations were followed by an increase in serum levels of calcium (an indirect index of blood vitellogenin) and the subsequent appearance of yolk granules in the developing oocytes.

Although 17β-oestradiol is known to stimulate vitellogenin production, the role of testosterone during this period is unknown. It is probable however, that testosterone may serve as a precursor for the production of oestrogens.

Vitellogenic growth of the ovary appeared to continue throughout the winter months albeit at a slower rate than the summer months. This is unlike the situation in another cyprinid, the carp, in which oocyte development is arrested during the winter due to cold water temperatures. It is possible that the high serum levels of 17β-oestradiol observed between September and December in the dace were required to maintain vitellogenin production by the liver, which may otherwise have been halted by the cold water temperatures.

Blood calcium levels had dropped considerably as the follicles approached final maturation and ovulation. However, during this period oocytes continued to increase in size (upto a mm in diameter). It is likely therefore that growth during this period occurs largely due to the uptake of water. Similar conclusions have been drawn in studies on the rainbow trout (Riazi and Fremont, 1988) and the killifish (Wallace and Selman, 1981).

In contrast to serum 17β-oestradiol and calcium levels, serum testosterone levels remained elevated prior to final maturation. Testosterone is the precursor of many other steroids and high testosterone levels at this time probably provide a 'pool' of precursors(s) for other steroids to be produced. Likely products at this time include steroid glucuronides involved in courtship behaviour and the attraction of a mate (see Chapter 4) and closer to ovulation the maturation-inducing steroid 17α-hydroxy,20β-dihydroxy progesterone.

Ovulation and spawning under natural conditions occurred at approximately the same time each year (within a week) suggesting that the processes of ovarian development are precisely timed. As water temperatures at the time of spawning are still cool and indeed may vary considerably from year to year, it is unlikely that temperature plays a major role in the timing of ovulation and spawning in this species. It is likely, however, that daylength which does not vary from year to year is the most important environmental cue involved in the timing of reproduction in the dace.

Under captive conditions, final maturation beyond the germinal vesicle migration stage, failed to occur spontaneously. It is unclear why G.V.B.D. and ovulation failed to occur, however it is likely that the specific environmental stimuli required for final maturation beyond G.V.M. were absent under captive conditions. Alternatively,

the stress of handling the animals and/or confinement in tanks inhibited these events. It was possible to artificially induce G.V.B.D. and ovulation in captivity however, by injecting fish with Carp Pituitary Extract; only a single injection was required which induced ovulation 6-18 hours after treatment.

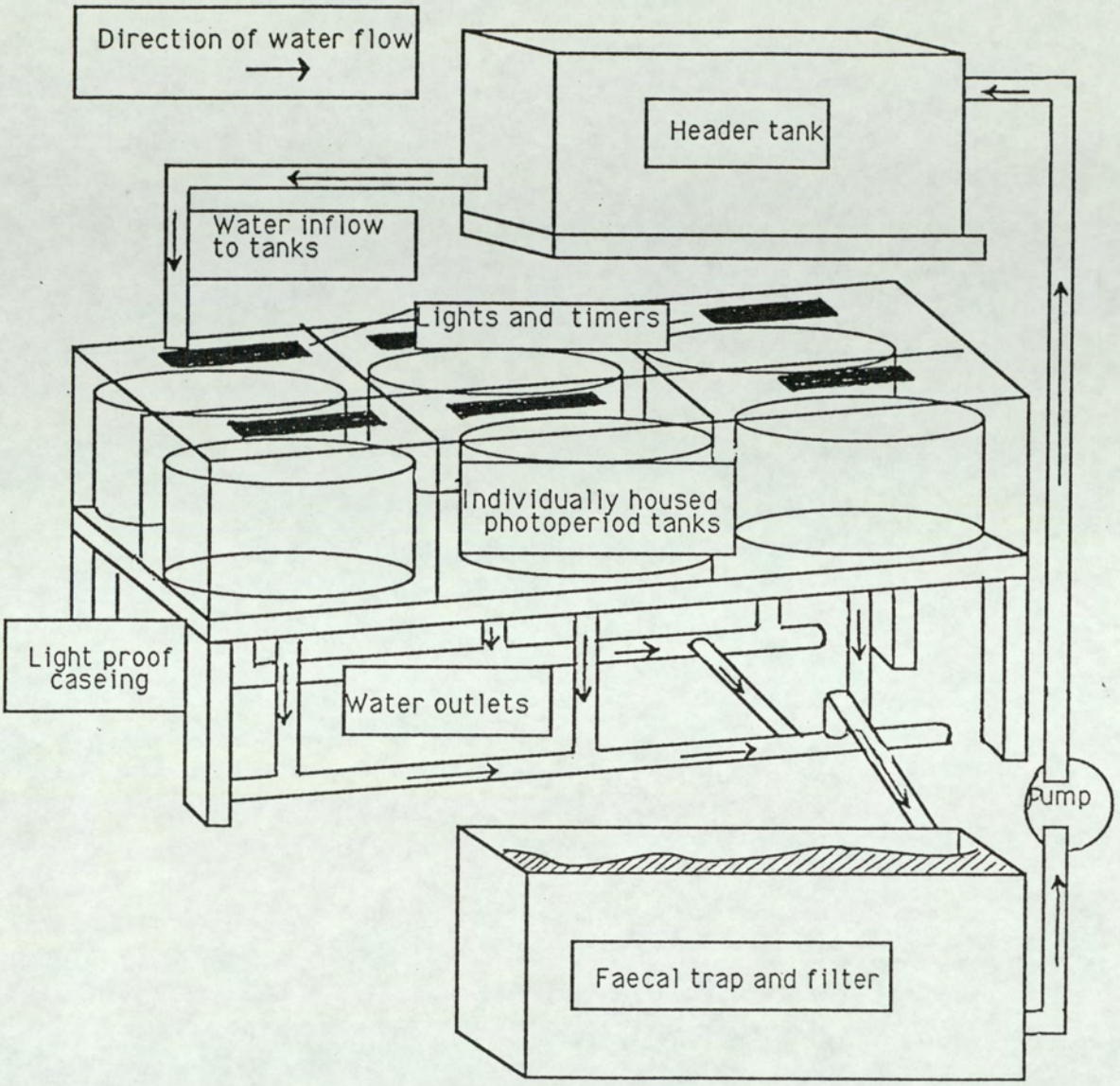
Under natural conditions, it is likely that reproduction is timed by seasonally changing daylengths. However, under experimental conditions, final maturation could also be achieved under constant daylengths suggesting that seasonally changing daylengths are not essential to time reproductive development. Investigations into the role of photoperiod on ovarian development and final maturation demonstrate that short and increasing daylengths are the most stimulatory. Under natural conditions, it is proposed therefore that a new ovarian cycle is initiated immediately after the previous spawning in March when daylengths although increasing are still short and final maturation in spring when daylengths are increasing. If ovarian development is initiated immediately after the previous spawning this would also suggest that there is no refractory period in the dace.

Final maturation was advanced (up to 5 months) in fish exposed to short daylengths earlier in the year than they would normally be exposed to them. This suggests that short photoperiods at this time are probably perceived as 'running behind' time and to compensate, final maturation was advanced. Because final maturation occurred at approximately the same time (in September) in all experimental groups irrespective of the time of exposure to a short day, it is suggested that oocytes have to develop for a minimum period of time before they can mature.

The inability of dace to respond to daylengths of constant length from December onwards compared to earlier in the year suggests that like the stickleback, there is a seasonal variation in photosensitivity.

Analysis of blood melatonin demonstrated that the daily levels varied according to the daylength; they were elevated during the duration of the dark phase under both long and short daylengths, suggesting that the dace has the ability to distinguish between light periods of different duration and therefore "measure" daylength. This datum further supports the proposal that photoperiod is the dominant environmental cue involved in reproductive timing in the dace.

APPENDICES

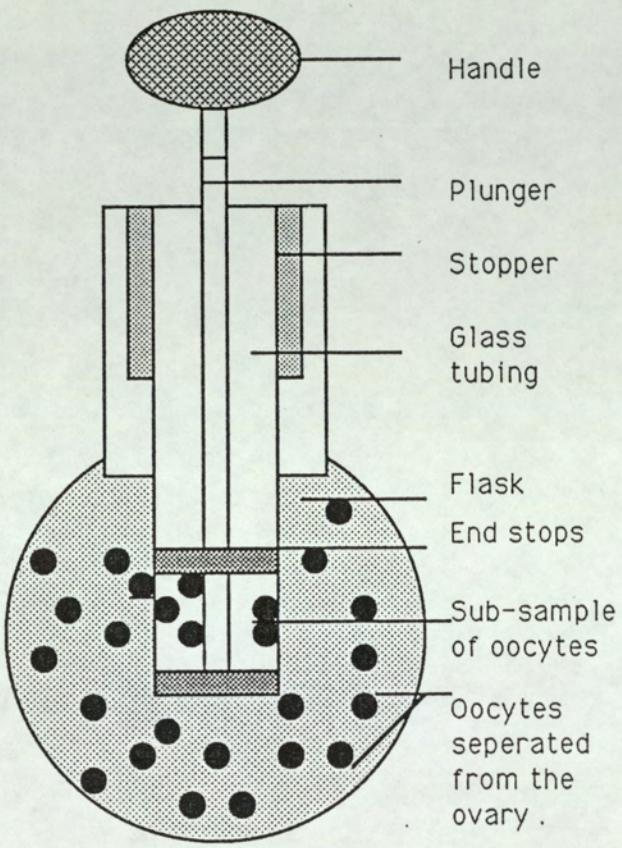


Appendix 1. Diagramatic representation of the recirculatory water tank systems used in this study.

Assay	Sample 1	Sample 2	Mean (x)	x	Std. dev.
1.	74	70	72	4	2.82
2.	93	93	93	0	0
3.	105	110	107.5	-5	3.53
4.	102	102	102	0	0
5.	105	95	100	10	7.07
6.	81	81	81	0	0
7.	78	82	80	-4	2.82
8.	70	70	70	0	0
9.	87	88	87.5	-1	0.7
10.	96	83	89.5	13	9.19
Average	89.1	87.4	88.25	1.7	4.04
Std. dev.	13	12.95	12.65	5.76	
SEM	4.11	4.09	4.0	1.82	

Intra assays	Std. dev.	Coefficient of variation
For single tube	4.04= sw	4.58
For means of duplicates	2.86	3.24
% coefficient of variation = 100 $\frac{sw}{x}$		
Inter assays		
For single tube	12.97	14.70
For means of duplicates	12.65	14.3

Appendix 2. An example of the method used to determine the inter– and intra–assay variances. (Ryan, 1981).



Appendix 3. Apparatus used for counting and sizing oocytes.

REFERENCES

8.1 References

Abraham, M., Hilge, V., Lison, S. and Tibika, H. (1984). The cellular envelope of oocytes in teleosts. Cell. Tiss. Res. 235: 403-420.

Ahsan, S.N. (1966). Effects of temperature and light on the cyclical changes in the spermatogenic activity of the lake chub, *Couesius plumbeus*. Can. J. Zool. 44: 161-171.

Almeida, O.F.X. and Lincoln, G.A. (1982). Photoperiodic regulation of reproductive activity in the ram: evidence for the involvement of circadian rhythms in melatonin and prolactin secretion. Biol. Reprod. 27:1062-75

Anderson, E. (1967). The formation of the primary envelope during oocyte differentiation in teleosts. J. Cell. Biol. 35: 193-212.

Anwand, K. (1963). Die wirkung von Hypophysean-und Gonabioninjektionen auf Hechtmilchner. Dtsch. Fischerei-z. 10(7): 202-207.

Arendt, J. (1983). Direct RIA for melatonin in plasma. Clinical chemistry, 29.

Bagenal, T.B. (1969). The relationship between food supply and fecundity in brown trout, *Salmo trutta* L. J. Fish. Biol. 1: 169-182.

Baggerman, B. (1972). Photoperiodic responses in the stickleback and their control by a daily rhythm of photosensitivity. Gen. Comp. Endocr. 3: 466- 476.

Baggerman, B. (1980). Photoperiodic and endogenous control of the annual reproductive cycle in teleost fishes. In: Ali, M (ed). Environmental Physiology of Fishes. Plennum Press, New York. pp 533-567.

Baggerman, B. (1988). Sticklebacks. In: Munro, A.D; Scott, A.P. and Lam, T.J. (eds). Reproduction in teleost fish: environment and control. CRC Press, Boca Raton, Florida.

Ball, J.N. (1981). Hypothalamic control of the pars distalis in fishes, amphibians and reptiles. Gen Comp. Endocr. 44: 135-170.

Bara, G. (1960). Histological and cytological changes in the ovaries of the mackerel, (*Scomber scomber*). Rev. Fac. Sci. Univ. Istanbul, Ser B. 25:49-91.

Barr,W.A. (1963). The endocrine control of the sexual cycle in the plaice, *Pleuronectes platessa* (L). I. Cyclical Changes in the normal ovary. Gen. Comp. Endocr. 3, 197- 204.

Barr, W.A. (1968). Patterns of ovarian activity. In: Barrington, E.J.W. and Jorgenson, C.B. (eds), Perspectives in Endocrinology. Hormones in the lives of lower vertebrates. Academic Press, New York, pp 163-237.

Baynes, S.M; Scott, A.P. and Dawson, A.P. (1981). Rainbow trout (*Salmo gairdneri*) spermatozoa: effects of cations and pH on motility. J. Fish Biol. 19:259-267.

Beams, H.W. and Kessel, R.G. (1973). Oocyte structure and early vitellogenesis in the trout *Salmo gairdneri*. Am. J. Anat. 136: 102-122.

Beumer, J.P. (1979). Reproductive cycles of two Australian freshwater fishes: the strangled perch, *Therapan unicolor* Gunther, 1859 and the East Queensland rainbow fish, *Nematocentris splendida* Peters, 1866. J.Fish. Biol. 15: 111-134.

Bienarz, B. and Epler, P. (1976). Prelimenary results of the *in-vitro* studies on ovarian resorption in carp, (*Cyprinus carpio* L.). J. Fish. Biol. 8:449-451.

Bienarz, K; Epler, P; Thuy, L.N. and Breton, B. (1980). Changes in blood gonadotropin level in mature female carp following hypophysial homogenate injections. Aquaculture, 20:65-69.

Billard, R. (1978). Testicular feedback on the hypothalamo-pituitary axis in rainbow trout, (*Salmo gairdneri*). Ann. Biol. Bioch Biophys. 18: 813-818.

Billard, R. (1982). The Reproductive Cycles in Teleost Fish. Estratta dalla Rivista Italienadi Piscicoltura e Ittiopatologia.

Billard, R. and Breton, B. (1977). Sensibilite a la temperature des

differentes etapes de la reproduction chez la truite arc-en-ciel. Cah. Lab. Hydrobiol. Montereau 5: 5-24.

Billard, R. and Breton, B. (1978). Rhythms of reproduction in teleost fish. In: Thorpe, J.E. (ed). Rhythmic activity in fishes, pp 31-53. Academic Press, London.

Billard, R., Bry, C. and Gillet, C. (1981). Stress, Environment and Reproduction in Teleost fish. In: Pickering, A.D. (ed). Stress and Fish. Freshwater Biological Association. Academic Press, London.

Billard, R., Breton, B., Fostier, A., Jalabert, B. and Weil, C (1978). Endocrine control of the teleost reproductive cycle and its relation to external factors; salmonid and cyprinid models. In: Gaillard, P.J; Boer, H.H. (eds). Comparative Endocrinology Elsevier/ North Holland, Amersham. pp37-48.

Binkley, S.A. (1976). Pineal gland biorhythms: N-acetyl transferase in chicken and rats. Fed. Proc 35:2347-2351.

Binkley, S.A. (1983). Rhythms in occular and pineal N-acetyltransferase. A portrait of an enzyme clock. Comp. Biochem. Physiol. A75: 123-129.

Bittman, E.L. and Karsch, F.J. (1984). Nightly duration of pineal melatonin secretion determines the reproductive response to inhibitory daylength in the ewe. Biol. Reprod, 30: 585-593.

Bittman, E.L., Dempsey, R.J. and Karsch, F.J. (1983). Pineal melatonin drives the reproductive response to daylength in the ewe. Endocr. 45:479-486.

Bogomolynaya, A. and Yaron, Z. (1984). Stimulation of *in-vitro* oestradiol secretion by the ovary of the cichlid fish, *Sarotherodon aureus*. Gen. Comp. Endocr, 53: 187-196.

Borg, B. (1982), Extraretinal photoreception involved in photoperiodic effects on reproduction in male three spined stickleback, *Gasterosteus aculeatus*. Gen. Comp. Endocr. 47: 84

Borg, B. and Ekstrom, P. (1981). Gonadal effects of melatonin in the three spined stickleback, *Gasterosteus aculeatus* L., during

different seasons and photoperiods. Reprod. Nutr. Develop. 21: 919.

Borg, B. and Van Veen, T. (1982). Seasonal effects of photoperiod and temperature on the ovary of the three spined stickleback, *Gasterosteus aculeatus*. Can. J. Zool. 60: 3387-3393.

Breder, C.M.Jr. and Rosen, D.E. (1966). Modes of reproduction in fish. Natural History Press, New York.

Breton, B. and Billard, R. (1977). Effects of photoperiod and temperature on plasma gonadotropin and spermatogenesis in the rainbow trout, *Salmo gairdneri,* Richardson. Annales de biologie animale, biochemie et biophysique, 17: 331-340.

Breton, B., Horoszewicz, L., Billard, R. and Bienarz, K. (1980). Temperature and reproduction in tench: the effect of a rise in the annual temperature regime on gonadotropin level, gametogenesis and spawning. 1. The male reproduction. Reprod. Nutr. Develop, 20: 105-118.

Breton, B., Fostier, A., Zohar, Y., Le Bail, P.Y. and Billard, B. (1983). Gonadotropine Glycoproteique Maturante et oestradiol-17b Pendant le cycle Reproducteur chez la Truite Fario (*Salmo trutta*). Femelle. Gen. Comp. Endocr. 49: 220-231.

Brighty, G.C., Macfarlane, N.A.A. and Easton, K.W. (1987). Plasma steroid profiles during gonad maturation and spawning in the dace, *Leuciscus leuciscus* (L.). In: Proceedings of the Third International Symposium on the Reproductive Physiology of Fish. St Johns, Newfoundland, Canada. pp -193.

Bromage, N.R. and Cumaranatunga, P.R.T. (1988). Egg production in the rainbow trout. In: Recent Advances in Aquaculture. Vol 3, Chapman and Hall publications.

Bromage, N.R., Whitehead, C. and Breton, B. (1982). Relationships between serum levels of gonadotropin, 17b-oestradiol and vitellogenin in the control of ovarian development in the rainbow trout. II. The effects of alterations in environmental photoperiod. Gen. Comp. Endocr, 47: 366-376.

Bromage, N.R., Elliott, J.A.K., Springate, J.R.C. and Whitehead, C. (1984). The effects of constant photoperiods on the

timing of spawning in the rainbow trout. Aquaculture, 43: 213-224.

Brommalaer, M.C., Billard, R. and Breton, B. (1981). Changes in plasma gonadotropin after ovariectomy and oestradiol supplementation at different stages at the end of the reproductive cycle in the rainbow trout, *Salmo gairdneri*. Reprod. Nutr. Develop. 21: 989-997.

Brook, A.J. and Bromage, N. R. (1988). Photoperiod: the principal environmental cue for reproduction in the dace, *Leuciscus leuciscus*. J. Interdisc. Cycle Res. 19: 165.

Brusle, S. (1980). Fine structure of early previtellogenic oocytes in *Mugil (Liza) auratus*. Risso, 1810 (Teleostei, Mugilidae). Cell. Tiss. Res. 207:123-124.

Bry, C. (1981), Temporal aspects of macroscopic changes in rainbow trout (*Salmo gairdneri*) oocytes before ovulation and of ova fertility during the post ovulation period: effect of treatment with 17α-hydroxy-20β dihydroxy progesterone. Aquaculture, 24: 153-160.

Bullough, W.S. (1939). A study of the reproductive cycle and some endocrine factors affecting the adult minnow. *Phoxinus phoxinus* (L.) J. Endocrinol 3:211-219.

Bullogh, W.S. (1942). Gametogenesis and some endocrine factors affecting the adult minnow *Phoxinus phoxinus*. J. Endocr. 3: 211-219.

Bunning, E. (1973). The physiological clock, Springer, New York.

Burzawa-Gerard, E. (1974). Etude biologique et biochimique de l'hormone gonadotrope d'un poisson teleosteen, la carpe (*Cyprinus carpio*). Mem. Mus. Hist. Nat. Ser. A. Zool. 86: 1-77.

Bye, V.J. (1984). The role of environmental factors in the timing of reproductive cycles. In: Potts, G.W. and Wootten, R.J. (eds). Fish reproduction; strategies and tactics. Academic Press, London.

Byskov, A.G. (1978). Follicular atresia. In: Jones, R.E. (ed). The Vertebrate Ovary. Plennum Press, New York and London. pp 533-562.

Cadusseau, J., Gaiilard, F. and Galand, G. (1979). Pineal response types in the frog's brain under white light exposure. Exp. Brain Res. 36: 41-51.

Callard, G.V. (1982). Aromatase in the teleost brain and pituitary. Role in hormone action. In: Reproductive Physiology of Fish. (C.J.J. Richter. and H.J.Th. Goos, comps). p 40-43. Pudoc, Wageningen.

Campbell, C.M. (1978). In-vitro stimulation of vitellogenin incorporation into trout oocytes by salmon pituitary extracts. Ann. Biol.. Anim. Bioch. Biophys. 18: 1013-1018.

Campbell, C.M. and Idler, D.R. (1976). Hormonal control of vitellogenesis in hypophysectomized winter flounder (*Pseudopleuronectes americanus*, Wallbum). Gen. Comp. Endocr. 28: 143-150.

Campbell, C.M., Walsh, J.M. and Idler, D.R. (1976). Steroids in the plasma of the winter flounder (*Pseudopleuronectes americanus* Wallbum). A seasonal study and investigation of steroid involvement in oocyte maturation. Gen. Comp. Endocrinol. 29: 14-20.

Carter, D.S. and Goldman, B.D. (1983). Antigonadal effects of timed melatonin infusion in pinealectomized male djungarian hamsters (*Phodopus sungorus-sungorus*)- Duration is the critical parameter. Endocr. 113: 1268-1273.

Cassone, V.M. and Menaker, M. (1983). Sympathetic regulation of chicken pineal rhythms. Brain Res. 272: 311-318.

Chan, K.(1976). A photosensitive daily rhythm in the female medaka, *Oryzias latipes*. Can. J. Zool. 54: 853-

Chan, K.K.S. (1977). Effect of synthetic leutenizing hormone-releasing hormone (LHRH) on ovarian development in Japenese medaka, *Oryzias latipes*. Can. J. Zool. 55: 155-160.

Clemens, H.P. and Reed, C.A. (1967). Testicular characteristics of goldfish, *Carrasius auratus*, in nature and under diet limitations. J. Morph. 122, 131-138.

Cockrem, J.F. and Follett, B.K. (1984). Rhythms of melatonin in

the Quail pineal gland *in vivo* and *in vitro*. Gen. Comp. Endocr. 53: 464.

Collins, J.P. (1981). Differentiation and regression of the cells of the sensory line in the epiphysis cerebri. In: Wolstenholme, G.E.W. and Knight, J. (eds). The Pineal Gland, pp79-125. Churchill Livingstone, Edinburgh.

Collins, J.P. and Okshe, A. (1981). Structural and functional relationships of the mammalian pineal gland. In: Reiter, R,J. (ed). The Pineal Gland, vol I. Anatomy and Biochemistry, pp27-67. CRC Press, Boca Raton, FL.

Colombo, L., Colombo Belvedere, P. and Arcarese, G. (1978). Emergence of ovarian 11-deoxycorticosteroid biosynthesis at ovulation time in the sea bass, *Dicentrarchus labrax* L. Annales de biologie animale, biochimie et biophysique 18: 937-941.

Colombo, L., Colombo Belvedere, P., Marconato, A. and Bentivegna, F. (1982). Pheromones in teleost fish. In: Richter, C.J.J. and Goos, H.J.Th (eds). Proc. Int. Symp. Reprod. Physiol. Fish. Wageningen, The Netherlands. 2-6th August, Pudoc, Wageningen, pp84-94

Co-operative Team for Hormone Application in Pisciculture, (1977). A new highly effective ovulating agent for fish reproduction. Scientia. Sin. 20: 469-474.

Cragg-Hine, D (1963). The natural history of several species of coarse fish in a small lowland stream. Proc. 1st Br. Coarse fish Conf. 3-7.

Cragg-Hine, D. (1964). The biology of the coarse fish of Willow Brook, Northhamptonshire. PhD thesis, University of Liverpool.

Crim, L.W. (1982). Control of gonadotropic hormone secretions by the rainbow trout pituitary gland. Evidence of GTH inhibition by catecholamine and stimulation of GTH release by some other neuroregulatory factors. In: Lederis, K. and Farner, D.S. (eds). Neurosecretion. . 446. Plennum, New York.

Crim. L.W. (1982). Environmental modulation of annual and daily

rhythms associated with reproduction in teleost fishes. Can. J. Fish. Aquat. Sci. 39: 17-21.

Crim, L.W. and Idler, D.R. (1978). Plasma gonadotropin, oestradiol and vitellogenin and gonad phosvitin levels in relation to the seasonal reproductive cycles of female brown trout. Ann. Biol. Anim. Bioch. Biophys. 18 (4): 1001-1005.

Crim, L.W., Peter, R.E. and Billard, R. (1981). Onset of gonadotropic hormone accumulation in the immature trout pituitary gland in response to oestrogen or aromatizeable steroid hormones. Gen. Comp. Endocr. 44: 374-381.

Cumaranatunga, P.R.T. (1985). The ovarian cycle of rainbow trout with special reference to atresia. PhD thesis, University of Aston.

Cushing, D.H. and Burd, A.C. (1956). Recruitment and maturity of the East Anglian herring, Nature, London 178: 86-87.

Day, J.R. and Taylor, M.H. (1983). Environmental control of the annual gonadal cycle of *Fundulus heteroclitus* L.: the pineal organ and eyes. J.Exp. Zool. 227: 453-

Day, J.R. and Taylor, M.H. (1984). Photoperiod and temperature interaction in the seasonal reproduction of female mummichogs. Trans. Am. Fish. Soc. 113:452-457.

Delahunty, G., Schreck, C.B. and de Vlaming, V. (1980). Effects of photoperiod on plasma corticoid levels in the goldfish, *Carrasius auratus*- role of the pineal organ. Comp. Biochem. Physiol. 65A, 355

Delahunty, G., Bauer, G., Prack, M. and de Vlaming, V. (1978). Effects of pinealectomy and melatonin treatment on liver and plasma metabolites in the goldfish, *Carrasius auratus*. Gen. Comp. Endocr. 35: 99-109.

Delahunty, G., Schreck, C.B., Specker, J., Olcese, J., Vodicnik, M.J. and Donaldson, E.M. (1973). Reproductive endocrinology of fishes. Am. Zool. 13:909-927.

Delahunty, G., Schreck, C.B., Specker, J., Olcese, J., Vodicnik, M.J. and de Vlaming, V.L. (1979). The effects of light reception on circulating oestrogen levels in female goldfish, *Carrasius auratus*:

importance of retinal pathways versus the pineal. Gen. Comp. Endocr. 38: 148-152.

Demain, I.J. and Taylor, D.H. (1977). Photoreception and locomotor rhythm entrainment by the pineal body of the newt, *Notopthalmus viridiscens*. J. Herpetol. 11: 131-140.

Desjardins, C. and Lopez, M.J. (1980). Testicular development, Structure and Function. Steinberger, A. and Steinberger, E. (eds). pp 381-388. Raven, New York.

Dickhoff, W.W. and Swanson, P. (1989). Functions of salmon pituitary glycoprotein hormones: the maturational surge hypothesis. XIth International Symposium on Comparative Endocrinology, Malaga.

Dodd, J.M. and Sumpter, J.P.(1982). Reproductive cycles of cyclostomes, elasmobranchs and bony fishes. In: Lamming, G.K. (ed). Marshall's Physiology of Reproduction. Vol 1. Chap 1, 4th ed. Churchill Livingston, London.

Donaldson, E.M. (1975). Physiological and physiochemical factors associated with maturation and spawning. EIFAC Workshop on controlled reproduction in cultivation of fishes. Hanberg. pp 53-71.

Donaldson, E.M. (1981). The pituitary-interrenal axis as an indicator of stress in fish. In: Pickering, A.D. (ed). Stress and Fish, pp 11-47. Academic Press, London.

Douglas, R.H. (1980). Visual adaptation and spectral selectivity in rainbow trout (*Salmo gairdneri*). PhD thesis, University of Sterling.

Duffey, R.J. and Goetz, F.W. (1980). The in-vitro effects of 17α hydroxy-20β dihydroprogesterone on germinal vesicle breakdown in brook trout (*Salvelinus fontinalis*) oocytes. Gen. Comp. Endocrinol. 41: 563-565.

Duston, J. and Bromage, N.R. (1986). Photoperiodic mechanisms and rhythms of reproduction in the female trout. Fish. Phys. and Biochem. 2: 35-51.

Duston, J. and Bromage, N. R. (1987). Constant photoperiod regimes and the entrainment of the annual cycle of reproduction in the female rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocr. 65: 373-384.

Eakin, R.M. and Westfall, J.A. (1960). Further observations on the fine structure of the parietal eye of lizards. J. Biphys. Biochem. Cytol. 8: 483-499.

Ebihara, S., Marks, T., Hudson, D.J. and Menaker, M. (1986). Genetic control of melatonin synthesis in the pineal gland of the mouse. Science, 231: 491-493.

Egami, N. and Hosokawa, K. (1973). Responses of gonads to environmental changes in the fish, *Oryzias latipes*. In: Chavin, W. (ed). Responses of fish to environmental changes, pp 279-301. Springfield. C.C.Thomas.

Eigenmann, C.H. (1892). *Cymatogaster aggregatus* Gibbons: A contribution to the ontogeny of viviparous fishes. Bull. U.S. Fish. Comm. 12: 401-478.

Elliott, J.A.K. (1982). Neuroendocrine mechanisms involved in the control of ovarian development and vitellogenesis in the rainbow trout. PhD thesis, Aston University, Birmingham.

Elliott, J.A.K., Bromage, N.R. and Whitehead, C. (1979). Effects of 17β-oestradiol on serum calcium and vitellogenin levels in the rainbow trout. J. Endocrinol. 83; 54-55.

Elliott, J.A.K., Bromage, N.R. and Springate, J.R.C. (1984). Changes in reproductive function of three strains of rainbow trout exposed to constant and seasonally changing light cycles. Aquaculture, 43: 23-34.

Epler, P. (1981). Effect of steroid and gonadotropic hormones on the maturation of carp ovaries. Part II. Effects of mammalian gonadotropins on the maturation of carp oocytes *in-vitro*. Pol. Arch. Hydrobiol. 28: 95-102

Epler, P. and Bienarz, K. (1983). Effects of triiodothyronine and of some gonadotropic and steroid hormones on the maturatin of carp (*Cyprinus carpio*) oocytes *in-vitro*. Reprod. Nutr. 23: 1011-1017.

Escaffre, A.M. and Billard, R. (1976). Cahiers Lab. Monterau 3: 43-46

Falcon, J., Balemans, M.G.M., Van Benthem, J. and Collin, J.P. (1985). *In-vitro* uptake and metabolism of (3H) indole compounds in the pineal organ of the pike. I. A radiochromatographic study. J. Pineal Res. 2: 341-356.

Falcon, J., Guerlotte, J.F., Voisin, R. and Collin, J. P. H. (1987). Rhythmic melatonin biosynthesis in a photoreceptive pineal organ: A study in the pike. Neuroendocrinology, 45: 470-486.

Fenwick, J.C. (1969). The Pineal Organ. In: Hoar, W.S. and Randall, D.J. (eds). Fish Physiology. vol 4, pp 91-108. Academic Press, New York.

Fenwick, J.C. (1970a). Demonstration and effect of melatonin in fish. Gen. Comp. Endocr. 14: 86-97.

Fenwick, J.C. (1970b). Effects of pinealectomy and bilateral enucleation on the phototactic response and the conditioned response to light of the goldfish, *Carrasius auratus* L. Can. J. Zool. 48: 175-182.

Flugel, H. (1967). Elektrnemikroskopische untersuch ungen an dian hullen der oozyten und eir des flussabanschues, *P fluviatalis*. Z. Zellforch. Mikrosk. Anat. 77: 244-256.

Follett, B.K. (1978). Photoperiodism and seasonal breeding in birds and mammals. In: Crighton, D,B; Haynes, B; Foxcroft, G. and Lamming, G.E. (eds). Control of ovulation, pp367-. Butterworths, London.

Follett, B.K. and Follett, D.E. (eds) (1981) Biological clocks in seasonal reproductive cycles. Wright, Bristol.

Follett, B.K. and Sharp, P.J. (1969). Circadian rhythmicity in photoperiodically induced gonadotropin release and gonadal growth in the quail. Nature (London). 223: 968-971.

Fontaine, M. (1976). Hormones and the control of reproduction in aquaculture. J. Fish. Res. Board. Can. 33:922-939.

Fontaine, M.E., Bertrand, E., Lopez, E. and Callamand, O. (1964). Sur la maturation des organes genitaux de l'anguille femelle (*Anguilla anguilla* L) et l'emission spontanee des oeufs en aquarium. C.R. Acad. Sci. 259: 2907-2910.

Fontaine, Y.A., Salmon, C., Fontaine-Bertrand, E., Burzawa-Gerard, E. and Donaldson, E.M. (1972). Comparison of the activities of 2 purified fish gonadotropins on adenyl cyclase activity in the goldfish ovary. Can. J. Zool. 50: 1673-1676.

Forberg, K.G. (1982). A histological study of development of oocytes in capelin, *Mallotus villosus* Muller. J. Fish Biol. 20: 143-154.

Fostier, A., Jalabert, B. and Terqui, M. (1973). Action d'une derive hydroxyle de la progesterone sur la maturation *in vitro* des ovocytes de la truite arc-en-ciel *Salmo gairdneri*. Comptes rendus lebdomadaines des Seances de l'Academie des Sciences 277D: 421-424.

Fostier, A., Breton, B. and Jalabert, B. (1979). Stimulation hypophysaire de la secretion d'oestradiol-17β chez la carpe commune (*Cyprinus carpio*). Ann. Endocrinol. 40: 83-84.

Fostier, A., Weil, C., Terqui, M., Breton, B. and Jalabert, B. (1976). Plasma oestradiol-17β and gonadotropin during ovulation in rainbow trout (*Salmo gairdneri* R,). Annales de biologie animale, biochemie et biophysique, 18:929-936.

Fostier, A., Jalabert, B., Billard, R., Breton, B. and Zohar, Y. (1983). The gonadal steroids. In: Hoar, W.S: Randall, D.J. and Donaldson, E.M. (eds). Fish Physiology, Vol IXa, pp 277-372.

Fostier, A., Breton, B., Jalabert, B. and Marcuzzi, O. (1981). Evolution des niveaux plasmatiques de la gonadotropine glycoprotique ne au cours de la maturation et de l'ovulation chez la truite arc on ciel, *Salmo gairdneri*? C.R. Lebd. Seances. Acad. Sci. 293D: 817-820.

Franchi, L.L., Mandl, A.M. and Zuckerman, S. (1962). Ovarian development and oogenesis. In: Zuckerman, S (ed). The Ovary. Academic Press, London, Vol I, pg 1-88.

Fujii, R. and Miyashita, Y. (1978). Receptor mechanisms in fish

chromatophores-IV. Effects of melatonin and related substances on dermal and epidermal melanophores of the siluroid, *Parasilurus asotus*. Comp. Biochem. Physiol. 59C: 59.

Gern, W.A. and Greenhouse, S. (1988). Examination of *in-vitro* melatonin secretion from superfused trout (*Salmo gairdneri*) pineal organs maintained under diel illumination or continuous darkness. Gen. Comp. Endocr. 71, 163-174.

Gern, W.A. and Karn, C.M. (1983). Evolution of melatonin's functions and effects. In: Reiter, R.J. (ed). Pineal Research Reviews, volI. pp 49-90. A.R. Liss, New York.

Gern, W.A. and Ralph, C.L. (1979). Melatonin synthesis by the retina. Science, 204: 183-184.

Gern, W.A., Owens, D.W. and Ralph, C.L. (1978). Plasma melatonin in the trout; Day-night change demonstrated by radioimmunoassay. Gen. Comp. Endocr. 34: 453-458.

Gillet, C. and Billard, R. (1977). Stimulation of gonadotropin secretion in goldfish by elevation of rearing temperature. Annales de biologie animale, biochemie et biophysique, 17:673-678.

Gillet, C. and Billard, R. (1981). Cah, Lab. Hydrobiol. Montereau 11: 41-48.

Goetz, F.W. (1983). Hormonal control of oocyte final maturation and ovulation in fishes. In: Hoar, W.S; Randall, D.J. and Donaldson, E.M. (eds). Fish Physiology. Vol IX/B. Reproduction/ Behaviour and Fertility Control, p 117-170.

Goetz, F.W. and Bergman, H.L. (1978). The effects of steroids on final maturation and ovulation of oocytes from brook trout (*Salvelinus fontinalis*) and yellow perch (*Perca flavascens*). Biol. Reprod. 18: 293-298.

Goetz, F.W. and Theofan, G. (1979). *In-vitro* stimulation of germinal vesicle breakdown and ovulation of yellow perch, (*Perca flavescens*) oocytes. Effects of 17α-hydroxy-20β dihydroprogesterone and prostaglandins. Gen. Comp. Endocr. 37:273-285.

Goetz, F.W., Smith, D.C. and Krickl, S.P. (1982). The effects of prostaglandins, Phosphodiesterase inhibitors, and cyclic AMP on ovulation of Brook trout (*Salvelinus fontinalis*) oocytes. Gen. Comp. Endocr. 48: 154-160.

Goldman, B.D., Darrow, J.M. and Yogev, L. (1984). Effects of timed melatonin infusions on reproductive development in the Djungarian hamster (*Phodopus sungorus*). Endocrinology 114: 2074.

Goldman, B.D., Carter, D.S., Hall, V.D.,m Roychoudhry, P. and Yellon, S.M. (1982). Physiology of pineal melatonin in three hamster species. In: Klein DC (ed) Melatonin Rhythm Generating System. Basel, Switzerland: S. Karger, pp210-31.

Goswami, S.V. and Sundararaj, B.I. (1971). Temporal effects of ovine LH and desoxycorticosterone acetate on maturation and ovulation of oocytes of the catfish, *Heteropneustes fossilis* (Bloch). An *in vivo* and *in vitro* study. J. Exp. Zool. 178: 457-466.

Gudger, E.W. (1918). Oral gestation in the gaff-topsail catfish, *Felichthys felis*. Corneige. Inst. Wash. Publ. 252:25-52.

Gupta, S (1975). The development of carp gonads in warm water aquaria. J. Fish. Biol, 7: 775-782.

Guraya, S.S. (1965). A comparative histochemical study of fish (*C. maruleus*) and amphibian (*Buto stomaticus*) oogenesis. Z. Zellforsch. Mikrosk. Anat. 65: 662-700.

Guraya, S.S., Toor, H.S. and Kumar, S. (1977), Morphology of ovarian changes during the reproductive cycle of the *Cyprinus carpio communis* (Linn). Zoologische Beitrage 23: 405-437.

Gwinner, E. (1973). Circannual rhythms in birds; their interaction with circadian rhythms and environmental photoperiod. J. Reprod. Fertil. Suppl. 19: 51-56.

Gwinner, E. (1978). Effects of pinealectomy on circadian locomoter activity rhythms in european starlings, *Sturnus vulgaris*. J. Comp. Physiol. 126: 123-129.

Gwinner, E. and Benzinger, I. (1978). Synchronization of a

circadian rhythm in pinealectomized european starlings by daily injections of melatonin. J. Comp. Physiol. 127: 209-213.

Hafeez, M.A. (1970). Effect of melatonin on body colouration and spontaneous swimming activity in rainbow trout, *Salmo gairdneri.* Comp. Biochem. Physiol. 36: 639-656.

Hafeez, M.A. (1971). Light microscopic studies on the pineal organ in teleost fish with special regard to its function. J. Morph. 134: 281-314.

Hafeez, M.A. and Zerihum, L. (1974). Studies on the central projections of the pineal nerve tract in rainbow trout, *Salmo gairdneri*, Richardson, using cobalt chloride iontophoresis. Cell Tiss, Res. 154: 485- 510.

Hamner, W. (1963). Diurnal rhythms and photoperiodism in testicular recrudescence of the house finch. Science, 142: 1294-1295.

Hanyu, I., Asahina, K. and Shimizu, A. (1982). The roles of light and temperature in the reproductive cycles of three bitterling species: *Rhodeus ocellatus ocellatus, Acheilognathus tabira* and *Pseudoperilampus typus*. In Richter, C.J. J; Goos, H.J.Th, (comps); Reproductive physiology of fish. pp. 123-127.

Hanyu, I., Asahina, K., Shimizu, A., Razani, H. and Kaneko, T. (1983). Environmental regulation of reproductive cycles in teleosts. Proc. 2nd N. Pac. Aquaculture Symp. Sept 1983, Tokyo, 173-188.

Harrington, R.W. Jr (1959). Photoperiodism in fishes in relation to the annual sexual cycle. In:Withrow, R.B (ed), Photoperiodism and related phenomena in plants and animals. Washington D.C. pp 651-657.

Hartley, P.H.T. (1947). Natural history of some freshwater fishes. Proc. Zool. Soc. Lond. 117:106-129.

Hartwig, H.G. and Van Veen, T. (1979). Spectral characteristics of visible radiation penetrating into the brain and stimulating extra-retinal photoreceptors. J. Comp. Physiol. 130: 277-282.

Healy, A (1956). Roach and dace in the Cork Blackwater. In: Dept's.

Rep. Sea and Inl. Fisheries. Dublin: Dept: Lands.

Hellawell, J.M. (1974). The ecology of populations of dace, *Leuciscus leuciscus* (L.), from two tributaries of the river Wye, Hertfordshire, England. Freshwat. Biol. 4: 577-604.

Henderson, N.E. (1963). Influence of light and temperature on the reproductive cycle of the Eastern brook trout, *Salvelinus fontinalis*. J. Fish. Res. Bd. Can. 20: 859-895.

Herwig, H.J. (1981). An ultrastructural and biochemical study of the pineal organ of *Hemmigrammus caudovittatus* and other closely related charcoid fish species, with reference to the mexican blind cave fish, *Astyanax mexicanus*. PhD thesis. Utrecht.

Herwig, H.J., Balemans, M.G.M. and Marien, A.G.H. (1981). Biochemical evidence for the synthesis of indoles in the pineal organ and retina of the teleost *Hemigrammus caudovittatus*. European pineal study group. Newsletter suppl. 3: 36 (abstract).

Hoffman, K. (1979). Photoperiodic effects in the Djungarian hamster: one minute of light during darktime mimics influence of long photoperiods on testicular recrudescence, body weight and pelage colour. Experimentia 35: 1529-1530.

Hoffman, K., Illernova, H. and Vanecek, J. (1985). Neurosci. Lett. 56: 39-43.

Hontela, A. and Peter, R.E. (1980). Effects of pinealectomy, blinding and sexual condition on gonadotropin levels in the goldfish. Gen. Comp. Endocr. 40: 168-179.

Htun-Han, M. (1975). The effects of photoperiod on maturation in the dab, *Limanda limanda* L. PhD thesis. University of East Anglia, Norwich, U.K.

Htun-Han, M. (1977). The effects of photoperiod on reproduction in fishes- an annotated bibliography. Ministry of Agriculture, Fisheries and Food, directorate of fisheries research, Lowestoft.

Hurk, R. Van den, and Peute, J. (1979). Cyclical changes in the ovary of the rainbow trout, *Salmo gairdneri* with special reference to sites of steroidogenesis. Cell. Tiss. Res. 199: 289-306.

Idler, D.R., Bazar, L.S. and Hwang, S. T. (1975). Fish gonadotropins. III. Evidence for more than one gonadotropin in chum salmon pituitary glands. Endocrine Research Communications, 2: 237-249.

Idler, D.R. and Ng, T.B. (1979). Studies on two types of gonadotropins from both salmon and carp pituitaries. Gen. Comp. Endocr. 38: 421-440.

Itoh, H., Susuki, K. and Kawauchi, H, (1988). The complete amino acid sequences of b-subunits of two distinct chum salmon GTHs. Gen. Comp. Endocrin. 71: 438-451.

Jalabert, B. (1976). *In-vitro* oocyte maturation and ovulation in rainbow trout (*Salmo gairdneri*), northern pike (*Esox lucius*) and Goldfish (*Carrasius auratus*). J. Fish. Res. Bd. Can. 33: 974-988.

Jalabert, B. and Breton, B. (1980). Evaluation de la gonadotropine plasmatique t-GTH apres ovulation chez la truite avec en ciel (*Salmo gairdneri*) et influence de la retention des ovules. C.R. Acad. Sci., Serie D 290: 799-801.

Jalabert, B., Bry, C., Breton, B. and Campbell, C. (1976). Action of 17α hydroxy-20 β dihydroxyprogesterone and of progesterone on the maturation and ovulation *in vitro* and on plasmatic gonadotropic hormone t-GTH level in the rainbow trout, *Salmo gairdneri*. C.R. Acad. Sci. 283: 1205-1208.

Jalabert, B., Breton, B., Brzuska, E., Fostier, A. and Wieniawaskij, L (1977). A new tool for induced spawning; the use of 17α hydroxy-20β dihydroprogesterone to spawn carp at low temperatures. Aquaculture 10: 353-364.

Jenkins, J.T. (1958). The Fishes of the British Isles. London, Warne.

Johannes, R.E. (1978). Reproductive strategies of coastal marine fishes in the tropics. Environ. Biol. and Fisheries. 3: 65-84.

Johnston, L.Y., Vaughan, M.K., Richardson, B.A., Petterborg, L.J. and Reiter, R.J. (1982). Proc. Soc. Exp. Biol. Med. 169: 416-420.

Kagawa, H., Takano, K, and Nagahama, Y. (1981). Correlation of plasma oestradiol-17β and progesterone levels with ultrastructure and histochemistry of ovarian follicles in the white spotted char, *Salvelinus leucomaenias*. Cell. Tiss. Res. 218: 315-329.

Kagawa, H., Young, G., Adachi, S. and Nagahama, Y. (1982). Oestradiol-17β production in Amago salmon (*Oncorhynchus rhodurus)* ovarian follicles. Role of the thecal and granulosa cells. Gen. Comp. Endocr 47: 440-448.

Kappers, J.A. (1971). The pineal organ: An introduction. In: Wolstenholme, G.E.W. and Knight, J. (eds). The pineal gland, pp3-34. Churchill Livingstone, Edinburgh.

Karla, P.S. and Karla, S.P. (1985). Control of gonadotropin secretion. In: Muro, H.I. (ed). The Pituitary Gland, pp 189-220- Raven Press, New York.

Karsch, F. J., Bittman, E.L., Foster, D.L., Goodman, R.L., Legan, S.J. and Robinson, J.E. (1984). Neuroendocrine basis of seasonal reproduction. Rec. Prog. Horm. Res. 40: 185-232.

Kavaliers, M. (1979). The pineal organ and circadian organization of teleost fish. Rev. Can. Biol. 38: 281-292.

Kavaliers, M. (1980). The pineal organ and circadian rhythms of fishes. In : Ali, M.A. (ed). Environmental Physiology of Fishes. pp 631-645, Plennum Press, New York.

Kavaliers, M. and Ralph, L.R. (1980). Pineal involvement in the control of behavioural thermoregulation of the white sucker, *Catostomus commersoni*. J. Exp. Zoo. 212: 303.

Kaya, C.M. (1973). Effects of temperature on responses of the gonads of green sunfish, (*Lepomis cyanellus*) to treatment with carp pituitaries and testosterone propionate. J. Fish Res. Bd. Can. 30: 905-912.

Kennaway, D.J., Frith, R.G., Phillpou, G., Mathews, C.D. and Seamark, R.F. (1977). A specific radioimmunoassay for melatonin in biological tissus and fluids and its validation by gas chromatography-mass spectrometry. Endocr. 101: 119-127.

Kennedy, M (1969). Spawning and early development of the dace, *Leuciscus leuciscus* (L). J.Fish. Biol, 1: 249-259.

Khoo, K.H. (1979). The histochemistry and endocrine control of vitellogenesis in goldfish ovaries. Can. J. Zool, 57: 617-926.

Kime, D.E. (1982). The control of gonadal androgen biosynthesis in fish. In: Richter, C.J.J. and Goos, H.J.Th (eds). The Reproductive Physiology of fish, Pudoc Press, Wageningen pp 95-98.

Kime, D.E. and Dolben, I.P. (1985). Hormonal changes during induced ovulation of the carp, *Cyprinnus carpio*. Gen. Comp. Endocr. 58: 137-149.

Klein, D.C. and Weller, J.L. (1970). Indole metabolism in the pineal gland: A circadian rhythm in N-acetyl transferase and hydroxyindole-O- methyl transferase: Control by the retinohypothalamic tract and suprachiasmatic nucleus. Brain Res. 174: 245-262.

Klein, D.C., Averbach, D.A., Namboodri, M.A.A. and Wheler, G.H.T. (1981). Indole metabolism in the mammalian pineal gland. In :Reiter, R.J. (ed). The pineal Gland, vol. I. Anatomy and Biochemistry, pp 199-243. CRC Press, Boca Raton, FL.

Kobayashi, M., Aida, K. and Hanyu, I. (1987). Hormone changes during ovulation and effects of steroid hormones on plasma gonadotropin levels and ovulation in goldfish. Gen Comp Endocr. 69:301-307.

Kobayashi, M., Aida, K. and Hanyu. I.(1988). Hormone changes during the ovulatory cycle in goldfish. Gen. Comp. Endocr. 69: 301-307.

Korf, H.W., Liesner, R., Meissl, H. and Kirk, A. (1981). Pineal complex of the clawed toad, *Xenopus laevis* Daudi. Structure and function. Cell. Tiss. Res. 216: 113-130.

Kossman, H (1975). Reproduction experiments on carp (*Cyprinus carpio*) EIFAC 25: 122-126.

Korfsmeier, K.H. (1966). Zur geness de dottersystmes in der oocyte

von *Brachydanio rerio*. Autoradiographische untersuchugen. Zell. Forsch, 71: 283-296.

Lam, T.J. (1982). Applications of endocrinology to fish culture. Can. J. Fish. Aquat. Sci. 39: 111-137.

Lam, T.J., Pandey, S., Nagahama, Y, and Hoar, W.S. (1976). Effect of synthetic leutenizing hormone-releasing hormone on ovulation and pituitary cytology of the goldfish, *Carrasius auratus*. Canad. J. Zool. 54: 816-824.

Lamba, V.J., Goswami, S.V. and Sundararaj, B.I. (1983). Circannual and circadian variations in plasma levels of steroids (cortisol, 17β-oestradiol, oestrone and testosterone) correlated with the annual gonadal cycle in the catfish, *Heteropneustes fossilis*. Gen. Comp. Endocr. 50: 205-225.

Lambert, J.G.D. (1970). The ovary of the guppy, *Poecilia reticulata*. The granulosa cells as sites of steroid biosynthesis. Gen. Comp. Endocr. 15: 467-476.

Lambert, J.G.D. (1978). Steroidogenesis in the ovary of *Brachydanio rerio* (Teleostei). In Gaillard, P.J. and Boer, H.H. (eds). Comp. Endocr. Amsterdam, pp 65-68.

Lambert, J.G.D. and Van den Hurk, R. (1982). Steroidogenesis in the ovaries of the Asian catfish, *Clarias lazera*, before and after an HCG induced ovulation. In: Richter, C.J.J. and Goos, H.J.Th (eds). Proc. Int. Symp. Reprod. Physiol. Fish. Wageningen. pp 99-102.

Lambert, J.G.D., Van den Hurk, R., Schoonen, W.G.E.J., Resink, J.W. and Van Oordt, P.G.W.J. (1986). Gonadal steroids and the possible role of steroid gluceronides as sex pheromones in 2 species of teleosts. Fish. Physiol. Biochem, 2: 101-107.

Leach, G.T. and Taylor, M. H. (1980). The role of cortisol in stress induced metabolic changes in *Fundulus heteroclitus*. Gen. Comp. Endocr.42: 219-227.

Liley, N.R. (1982). Chemical communication in fish. Can. J. Fish. Aquatic Sci. 39: 22-35.

Liley, N.R. and Stacey, N.E. (1982). Hormones, pheromones and

reproductive behaviour in fish. In: Hoar, W.S; Randall, D.J. and Donaldson, E.M. (eds). Fish Physiology. Vol IXB. pp1-63. Academic Press, New york.

Lincoln, G.A., Almeida, O.F.X. and Arendt, J. (1981). J. Reprod. Fertil. Suppl. 30: 23.

Lincoln, G.A., Ebling, F.J. and Almeida, O.F.X. (1985). Photoperiodism, melatonin and the pineal. pp 129-141. Pitman, London.

Lofts, B. and Marshall, A.J. (1957). Quart. J. Micro Sci. 98: 79-88.

Lynch, H.J., No, M. and Wurtman, R.J. (1977). The adrenal medulla may mediate the increase in pineal melatonin synthesis induced by stress, but not that caused by darkness. J. Neural. Transm. 40, 87-97.

Lynch, H.J., Ozaki, Y., Shakal, L. and Wurtman, R.J. (1975). Melatonin excretion of man and rats: effect of time of day, sleep, pinralectomy and food consumption. Int. J. Biometeorol. 19:267-279.

Lynch, H.J., Rivest, R.W., Ronsheim, P.M. and Wurtman, R.J. (1981). Light intensity and the control of melatonin secretion in rats. Neuroendocr. 33: 181-186.

Mann, R.H.K. (1974). Observations on the age, growth, reproduction and food of the dace, *Leuciscus leuciscus* (L) in two rivers in Southern England. J. Fish. Biol 6: 237-253.

Mann. R.H.K. and Mills, C.A. (1985). Variations in sizes of gonads, eggs and larvae of the dace, *Leuciscus leuciscus*. Environ. Biol. Fish. 13: 277-287.

Mathews, C.P. (1971). Contribution of young fish to total production of fish in the R. Thames, near Reading. J. Fish. Biol 3: 157-180.

Mcnulty,J.A. (1986). Uptake and metabolism of indole compounds by the goldfish pineal organ. Gen. Comp. Endocrinol. 61, 179-186.

Meier, A.H. (1975). Chronobiology of vertebrates. In: Elefetheriou, B.E. and Sprott, R.L. (eds). Hormonal Correlates of Behaviour, vol 2, pp

49-149. Plenum Press, New York.

Meissl, H. (1986). Photoneurophysiology of pinealocytes. In: O'Brien, P.J. and Klein, D,C. (eds). Pineal and retinal relationships, pp33-46. Academic Press, Orlando, FL.

Meissl, H. and Ueck, M. Extra-occular photoreception of the pineal gland of the aquatic turtle, *Pseudemys scriptaetegans*. J. Comp. Pysiol. 140: 173-179.

Menaker, M. and Wisner, S. (1983). Temperature compensated circadian clock in the pineal of *Anolis*. Proc. Natl. Acad. Sci. USA 80: 6119-6121.

Menaker, M. and Zimmerman, N. (1976). Role of the pineal in the circadian system of birds. Am. Zool. 16: 45-55.

Micha, J.C. (1975). Synthese des essais de reproduction, d'alevinage et de production chez un silure Africain: *Clarias lazera*. CIFA/75/SE5 (FAO.CIFA Symp. Aquacult. Africa. p23.

Michael, R.P. and Bonsall, R.S. (1977). A 3 year study of an annual rhythm in plasma androgen levels in male rhesus monkeys (*Malaca mulatta*) in a constant laboratory environment. J. Reprod. Fertil. 49: 121-131.

Meissl, H. and Ueck, M. (1980). Extraocular photoreception of the pineal gland of the aquatic turtle, *Pseudemys scripta elegans*. J. Comp. Physiol. 140: 173-179.

Morawaska, B. (1984). The effect of water temperature elevation on incipient and cumulative fecundity of batch spawning tench, *Tinca tinca* (L). Aquaculture 42: 272-290.

Moser, H.G. (1967). Seasonal histological changes in the gonads of *Sebastodes paucipinus*. A: an ovoviparous teleost. J. Morph. 123: 329-394.

Munro, J.L., Gaut, V.C., Thomson, R. and Reeson, P.H. (1973). The spawning seasons of carribean reef fishes. J. Fish. Biol. 5: 69-84.

Nagahama, Y, (1987a). Gonadotropin action on gametogenesis and steroidogenesis in teleost gonads. Zool. Science 4: 209-222.

Nagahama, Y. (1987b). 17α,20β-Dihydroxy-4-pregnen-3-0ne. A teleost maturation inducing hormone. Develop. Growth. Differ, 29: 1-12.

Nagahama, Y., Kagawa, H. and Tashiro, F (1980). Bull. Jap. Soc. Scient. Fish. 46: 1097-1102.

Nagahama, Y., Young, G. and Kagawa, H. (1982). Steroidogenesis in the amago salmon (*Oncorhynchus rhodurus*) ovarian follicle: a two cell type model. In: Proceedings of the IX Int. Symp. on Comp. Endocr. Hong kong.

Ng, T.B., Campbell, C.M. and Idler, D.R. (1980). Antibody inhibition of vitellogenesis on oocyte maturation in salmon and flounder. Gen. Comp. Endocr. 41: 233-239.

Ng, T., and Idler, D.R. (1983). Yolk formation and differentiation in teleost fishes. In Hoar, W.S; Randall, D.J. and Donaldson, E.M. (eds). Fish Physiology, Vol IX. Reproduction-Part A, Endocrine tissue and hormones. Academic Press, New York, pp 373-404.

Nikolsky, G.V. (1969). Theory of fish population dynamics. Oliver and Boyd, Edinburgh.

Oguri, M. and Omura, Y. (1973). Ultrastructure and functional significance of the pineal organ in teleosts. In: Chavin, W (eds). Responses of fish to environmental changes. Thomas Springfield. pp412-434.

Okshe, A, and Hartwig, H.G. (1975). Photoneuroendocrine systems and the third ventricle. In: Knigge, K.M; Scott, D,E; Kobayashi, K. and Ishii, S. (eds). Brain Endocrine Interaction II. The Ventricular System, pp50-53. Basel, Krager.

Oliver, J. and Bayle, J.D. (1982). Brain photoreceptors for photo-induced testicular responses in birds. Experementia 38: 1021.

Omura, Y. and Oguri, M. (1969). Histological studies on the pineal organ of 15 species of teleosts. Bull. Jap. Soc. Sci. Fish. 35: 991-1000.

Owman, C.H. and Rudeberg, C. (1970). Light, fluorescence and

electron microscope studies on the pineal organ of the pike, *Esox lucius*, with special regard to 5-hydroxy tryptamine. Z. Zellforsch. Mikrosk. Anat. 107: 522-550.

Pandey, S; Hoar, W.S. (1972). Induction of ovulation in goldfish by clomiphene citrate. Can. J. Zool. 50: 1679-1680.

Pandey, S., Stacey, N. and Hoar, W.S. (1973). Mode of action of clomiphene citrate on indicating ovulation in goldfish. Can. J. Zool. 51: 1315-1316.

Pang, P.K.T. (1971). The effects of complete darkness and vitamin C supplement on the killifish, *Fundulus heteroclitus* adapted to sea water.1. Calcium metabolism and gonadal maturation. J.Exp. Zool. 178: 15-22.

Panke, E.S., Rollag, M.D. and Reiter, R.J. (1979). Pineal melatonin concentrations in the Syrian hamster. Endocr. 104: 194-197.

Pankhurst, N.W. and Stacey, N.E. (1985). The effect of 17β-oestradiol on spontaneous ovulation in the goldfish, *Carrasius auratus*. Can. J. Zool. 63: 2979-2981.

Pantin, C.F.A. (1960). Notes on microscopical technique for zoologists. 4th edn. Cambr. Univ, Press, London.

Patzner, R.A. (1983). The reproduction of *Blennius pavo* (Teleostei, Blennidae) 1. Ovarial cycle, environmental factors and feeding. Helgolander Meeresunters, 36: 105-114.

Peter, R.E. (1980). Serum gonadotropin levels in mature male goldfish in response to leutenizing hormone-releasing hormone (LHRH) and des-Gly^{10} -[D-Ala^{6}]-LHRH ethylamide. Can. J. Zool. 58: 1100-1104.

Peter, R.E. (1982). Neuroendocrine control of reproduction in teleosts. Can. J. Fish. Aquat. Sci. 39: 48-55.

Peter, R.E. and Crim, L.W. (1979). Reproductive endocrinology of fishes: Gonadal cycles and gonadotropin in teleosts. Ann. Rev. Physiol. 41: 323-335.

Peter, R.E. and Hontela, A. (1978). Annual gonadal cycles in teleosts: Environmental factors and gonadotropin levels in blood. In: Assenmacher, I. and Farner, D.S. (eds). Environmental Endocrinology, pp 20-25. Springer-Verlag, Berlin.

Peter, R.E. and Nagahama, Y. (1974). A light and electron microscope study on the structure of the nucleus preopticus and nucleus lateral tuberis of the goldfish, *Carrasius auratus*. Can. J. Zool. 54: 1423-1437.

Peter, R.E., Chang, J.P., Cook, A.F. and Nahorniak, C.S. (1982). Neuroendocrine regulation of gonadotropin and growth hormone secretion in goldfish. In: Lofts, B. (ed). Proceedings of the ninth International Symposium on Comparative Endocrinology. University of Hong Kong Press, Hong Kong. Abst, 64.

Peter, R.E., Kah, D., Paulencu, C.R., Cook, H. and Kyle, A.L. (1980). Brain lesions and short term endocrine effects on monosodium glutamate in goldfish, *Carrasius auratus*. Cell.Tiss. Res. 212: 429-442.

Peute, J., Huiskamp, R. and Van Oordt, P.G.W.J. (1985). Quantitative analysis of oestradiol-17β induced changes in the ultrastructure of the liver of the male zebrafish, *Brachydanio rerio*. Cell. Tiss. Res. 242: 377-382.

Peute, J., de Bruyn, M.G.A., Seldenryk, R., Van Oordt, P.G.W.J. (1976). Cytophysiology and innervation of gonadotropic cells in the pituitary of the black molly (*Poecilia latipinna*). An electron microscopical study. Cell. Tiss. Res. 174: 35-54.

Peute, J., Goos, H.J.Th., de Bruyn, M.G.A. and Van Oordt, P.G.W.J. (1978). Gonadotrophic cells of the rainbow trout pituitary during the annual cycle. Ultrastructure and hormone content. Annales de biologie, biochimie et biophysique 18: 905-910.

Phillipart, J.A. (1981). Ecologie d'un population de vandoises, *Leuciscus leuciscus* (L) dans la riviere Ourthe (basin de la Meuse, Belgique). Ann. Limmol. 17:41-62.

Pickering, A.D., Pottinger, T.G., Carragher, J. and Sumpter, J.P. (1987). The effects of acute stress on the levels of reproductive

hormones in the plasma of mature male brown trout, *Salmo trutta*. Gen.Comp. Endocr. 68:249-259.

Pittendrigh, C.S. (1974). Circadian oscillations in cells and the circadian organization of multicellular systems. In: Schmidt, F.O. and Worden, F.G. (eds). The neurosciences: Third study program, pp437-458. M.I,T, Press, Cambridge, Mass.

Pittendrigh, C.S. (1976).Hastings, J.W. and Schweiger, H.G. (eds). The molecular basis of circadian rhythms. pp11-49. Dahlem Konferenzen, Berlin.

Pittendrigh, C.S. (1981). Circadian systems: Entrainment. In: Aschoff, J. (ed). Biological rhythms (Handbookj of behavioural neurobiology, Vol 4). Plennum Press, New York, pp 95-124.

Polder, J.J.W. (1961). Cyclical changes in testis and ovary related to maturity stages in the North Sea herring, *Clupea harengus*, L. Archives neerlandaises de zoologie 14:45-60

Poncin, P. and Phillipart, J.C. (1986). Inhibiting effect of a decreasing photoperiod on the sexual maturation of captive *Barbus barbus* (L.) (Pisces: Cyprinidae) at a constant 20°C temperature. 8th Conference of the European Society for Comparative Physiology and Biochemistry. Comparative Physiology of Environmental Adaption. Strasbourg (Franve), August 31-september 2, 1986.

Poncin. P., Phillipart, J.C. and Melard, C.L. (1985). Induction of repeated spawnings in female barbel, *Barbus barbus* (L). Fish Culture. European Society for Comparative Physiology and Biochemistry, 7th conference, Barcelona.

Pottinger, T.G. and Pickering, A.D. (1985). Changes in skin structure associated with elevated androgen levels in maturing male brown trout, *Salmo trutta* L. J.Fish. Biol. 26: 745-753.

Ralph, C.L. (1976). Correlations of melatonin content in pineal gland, blood and brain of some birds and mammals. Amer. Zool. 16:35-43.

Ralph, C.L. (1978). Pineal control of reproduction: non-mammalian vertebrates. In: Reiter, R.J.(ed). The pineal and Reproduction, pp30-. S Karger, Basel.

Ralph, C.L. (1983). Evolution of pineal control of endocrine function in lower vertebrates. Am. Zool. 23: 597-605.

Randall, C.F., Duston, J., Bromage, N.R. (1987). Photoperiodic history and the entrainment of the annual reproductive cycle in the female rainbow trout. In: Idler, D.R; Crim, L.W. and Walsh, J.M. (comps).Proc. 3rd. Int. Symp. Reprod. Physiol. Fish, Newfoundland, Canada. pp 310.

Razani, H., Hanyu, I. and Aida, K. (1987). Critical daylength and temperature level for photoperiodism in gonadal maturation of goldfish. In: Idler, D.R; Crim. L.W. and Walsh, J.M. (comps). Proc. 3rd Int. Symp. Reprod. Physiol. Fish. Newfoundland, Canada. pp311

Reagan, T (1911). The freshwater fishes of the British Isles. London. Methuen.

Reiter, R.J. (1982). Neuroendocrine effects of the pineal gland and of melatonin. In: Martini, G (ed). Frontiers of endocrinology, vol. 7. pp 287-316. Raven Press, New York.

Reiter, R.J. (1983). In: Axelrod, J; Fraschini, F. and Velo, G.P (eds). The pineal Gland and its Endocrine Role. Plennum Press, New York. pp 227-241.

Reiter, R.J. (1985). The Pineal Gland: Endocrine aspects. Brown, G.M. and Wainwright, S.D. (eds). pp 163-173., Pergamon, New York.

Reiter, R.J. (1986). Advances in Pineal Research, Vol. 1. Reiter, R.J. (ed). pp 77-87, John Libbey, London.

Reiter, R.J. (1987). Mini-review. The melatonin message: Duration versus coincidence hypothesis. Life sciences. 40: 2119-2131.

Reiter, R.J., Lukaszyk, A.J., Vaughan, M.K. and Blask, D.E. (1976). New horizons of pineal research. Am. Zool. 16: 93-101.

Reiter, R.J., Petterborg, L.J., Trakulrungsi, C. and Trakulrungsi, W.K. (1980). Surgical removal of the olfactory bulbs increases sensitivity of the reproductive system of female rats to the inhibitory effects of late afternoon melatonin infusions. J. Exp. Zool. 212: 47-54.

Reiter, R.J., Steinlechner, S., Richerdson, B.A. and King, T.S. (1983). Differential response of pineal melatonin levels to light at night in laboratory raised and wild captured 13-lined ground squirrels (*Spermophilus tridecernitineatus*). Life Sci. 32: 2625-2629.

Riazi, A. and Fremont, L. (1988). Serum vitellogenin and yolk proteolipid complex composition in relation to ovarian growth in rainbow trout, *Salmo gairdneri* (Rich.). Comp. Bioch. Physiol. 89B: 525-530.

Rivas, L.R. (1953). The pineal apparatus of tunas and related scombid fishes as a possible light receptor controlling phototaxic movements. Bull. Mar. Sci. Gulf. Carrabean, 3: 168-180.

Rizkalla, W. (1970). The morphology of the pineal organ in the teleost, *Clarias lazera* C.V. Acta Biol. Acad. Sci. Hungary 21: 25-35.

Rodbard, D. (1974). Statistical quality control and routine data processing for RIA and immunoradiometric assays. Clin. Chem. 20/10: 1255-1270.

Rollag, M.D. and Niswender, G.D. (1976). Radioimmunoassay of serum concentrations of melatonin in sheep exposed to different lighting regimes. Endocr. 98: 482-489.

Ryan, R.J. (1981). Follicular atresia. Some speculations of biochemical marks and mechanisms. In: Schwartz, N.B. and Hunziker-Dunn, M. (eds). Dynamics of Ovarian Function. Raven Press, New York.

Ryan, A.T., Joiner, B.L. and Ryan, B.F. (1981). Minitab reference manual. Duxbury Press, Boston, USA.

Sagi, G. and Abraham, M. (1984). Fine structure of the pineal body and effect of shprt photoperiod, pinealectomy and melatonin treatment in *Liza ramada*. Gen. Comp. Endocr. 53: 460

Saidpur, S.K. (1978). Follicular atresia in the ovaries of nonmammalian vertebrates. Int. Rev. Cytol. 54: 225-244.

Saxena, P.K. and Anand, K. (1977). A comparison of ovarian recrudescence in the catfish, *Mystus tengara* (Ham) exposed to short photoperiods, to long photoperiods and melatonin, Gen. Comp. Endocr.

33: 506

Schmidt, P.J. and Idler, D.R. (1962). Steroid hormones in the plasma of salmon at various states of maturation. Gen. Comp. Endocr. 2: 204-214.

Schoonen, W.G.E.J. and Lambert, J.G.D. (1986). Steroid metabolism in the testes of the African catfish, *Clarias gariepinus* (Burchell), during the spawning season under natural conditions and kept in ponds. Gen. Comp. Endocr. 61: 40-52.

Schuetz, A.W. (1988).. Local control mechanisms during oogenesis and folliculogenesis. In: Browder, L.W (ed). Developmental Biology. Vol I. Oogenesis. Plennus Publishing Corp. New York, p 3-83.

Schwassman, H.O. (1971). Biological Rhythms. In: Hoar, W.S. and Randall. D.J. (eds). Fish Physiology, Vol 6, pp371-428. Academic Press, New York.

Scott, D.B.C. (1979). Environmental timing and the control of reproduction in teleost fish. Symposia of the Zoological Society of London, 44: 105-132.

Scott, A.P. (1987). Reproductive endocrinology of fish. In: Chester-Jones, I; Ingleton, P.M. and Phillips, J.G. (eds). Fundamentals of Comparative Vertebrate Endocrinology. pp 223-256. Plennum Press, New York.

Scott, A.P. (1988). Environmental control of spawning time in salmonids. In press (CRC book).

Scott, A.P. and Baynes, S.M. (1982). Plasma levels of sex steroids in relation to ovulation and spermiation in rainbow trout (*Salmo gairdneri*). In: Richter, C.J. and H.J. Th. Goos.(eds). Proceedings of the International Symposium on Reproductive Physiology of Fish. Wageningen, Netherlands, Pudoc Wageningen. pp103-106.

Scott, A.P., Bye, V.J. and Baynes, S.M. (1980). Seasonal variation in sex steroids of female rainbow trout, *Salmo gairdneri* (Richardson). J. Fish. Biol. 17: 587-592.

Scott, A.P., Mackenzie, D.S. and Stacey, N.E. (1984). Endocrine

changes during natural spawning in the white sucker, *Catosomus commersani*. II. Steroid hormones. Gen. Comp. Endocr. 56: 349-359.

Scott, A.P., Sheldrick, E.L. and Flint, A.P. (1982). Measurement of 17α20β-dihydroxy-4-pregnen-3-one in plasma of trout (*Salmo gairdneri* Richardson). Seasonal changes and response to salmon pituitary extracts. Gen. Comp. Endocr. 46: 444-451.

Scott, A.P., Sumpter, J.P. and Hardiman, P.A. (1983). Hormone changes during ovulation in the rainbow trout (*Salmo gairdneri*, Richardson). Gen. Comp. Endocr. 49: 128-134.

Selman. K. and Wallace, R.A. (1983). Oogenesis in *Fundulus heteroclitus*. III. Vitellogenesis. J.Exp. Zool. 226: 441-457.

Selman, K., Wallace, R.A. and Barr, V. (1986). Oogenesis in *Fundulus heteroclitus* IV. Yolk vesicle formation. J. Exp. Zool. 239: 277-288.

Shackley, S.E. and King, P.E. (1977). The reproductive cycle and its control; frequency of spawning and fecundity in *Blennius pholis* L. J. Exp. Mar. Biol. Ecol. 30: 73-78.

Smith, J.R. and Weber, L.J. (1976). The regulation of day-night changes in HIOMT activity in the pineal gland of steelhead trout, *Salmo gairdneri*. Can. J. Zool. 54: 1530-1534.

Sorrenti, S. Jr., Reiter, R.J. and Schalch, D.S. (1971). Interactions of pineal gland, blinding and under feeding on reproductive organ size and radioimmunoassayable growth hormone. Neuroendocr. 7: 105-115.

Sporn, M.B. and Roberts, A.B. (1988). Peptide growth factors are multifunctional. Nature, 332: 217-218.

Stacey, N.E. (1984). Control of the timing of ovulation by exogenous and endogenous factors. In: Potts, G.W. and Wootton, R.F. (eds). Fish Reproduction. Strategies and Tactics, Academic Press, London.

Stacey, N.E. and Goetz, F.W. (1982). Role of prostaglandins in fish reproduction. Can. J. Fish. Aquat. Sci. 39: 92-98.

Stacey, N.E. and Pandey, S. (1975). Effects of indomethacin amd

prostaglandins on ovulation of goldfish. Prostaglandins, 9: 597-607.

Stacey, N.E., Cook, A.F. and Peter, R.E. (1979a). Ovulatory surge of gonadotropin in the goldfish, *Carrasius auratus*. Gen. Comp. Endocr. 37: 246-249.

Stacey, N.E., Coof, A.F. and Peter, R.E. (1979b). Spontaneous and gonadotropin induced ovulation in the goldfish, *Carassius auratus* L. effects of external factors. J. Fish. Biol. 15: 349-361.

Stacey, N.E., Peter, R.E., Cook, A.F., Truscott, B., Walsh, J.M. and Idler, D.R. (1983). Changes in plasma concentrations of gonadotropin, 17β-oestradiol, testosterone, and 17α-hydroxy-20β dihydroprogesterone during spontaneous and brain lesioned induced ovulation in goldfish. Can. J. Zool, 61: 2646-2652.

Stantos, A.J.G., Furukawa, M., Kobayashi, K., Bando, K., Aida, K. and Hanyu, I (1986). Plasma gonadotropin and steroid hormone profiles during ovulation in the carp, *Cyprinus carpio*. Bull. Jap. Soc. Scient. Fish. 52: 1159-1166.

Steffens, W. (1956). Laichreife Aschen durch Hypophyseninjektion. Dtsch. Fischerei Z, 3: 59-61.

Strange, R.J., Schreck, C.B. and Golden, J.T. (1977). Corticosteroid stress response to handling and temperature in salmonids. Trans. Amer. Fish. Soc. 106: 213-218.

Sumpter, J.P., Scott, A.P., Baynes, S.M. and Witthanes, R.R. (1984). Early stages of the reproductive cycle in virgin female rainbow trout (*Salmo gairdneri*). Aquaculture 43: 235-242.

Sundararaj, B.I. and Goswami, S.V. (1977). Hormonal regulation in vivo and *in vitro* oocyte maturation in the catfish, *Heteropneustes fossilis* (Bloch). Gen. Comp. Endocr. 32: 17-28.

Sundararaj, B.I. and Keshavanth, P. (1976). Effects of melatonin and prolactin treatment on the hypophyseal- ovarian system in the catfish, *Heteropneustes fossilis* (Bloch). Gen Comp. Endocr. 29:84

Sundararaj, B.I. and Sehgal, A. (1970). Effects of a long or an increasing photoperiod on the initiation of ovarian recrudescence

during the preparatory period in the catfish (*H. Fossilis Bloch*). Biol. Reprod. 2: 413-424.

Sundararaj, B.I. and Vasal, S. (1976). Photoperiod and temperature control in the regulation of reproduction in the female catfish, *Heteropneustes fossilis*, J Fish. Res. Bd. Can. 33: 959- 973.

Sundararaj, B.I., Vasal. S. and Halberg, F. (1983). Circannual rhythmic ovarian recrudescence in the catfish, *Heteropneustes fossilis*. Int. J. Chronobiol. 1: 362-363.

Susuki, K., Nagahama, Y. and Tamaoki, B. (1981). *In vitro* synthesis of an inducer for germinal vesicle breakdown of fish oocytes, 17α20β-dihydroxy-4-pregnen-3-one by ovarian tissue preparation of Amago salmon (*Oncorhynchus rhodurus*). Gen. Comp. Endocr. 45: 473-481.

Tabata, M., Niwa, H. and Tamura, T, (1971). On a slow potential from the epiphysis cerebri of fishes. Bull. Japan. Soc. Sci. Fish. 37: 486-496.

Takahashi, H. (1969). Light and electron microscope studies on the pineal organ of the goldfish, *Carrasius auratus* L. Bull. Fac. Fish. Hokkaido. Univ. 20: 143-157.

Takahashi, J.S., Hamm, H. and Menaker, M. (1980). Circadian rhythms of melatonin release from individual superfused chicken pineal glands *in-vitro*. Proc. Natl. Acad. Sci. USA, 77: 2319-2322.

Tamarkin, L. Reppert, S.M. and Klein, D.C. (1979). Regulation of pineal melatonin in the Syrian hamster. Endocrinology 104: 385- 89.

Tata, J.R. (1978). Regulation of vitellogenesis by oestrogen. In: Dumont, J. and Numez, J. (eds). Hormones and cell regulation. vol. 2: 35-54. Elsevier/North Holland, Biomedical Press.

Thibault, R.E. and Shultz, R.J. (1978). Reproductive adaptations among viviparous fish (Cyprinidontiformes: Poeciliidae). Evolution, 32: 320-333.

Thorpe, J.E. (1978). The rhythmic activity of fishes, Academic Press, New York.

Tokarz, R.R. (1978). Oogonial proliferation, oogenesis and folliculogenesis in non-mammalian vertebrates. In: Jones. R.E. (ed). The Vertebrate Ovary. pp 145-180. Plennum Press, New York and London.

Truscott, B., Idler, D.R., Sundararaj, B.J. and Goswami, S.V. (1978). Effects of gonadotropins and adrenocorticotropin on plasmatic steroids of the catfish, *Heteropneustes fossilis* (Bloch). Gen. Comp. Endocr. 34: 149-157.

Turek, F., Swann, T. and Earnest, D. (1984). Role of the circadian system in reproductive phenomena. Recent Prog. Horm. Res. 40: 143-

Tyler, C.R., Sumpter, J.P. and Bromage, N.R. (1987a). The hormonal control of vitellogenin uptake into cultured ovarian follicles of the rainbow trout. In: Reproductive Physiology of Fish. (D.R. Idler; L.W. Crim and J.M. Walsh. comps), p220. Newfoundland, Canada.

Tyler, C.R., Sumpter, J.P. and Bromage, N.R. (1987b). Uptake of vitellogenin into cultured ovarian follicles of rainbow trout. In: Reproductive Physiology of Fish. (D.R. Idler; L.W. Crim. and J.M. Walsh, comps). p221. Newfoundland, Canada.

Tyler, C.R., Sumpter, J.P. and Bromage, N.R. (1988a). *In vivo* ovarian uptake and processing of vitellogenin in the rainbow trout, *Salmo gairdneri*. J. Exp. Zool. 246: 171-179.

Tyler, C.R., Sumpter, J.P. and Bromage, N.R. (1988b). Selectivity of protein sequestration by vitellogenic oocytes of the rainbow trout, *Salmo gairdneri*. J. Exp. Zool. 248: 199-206.

Tyler, C.R. and Sumpter, J.P. (1989). The development of a RIA for carp vitellogenin. Fish. Physiol. and Biochem. in press.

Tyler, C.R. and Sumpter, J.P. (1989b). Dynamics of follicle growth during vitellogenesis in the rainbow trout. In: XIth International Symposium on Comparative Endocrinology, Malaga, Spain.

Tyler, C.R., Sumpter, J.P. and Kawauchi, H. (1989). Involvement of GTH on the uptake of vitellogenin into vitellogenic follicles of the rainbow trout, *Oncorhynchus mykiss*. In press.

Underwood, H. (1973). Retinal and extraretinal photoreceptors mediate entrainment of the circadian locomotor rhythm in lizards. J, Comp. Physiol. 83: 187-222.

Underwood, H. (1979). Extraretinal photreception. In: Burtt, E.H. (ed). The Behavioural Significance of colour, Garland Press, New York. 172.

Underwood, H. (1982). The pineal and circadian organization in fish, amphibians and reptiles. In: Reiter, R.J. (ed). The Pineal Gland:VIII. Extra-reproductive effects, pp 1-. CRC Press, Boca Raton, FL.

Underwood, H. (1983). Circadian pacemakers in lizards. Phase Response curves and effects of pinealectomy. Am. J. Physiol. 244: 857-864.

Underwood, H. (1985). Pineal melatonin rhythms in the lizard, *Anolis carolinensis*: effects of light and temperature cycles. J. Comp. Physiol. A 157: 57-65.

Upadhyay, S.N., Breron, B. and Billard, R. (1978). Ultrastructural studies on experimentally induced vitellogenesis in juvenile rainbow trout (*Salmo gairdneri*). Ann. Biol. Anim. Biochem. Biophys. 18: 1019-1025.

Urasaki, H. (1972). Effects of pinealectomy on gonadal development in the Japenese killifish (Medaka), *Oryzias latipes*. Chronobiologia, 3: 228.

Urasaki, H. (1973). Effect of pinealectomy and photoperiod on oviposition and gonadal development in the fish, *Oryzias latipes*. J. Exp. Zool. 185: 241-246.

Van den Ekoudt, J.P. (1947). Resurches sur l'influence de la luminere sur le cycle de l'epinoche (*Gasterosteus aculeatus*). Ann. Soc. Zool. Belg. 77: 83-89.

Van den Hurk, R. (1974). Steroidogenesis in the testis and gonadotropic activity in the pituitary during post-natal development of the black molly, *Mollieniesia latipinna*. Proc. K. Nad. Akad. Wet. 77: 193-200.

Van den Hurk, R. and Lambert, J.G.D. (1983). Ovarian steroid glucuronides functions as sex pheromones for male zebrafish, *Brachydanio rerio*. Can. J. Zool. 61: 2381-2387.

Van der Waal, B.C.W. (1974). Observations on the breeding habits of *Clarias gariepinus* (Burchel). J. Fish. Biol. 6: 23-27.

Van Mullen, P.J. (1967). On synchronisation in the reproduction of the stickleback (*Gasterosteus aculeatus* L.; forma leiura Cuv). Arch. Neerl. Zool., 17: 256.

Van Ree, G.E., Lok, D. and Bosman, G.J.G.M. (1977). *In vitro* induction of nuclear breakdown in oocytes of the zebrafish, *Brachydanio rerio*. Effects of the composition of the medium and of protein and steroid hormones. Proc. Kon. Ned. Akad. Wetensch. 80: 353-371.

Van Veen, T., Hartwig, H.G. and Muller, K. (1976). Light dependent motor activity and photonegative activity in the eel, (*Anguilla anguilla* L.). J Comp. Physiol. 111: 209-219.

Vivien, J.H. (1939). Role de l' hypophyse dans le determinisme du cycle genital femelle d'un teleostean, *Gobius paganellus* L. C.R. Acad. Sci. Ser. D 208: 948-949.

Vivien-Roels, B. and Arendt, J. (1983). How does the indoleamine production of the pineal gland respond to variations of the environment in a non-mammalian vertebrate, *Testudo hermanni*, Guelin? Psychoneuroendocr. 8: 327-332.

Vivien-Roels, B., Arendt, J. and Bradtke, J. (1979). Circadian and circannual fluctuations of pineal indoleamines (serotonin and melatonin) in *Testudo hermanni*, Guelin (Reptila, chelonia). I. Under natural conditions of photoperiod and temperature. Gen. Comp. Endocr. 37: 197- 210.

Vivien-Roels, B., Pevet, P., Dubois, M.P., Arendt, J. and Brown, G. (1981). Immunohistochemical evidence for the presence of melatonin in the pineal gland, the retina and the Harderian gland. Cell Tiss. Res. 217: 105-115.

de Vlaming, V.L. (1972). Environmental control of teleost

reproductive cycles: a brief review. J. Fish Biol. 4: 131-140.

de Vlaming, V.L. (1974). Environmental and endocrine control of teleost reproduction. In: Schreck, C.B. (ed). Control of sex of fishes. Virginia Polytechnic Institute and State University, Blackburg pp 13-83.

de Vlaming, V.L. (1975). Effects of photoperiod and temperature on gonadal activity in the cyprinid teleost, *Notemigonus crysoleucas*. Biol. Bull. 148: 402-415.

de Vlaming, V.L. (1979). The effects of light reception on circulating oestrogen levels in female goldfish, *Carrasius auratus*; Importance of retinal pathways versus the pineal. Gen. Comp. Endocr, 38: 148

de Vlaming, V. and Olcese, J. (1981). The pineal and reproduction in fish, amphibians and reptiles. In: The Pineal Gland. Vol II. Reproductive Effects, pp 1-29. R.J. Reiter (Ed). CRC Press, Inc. Boca Raton, FL.

de Vlaming, V.L. and Paquette, G. (1977). Photoperiod and temperature effects on gonadal regression in the golden shiner, *Notemigonus crysoleucas*. Copeia, 793-796.

de Vlaming, V.L. and Vodicnik, M.J. (1977). Effects of pinealectomy on pituitary gonadotrophs, pituitary gonadotropin potency and hypothalamic gonadotropin releasing activity in *Notemigonus crysoleucas*. J. Fish. Biol. 10: 73.

de Vlaming, V.L. and Vodicnik, M.J. (1978). Seasonal effects of pinealectomy on gonadal activity in the goldfish, *Carassius auratus*. Biol Reprod 19: 57-63.

de Vlaming, V.L., Delahunty, G. and Wallace, R.A. (1980). Goldfish (*Carassius auratus*) vitellogenin induction, isolation, properties and relationship to yolk proteins. Comp. Biochem. 67: 613-623.

de Vlaming, V.L., Kuris, A. and Parker, F.R.Jr. (1978). Seasonal variation of reproduction and lipid reserves in some subtropical cyprinidontids. Trans. Amer. Fish. Soc. 107: 464-472.

de Vlaming, V.L., Sage, M. and Charlton, C.B. (1974). The effects of melatonin treatment on gonadosomatic index in the teleost,

Fundulus similis and the tree frog, *Hyla cinerea*. Gen. Comp. Endocr, 22: 433-438.

de Vlaming, V.L., Vodicnik, M.J., Bauer, T., Murphy, T. and Evans, D. (1977). 17β-oestradiol effects on lipid and carbohydrate metabolism and on the induction of a yolk precursor in goldfish, *Carrasius auratus*. Life Sci. 20: 1945-1952.

Vodicnik, M.J., Kral, R.E. and de Vlaming, V. L. (1978). Effects of pinealectomy on pituitary and plasma gonadotropin levels in *Carassius auratus* exposed to various photoperiod/temperature regimes. J.Fish. Biol. 12: 187-196.

Von Frisch, K. (1911). Betrage zur physiologie der pigmentzellen in der fischthaut. Pflugers Arch. Ges. Physiol. 138: 319-387.

Wallace, R.A. (1978). Oocyte growth in nonmammalian vertebrates. In: Jones, R.E. (ed). The Vertebrate Ovary. Plennum Press, New York,pp 469-502.

Wallace, R.A. (1985). Interactions between somatic cells and the growing oocyte of *Xenopus laevis*. In: Mclaren, A. and Wylie, C.C. (eds). Current Problems in Germ Cells. Cambridge University Press, Cambridge. p 285-306.

Wallace, R.A. and Misulovin, Z (1978). Long term growth and differentiation of *xenopus* oocytes in a defined medium. Proc. Nat. Acad. Sci. USA. 75: 5534-5538.

Wallace, R.A. and Selman, K. (1978). Oogenesis in *Fundulus heteroclitus*. I. Preliminary observations on oocyte maturation *in-vivo* and *in-vitro*. Develop. Biol. 62: 354-369.

Wallace, R.A. and Selman, K. (1979). Physiological aspects of oogenesis in two species of sticklebacks, *Gasterosteus aculeatus* L. and *Apeltes quadracus*. (Mitchill). J. Fish Biol. 14: 613-623.

Wallace, R.A. and Selman, K. (1981). Cellular dynamic aspects of oocyte growth in teleosts. Amer. Zool. 21: 325-343.

Wallace, R.A., Misulovin, Z. and Eakin, L.D. (1981). Long term growth and differentiation of Xenopus oocytes in a defined medium.

Proc. Natl. Acad. Sci., U.S.A. 75: 5534-5538.

Wallace, R.A., Selman, K., Greeley, M.S., Begovac, R.C.Jr., Lin, Y-W.P., Mcpherson, R. and Petrino, T.R. (1987). Current status of oocyte growth. In: Reproductive Physiology of Fish. (D.R. Idler; L.W.Crim and J.M. Walsh. comps), Newfoundland, Canada. pp 167-177.

Wauldhauser, F., Lynch, H.J. and Wurtman, R.J. (1984). The Pineal Gland. Reiter, R.J. (ed). pp 345-370, Raven, New York.

Weibe, J.P. (1968). The effects of temperature and daylength on reproductive physiology of the viviparous sea perch, *Cymatogaster aggregata*. Can. J. Zool. 46: 1207-1219.

Weil, C., Fostier, A., Horvath, L., Marlot, S. and Berscenyi, M. (1980). Profiles of plasma gonadotropin and 17β-oestradiol in the common carp, *Cyprinus carpio* L; as related to spawning induced by hypophysation or LHRH treatment. Reprod. Nutr. Develop.20: 1041-1050.

Whitehead, C. (1979). The control of reproduction in rainbow trout. Commercial and technical aspects. PhD thesis. University of Aston.

Whitehead, C. and Bromage, N.R. (1980). Effects of constant long and short day photoperiods on the reproductive physiology and spawning of the rainbow trout. Ann. Biol. Anim. Biochim. Biophys. 18: 1035-1043.

Whitehead, C., Bromage, N.R., Forster, J.R.M. and Matty, A.J. (1978). The effects of alterations in photoperiod on ovarian development and spawning time in the rainbow trout (*Salmo gairdneri*). Ann. Biol. anim. Bioch. Biophys, 18:1035-1043.

Wiegand, M.D. (1982). Vitellogenesis in Fishes. In: Reproductive Physiology of Fish. (C.J.J/ Richter and H.J.Th. Goos. comps). p 136-146. Pudoc, Wageningen.

Wilkinson, D.R. and Jones, J.W. (1977). The fecundity of dace, *Leuciscus leuciscus* (L) in Emral Brook, Clwyd, North Wales. Freshwat Biol. 7: 135-145.

Wingfield, J.C. (1984). Influence of weather on reproduction. J. Exp.

Zool. 232:589.

Wingfield, J.C. and Grimm, A.S. (1977). Seasonal changes in plasma cortisol, testosterone and oestradiol-17β in the plaice, *Pleuronectes platessa* L. Gen. Comp. Endocr. 31: 1-11.

Wooton, R.J. (1977). Effect of food limitation during the breeding season on the size, body components and egg production of female sticklebacks, *Gasterosteus aculeatus*. J. Anim. Ecol. 46: 823-834.

Worthington, A.P. (1983). Studies on the reproduction and culture of the roach, *Rutilus rutilus*. PhD thesis. Trent polytechnic, Nottingham.

Worthington, A.P., Macfarlane, N.A.A. and Easton, K. (1982). Controlled reproduction in the roach, (*Rutilus rutilus* L). In: Richter, C.J.J. and Goos, H.J. Th (comps). International Symposium on Reproductive Physiology of fish, Wageningen, The Netherlands.

Wourms, J.P. and Sheldon, H. (1976). Annual fish oogenesis. II. Formation of the secondry egg envelope. Dev. Biol. 50: 355-366.

Yamamoto, K., Oota, I., Takano, K. and Ishikana, T. (1966). Studies on the maturing process of the rainbow trout, *Salmo gairdneri irideus*.1. Maturation of the ovary of one year old fish. Bull. Jap. Soc.Sci. Fish 31: 123-132.

Yamazaki, F. (1965). Endocrinological studies on the reproduction of the female goldfish, Carrasius auratus with special reference to the function of the pituitary gland. Mem. Fac. Fish, Hokkaido Univ. 13: 1-64.

Yaron, Z. and Levevi-Zermonsky, B. (1986). Fluctuations in gonadotropin and ovarian steroids during the annual cycle and spawning of the common carp. Fish. Physiol. Biochem (in press).

Yaron, Z., Bogmolynaya, A. and Donaldson, E.M. (1982). Stimulation of 17β-oestradiol secretion by the ovary of a teleost, *Sarotherodon aureus*. In: Proceedings. IX Int. Symp. Comp. Endocr., Hong kong.

Yaron, Z., Cocos, M. and Salzer, H. (1980). Effects of temperature

and photoperiod on ovarian recrudescence in the cyprinid fish *Mirogrex terrae-sanctae*. J. Fish. Biol. 16: 371-382.

Zohar, Y., Breton, B. and Fostier, A. (1982). Gonadotropic function during the reproductive cycle of the female rainbow trout, *Salmo gairdneri*, in relation to ovarian steroid secretion: *in-vivo* and *in-vitro* studies. In: Reproductive Physiology of Fish. (C.J.J. Richter. and H.J.Th. Goos. comps). p14-18. Pudac, Wageningen.