# CIRCANNUAL RHYTHMS OF MATURATION IN THE FEMALE RAINBOW TROUT AND THEIR ENTRAINMENT BY CHANGES IN PHOTOPERIOD

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## Summary

The importance of endogenous rhythms in the photoperiodic control of the annual reproduction cycle in female rainbow trout were investigated. The effect of photoperiod regimes on the different stages of maturation was assessed by recording the timing of ovulation and from quantifying associated changes in serum oestradiol-17ß, testosterone and total calcium. Maintained under constant 6L:18D and constant temperature for up to four years, rainbow trout exhibited an endogenous rhythm of maturation with a periodicity of approximately one year. This rhythm of maturation appears to be driven by an autonomous circannual oscillator or clock which can be dissociated from the neuroendocrine mechanisms controlling gonadal maturation. Under conditions of constant 18L:6D or LL the periodicity of the maturation rhythm was 5.5-6 months; it is suggested that this periodicity may be caused by a splitting or uncoupling of at least two circannual clocks involved in the control of maturation.

Abrupt changes in the length of the photoperiod act as a zeitgeber to entrain the endogenous rhythm of maturation. Whether the timing of maturation is advanced or delayed depends primarily on the direction of the change in photoperiod and its timing in relation to the phase of the rhythm, with the magnitude of the alteration in photoperiod having only a supplementary effect. The effect of specific changes in photoperiod on the entrainment of the maturation cycle can be described in terms of a phase-response curve. Photic information is transduced, probably by the pineal gland, into a daily rhythm of melatonin; exposure of rainbow trout to skeleton and resonance photoperiod regimes indicated that daylength measurement is effected by endogenous circadian clock(s) rather than by hour-glass mechanisms. A gating mechanism is closely associated with the circannual clock which determines the timing of onset of maturation in virgin female rainbow trout, only allowing fish that have attained a threshold stage of development to undergo gonadal maturation.

Collectively the results support the hypothesis that the female rainbow trout exhibits an endogenous circannual rhythm of maturation which can be entrained by changes in photoperiod.

Keywords: circannual, entrainment, photoperiod, reproduction, trout.

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# CHAPTER ONE

# GENERAL INTRODUCTION

Reproduction of the rainbow trout (*Salmo gairdneri*) is characteristically an annual event confined to a specific period of the year with spawning generally occurring in the autumn and winter months. The phenomenon of seasonal breeding is widespread throughout the animal kingdom and is generally accepted as being an adaptation to the annual variation in environmental conditions; it enables animals to produce offspring at a time of year when their chances of survival are greatest. As with many other vertebrates indigenous to higher latitudes it has been established for the rainbow trout and other salmonids that the annual cycle of daylength has an important role in controlling the timing of maturation (Hoover and Hubbard, 1937; Hazard and Eddy, 1951; Corson, 1955; Combs *et al*, 1959; Nomura, 1962; Henderson, 1963; Shiraishi and Fukuda, 1966; Whitehead *et al*, 1978; Bromage *et al*, 1982 a,b; Takashima and Yamada, 1984). However, only limited progress has been made in elucidating the mechanisms by which daylength influences this process. Thus, using the female rainbow trout as an experimental animal, the central aim of this thesis was to further the understanding on how the timing of the annual maturation cycle is controlled by changes in daylength. Evidence is presented which supports the hypothesis that changes in daylength serve to entrain an endogenously driven rhythm of maturation.

## 1.1 The control of seasonal breeding in vertebrates: the involvement of exogenous factors and endogenous timing mechanisms

Seasonal breeding has evolved as an adaptation to the annual change in environmental conditions that occurs away from the equator. These changes are due to the variation in the orientation of the Earth's axis in relation to the radiation coming from the Sun. The Earth rotates around its polar axis which is tilted 23.5° to the plane of the orbit around the Sun. For the northern hemisphere the axis of rotation leans most towards the Sun on 22nd June, and for the southern hemisphere most towards the Sun on 22nd December (Figure 1.1). This ecliptic orbit of the Earth around the Sun together with the 24 hour rotation around its own axis causes there to be an annual cycle in daylength which is more pronounced the further away from the equator (Figure 1.2). The experiments described in the present study were performed at latitudes of between 51-53°N where the natural daylength oscillates between slightly less than 8 hours at the winter solstice (22nd December) to over 16.5 hours at the summer solstice (22nd June). In addition to the regular oscillation in daylength, the annual variation in solar radiation results in a cycle of ambient temperature and more subtle effects on rainfall and a host of other climatic variables.

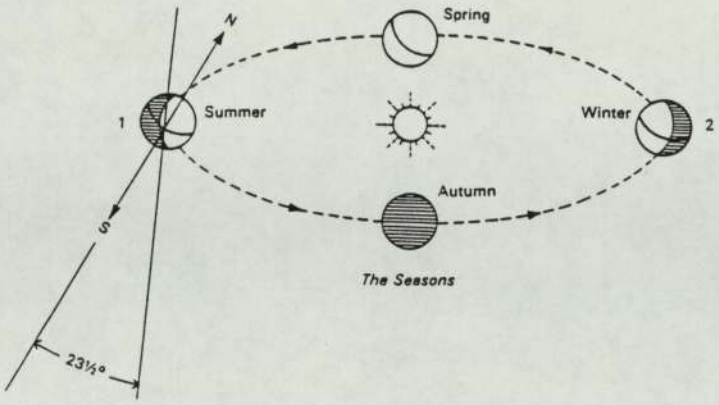



Fig 1.1. The ecliptic orbit of the Earth around the Sun ; note the change in orientation of the axis in relation to the solar radiation which results in the seasonal variation in climatic conditions. (From Saunders, 1977).

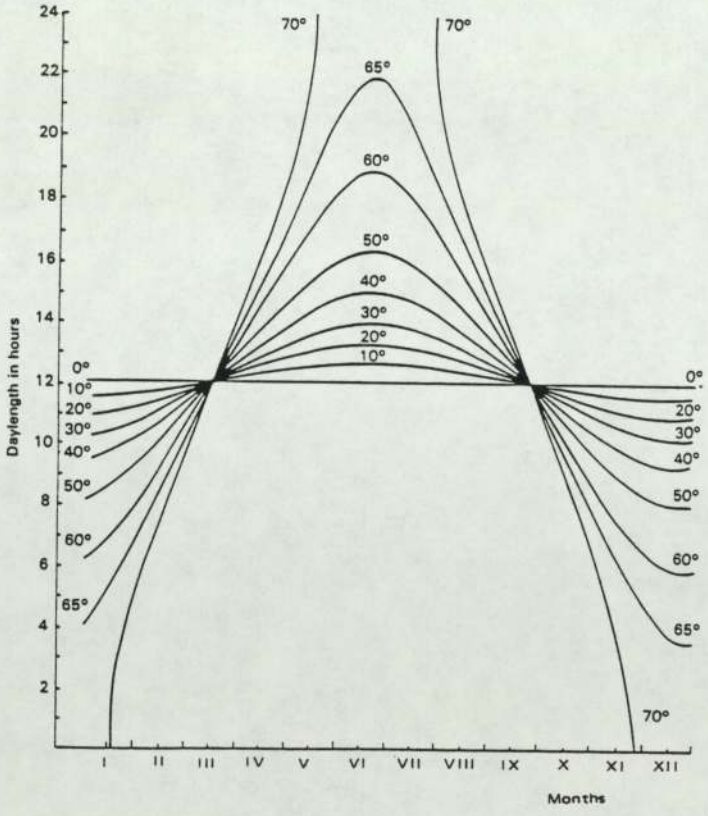



Fig 1.2 . Seasonal changes in daylength at different latitudes in the northern hemisphere. (From Danilevskii , 1965).

The survival of any species in a non-uniform environment is dependent on the development of mechanisms that are capable of perceiving these changes and effecting adjustments in behavioural and physiological functions to optimise the chances of survival. Possibly the most important of these functions is reproduction. In temperate climates the offspring of many species are produced in the spring enabling them to take advantage of the abundant food resources available at this time of year and be sufficiently developed to survive the environmental hardships of winter. Although the offspring of most animals are born or emerge in the spring, the timing of the commencement of breeding varies markedly between species. For example many rodents and birds have a short interval from conception to birth and therefore commence their breeding season in the spring of the same year that they bear their young. In contrast sheep and deer have gestation periods of about 5-6 months, and consequently they enter their breeding season in late summer or autumn to allow birth to occur in the spring. In many salmonids gonadal development is a prolonged process which commences approximately 12 months before full maturation. Spawning generally occurs in the winter but the fry do not emerge until the spring because embryonation up to hatching takes many weeks.

To ensure that reproduction occurs at the most suitable time of year, seasonal breeders synchronise their maturation cycles with the annual changes in the environment. In the following review the control of reproduction in the major vertebrate groups is described, illustrating how external factors such as light and temperature and also endogenous long-term timing mechanisms co-operate in regulating the breeding cycles of many animals. The review will emphasise that in attempting to gain a fundamental understanding of the control of seasonal breeding in any particular animal is of vital importance to take into account the contribution of both exogenous factors and endogenous mechanisms.

Of all the external factors, the annual cycle of daylength is the most dependable and regularly-changing variable and consequently many animals and plants have developed mechanisms which are capable of measuring photoperiod and synchronising the timing of reproduction accordingly. Modern scientific study of the photoperiodic control of seasonal reproduction cycles commenced with the work of Garner and Allard (1920) who first quantitatively described the effects of daylength on a variety of plants. It was they who first introduced the terms photoperiod, photoperiodism and the notion of critical daylength, a phenomena which is discussed in Chapter 3. Experimental work on vertebrates began with the observations of Rowan (1926) on gonadal growth and migration in the snow-bird (*Junco hyemalis*). It was shown that artificially lengthening the photoperiod induced maturation and a tendency to migrate northwards, whereas

shortening of the daylength led to gonadal regression and a southerly movement. Also based on studies on the maturation cycle of birds, Baker (1938) recognised that there were two separate exogenous influences in the timing of annual breeding; these he called ultimate and proximate factors. Ultimate factors exert selective pressures to ensure that populations breed during the optimal season. In many animals the most important ultimate factor is the availability of suitable food supplies as this determines the number of offspring that can be reared to a reproductive age. Proximate factors are the changes in the environment used by the animals to time reproduction to occur at the most suitable time of year. They allow the organism to predict the time at which the ultimate factors will appear. In many seasonally-breeding animals the annual change in photoperiod is the most important proximate factor.

The involvement of internal or endogenous timing mechanisms in the control of seasonal maturation was first suggested following the observations that birds indigenous to equatorial regions exhibited distinct breeding cycles even though photoperiod and climate remain almost constant all year round (Moreau, 1931; Chapin, 1932; Baker, 1938). Subsequently, Pengelley and Fisher (1957, 1963) clearly demonstrated the existence of endogenously-driven rhythms with an approximately annual periodicity in golden mantled ground squirrels (*Citellus lateralis*) maintained for long periods under constant environmental conditions. These long-term rhythms have since been demonstrated in a wide range of animals (Gwinner, 1981a; Farner, 1985). The phenomenon of endogenous rhythmicity is ubiquitous amongst living organisms. In addition to rhythms that are annually-occurring, oscillations associated with a 24 hr periodicity are almost universally exhibited by eukaryotes, and certain animals exhibit rhythms entrained to the tides or the phases of the moon (Saunders, 1977). In each case the particular rhythms continue to be expressed under artificially constant conditions indicating that they are driven by endogenous mechanisms (Aschoff, 1967). Under constant conditions the periodicity of the rhythm usually deviates slightly from the environmental cycle to which it is normally synchronised or entrained. Because of this 'free-running' behaviour, the prefix *circa* was introduced by Halberg (1959) to characterise 'approximately 24 hr' as 'circadian rhythms'. The same prefix has also been used to describe circatidal, circalunar and circannual rhythms (Aschoff, 1967).

The following review on the control of seasonal breeding in vertebrates emphasises the involvement of both exogenous proximate factors and endogenous timing mechanisms in this process and illustrates the difficulties in interpreting the results from short-term experiments. For although endogenous circannual mechanisms have long been suggested to be involved in seasonal breeding (Moreau, 1931; Chapin, 1932;

Baker, 1938) most contemporary investigators have emphasised the direct inductive effects of proximate factors such as photoperiod and temperature on the onset and course of gonadal development with the possibility of the involvement of an endogenous rhythm of maturation receiving little attention. However, when the relative importance of endogenous and exogenous factors in the different vertebrate groups are considered, it is apparent that the involvement of endogenous long-term rhythms in the control of seasonal breeding may be more important than presently thought. For example, evidence will be presented which indicates that in certain species changes in photoperiod serve to synchronise or entrain an endogenous rhythm of maturation rather than having a direct inductive effect. Overall, the research on the control of reproduction in other vertebrates reviewed below provided the background from which an experimental rationale for the present study on the breeding cycle of the rainbow trout could be based.

Following the pioneer studies of Rowan (1926) more research has been conducted on the control of seasonal breeding in birds than any other group of vertebrates and hence consideration of this group is positioned at the beginning of this review. It is generally accepted that daylength is the most important proximate factor in controlling the maturation cycle of birds indigenous to temperate regions (reviews: Wolfson, 1959; Farner, 1975; Murton and Westwood, 1977; Farner and Follett, 1979). In many birds gonadal development is stimulated in the spring when the photoperiod exceeds a certain critical value. This gonadal recrudescence is effected by endogenous circadian timing mechanisms which are capable of accurately measuring the photoperiod (Chapter 3; Hamner, 1963; Follett and Sharp, 1969; Turek, 1974; Gwinner and Eriksson, 1977). Overall these results support the hypothesis that photoperiod has a direct inductive effect on maturation. Following this induction in the spring, the breeding season of many temperate region birds is terminated in the summer by the onset of photorefractoriness. Photorefractoriness, which was first noted by Bissonnette and Waglund (1931) in *Sturnus vulgaris* and then by Riley (1936) in *Passer domesticus*, is generally defined as the regression of the gonads under photoperiods that were previously stimulatory. Apart from the observations that refractoriness to the stimulatory effect of long photoperiods can be dissipated by a species specific period of short days and that the primary site of photorefractoriness is at a hypothalamic or higher neural level, very little is known about this phenomenon despite it receiving considerable attention (reviews: Farner and Follett, 1966, 1979; Murton and Westwood, 1977; Farner *et al*, 1983).

It is suggested that in some species of birds the timing of the onset and possibly the duration of photorefractoriness may be explained by implicating the involvement of endogenous long-term, possibly circannual timing mechanisms. Maintained for long periods under constant conditions, circannual rhythms of gonadal maturation have been shown to be exhibited by a variety of species including the starling (*Sturnus vulgaris*; Schwab, 1971; Gwinner, 1981b), duck (*Anas platyrhynchos*; Assenmacher, 1974; Sharp, 1984) red billed quelea (*Quelea quelea*; Lofts, 1964) and spotted munia (*Lonchura punctulata;* Chandola *et al*, 1983). In addition Gwinner (1977) synchronised the circannual rhythm of gonadal development and moult in starlings with photocycles ranging from 1 year to less than 3 months. Similarly two cycles of gonadal maturation have been observed in the spotted munia (Chandola *et al*, 1985) and two species of warbler (*Sylvia borin, S.melanocephala*; Berthold, 1979) maintained under photocycles with a periodicity of 6 months. In each case the phase relationship between the photocycle and gonadal development supported the hypothesis that photoperiod was entraining an endogenous rhythm of maturation (the entrainment behaviour of endogenous rhythms is covered in detail in Chapter 4). Despite this evidence, the majority of investigators on the environmental control of maturation in birds continue to emphasise the direct effects of photoperiod. For example, even in a species such as the starling which exhibits an autonomous rhythm of maturation, the involvement of an endogenous circannual timing mechanism in controlling breeding under natural conditions is not universally accepted (e.g. Hamner, 1971; Nicholls *et al*, 1984). This is due to the fact that the starling only exhibits a circannual rhythm of maturation when maintained under 12L:12D, under longer or shorter constant photoperiod regimes no cyclicity is observed (Schwab, 1971). If starlings are maintained under a stimulatory 15L:9D photoperiod they become sexually mature and then the gonads regress due to the onset of photorefractoriness a condition in which the birds remain permanently immature (Hamner, 1971). Only by exposing the birds to a period of short days is their photosensitivity restored (Burger, 1949). Under natural conditions starlings, like many other temperate zone birds, become spontaneously photorefractory in the summer with the shorter photoperiods of autumn and winter terminating this condition (Murton and Westwood, 1977). The increasing daylengths of spring are highly stimulatory to gonadal development with the literature emphasising the direct inductive effect of photoperiod on maturation (Gwinner and Eriksson, 1977). However, there is evidence that the increase in testicular size in male starlings is also due to autonomous increase in reproductive activity as it can occur before, or in the absence, of the spring increase in photoperiod (Rutledge, 1974; Rutledge and Schwab, 1974; Gwinner and Ganshirt,

1982). Thus, in the starling the commencement of refractoriness and subsequent gonadal development appears to involve a complex interaction between the direct effects of photoperiod and an underlying rhythm of reproductive activity.

In conclusion, the involvement of endogenous circannual rhythms in the control of bird reproduction is not generally accepted with most investigators emphasising the direct effects of photoperiod on gonadal development in temperate species (Murton and Westwood, 1977; Farner and Follett, 1979; Follett, 1984). However the research on the 'model' temperate zone bird the starling, described above, and other avian species has indicated that autonomous cycles of maturation do occur in birds (Farner *et al*, 1983; Sharp, 1984; Farner, 1985) and that changes in photoperiod can serve to entrain rather than drive a rhythm of gonadal development (Gwinner, 1977; Berthold, 1979; Chandola *et al*, 1985). It might be suggested that a more general acceptance of the involvement of endogenous circannual timing mechanisms in the control of seasonal breeding in at least some species of birds may help in the elucidating the control of gonadal development and also explain the phenomenon of photorefractoriness.

Research into the environmental control of seasonal reproduction in mammals is dominated by studies on the effects of photoperiod on gonadal development in the sheep and a small number of rodent species, notably hamsters. The control of reproduction in some rodents is similar to temperate zone birds in that the mechanism involves the inductive effects of long photoperiods and the phenomenon of photorefractoriness. However the important difference is that birds become refractory to the stimulatory effects of long photoperiods, whereas rodents become refractory to the inhibitory effect of short daylengths (Reiter, 1980). In the Syrian (golden) hamster (*Mesocricetus auratus*) testicular function is maintained indefinitely provided that the photoperiod remains greater than a critical ~ 12.5 hrs (Gaston and Menaker, 1967; Elliott, 1976). Unlike many birds, the breeding season in this hamster is not terminated by photorefractoriness but only occurs in autumn when the daylength falls below 12.5 hrs. Experiments have provided evidence that photic information is transduced by the pineal gland into a daily rhythm of melatonin (Chapter 5; Carter and Goldman, 1983, a,b) with daylength measurement being effected by an endogenous circadian clock (Chapter 3; Elliott *et al*, 1972; Stetson *et al*, 1976). Following this photoperiod-induced degeneration of the gonads in the autumn, spontaneous recrudescence occurs 4-5 months later (Reiter, 1980). This regeneration of the reproductive system does not require photic input since Syrian hamsters kept on continuous short days, or are blinded, still exhibit gonadal recrudescence (Reiter, 1972, 1975; Turek *et al*, 1975a). A similar mechanism for the control of seasonal reproduction has been adopted by the Djungarian

hamster (*Phodopus sungorus sungorus*) although there is a slight difference in the duration of its critical photoperiod (Hoffman, 1979; Duncan *et al*, 1985). As both these species of hamster spend the winter hibernating in a burrow, it may be important from an adaptive viewpoint that gonadal activity is restored in advance of the interruption of hibernation so that there is no delay in the commencement of breeding when they emerge in the spring. The mechanism controlling the spontaneous regrowth of the gonads after several months of quiescence is unknown, however similar to birds it might be suggested that some form of endogenous long-term or circannual timing mechanism is involved.

Spontaneous regrowth of gonads due to the onset of refractoriness to the inhibitory effects of short photoperiods has also been observed in a mouse (*Perognathus parvus*; Kenagy and Barnes, 1984), the rabbit (*Oryctolagus cuniculus*; Boyd, 1985) and the ferret (*Mustela furo*; Herbert and Klinowska, 1978). However in contrast to hamsters these three species also become refractory to the stimulatory effects of long daylengths resulting in the termination of breeding in the summer when the photoperiod is still extended. The 'dual' refractoriness to both the stimulatory and inhibitory effects of long and short photoperiods may explain why both the ferret (Vincent, 1970) and the mouse (Kenagy and Barnes, 1984) exhibit long term rhythms of gonadal development and regression under constant environmental conditions. Because these rhythms were neither regular nor persistent the authors did not class them as being truly 'circannual', however it can be concluded that the seasonal breeding cycle in these species appears to be controlled by a complex interaction between external factors such as daylength and also some form of long-term endogenous timing mechanism.

A similar combination of exogenous factors and endogenous control also appears to be involved in the control of annual maturation in the sheep (*Ovis aries*). Under natural conditions the shortening daylengths of autumn stimulate gonadal maturation in this species and the lengthening days of spring terminate the breeding season (Yeates, 1949; Karsch *et al*, 1984). The converse effects of 'long' and 'short' photoperiods on maturation are emphasised by the observation that sheep maintained on alternate 16 week periods of 16L:8D and 8L:16D exhibited successive phases of gonadal regression and maturation respectively (Lincoln, 1978). However, the effects of these photoperiods were not permanent, for after approximately 4 months of either 8L:16D or 16L:8D the sheep become refractory to the stimulatory or inhibitory influence of short and long days respectively (Lincoln, 1978). This refractoriness to both long and short photoperiods was confirmed in subsequent studies (Robinson and Karsch, 1984; Robinson *et al*, 1985) indicating that analogous mechanisms for controlling seasonal

reproduction may operate in the sheep as has been suggested for the ferret, rabbit and mouse (*P.parvus*) except in these species it is a long photoperiod that is initially stimulatory to gonadal development.

When maintained under constant environmental conditions for long periods sheep exhibit a free-running rhythm of gonadal maturation (Ducker *et al*, 1973; Howles *et al*, 1982; Almeida and Lincoln, 1984). Some authors (e.g. Almeida and Lincoln, 1984) are reluctant to describe these rhythms as 'circannual' because their periodicity showed considerable variation. However, whatever name is ascribed to this phenomenon it is clear that there is some form of long-term internal timing mechanism in sheep which is capable both of maintaining a rhythm of maturation under constant conditions, and possibly also controlling the timing of onset of refractoriness under natural conditions. Certainly the importance of endogenous timing mechanisms in sheep are now more widely recognised, with ideas involving the 'driving' influence of photoperiod being replaced with the concept of an autonomous circannual rhythm of maturation being entrained by changes in the daylength (Robinson *et al*, 1985; Karsch *et al*, 1986). There is evidence that this entrainment of the maturation cycle in sheep is mediated by the pineal gland which converts photic information into a daily rhythm of the hormone melatonin which influences the activity of the hypothalamo-pituitary-gonadal axis (Bittman *et al*, 1983; Karsch *et al*, 1984, 1986).

There is evidence for the involvement of both exogenous factors and internal timing mechanisms in the control of seasonal breeding in lower vertebrates. In several species of reptiles endogenous circannual timing mechanisms have an important role in the control of reproduction. The most convincing evidence comes from studies where *Cnemidophorus uniparens* (Cuellar, 1981) and *Dipsosaurus dorsalis* (Licht, 1972a, 1973a) maintained under constant photothermal conditions for several years continued to exhibit long-term cycles of gonadal recrudescence and regression. Furthermore, similar to certain birds and mammals, in several species of lizard the breeding season is followed by a refractory period of gonadal quiescence. In *D.dorsalis* and *Xantusia vigilis* the cessation of spermatogenesis is independent of photoperiod and temperature indicating the involvement of endogenous mechanisms (Licht, 1973a). Similarly in female *Lacerta sicula* (Botte *et al*, 1976) and female *Anolis carolinensis* (Licht, 1971) the development of refractoriness cannot be changed appreciably by the manipulation of light or temperature. The duration of refractoriness varies among species but it generally persists for several months. In female *A.carolinensis* the refractory period exhibits a gradual termination with the ovaries showing an increased responsiveness to standardised photothermal stimuli as the season progresses, the 'strength' of the

stimulus required to initiate ovarian recrudescence decreasing as the normal breeding season approaches (Licht, 1973b, Crews and Garrick, 1980).

Although the reproductive cycles of some species of reptiles indigenous to temperate regions include a refractory period it is apparent that this phenomenon is not universal (reviews: Duvall *et al*, 1982; Licht, 1984). The onset of a reproductive quiescent period may result from the absence of appropriate exogenous cues; for example in the lizard *A.carolinensis* (Licht, 1971) and snake *Thamonphis sirtalis* (Hawley and Aleksiuk, 1976) spermatogenic activity can be maintained all-year-round by appropriate manipulation of photothermal conditions. In tropical regions there is evidence that species remain sexually-mature all-year-round because of favourable environmental conditions (Hendrickson, 1958; Berry and Lim, 1967; Limpus, 1971; Gorman and Licht, 1975). Even where there appears to be an endogenous rhythm involved in seasonal breeding, environmental factors have an important role in determining the timing of reproduction. Studies on the annual breeding in reptiles indigenous to temperate regions have concentrated on the major proximate cues of temperature and photoperiod, and many field studies have correlated gonadal growth with seasonal changes in these two parameters (reviews: Duvall *et al*, 1982; Licht, 1984). However in field studies it is difficult to isolate the potential effects of temperature and light. Unfortunately many laboratory experiments have also had coincident variation in photoperiod and temperature making it difficult to draw any firm conclusions on the environmental control of reproduction on reptiles (Licht, 1972b). Despite these problems it is generally considered that temperature is the most important environmental variable although there is considerable variation amongst different species in their dependence on this factor (Licht, 1972b, 1984; Lofts, 1978). As reptiles are poikilotherms it is not surprising that temperature has such important effects on all components of the reproductive system. However, the fact that high temperatures cannot prevent the onset of refractoriness in some species indicates that this factor may interact with an autonomous cycle of maturation.

Temperature is also important in controlling seasonal reproduction in amphibians (review: Lofts, 1984). Although only a few species have been studied, the onset of breeding in temperate-zone amphibians can be correlated with the elevation of environmental temperatures. In *Rana esculenta* for example, recrudescence of spermatogenic activity can be stimulated in sexually regressed winter frogs by increasing the temperature to about 20°C (Jorgensen *et al*, 1979). Conversely, lowering the temperature during the summer causes a degeneration of the gonads (Galgano, 1934, 1936). Similarly in *Triturus viridescens* a degeneration of spermatids occurs when

temperatures fall below 12°C, but above this temperature spermatogenesis can occur even in winter (Ifft, 1942). Thus, there is good evidence that temperature exerts a direct driving influence on the timing of reproduction in amphibians. Photoperiod is not thought to be involved in the control of the timing of reproduction in this group as neither constant light nor steadily increasing photoperiods have any effect on spermatogenic activity (Ifft, 1942; van Oordt, 1956).

There is evidence that some amphibians exhibit a form of refractoriness to the stimulatory effect of temperature, a response that is in some ways analogous to the photorefractoriness of birds and mammals. After spawning some temperate-zone anurans enter a sexually quiescent period where the males are spermatogenically insensitive to elevated temperature (Cei, 1944; van Oordt, 1956, 1960). Although it might be suggested that this refractory period is controlled by some form of internal timing mechanism similar to higher vertebrates there is evidence that it is related to the nutritional state of the animal rather than an internal 'clock'. If recently spawned female *Bufo bufo* are taken into the laboratory and fed, the quiescent period does not occur and the next ovarian cycle resumes almost immediately (Jorgensen, 1973). Thus, under natural conditions it would appear that the development of a new batch of oocytes is postponed until the body food reserves have become restored after the energetically demanding breeding season. After this resting period gametogenesis proceeds at a rapid rate and is mostly completed before the falling temperatures of autumn result in the animals entering hibernation (Lofts, 1984). In conclusion, there is little evidence of an endogenous rhythm of maturation in amphibians, with temperature appearing to have a dominant role in controlling the timing of seasonal breeding.

In contrast to amphibians, the environmental control of reproduction in fish has been the subject of considerable research (reviews: deVlaming, 1972; Scott, 1979; Lam, 1983; Dodd and Sumpter, 1984). Collectively the data indicate that both photoperiod and temperature are important proximate factors, however much of this research comprised of short-term experiments which have failed to consider the possible involvement of an endogenous rhythm of maturation. Research on the catfish (*Heteropneustes fossilis*) is exempt from this criticism although it does serve to illustrate the difficulties of drawing conclusions from experiments performed over limited time periods. Short-term experiments on this species indigenous to the monsoon regions of India established that photoperiod, and possibly more importantly temperature are involved in controlling the timing of maturation (Sundararaj and Sehgal, 1970a,b; Sundararaj and Vasal, 1976). In a subsequent experiment female catfish maintained under conditions of constant photoperiod and temperature for up to 3 years exhibited a

circannual rhythm of ovarian development (Sundararaj *et al*, 1982). This result indicated that seasonal variations in photoperiod and temperature do not drive the breeding cycle but may only serve to entrain or allow the expression of an endogenous rhythm of maturation. Endogenous circannual rhythms have also been implicated in the control of the reproduction cycle of the stickleback (*Gasterosteus aculeatus*; Baggerman, 1980). Following a comprehensive series of experiments Baggerman (1980) proposed that the annual reproduction cycle of this fish was controlled by a complex mechanism involving endogenous circadian and circannual rhythms, with photoperiod having an inductive but also an entraining role in the timing of maturation (see Chapters 3 and 4). This work on the stickleback and catfish serve to emphasise that the complexities of the mechanisms controlling reproduction are only revealed after long-term studies. The less detailed studies described below illustrate the difficulties in drawing conclusions as to the overall mechanisms controlling seasonal reproduction in other species of fish.

In goldfish (*Carassius auratus*), Fenwick (1970a) found that long photoperiods (16L:8D and 24L:OD) at 11-12°C stimulated gonadal development only in the spring. In contrast Kawamura and Otsuka (1950) reported a stimulation of ovarian development in goldfish exposed to long photoperiods and warm temperatures in both the winter and spring. In another study on goldfish Gillet *et al*, (1978) demonstrated that a long photoperiod (16L:8D) in winter promoted goldfish ovarian growth at both 10°C and 20°C. In autumn the 16L:8D (10°C) treatment appeared to be stimulatory but 8L:16D (10°C) proved more effective. However, in spring 8L:16D (10°C) was inhibitory but 16L:8D (10°C) remained stimulatory (Gillet *et al*, 1978). The difficulties in drawing conclusions from these results are evident. It is suggested that the discrepancies in the results between the studies may have been due to the gonadal condition of the animals at the start of the experiments. In Fenwick's experiments the fish came from a stock tank maintained at 8L:16D and 11-12°C, whereas those used by Gillet *et al* came from ponds under natural conditions. Although these studies tended to emphasise the direct, inductive effect of photoperiod and temperature on gonadal development, it is possible that an endogenous rhythm is involved, for goldfish maintained under conditions of constant photoperiod and temperature initially underwent gonadal regression but after 4 months exhibited spontaneous recrudescence (Gillet *et al*, 1978).

The involvement of long-term endogenous timing mechanisms in the control of seasonal reproduction is also suggested from experiments on other teleosts including the bridle shiner (*Notropis bifrenatus*; Harrington, 1957), the green sunfish (*Lepomis cyanellus*; Kaya, 1973), the minnow (*Phoxinus phoxinus*; Scott, 1979) and the tench (*Tinca tinca;* Breton *et al*, 1980a,b). The evidence is based primarily on the finding that

continuation of environmental conditions conducive to gonadal recrudescence cannot prevent gonadal regression. Similar results were recorded by Baggerman (1980) in the stickleback and by Sundararaj and co-workers on the catfish (Sehgal and Sundararaj, 1970; Sundararaj and Sehgal, 1970a,b). As described earlier, these workers backed up their initial observations with long-term experiments which provided more convincing evidence of the involvement of a circannual rhythm. It is unfortunate that similar prolonged studies have not been performed on the other species listed above.

The overall conclusion that can be drawn from studies on the control of reproduction in the various groups of vertebrates is that in addition to exogenous factors such as light and temperature, some form of autonomous rhythmicity also appears to be involved in timing of seasonal breeding of many species. Any detailed study of seasonal reproduction in a particular species must attempt to quantify the relative contribution of endogenous and exogenous factors. Experiments must be carefully controlled and sufficiently long-term to reveal the potential involvement of automonous circannual rhythms in the control of maturation. It is proposed that where such a rhythm is identified, its entrainment by changes in the environment such as photoperiod can then be investigated. With consideration to these important points the following section describes research on the control of annual reproduction in salmonids and sets out the aims of the present thesis.

It is established that photoperiod has an important role in controlling the timing of the annual reproduction cycle of the rainbow trout and other salmonids. Salmonids maintained under artificial light regimes where the normal 12 month photocycle was compressed into a period of less than a year exhibited an advance in the timing of maturation (Hoover and Hubbard, 1937; Hazard and Eddy, 1951; Corson, 1955; Combs *et al*, 1959; Nomura, 1962; Henderson, 1963; Whitehead *et al*, 1978; Bromage *et al*, 1982a). Conversely maintaining salmonids under a photocycle with a periodicity of greater than 12 months caused a delay in the time of maturation compared to fish under natural conditions (MacQuarrie *et al*, 1979; Bromage and Duston, in press). Although these results illustrate the ability of photoperiod to alter the timing of maturation it is difficult to analyse the role of daylength in the control of spawning when it is continuously-changing. In this respect the use of constant photoperiods have proved to be a useful experimental technique. Allison (1951) discovered that a 6 week delay in the time of spawning could be elicited by maintaining brook trout (*Salvelinus fontinalis*) on a natural photoperiod until mid-summer followed by constant 17L:7D. This delay in maturation by 'long days' after the summer solstice was confirmed by other studies on salmonids (Shiraishi and Fukuda, 1966; Eriksson and Lundqvist, 1980). Conversely,

abruptly reducing the photoperiod during the summer to a constant 'short' daylength (≤ 8L:16D) resulted in an advancement in the timing of maturation compared to controls (Shiraishi and Fukuda, 1966; Eriksson and Lundqvist, 1980; Lundqvist, 1980; Whitehead and Bromage, 1980; Takashima and Yamada, 1984). The use of constant photoperiods has been extended to cover the entire period from spring when early maturation is occurring (Scott and Sumpter, 1983) until spawning. The results of these experiments are discussed in detail in Chapter 4, however the overall conclusion is that gonadal development is stimulated by a 'long' or increasing photoperiod early in the process of maturation followed by 'short' or decreasing photoperiods being stimulatory later in the cycle. These results have led to the development of a hypothesis that the rainbow trout, and possibly other salmonids, have a requirement for specific photoperiods at particular stages of the maturation cycle (Whitehead and Bromage, 1980; Bromage *et al*, 1982a, 1984; Scott and Sumpter, 1983). Although the hypothesis has not been clearly defined it implies that photoperiod has a direct inductive effect on gonadal maturation in rainbow trout. However, similar to research on the control of seasonal breeding in many other vertebrates described earlier, to date studies have failed to investigate the possibility that the rainbow trout exhibits an endogenously driven rhythm of maturation. Thus one of the major aims of the present thesis was to maintain rainbow trout under constant environmental conditions for extended periods of time to investigate if the fish continue to show a circannual rhythm of gonadal maturation. The results to these experiments described in Chapter 3 provide good evidence that there is an autonomous rhythm of maturation and that photoperiod does not have a direct inductive effect on maturation as suggested above.

In certain other vertebrates which exhibit a circannual cycle of maturation, changes in photoperiod have been shown to entrain the endogenous rhythm (Goss, 1969a,b; Gwinner, 1977; Berthold, 1979; Karsch *et al*, 1984, 1986). Entrainment is vitally important to the survival of any species as it ensures that circannual rhythms of maturation for example, remain synchronised to the annual changes in the daylength so that breeding occurs at the time of year optimal to the survival of the offspring. Experiments described in Chapter 4 investigate the effects of daylength on the timing of maturation in the rainbow trout and provide good evidence that changes in photoperiod entrain an endogenous rhythm of maturation.

As revealed, in some species of mammals there is good evidence that the pineal gland has an important role in transducing daylength information into a daily rhythm of the hormone melatonin which mediates in the control of the timing of seasonal maturation (Carter and Goldman, 1983a,b; Bittman *et al*, 1983; Karsch *et al*, 1984). In

Chapter 5 of this thesis the physiological role of the pineal gland is discussed in detail and experiments are described which investigate the role of melatonin in the control of reproduction of the rainbow trout.

The female rainbow trout is used as an experimental animal because a considerable number of investigations have already been performed on the control of reproduction in this species and this study represents a continuation of an on-going research programme. Although this thesis is devoted to pure fundamental research into the control of maturation, there is considerable commercial interest in the ability to manipulate the timing of spawning of rainbow trout. Indeed, most of the experiments described in this thesis were performed on commercial fish farms with the kind consent and co-operation of the proprietors. In most cases the experimental design aimed to consider both our scientific requirements and the requirements of the fish farmer. For example, in Chapter 4, Experiments 1-3 all adopt photoperiod regimes which produced an advance in the time of spawning; this was to satisfy the fish farmers' requirements for eggs 2-3 months prior to trout raised under natural conditions. Nevertheless, the experimental rationale still serve to provide rigorous tests of the proposed hypotheses regarding entrainment.

## 1.2 Summary of Aims of Thesis

(1) Investigate the role of endogenous circannual timing mechanisms in the control of the annual maturation cycle of the female rainbow trout (Chapter 3).

(2) Quantify the effects of changes in photoperiod on the entrainment of the maturation cycle (Chapter 4).

(3) Investigate the mechanisms by which rainbow trout measure daylength and how photic information is converted into a neuroendocrine signal which influences the timing of gonadal development (Chapters 3 and 5).

## 1.3 The control of Ovarian Development in Teleosts

Although the central aim of this thesis is the study of the effects of changes in the photoperiod on the timing of maturation and not the endocrine control of ovarian development *per se*, any alteration in the timing of spawning implicitly involves the neuroendocrine system. In order to properly understand the interrelationships of these processes, there follows a review of the developmental stages of the rainbow trout ovary and the endocrine control of maturation. A number of experiments described in this thesis measure serum levels of oestradiol-17ß, testosterone and total calcium as indices of the stage of maturation of the ovaries. The role of these three parameters in the control of gonadal development will be discussed in the review and their inclusion in the experimental protocol justified.

### 1.3.1 The developmental stages of the rainbow trout ovary

For detailed accounts of the developmental stages of the rainbow trout ovary the reader is referred to a series of comprehensive studies (Yamamoto *et al*, 1965; Hurk and Peute, 1979; Wallace and Selman, 1981; Nagahama, 1983; Cumaranatunga, 1985). Presented here is a brief description of the important features of the ovary and summarises the major stages in the maturation of the oocytes.

The rainbow trout ovary is a paired organ which varies greatly in size and appearance according to the stage of the maturation cycle; in immature and post-spawned fish the gonadal weight:somatic weight ratio (gonado-somatic index, GSI) is less than 1% whereas in mature animals the GSI can approach 15%. This increase in GSI is due to the growth of the oocytes which enlarge from a diameter of 20 µm to around 5mm at the fully developed stage at which time there can be approximately 3000 mature eggs $kg^{-1}$ body weight in the ovary. In the annually spawning rainbow trout the development of primary oogonia into mature oocytes takes approximately 12 months and their increase in size is primarily attributable to the uptake of an exogenous yolk material (vitellogenin) synthesised in the liver. The transformation from diploid oogonia into haploid fertilisable ova can be divided into 7 stages of development which are classified according to size of the oocytes, the shape, number and position of nucleoli within the nucleus, the position of the nucleus and lastly the presence of other organelles within the ooplasm as follows:

Stage 1 - chromatin nucleolar stage: cytoplasm contain a large nucleus with centrally located nucleolus. The oocytes are just commencing meiotic prophase.

Stage 2 - early perinucleolar stage: this stage is characterised by the appearance of shallow undulations in the nuclear envelope and electron dense substances known as Balbiani bodies accumulating in the juxtanuclear region. The function of the Balbiani bodies is unclear.

Stage 3 - late perinucleolar stage: this stage is distinguished by the presence of small spherical nucleoli closely situated to the nuclear membrane. The Balbiani bodies migrate to the periphery of the oocytes and then disappear. By the end of this stage follicle cells multiply and form a continuous follicular layer (granulosa layer) around each oocyte. Simultaneously the surrounding stromal connective tissue elements also become organised to form the distinct layer of the follicular envelope (the thecal layer). Thus vitellogenic oocytes are surrounded by 2 major cell layers, an outer thecal layer and an inner granulosa layer which are separated by a distinct basement membrane. The thecal layer contain fibroblasts, collagen fibres, capillaries and special steroid producing cells.

Stages 1-3 are known as the primary growth phase. After the late perinucleolar stage there is a complex series of changes within the oocytes known as the secondary growth or vitellogenic phase which can be sub-divided into endogenous and exogenous stages.

Stage 4 - endogenous vitellogenesis: during this stage vesicle formation occurs within the ooplasm. Their structure, synthesis and functional importance are not fully understood although it is established that these vesicles are synthesised within the oocyte (autosynthetic). The vesicles initially fill the oocytes but are pushed to the periphery during the later stages of vitellogenesis.

Stages 5, 6 and 7 - exogenous vitellogenesis: this stage can take approximately 6 months to complete. It is characterised by high circulating levels of oestradiol-17ß, oestrone, testosterone and vitellogenin. Oestrogens stimulate the production of vitellogenin in the liver and its release into the bloodstream. In the rainbow trout oocytes undergoing exogenous vitellogenesis can be divided into stages 5, 6 and 7:

Stage 5 - peripheral yolk granular stage: yolk granules become visible at the periphery of the oocytes. The nucleus to each oocyte is situated centrally.

Stage 6 - migrating yolk granule stage: during this stage, in addition to the yolk granules becoming visible at the periphery, the nucleus also begins its migration towards the outer membrane of each oocyte.

Stage 7 - final maturation: oocytes at this stage reach the end of yolk deposition. There is a breakdown of the nuclear membrane (germinal vesicle breakdown) and there is presumed to be a resumption of meiosis although the evidence for this occurring in teleosts is poor. Following the completion of maturation the follicular layers breakdown and the oocytes are ovulated into the body cavity. Under natural conditions muscular contractions of the body wall result in the rainbow trout ovipositing the eggs into the surrounding water. However in captivity, due to the absence of specific cues yet to be determined, the eggs fail to be oviposited and have to be manually 'stripped' by applying gentle hand pressure to the abdomen of the fish.

The stages of oocyte development are summarised in Figures 1.3 and 1.4. The following section describes the endocrine control of the maturation of the oocytes.

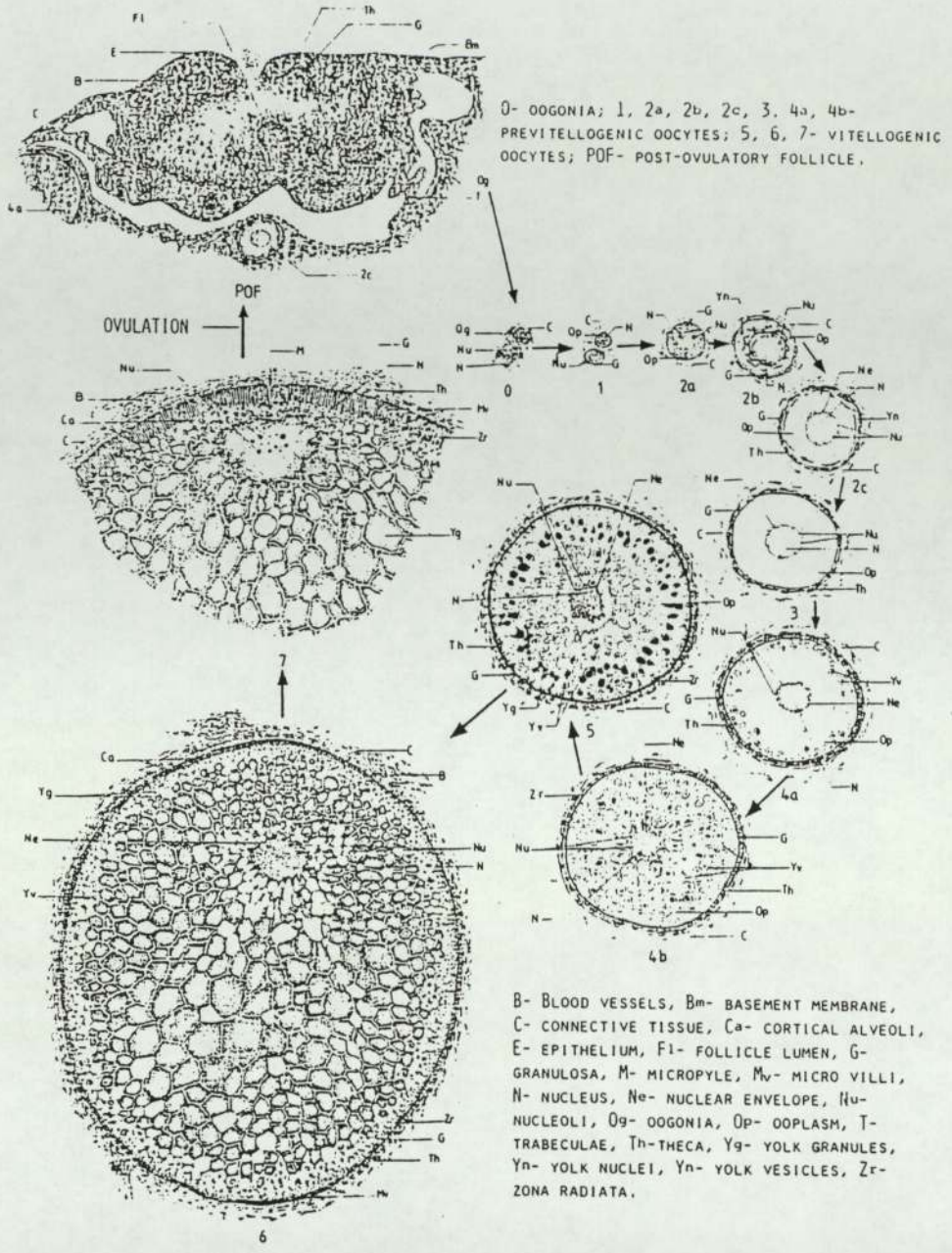



Fig 1.3. Developmental stages and cytological changes of rainbow trout oocytes. (From Cumaranatunga, 1985).

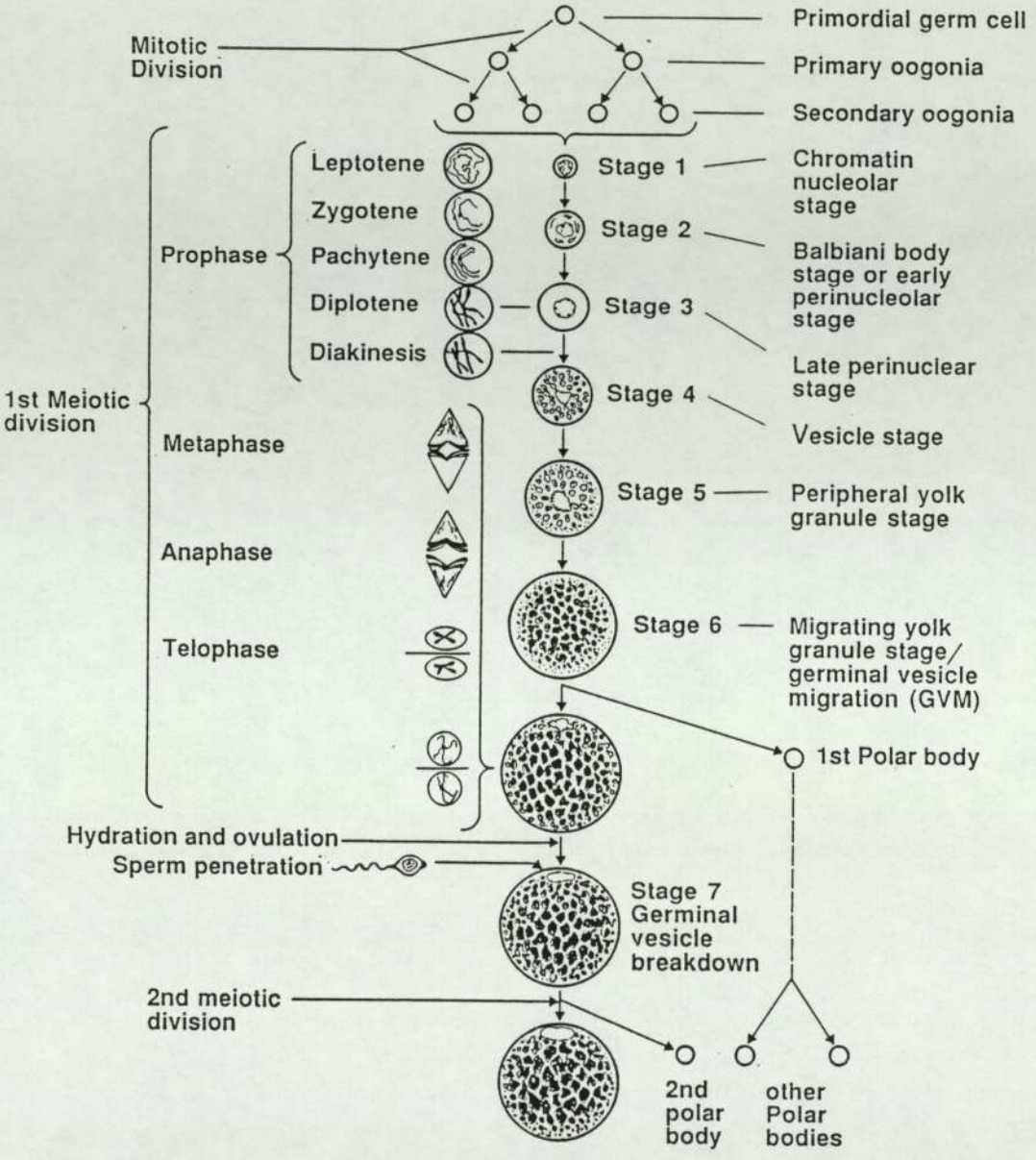



Fig 1.4. Schematic diagram showing the cytological events associated with oocyte development in the rainbow trout. (From Cumaranatunga, 1985).

1.3.2 The endocrine control of ovarian development

The maturation of the oocytes is under direct control of steroid hormones produced by the ovary. The secretion of these hormones is controlled by gonadotropin released from the pituitary gland which is in turn controlled by the hypothalamus. In addition to the hierarchial hypothalamo-pituitary-gonadal control of oocyte development, the release of hormones from each 'level' is regulated by complex feedback mechanisms. This review provides a basic description of the hormones involved in ovarian maturation in teleosts with emphasis on the salmonids. More comprehensive reviews are provided by Dodd and Sumpter (1984) and Hoar, Randall and Donaldson (1983).

It has long been recognised that the hypothalamus (HE) has an important role in the control of vertebrate reproduction (Harris, 1955). More specifically small peptide molecules released from neurosecretory cells in the HE have been shown to stimulate the secretion of gonadotropin (GtH) from the pituitary gland. In mammals the 'gonadotropin-releasing hormone' (GnRH) is luteinizing-hormone-releasing-hormone (LHRH), a decapeptide the structure of which is shown below (reviews: Vale *et al*, 1977; Guillemin, 1978).

Pyro Glu$^{1}$-His$^{2}$-Tryp$^{3}$-Ser$^{4}$-Tyr$^{5}$-Gly$^{6}$ -Leu$^{7}$-Arg$^{8}$-Pro$^{9}$-Gly$^{10}$-amide

Mammalian GnRH

where Glu = Glutamic acid; His = Histidine; Tryp = Tryptophan; Ser = Serine; Tyr = Tyrosine; Gly = Glycine; Leu = Leucine; Arg = Arginine; Pro = Proline.

In fish, Breton *et al*, (1971) were the first to demonstrate GnRH activity in the hypothalamus. The addition of carp (*Cyprinus carpio*) HE to carp pituitary cultures caused an increase in GtH levels in the medium. Further experiments in which crude hypothalamic extracts, mammalian LHRH and synthetic LHRH analogues stimulated the release of GtH from teleost pituitaries *in vitro* and *in vivo* supported the hypothesis of GnRH having a functional role in the control of maturation in fish (Crim *et al*, 1976; Crim and Evans, 1980; Ball, 1981; van der Kraak *et al*, 1983). Evidence that teleost and mammal GnRH were of a similar structure was provided by King and Millar (1979, 1980) who showed that a hypothalamic substance in fish was immunoreactive with antisera against mammalian LHRH. Salmon (*Oncorhynchus keta*) GnRH has since been characterised and is identical to mammalian LHRH except for Tryp$^{7}$ and Leu$^{8}$ substituted for Leu$^{7}$ and Arg$^{8}$ (Sherwood *et al*, 1983).

Pyro Glu$^{1}$-His$^{2}$-Tryp$^{3}$-Ser$^{4}$-Tyr$^{5}$-Gly$^{6}$-Tryp$^{7}$-Leu$^{8}$-Pro$^{9}$-Gly$^{10}$-amide

Salmon GnRH

This structure known as sGnRH is common to a number of teleosts including the rainbow trout (Sherwood *et al*, 1984). The increase in secretion of GtH following the injection of sGnRH into rainbow trout has confirmed that this hypothalamic peptide has an important role in stimulating the release of gonadotropin from the pituitary gland in this species (Weil *et al*, 1986). The recent development of a radioimmunoassay for sGnRH (Breton *et al*, 1986) will no doubt prove to be an invaluable tool in elucidating the role and mode of action of this hormone.

In addition to a stimulatory GnRH, there is some evidence that the hypothalamic region of some cyprinids also secrete gonadotropin-release-inhibiting-hormone (GnRIH) which, as the name indicates, inhibits the release of gonadotropin from the pituitary gland of fish (Peter, 1982, 1983; Peter *et al*, 1984). Although teleost GnRIH has not yet been isolated or characterised, there is good evidence that it is the neurotransmitter dopamine (Chang and Peter, 1983; Dufour *et al*, 1984; Peter *et al*, 1984; de Leeuw *et al*, 1986). How the inhibitory and stimulatory releasing hormones from the hypothalamus modulate the maturation cycle in teleosts remains to be discovered, but it is clear that they have a central role in controlling the secretions from the pituitary gland.

The paramount importance of the pituitary gland itself in the control of teleost gonadal maturation has been extensively reviewed (Pickford and Atz, 1957; Dodd, 1960; Hoar, 1969; Dodd and Sumpter, 1984). Hypophysectomy leads to gonadal atrophy. Fish gonadotropins have been isolated from the pituitaries of a number of species including carp (*Cyprinus carpio*; Burzawa-Gerard, 1971; Idler and Ng, 1979), salmon (*Oncorhynchus* sp; Donaldson *et al*, 1972; Idler *et al*, 1975 a,b; Idler and Ng, 1979). American plaice (*Hippoglossoides platessoides*; Ng and Idler, 1978; Ng and Idler, 1979), winter flounder (*Pseudopleuronectes americanus*; Ng and Idler, 1979), *Tilapia* (Farmer and Papkoff, 1977) and the rainbow trout (*Salmo gairdneri*; Breton *et al*, 1976). Despite this extensive research there is still uncertainty regarding the number of gonadotropic hormones in teleosts because purification of pituitary extracts is often incomplete and there is an absence of suitable bioassays to differentiate between different presumptive forms of GtH (Idler, 1982; Dodd and Sumpter, 1984). The most compelling evidence in support of there being more than one teleost gonadotropin comes from the work of Idler and associates (reviews: Idler, 1982; Idler and Ng, 1983). Using the technique of affinity chromatography with Con-A-sepharose, chum salmon (*Oncorhynchus keta*) pituitary extract yielded a broad unadsorbed peak (designated Con AI fraction) and an adsorbed fraction (Con AII fraction; Idler *et al*, 1975b). Similarly Con AI and Con AII fractions have been isolated from pituitary extracts of the carp (*Cyprinus carpio*), plaice (*Hippoglossoides platessoides*) and flounder

(*Pseudopleuronectes americanus*) with both fractions exhibiting gonadotropic activity (Idler and Ng, 1979; Ng and Idler, 1979). Idler (1982) proposed that the Con AI and Con AII fractions each contained a gonadotropin which stimulated vitellogenesis and final maturation respectively, however this is still to be ratified. Thus it remains to be confirmed whether the ovarian cycle of teleosts including the rainbow trout is modulated by one or two gonadotropins.

The problem in elucidating the precise role of putative gonadotropins in the control of maturation is the uncertainty as to what existing salmonid GtH radioimmunoassays (RIA) actually measure. Certainly all of the current salmonid GtH RIAs depend on an antigenicity towards the Con AII fraction; however the GtH used to raise the antibody was not fully purified and therefore may well be cross-reacting with other gonadotropins (Breton *et al*, 1976; Stuart-Kregor *et al*, 1981; Idler, 1982). Thus, caution must be exercised when interpreting the results from any study involving the quantification of salmonid gonadotropin (discussion: Dodd and Sumpter, 1984). Another factor which must be taken into account when interpreting such data is that like higher vertebrates (Brinkley, 1981; Desjardins, 1981) there are diel fluctuations in the circulating levels of GtH in rainbow trout (Zohar *et al*, 1986 a,b) and other teleosts (Hontela, 1984). This episodic secretion of GtH indicates that the numerous studies where blood samples are only taken at approximately monthly intervals (see below) may not be particularly informative in the elucidation of the mechanism of GtH action. Importantly in goldfish (*Carassius auratus*), changes in photoperiod and temperature have been shown to alter the diel secretion patterns of GtH and subsequent gonadal development (Hontela and Peter, 1983; Hontela, 1984). Although not investigated in this thesis, future research on the entrainment of GtH secretion in teleosts may result in a better understanding at the physiological level of how changes in photoperiod alter the timing of maturation.

Despite the difficulties in measuring gonadotropin(s) it is established that this hormone secreted from the pituitary gland has an essential role in controlling the maturation of the gonads. In female fish exogenous vitellogenesis is regulated indirectly by the pituitary through the action of oestrogen hormones produced by the ovary (reviews: Peter and Crim, 1979; Wallace and Selman, 1981; Dodd and Sumpter, 1984). A number of studies have shown that gonadotropin is responsible for stimulating the secretion of oestrogens in female salmonids (Crim and Idler, 1978; Kagawa *et al*, 1982; Young *et al*, 1982). Small increases in the serum levels of GtH have been detected at the onset of vitellogenesis, these being closely followed by increases in the circulating levels of oestrogens (Figure 1.5; Billard *et al*, 1978; Bromage *et al*, 1982 a,b; Sumpter *et al*,

1984). In the rainbow trout oestradiol-17ß (E2) and oestrone (E1) are the naturally occurring oestrogens with E2 having the major role of stimulating the synthesis and secretion of vitellogenesis in the liver (van Bohemen and Lambert, 1981; Wallace and Selman, 1981; Elliott *et al*, 1979). Circulating levels of E2 increase from basal levels of less than 5 ng $ml^{-1}$ to over 30 ng $ml^{-1}$ during maximal vitellogenesis. (Bromage *et al*, 1982a; Scott and Sumpter, 1983). Because increases in the circulating levels of E2 provide a good indicator of the onset and course of vitellogenesis, the measurement of this hormone was included in a number of experiments described in this thesis. The circulating oestrone levels follow a similar secretory profile to oestradiol-17ß in salmonids (Elliott et al, 1984). The relative importance of oestrone in the rainbow trout is unclear although it has been suggested that it 'primes' the liver for subsequent stimulation of vitellogenin by oestradiol-17ß (van Bohemen and Lambert, 1981; van Bohemen *et al*, 1982).

The stimulation of vitellogenesis by oestrogen is reflected in concomitant increases in circulating levels of oestradiol-17ß and vitellogenin (Elliott *et al*, 1984; Sumpter *et al*, 1984). Serum titres of protein-bound calcium also change in parallel with changes in vitellogenin as this component forms an integral part of the yolk molecule (Bromage *et al*, 1982a). Because of the high degree of correlation between serum calcium and vitellogenin levels (Figure 1.5) and the ease and speed with which the former can be measured (Chapter 2), serum calcium analyses have been adopted in experiments described in this thesis as an index of vitellogenin secretion.

In salmonids, as maturation of the oocytes proceeds, dramatic changes occur in the circulating levels of hormones associated with this process. Approximately 2 months before full maturation oestradiol-17ß levels begin to fall, returning to basal levels around the time of ovulation (Figure 1.5; Whitehead *et al*, 1978; Scott *et al*, 1980). This decrease in serum oestradiol-17ß concentration is accompanied by a large increase in the blood levels of testosterone. Testosterone has been identified in a number of female teleosts (Schmidt and Idler, 1962; Katz and Eckstein, 1974; Schreck and Hopwood, 1974; Wingfield and Grimm, 1977) including the rainbow trout (Campbell *et al*, 1980; Scott *et al*, 1980), however, its functional significance remains uncertain. Testosterone is the biosynthetic precursor of oestradiol-17ß (Figure 1.6); the inverse relationship between the circulating concentrations of these two steroids appears to be due to the inhibition of the aromatase enzyme involved in conversion of this androgen to oestrogen (Kagawa *et al*, 1983). *In vitro* studies on salmon ovaries (*Oncorhynchus rhodurus*) have provided evidence that the thecal layer of the follicle is the site of aromatisable androgen synthesis and the granulosa layer converts this 'precursor' into oestradiol-17ß

(Kagawa *et al*, 1983; Young *et al*, 1983). Although the word 'precursor' has been used here to describe testosterone, implying that it is without a physiological role, *in vitro* studies have suggested that this hormone enhances the effectiveness of gonadotropin to induce final oocyte maturation in the female rainbow trout (Jalabert, 1976; Jalabert and Fostier, 1984) and salmon (*O.rhodurus*; Young *et al*, 1982). That circulating levels in female rainbow trout reach over 150 ng $ml^{-1}$ just prior to ovulation (Scott *et al*, 1983) suggests that testosterone does have a physiological role in the control of maturation, it just remains to be elucidated. The fact that circulating testosterone levels reach such high levels just prior to ovulation before rapidly decreasing to basal means it can serve as an accurate indicator of the timing of final maturation. Hence the quantification of this hormone was included in some of the experiments described in this thesis.

Prior to ovulation there is a large increase in the circulating concentration of gonadotropin (Bromage *et al*, 1982 a,b; Scott *et al*, 1983) due primarily to the influence of GnRH on the pituitary (Peter, 1983), but also partly due to the decrease in levels of oestradiol-17ß which is known to have a negative feedback effect on GtH secretion (Bommelaer *et al*, 1981). The surge in gonadotropin indirectly causes the resumption of meiosis and germinal vesicle breakdown (GVBD) by stimulating the secretion of maturation inducing progestagens within the ovary which act directly on the oocyte surface to cause ovulation (review: Goetz, 1983). In addition to the hormone changes shown in Figure 1.5 amongst salmonid species there are large increases in blood levels of 17α-hydroxy-20ß-dihydroprogesterone (17α-20ß-dihydroxy-4-pregan-3-one; 17α 20ß-P) prior to ovulation with this hormone being generally considered as the natural maturation-inducing steroid (Jalabert, 1976; Nagahama *et al*, 1980, 1983; Goetz, 1983). In addition, 17α hydroxyprogesterone (17αOH-P) levels are greatly elevated during the period of ovulation in the rainbow trout (Figure 1.7; Campbell *et al*, 1980) and although stimulatory to GVBD it is not as effective as 17α20ß-P (Jalabert, 1976; Nagahama *et al*, 1983). It has been suggested that the apparent activity of 17αOH-P and other hormones such as progesterone in stimulating GVBD may be due to their conversion to 17α20ß-P (Nagahama *et al*, 1983).

The actual process of ovulation involves the oocyte being separated and expelled from the follicle. The hormonal control of ovulation in fish is poorly understood. *In vitro* studies on rainbow trout ovaries have indicated that although 17α20ß-P is involved in final maturation and GVBD it does not induce ovulation unless followed by prostaglandin treatment, thus implicating this group of hormones in the expulsion of the oocytes from the ovary (Jalabert and Szollosi, 1975; Jalabert, 1978). Ultrastructural

studies of teleost follicles have found microfilaments similar in appearance to smooth muscle present in the thecal layer prior to ovulation (Nagahama *et al*, 1976; Pendergrass and Schroeder, 1976; Szollosi *et al*, 1978). There is some evidence to indicate that the contraction of these fibres, possibly due to prostaglandin stimulation, forces the mature oocytes out of the follicle and into the body cavity (Jalabert and Szollosi, 1975; Schroeder and Pendergrass, 1976).

In conclusion, research has shown that the endocrine control of ovarian maturation in teleosts is a highly complex process involving many different hormones all interacting with each other to produce the final end product, fertilisable ova. The present investigation monitored changes in the blood levels of oestradiol-17ß, testosterone and total calcium to provide indices of the effect of experimental photoperiod regimes on the timing of maturation in the female rainbow trout. As described above, these three parameters undergo significant changes over the course of maturation and are involved in mediating the effects of exogenous and endogenous control mechanisms on the timing of seasonal breeding in this species. As summarised on page 26, the major aim of the present study was to investigate how changes in the environmental photoperiod and endogenous timing mechanism co-operate in the control maturation in the rainbow trout.

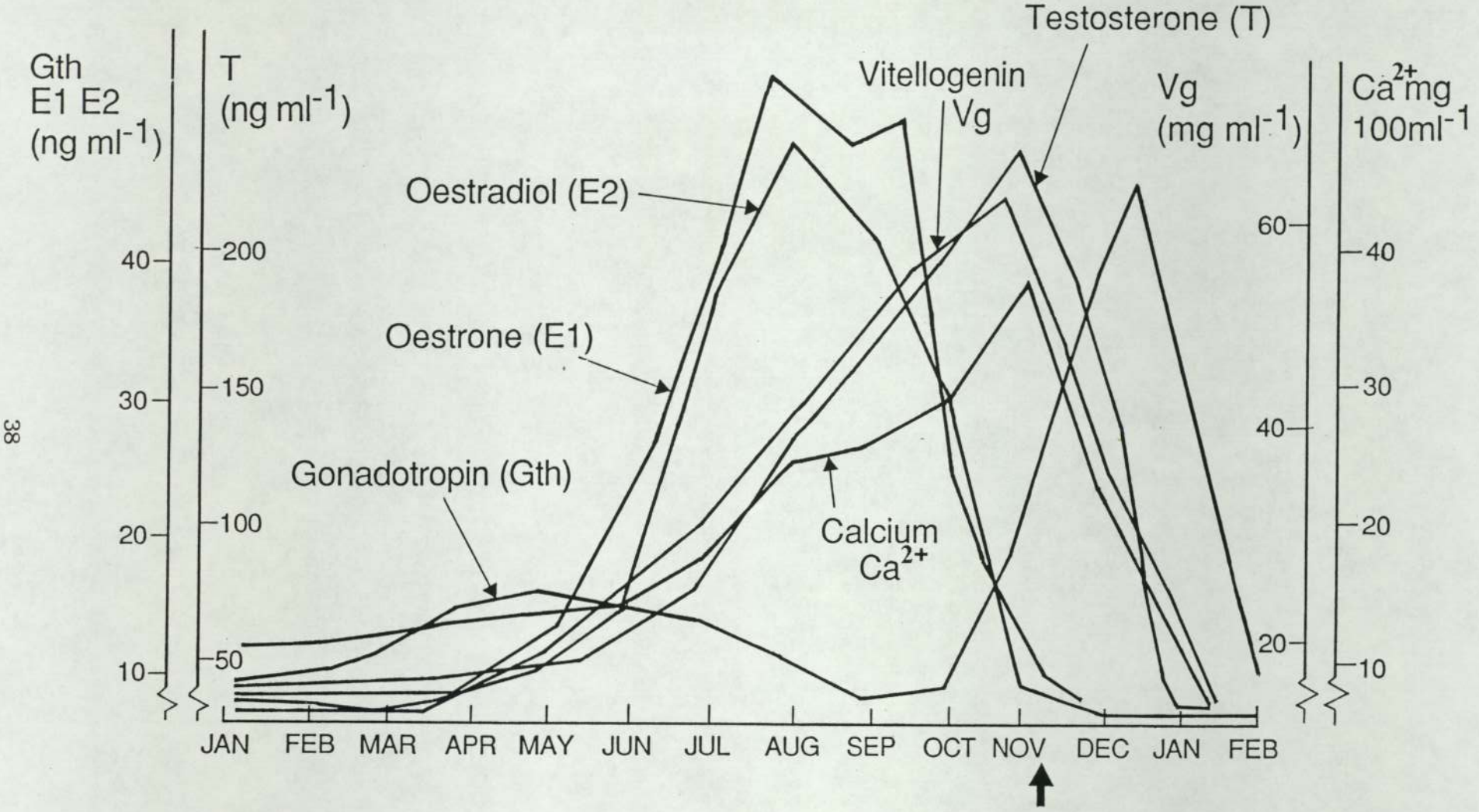



Fig.1.5 Composite graph showing the changes in serum concentration of a number of components associated with gonadal maturation in the female rainbow trout. The arrow(⬆) denotes the timing of ovulation. The vertical lines on the x-axis refer to the first day of the month as labelled.(From Bromage and Cumaranatunga, in press).

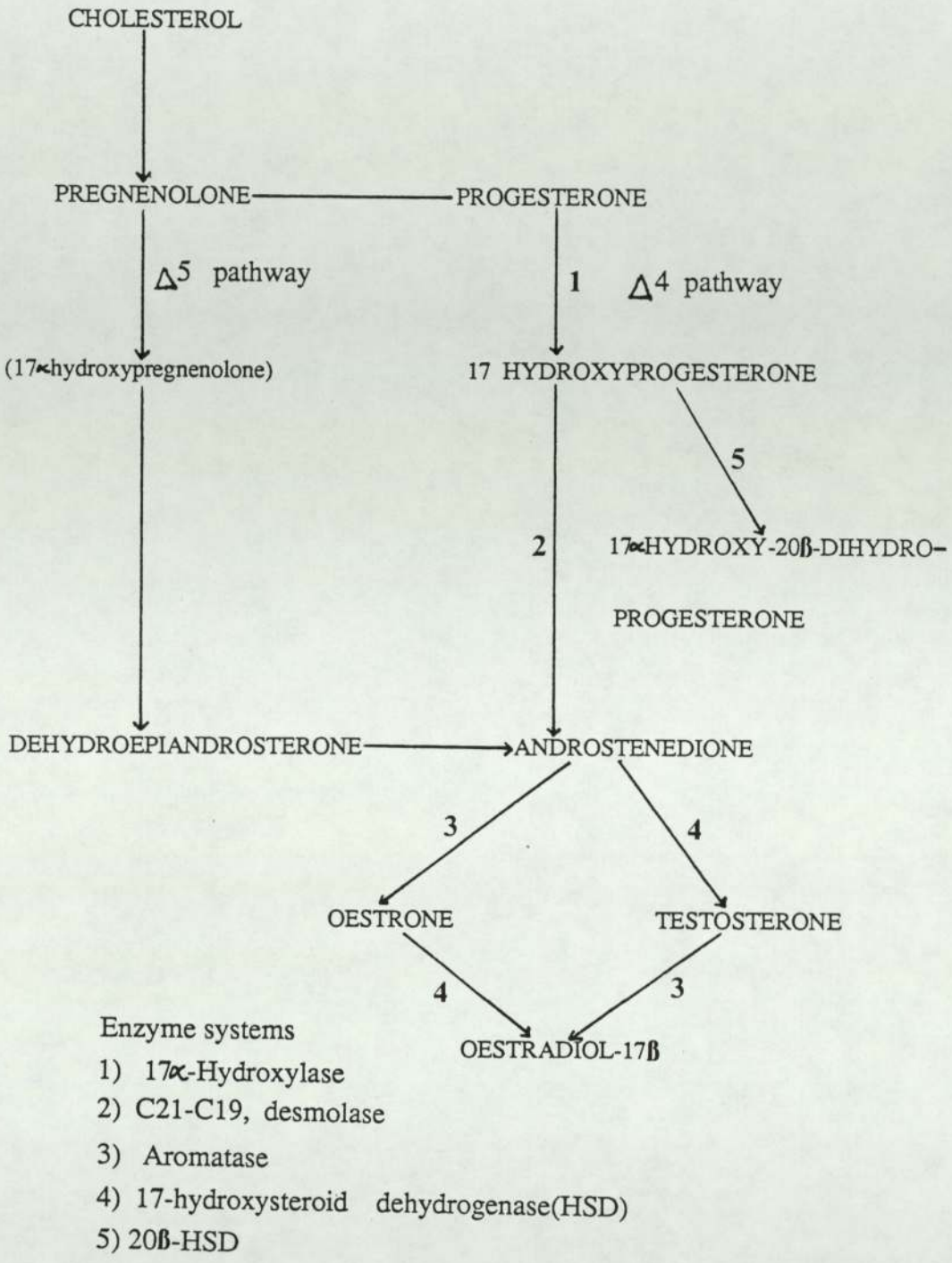



Fig 1.6. Pathways of steroid biosynthesis in the female rainbow trout.

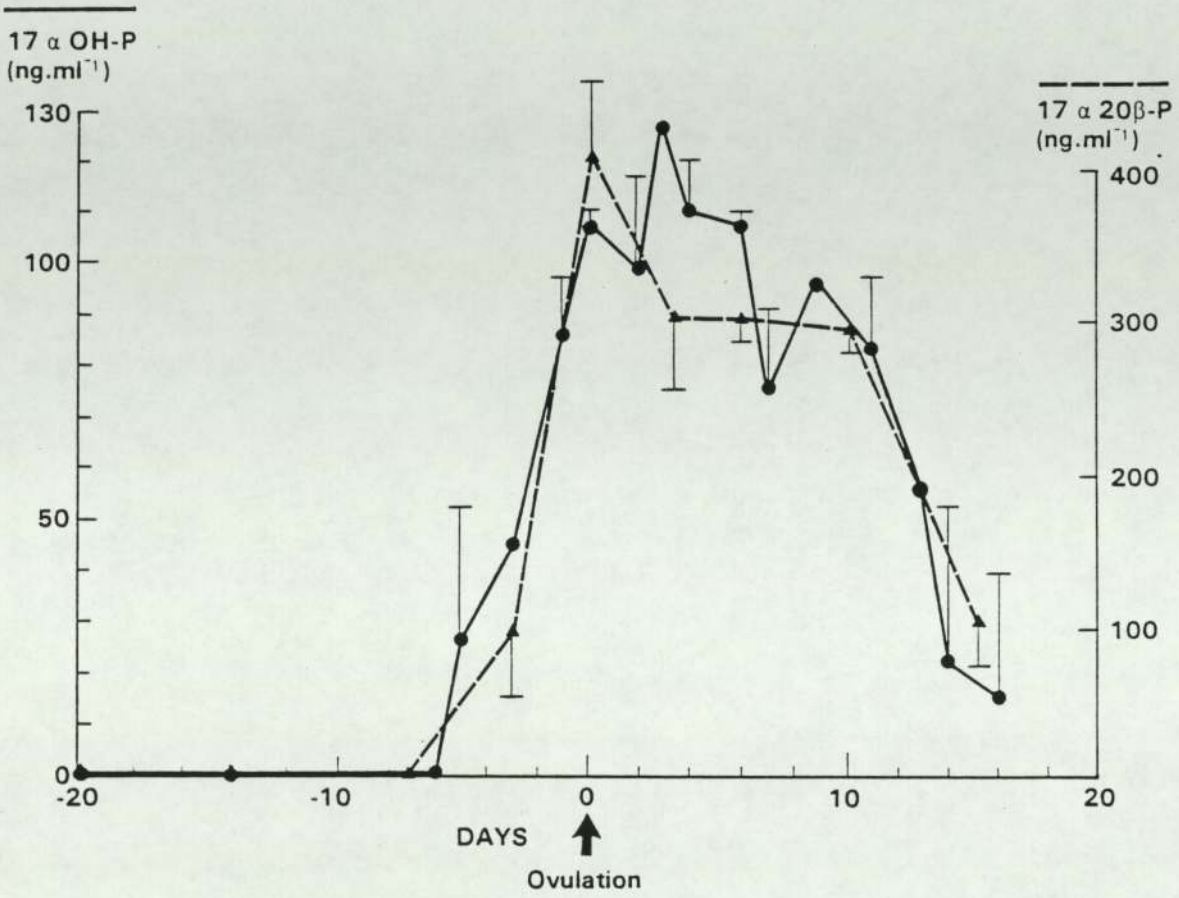



Fig. 1.7 Changes in serum levels of 17∝-hydroxy-20β-dihydroprogesterone (17∝20β-OH) and 17∝hydroxyprogesterone (17∝OH-P) associated with final maturation in the female rainbow trout. Coordinates represent mean values ± one standard error of the mean. (From Springate, 1985).

# CHAPTER TWO

# GENERAL MATERIALS AND METHODS

This chapter includes the materials and methods relevant to more than one experiment described in this thesis.

## 2.1 Experimental animals; handling, blood sampling, tagging and assessment of maturity

All experiments were performed on domesticated stocks of female rainbow trout (*Salmo gairdneri*). Fish from different stocks were used during the course of the study but it was ensured that fish from the same source were used in related experiments. Unless otherwise specified, the fish were fed daily a dry trout diet of an appropriate ration and pellet size as recommended by the manufacturers (Mainstream, BP Nutrition U.K. Ltd., Witham, U.K.). Fish were starved for 24 hrs prior to blood sampling, tagging or stripping. To allow easy handling, fish were anaesthetised by immersion in a solution of 2-phenoxyethanol (Sigma Chemicals Ltd., Poole, U.K.) at a concentration of 1 part per 2000 parts water. Post-sampling mortalities were a rare occurrence.

Blood sampling: Blood samples were withdrawn from the Cuverian duct of anaesthetised fish using a 5 ml serum monovette (Sarstedt Ltd., Leicester, U.K.) fitted with a 19G 5cm needle. Monovettes are combined syringe and centrifuge tubes which contain glass beads creating a greater surface area on which a clot can form. The blood obtained was allowed to stand for at least 1 hr to allow the clot to form and then centrifuged at 2,000 rpm for 15 minutes. The resulting serum was decanted into clean polystyrene tubes (LP3, Luckhams Ltd., Burgess Hill, U.K.) stoppered and stored at -20°C until analysis.

Fish identification: Individual fish were identified by small plastic numbered tags (Charles Neal Ltd., East Finchley, U.K.) attached to the base of the dorsal fin by two plastic pins punched through the tag and the body muscle by a special tagging gun (Kimbal Systems Ltd., Leicester, U.K.).

Assessment of the timing of maturation: Experimental animals were routinely checked at monthly intervals for mature animals and at approximately fortnightly intervals during the spawning period. External signs of the female rainbow trout approaching maturity are the extrusion of the anal papilla and reddening and softening of the abdomen. In captivity although rainbow trout ovulate successfully they fail to expel their eggs from the abdominal cavity into the water, consequently they must be

manually forced from the fish. This process is called 'stripping' and involves a gentle squeezing of the abdomen of the anesthetised fish which forces the ovulated eggs towards and out through the anal papilla. Throughout the text the point at which eggs could be stripped was defined as the time of spawning. As experimental animals were checked for maturity at approximately fortnightly intervals the fish may have reached full maturation (ovulation) at any period between successive sampling times.

2.2 Quantification of egg size, total fecundity and relative fecundity

The eggs from individual mature fish were stripped into clean, dry plastic bowls. They were then fertilised by adding sperm from a number of mature males at a concentration of 1 ml to approximately 10,000 eggs and gently mixed. After allowing approximately 2 minutes for the sperm to fertilise the eggs, they were rinsed and covered with water. The eggs absorb the water and become spherical in shape. This process is referred to as 'water hardening'.

Egg size: Mean egg size of individual fish was estimated by aligning a sample of the water hardened eggs along a 120mm measuring groove. The number of eggs aligned along the groove were counted to the nearest $^1/_2$ of an egg. Mean egg diameter was then calculated as follows:

$$\text{Mean egg diameter, OD (mm)} = \frac{120}{\text{No. eggs along groove}}$$

The accuracy of this method for calculating mean egg diameter has been validated by Springate (1985). There was no significant difference (Student's t-test) between the mean egg diameter calculated using the above method and those individual eggs measured directly with calipers.

Total fecundity: After measuring the egg diameter (OD) the volume of eggs produced from each fish was determined by carefully pouring the water hardened eggs into a graduated beaker and quantifying the volume to the nearest 10 ml. As the eggs from an individual fish were all the same diameter and were hexagonally close packed in a regular manner, it was possible to calculate the total fecundity from the total volume using the following equation:

$$Y = -0.283\,X + 5.41$$

$$\text{Total fecundity (TF)} = \text{Antilog } Y \left(\frac{Z}{100}\right)$$

where X = OD (mm)

Z = volume of eggs (ml)

$Y = \log_{10}$ No eggs litre$^{-1}$

This equation for calculating total fecundity was first derived by Von Bayer (1950) (In Leitritz and Lewis, 1976) and has been validated for use on rainbow trout eggs by Springate (1985). There was a highly significant correlation ($r^2 = 0.996$, $P \leq 0.001$) between actual counts of egg numbers and 'Von Bayer' determinations.

Relative fecundity: Relative fecundity was calculated by dividing total fecundity by the post-stripped weight of the individual fish:

$$\text{Relative fecundity (No eggs kg}^{-1}) = \frac{\text{Total fecundity}}{\text{Post-stripped weight of fish (kg)}}$$

## 2.3 Experimental Fish Holding Tanks

Experimental fish were maintained in a variety of different systems depending on the locations at which the experiments were performed and the particular experimental design. There follows a description of each system used with the type used in each experiment specified.

1200 litre photoperiod tanks (non-recirculatory): This type of system was used in all experiments described in Chapter 3 and Experiments 1, 2 and 3 in Chapter 4. The system consisted of a row of 6 identical tanks each rendered lightproof by black polythene sheeting secured over a wooden framework. Each tank was illuminated by a 40W bulb suspended 1.5m above the water surface providing a light intensity of 25 lux at the water surface. Photoperiod was controlled by mains driven electronic timers; in the case of 24 hr photocycles by a commercially available clock (Smiths Industries Ltd., London, U.K.) or by a specially constructed timer to control non-24 hr photocycles. The tanks, constructed of fibreglass were circular with a diameter of 1.6m and a water depth of 0.6m (Figure 2.1). The tanks were supplied with gravity

fed spring water (8.5-9.0°C) at the rate of approximately 50 litres $min^{-1}$ $tank^{-1}$. The water flowed out through the central drain carrying away any waste products. Each tank could maintain up to 35 kg of rainbow trout in a healthy condition. Each tank was covered with wire netting to prevent fish from jumping out.

1200 litre photoperiod tanks (recirculatory): This type of system was used in Experiment 4(a) in Chapter 4 and Experiment 1 and 2(b) in Chapter 5. The system consisted of two 1200 litre fibreglass tanks as described above, each with a fibreglass lid to render them lightproof. Each tank was illuminated by a 40W bulb suspended 0.5m above the water providing a light intensity of 30 lux at the surface. Photoperiod was controlled by mains driven electronic timers (Sangamo Weston Ltd., Enfield, U.K.). The tanks were supplied with water at a rate of 30 litres $min^{-1}$ from a header tank which then drained out via a central waste pipe, through a faecal trap and through a gravel filter bed (Figure 2.2). The water was purified by a combination of the filter action and the nitrifying bacteria inhabiting the surface of the gravel. The filtered water was then pumped (Pump model PV21, Beresford & Son Ltd., Birmingham, U.K.) back up to the header tank. The system included a ball-cock controlled top-up system which compensated for water losses due to leakage, spillage and evaporation. Water temperature varied between 11°C (winter) and 18°C (summer).

700 litre photoperiod tanks (recirculatory): This type of system was used in Experiment 2(a) in Chapter 5. The system consisted of three 700 litre fibreglass tanks rendered lightproof by black polythene sheeting secured over a wooden framework. Each tank was illuminated by a 3W fluorescent strip providing 30 lux at the water surface. The water recirculation and purification system was identical to that described above for the 1200 litre tanks and as illustrated in Figure 2.2. Water temperature was maintained at a constant 10°C by an electric powered chiller unit.

Oxyder tanks: This type of system was used in Experiment 4(b) in Chapter 4. An Oxyder is a commercially available (Field Stream and Covert Ltd., Meriden, U.K.) fibreglass fish tank. The tank is oblong shaped with rounded and sections and a centrally located screen (Figure 2.3). Spring water entered the tank at a rate of 90 litres $min^{-1}$ and was aerated and circulated by means of a purpose built air pump. Water depth was approximately 0.7m with excess water and waste products exiting via a drain pipe. Water temperature varied between 7°C (winter) and 15.3°C (summer). The tanks were sub-divided into 6 sections by fitting net screens to allow different experimental groups to be segregated.

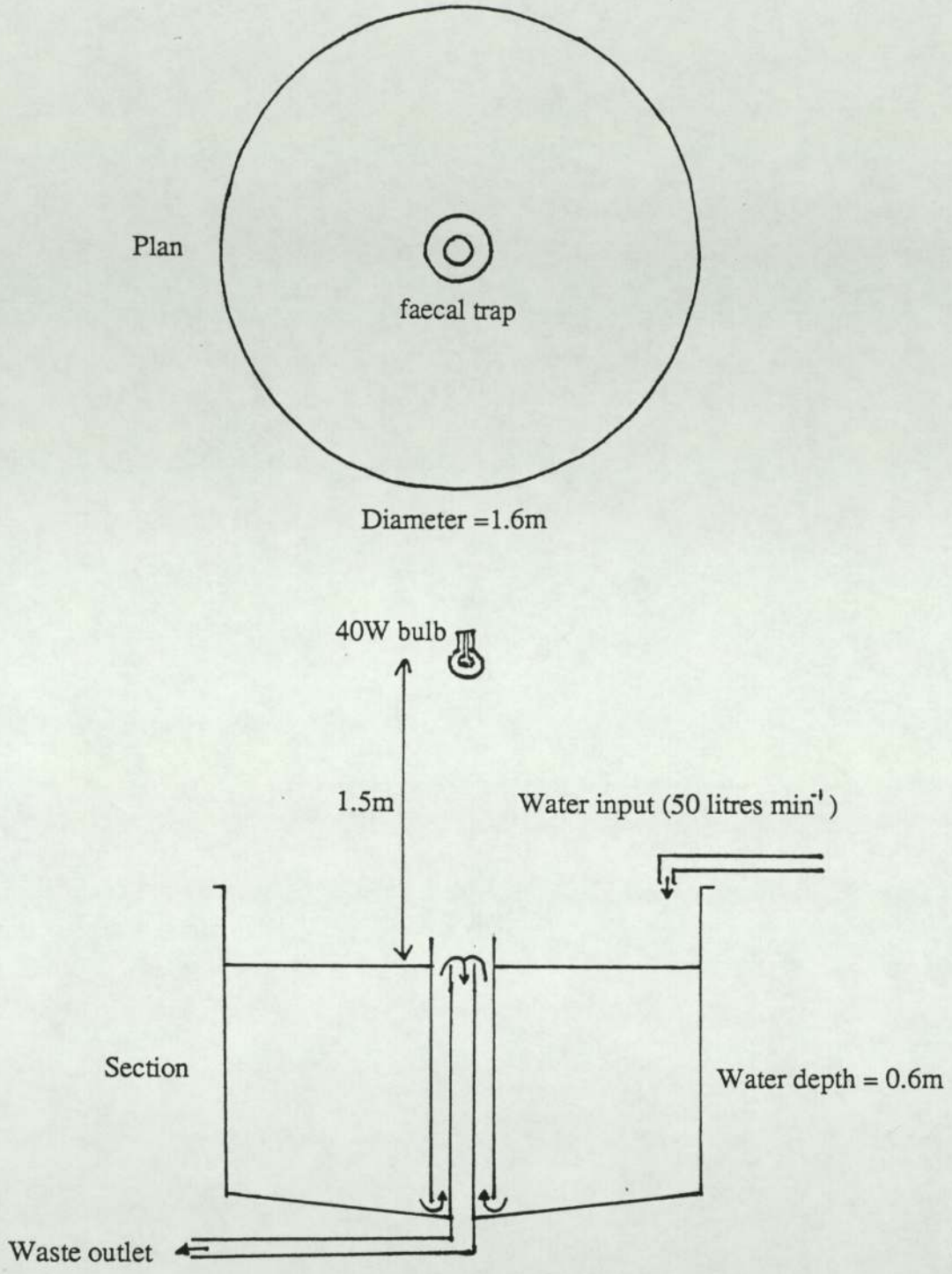



Fig 2.1 Diagram illustrating a 1200 litre capacity photoperiod tank with a non-recirculatory water supply.

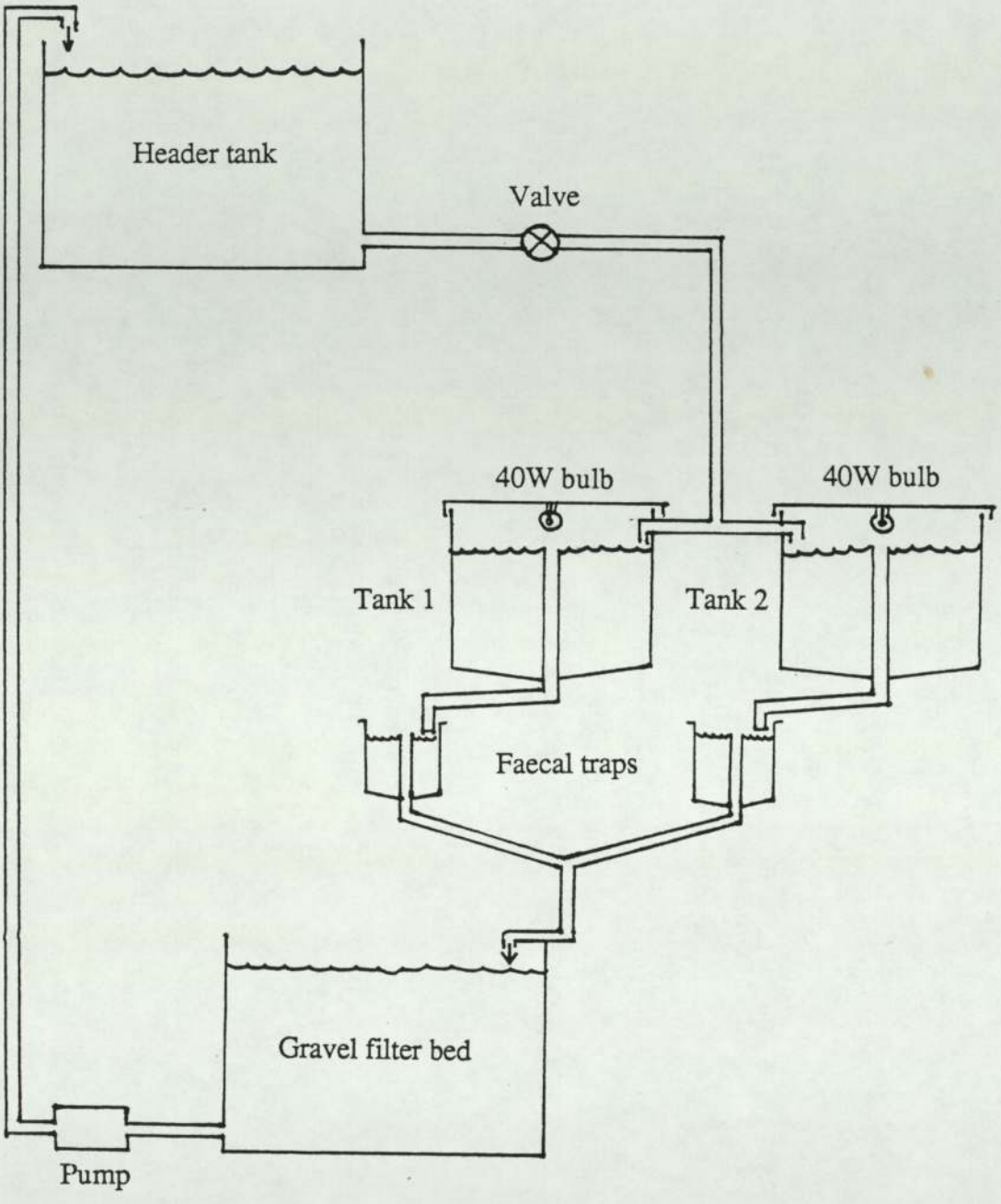



Fig 2.2 Diagram illustrating the basic construction of photoperiod tanks with a recirculatory water supply (not to scale).

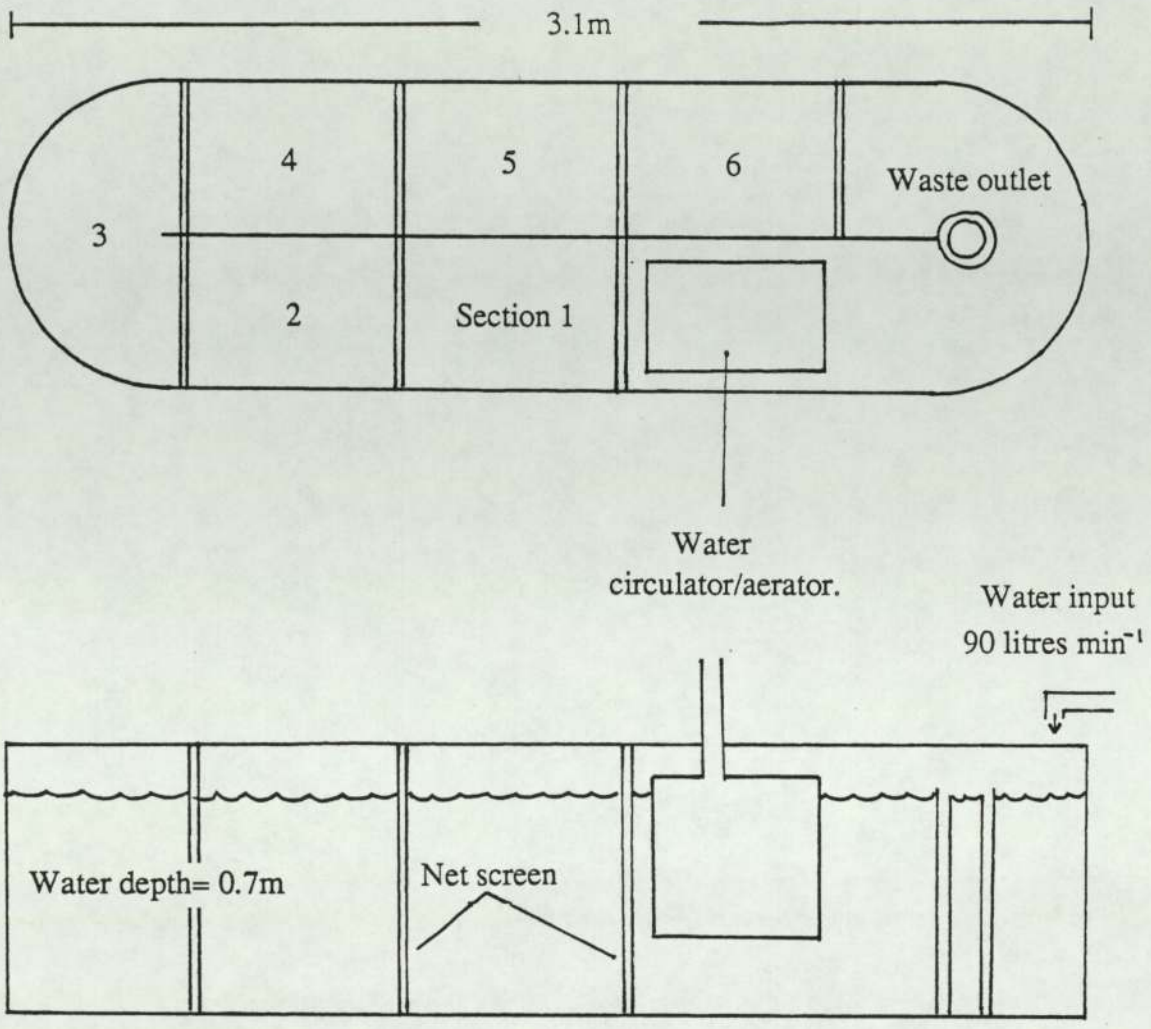



Fig. 2.3 Diagram illustrating an 'Oxyder' fish holding tank (not to scale).

2.4 Serum analysis

Prior to analysis serum samples were stored at -20°C. Storage at this temperature caused no significant changes with time in the measured levels of any of the serum parameters.

2.4.1 Assay for total serum calcium

Total serum calcium was measured using a Corning Calcium analyser Model 940 (Evans Electroselenium Ltd., Halstead, U.K.). The process involves a complexiметric titration and is based on the measurement of the fluorescence of a dye when associated with calcium ions. The dye, calcein, is a fluorescein derivative and forms an intensely fluorescent complex with calcium ions when in an alkaline medium. The analytical procedure incorporated in the Model 940 is based upon the quenching of this fluorescence by chelating the calcium ions with the titrant EGTA. The procedure was as follows:

1) Switch machine 'on' and allow to warm up for 15 minutes.
2) Fill cuvette with 1M KOH to appropriate level; check magnetic stirrer is working correctly.
3) Add 100 µl (10 µg) calcein dye to cuvette.
4) Add 100 µl (10µg) calcium standard to cuvette.
5) Press 'Titrate' button - which continues to flash.
6) Calibrate machine by repeatedly adding 20 µl aliquots of calcium standard and pressing 'titrate' button until the digital readout on successive samplings varies less than 4.0%.
7) Press 'calibrate'.
8) 20 µl serum samples are added successively and titrated automatically. Digital readout gives calcium concentration in milligrams per 100 ml serum (mg 100 $ml^{-1}$).

The inter- and intra- coefficient of variation were 1.3% and 1.27% respectively.

2.4.2 Radioimmunoassay for serum oestradiol-17ß

Serum oestradiol-17ß levels were quantified by means of a validated radioimmunoassay. The assay protocol was as follows:

1) Pipette 100 µl serum into polypropylene tube (Hughes and Hughes Ltd., Romford, U.K.).

2) Add 2 ml ethyl acetate (Analar).
3) Mix on rotary mixer for 1 hr.
4) Centrifuge at 1500 rpm for 10 minutes.
5) Transfer 100 µl aliquot into glass assay tube (in duplicate)
6) Prepare serial dilution of oestradiol-17ß standard in duplicate in similar assay tubes. Range = 1000 pg- 0 pg.
7) Dry down extracts and standards in vacuum oven at less than 35°C.
8) Cool tubes to 4°C.
9) Add 100 µl pH-buffered oestradiol-17ß antiserum to each tube.
10) Add 100 µl pH-buffered tritiated oestradiol-17ß label to each tube.
11) Vortex mix tubes for 10 seconds and incubate at 4°C overnight.
12) Add 500 µl dextran coated charcoal suspension (4°C) to each tube.
13) Vortex mix and incubate tubes for 10 minutes at 4°C.
14) Centrifuge for 10 minutes at 2000 rpm (4°C).
15) Transfer 400 µl supernatant from each tube into a scintillation vial.
16) Add 9ml scintillation fluid (Optiphase 'Safe', LKB Ltd., Croydon, U.K.).
17) Count each vial for 5 minutes in scintillation counter (Packard Tri-Carb 2660).
18) Calculate amount of hormone in each tube from the dose response curve.

Assay Details

Assay buffer:

| | |
|---|---|
| Sodium chloride | 4.5gms |
| Gelatine | 0.5gms |
| Disodium hydrogen phosphate | 8.88gms |
| Sodium dihydrogen phosphate | 5.82gms |

Made up to 500ml with distilled water. All buffer constituents supplied by BDH Chemicals Ltd.,(U.K.).

Dextran coated charcoal: One 'Separex' (Steranti Research Ltd., St Albans, U.K.) dextran coated charcoal tablet suspended in 50 ml assay buffer (4°C).

Label: (2,4,6,7-$^{3}$H)-oestradiol-17ß supplied by Amersham International Ltd., (Amersham U.K.). The label was stored at 10µCi $ml^{-1}$ ethanol. The working solution was prepared in RIA-buffer giving approximately 20,000 dpm per 100µl aliquot. This is the concentration recommended by Abraham (1974).

Antiserum: A highly specific antiserum (Steranti Research Ltd., St Albans, UK) was produced by injecting white rabbits with 17ß-oestradiol-6 (CMO)-BSA antigen. The specificity of the antiserum was checked against a number of steroids that are found in teleost serum (Table 2.1). The cross reactivity of each steroid is expressed as the diminution in the proportion of bound label produced by 100 pg of the steroid relative to that produced by 100 pg of oestradiol-17ß.

Extraction: Ethyl acetate extracted between 90-100% of the oestradiol-17ß from the serum and therefore a recovery step was not included in the routine assay procedure.

Sensitivity: The smallest amount of steroid statistically distinguishable from zero was 8pg per assay tube.

Accuracy: Known amounts of unlabelled oestradiol-17ß were added to serum aliquots, allowed to equilibrate overnight (4°C) and then assayed using the above procedure. There was a high degree of correlation between the added and detected amounts of oestradiol-17ß ($r^2 = 0.998$; $P \leq 0.001$) which indicated that the assay was very accurate.

Precision: The inter- and intraassay coefficients of variations for the oestradiol-17ß assay were 9.5 and 6.2% respectively.

2.4.3 Radioimmunoassay for serum testosterone

The basic procedure for this assay was identical to that described for the oestradiol-17ß assay except for the following steps:

Step

5) Transfer 50 µl aliquot into glass assay tube (in duplicate)
9) Add 100 µl pH-buffered testosterone antiserum to each tube
10) Add 100 µl pH-buffered tritiated testosterone label to each tube.

Only 50 µl of the extractant was assayed became of the comparatively high levels of testosterone in female rainbow trout blood.

Assay details

Label: (1,2,6,7)-$^3$H-testosterone (Amersham International Ltd)

Antiserum: Testosterone antiserum (Steranti) was raised by injecting rabbits with testosterone-6-(CMO)-BSA antigen. The cross reactivity was calculated by quantifying the diminution in the proportion of bound label by 100 pg of the steroid relative to that produced by 100 pg of testosterone (Table 2.1).

Extraction: Ethyl acetate extracted between 90-100% of testosterone from the serum and therefore a recovery step was not included in the routine assay procedure.

Sensitivity: The smallest amount of steroid statistically distinguishable from zero was 8pg per assay tube.

Accuracy: Known amounts of testosterone were added to serum aliquots, allowed to equilibrate overnight (4°C) and then assayed using the above procedure. There was a high degree of correlation between the added and detected amounts of testosterone ($r^2 = 0.988$, $P \leq 0.001$) which indicated that the assay was very accurate.

Precision: The inter- and intraassay coefficients of variation for the testosterone assay were 6.6 and 5.5% respectively.

| | Oestradiol-17ß antiserum . (%) | Testosterone antiserum (%) |
|---|---|---|
| Oestradiol -17ß | 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-20β-dihydroprogesterone | 1.0 | 1.66 |
| 17α-hydroxyprogesterone | 1.0 | 1.0 |
| Pregnenolone | 1.0 | 3.3 |
| Cortisol | 1.0 | 1.0 |

Table 2.1 Cross reactivity of oestradiol-17ß and testosterone antisera with a number of structurally similar steroids.

| | % |
|---|---|
| Melatonin | 100 |
| N-Acetyl tryptamine | 0.91 |
| 6-hydroxymelatonin | 0.33 |
| N-Acetyl tryptophan | 0.22 |
| N-Acetyl serotonin | < 0.06 |
| 6-hydroxymelatonin sulphate | < 0.06 |
| Tryptophol | < 0.06 |
| 5-Methoxy tryptophol | < 0.06 |
| 5-Hydroxy tryptamine | < 0.06 |
| 5-Hydroxy indole acetic acid | < 0.06 |
| 5-Methoxy tryptamine | < 0.06 |
| Tryptophan | < 0.06 |
| Tryptamine | < 0.06 |

Table 2.2 Cross reactivity of melatonin antiserum to structurally similar molecules (Data from Guildhay Antisera Ltd.).

2.4.4 Radioimmunoassay for serum melatonin

This assay was originally developed by Fraser *et al* (1983) for measuring melatonin in mammalian plasma. Great difficulties were encountered in getting the radioimmunoassay to work reliably in our laboratory and as a result it was not possible to properly validate the assay for measurement of melatonin in rainbow trout serum. However the antibody was highly specific (Table 2.2) and therefore the results from melatonin radioimmunoassays were included. The assay protocol was as follows:

1) Add 500 µl serum samples (in duplicate) to plastic assay tubes (LP3, Luckhams Ltd., Burgess Hill, U.K.).
2) Add melatonin standard (0-1000 pg, in duplicate) in 500 µl aliquots to plastic assay tubes.
3) Add 200 µl of antiserum to assay tubes, vortex mix and incubate at room temperature for 30 minutes.
4) Add 100 µl tritiated-melatonin to assay tubes, vortex mix and incubate overnight (4°C).
5) Add 500 µl dextran coated charcoal suspension (4°C) to each tube.
6) Vortex mix and incubate tubes at 4°C for 15 minutes.
7) Centrifuge for 10 minutes at 2000 rpm (4°C).
8) Transfer 1ml supernatant from each tube into a scintillation vial.
9) Add 9ml scintillation fluid (Optiphase 'Safe', LKB, Ltd., Croydon, U.K.).
10) Shake vials thoroughly.
11) Count each vial for 5 minutes in scintillation counter (Packard Tri-Carb 2660).
12) Calculate amount of hormone in each tube from the dose-response curve.

Assay details

It is essential that all glassware and plastic used in the assay are very clean and free from any form of contamination.

Assay buffer:

| | |
|---|---|
| Tricine (Sigma Chemicals, Poole, U.K.) | 8.96gms |
| Sodium chloride | 4.5gms |
| Gelatine | 0.5gms |

Made up to 500 ml with double distilled water.

Label: Tritiated melatonin (Acetyl-5-methoxytryptamine, N-[2-amino-methyl-2-$^{3}$H]) stock solution was supplied by New England Nuclear Ltd. (Boston, USA). A intermediate solution of $^{3}$H-melatonin was prepared by diluting 20 µl of this stock label to 2ml with ethanol. The working solution was freshly prepared before each assay by further diluting the intermediate solution with assay buffer to give approximately 8,000 cpm per tube.

Antiserum: A highly specific antiserum (Guildhay Antisera Ltd., Guildford, U.K.) was produced by injecting sheep with a melatonin antigen complex. The specificity of the antiserum was assessed by quantifying the alteration in the % binding of labelled melatonin when challenged with chemicals of a similar molecular structure (Table 2.2). Supplied in freeze-dried form the antiserum was reconstituted with 2ml of double distilled water to provide an intermediate solution of 1:10. This was aliquoted into 100 µl portions and stored at -20°C. The working solution was prepared by diluting one 100 µl portion to 20 mls in assay buffer. This provides sufficient reagent for 100 tubes with an initial dilution of 1:2000.

Melatonin standard: Standard melatonin (Sigma) 1mg ml$^{-1}$ was prepared by dissolving 10mg melatonin in 500 µl absolute ethanol and adjusting the volume to 10 ml with double distilled water. The standards are freshly prepared for each assay:

100 µl (1mg ml$^{-1}$) made up to 100 ml in double distilled water (Solution A)
500 µl (A) made up to 50 ml in double distilled water (Solution B)
125 µl (B) made up to 2.5ml in assay buffer (Solution C)

Further serial dilution of intermediate solution (C) with assay buffer provides standards of 0-1000pg melatonin per 500 µl aliquot. A curve prepared using assay buffer (as above) produced an identical standard curve to a curve prepared using melatonin-free serum and therefore was adopted in the routine assay procedure as it did not require the use of limited serum sources.

## 2.5 Statistical methods

The statistical techniques used in the analyses of results in this thesis are listed below. The methods are comprehensively described in Snedecor and Cochrane (1980).

2.5.1 Estimation of the mean

The arithmetic mean of a sample represents an estimate of the true population mean ($\mu$). Throughout this thesis an estimate of the population mean is written as the arithmetic mean $\pm$ one standard error of the mean ($\bar{x} \pm$ sem).

$$\text{Arithmetic mean} = \bar{x} \frac{\Sigma x}{n}$$

$$\text{Standard error of sample mean} = \sqrt{\frac{s^2}{n}}$$

where n = total number of sampling units

$s^2$ = variance of sample

$\Sigma x$ = sum of independent variables.

Coefficient of variation: This coefficient is used to compare the relative variability of samples. It is applied to the standard deviation(s) when it is expressed as a percentage of the sample mean.

$$\text{Coefficient of variation (C)} = s \left(\frac{100}{\bar{x}}\right)$$

2.5.2 Comparison of sample means

The means of two samples will rarely be the same, and several samples with different means may all be from the same population. The statistical tests listed below indicate whether a difference between sample means is significant, or whether the difference is covered by the error of the estimated population mean.

Comparison of two samples: The means of two samples were compared using Student's t-test where applicable. For this test to be applicable there must be no significant difference between the variances ($s^2$) of the two groups being compared. The equality of the variances is first tested by the F-test. If the calculated value for F is below the tabulated value at the 5% level (Pearson and Hartley, 1966) the variances are not significantly different and the means can be compared using Student's t-test. If the calculated value for t is greater than the tabulated value (Pearson and Hartley, 1966) at, for example the 5% level, then one can be 95% confident that the means of the two samples are derived from different populations.

If the F-test revealed that the variances of the two samples being compared were significantly different the Student's t-test was not applicable. When this occurred the means of the two samples were compared using an alternative test (see Bailey, 1959) which involved calculating d and treating it as being distributed like Student's t with f degrees of freedom, the latter being given by:

$$f = \frac{1}{\frac{\mu^2}{n_1 - 1} + \frac{(1 - \mu)^2}{n_2 - 1}}$$

$$\text{where } \mu = \frac{\frac{s_1^2}{n_1}}{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}$$

Comparison of more than two samples: One way analysis of variance (ANOVA) was used to compare the data from experiments where there were more than two samples. ANOVA calculations were carried out using a 'Minitab' statistical package (Ryan *et al*, 1981) on a Harris mainframe computer. The principles and methods of calculating an ANOVA table are fully described in Snedecor and Cochran (1980). Following the completion of the ANOVA table it was then possible to compare the difference in the mean values of the respective groups. Because many of the samples analysed were of unequal size (unequal n), the means were compared using the following equation:

$$\text{t value} = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{S^2 \left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

$$\text{degrees of freedom} = n_1 + n_2 - 2$$

where $n_1$, $n_2$ = number of observations in each sample.

$\bar{x}_1$, $\bar{x}_2$ = respective mean values

$S^2$ = residual mean square value from ANOVA table.

If the calculated value for t is greater than the tabulated value for t, at for example the 5% level (Pearson and Hartley, 1966) one can be 95% confident that the means of two respective samples being compared are derived from different populations.

# CHAPTER THREE

# ENDOGENOUS CIRCANNUAL AND CIRCADIAN TIMING MECHANISMS AND THE CONTROL OF MATURATION IN THE FEMALE RAINBOW TROUT

## 3.1 INTRODUCTION

The reproduction of salmonids is characteristically an annual event with gonadal development commencing in the spring and final maturation and ovulation occurring in the autumn and winter months. The phenomenon of annual breeding is an adaptation to the seasonal variation in the environmental conditions experienced in temperate regions of the world enabling plants and animals to produce offspring at a time of year when their chances of survival are greatest.

Investigations have established that the annual variation in daylength has an important role in synchronising the maturation cycle in salmonids (Hoover and Hubbard, 1937; Hazard and Eddy, 1951; Corson, 1955; Combs *et al*, 1959; Nomura, 1962; Henderson, 1963; Shiraishi and Fukuda, 1966; Whitehead *et al*, 1978; MacQuarrie *et al*, 1979; Bromage *et al*, 1982a,b; Takashima and Yamada, 1984). Figure 3.1 clearly shows that the timing of ovulation in female rainbow trout (*Salmo gairdneri*) can be significantly altered by subjecting fish to photocycles with a period other than 12 months. Although such experiments employing artificial light cycles have resulted in an understanding of how the timing of reproduction can be manipulated by particular changes in photoperiod, little is known of the mechanisms by which this animal measures daylength and hence registers time and season.

Similar to rainbow trout, many plants (Vince-Prue, 1975) and animals (Lofts, 1970) maintain synchronisation with periodic environmental changes by measuring the daylength (or night length). This is not surprising since compared to other geophysical variables the regular and systematic change in daylength offers the most reliable indicator of the phase of the seasonal cycle. A substantial amount of research on the effects and measurement of photoperiod has been performed on a wide range of organisms. These studies have resulted in the development of fundamental hypotheses on how daylength measurement is achieved. This chapter will first review these ideas and then go on to describe experiments designed to investigate the nature of the time measuring mechanism in the rainbow trout.

Some of the most important early research on photoperiodism was performed by plant physiologists. Garner and Allard (1920) studied the effects of daylength on the flowering of a variety of plants. These authors introduced the terms photoperiodism, photoperiod, short and long day plants and the notion of critical photoperiod. They found that certain plants only flowered when daylength exceeded a certain critical value, while other species were 'short day' plants only flowering when the photoperiod was shorter than a particular value. Research on

the effects of photoperiod on vertebrates began with Rowan (1926) who worked on snowbirds (*Junco hyemalis*). He showed that artificial lengthening of the photoperiod caused maturation and a tendency to migrate northwards even at the coldest part of the year, whereas shortening of the photoperiod led to gonadal regression and a southerly movement. The effects of different lengths of photoperiod on a wide range of physiological processes have since been described in a wide range of plants and animals. Whether long or short day species, many show a well defined critical photoperiod which separates two opposite physiological pathways, for example, flowering versus vegetative growth, gonadal growth versus regression (Figure 3.2). The critical daylength not only varies with species (Figure 3.2) but also within species; the length of the critical photoperiod being adapted to the latitude at which a particular organism resides. This adaptation ensures that the appropriate metabolic processes occur at the correct time of the year. For example, the critical daylength for diapause induction in the knot grass moth (*Acronycta rumicis*) was found to be around 14.5 hrs at a latitude of 43$^{o}$N whereas populations from 60$^{o}$N showed a critical photoperiod of 19.5 hrs (Danilevskii, 1965). Whatever the position of the critical photoperiod it is important to note that the steepness of the response curve around the threshold daylength indicates that the organisms are capable of precise time measurement.

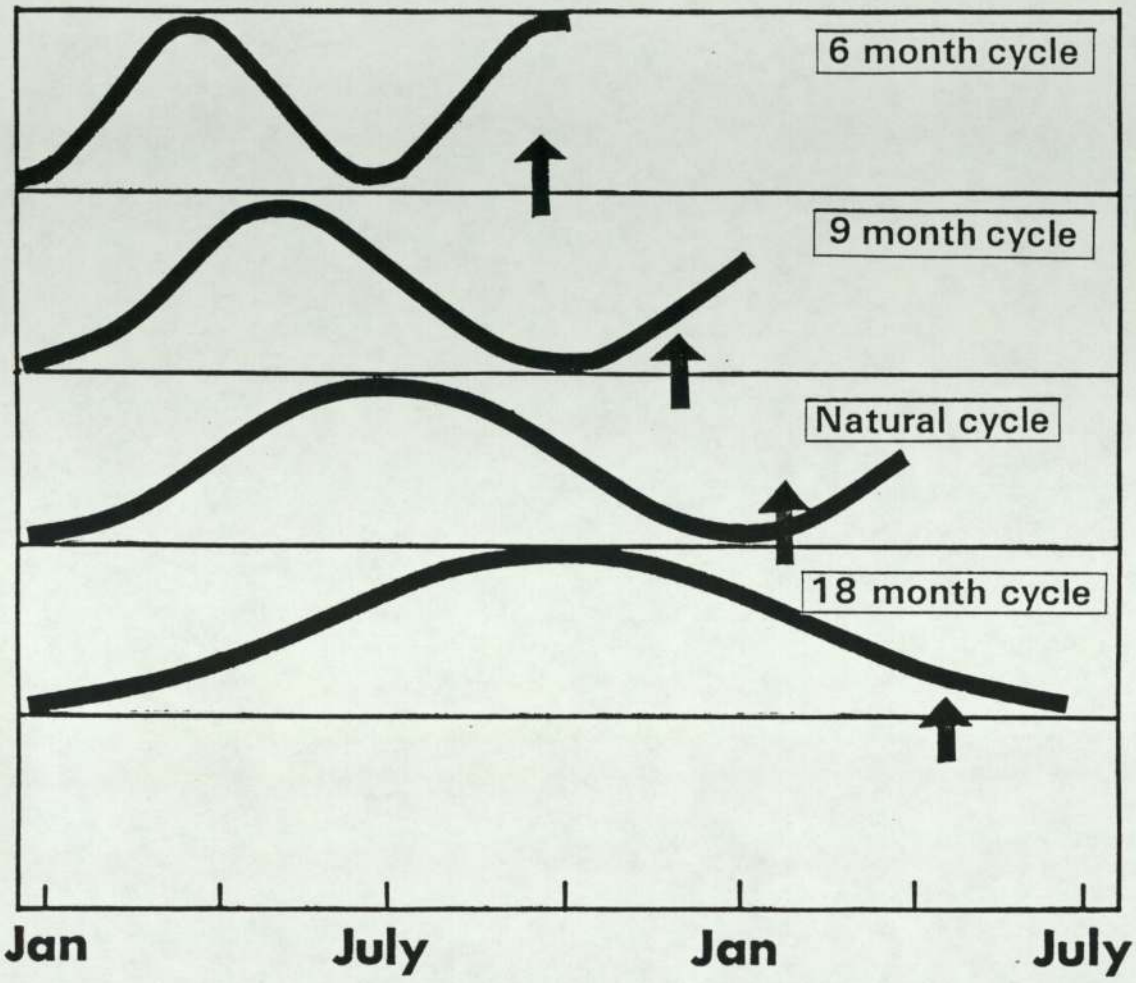



Fig. 3.1 Effect of photoperiod cycles with periodicities of 6, 9, 12 and 18 months respectively on the timing of spawning ( ⬆ ) of female rainbow trout. The vertical lines on the x-axis refer to the first day of the month as labell ed. ( From Bromage and Duston, in press).

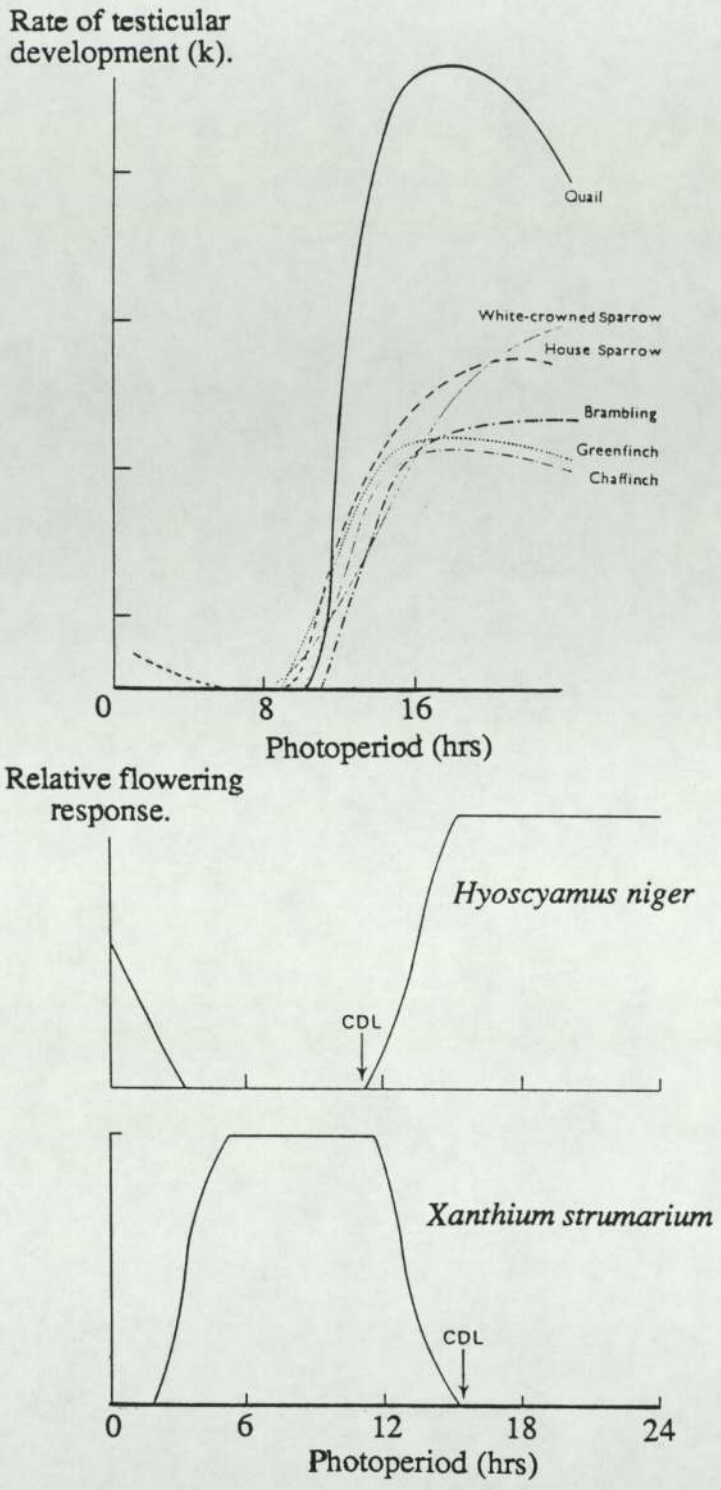



Fig. 3.2 Examples illustrating the phenomenon of critical daylength which separates opposite physiological responses. Top: rate of testicular development (k, arbitary units) as a function of the daily photoperiod in six different species of birds.( From Lofts *et al* , 1970). Bottom: long day plant *Hyoscyamus niger* flowers on photoperiods longer than the critical daylength (CDL), whereas short day plant *Xanthium strumarium* flowers on photoperiods shorter than the critical daylength. In both species this relationship does not apply to very short photoperiods. ( From Vince-Prue, 1975).

Before proceeding further it should be stated that despite being the subject of considerable research the physiology of biological time measuring mechanisms remains to be discovered. Perhaps because of this, research on biological 'clocks' often treats the organism as a 'black box', varying the input (the light-dark regime) on the one hand and noting the output (the photoperiodic response) on the other. From this type of research, two fundamentally different hypotheses have been developed to explain how time measurement is achieved:

(i) Hour glass model: this model proposes that photoperiodic effects result from a light (or dark) dependent reaction that produces an essential compound whose accumulation, duration of physiological activity or release from a site of production is a function of the daily photoperiod. Such a linear system of time measurement appears to be employed by only a few insects (Lees, 1973; Saunders, 1981) and a lizard (Underwood, 1981).

(ii) Endogenous circadian time measuring models: this basic hypothesis has been developed into two separate models, (a) external and (b) internal coincidence.

The external coincidence model was originally formulated by Erwin Bunning (1936, 1960) and extended by Pittendrigh and Minis (1964). He suggested that organisms possess an internal or endogenous rhythm consisting of two half cycles each of approximately 12 hrs in duration, one of them 'light requiring' (photophil) and the second 'dark requiring' (scotophil). Photoperiodic induction by long days is effected when day light extends into the scotophil part of the endogenous cycle; it fails when it is restricted to photophil. In this model light has the dual role of entraining the endogenous rhythm and also acting as the inducer of the photoperiodic response.

The internal coincidence model was developed independently by Pittendrigh (1960, 1972, 1974) and Tyschenko (1966). Similar to the external coincidence model this hypothesis involves endogenous circadian oscillators. However, it is proposed that in this system light only serves as an entraining agent to the endogenous rhythms and has no direct role in the induction process. Recognition of a specific photoperiod depends on the phase relationship between at least two internal oscillators coupled separately to sunrise and sunset.

Before describing these hypotheses in any more detail it is necessary to introduce the phenomena of endogenous rhythmicity which underpin both the internal and external coincidence models.

### 3.1.1 Endogenous Rhythms

Daily and annual changes in environmental variables such as light and temperature have accompanied evolution throughout its course. Inevitably, 24 hr rhythmicity has become an almost ubiquitous characteristic of life. In addition, organisms with a lifespan longer than one year have adapted to the annual variations in the environment. Yet another class of rhythms are exhibited by creatures inhabiting coastal habitats which have adapted their lifestyles to the rhythms of the tides (Saunders, 1977). That such cycles could persist with no less vigour in the laboratory under constant environmental conditions was one of the most significant discoveries in the study of rhythms in plants and animals. The first documented recording of this phenomenon was made by Jean Jacques de Mairan in 1729 who observed that the daily periodic leaf movements of *Mimosa* plants persisted when kept in constant darkness. Kleinhoonte (1929) noted that leaf movements of *Canavalia* plants which had a periodicity of 24 hrs under normal light-dark conditions oscillated spontaneously with a period of about 25 hrs under conditions of constant darkness. Endogenous rhythms with such a 'free running' period of approximately 24 hrs have been recorded in a whole host of plants and animals maintained under either constant dark or constant light and appear to be innate in all organisms except bacteria and blue green algae (Brady, 1978). Halberg (1959) proposed that such rhythms should be described as 'circadian'. Circadian rhythms (examples: Figure 3.3) exhibit four important properties:

(a) The difference between the unique 24 hr periodicity of earthly environmental phenomena and the non-24 hr periodicity of circadian rhythms in constant conditions indicates that such rhythms are driven from within the organism by some kind of self-sustained oscillator that times the system. Indeed, the 'free-running' of any biological rhythm under constant conditions is generally accepted as proof of its endogeneity.

(b) Although the free-running period of circadian rhythms is not exactly 24 hrs, the day to day variability in the period of the rhythm is very small. For example, in constant darkness the free running period of activity in flying squirrels (*Glaucomys volans*) only varied 0.1-1.0% of their mean period length (DeCoursey, 1961).

(c) Under natural light-dark cycles circadian rhythms are entrained to a period of exactly 24 hrs ensuring that endogenous rhythms remain synchronised with local time. Environmental synchronisers of endogenous rhythms are often referred to as 'zeitgebers'. The entrainment of circadian oscillators is covered in detail in Chapter 4.

(d) Endogenous circadian rhythms are temperature compensated. Most physiological processes have a $Q_{10}$ between 2-3 (i.e. for a 10°C rise in temperature the rate of the physiological process increases 2-3 fold) but remarkably the mechanism underlying circadian rhythms have a $Q_{10}$ very close to 1 (Sweeney and Hastings, 1960). It was Pittendrigh (1954) who recognised that this phenomenon was essential if circadian rhythms were to be involved in accurate time measurement; an oscillator which ran faster as temperature rose would be almost useless as a clock.

Perhaps because of their ubiquity circadian rhythms have received much more attention than other endogenously driven cycles. However in a variety of organisms both tidal and annual entrained cycles in behaviour and physiology have been shown to exhibit circatidal (Enright, 1963) and circannual rhythms respectively when maintained under constant conditions (Gwinner, 1981a; Farner, 1985). Circannual rhythms are of particular relevance to the present study because they are involved in the timing of annual cycles and may be important in the control of the annual reproduction of the rainbow trout. The existence of endogenous circannual rhythms was first suggested in the studies on the breeding cycles of birds in equatorial regions where photoperiod and climate are almost constant all year round (Moreau, 1931; Chapin, 1932; Baker, 1938). They were first rigorously demonstrated by Pengelley and Fisher (1957, 1963) who recorded circannual rhythms of hibernation in golden mantled ground squirrels (*Citellus lateralis*). Some of the most impressive examples of circannual rhythms are those recorded from work on birds. For example Berthold (1978) followed the circannual rhythm of plumage moults in a blackcap (*Sylvia atricapilla*) and a garden warbler (*S. borin*) for over 8 years. Circannual rhythms have been demonstrated in other species of animals including a beetle (*Anthnenus verbasci*; Blake, 1959), a slug (*Limax flavus*; Segal, 1960), a lizard (*Cnemidophorous uniparens*, Cuellar, 1981) and a variety of birds and mammals (Gwinner, 1981a; Farner, 1985). Notably Sundararaj *et al* (1982) indicated a circannual rhythm in gonadal development in the catfish (*Heteropneustes fossilis*).

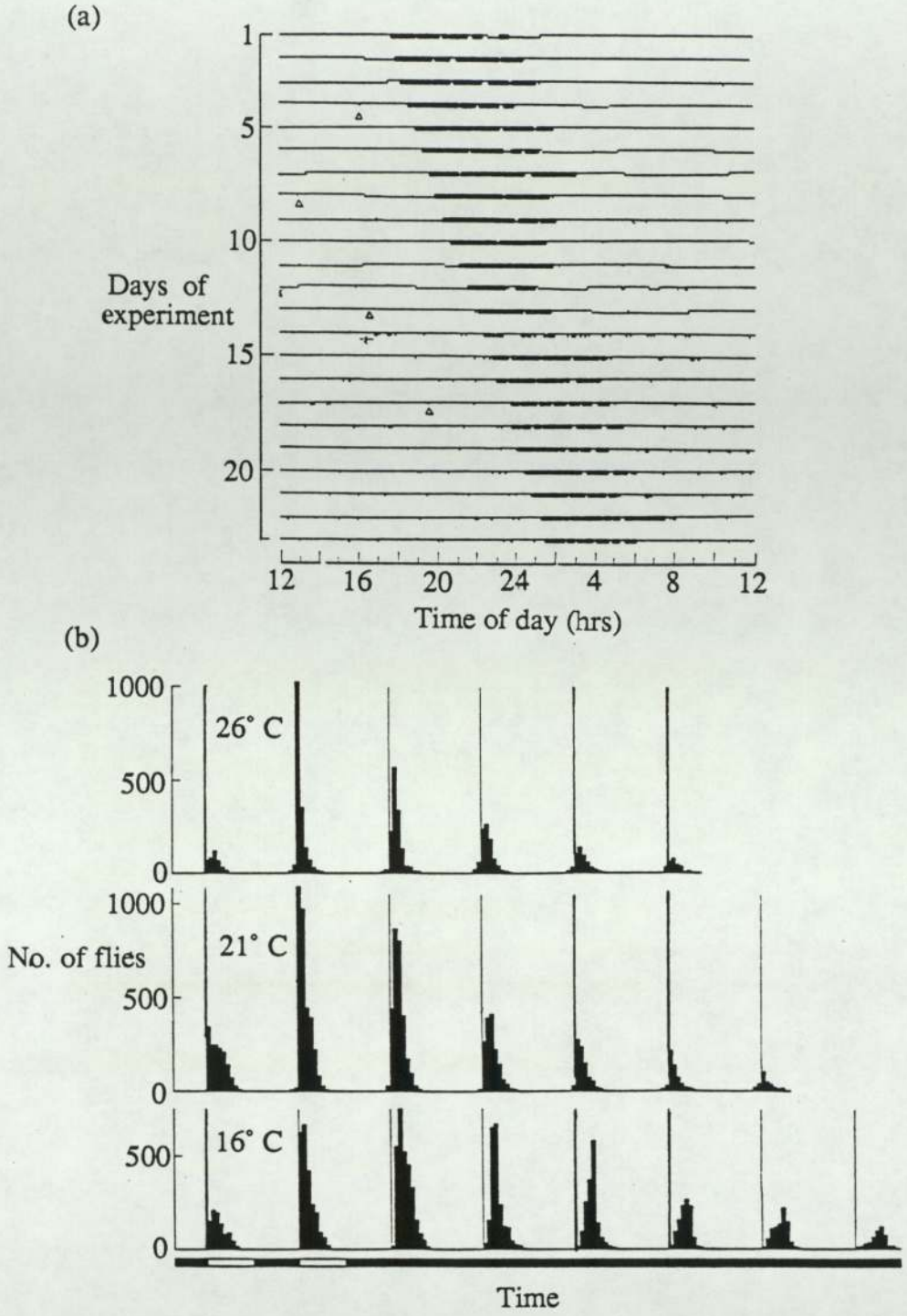



Fig. 3.3 Two examples of circadian rhythmicity. (a): Free-running activity rhythm of a flying squirrel ( *Glaucomys volans* ) maintained in constant darkness(DD). The thicker bands indicate periods of activity. Note the constant periodicity of the rhythm of 24hrs 21mins ± 6mins. (From DeCoursey, 1961). (b): The circadian rhythm of pupal eclosion in a population of *Drosophila pseudoobscura* transferred from a light-dark cycle into constant darkness(DD). Vertical lines are 24hr apart. Note that the periodicity of the rhythm is broadly unaffected by temperature, indicating that the timing mechanism is temperature compensated. (From Pittendrigh, 1954 ).

Circannual rhythms are analogous to circadian rhythms in some respects in that they free-run under constant conditions they are normally entrained by environmental zeitgebers, particularly the annual cycle in daylength, and there is some evidence that they are temperature compensated (Gwinner, 1981a). However, one significant difference is that where circadian rhythms are only exhibited under constant light (LL) or dark (DD), circannual rhythms may be expressed under constant photoperiods such as 12L:12D (Pengelley and Fisher, 1963) and 10L:14D (Berthold, 1978) in addition to DD and LL (Pengelley *et al*, 1976). There are examples of species which will exhibit circannual rhythms under certain photoperiods but not others. The sika deer (*Cervus nippon*) shows a well defined circannual rhythm of antler growth under LL, 16L:8D or 8L:16D but the rhythm disappears under a 12L:12D photoperiod (Goss, 1969b). On the other hand the European starling (*Sturnus vulgaris*) will exhibit a circannual rhythm of gonadal development when kept under 12L:12D but not when the photoperiod is less than 11L:13D or more than 13L:11D (Schwab, 1971). This behaviour remains to be explained and has been quoted as evidence to suggest that circannual rhythms may not be true oscillators in the same sense as circadian rhythms (Hamner, 1971; Mrosovsky, 1978).

Whatever the mechanisms of circannual rhythms it is apparent that they have an important role in controlling the seasonal cycles in certain species. Their existence brings into question the role of photoperiod in the control of the reproduction cycle of the rainbow trout; photoperiod may drive the reproduction cycle or it may serve as an entraining zeitgeber to a circannual rhythm of maturation. In addition to circannual rhythms it is also evident that circadian or hour-glass mechanisms are involved in measuring the duration of the photoperiod in some species and hence could also be involved in control of reproduction in the rainbow trout. Thus, the experiments described in this chapter investigate the following two questions:

(i) Are circadian or hour-glass mechanisms involved in the control of rainbow trout reproduction?

(ii) Under constant environmental conditions do rainbow trout exhibit a circannual rhythm of reproduction?

In an attempt to answer these questions a series of experimental procedures were used for which the underlying rationale are described below:

(i) Are circadian or hour-glass mechanisms involved in the measurement of daylength?

Earlier in this chapter reference was made to the three alternative hypotheses for daylength measurement namely the hour-glass and the external and internal coincidence models. Reiterating Bunning's external coincidence model, he envisaged that daylength measurement was achieved by light interacting with an endogenous circadian rhythm. In its most explicit form Bunning's hypothesis is summarised by Figure 3.4 which is taken from his 1960 paper. This model was originally developed to explain how certain flowering plants responded to photoperiod. 'Long day' plants only flowered when the photoperiod extended into the dark requiring or scotophil part of the circadian rhythm. On the other hand in 'short day' plants induction fails when the photoperiod extends into the scotophil and succeeds when it is restricted to the photophil.

It is clear that this hypothesis cannot be tested simply by varying the length of the photoperiod in a 24 hr light-dark cycle; photoperiodic induction occurring when a particular critical photoperiod is exceeded could equally well be explained by the organism possessing an hour-glass mechanism. A class of experiments which have been particularly valuable in distinguishing between hour-glass and circadian based timing mechanisms are those which have relied on 'night interruptions' with short light pulses (Bunning and Joerrens, 1960). These simulate the action of a complete photoperiod and are often referred to as skeleton photoperiods. Figure 3.5 illustrates the effect of a skeleton regime consisting of a main 6 hr photoperiod plus a 15 minute pulse of light at various times during the dark period on the gonadal development of the Japanese quail (*Coturnix coturnix*; Follett and Sharp, 1969). The total amount of light in each treatment was well below the critical photoperiod of ~ 12 hrs emphasising that it was the distribution of the photoperiod that was important. In the treatments where the light pulse coincided with the scotophil or photo-inducible phase, gonadal development resulted. Bunning originally suggested that the photo-inducible phase had a duration of approximately 12 hrs, however Pittendrigh and Minis (1964) emphasised that this phase may not last for a full 12 hrs and in this example it is clear that the photosensitive phase has only a short duration between 12-16 hrs after the start of the main photoperiod.

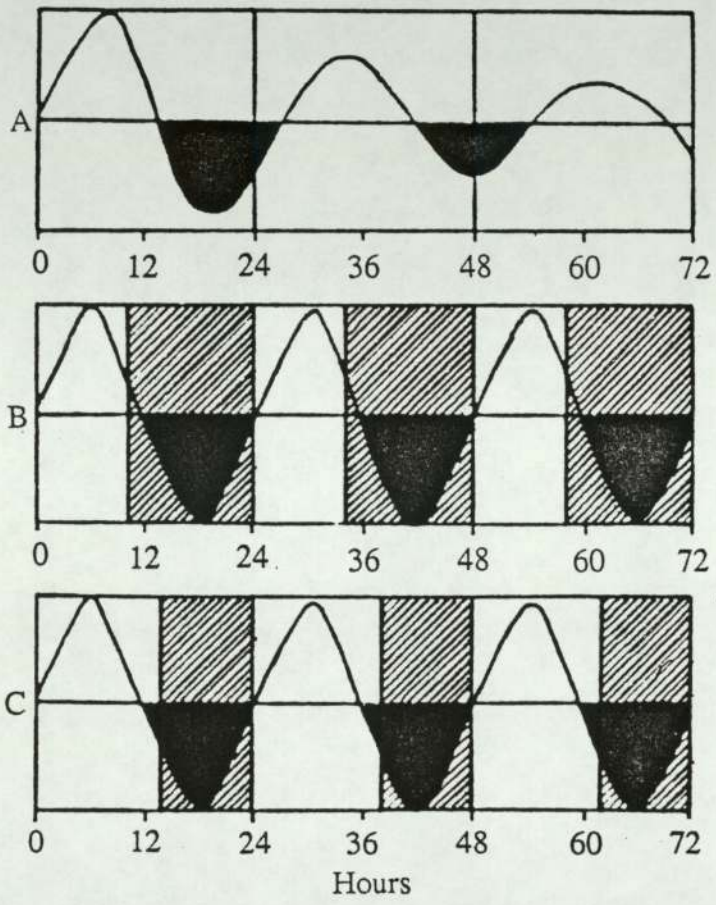



Fig. 3.4 Bunning's model for the execution of the photoperiodic time measurement by a circadian oscillation. The clock causes the alternation of half cycles with qualitatively different light sensitivity ( white versus black ). A: The free-running clock in continuous light (LL) or continuous darkness (DD). B: Short photoperiod. C: Long photoperiod. Under the long photoperiod conditions light illuminates the 'black' half cycle. (From Bunning, 1960).

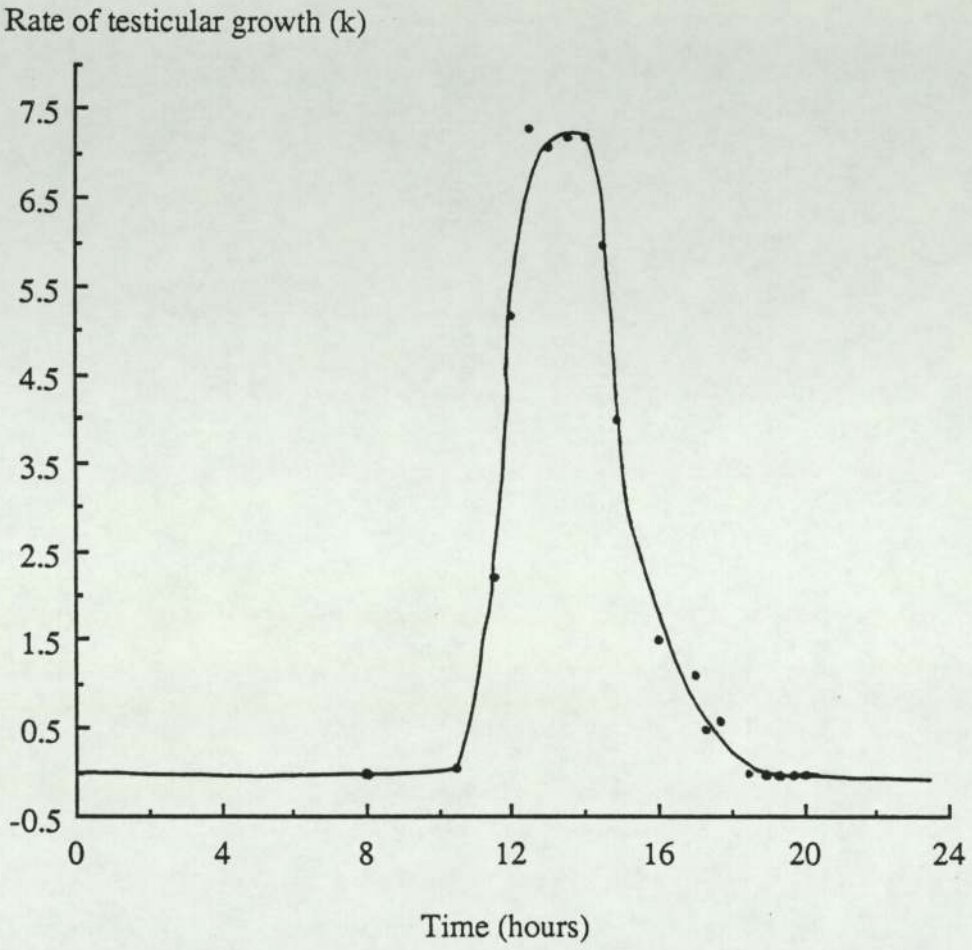



Fig. 3.5 The effect of skeleton photoperiod regimes on the rate of gonadal development (k, arbitary units) in the Japanese quail (*Coturnix coturnix* ). Each coordinate represents the mean value of k for a schedule consisting of 6L:18D plus a 15 minute light pulse given at the times indicated on the x-axis. (From Follett and Sharp, 1969).

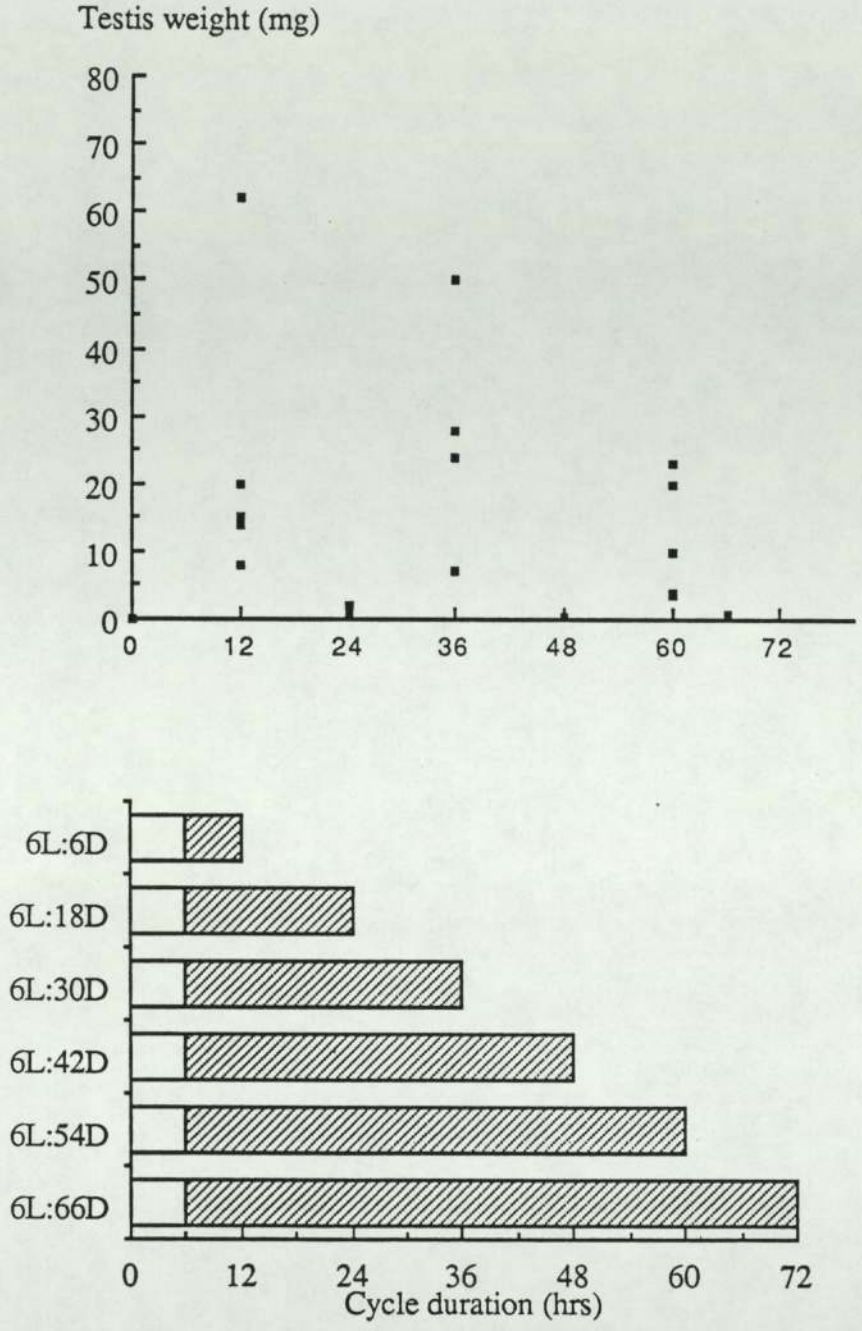



Fig. 3.6 The effect of resonance photoperiod regimes (6L:6D, 6L:18D, 6L:30D, 6L:42D, 6L:54D and 6L:66D) on gonadal development in the house finch (*Carpodacus mexicanus* ). Note that the gonadal growth under 12, 36 and 60 hour cycles is generally greater than the other schedules. (Drawn from Hamner, 1963).

Without going into further details at this stage, it is clear that a positive result from this type of experimental procedure supports the external coincidence model. However, used in isolation skeleton photoperiods do not prove the involvement of a circadian rhythm in photoperiodism. For example it could still be argued that the light period somehow embodied a rate limiting process (if the hour-glass principle be adopted) with a carry over effect which was removed by the provision of a pulse of light at night (Kirkpatrick and Leopold, 1952). It might also be claimed that the short light pulse was interacting somehow with the preceding or succeeding main light period. Only by employing experimental photoperiods with cycles longer than 24 hrs can the question of an hour-glass or circadian timing mechanism be resolved. Resonance experiments where a non-inductive short photoperiod is coupled in different experimental groups with different lengths of darkness were first introduced by K.C. Hamner on plants (Nanda and Hamner, 1958) and W.M. Hamner (1963) on vertebrates. It is envisaged that a circadian rhythm free-runs throughout the dark period and the light sensitive phase may be engaged or illuminated if light pulses are given at appropriate times of the endogenous cycle; the phase relationship between the light pulse and the free running oscillation is critical to the inductive mechanism. As an example Figure 3.6 summarises the response of the house finch (*Carpodacus mexicanus*) kept under such a resonance regime (Hamner, 1963). Gonadal growth under 12, 36 and 60 hour cycles is significantly greater than other schedules. In agreement with the external coincidence theory, maximal gonadal growth only occurs where the recurrent photoperiods coincide with the photosensitive scotophase. There is no way that this kind of result can be explained in terms of an hour-glass mechanism.

It is clear that skeleton and resonance photoperiod regimes can be used to indicate whether an hour-glass or circadian based system is involved in daylength measurement and can be used to test the external coincidence model. It was thus pertinent to include both experimental procedures in the present study on daylength measurement in rainbow trout.

The third hypothesis to explain how organisms measure daylength is the internal coincidence model (Pittendrigh, 1960, 1972, 1974; Tyschenko, 1966). This system relies on two circadian oscillators, each of a circadian periodicity, one entrained to the dawn transition in the photoperiod, the other to dusk. As the length of the photoperiod changes the phase relationship of the two oscillators also changes and induction occurs when the 'active' phase points of the endogenous rhythms coincide. Support for a model of this type comes primarily from observations that the circadian systems in a variety of organisms appear to consist

of a population of oscillators which separate or split when maintained under constant conditions (Pittendrigh and Daan, 1976). As yet there is no specific test for this hypothesis of daylength measurement but results from particular photoperiod and other experimental procedures can be better explained by the internal rather than the external coincidence model (Meier *et al*, 1971; Meier and Wilson, 1985). A notable piece of evidence in support of an internal coincidence system was produced by Saunders (1973). Where internal coincidence does underlie the photoperiodic induction in poikilotherms light should be entirely dispensable in time measurement; it should be possible to create the necessary phase relations between internal oscillators by a changing 'thermoperiod'. Saunders tested this hypothesis on the wasp *Nasonia vitripennis* and indeed found that diapause could be controlled as effectively with thermoperiod as photoperiod. This result clearly rules out any model for a clock which includes an inductive function for the environmental light cycle. Thus, in the interpretation of the results from the present study on daylength measurement in rainbow trout, consideration will be given to both the internal and external coincidence models in addition to the hour-glass hypothesis.

(ii) Under constant environmental conditions do rainbow trout exhibit a circannual rhythm of maturation?

As described earlier, a variety of organisms have been observed to exhibit a circannual rhythm of maturation when maintained under constant environmental conditions. To be accepted as being truly endogenous, such a rhythm should be observed to free run for at least two full cycles to indicate that it is self-sustaining, and should exhibit temperature compensation (Gwinner, 1981a). In the present study groups of female rainbow trout were maintained under conditions of constant photoperiod and temperature for at least three spawning cycles. Because some animals exhibit circannual rhythms under certain constant photoperiods but not others (Goss, 1969b; Schwab, 1971) it was decided to adopt three regimes. One group of fish were maintained on constant light (LL), one group on 18L:6D and the other on 6L:18D. Water temperature was constant.

In order to quantify the effects of the photoperiod treatments on the timing of maturation, fish were checked at approximately monthly intervals to assess whether they had ovulated. In addition, in the circannual rhythm experiments the maturation cycles were followed by blood sampling the fish at monthly intervals and subsequently assaying the serum for oestradiol-17ß, testosterone and total calcium. The role of these three parameters in the control of maturation has been

described in Chapter 1.

## 3.2 MATERIALS AND METHODS

Groups of between 20 and 40 one or two year old female rainbow trout, *Salmo gairdneri*, with a natural spawning time in December (Lat 52° 30 N) were maintained in 1.6m diameter light-proof tanks, each illuminated with a 40W bulb providing 25 Lux at the water surface. daylength was controlled by either conventional 24 hr time clocks or electronic devices accurate to within 1 s of light $day^{-1}$. Three experimental series were conducted and each began in February. The procedures were as follows:

### (i) Skeleton photoperiods

Over a 2 year period, groups of 1 year-old fish were exposed in separate tanks to one of the following photoperiod regimes: 6L:18D, 6L:42D, 6L:4D:2L:12D, 6L:6D:2L:10D or 6L:8D:2L:8D.

### (ii) Resonance procedures

Over a 2 year period, groups of 2 year-old fish were exposed in separate tanks to one of the following photoperiod regimes: 6L: 18D, 18L:6D, 6L:48D or 6L:54D. The total number of hours of light and dark and the number of light-dark cycles offered during the year by each of these regimes and the natural cycle are compared in Table 3.1.

### (iii) Constant light regimes

Over a 2-4 year period, 3 groups of 2 year-old fish, maintained in separate tanks, were subjected to short days (6L:18D), continuous light (LL) or long days (18L:6D). For each tank of fish the reproductive cycles were followed for at least three successive spawning periods.

Under each of the three series of experiments appropriate controls were maintained under ambient light conditions. Water temperature was a constant 8.5-9°C and fish were fed throughout at 0.5% body weight $day^{-1}$ with a commercial trout diet. The fish under the resonance procedures received equivalent amounts of food but this was only given while the lights were on. Small numbers of male fish were also stocked in each tank to provide sperm for fertilization. All fish were identified with small numbered plastic discs attached through the dorsal fin. At approximately monthly intervals a number of female fish from each tank were anaesthetised with 2-phenoxyethanol (1:2000) and blood

samples taken from the Cuverian sinus for measurement of serum levels of testosterone, oestradiol-17ß and calcium (as an index of vitellogenin) using methods already described (see Chapter 2 for details). Sampling was always performed 3-4 hours after 'lights on', LL treatment excepted. As the time of expected spawning approached fish were examined more frequently for the presence of ripe eggs.

| Photoperiod regime | No. light-dark cycles | Hr.light $year^{-1}$ | Hr.dark $year^{-1}$ |
|---|---|---|---|
| Natural cycle | 365 | 4380 | 4380 |
| 18L:6D(long day) | 365 | 6570 | 2190 |
| 6L:18D(short day) | 365 | 2190 | 6570 |
| 6L:42D | 183 | 1095 | 7665 |
| 6L:48D | 162 | 973 | 7787 |
| 6L:54D | 146 | 876 | 7884 |

Table 3.1 . Total numbers of light-dark cycles and the daylight hours under various photoperiod regimes.

## 3.3 RESULTS

### (i) Skeleton photoperiods

None of the 1+ year old fish in this experiment spawned at the end of the first year of treatment. Subsequently, all the fish matured during the next cycle (Figure 3.7). The spawning of the fish subjected to 6L:6D:2L:10D began in December with a mean time for this experimental group significantly in advance of the fish under 6L:18D ($P \leq 0.025$, Student's t-test), 6L:4D:2L:12D ($P \leq 0.05$) and 6L:8D:2L:8D ($P \leq 0.001$). The 6L:8D:2L:8D fish were also delayed in comparison with the 6L:4D:2L:12D treatment ($P \leq 0.01$).

### (ii) Resonance procedure

During the first treatment cycle the fish under 6L:42D , 6L:48D and 6L:54D commenced spawning in late October/early November, significantly in advance of the fish under 6L:18D ($P \leq 0.001$) where spawning began in December and peaked between mid-January and early February (Figure 3.8). The fish under 6L:48D and 6L:54D began spawning at the same time as a parallel group under 18L:6D. The second spawning of the fish, maintained under 6L:18D for a year, occurred almost exactly one year later again with a median time of early February. The 6L:54D regime produced a second spawning peak in mid-October approximately 10 months after the first. The 6L:48D photoperiod produced an erratic spawning period which extended from mid-August until the following January. Unfortunately, persistent malfunction with the 6L:42D electronic timer caused this treatment to be prematurely terminated in mid-summer of the second year. When the 6L:42D and 6L:18D treatments were repeated, fish spawned between mid-December and mid-January, and March respectively; a difference which was statistically significant ($P \leq 0.001$). Subsequently the fish maintained under 6L:42D spawned again almost exactly 12 months later (Figure 3.9).

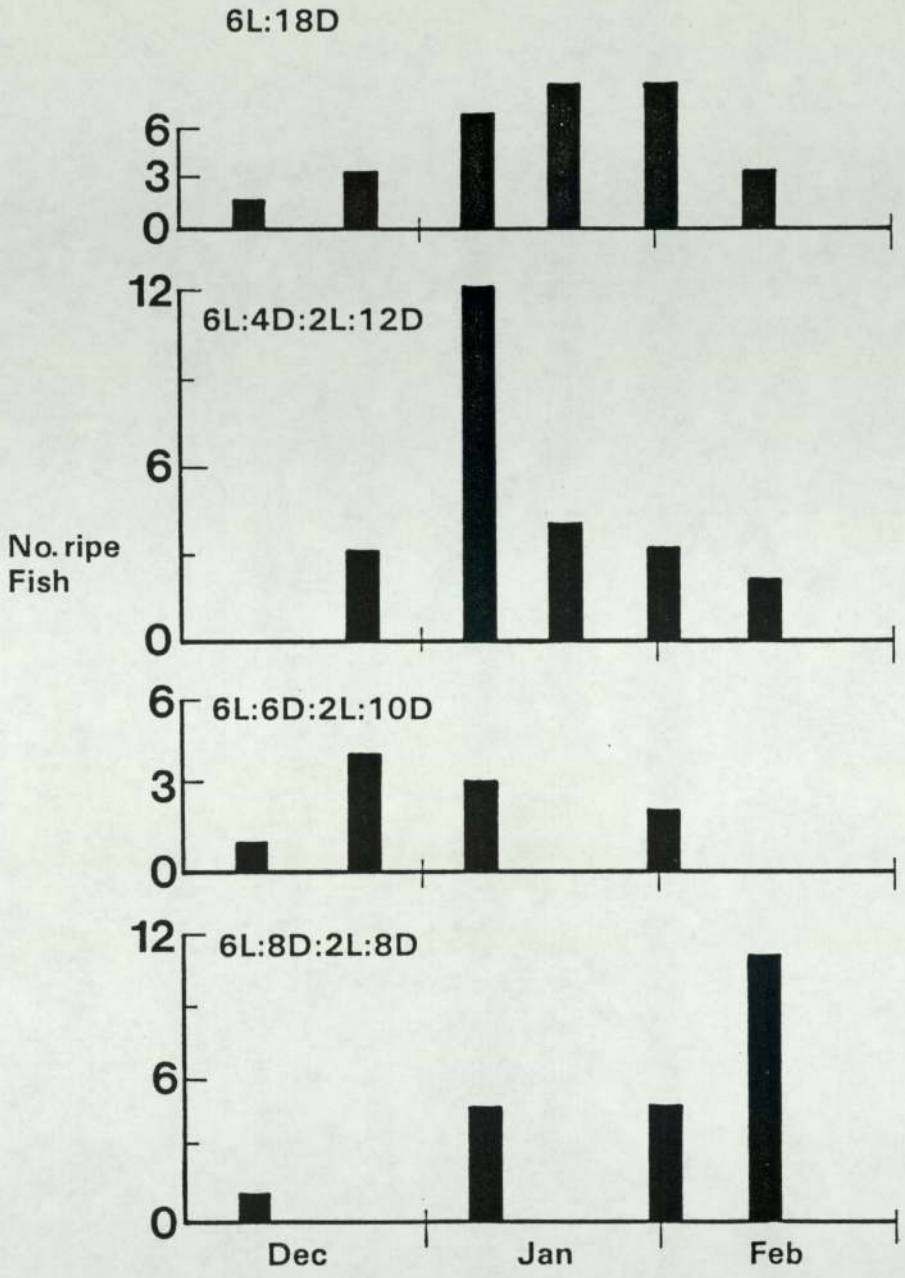



Fig. 3.7 The effect of skeleton photoperiod regimes (6L:4D:2L:12D, 6L:6D:2L:10D and 6L:8D:2L:8D) and constant 6L:18D on the timing of maturation of female rainbow trout. Histograms illustrate the number of mature females ( No. ripe fish) at each sampling time. The vertical lines on the x-axis refer to the first day of the month as labelled.

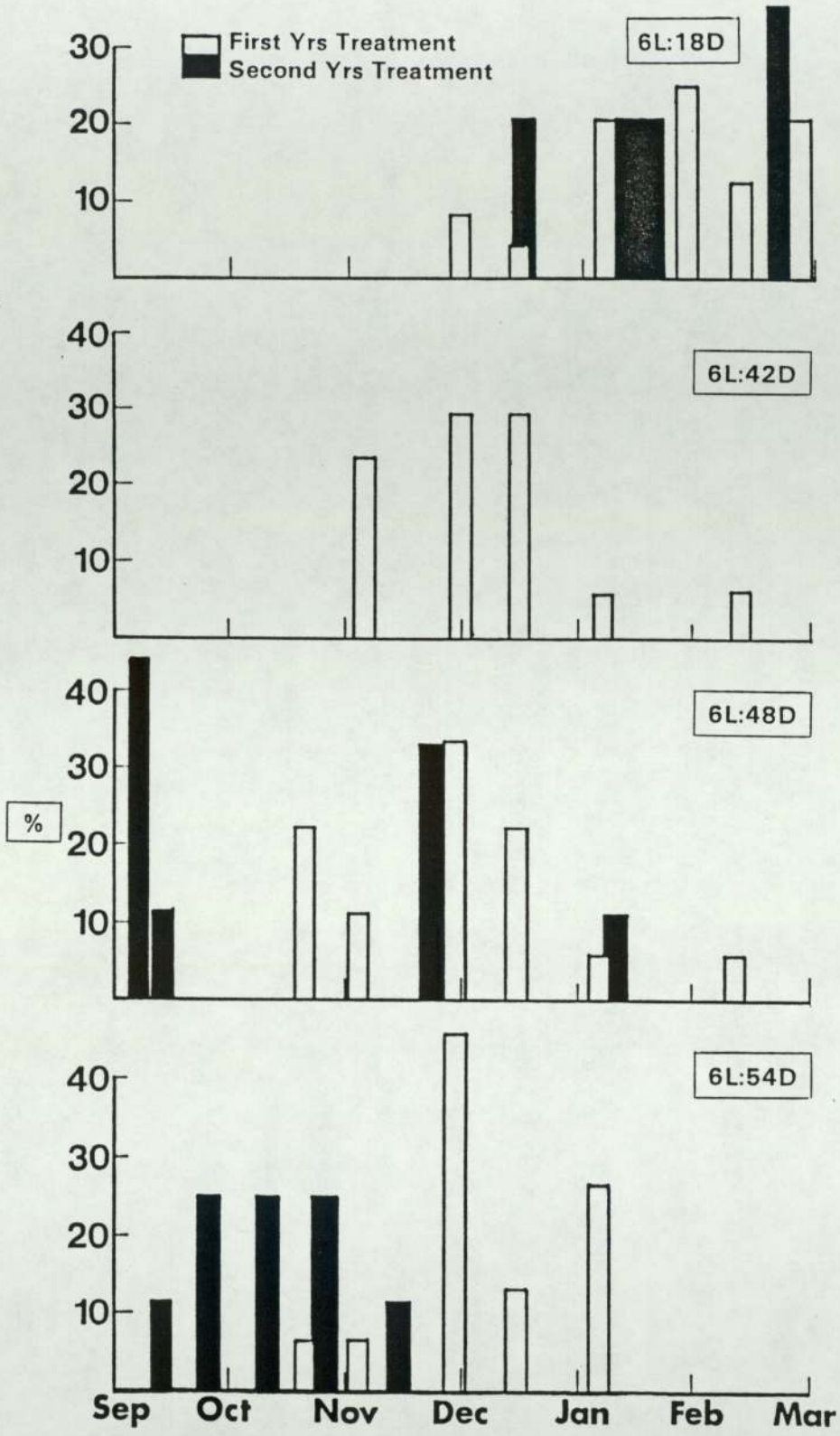



Fig. 3.8 The effect of resonance photoperiods (6L:42D, 6L:48D and 6L:54D) and constant 6L:18D on the timing of maturation of female rainbow trout over a two year period. Histograms illustrate the percentage of mature females (n= 20 in each group) at each sampling time. The open histograms are the first year's spawning under these photoperiods and the filled histograms the second years spawning. The 6L:42D treatment was discontinued before the end of the second reproductive cycle. The vertical lines on the x-axis refer to the first day of the month as labelled.

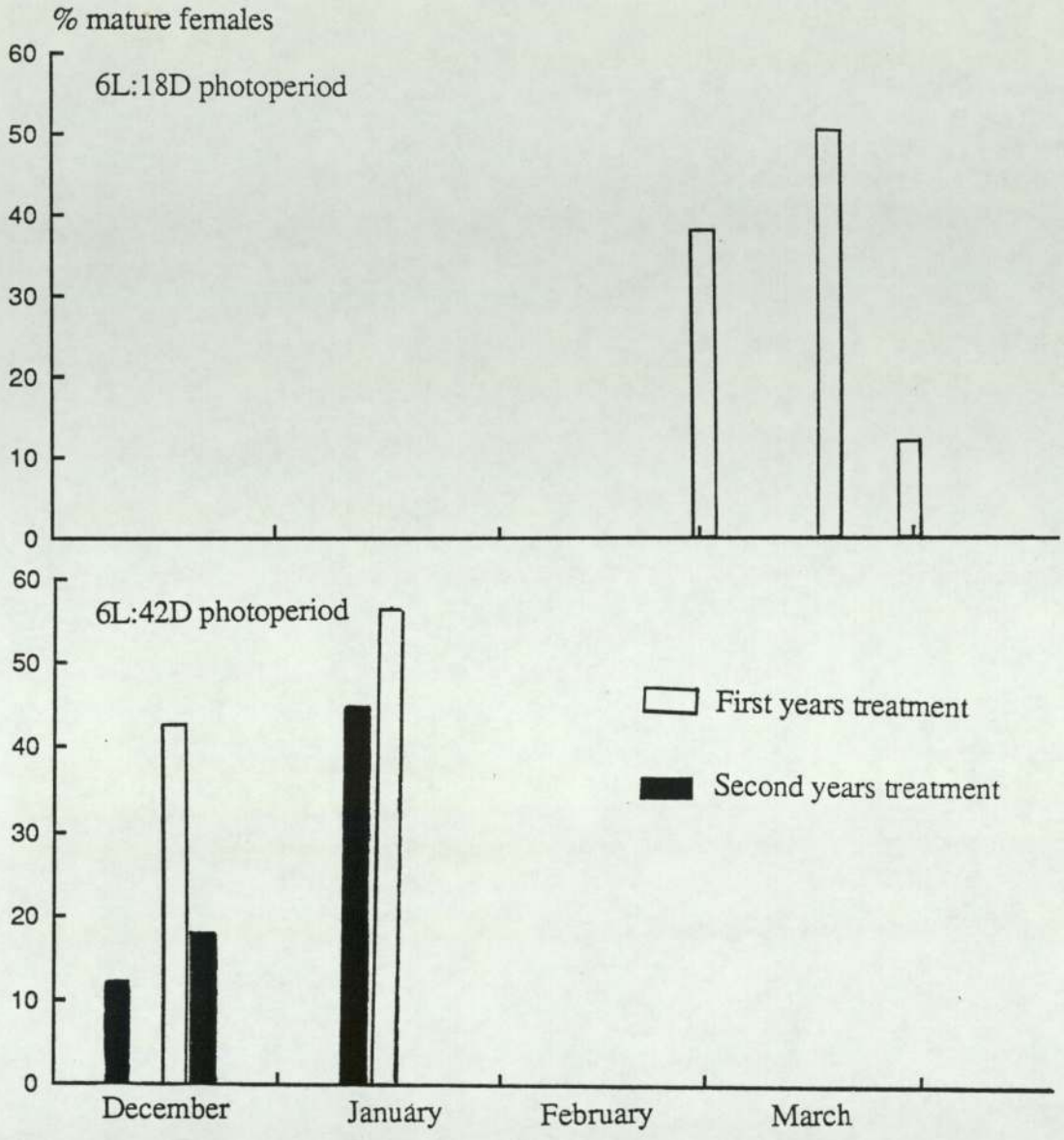



Fig. 3.9 Repeat of experiment investigating the effect of 6L:18D and 6L:42D photoperiod regimes on the timing of maturation of female rainbow trout. Histograms illustrate the percentage of mature females at each sampling time (n= 20 in each group). The open histograms are the first years spawning under these photoperiods and the filled histograms the second year spawning. The 6L:18D treatment was discontinued before the end of the second reproductive cycle. The vertical lines on the x-axis refer the first day of the month as labelled.

(iii) Constant light regimes

During the first year's exposure to LL (continuous light) and constant 18L:6D spawning occurred in October and November up to 2 months in advance of control fish under ambient lighting and up to 7 months before some of the fish under 6L:18D (short days). Under both LL and constant 18L:6D spawning was repeated at intervals of approximately 160 days (Figure 3.10a,b) although as the experiment progressed the loss of tags made it difficult to provide reliable information of the period length between the successive spawnings of each individual fish. Much more convincing data were provided by the serum analyses of testosterone, oestradiol-17ß and calcium. Here the spawning periodicities were reflected by the distances between the peak heights of the three parameters measured. Under both LL (Figure 3.11) and 18L:6D (Figure 3.12), periodicities of approximately 150-170 days are clearly shown. Furthermore, these period lengths were repeated over 3 or 4 successive cycles. Some data are available for the multiple spawning of individual fish under LL and 18L:6D and a typical example is shown in Figure 3.13. Under both photoperiod regimes testosterone, oestradiol-17ß and calcium levels all peaked before spawning and had begun to fall before ripe eggs were stripped from the fish. Within a month of spawning testosterone and oestradiol-17ß levels had begun to increase and this was followed a few weeks later by increases in serum calcium.

Under 6L:18D, most fish spawned in February or March, representing delays of up to 4 and 7 months when compared with fish maintained under ambient or 18L:6D and LL respectively. Maintenance of the fish under 6L:18D for at least another two reproductive cycles resulted in peaks of spawning at approximately annual intervals (Figure 3.14). There was considerable inter- and intra-individual variation in the periodicity of the maturation rhythm ranging from approximately 11 to 15 months (Figure 3.15). Changes in the serum concentrations of oestradiol-17ß, testosterone and total calcium corresponded to the periodicity of the spawning rhythm (Figure 3.16). Figure 3.17 illustrates the hormone profiles of individual fish maintained for at least 2 spawnings under constant 6L:18D.

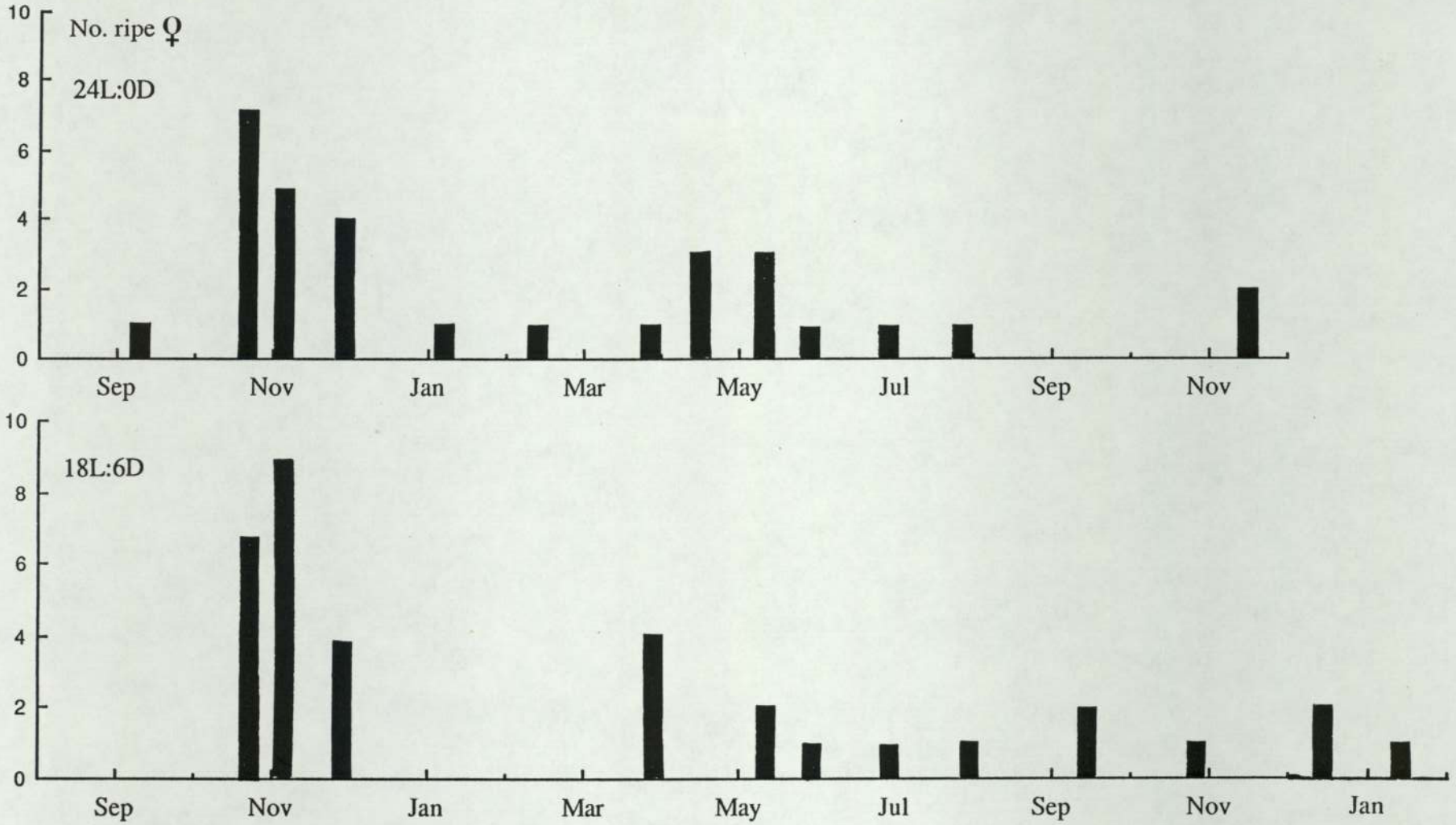



Fig. 3.10 The effect of (a) continuous light(24L:0D) and (b) constant 18L:6D over a two year period on the timing of spawning of female rainbow trout. Histograms illustrate the number of mature females (No. ripe ♀) at each sampling time. The vertical lines on the x-axis refer to the first day of the month as labelled.

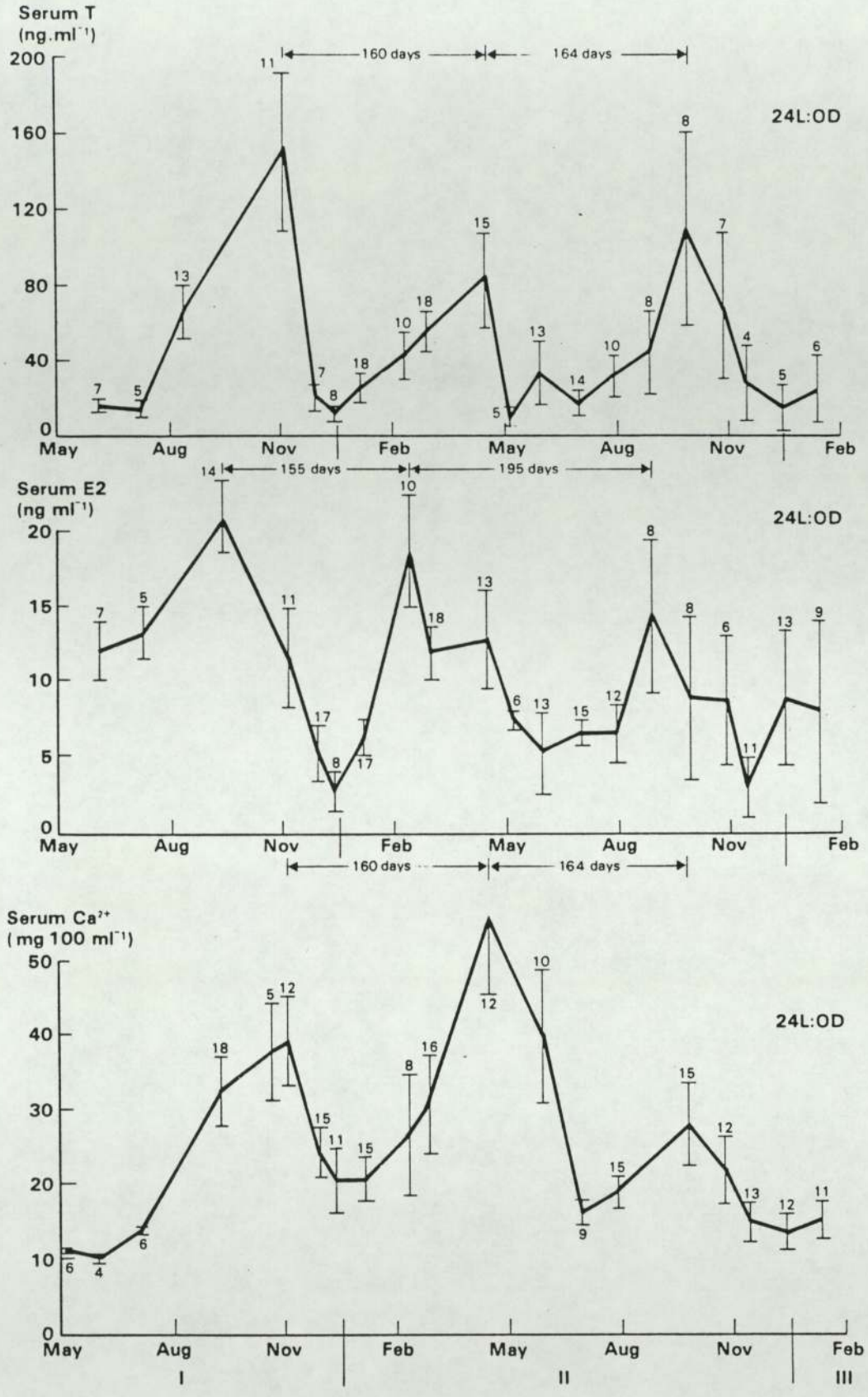



Fig. 3.11 The sequential changes in the mean levels of serum testosterone (T, ng $ml^{-1}$) oestradiol-17ß (E2, ng $ml^{-1}$) and total serum calcium ($Ca^{2+}$, mg $100ml^{-1}$) in female rainbow trout maintained under continuous light (LL) over a two year period. Period lengths between the peak heights of these three components are arrowed and the time interval indicated. The integer above each coordinate indicates the number of fish sampled(n), with the vertical bars representing ± one standard error of the mean. The vertical lines on the x-axis refer to the first day of the month as labelled.

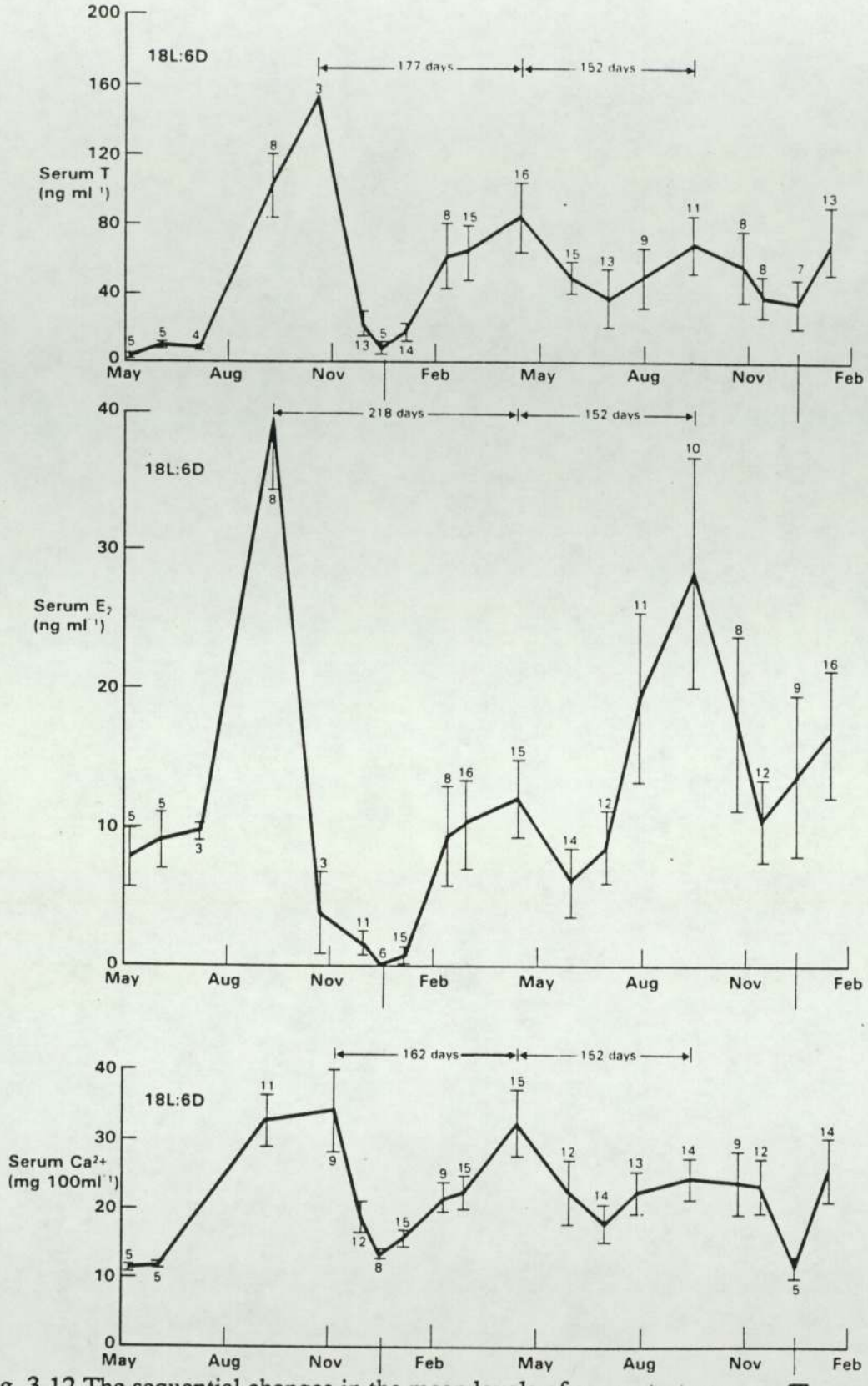



Fig. 3.12 The sequential changes in the mean levels of serum testosterone (T, ng $ml^{-1}$) oestradiol-17ß (E2, ng $ml^{-1}$) and total serum calcium ($Ca^{2+}$, mg $100ml^{-1}$) in female rainbow trout maintained under constant 18L:6D over a two year period. Period lengths between the peak heights of these three components are arrowed and the time interval indicated. The integer above each coordinate indicates the number of fish sampled (n), with the vertical bars representing $\pm$ one standard error of the mean. The vertical lines on the x-axis refer to the first day of the month as labelled.

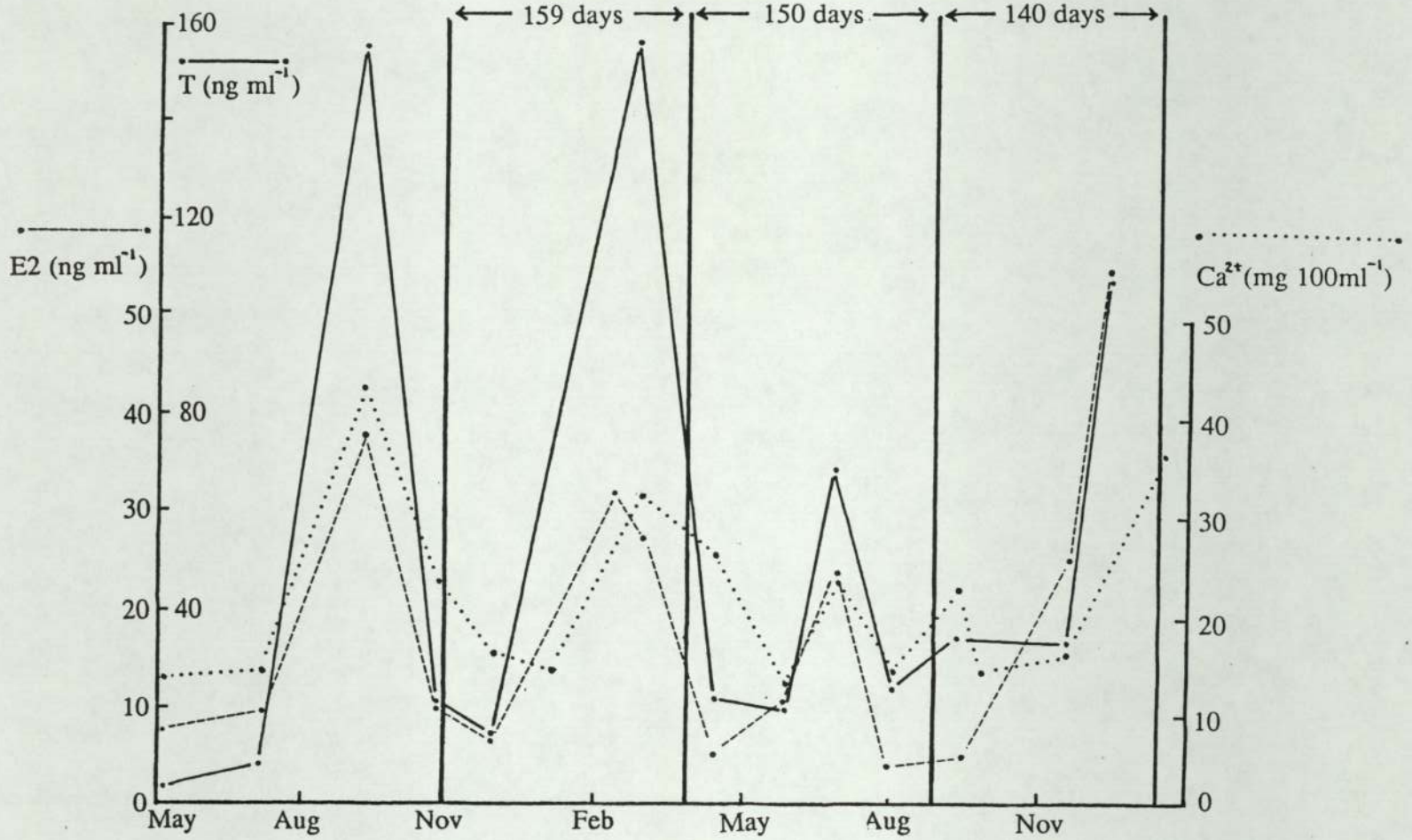



Fig. 3.13 The sequential changes in serum testosterone(T, ng $ml^{-1}$), oestradiol-17$\beta$(E2, ng $ml^{-1}$) and total serum calcium($Ca^{2+}$, mg $100ml^{-1}$) in an individual female rainbow trout maintained under constant 18L:6D for two years. The vertical lines show the timing of successive spawnings, and the periodicity is indicated. The vertical lines on the x-axis refer to the first day of the month as labelled.

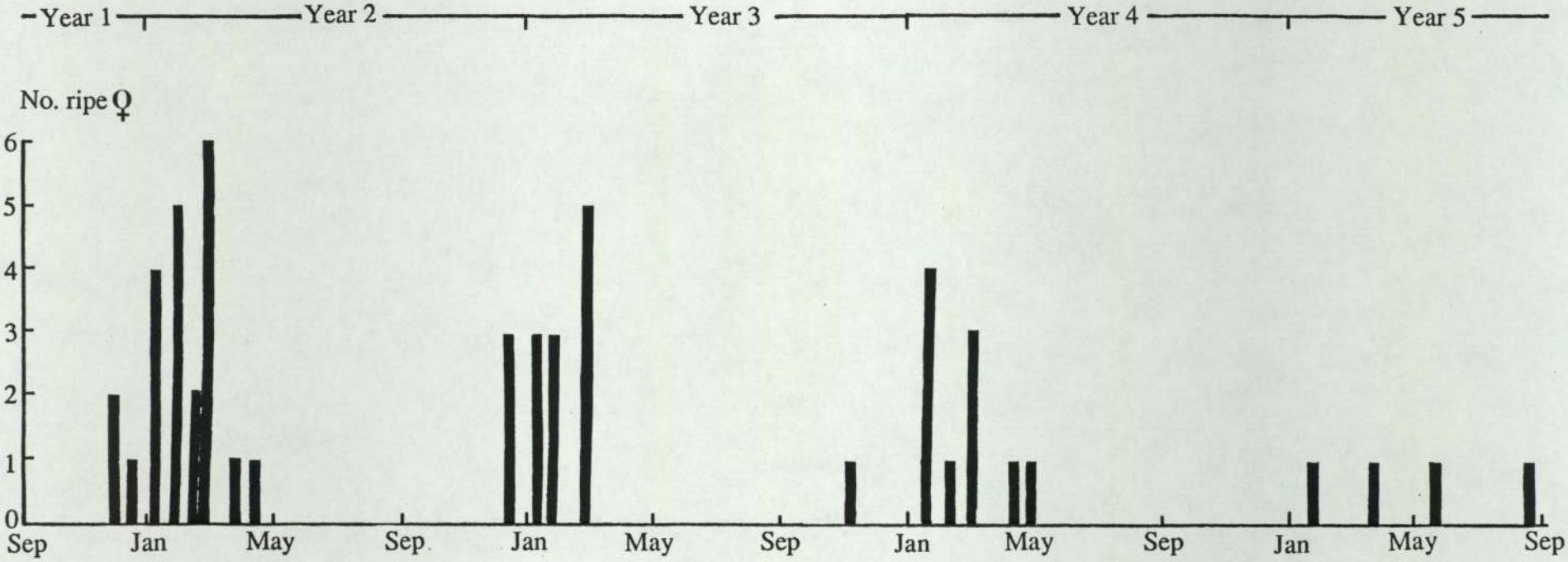





Fig. 3.14 The effect of constant 6L:18D for over four years on the timing of spawning of female rainbow trout. Histograms illustrate the number of mature females (No. ripe ♀) at each sampling time. The vertical lines on the x-axis refer to the first day of the month as labelled.

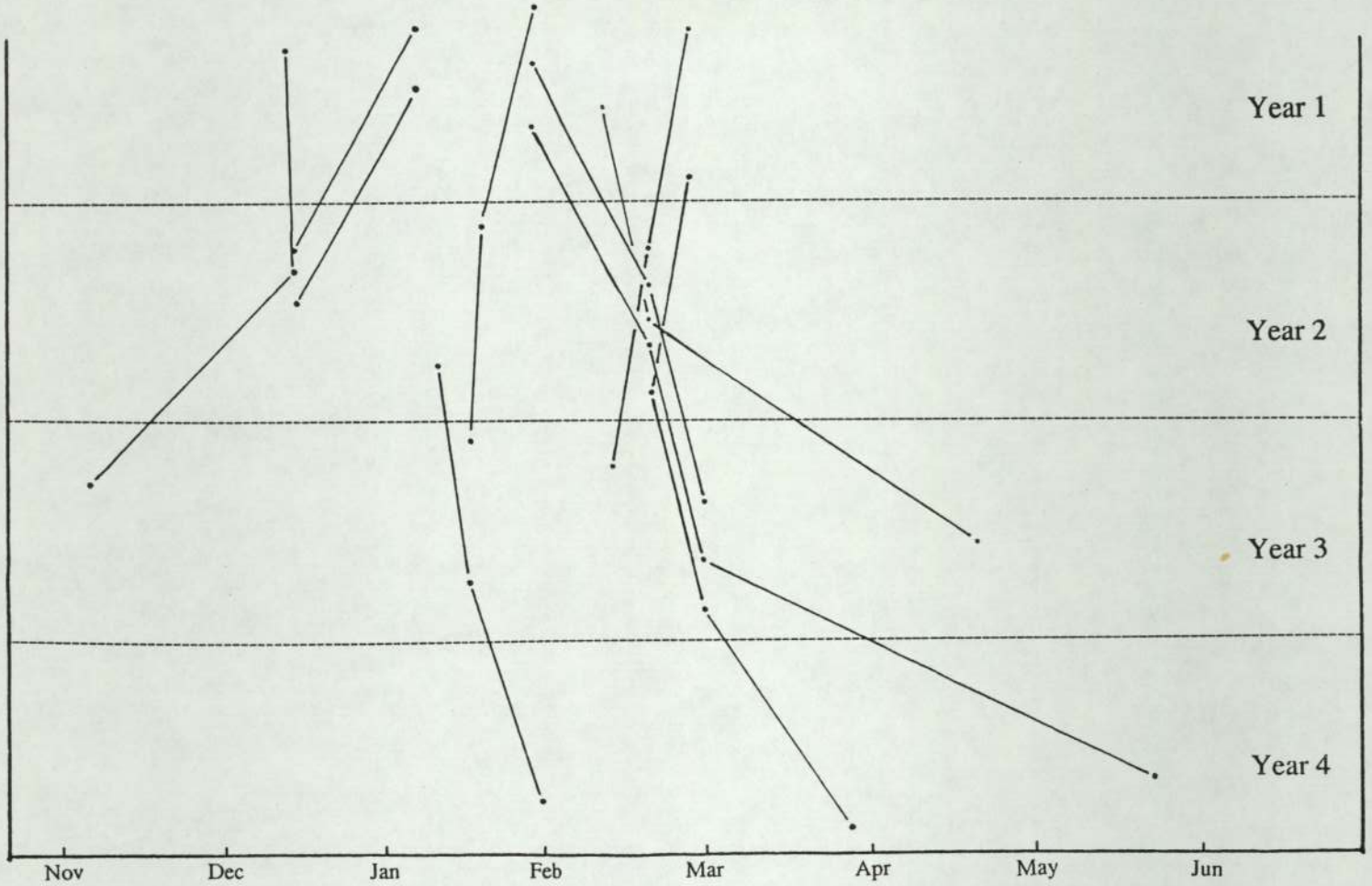



Fig. 3.15 The periodicity of the timing of spawning of individual female rainbow trout maintained for up to four years under constant 6L:18D. Each coordinate indicates the timing of maturation of an individual fish with the lines linking successive years spawnings. Note that in most fish the periodicity between spawnings differs from 12 months indicating that the rhythms are free-running. The vertical lines on the x-axis refer to the first day of the month as labelled.

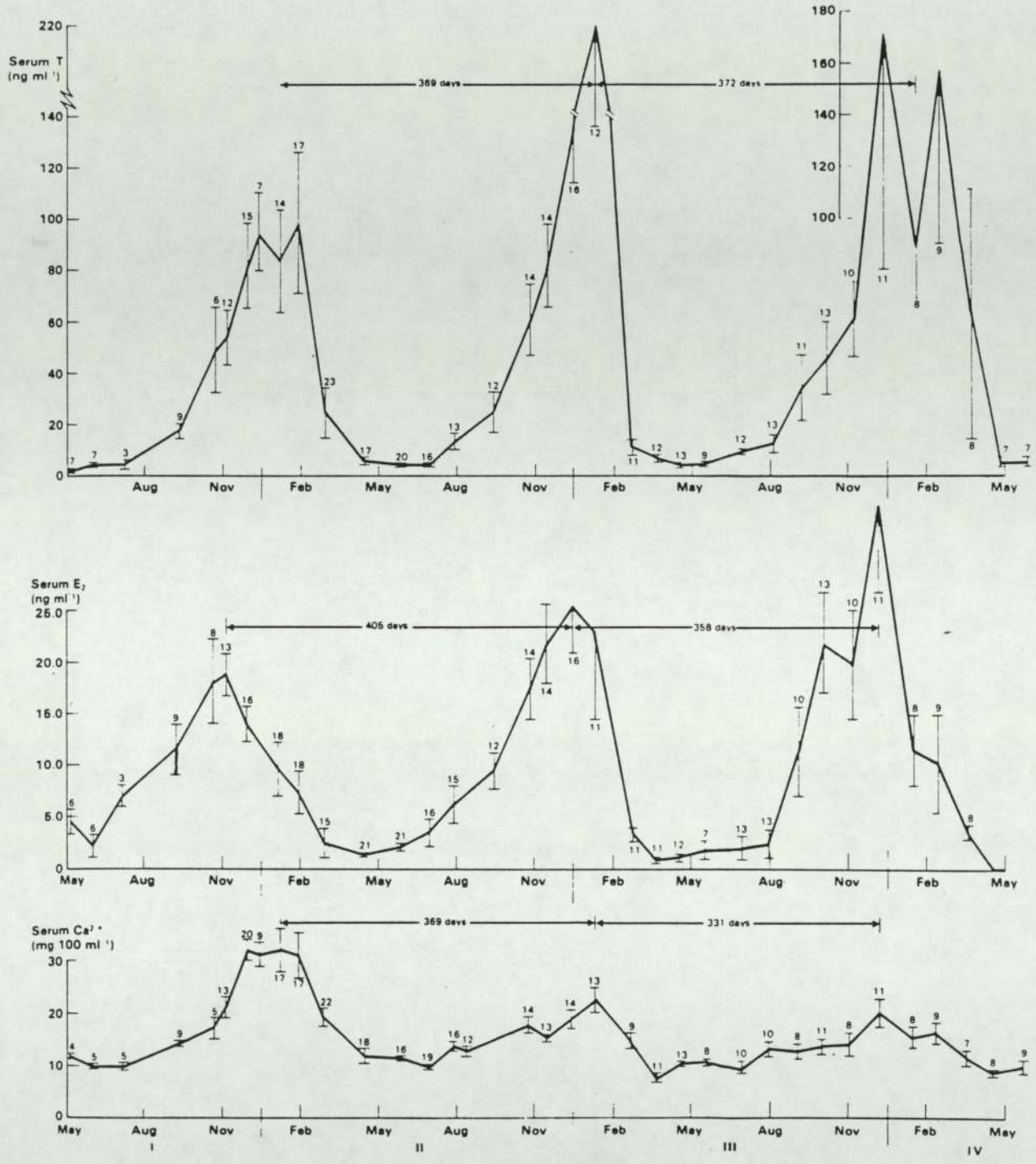



Fig. 3.16 The sequential changes in the mean levels of serum testosterone (T, ng $ml^{-1}$), serum oestradiol-17ß (E2, ng $ml^{-1}$) and total serum calcium ($Ca^{2+}$mg $100ml^{-1}$) in female rainbow trout maintained under constant 6L:18D for over three years. Period lengths between the peak heights of these three components are arrowed and the time interval indicated. The integer above each coordinate indicates the number of fish sampled(n) with the vertical bars representing ± one standard error of the mean. The vertical lines on the x-axis refer to the first day of the month as labelled.

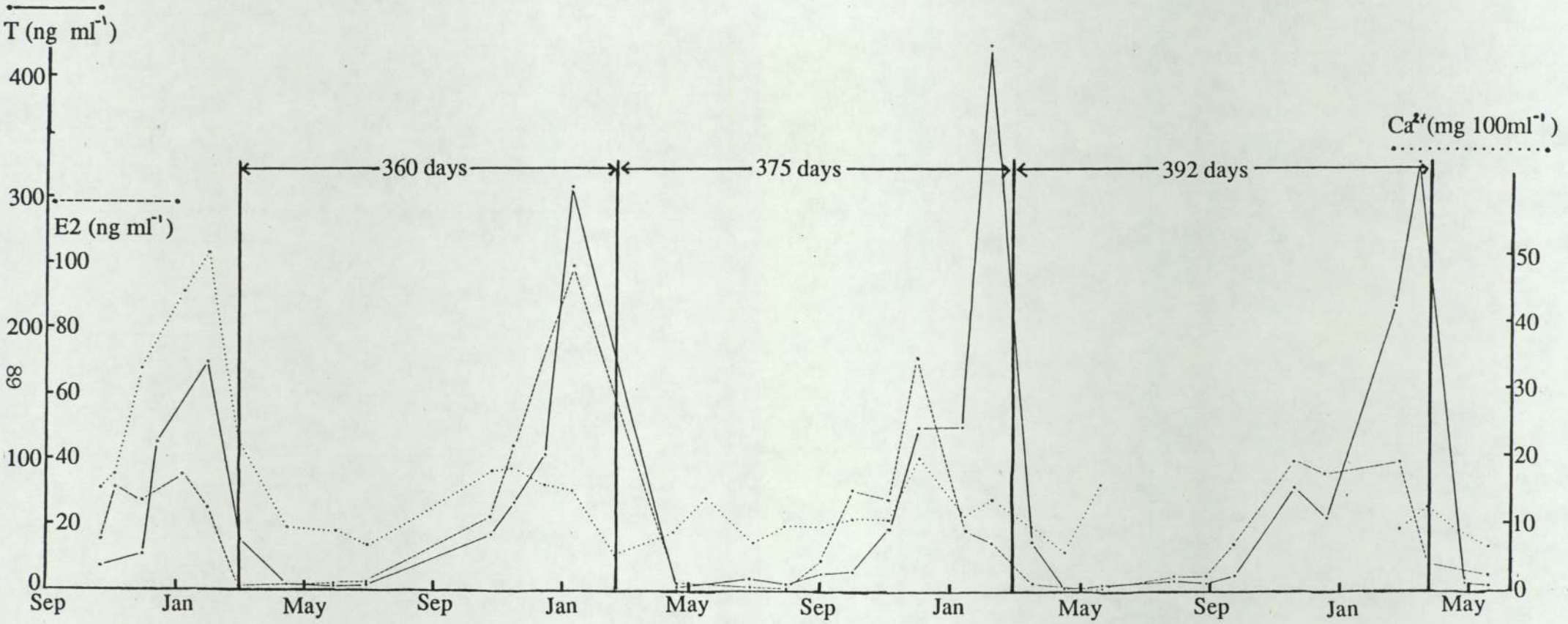



Fig. 3.17a The sequential changes in serum testosterone(T, ng $ml^{-1}$), oestradiol-17$\beta$(E2, ng $ml^{-1}$) and total calcium ($Ca^{2+}$, mg $100ml^{-1}$) in an individual female rainbow trout maintained under constant 6L:18D for over 4 years. The vertical lines show the timings of successive spawnings and the periodicity indicated. The vertical lines on the x-axis refer to the first day of the month as labelled.

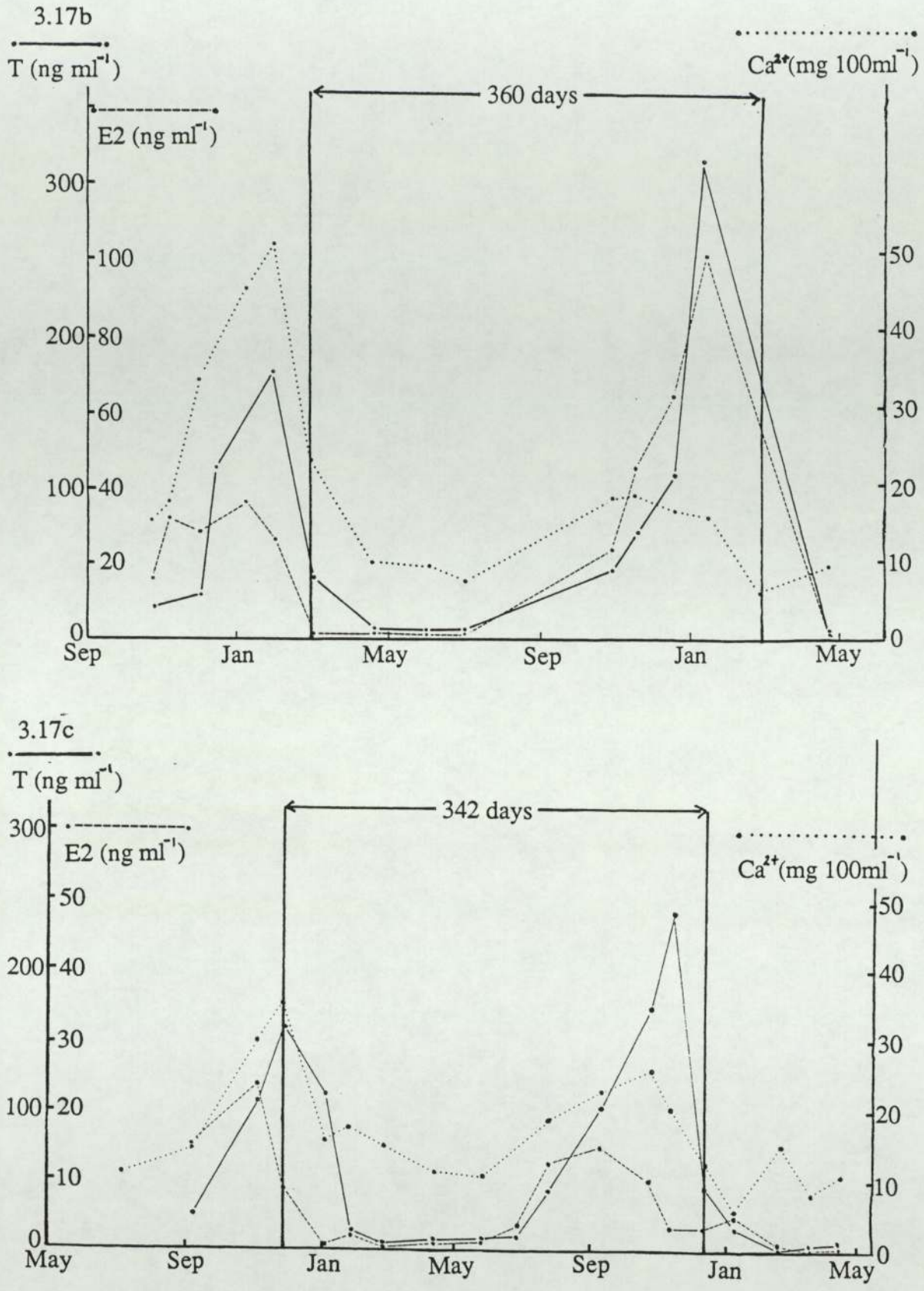



Fig. 3.17b-c. The sequential changes in serum testosterone (T, ng $ml^{-1}$), oestradiol-17ß (E2, ng $ml^{-1}$) and total calcium ($Ca^{2+}$, mg $100ml^{-1}$) in individual female rainbow trout maintained under constant 6L:18D for over two years. The vertical lines show the timing of successive spawnings, and the periodicity is indicated. The vertical lines on the x-axis refer to the first day of the month as labelled.

## 3.4 GENERAL DISCUSSION

The results from the present set of experiments provide evidence for the involvement of circadian and circannual processes in the control of the timing of reproduction in the rainbow trout.

### 3.4.1 The involvement of circadian rhythms in the control of maturation

The results from the skeleton photoperiod experiment indicate that the timing of maturation in the rainbow trout can be influenced by the distribution rather than the duration of the light (or dark) in each 24 hr period. The regime most stimulatory to the advancement of spawning was a 2 hr light pulse 12-14 hrs after the start of the main light period (i.e. 6L:6D:2L:10D). The 6L:4D:2L:12D treatment also produced an advancement in the timing of ovulation compared to fish on 6L:8D:2L:8D and constant 6L:18D. These results support the hypothesis that rainbow trout possess a photo-inducible phase, the illumination of which results in an advancement in the time of spawning.

Similar asymmetric skeleton photoperiod experiments have been performed on other teleosts (Chan, 1976; Sundararaj and Vasal, 1976; Baggerman, 1980) and a number of other animal species (Bunning and Joerrens, 1960; Hamner, 1963; Menaker, 1965; Follett and Sharp, 1969). All have produced results that can be explained by the external coincidence model. Chan (1976) working on the medaka (*Oryzias latipes*) found significant increases in the gonadosomatic index (GSI) after 8 weeks exposure of the fish to a skeleton photoperiod where the light pulses fell 15-16 hrs after the start of the main photoperiod. In a parallel experiment which ran for 12 weeks, significant increases in the GSI were recorded where the pulses occurred 13-14 and 15-16 hrs after the start of the main photoperiod. Sundararaj and Vasal (1976) maintained postspawned catfish (*Heteropneustes fossilis*) for 45 days on skeleton regimes. Where the 1 hr light pulses fell between 16-17 hrs and 20-21 hrs after the start of the main photoperiod respectively, significant increases were detected in the size of the ovary. However, when the experiment was repeated with catfish in the 'preparatory' phase of the maturation cycle all light pulses between 13 and 21 hrs produced significant increases in ovarian weight. Similarly Baggerman (1980) studying the response of sticklebacks (*Gasterosteus aculeatus*) to skeleton regimes produced good evidence that there was a photo-inducible phase whose position and width changed over the course of the year. Thus, although the present study on the rainbow trout provides evidence for a photo-inducible phase, it is possible that it does not remain positioned 11-14 hrs after the start of the main

photoperiod throughout the year. There is evidence that seasonal shifts in the photo-inducible phase also occurs in birds (Hamner, 1968; Lofts and Lam, 1973; Robinson and Follett, 1982). This phenomenon may be important in the control of annual reproduction cycles and will be discussed more fully later in the chapter.

Not only is it evident that the photo-inducible phase can alter position, it is also possible for the endogenous circadian rhythm of photo-sensitivity to entrain to either the long or short light periods in the skeleton regime, making interpretation of the results even more hazardous. Implicit in Bunning's (1960) original hypothesis of external coincidence was the assumption that the endogenous circadian rhythm is 'locked on' or entrained to the beginning of the main light period ('dawn'). Pittendrigh and Minis (1964) were the first to emphasise that this assumption was erroneous. They concluded this from a series of asymmetric and symmetric (light pulses of equal length) skeleton photoperiod experiments performed on the insect *Drosophila pseudoobscura*. For particular skeleton regimes the animal would read the first light pulse as the presumptive dawn and entrain its circadian rhythm to that photoperiod. However, when the dark period between the two light periods is extended to a certain duration the system becomes unstable and the circadian rhythm entrains onto the second pulse. For example in *Drosophila* a 10 hr photoperiod can be simulated by two 15 minute light pulses 10 hr apart, the animal reading the first pulse as dawn. However, when one attempts to entrain the animals to the skeleton of a 14 hr photoperiod the circadian oscillator phase shifts and entrains to the second light pulse, effectively assuming the phase characteristic of a 10 hr skeleton photoperiod (Pittendrigh and Minis, 1964). Subjecting pink boll worms (*Pectinophora gossypiella*) to assymetric skeleton photoperiods resulted in two maxima of diapause, one early and one late in the dark phase (Adkisson, 1964). Pittendrigh and Minis (1964) interpreted these results as a good example of a phase shifting in the circadian rhythm underlying the photoperiodic response. In addition to insects there is good evidence that similar phase shifts occur in birds in response to skeleton photoperiods (Menaker, 1965; Murton, 1975; Follett, 1981). Indeed it may also occur in teleosts, although not mentioned by the authors it is possible that the bimodal response of catfish to skeleton photoperiods was due to the phase shifting in the circadian rhythm (Sundararaj and Vasal, 1976).

It is clear that although the skeleton photoperiods used in the present study indicate that the rainbow trout possess a photo-inducible phase it is difficult to reach any firm conclusions. As stated in the introduction to this chapter, photoperiod regimes based on 24 hr cycles are open to criticism when they are

quoted in support of the hypothesis that the photo-inducible phase is associated with, or driven by an endogenous circadian oscillator. Resonance photoperiod regimes are acknowledged as a more powerful test of the external coincidence model and it was hoped that their inclusion in the present study would help in the elucidation of the timing mechanism. Unfortunately the results were somewhat equivocal. Figure 3.18 illustrates the position of the 6 hr photoperiods used in the resonance experiments in relation to 24 hr periods of time. If rainbow trout conform to the external coincidence model it would be expected that the 6L:18D and 6L:42D photoperiods would produce a similar response, certainly this is true of other species which have been subjected to resonance regimes (Nanda and Hamner, 1958; Follett and Sharp, 1969; Elliott *et al*, 1972; Gwinner and Eriksson, 1977). However, the results clearly show that the fish maintained under 6L:42D spawned ahead of those on 6L:18D. To eliminate any doubt about the validity of this result the 6L:42D and 6L:18D treatments were subsequently repeated on different batches of fish, again producing the same results; the fish on 6L:42D spawned during December and January while those on 6L:18D spawned in February and March. It is not possible to reconcile these observations with the external coincidence model. However, it is of interest to note that in other resonance experiments (Stetson *et al*, 1976; Gwinner and Eriksson, 1977) the 6L:42D treatment is not as non-stimulatory as 6L:18D, indicating a possible weakness in the external coincidence model. The spawning profiles of the fish maintained under 6L:48D and 6L:54D also did not provide any positive evidence in support of external coincidence. Hypothetically both these photoperiod regimes illuminate the photo-inducible phase and in other species do produce a 'long day' response (Follett and Sharp, 1969; Stetson *et al*, 1976). In both 6L:48D and 6L:54D groups the timing of the first years spawning was advanced compared to those on 6L:18D thus supporting the results from the skeleton photoperiod experiment. However, the succeeding spawning of fish under both regimes occurred approximately one year later. It is established that rainbow trout maintained under long days for long periods of time spawn at approximately 6 month intervals (Bromage *et al*, 1984; Scott *et al*, 1984; Chapter 3). Therefore if the fish had perceived 6L:48D or 6L:54D as a long day they would have been expected to mature at twice yearly intervals. This was clearly not the case and thus the results do not support the external coincidence model.

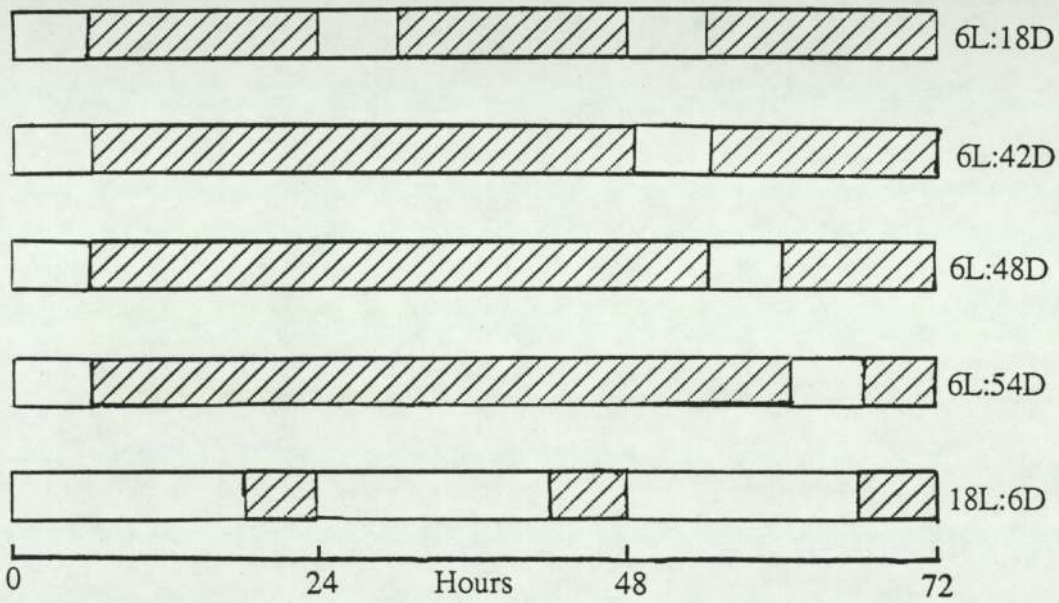



Fig. 3.18 The position of the 6-hour daylengths of the resonance photoperiod regimes (6L:18D, 6L:42D, 6L:48D, 6L:54D ) and 18L:6D in relation to 24-hour periods of time.

One of the major difficulties in interpreting the results from both the resonance and the skeleton photoperiod experiments is that the spawning of the rainbow trout is never completely arrested by non-stimulatory photoperiods. Similar photoperiod experiments on other species tend to be specifically designed to produce an 'all or nothing' response. For example in many species of birds the gonads will only mature if the animals are exposed to long photoperiods. Spontaneous gonadal regression will then occur even though the animals are maintained on photostimulatory long days. This physiological state in which an animal is incapable of responding to a once stimulatory photoperiod is referred to as the refractory period. In order to become responsive again to long days the birds must first be exposed to short days for a species specific period of time (Farner and Follett, 1966; Follett, 1981). To ensure that birds are not refractory and will respond 'classically' to resonance and skeleton photoperiod regimes they are pretreated by maintaining them under short days (Hamner, 1963; Follett and Sharp, 1969). Similarly for the golden hamster (*Merocricetus auratus*) to become responsive to the inhibitory effects of short days, mature animals must be exposed to long days for about 11 weeks. At this stage of the maturation cycle a long day can be substituted by a 'long' resonance photoperiod (6L:30D or 6L:54D) producing a result that supports the external coincidence model (Stetson *et al*, 1976). However hamsters with regressed gonads will exhibit spontaneous gonadal recrudescence regardless of the photoperiod under which they are maintained (Reiter, 1972, 1975; Turek *et al*, 1975a). Subjecting animals to resonance photoperiod regimes at this stage in the maturation cycle would not provide good evidence for the involvement of a circadian rhythm in controlling gonadal growth. If a more complete knowledge of the responses of rainbow trout to normal 24 hr photoperiods over the whole maturation cycle had been known beforehand, it may have been possible to design an experiment which would have produced less equivocal results. For example, like the rainbow trout, sheep (*Ovis aries*) do not exhibit an all or nothing response but will continue to show a cycle of maturation even under constant environmental conditions (Ducker *et al*, 1973; Howles *et al*, 1982; Almeida and Lincoln, 1984). However, by maintaining mature soay rams under an inhibitory long photoperiod for 16 weeks and then subjecting them to resonance regimes, Almeida and Lincoln (1982) produced some reasonably good evidence in support of the external coincidence model.

In spite of the equivocal results from the resonance experiment these data indicate that the measurement of photoperiod in connection with the control of maturation in the rainbow trout is not governed by an hour-glass mechanism. Lees (1973) produced convincing evidence that the photoperiodic response in the aphid *Megoura viciae* was controlled by an hour-glass which measured the total length of the dark period. If such a system was employed by the rainbow trout it might be expected that the resonance photoperiods with different lengths of light and dark would have resulted in different spawning times. However, the observation that the fish under these treatments spawned at about the same time of year, together with the ability of the fish to spawn successfully under constant light (this chapter) negates the possibility that a night length measuring hour-glass is involved in the photoperiodic control of maturation. These results also fail to be explained in terms of an hour-glass that measures the light period, or by a system that summates light-dark cycles because these parameters showed considerable variation between the experimental groups (Table 3.1). Another strong argument against the hour-glass hypothesis is the observation that the rainbow trout continue to exhibit a rhythm of maturation under these photoperiod regimes. It is difficult to see how a linear time-measuring system such as an hour-glass could generate such an oscillation unless it was somehow reset after each spawning.

An alternative hypothesis to explain photoperiodism and daylength measurement is the internal coincidence model (Pittendrigh 1960, 1972). This is thought to consist of at least two circadian oscillators, one possibly entrained by dawn, the other by dusk. Photoperiodic induction occurs when the two oscillators are in a particular phase relationship with each other. Unfortunately the resonance and skeleton photoperiod experiments cannot give any indication whether such a system is operating in the rainbow trout. Pittendrigh and Daan (1976) described how an internal coincidence system could time such events as seasonal reproduction; certainly under natural photoperiod conditions where the phase relationship between sunrise and sunset changes throughout the year the hypothesis applies. Indeed, recent work on mammals (Earl *et al*, 1985; Stetson *et al*, 1986) described in Chapter 5 has provided evidence that an internal coincidence mechanism is involved in controlling gonadal development. However, the most striking feature of the results of the resonance experiment was that under 'constant' photoperiod regimes the rainbow trout continued to exhibit a circannual rhythm of maturation. It is impossible to explain this observation in terms of internal, external or hour-glass mechanisms without including in the hypothesis that seasonal reproduction in the rainbow trout is controlled by some form of

endogenous timing mechanism with an annual periodicity.

### 3.4.2 The involvement of circannual timing mechanisms in the control of maturation

Evidence for an endogenous circannual timing mechanism was provided by the experiments where rainbow trout were held for long periods of time under conditions of constant photoperiod and temperature. If truly endogenous the rhythm of maturation would be expected to free-run with a periodicity approximating to, but significantly different from one year. Circannual rhythms should also be capable of entrainment by light cycles and show temperature compensation. More importantly, to ensure that the rhythm is self-sustaining it should be demonstrable over at least two cycles (Farner and Follett, 1966; Gwinner, 1981a). The results clearly showed that rainbow trout are capable of exhibiting a rhythm of maturation with at least two periodicities; under constant 6L:18D successive spawnings were approximately one year apart while those fish maintained under 18L:6D and LL had a rhythm of spawning with a periodicity of approximately 5.5 months. Overall these results satisfy the criteria that the maturation cycle of the rainbow trout is controlled by some form of self-sustained internal timing mechanism. Whether the spawning periodicities of approximately 5.5 and 12 months are manifestations of the same circannual timing mechanism will be discussed later. It must be conceded that after the first two reproductive cycles under 6L:18D there was some doubt as to the endogeneity of the rhythm. Because the fish were only examined at approximately monthly intervals it was impossible to resolve whether or not the periodicity was significantly different from 1 year. This left open the possibility that the maturation cycle was being driven by some geophysical variable other than temperature or photoperiod (Weihaupt, 1964). However, as the experiment progressed for two further spawning cycles it became clear that the periodicity of the rhythm in individual animals was truly circannual (Figure 3.15).

It is possible that the first two spawnings under 6L:18D of an annual periodicity were examples of transient cycles. Pengelley and Asmundson (1970) noted that the influence of constant environmental conditions on the free-running period of hibernation in golden mantled ground squirrels (*Citellus lateralis*) takes time to show any effect; in the trout it was not until the third and fourth cycles under 6L:18D that a clear circannual rhythm was observable. Ewes (*Ovis aries*) raised from birth under a range of constant photoperiods exhibited a transitory

mean duration of 112 to 162 days between the first and second breeding seasons before showing a circannual period between the second and third breeding seasons (Ducker *et al*, 1973). Similarly Berthold (1978) observed two transitory cycles of moult in migratory warblers under constant conditions before they stabilised to exhibit a classic circannual rhythm which continued for over eight years. The phenomena of transient cycles are important because many studies on circannual cycles are terminated after two, or even one complete cycle and thus the results have to be treated with caution because it is not always clear whether they refer to the steady state properties of the rhythm. Indeed this caution can be levelled at all other studies on circannual rhythms in teleosts; Sundararaj *et al* (1982) provided evidence that catfish (*Heteropneustes fossilis*) maintained under LL exhibited a circannual rhythm of maturation with a period significantly shorter than 1 year whereas under DD the period was not significantly different from 1 year. If the experiment had been allowed to run longer than one cycle a circannual rhythm may have been observed under both LL and DD regimes. Baggerman (1980) maintained sticklebacks (*Gasterosteus aculeatus*) under constant 16L:8D and observed that there was approximately 220 days between successive reproductive phases. Again the results suggested that the fish were exhibiting an endogenous circannual cycle of maturation but the data did not satisfy the criteria for endogeneity because the rhythm was only observed over one cycle. The other studies on circannual rhythms in fish by Eriksson and Lundqvist (1982) and Wagner (1974) are also equivocal because the period lengths are indistinguishable from 1 year. Once again, continuing the experiments for a longer period of time may have revealed a steady state rhythm with a circannual periodicity.

Better examples of circannual rhythms have been produced in studies on birds and mammals. Before comparing these results with the present observations on rainbow trout it should be stated that only a small number of species have been studied and it appears that circannual rhythms are not as ubiquitous as circadian rhythms. One reason for this is that circannual rhythms would primarily be expected to occur among the small percentage of animals that have a life span of at least one year. Moreover, animals that are sufficiently long-lived for circannual rhythmicity to be of potential significance have evolved relatively recently and independently in various groups of organisms (Farner, 1970). It is to be expected therefore that circannual rhythms are of a heterogeneous origin. As a result, one has to be wary of generalisations about common features of circannual phenomena because formal similarities do not necessarily indicate identical mechanisms.

The maintenance of rainbow trout under constant 6L:18D showed that there was individual variation in the periodicity of the circannual rhythm from slightly shorter to considerably longer than 12 months. There was also variation in the time period between successive spawnings of individual fish. Such variation has been recorded in other species; Pengelley and Asmundson (1969) observed that the circannual rhythm of onset of hibernation in a group of golden mantled ground squirrels (*Citellus lateralis* ) varied from 229 to 445 days, however of the 61 complete cycles recorded, only 13 were longer than 12 months. In several studies on circannual rhythms, the cycles tend to be very erratic or even fail to be observed in certain species under certain experimental conditions. For example ferrets (*Mustela furo*) maintained under constant 6L:18D showed recurrent oestrous periods of approximately 1 year but those on 14L:10D had irregular oestrous cycles and frequently became permanently anoestrous (Vincent, 1970). Rams on constant 16L:8D showed rhythms of testis volume and plasma prolactin with periodicities of approximately 35 weeks, however those animals on 8L:16D showed some irregular excursions of testis growth but no rhythm of prolactin (Howles *et al*, 1982). Gwinner (1971) compared circannual rhythms of moult, body weight and *Zugunruhe* (nocturnal hopping activity typically shown by noctural migrants during the migratory season) in two species of migratory birds under 12L:12D; whereas the willow warbler (*Phlloscopus trochilus*) exhibited clear circannual rhythms in all three parameters, in the chiffchaff (*P.collybita*) the rhythms were very erratic with no body weight rhythm whatsoever. Thus even in very closely related species the occurrence and persistence of circannual rhythms can vary considerably. In another bird, the starling (*Sturnus vulgaris*) exhibited a typical circannual cycle of testis growth when maintained under 12L:12D, however such a rhythm failed to be observed when the photoperiod was less than 11L:13D or greater than 13L:11D (Schwab, 1971). Conversely the sika deer (*Cervus nippon*) failed to exhibit a circannual rhythm under 12L:12D, however under 8L:16D, 16L:8D and LL they shed and regrew their antlers with a period that was generally around 10 months, although like the rainbow trout there was variation between individuals (Goss, 1969b). These observations on the expression or non-expression of circannual rhythms in certain species brings into focus an important question; if such rhythms are driven by an endogenous oscillator or pacemaker then when a circannual rhythm fails to be observed is it because the internal driver stops or is it because the pacemaker becomes uncoupled from the overt rhythm? Some workers are of the opinion that because circannual

rhythms are not always expressed in some species they should not be classed as true internally driven oscillations (Mrosovsky, 1978). This argument is valid but remains hypothetical until evidence is provided on the working of the internal clock itself. As far as the present study on the rainbow trout is concerned, it is proposed that the rhythm of maturation is indeed driven by an autonomous circannual oscillator. In addition to this proposition being supported by the evidence presented in this chapter, the entrained behaviour of the rhythm presented in Chapter 4 also supports the internal oscillator concept. In Chapter 4 evidence is presented which indicates that the internal oscillator can be dissociated from the overt maturation cycle and can still be subject to entrainment when the spawning rhythm is not expressed (page137).

Returning to the free-running behaviour of the rhythm, rainbow trout maintained under both LL and 18L:6D exhibited a self-sustained rhythm of spawning with a periodicity of approximately 5.5 months. This periodicity of spawning was confirmed by the cyclical alterations in the serum levels of testosterone, oestradiol-17ß and total calcium. Spawning at intervals of 4-6 months under constant long days have been reported in sticklebacks (Baggerman, 1972) and other studies on rainbow trout (Bromage *et al*, 1984; Scott *et al*, 1984). There are also reports of cultivated strains of rainbow trout spawning twice a year under completely natural photoperiodic conditions indicating that constant long days are not always essential for this phenomenon to occur (Hume, 1955; Lou *et al*, 1984). Twice yearly phases of gonadal activity have also been reported in other vertebrate groups. The lizard (*Cnemidophorus uniparens*) maintained under constant 12L:12D exhibited an initial mean refractory period of 240 days between egg laying before stabilising to show a twice yearly reproductive cycle (Cuellar, 1981). Some adult sika deer on 16L:8D and LL exhibited a 6 month cycle of antler growth (Goss, 1969b) and some sheep maintained on constant 6L:18D appeared to exhibit a twice yearly rhythm in testicular volume (Howles *et al*, 1982). Benoit *et al* (1956a,b) maintained Pekin ducks (*Anas platyrhynchos*) under LL and DD for several breeding cycles. Under both regimes the ducks clearly exhibited a 5-5.5 month cycle of gonadal growth. Like the rainbow trout however, some individuals from the same strain exhibited a biphasic cycle of gonadal growth under natural photoperiods, the first peak occurring in the spring and a second phase of testicular growth taking place from around August to October (Assenmacher, 1974). The blackcap (*Sylvia atricapilla*) is a summer visitor to northern Europe, migrating to Africa for the winter months. Birds held under constant 10L:14D generally exhibited a circannual rhythm of moult and

gonadal growth, however, some individuals exhibited two peaks of testis size per year (Berthold *et al*, 1972). In natural conditions, in contrast to other migratory warblers, the blackcap has been observed to undergo autumnal moult and courtship displays, and in resident populations of this species on the Cape Verde Islands (Lat 15°N) breed in spring and autumn (Bannerman and Bannerman, 1968). Berthold (1974) suggested that the 6 month gonadal cycle of the experimental birds in Germany was an expression of an original disposition; in the distant past all blackcaps may have bred twice a year for which the animal evolved some form of internal semi-circannual timing mechanism. As the climate changed or perhaps when the birds spread into more northerly latitudes it only became profitable for the species to breed once per year, the second gonadal cycle being suppressed either by environmental factors or internal factors such as migratory behaviour which is known to exert strong effects on gonadal development (Berthold, 1969). This explanation may also apply to other species exhibiting twice yearly maturation cycles. It may seem unlikely that salmonids in the wild would ever show a 6 month spawning cycle because they are thought to have evolved at high latitudes which are subject to considerable seasonal change in environmental conditions (Tchernavin, 1939). However the Salmonids are thought to have existed as a genus since the Upper Cretaceous (Young, 1981) and it is possible that at some time since this epoch a 6 monthly spawning rhythm may have been of adaptive advantage to the fish. In present stocks of rainbow trout the second spawning cycle is normally inhibited from occurring and is only exhibited under particular experimental conditions (present study; Bromage *et al*, 1984; Scott *et al*, 1984) or after prolonged cultivation (Hume, 1955; Lou *et al*, 1984).

### 3.4.3 The mechanism controlling seasonal breeding cycles

The mechanism controlling periodic breeding cycles in vertebrates is poorly understood although several hypotheses have been proposed to explain how such a system might operate. It is relevant to the present study to discuss these theories in relation to the present results on the endogenously driven reproduction cycle of the rainbow trout.

The introduction to this chapter described the general properties of circannual rhythms and indicated that they were similar in certain respects to circadian rhythms in their formal properties; under constant conditions they both exhibit free-running rhythms, they can both be synchronised by environmental variables such as photoperiod and they both appear to exhibit temperature compensation (Saunders, 1977). In trying to develop hypotheses about possible

mechanisms controlling circannual rhythms, the long time constants are often difficult to reconcile with those physiological processes known to be involved in seasonal phenomena. In an attempt to overcome these difficulties it has been suggested that circannual rhythms might be generated by rhythms with higher frequencies, in particular circadian rhythms (King, 1968; Hamner, 1971; Gwinner, 1973; Mrosovsky, 1978). One of the possible ways an organism could transform circadian frequencies into circannual rhythms is by a process called frequency demultiplication (Gwinner, 1973; Mrosovsky, 1978; Farner and Follett, 1979). In effect such a mechanism would amount to the organism counting circadian cycles 'knowing' that a year consists of 365 such cycles. An analogue of such a device is the electrical clock which generates a 12 hour cycle from 50 Hz mains alternating current. This theory predicts that the period of the circannual rhythm is proportional to the period of the circadian rhythm. Gwinner (1981b) tested the hypothesis by exposing groups of starlings (*Sturnus vulgaris*) and garden warblers (*Sylvia borin*) for long periods of time to photoperiods of 11L:11D, 12L:12D and 13L:13D and observed the periodicity of the circannual rhythms. According to Gwinner the circadian rhythms of the birds would have entrained to these photoperiods of approximately 22, 24 and 26 hours and if a frequency demultiplication model was in operation then one would expect to observe differences in the periodicities of the circannual rhythms in the respective groups. No such differences were found indicating that this particular model was not applicable to starlings or garden warblers. The frequency demultiplication model is also inapplicable to the rainbow trout because the fish maintained under 6L:18D and 18L:6D exhibited two completely different spawning periodicities even though they were exposed to 24 hr light-dark cycles which would probably have entrained any endogenous circadian rhythms to a period of 24 hours. In addition, the results from the resonance experiment also fail to support the frequency demultiplication model; the fish in the respective groups all spawned at approximately the same time of the year even though the number of light-dark cycles varied between 146 and 365 (Table 3.1). Apart from the direct experimental evidence against the frequency demultiplication hypothesis there are general properties of circannual rhythms that are difficult to reconcile with this model. Firstly, individual animals exposed to the same constant photoperiods can show very different circannual periodicities (e.g. Pengelley and Asmundson, 1969). Secondly, the periodicity of the circannual rhythm during the first cycle is often different from the period in the steady state (e.g. Berthold, 1978). Thirdly, circannual rhythms can be compressed up to one fourth their spontaneous

periodicity by exposing animals to photocycles with periods shorter than 1 year even though the number of light-dark cycles remains 365 per year (Gwinner, 1977; Chapter 4).

An alternative hypothesis to explain the occurrence of circannual rhythms has been developed from the external coincidence model. This theory proposes that the phase relationship between a circadian rhythm of photosensitivity and the light-dark cycle to which it is entrained is subject to a circannual oscillation (Gwinner, 1973, 1981a, Figure 3.19). Subjecting some species of fish (Sundararaj and Vasal, 1976; Baggerman, 1980) and birds (Hamner, 1968; Lofts and Lam, 1973) to skeleton photoperiod regimes at different times of the year provides support for the idea of seasonal shifts in position of the photo-inducible phase. Additional evidence for a seasonal variation in position of the photo-inducible phase or at least the length of the critical photoperiod comes from work on the maturation cycle of a number of temperate zone birds. It must be emphasised that many of these species do not exhibit a circannual rhythm of maturation because of the phenomenon of photorefractoriness (Farner and Follett, 1966; Lofts and Murton, 1968). Photorefractoriness is characterised by birds becoming insensitive to long daylengths that were previously photostimulatory resulting in a collapse of the gonads. In many passerine species this refractory period cannot be overriden, not even by constant light and the birds have an obligate requirement for exposure to short days before photosensitivity is once more regained (Farner, 1959). Of relevance to the hypothesis is the observation that the transition from absolute refractoriness is characterised by a period of time when the ability to respond photoperiodically is totally lost through a state of relative refractoriness when the birds will respond to quite long photoperiods although not initially to the rather shorter ambient photoperiod (Hamner, 1968). This can be interpreted as a result of a gradual shift in the position of the photo-inducible phase although it fails to explain the period of total refractoriness. The Japanese quail (*Coturnix coturnix*) exhibits 'relative' refractoriness which means that during the refractory period the gonads will recrudesce if the birds are subjected to a long daylength. In a comprehensive study of this phenomenon, Robinson and Follett (1982) concluded that there was a seasonal shift in the critical daylength between 12-15 hrs. Although there is no evidence that this species exhibits a circannual rhythm of gonadal development, the results indicate that there is some form of endogeneous timing mechanism causing the seasonal change in the duration of the critical photoperiod.

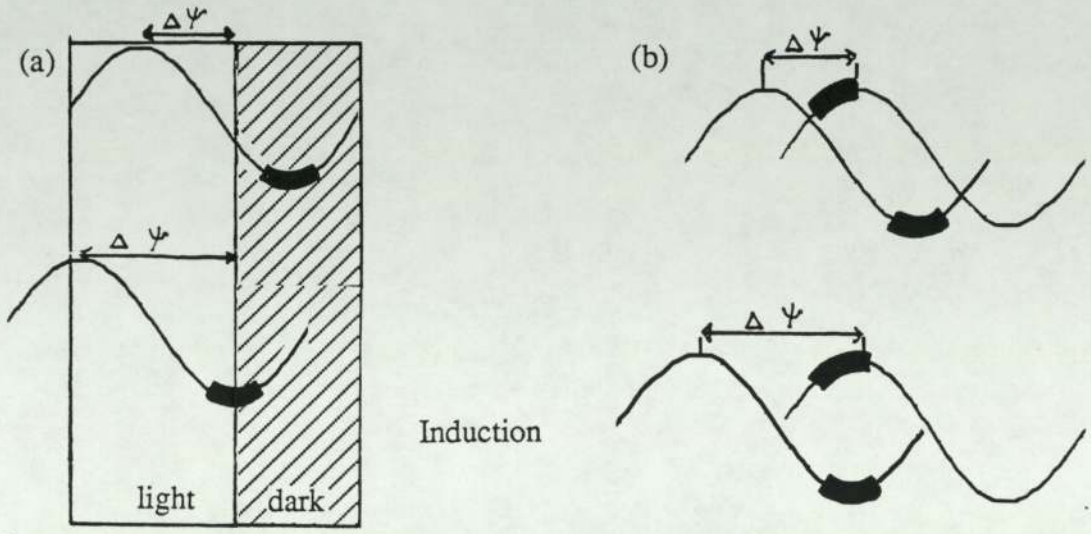



Fig. 3.19 Schematic representation of two hypotheses which derive circannual rhythms from spontaneous variations in an organism's circadian system. (a) External coincidence model: A circannual rhythm results from changes in the phase-angle difference( $\Psi$ ) between a circadian rhythm and its entraining light-dark cycle. In the above diagram induction occurs when the photoperiod illuminates a particular phase of the circadian rhythm (thickened part of the curve). (b) Internal coincidence model: A circannual rhythm results from changes in the phase-angle difference ($\Psi$) between two circadian rhythms. Induction only occurs when the two oscillators are in a particular phase relationship with each other. (Redrawn from Gwinner, 1973).

There are aspects of the maturation cycle of the rainbow trout maintained under constant photoperiods that can be explained by a circannual rhythm based on a circadian external coincidence system. The fish maintained under constant 18L:6D and LL exhibited a rhythm of maturation with a semi-circannual periodicity; it can be imagined that despite any circannual movement, these photoperiods permanently illuminated the photo-inducible phase. As soon as the oocytes had reached full maturation another batch of primary oocytes were almost immediately recruited into vitellogenesis. This view is supported by the serum hormone profiles of the fish in these treatments (Figures 3.11, 3.12) which increase within weeks of the ovulation of a previous batch of mature oocytes. In contrast the 6L:18D photoperiod resulted in the endogenous seasonal movement of the photo-inducible phase, only being illuminated at approximately yearly intervals resulting in the observed circannual rhythm in spawning. If this model is correct, the timing of spawning under the 6L:18D treatment is governed by the circannual movement of the photoinducible phase, whereas under the LL and 18L:6D regimes this circannual control is masked by the long photoperiods and the periodicity of the rhythm is governed by the rate at which a batch of oocytes can reach maturity. It is of interest to note that the rainbow trout does not appear to exhibit any form of refractoriness, under constant long days the fish continue to go through successive spawnings. From an adaptive point of view this is explainable; whereas in the bird refractoriness prevents breeding when the days are still 'long' but too late in the year to ensure the survival of the offspring and parents, the rainbow trout normally produces only one batch of eggs and takes approximately the whole year to mature them and thus has no need for any form of refractory mechanism. The attraction of this external coincidence model for circannual rhythms is that not only can it explain the observed spawning rhythms under constant photoperiods but is also incorporates circadian oscillators which were implicated in the control of spawning by the skeleton photoperiod experiments (Figure 3.7). However, a major criticism of the model is that it cannot explain how certain strains of rainbow trout can spawn at twice yearly intervals under natural photoperiod conditions (Lou *et al*, 1984). Other important observations are that brook trout (*Salvelinus fontinalis*) and catfish (*Heteropneustes fossilis*) maintained under continuous darkness mature successfully (Pyle, 1969; Sundararaj *et al*, 1982). These results are not compatible with the external coincidence model even though Sundararaj and Vasal's (1976) skeleton photoperiod experiments do indicate that such a system was involved in controlling the maturation of the catfish. Unfortunately, during the present study

it was not possible to test the external coincidence model by maintaining rainbow trout under constant darkness but hopefully this experiment will be performed in the near future.

Just as the external coincidence model can be extended to explain a possible mechanism for circannual rhythms so can the internal coincidence model. Pittendrigh and Daan (1976) described how two circadian oscillators, one entrained to dawn and the other to dusk could account for annual cycle of activity of the brown trout (*Salmo trutta*, Eriksson, 1973). They cited this example as a possible candidate for internal coincidence because it exhibited the phenomenon of splitting indicative of a control mechanism composed of two coupled circadian oscillators. To drive annually occurring events under constant environmental conditions it must be assumed such a system has a self-sustained circannual rhythm in the internal phase relationship between two or more circadian oscillators (Figure 3.19; Gwinner, 1973, 1981a). The best evidence in support of this hypothesis comes from work on the relationship between the circadian rhythms in the production and secretion of prolactin and corticosterone and annual cycles in the white throated sparrow (*Zonotrichia albicollis*). It is proposed that the changes in the phase relationship between these two hormones are responsible for seasonal variations in testicular growth, migratory fat deposition and also migratory orientation (Meier, 1976). Apart from Eriksson's (1973) work on the locomotor activity of the brown trout there is no indication from the present study whether an internal coincidence mechanism is operative in the control of the maturation cycle in fish. However, there is evidence that multiple oscillators are involved in the control of circannual rhythms in a variety of species and offer an explanation of the semi-circannual and circannual spawning cycles observed in the rainbow trout under constant conditions. The three models which have been discussed so far are all rather tentative and with the exception of the frequency demultiplication model still require a circannual oscillation in the photo-inducible phase or in the phase relationship between two circadian oscillators and thus they do not address the fundamental question of how the circannual rhythm itself might be generated. As circannual rhythms can be entrained by changes in the 24 hr photoperiod (see Chapter 4), and circadian rhythms are involved in daylength measurement in a variety of species, it is logical to suggest that circadian rhythms are closely linked if not an integral part of circannual rhythms. However, it is quite possible that circannual rhythms originate independently of circadian rhythms and the latter may only be of significance in connection with the process of synchronisation which may a separate physiological phenomenon.

Turning to circannual rhythms as oscillators in their own right there is evidence in some species that there exists independent, but normally synchronised circannual rhythms in the range of physiological parameters. Gwinner (1981b) noted that in some starlings (*Sturnus vulgaris*) maintained under 12L:12D a circannual rhythm of moult persisted for the whole experimental period whereas the rhythm of testis size waned after about one year; in contrast one individual showed a persistent rhythm in testicular size and an absence of a moult rhythm. Golden mantled ground squirrels (*Citellus lateralis*) maintained under 12L:12D and 35°C failed to hibernate but continued to show a circannual rhythm of food consumption and body weight; only when the temperature was dropped to 0°C did the animals proceed to exhibit a circannual rhythm of hibernation (Pengelley and Fisher, 1963). If, as their observations suggest, organisms can possess multiple circannual oscillators, it is conceivable that analogous to the internal coincidence model of circadian rhythms, coupled circannual oscillators are involved in the control of annually occurring phenomena such as gonadal maturation. The most convincing evidence that coupled circadian oscillators are involved in controlling 24 hr based rhythms is when they 'split' to produce two distinct phases. For example when hamsters (*Mesocricetus auratus*) are transferred to constant light the single circadian activity band commonly splits into two components that initially persist at different frequencies until they become locked 180° antiphase to each other (Pittendrigh, 1974). Support for this splitting theory comes from mathematical demonstrations that mutually coupled pacemakers do indeed have two alternative steady states, one with nearly 0° phase difference and the other with a 180° phase difference (Daan and Berde, 1978). It is tempting to speculate that the annual reproduction cycle of the rainbow trout is controlled by two mutually coupled circannual oscillators which under conditions of 18L:6D and LL split to be 180° out of phase with each other; thus explaining the observed semi-circannual rhythm of spawning. Obviously, this theory is very tentative and cannot explain why splitting fails to occur under constant 6L:18D but it remains a possible mechanism to explain semi-circannual rhythms of maturation in the rainbow trout and other species that exhibit this same phenomenon.

In conclusion, the numerous models based on oscillators serve to indicate the considerable uncertainty as to how circannual rhythms are generated. However, as has already been discussed it is probable that circannual rhythms evolved independently in a wide range of animal groups and thus it is possible that there are indeed a variety of mechanisms involved in their control and production. It has even been suggested that circannual rhythms are not generated by oscillators

but rather a sequence of linked stages, each one leading to the next (Mrosovsky, 1970, 1978). Menaker (1974) emphasised that circannual and circadian oscillators themselves must consist of sequences of interdependent steps and the question is, what are the steps and at what level of organisation do they occur? Mrosovsky's argument was based solely on data collected on the hibernation cycle of ground squirrels and it was suggested that the circannual rhythms of this event were rather too irregular in their periodicity to be considered to be driven by an internal oscillator. In support of the 'sequence of stages' hypothesis he suggested that the attainment of a heavy body weight is a prerequisite or a trigger to the onset of hibernation. This approach to explaining annual cycles is very interesting and in a certain respect is correct, for example, in the rainbow trout the process of vitellogenesis must precede the process of ovulation. However, it has already been revealed that in some species there is a high degree of independence among various circannual functions within individual animals arguing against the sequence of stages hypothesis. As far as the female rainbow trout is concerned the behaviour of the maturation cycle under constant environmental conditions and the response to a changing photoperiod regime (Chapter 4) can best be explained by the animal possessing some form of internal oscillator. A model based upon external coincidence is attractive because it can with some modification explain the approximate 5.5 and 12 month spawning cycles and accommodate both circadian and circannual oscillators, both of which were implicated by the experimental observations. However, it is possible that some form of internal coincidence and a sequence of physiological events involving the maturation rate of the ovary are also intimately in the control of the reproduction cycle. Only through further experimentation, some of which will be described in the proceeding chapters, will it be possible to gain a better understanding of the mechanism involved in the control of this complex process.

### 3.4.4 The adaptive importance of circannual rhythms

Having spent considerable time discussing the occurrence and possible mechanisms of circannual rhythms it is relevant to consider why animals possess internal timing mechanisms with a periodicity of approximately one year. The potential adaptive advantage of circannual rhythms is not immediately apparent simply because over most of the Earth's surface, with the exception of the equator, the annual cycle of daylength offers a reliable and noise free indicator of the time of year. Many organisms have the ability to measure daylength by means of either circadian or hour-glass mechanisms and could possibly rely solely on this

method to determine the time of year and to synchronise their annual behavioural and physiological rhythms accordingly.

A possible functional role of endogenous circannual rhythms is more understandable when the natural life cycle of the animal exhibiting the rhythm is considered. For example Pengelley and Kelly (1966) studied the circannual rhythms of hibernation in four closely related species of ground squirrels maintained under 12L:12D and either 12°C or 3°C. The rhythms ranged from strong and persistent in *Citellus lateralis* to almost non-existent in *C.tereticaudus*. *C.lateralis* generally live at altitudes ranging from 5,000 to 12,000 ft and are obliged to hibernate in deep burrows to avoid the harsh weather conditions of winter. While hibernating the animals would not experience any environmental indicators as to the time of the year and thus the possession of an endogenous circannual clock would be of significant adaptive importance. At the other extreme *C.tereticaudus* occupies a niche at a much lower altitude in the Colorado desert where the winters are more clement. Thus this species has almost abandoned a period of obligatory hibernation and retains only traces of an endogenous circannual rhythm. *C. mohavensis* exhibited an endogenous rhythm of hibernation of intermediate persistence compared to the two species already discussed. This species lives in low altitude desert regions, nevertheless the winter is quite hostile and the animal does exhibit a short period of obligatory hibernation and thus retains an endogenous timing mechanism. Finally *C. beecheyi* spans the range of physiological behaviour in *Citellus* in that it may undergo a period of obligatory hibernation as a result of an endogenous circannual rhythm or may remain strictly homeothermic. It is therefore most interesting to note that this species also spans the physical range of the other species. Thus, *C. beecheyi* is sympatric with *C.lateralis* in much, if not all, of the boreal life zone and is also sympatric with *C.tereticaudus* in the lower sonoran life zone. It is possible that the adaptability of *C.beecheyi* is in part the result of its ability to make use of an endogenous circannual rhythm in the form of hibernation where necessary or to abandon it and remain homeothermic where advantageous.

Birds that migrate over long distances would require rather complicated mechanisms to achieve precise timing of seasonal events if they relied exclusively on information provided by photoperiod because of the variation of daylength with latitude (Engels, 1959; Marshall, 1960; Gwinner, 1971). This is especially true with respect to the initiation of winter moult and spring migration in those individuals that winter close to the equator where seasonal changes in the environment are almost non-existent. Gwinner (1971) provided evidence that

birds migrating to equatorial regions make use of an endogenous circannual timing mechanism to maintain temporal orientation. He compared the circannual rhythms of *Zugunruhe*, body weight and moult in two palaearctic species of warbler, the willow warbler (*Phylloscopus trochilus*) which migrates from Europe to Central Africa close or beyond the equator, and the chiffchaff (*P. collybita*) which only migrates to the Mediterranean or North Africa. The willow warbler exhibited strong and persistent circannual rhythms in all three parameters whereas the chiffchaff produced only weak and erratic variations. The adaptive significance of the internal timing mechanism in the willow warbler is evident, it could control the preparation for the northward migration in the absence of any significant changes in the equatorial photoperiod. On the other hand, the chiffchaff remaining all year round in latitudes where there is a reliable and distinct annual change in the photoperiod would not have such a requirement for a circannual timing mechanism. In a development of these studies it has been shown that endogenous circannual clocks not only ensure the correct timing of physiological and behavioural changes but also have a very important role informing long distance migratory birds when to begin and end their flight and also assist in finding the correct destination (Gwinner and Wiltschko, 1978, 1980; Gwinner, 1986).

Thus by carefully considering the life cycles of animals that exhibit circannual cycles it is possible to ascertain why the possession of such a timing mechanism can be an important adaptive advantage. The pertinent question to ask is: what is the functional role of endogenous circannual rhythms in the rainbow trout? It is reasonable to assume that this phenomenon has not evolved as an adaptation to life under fish farm conditions and therefore one has to consider the life cycles of wild stocks. The rainbow trout (*Salmo gairdneri*) comprise of a very variable and complex group of related forms. Although they all breed in freshwater some strains such as the steelhead trout (*Salmo gairdneri gairdneri*) migrate to sea between spawnings while other strains such as the kamloop (*Salmo gairdneri kamloops*) spend their entire life in freshwater. According to Worthington (1940, 1941) the import of rainbow trout into Britain prior to 1890 were of the race that spend all their life in freshwater often referred to as shasta rainbow trout, but subsequently the crossing with other races such as the anadromous steelhead has led to mixed strains. To add to the confusion Needham and Behuke (1962) produced evidence to show even the original shasta rainbow trout were the result of a cross between a resident form and the anadromous steelhead. Thus it is possible that circannual rhythms in rainbow trout have evolved as an adaptation to a resident freshwater lifestyle or a migratory lifestyle

or both. Indigenous populations of rainbow trout generally inhabit waters at latitudes greater than 30°N and are normally exposed to a distinct annual variation in the photoperiod which would conceivably provide all the necessary information as to the time of year and make an endogenous circannual clock superfluous. It is reasonable to suggest then that a circannual timing mechanism would be of value to rainbow trout if they were unable to detect the changing photoperiod or if they migrated in a north-south plane where they would experience a rather complex variation in the daylength cycle. However, studies on the migratory movements of steelhead rainbow trout from the rivers of America and Canada into the Pacific ocean indicate that the fish tend to travel north-westwards between latitudes 40°N and 60°N (Baker, 1978) and would experience a regularly changing photocycle for the entire year. Seawater is optically quite clear with light penetrating to considerable depths (McFarland, 1986) indicating that the fish would be able to detect the changes in photoperiod satisfactorily. Thus, the role of a circannual clock in anadromous strains of rainbow trout when they are at sea is not apparent, however, like some migratory birds it may assist the fish in locating the correct destination. A more convincing explanation for the function of circannual timing mechanisms can be suggested for rainbow trout inhabiting exclusively freshwater habitats from where the Salmonids are thought to have evolved (Tchernavin, 1939). As these fish are found in temperate regions it is quite possible that during the winter the water surface is frozen and covered with a layer of snow effectively cutting the fish off from photic information for several months. Larson (1972) found that the timing of gonad maturation in brook trout (*Salvelinus fontinalis*) was similar under these conditions as those fish in ice-free lakes, suggesting that an endogenous circannual clock may have helped maintain them in temporal synchronisation with the changes in the environment. Similarly fish might be cut off from the photoperiod when the rivers are in flood and clouded by suspended sediments and other debris.

In conclusion, it is clear that under constant conditions, female rainbow trout exhibit a self-sustained, free-running rhythm of maturation indicating that they possess an internal circannual timing mechanism. The adaptive importance of this timing mechanism is open to speculation. Under most natural environmental conditions this internal clock is entrained to the annual change in the photoperiod and possibly other geophysical variables, maintaining the fish in temporal synchronisation with the seasons. The following chapter will study the entrainment of the maturation cycle of rainbow trout in more detail and will produce additional evidence to support the hypothesis of the involvement of an

endogenous circannual clock in this process.

# CHAPTER FOUR

# THE PHOTOPERIODIC ENTRAINMENT OF THE ANNUAL CYCLE OF MATURATION IN THE FEMALE RAINBOW TROUT

## 4.1 INTRODUCTION

The previous chapter introduced the concept of endogenous timing mechanisms and provided evidence that internally generated circannual and probably also circadian rhythms are involved in the control of the maturation cycle of the rainbow trout. In order to detect such endogenous rhythms in any organism it is necessary to maintain the experimental animals under constant environmental conditions. When isolated from environmental time cues the rhythms free-run and reveal an innate self-sustained periodicity. It must be emphasised that under natural conditions endogenous oscillators, be they circadian or circannual, do not free-run but are entrained to the changes in the environment, the most reliable indicator of which is the daily change in light intensity and the annual change in the photoperiod. When exposed to such entraining agents the endogenous oscillation adopts the exact periodicity of the environmental zeitgeber and also exhibits a particular phase relationship to it. This entrainment is very important to the survival of organisms, not only does it ensure that physiological and behavioural functions remain synchronised with changes in the environment, but it also keeps individuals in phase with each other. For example,in seasonally-breeding animals in temperate regions it is vital that males and females reach maturity at the same time and that the offspring are produced at a time of the year when their chances of survival are optimal.

Quantitative research on the entrainment of endogenous rhythms has been almost exclusively confined to oscillators of a circadian periodicity with studies on the pupal eclosion rhythm of the fruit fly *Drosophila pseudoobscura* being notable (Pittendrigh, 1960, 1965, 1966, 1981; Saunders, 1977). Although the experiments described in this chapter will be concerned with the synchronisation of the annual maturation cycle of the rainbow trout, because of the possible similarities in behaviour of circadian and circannual rhythms it will be helpful to our understanding to describe the important features of the entrainment of endogenous circa-24 hour oscillators.

One of the prerequisites for entrainment is a periodically changing sensitivity to the stimulus provided by the zeitgeber, the signal causing adjustments (or phase-shifts) of different magnitude or sign depending on the phase at which the circadian oscillation is perturbed. Under natural conditions the most significant entraining responses occur at dawn or dusk at times of the greatest changes in the light intensity. The usual experimental protocol used to elucidate the phase responses of a circadian oscillator to such signals is to place the organism in constant darkness and then expose it at different phases of its free-running rhythm to a short pulse of light (~ 15 minutes) and observe how the rhythm phase shifts. Since different organisms have widely different free-running periods, for analysis it is convenient to divide any circadian cycle into $360^{o}$

so that all comparisons are based on the same notational time scale. As a reference point, the phase that under 12L:12D would coincide with the light-dark transition is called $180^{o}$; the part of the cycle covering phases $0\text{-}180^{o}$ is then called the subjective day and the part covering $180\text{-}360^{o}$, the subjective night. A good example of a phase response of a free-running circadian rhythm exposed to a pulse of light is provided by a study of the activity rhythm of a nocturnal vole, *Microtus arvalis* (Daan, 1982). If $180^{o}$ represents the onset of nocturnal activity then a light pulse at $200^{o}$ (i.e. ~ 1 hr after the onset of activity under a 12L:12D regime) is interpreted by the animal as meaning that the circadian cycle has drifted foward relative to the light-dark cycle and it responds with a correcting backward shift in the rhythm, i.e. a phase delay (Figure 4.1a). Conversely if the vole is subjected to a pulse of light at $340^{o}$, about 1 hr before the subjective dawn, the animal interprets this as meaning that the circadian clock is running 'behind time' and compensates with a phase advance (Figure 4.1b). This vole is not exclusively nocturnal but emerges about every 2 hrs during the daytime for feeding trips, thus receiving a series of light pulses. It is clear then why such pulses during the subjective day ($0\text{-}180^{o}$) in constant darkness elicit no phase response (Figure 4.1c), the animal does not interpret these light periods as being indicative of circadian rhythm being out of phase with the zeitgeber. By scanning the entire circadian cycle with light pulses it is possible to draw a complete phase-response curve from $0\text{-}360^{o}$. This has been done for many plants and animals, several examples are shown in Figure 4.2. All are similar in having a phase delay in the beginning of the subjective night, phase advance in the late subjective night and a prolonged zone of near insensitivity to light pulses during the subjective day.

Once a phase response curve has been established for a particular organism it can be used to analyse the response of a circadian rhythm to a driving zeitgeber with a periodicity T (Pittendrigh and Minis, 1964; Eskin, 1971). When a circadian oscillation with a natural period $\tau$ is entrained by a zeitgeber with a period T, the former assumes the periodicity of the latter by undergoing phase shifts in each cycle ($T - \tau = \Delta\emptyset$, where $\Delta\emptyset$ = the magnitude of the phase shift). Taking the study of Pittendrigh and Minis (1964) as an example, these workers entrained the eclosion rhythm of *D.pseudoobscura* to recurrent 15 minute light pulses. For example, pulses 23 hrs apart defined a 23 hr cycle (T = 23) whereas pulses 25 hr apart defined T = 25 hrs. A general property of entrained rhythms is that when T is shorter than $\tau$ the endogenous cycle phase lags ($-\psi$) behind the zeitgeber (Figure 4.3a); conversely when T is longer than $\tau$ the endogenous rhythm will phase lead ($+\psi$) the zeitgeber (Figure 4.3b). This phase relationship between the zeitgeber and the endogenous oscillation is crucial to the process of

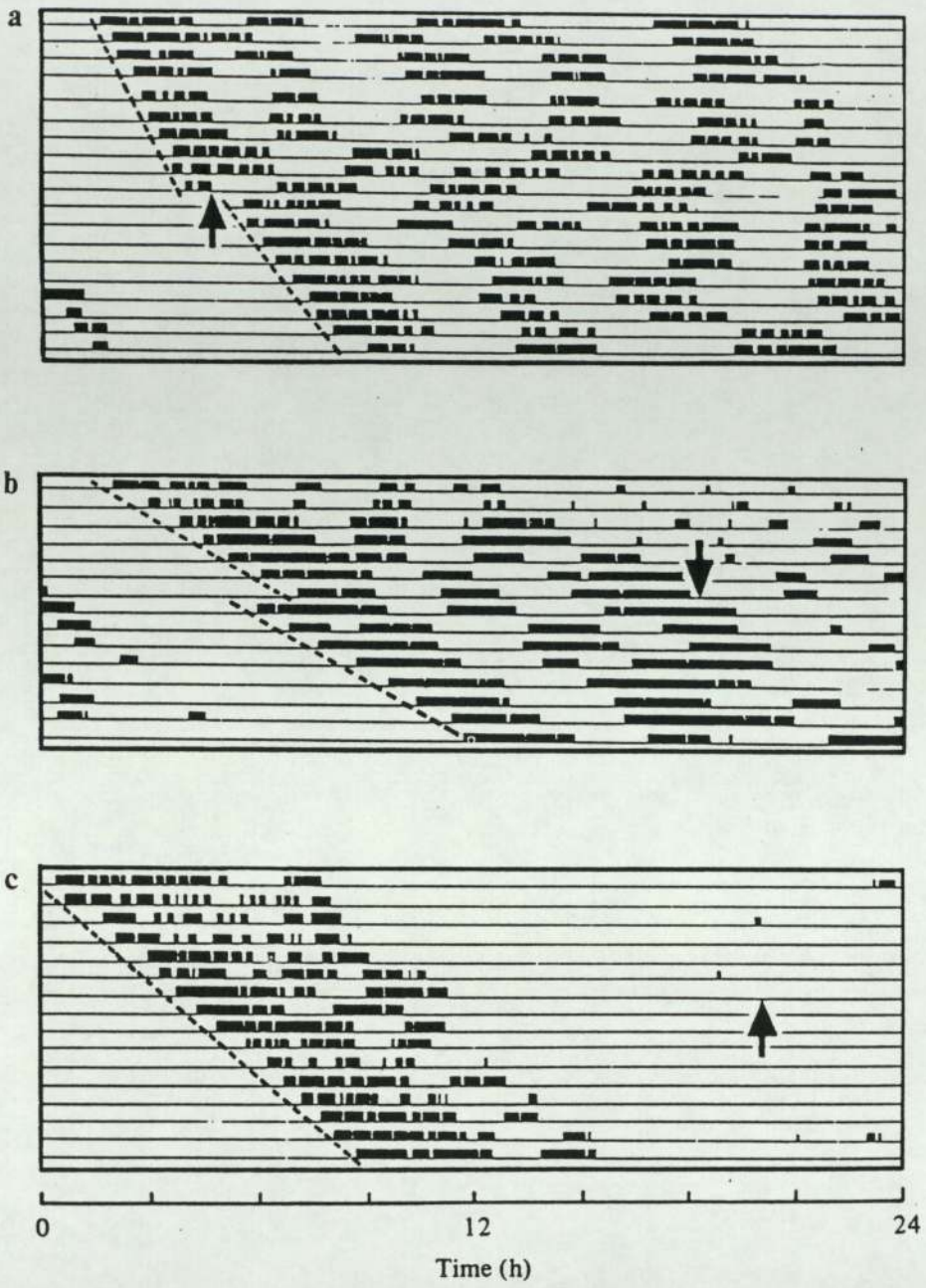



Fig 4.1. Phase-responses in the free-running locomotor activity rhythms in constant darkness of a vole (*Microtus arvalis*) to single light pulses of 20 mins(at arrows). The black boxes indicate times when the vole was active in its running wheel. 4.1a : around the time of activity onset the pulse produces a phase delay. 4.1b : later in the activity period the pulse produces a phase advance. 4.1c : in the rest period the pulse produces no response.(From Daan,1982).

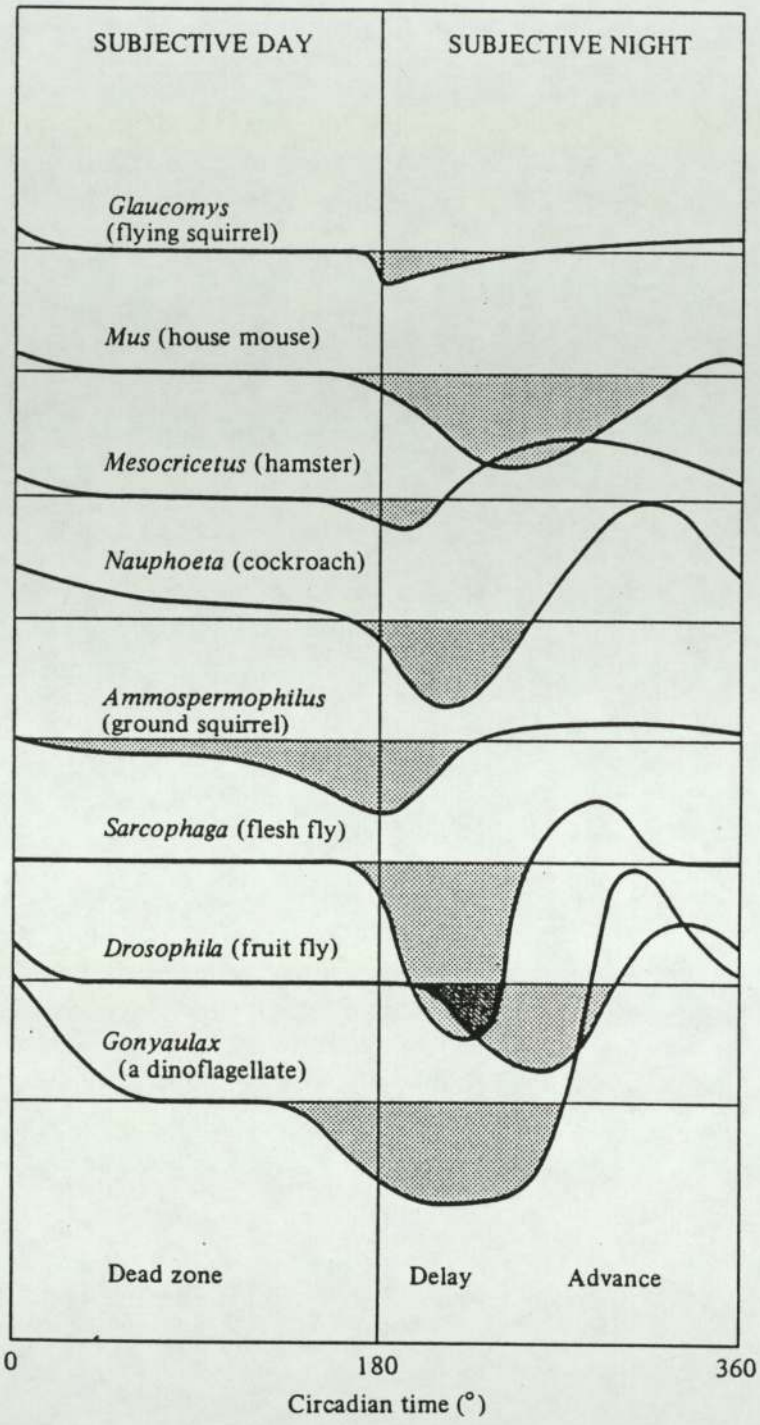



Fig 4.2. Phase-response curves in eight different organisms. The curves show the changing magnitude of the phase shifts in the various free-running rhythms to light pulses given at several different phases in the circadian cycle. Delay shifts(shaded) are plotted downwards, advance shifts upwards. (From Daan, 1982).

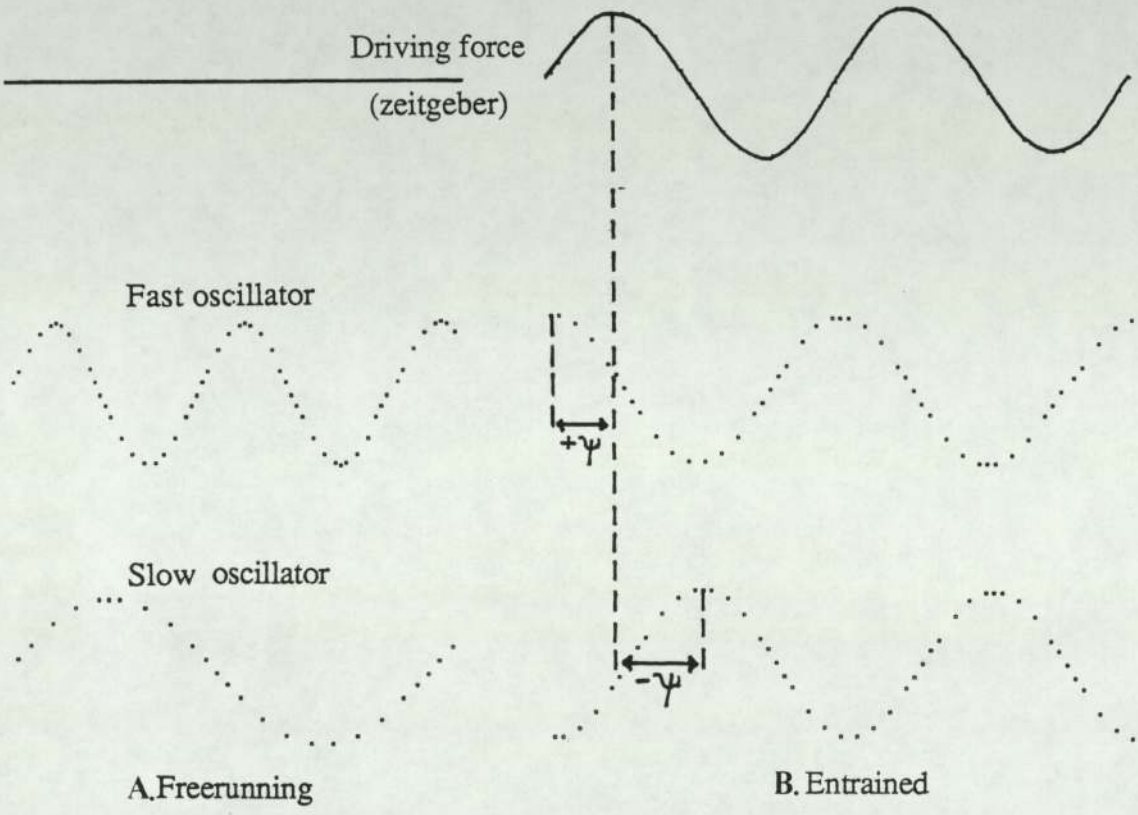



Fig. 4.3 Schematic illustration of the principles of entrainment. A) Two oscillations (dashed curves) freerun under constant conditions with different frequencies (fast and slow). B) When entrained to the same zeitgeber of medium frequency (solid curve), the fast oscillation phase leads ($+\psi$), and the slow oscillation phase lags ($-\psi$) the zeitgeber.

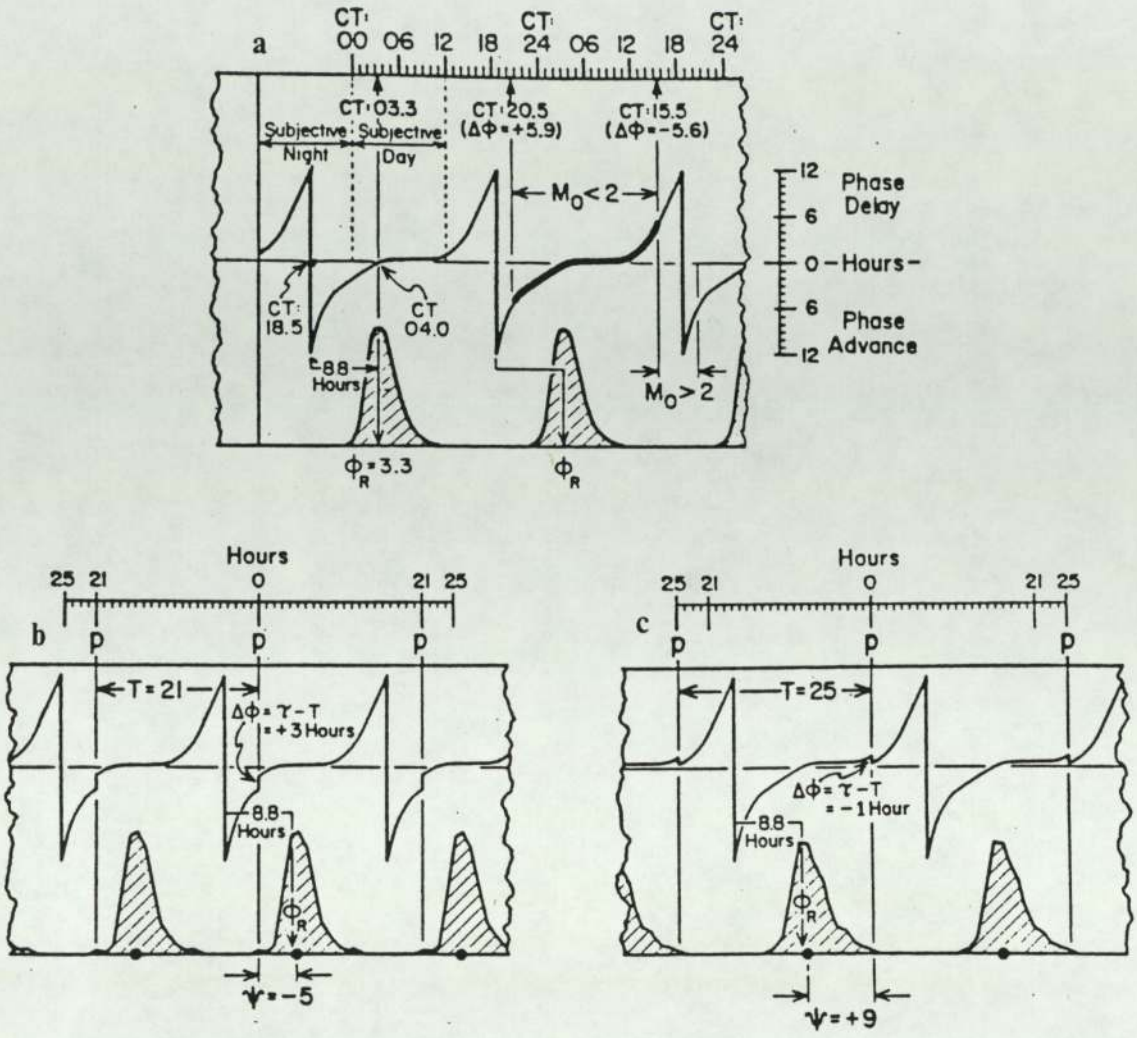



Fig. 4.4. Entrainment of the *D.pseudoobscura* eclosion rhythm to one 15-minute light pulse per cycle. (a) The "standard" phase-response curve; entrainment is only possible when the light pulse in the steady state falls at points on the phase-response curve where its slope (Mo) is -2 or less (heavy line). (b) Entrainment by light cycles in which a 15-minute pulse recurs every 21 hours(T=21). In T=21 the pulse causes a phase advance of 3 hours in each cycle, and falls at circadian time 23.3 (Ct= 23.3) in the late subjective night. (c) In T=25 the pulse causes a phase delay of 1 hour and falls at Ct 12.3 in the early subjective night. (From Pittendrigh, 1966).

entrainment. When T is shorter than $\tau$ the light pulse falls in each cycle during the late subjective night resulting in a phase advance (+ $\Delta\emptyset$) which serves to 'shorten' the period of the oscillator to that of the zeitgeber. When $\tau$ is shorter than T and the oscillation phase-leads the zeitgeber, the light pulse falls earlier in the subjective night, which causes a phase delay (- $\Delta\emptyset$); both result in entrainment. Pittendrigh's model for the entrainment of the eclosion rhythm of *Drosophila pseudoobscura* is summarised in Figure 4.4, it shows the relationship between the phase response curve and the eclosion rhythm, it also accommodates the observed entrainment of the endogenous rhythm to zeitgebers with periodicities shorter and longer than $\tau$. It is evident that the maximum values of $\Delta\emptyset$ of the phase response curve are determined by the limits of T to which a circadian oscillator can be entrained. Although Figure 4.4a shows that the maximum $\Delta\emptyset$ values approach 12 hrs, in practice entrainment is only possible when light pulse falls at points on the phase response curve where the slope is less than -2.0 (represented by the thick black line Figure 4.4a). Thus, the maximum utilizable $+\Delta\emptyset$ is 5.9 hrs and the maximum utilizable $-\Delta\emptyset$ is 6.6 hrs, and the range of entrainment for *D.pseudoobscura* is from about 18 to 30 hrs. This range of realizable T-values on either side of the natural period ($\tau$) is called the primary range of entrainment. In other organisms the range of entrainment is also dictated by the phase shifts which a light signal can generate and therefore can be determined from the phase-response curve. For example, in contrast to *D.pseudoobscura*, the flying squirrel (*Glaucomys volans*) exhibits a small amplitude phase response curve (Figure 4.2) and consequently has only a limited primary range of entrainment. The house finch (*Carpodacus mexicanus*) with a similar low amplitude phase response curve also has a correspondingly narrow range of entrainment of T = 21-26 hrs (Enright, 1965).

To summarise, there is good evidence that the entrainment of circadian rhythms involves a periodically-changing sensitivity to the stimulus provided by an environmental zeitgeber, this results in phase advances or delays of different magnitude depending on the phase at which the circadian oscillation is perturbed. The similarities in behaviour of circadian and circannual rhythms in that they both free-run under constant conditions, are self sustaining and both appear to be temperature compensated (Chapter 3), suggests that there may be analogies in the entrainment of these two types of rhythms. Indeed, there is evidence to indicate that circannual rhythms do have similar entrainment properties to circadian cycles. Goss *et al* (1974) showed that the circannual rhythm of antler growth in the sika deer (*Cervus nippon*) could be entrained to photocycles with periodicities ranging from 4 to 24 months. As the period of the photocycle was shortened, the rhythm of antler growth was observed to be progressively phase delayed. When

maintained under 2-month photocycles entrainment broke down and reverted to a circannual periodicity suggesting that the primary range of entrainment had been exceeded (Figure 4.5). Similar entrainment effects have been observed in studies on birds; Gwinner (1977) synchronised the circannual rhythm of gonadal development and moult in starlings (*Sturnus vulgaris*) with photocycles of periodicities ranging from 1 year to less than 3 months. As the periodicity of the photocycle decreased the phase of the testicular and moult rhythm became progressively delayed in agreement with general oscillator theory. Similarly, two cycles of gonadal development instead of the usual single breeding season have been observed in spotted munia (*Lonchura punctulata*; Chandola *et al*, 1985) and two species of warbler (*Sylvia borin, S.melanocephala*; Berthold, 1979) maintained under photocycles with a periodicity of 6 months.

In contrast to circadian rhythms which can be entrained by pulses of light, there is evidence that abrupt changes in the length of the photoperiod can cause phase shifts in the timing of annually occurring phenomena. Willow warblers (*Phylloscopus trochilus*) transferred from natural lighting conditions to 12L:12D in spring and early summer showed a phase advance of about 10 days in the ending of post-juvenile moult, whereas those transferred in late September showed a phase delay of 20-40 days in the onset of this process (Gwinner, 1971). Conversely birds transferred to 18L:6D in the spring and autumn showed a phase delay of about 10 days and a phase advance of 20-40 days respectively (Gwinner, 1971). As this species of bird has been shown to exhibit a circannual rhythm of moult (Gwinner, 1971), these results support the hypothesis that the entrainment of such long term endogenous rhythms conform to the oscillator theory developed primarily from circadian studies.

The main aim of the experiments described in this chapter is to test the hypothesis that the endogenous rhythm of maturation in the female rainbow trout also conforms to this entrainment theory. As described below, photoperiod has an important role in controlling the timing of maturation in salmonids and the present study attempts to quantify some of these effects.

Following the pioneer studies of Rowan (1926, 1930) and Bissonnette (1930, 1932) who manipulated the timing of sexual maturation in birds by the use of light, Hoover and Hubbard (1937) were the first to provide evidence that the timing of spawning in fish could be modified by photoperiod. Starting the experiment in early spring, brook trout (*Salvelinus fontinalis*) subjected to an artificial seasonal light cycle compressed into about 25 weeks spawned approximately 4 months ahead of control fish maintained under an ambient photoperiod. Subsequent studies on brook trout (Hazard and Eddy, 1951; Corson, 1955; Henderson, 1963), blueback salmon (*Oncorhynchus nerka*; Combs *et al*, 1959) and rainbow trout (Nomura, 1962) adopted similar accelerated

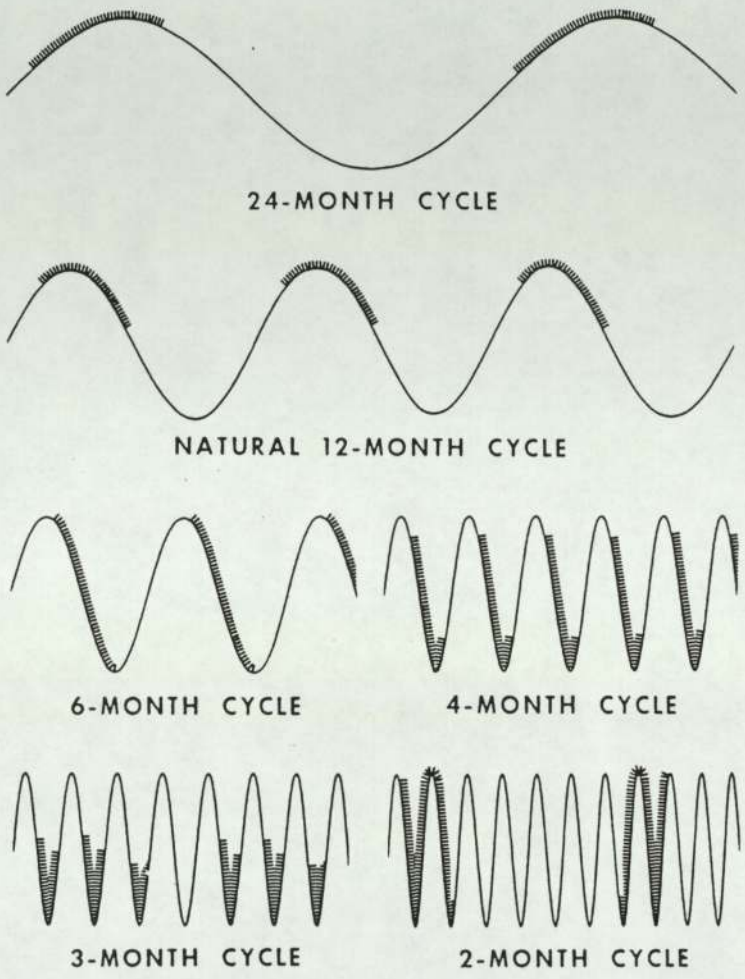



Fig. 4.5 Entrainment of the antler growth cycle of the sika deer (*Cervus nippon* ) to photocycles of different frequencies. Each curve represents a cycle of daylength changes to which the deer were exposed. Their periods in velvet, when the antlers were in the growing phase are indicated on each of the curves. Note the alteration in the phase angle between the entrained rhythm and the zeitgeber rhythm as the periodicity of the light cycle is reduced. The deer did not entrain to the 2-month photocycle indicating that the primary range of entrainment had been exceeded. (From, Goss *et al*, 1974 ).

seasonal cycles of daylength to produce advancements in the time of spawning. In more detailed investigations rainbow trout subjected to seasonal photocycles of 6 and 9 months proceeded to spawn 12 and 6 weeks respectively ahead of controls (Whitehead *et al*, 1978; Bromage *et al*, 1982a) while fish maintained under an 18 month cycle of daylength spawned approximately 3 months after trout on a natural photoperiod (Bromage *et al*, 1984). These particular studies on the rainbow trout provide good evidence that photoperiod entrains an endogenous rhythm of maturation and will be analysed in more detail in the discussion to this chapter. MacQuarrie *et al*, (1979) similarly advanced or delayed maturation respectively by subjecting Pacific salmon (*O.gorbuscha*) to a photocycle with a periodicity shorter or longer than normal for the first year of life and then at an out of phase 12 month cycle until spawning.

Even though some of the studies cited above monitored changes in the concentration of serum components associated with maturation (Whitehead *et al*, 1978; Bromage *et al*, 1982a) it is difficult to analyse the role of daylength in the control of spawning when continuously changing photoperiods are used in the experimental protocol. In this respect the use of constant photoperiods have proved to be a useful experimental technique. Allison (1951) induced a 6 week delay in the spawning of brook trout by maintaining fish under a natural photoperiod until August followed by constant 17L:7D. This delay in maturation achieved by 'long days' ($\geq$ 16L:8D) after the summer solstice has been observed in other studies on salmonids (Shiraishi and Fukuda, 1966; Eriksson and Lundqvist, 1980). Conversely maintaining salmonids on a natural photocycle until summer and then abruptly reducing the photoperiod to a constant short day ($\leq$ 8L:16D) resulted in an advancement in the timing of maturation compared to controls (Shiraishi and Fukuda, 1966; Eriksson and Lundqvist, 1980; Lundqvist, 1980; Takashima and Yamada, 1984). The use of constant photoperiods has been extended to cover the entire period from spring when early maturation is occurring (Scott and Sumpter, 1983) until spawning. A general conclusion drawn from these studies is that a long photoperiod from early in the year until spawning is stimulatory to gonadal development whereas a short photoperiod is inhibitory (Bromage *et al*, 1982b, 1984; Takashima and Yamada, 1984). The regime most stimulatory to gonadal maturation is a constant long photoperiod from early spring until around May-July followed by an abrupt reduction to a constant short photoperiod until spawning. This experimental procedure can advance the time of spawning by 3-4 months compared to controls (Bromage *et al*, 1982b, 1984; Takashima and Yamada, 1984). Conversely maintaining salmonids on a constant short photoperiod followed by a long photoperiod will result in a delay of up to 3 months in the time of spawning (Bromage *et al*, 1984). The overall conclusion that can be drawn from these investigations on salmonids is that a constant

long or an increasing photoperiod during the early period of gonadal maturation followed by a decreasing or constant short photoperiod can advance the time of spawning. Short photoperiods during early maturation, or long photoperiods during the later stages of gonadal development can result in a delay in the time of spawning. The degree to which spawning is advanced or delayed by a particular photoperiod appears to depend on the stage of maturation of the fish and also the photoperiod previously experienced by the experimental animals. A hypothesis proposed to explain these observations is that rainbow trout and possibly all salmonids have a requirement for different lengths of photoperiod at different stages of the annual reproductive cycle (Whitehead and Bromage, 1980; Bromage *et al*, 1982b,1984; Scott and Sumpter, 1983). Alternatively, having established that the rainbow trout exhibits an endogenously driven circannual rhythm of maturation (Chapter 3) it can be suggested that changes in photoperiod act as an entraining agent or zeitgeber to the internal rhythm. Thus, rather than photoperiod directly initiating and modulating the spawning cycle, its major role may be to entrain an endogenous oscillator or clock which ultimately controls maturation.

In an attempt to resolve whether light 'drives' or entrains the maturation cycle, this chapter describes a series of four experiments which study the effects of a range of photoperiod regimes on the timing of spawning in female rainbow trout. Experiments 1, 2 and 3 are directly comparable with each other in that they were performed in successive years on two year old female fish derived from the same breeding stocks; they were also carried out under identical experimental conditions (the only variable being photoperiod), and all of the experiments were started at the same time of the year (19.1.84 (Expt 1), 18.1.85 (Expt 2), 17.1.86 (Expt 3)). Experiment 4 was performed under different experimental conditions but the results are of relevance to the discussion on how photoperiod controls the timing of maturation. The account of each experiment takes the format of (i) a brief introduction, (ii) materials and methods, (iii) results and (iv) main conclusions. The main discussion to the chapter brings together the results and conclusions from all four experiments and compares these with previous studies on the effects of photoperiod on the timing of maturation in salmonids and other species. An attempt will be made to develop a hypothesis for the mechanism of the environmental and internal control of maturation in the rainbow trout which can be tested in future investigations.

## 4.2 Experiment 1: The effect of a reduction from 18L:6D to a range of shorter photoperiods (14L:10D, 10L:14D, 6L:18D) on the timing of maturation in the female rainbow trout

The introduction to this chapter reviewed the literature relating to the effects of photoperiod on salmonid maturation and concluded that there are two major hypotheses to explain how this control is mediated. One possible mechanism is that the rainbow trout and possibly all salmonids have a requirement for different lengths of photoperiod at different stages of the annual reproduction cycle (Whitehead and Bromage, 1980; Bromage *et al*, 1982b, 1984; Scott and Sumpter, 1983). Although this hypothesis has never been explicitly described it suggests that changes in the photoperiod have an essential 'driving' influence on the control of maturation. The alternative hypothesis is that photoperiod serves to entrain an internal circannual rhythm of maturation with abrupt changes in the daylength causing phase advances or delays in this rhythm.

In an attempt to resolve whether light drives or entrains the maturation cycle the present experiment studied the effect of a constant long photoperiod followed by direct reductions to a range of shorter photoperiods on the timing of maturation of female rainbow trout. If maturation is controlled by the length of photoperiod one would expect there to be a significant difference in the time of spawning between the groups. Alternatively if a direct reduction from a long to a shorter photoperiod acts only as an entraining cue for a circannual rhythm then the fish in each of the groups would be expected to attain maturation at similar times.

In order to follow maturation more closely, fish were blood sampled at approximately monthly intervals and the serum subsequently assayed for oestradiol-17ß, testosterone and vitellogenin (as calcium). It has already been established that these three serum components exhibit significant changes in concentration over the course of maturation (Scott *et al*, 1980; Bromage *et al*, 1982b; Elliott *et al*, 1984); their physiological role is discussed more fully in Chapter 1.

### Materials and Methods (Expt 1)

Four groups of 30 and one group of 18 (Grp A) virgin female 2 year old rainbow trout raised under ambient photoperiod (Lat 52° 30'N) and water temperature (8.5-9.0°C) with a natural spawning time starting in early December were maintained from 19th January (1984) in lightproof 1200 litre tanks under the following photoperiods:

Group A - 6L:18D constant (short day) until spawning
Group B - 18L:6D until 8th May followed by 6L:18D until spawning

Group C - 18L:6D until 8th May followed by 10L:14D until spawning
Group D - 18L:6D until 8th May followed by 14L:10D until spawning
Group E - 18L:6D constant (long day) until spawning

The photoperiod in each tank was timed by an electronic clock controlling a 40W bulb providing 25 lux at the water surface. Water temperature was 8.5-9.0$^{o}$C. Fish were fed 0.5% body weight day$^{-1}$ with a commercial trout diet. All fish were checked at monthly intervals to assess their state of maturity, and fortnightly once spawning had commenced. Blood samples were taken at approximately monthly intervals from a random sample of animals in each group. After centrifugation the serum was stored at -20$^{o}$C until analysis for oestradiol-17ß, testosterone and vitellogenin (as calcium). The eggs from mature fish were collected and after fertilisation and water hardening their total volume and mean diameters measured. One way analysis of variance (ANOVA) was used to analyse the statistical relationship between the hormone profiles. Differences in spawning time between the groups were compared by Student's t-test or by d and treating it as t if the variances were statistically dissimilar. For an account of these materials and methods see Chapter 2.

## Results (Expt 1)

The effects of the photoperiod treatments on the timing of spawning and the serum levels of oestradiol-17ß, testosterone and vitellogenin (as calcium) are described below:

Spawning: The time of spawning of individual fish is illustrated in Figure 4.6. In the groups in which the photoperiod was reduced from 18L:6D to 6L:18D (Grp B), 10L:14D (Grp C) and 14L:10D (Grp D) on 8th May, the first fish commenced spawning on the 3rd, 13th and 24th of September respectively. Subsequently, the remaining fish in each of these groups spawned within 6 week periods. Unfortunately, only four fish from Grp D survived to spawning as a result of an interruption of the water supply to this tank on 20th September; examination of the ovaries of the dead fish revealed that they were all within 2 weeks of ovulation. Under the constant 18L:6D regime (Grp E), the first fish spawned on 24th October but the last fish in this group did not spawn until 12 weeks later. Fish in Grp A (6L:18D) commenced spawning on the 1st March the following spring, approximately 5 months after Grp B. Excluding Grp D, the differences in spawning times between the groups were statistically significant (Grp B vs Group C: $P \leq 0.025$ and the remainder $P \leq 0.001$).

Oestradiol-17ß: Between the start of the experiment and the reductions in photoperiod on 8th May there were no statistically significant increases in serum oestradiol-17ß levels in any of the groups (Figure 4.7). On 15th May Grps B (18L:6D —> 6L:18D) and C (18L:6D —> 10L:14D) had significantly higher ($P \leq 0.001$) mean oestradiol-17ß levels than the other three groups. Between the May and June samples there were significant increases in the mean oestradiol-17ß levels of Grps B ($P \leq 0.001$), C ($P \leq 0.01$), D (18L:6D —> 14L:10D; $P \leq 0.001$) and E (18L:6D constant: $P \leq 0.05$). Grps B, C and D exhibited similar rates of increase in oestradiol-17ß titres reaching mean peak levels of 30-40ng $ml^{-1}$ in late July, early September and mid-September (estimate) respectively. By comparison the rate of increase of oestradiol-17ß in Grp E was slower and maximal levels were not reached until late September. The oestradiol-17ß levels of fish under constant 6L:18D (Grp A) remained at basal values until significant increases ($P \leq 0.05$) were detected between the September and October samples. Thereafter, the oestradiol-17ß levels increased rapidly to reach a peak of over 45ng $ml^{-1}$ in early December. This level was significantly higher than the oestradiol-17ß peak in Grp B ($P \leq 0.05$) but was not different from the corresponding values of the other groups. In all groups the oestradiol-17ß levels returned to basal values within three months of attaining their highest levels.

Testosterone: Serum testosterone levels remained basal in all groups until the middle of May (Figure 4.8). By late June the testosterone levels of Grp B were significantly higher ($P \leq 0.05$) than all the other treatments. By late June testosterone levels in Grps C and D were significantly higher ($P \leq 0.01$) than Grp A, and Grp C was significantly higher ( $P \leq 0.01$) than Grp E. Testosterone levels in Grps B and C increased at similar rates and both reached peak levels of 250-300 ng $ml^{-1}$ in early September. The testosterone levels of fish in Group D exhibited a similar profile to those in B and C but it was delayed by approximately 2-3 weeks. The constant 18L:6D treatment (Grp E) did not produce a significant increase in testosterone until August ($P \leq 0.05$) with peak levels of 250-300 ng $ml^{-1}$ being attained in early November. The testosterone levels of fish in Grp A (6L:18D) which remained basal until October, were significantly increased ($P \leq 0.001$) between the November and December samples and reached peak levels of over 400 ng $ml^{-1}$ in early February. There was no significant difference in the peak testosterone levels attained by the five different groups which in each case were reached just prior to ovulation before returning to basal values within two months.

Total serum calcium: The calcium levels in all groups remained at basal values (~ 10 mg 100 $ml^{-1}$ serum) until the middle of May (Figure 4.9). Grps B and C showed significant increases ($P \leq 0.05$) in calcium levels between the May and July samples. These two groups exhibited almost identical profiles of total calcium levels, both reaching peak levels of approximately 35mg 100 $ml^{-1}$ in late September before falling to basal levels in December. The calcium levels of Grp D increased significantly ($P \leq 0.05$) between May and July although the rate of increase was slower than Grps B and C and with the levels in early September being only half those of Grps B and C. The calcium levels of fish held on a constant long day (Grp E) became significantly elevated ($P \leq 0.05$) above basal during August to reach a mean peak level of 29 mg 100 $ml^{-1}$ in December. The calcium levels of the fish maintained on constant short days (Grp A) remained at basal values until October when they rose significantly ($P \leq 0.01$) to a level of approximately 20 mg 100 $ml^{-1}$. This concentration, which was significantly lower ($P \leq 0.05$) than the peak $Ca^{2+}$ level in Grps B and C, was maintained for the months of November through to April before falling towards basal.

Egg size: Mean egg diameter (± standard error mean) of September-October spawning Grps B, C and D were 4.16 ± 0.03mm (n=30), 4.19 ± 0.03mm (n=28) and 4.17 ± 0.06mm (n=4) respectively. Fish maintained under constant 18L:6D (Grp E) had a mean egg diameter of 4.49 ± 0.17mm (n=29), and the March spawning fish in Grp A, 4.85 ± 0.04mm (n=8).

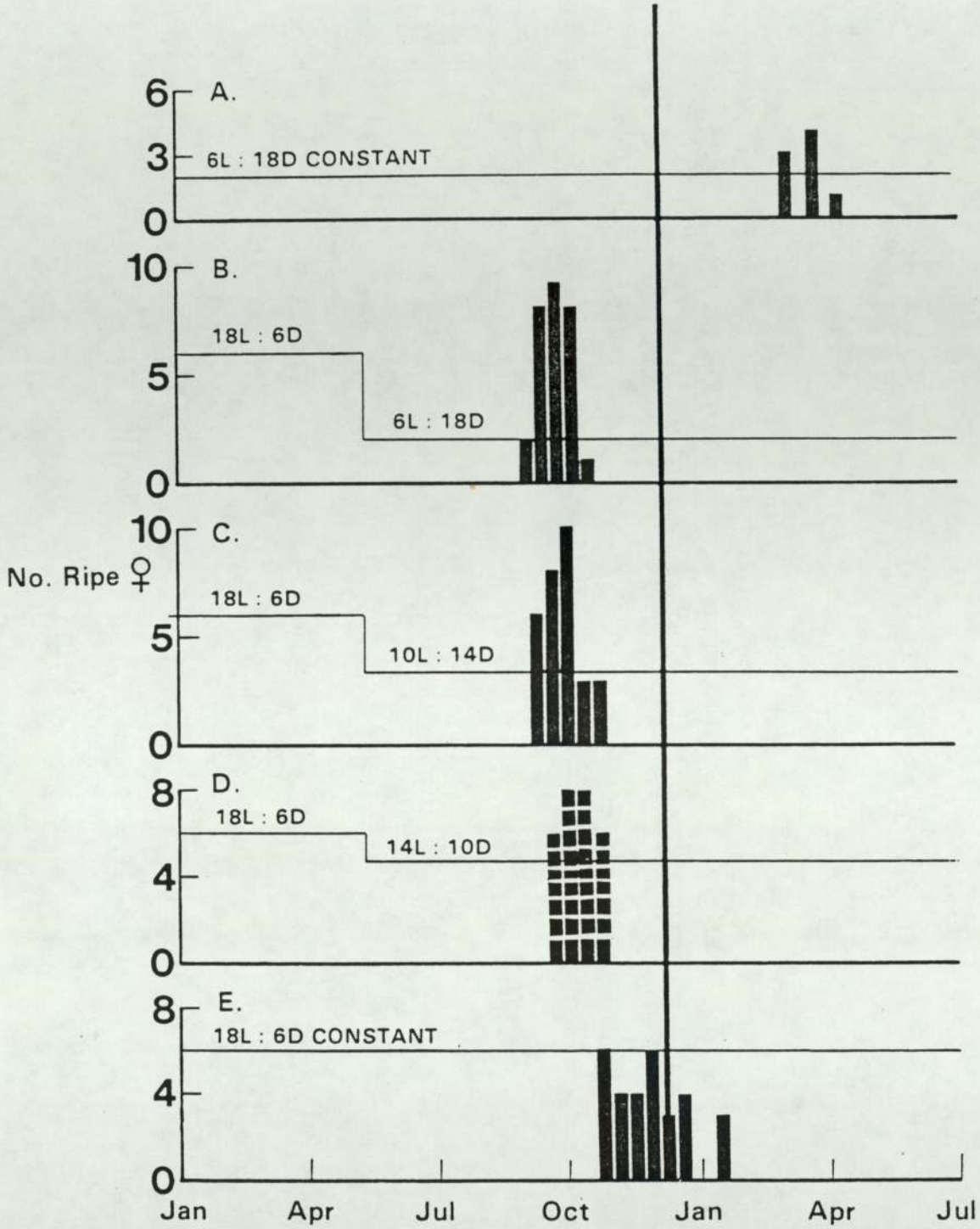



Fig. 4.6. The effect of five photoperiod regimes (Groups A-E) on the timing of maturation of female rainbow trout. Histograms illustrate the number of mature females (No. ripe ♀) at each sampling time. The broken histogram in Group D represent an estimate of the spawning time (see Results). The vertical line running through all five histograms indicates the onset of spawning of rainbow trout of the same stock maintained under natural photoperiod. The photoperiod regime in each treatment is indicated by the horizontal line. The vertical lines on the x-axis refer to the first day of the month as labelled.

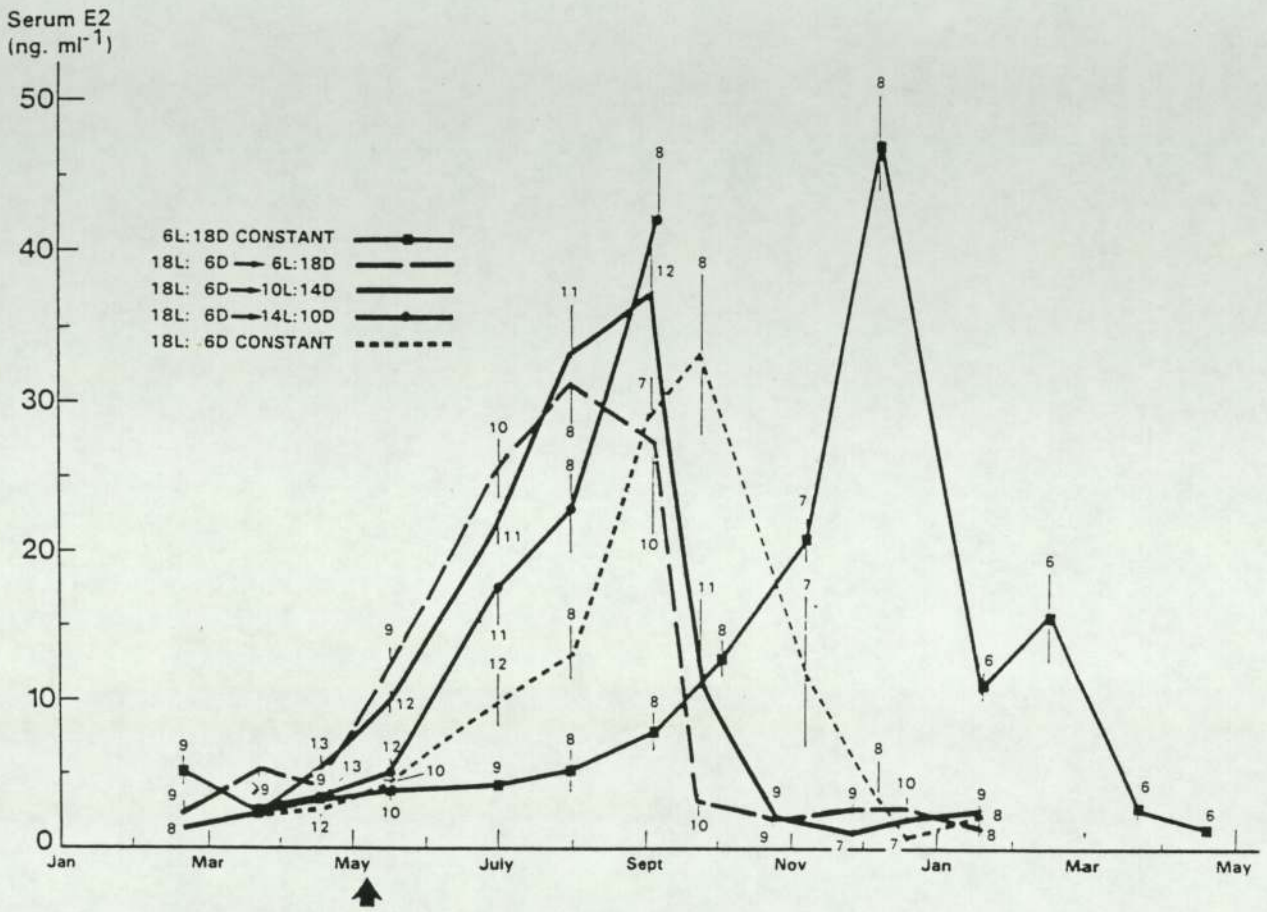



Fig. 4.7. The sequential changes in the mean levels of serum oestradiol-17ß (E2, ng ml$^{-1}$) in female rainbow trout maintained under the five photoperiod regimes: 6L:18D constant (Group A), 18L:6D → 6L:18D(Group B), 18L:6D → 10L:14D(Group C), 18L:6D → 14L:10D(Group D), and 18L:6D constant (Group E). The integer above each coordinate indicates the number of fish sampled (n) with the vertical bars representing ± one standard error of the mean. The arrow(⬆) indicates the time of the reduction in photoperiod(Groups B,C and D only). The vertical lines on the x-axis refer to the first day of the month as labelled.

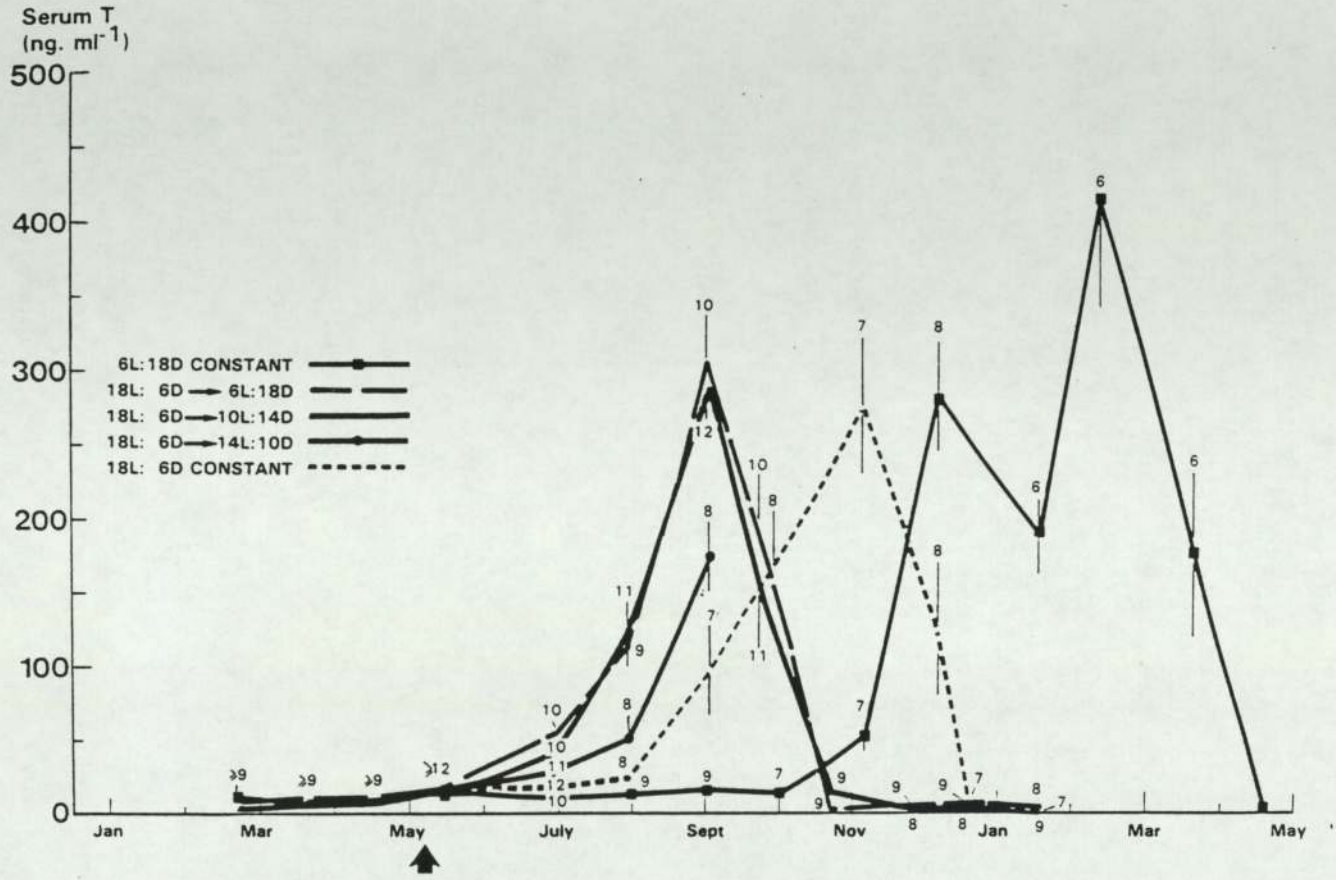



Fig. 4.8. The sequential changes in the mean levels of serum testosterone(T, ng ml$^{-1}$) in female rainbow trout maintained under five photoperiod regimes: 6L:18D constant(Group A), 18L:6D → 6L:18D(Group B), 18L:6D → 10L:14D(Group C), 18L:6D → 14L:10D (Group D), and 18L:6D constant (Group E). The integer above each coordinate indicates the number of fish sampled(n) with the vertical bars representing ± one standard error of the mean. The arrow (⬆) indicates the time of the reduction in photoperiod(Groups B,C and D only). The vertical bars on the x-axis refer to the first day of the month as labelled.

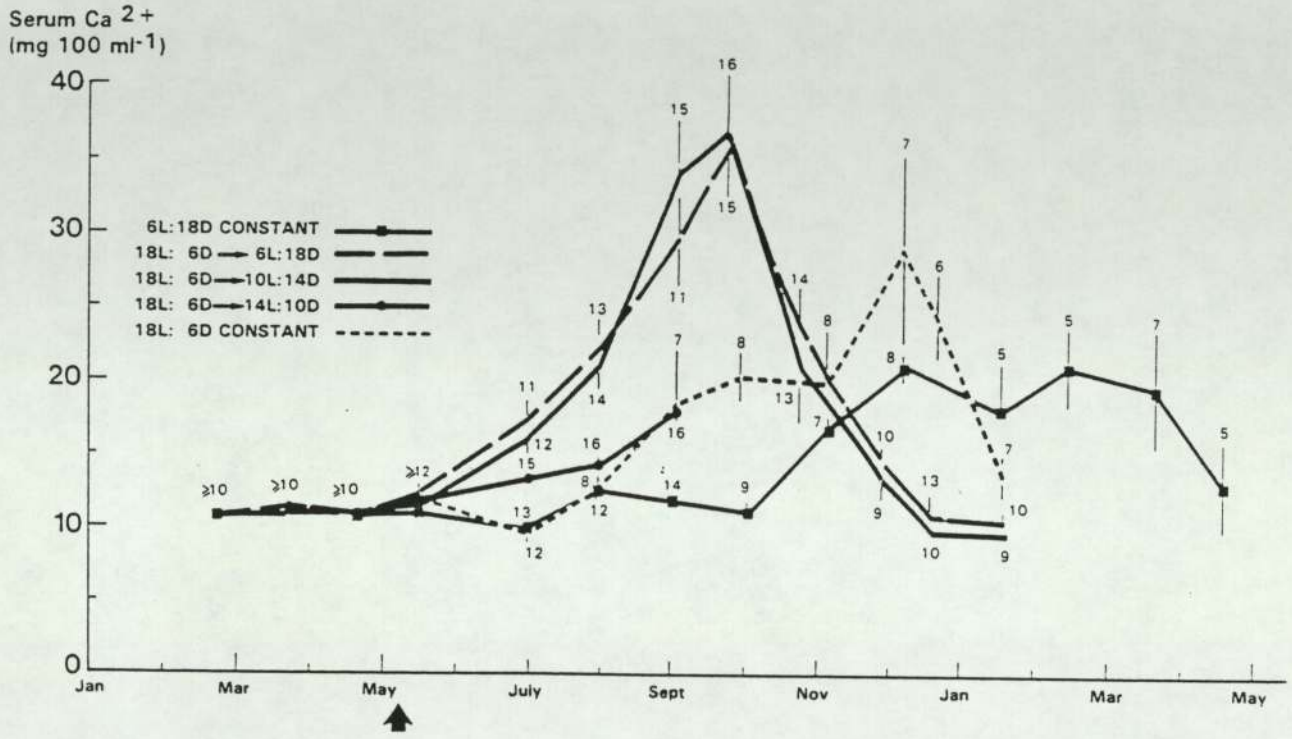



Fig. 4.9. The sequential changes in total serum calcium ($Ca^{2+}$; mg $100ml^{-1}$) in female rainbow trout maintained under the five photoperiod regimes: 6L:18D constant(Group A), 18L:6D → 6L:18D(Group B), 18L:6D →10L:14D(Group C), 18L:6D → 14L:10D(Group D) and 18L:6D constant(Group E). The integer above each coordinate indicates the number of fish sampled(n) with the vertical bars representing ± one standard error of the mean. The arrow (↑) indicates the time of the reduction in photoperiod (Groups B, C and D only).The vertical lines on the x-axis refer to the first day of the month as labelled.

Conclusions (Expt 1)

The results clearly show that the abrupt reduction in photoperiod produced an advancement of the timing of spawning in the rainbow trout. Similar photoperiod-induced advancements in the timing of maturation in salmonids have been observed by other groups (Henderson, 1963; Shiraishi and Fukuda, 1966; Bromage *et al*, 1984; Takashima and Yamada, 1984). The fact that the groups subjected to a reduction in photoperiod commenced spawning at least 2 months before fish on an ambient daylength, but all within 3 weeks of each other indicates that the magnitude of the reduction in photoperiod had only a minor or supplementary role in modifying the time of spawning. The reduction in photoperiod had the additional effect of synchronising the time of spawning of individual animals within each treatment so that the period of time between the spawning of the first and last fish was only 6 weeks; this is similar to rainbow trout maintained under ambient conditions (Kato, 1973). In contrast the corresponding spawning period of the fish under constant 18L:6D was 12 weeks.

In general the effects of photoperiod on the time of spawning were confirmed by the changes in the levels of serum oestradiol-17ß, testosterone and total calcium. Within a week of the reduction in photoperiod on the 8th May increases were detected in serum oestradiol-17ß levels. Figure 4.7 clearly shows that the increases in the oestradiol-17ß levels were more pronounced the greater the reduction in photoperiod. By late June a clear gradation in the values of serum oestradiol-17ß were followed by increases in testosterone and total calcium. Over the course of maturation the relationship between the changes in these three parameters within each group were similar to those observed in previous studies (Scott *et al*, 1980; Scott and Sumpter, 1983; Elliott *et al*, 1984), however there were differences between the experimental groups in the timing of these changes which resulted in the different spawning times. The oestradiol-17ß and testosterone levels in each group reached peak levels approximately one month and one week before ovulation respectively. The slightly broader and flatter profiles of the serum components of the fish under the constant 18L:6D treatment were almost certainly due to the desynchronisation of maturation and spawning of individual animals in this group rather than any effect of photoperiod on serum hormone levels. Total calcium levels peaked around the time of spawning but did not return to basal until approximately 3 months after ovulation. The group that spawned last (Grp A) had a significantly higher mean oestradiol-17ß peak ($P \leq 0.05$) and a significantly lower mean calcium peak ($P \leq 0.05$) compared to the September spawning Group B. Similar observations have also been made by Elliott *et al* (1984) and it is suggested that these differences in hormone levels may be related to the increase in egg size which invariably occurs when the timing of spawning is delayed. The fish spawning in September and October had oocyte

diameters that were at least 0.5mm smaller than those produced by fish spawning later in the following March. The changes in the levels of these serum components together with other hormones are of importance in the control of growth of the ovary as have been discussed in Chapter 1. However, as the physiological mechanisms in the central nervous system controlling maturation remain to be elucidated, at this stage it is convenient to consider the rainbow trout as a 'black box' and construct a hypothesis for the control of this process based on photoperiod 'input' and timing of spawning 'output'.

Although experimental changes in daylength can significantly alter the timing of maturation, the ability of the trout in Groups A and E to spawn successfully under constant short and long days respectively indicates that changes in the environmental photoperiod are not essential for maturation. This view confirms the results presented in Chapter 3 which showed that when maintained under constant environmental conditions rainbow trout continued to exhibit an endogenously driven circannual rhythm of maturation. This evidence alone strongly indicates that the maturation cycle of the rainbow trout does not have specific requirements for different photoperiods at different stages of the reproductive cycle as suggested by some earlier work (see Introduction). This view is also supported by the results of the three treatments groups which were subjected to constant long and then different lengths of shorter photoperiods; they all proceeded to spawn at similar times. These results suggest that the reduction in photoperiod *per se* could be serving as an entrainment cue, advancing the time of spawning by causing a phase advancement in an endogenous circannual timing mechanism associated with the control of maturation. This entrainment hypothesis is supported by the observation that the abrupt reduction in photoperiod synchronised the spawning of the individual animals in each treatment so that the spawning period for each of these groups was confined to 6 weeks. In contrast the spawning period of the fish maintained under constant 18L:6D lasted for 12 weeks. A desynchronisation of spawning time was also reported in Chapter 3 and in an earlier study of rainbow trout maintained under constant photoperiods (Bromage *et al*, 1984). The similarity of these results to the behaviour of circannual rhythms in other vertebrates (Pengelley and Asmundson, 1974) indicates that the loss of synchrony is due to the free-running of the endogenous rhythm which then becomes entrained if the experimental animals are subjected to a change in photoperiod.

If the direct reduction in photoperiod in May served to advance the phase of the circannual rhythm of maturation, it is apparent that the change in photoperiod experienced by the fish at the start of the experiment in January when they were taken from a short ambient photoperiod into 18L:6D (Grps B-E) or 6L:18D (Grp A) was also

having an entraining influence on the endogenous rhythm. This may explain why rainbow trout on constant 18L:6D (Grp E) and those on constant 6L:18D (Grp A) spawned 6 weeks before and 3 months after fish on an ambient photoperiod respectively. The direct increase from an ambient photoperiod of ~ 8.5 hrs in mid-January (Lat 52° 30'N) to 18L:6D (Grp E) may have been responsible for the phase advance in the circannual rhythm whereas the reduction to 6L:18D (Grp A) may have caused a phase delay. These results are still compatible with the idea that direct changes in the photoperiod are serving to synchronise the maturation rhythms of individual animals within an experimental group. A possible reason why the constant 18L:6D group exhibited a desynchronised spawning profile is that they received their only entraining cue in mid-January and the maturation rhythm proceeded to free-run from that date. A desynchronised spawning profile perhaps would have been more clearly observed in the constant 6L:18D group if a larger number of fish had been stocked in this treatment. In contrast, the fish in groups B, C, D all received an additional entraining cue on 8th May in the form of a direct reduction in the photoperiod. It is suggested that the endogenous rhythms of maturation in these animals proceeded to free-run after this date but with only 4 months or so remaining until their projected ovulation they did not have as much time to lose synchrony as those maintained under constant conditions from 19th January.

The hypothesis that abrupt changes from one constant photoperiod to another provide entraining cues for an endogenous circannual rhythm of maturation appears to provide the best explanation of the experimental results. However, the fact that there was a relationship between the magnitude of the reduction in photoperiod and the timing of the changes in serum oestradiol-17ß, testosterone and calcium, resulting in small differences in the onset of spawning between the groups, indicates a more complex mechanism. Results from skeleton photoperiod experiments described in Chapter 3 and similar studies on other fish (Chan, 1976; Sundararaj and Vasal, 1976; Baggerman, 1980) suggest that circadian rhythms of photosensitivity may also be involved in the control of the timing of maturation. These ideas will be given further attention in the discussion to this chapter. At this stage the hypothesis that abrupt changes in the photoperiod can entrain a circannual rhythm of maturation provides a working model which can be tested in the next experiment.

## Summary

(i) An abrupt reduction from constant 18L:6D to a range of shorter constant photoperiod provided an important entraining cue in advancing the time of spawning of female rainbow trout. The reduction in photoperiod also synchronised the timing of spawning of individual animals within an experimental group.

(ii) The magnitude of the reduction in photoperiod had only a minor or supplementary role in advancing the time of spawning.

(iii) Rainbow trout do not have specific requirements for certain photoperiods at different stages of the reproductive cycle.

(iv) The results support the hypothesis that changes in the photoperiod serve to entrain an endogenous circannual rhythm of maturation rather than having a direct, driving influence on the control of reproduction.

## 4.3 Experiment 2: The effect of a reduction in photoperiod from 18L:6D to 6L:18D at different times of the year on the timing of maturation in the female rainbow trout.

The results from the previous experiment showed that a reduction from a constant long to a constant shorter photoperiod advanced the time of spawning in female rainbow trout. It was hypothesised that this advance in the timing of ovulation was due to the abrupt reduction in photoperiod in May causing a phase advance in an endogenous circannual rhythm of maturation. To investigate more fully the effect of a reduction in photoperiod on the advance of maturation, the current experiment maintained groups of fish from mid-January on 18L:6D for 6, 10, 14 or 18 weeks before abruptly reducing the photoperiod to 6L:18D. In addition one group of fish remained on constant 18L:6D throughout the experiment (control). If the entrainment hypothesis stated above is correct, then these experimental treatments would be expected to result in advances in the timing of spawning which were directly related to the timing of the reduction in photoperiod.

### Materials and Methods (Expt 2)

Five groups of 18-25 virgin female 2 year old rainbow trout raised under ambient photoperiod (Lat 52° 30'N) and temperature (8.5-9.0°C) with a natural spawning time starting in early December were maintained from 18th January (1985) in lightproof tanks under the following photoperiods:

Group A - 18L:6D until 1st March followed by 6L:18D until spawning
Group B - 18L:6D until 1st April followed by 6L:18D until spawning
Group C - 18L:6D until 1st May followed by 6L:18D until spawning
Group D - 18L:6D until 1st June followed by 6L:18D until spawning
Group E - 18L:6D constant (long day) until spawning.

The photoperiod in each tank was timed by an electronic clock controlling a 40W bulb providing 25 lux at the water surface. Water temperature was 8.5-9.0°C. Fish were fed 0.5% body weight $day^{-1}$ with a commercial trout diet. All fish were checked at monthly intervals to assess their state of maturity, and fortnightly once spawning had commenced. Blood samples were taken at approximately monthly intervals from a random sample of animals in each group. After centrifugation the serum was stored at -20°C until analysis for oestradiol-17ß and vitellogenin (as calcium). The eggs from each mature fish were collected and after fertilisation and water hardening the

mean oocyte diameter was measured. One way analysis of variance (ANOVA) was used to analyse the statistical relationship between the hormone profiles. Differences in spawning time between the groups were tested by Student's t-test or by d and treating it as t if the variances were statistically dissimilar. For an account of these materials and methods see Chapter 2.

Following the maturation of the experimental animals in autumn 1985, a small sample of fish from Grps A-D were maintained for a further year on 6L:18D. This sample (designated Grp F) included both spawners and non-spawners from 1985. The purpose of retaining these fish was to investigate the effect of the reduction in photoperiod on the timing of subsequent spawnings.

## Results (Expt 2)

Spawning: The time of spawning of individual fish is illustrated in Figure 4.10. In the groups in which the photoperiod was reduced from 18L:6D to 6L:18D on 1st March (Grp A), 1st April (Grp B), 1st May (Grp C) and 1st June (Grp D) spawning commenced on 31st July, 13th and 30th August and the 16th September respectively. Under constant 18L:6D (Grp E) the fish commenced spawning on 10th October. The differences in the spawning times between groups were all statistically significant at the $P \leq 0.001$ level with the exception of Grp B vs Grp C ($P \leq 0.05$) and Grp A vs Grp B ($P \leq 0.05$). The remaining fish in groups A, B, C, D and E spawned within 6, 8, 7, 5 and 10 weeks respectively. Using an F-test (2-tailed) the variance of the spawning profile of Grp E was found to be significantly greater ($P \leq 0.025$) than those of Grps C and D. A comparison of the variances of the other groups revealed no significant differences.

Figure 4.10 also illustrates the proportion of females in each treatment which did not mature. In groups A, B, C and D 74%, 48%, 23% and 8% of the fish respectively failed to mature in 1985. Of those fish maintained on constant 18L:6D (Grp E) 22% of the fish had not matured by the end of 1985. Figure 4.11 illustrates the timing of spawning of fish in Grp F. With the exception of fish numbers 348 and 364 which exhibited a semi-circannual rhythm of spawning, those trout which matured in August-September 1985 ovulated again in 1986 with a periodicity which was generally slightly shorter than 12 months. The sample of 5 fish which failed to mature in 1985 were spawned either on 6.8.86 or 3.9.86.

Serum oestradiol-17ß and vitellogenin (as calcium):

Group A: The mean changes in the serum levels of oestradiol-17ß and total calcium in spawning and non-spawning fish are shown in Figure 4.12a. Of the maturing fish significant increases ($P \leq 0.05$) in serum oestradiol-17ß were detected between 18th March and 4th July on which date they reached a mean peak level of 23.9 ng $ml^{-1}$ before returning to basal values in late August. The total serum calcium levels increased significantly ($P \leq 0.05$) between the 22nd May and 4th July samples on which date they reached a peak level of over 60mg 100 $ml^{-1}$ before returning to basal values in September. Of those fish which failed to spawn in 1985, serum oestradiol-17ß and calcium levels remained at basal levels.

Group B: After the reduction in photoperiod on 1st April, those fish which subsequently matured that year exhibited a significant increase ($P \leq 0.05$) in mean serum oestradiol-17ß levels between the 19th April and 22nd May samples reaching a mean peak level of 25.9 ng $ml^{-1}$ on 31st July before returning to basal by late September (Figure 4.12b). Mean total calcium levels increased significantly during July ($P \leq 0.01$) reaching a peak level of 101 mg 100 $ml^{-1}$ in late August before returning to basal by late November. Those fish that failed to spawn did not exhibit any significant changes in serum oestradiol-17ß levels, however the total calcium levels in September were significantly higher than the basal levels of the April serum samples.

Group C: Serum oestradiol-17β levels remained basal until the reduction in photoperiod on 1st May after which significant increases ($P \leq 0.001$) were detected between the 22nd May and 4th July samples (Figure 4.12c). Peak levels of 27.9 ng $ml^{-1}$ were reached on 31st July before returning to basal levels in mid-September. Similarly total calcium levels increased significantly ($P \leq 0.05$) between the May and early July samples, but the mean peak level of 82.5mg $100ml^{-1}$ did not occur until mid-September approximately 1.5 months after the serum oestradiol-17ß peak subsequently returning to around basal levels in December. Of the non-spawning fish in Grp C, no statistically significant changes were detected in serum oestradiol-17ß or total calcium levels.

Group D: Serum oestradiol-17ß levels remained at basal values up to the reduction in photoperiod after which significant increases ($P \leq 0.01$) were detected between the 1st June and 4th July samples (4.12d). Oestradiol-17ß levels reached a peak of 26.6 ng $ml^{-1}$ on 31st July before returning to basal in late October. Total calcium levels of maturing fish increased significantly during July ($P \leq 0.01$) reaching peak values of

65-70 mg 100 $ml^{-1}$ in late September, almost two months after the oestradiol-17ß peak before returning to around basal levels in December. There were insufficient data from the non-spawning fish in this group to analyse the changes in serum levels of oestradiol-17ß or total calcium.

Group E: Those fish which remained on constant 18L:6D throughout the experiment exhibited significant increases ($P \leq 0.01$) in serum oestradiol-17ß levels during July reaching a mean peak value of 17.5 ng $ml^{-1}$ in late August before slowly returning to basal levels in December (Figure 4.12e). Total serum calcium levels became significantly elevated ($P \leq 0.05$) during August reaching a mean peak of 68.3 mg 100 $ml^{-1}$ in late October approximately two months after the oestradiol-17ß peak. By the end of the experiment in December total calcium levels were returning to basal levels. There were insufficient data from the non-spawning fish in this group to make any analysis of their serum levels of oestradiol-17ß or total calcium.

Group F: Figure 4.13 shows the increases in the total serum calcium levels of this group of fish which were retained for a further 12 months under constant 6L:18D. In the fish that spawned successfully and also those which failed to spawn in 1985, subsequent increases ($P \leq 0.05$) in total calcium were detected from around basal in late January (1986) to over 20 mg 100 $ml^{-1}$ by the beginning of May.

Egg Size

Figure 4.14 shows the relationship between mean (± standard error mean) egg diameter and the time of spawning of data pooled from Grps A-E. Mean egg diameter increased progressively from 3.64 (± 0.8)mm in those trout spawning in late August to over 4.5mm in those trout spawning in December.

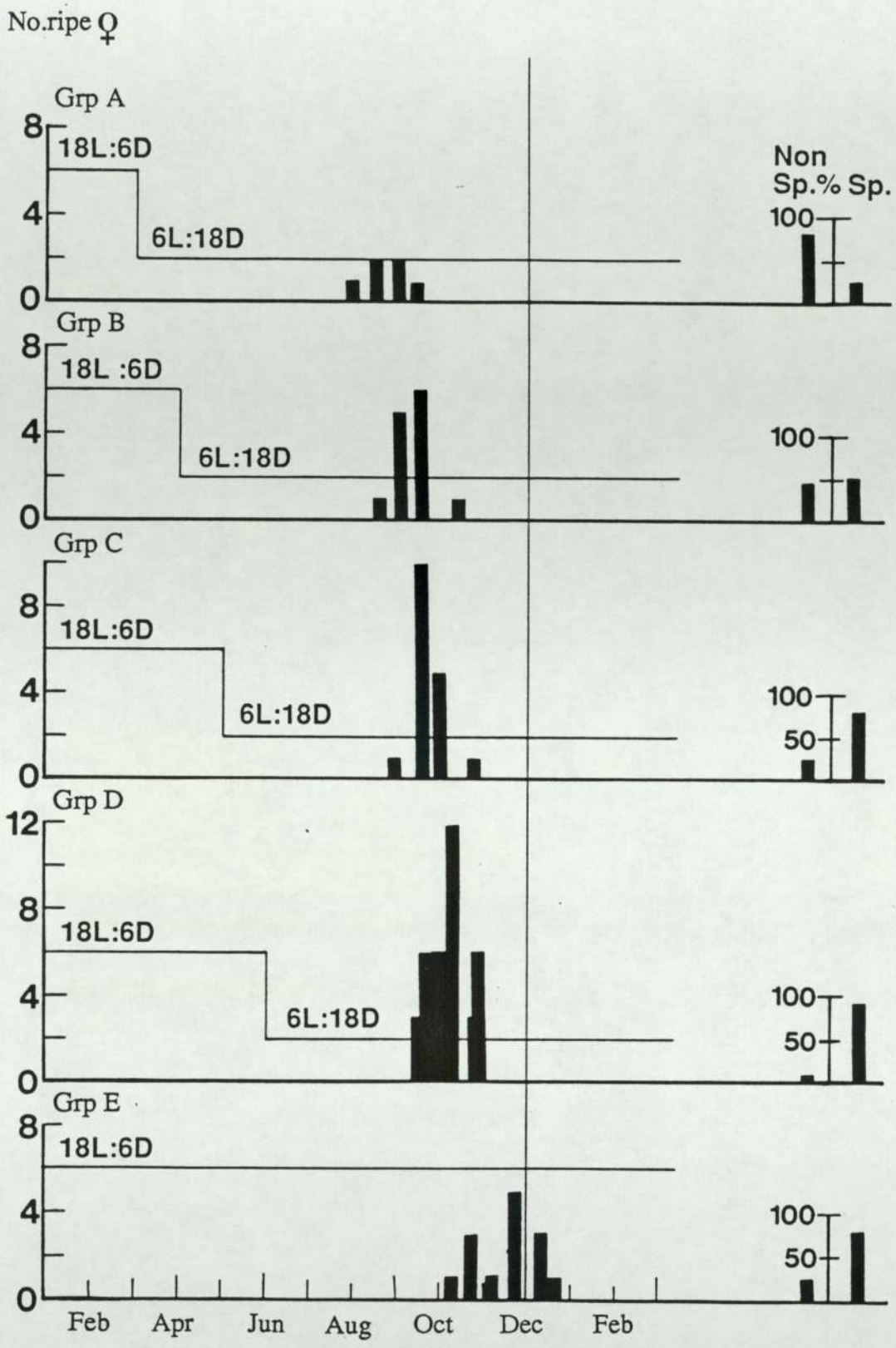



Fig. 4.10. The effect of five photoperiod regimes(Groups A-E) on the timing of maturation of female rainbow trout. Histograms illustrate the number of mature females (No. ripe ♀) at each sampling time. The percentage of females attaining maturation in each group are shown by the histograms at the right hand side of the figure. The photoperiod regime in each treatment is indicated by the horizontal lines. The vertical line running through all five histograms indicates the timing of onset of spawning of rainbow trout of the same stock maintained under natural photoperiod. The vertical lines on the x-axis refer to the first day of the month as labelled.

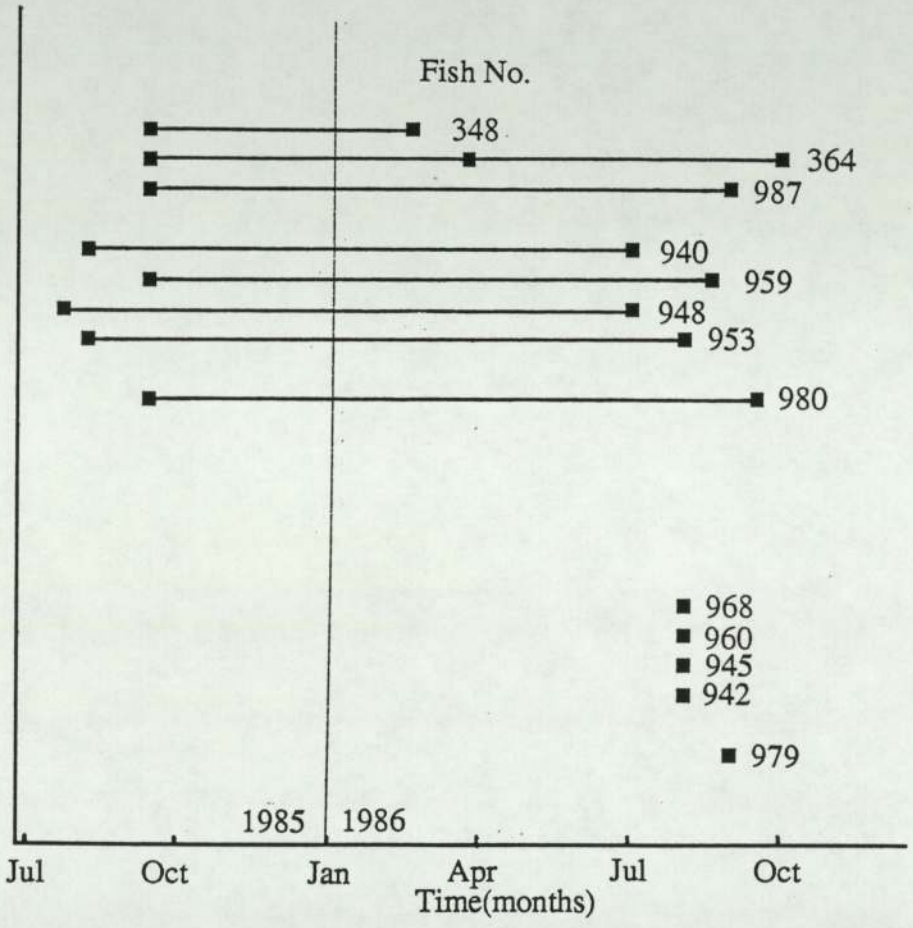



Fig. 4.11. The timing of maturation of individual rainbow trout in Group F subjected to a reduction of 18L:6D→ 6L:18D in March-May 1985 then remaining on constant 6L:18D until the end of the experiment. The upper section shows the periodicity between successive spawning of those fish which matured in 1985 and 1986. The fish which failed to mature in 1985 (lower section) exhibited a 4-month advancement in the time of spawning in 1986. The vertical bars on the x-axis refer to the first day of the month as labelled.

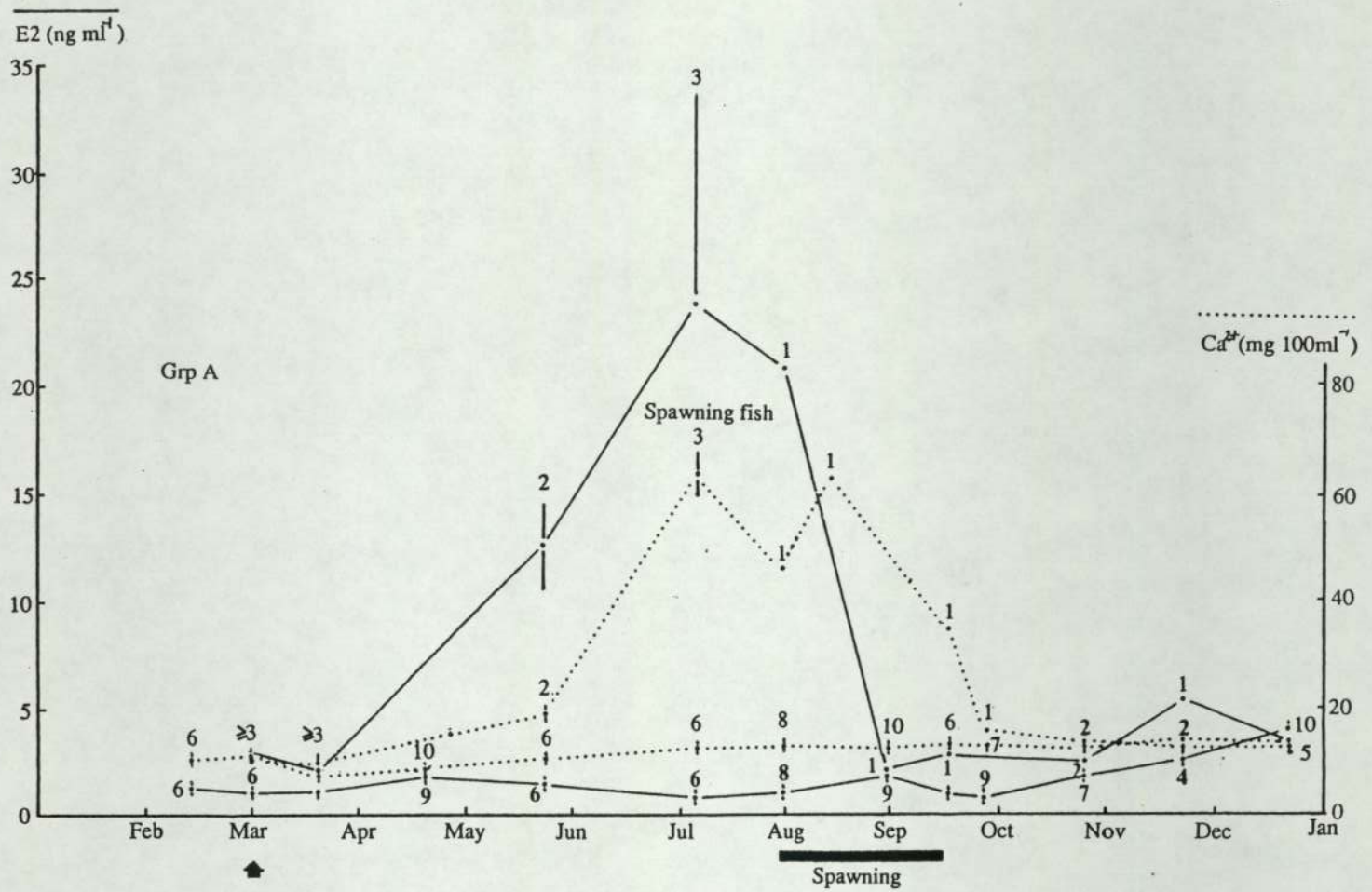



Fig. 4.12a The sequential changes in the mean levels of serum oestradiol-17β(E2,ng $ml^{-1}$) and total serum calcium ($Ca^{2+}$,mg $100ml^{-1}$) in female rainbow trout subjected to a reduction in photoperiod from 18L:6D to 6L:18D at the date shown(⬟), illustrating alterations in spawning and non-spawning fish. The integer above each coordinate indicates the number of fish sampled(n) with the vertical bars representing ± one standard error of the mean. The horizontal bar below the x-axis indicates the time of spawning. The vertical lines on the x-axis refer to the first day of the month.

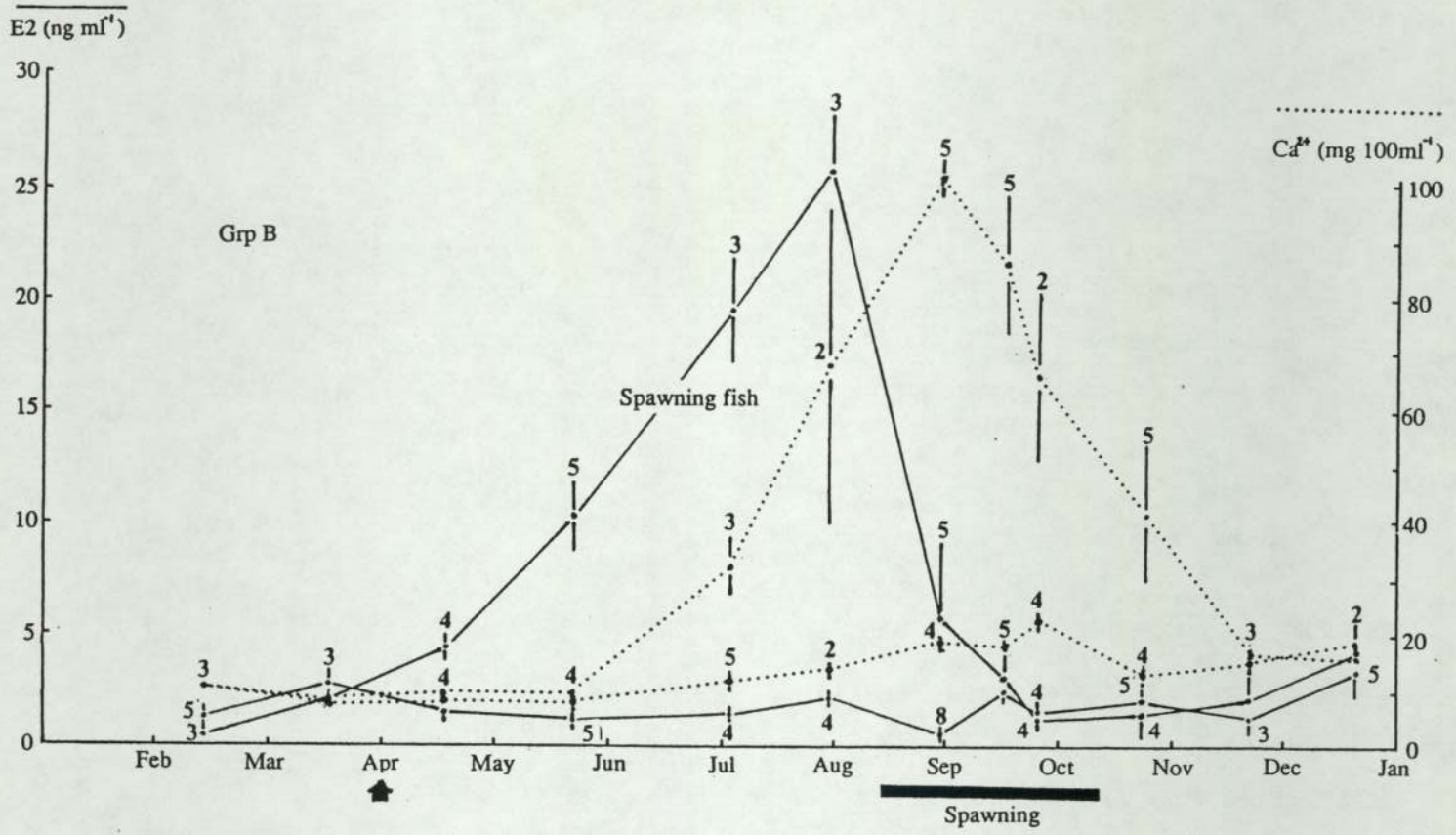



Fig. 4.12b The sequential changes in the mean levels of serum oestradiol-17β (E2, ng $ml^{-1}$) and total serum calcium ($Ca^{2+}$, mg $100ml^{-1}$) in female rainbow trout subjected to a reduction in photoperiod from 18L:6D to 6L:18D at the date shown(⬆) illustrating alterations in spawning and non-spawning fish. The integer above each coordinate indicates the number of fish sampled(n) with the vertical bars representing ± one standard error of the mean. The horizontal bar below the x-axis indicates the time of spawning. The vertical lines on the x-axis refer to the first day of the month.

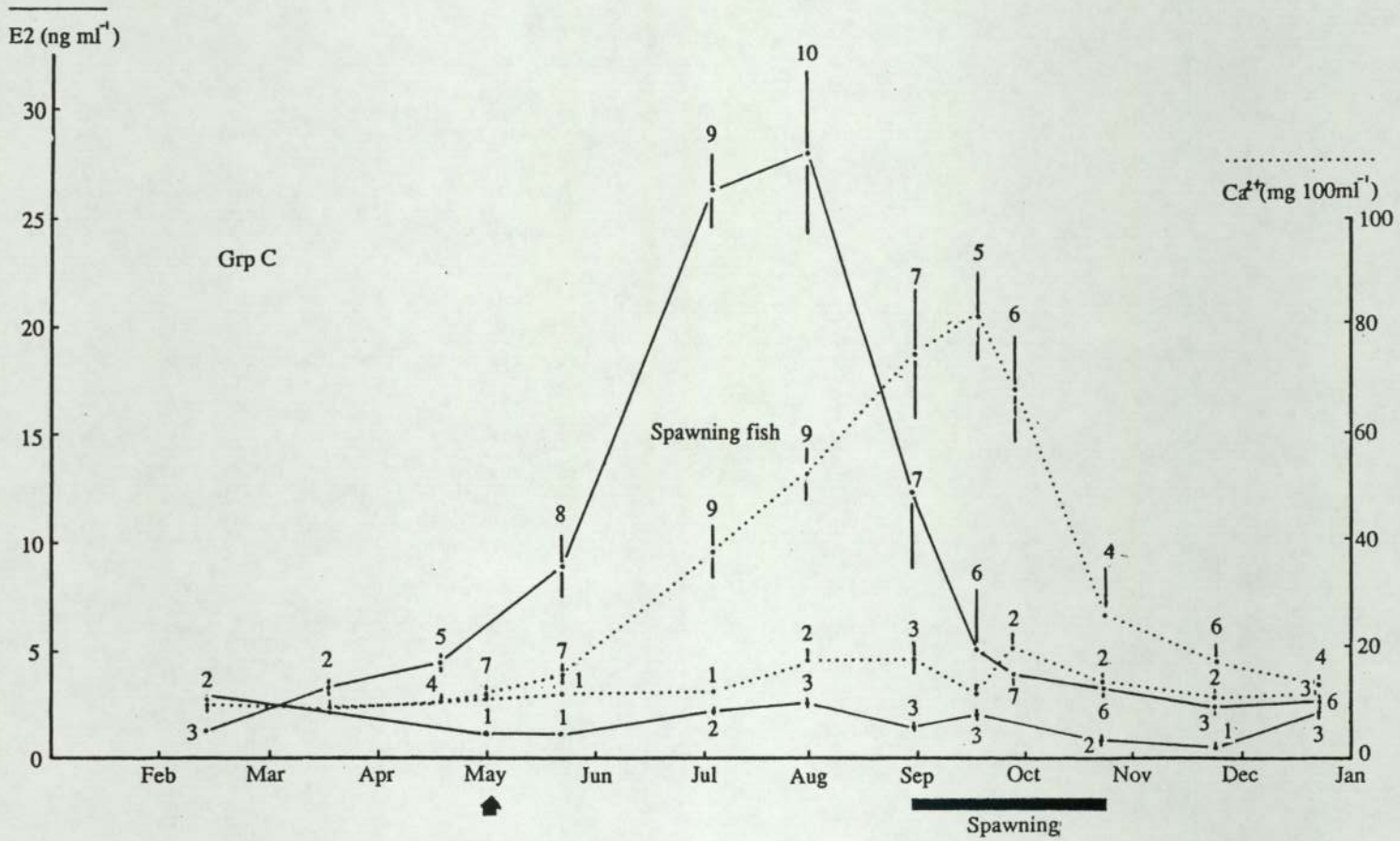



Fig. 4.12c The sequential changes in the mean levels of serum oestradiol-17β (E2, ng $ml^{-1}$) and total serum calcium ($Ca^{2+}$, mg $100ml^{-1}$) in female rainbow trout subjected to a reduction in photoperiod from 18L:6D to 6L:18D at the date shown(⬟), illustrating alterations in spawning and non-spawning fish. The integer above each coordinate indicates the number of fish sampled(n) with the vertical bars representing ± one standard error of the mean. The horizontal bar below the x-axis indicates the time of spawning. The vertical lines on the x-axis refer to the first day of the month.

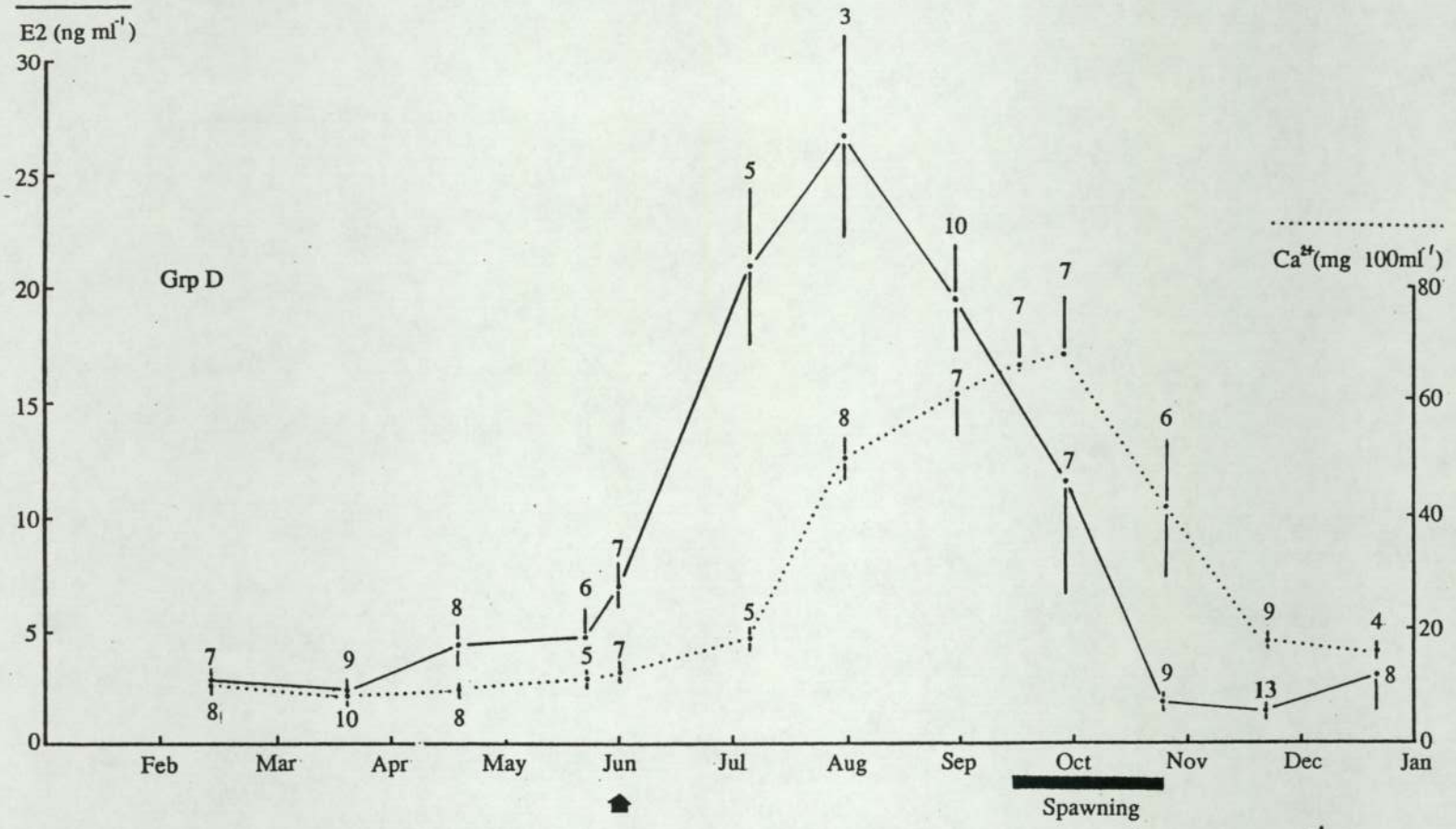



Fig. 4.12d The sequential changes in the mean levels of serum oestradiol-17β (E2, ng $ml^{-1}$) and total serum calcium ($Ca^{2+}$, mg $100ml^{-1}$) in female rainbow trout subjected to a reduction in photoperiod from 18L:6D to 6L:18D at the date shown(⬟). There were insufficient non-spawning trout in this group to produce a graph of changes in serum parameters in these fish.The integer above each coordinate indicates the number of fish sampled(n) with the vertical bars representing ± one standard error of the mean. The horizontal bar below the x-axis indicates the time of spawning. The vertical lines on the x-axis refer to the first day of the month.

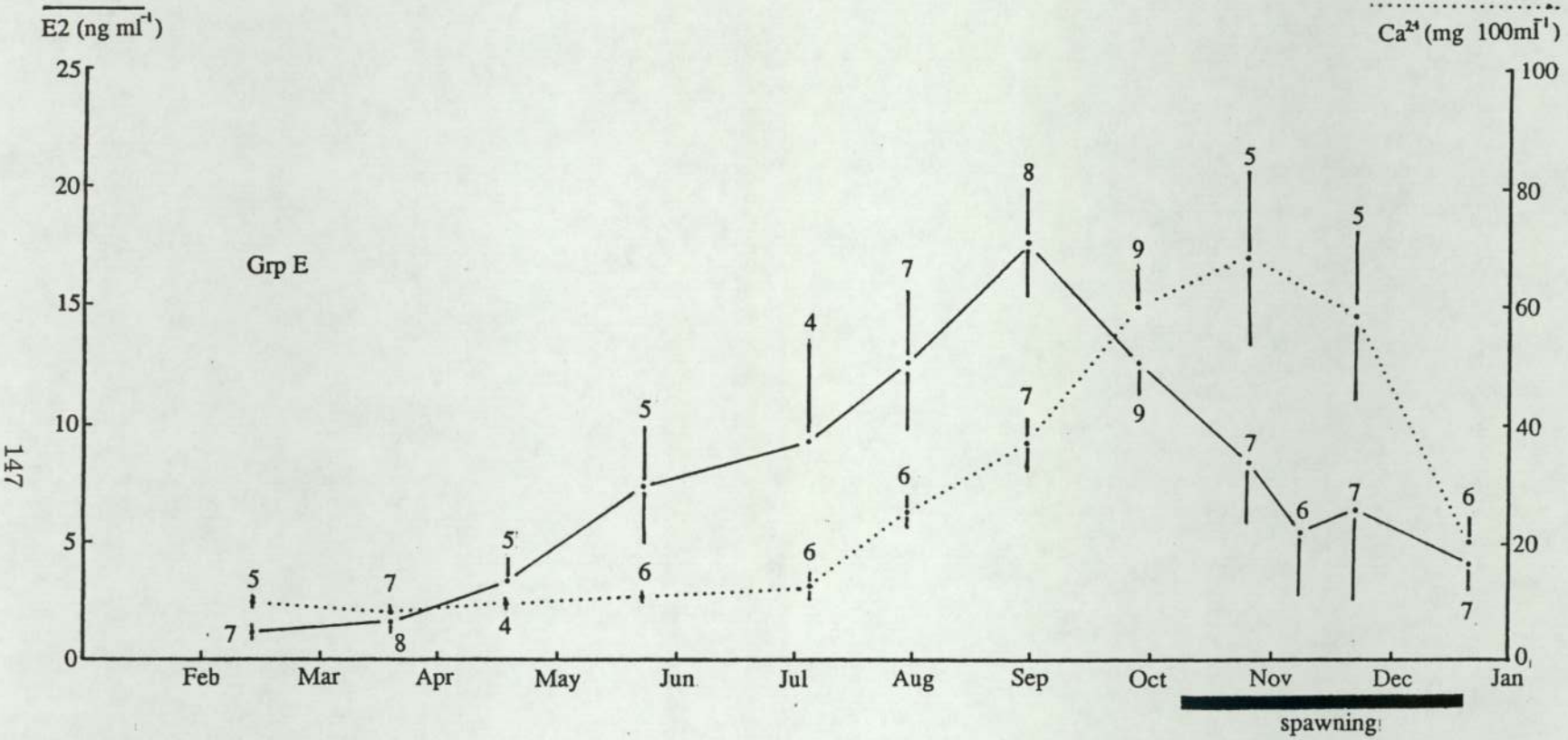
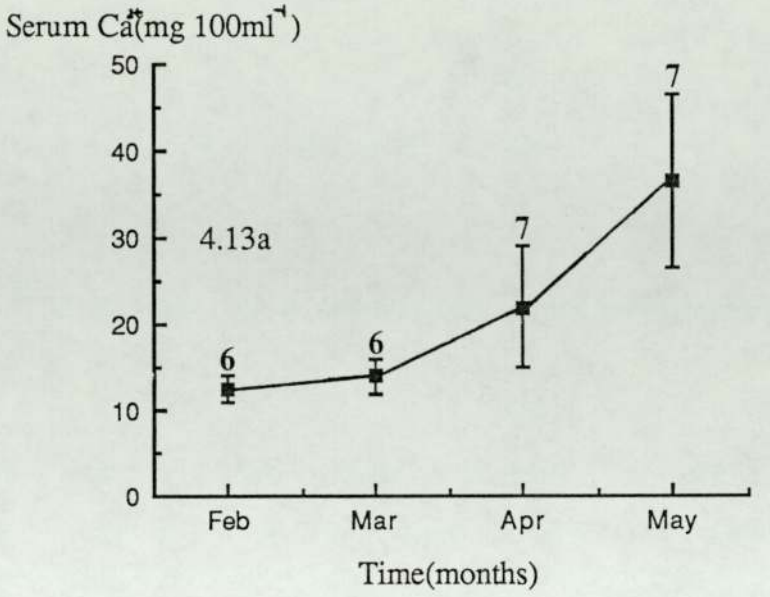



Fig. 4.12e The sequential changes in the mean levels of serum oestradiol-17β (E2, ng $ml^{-1}$) and total serum calcium ($Ca^{2+}$, mg $100ml^{-1}$) in female rainbow trout maintained under 18L:6D constant. There were insufficient non-spawning trout in this group to produce a graph of changes in serum parameters in these fish. The integer above each coordinate indicates the number of fish sampled(n) with the vertical bars representing ± one standard error of the mean. The horizontal bar below the x-axis indicates the time of spawning. The vertical lines on the x-axis refer to the first day of the month.



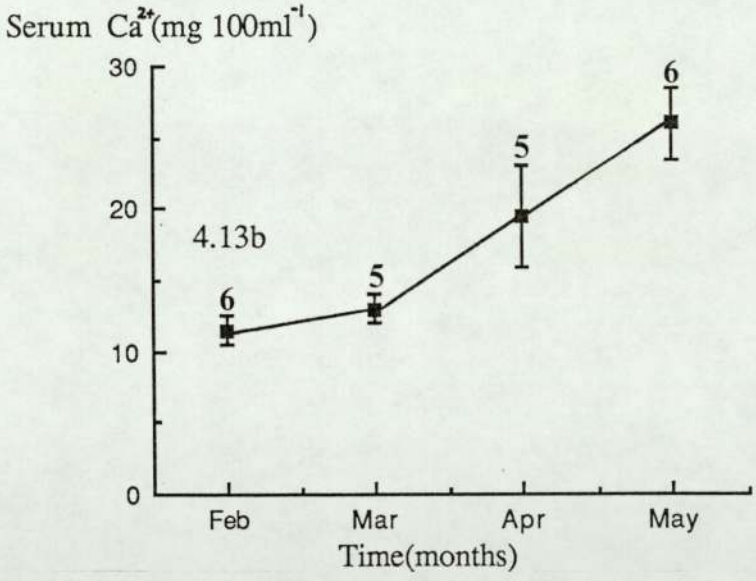




Fig. 4.13. Sequential changes in total serum calcium (mg 100ml $^{-1}$) in female rainbow trout which failed to spawn in 1985 (4.13a), and those which spawned in 1985 (4.13b) maintained for a subsequent year under constant 6L:18D (Group F). In both groups significant increases ($P \leq 0.05$) in serum calcium levels were detected between January and May 1986. The integer above each coordinate indicates the number of fish sampled(n), with the vertical bars representing $\pm$ one standard error of the mean. The vertical lines on the x-axis refer to the first day of the month as labelled.

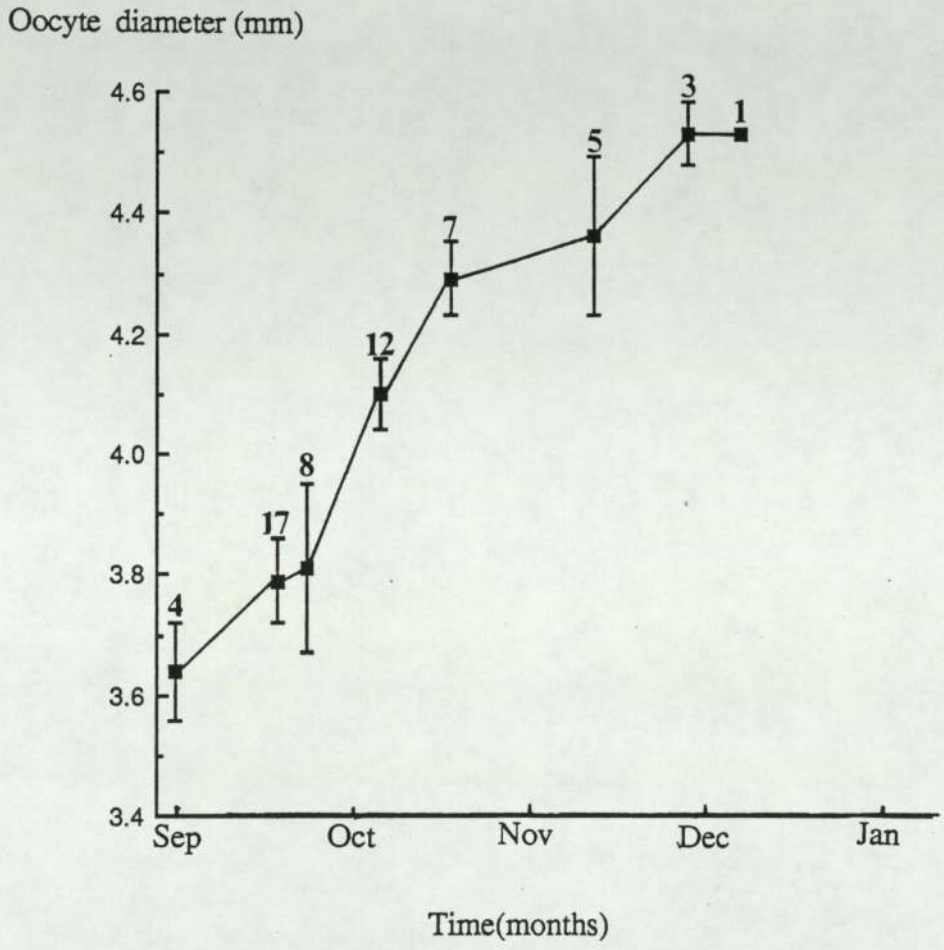



Fig.4.14. Relationship between the time of spawning and mean oocyte diameter(m.m.). Data pooled from fish spawning in Groups A-E. The integer above each coordinate indicates the number of fish sampled(n) with the vertical bars representing ± one standard error of the mean. The vertical lines on the x-axis refer to the first day of the month as labelled.

Conclusions (Expt 2)

The results from Experiment 1 indicated that the reduction in photoperiod provided an entraining cue to an endogenous rhythm of maturation and it was suggested that the timing of the reduction dictated the timing of the onset of ovulation. To test this hypothesis the present experiment subjected groups of rainbow trout to abrupt reductions in photoperiod at different times of the year. In agreement with the hypothesis the groups commenced spawning in sequence according to when they were subjected to a reduction in photoperiod. However, as shown in Table 4.1 the timing of the onset of spawning was not directly proportional to the timing of the reduction in the photoperiod.

| Time of reduction in photoperiod | Time of onset of spawning | Time difference between reduction in photoperiod and onset of spawning |
|---|---|---|
| 1st March (Grp A) | 31st July | 153 days |
| 1st April (Grp B) | 13th August | 135 days |
| 1st May (Grp C) | 30th August | 122 days |
| 1st June (Grp D) | 16th September | 108 days |

Table 4.1: The relationship between the timing of the reduction in photoperiod from 18L:6D to 6L:18D and the timing of onset of spawning

Consider the timing of maturation of fish subjected to a reduction in photoperiod on 1st June; this group (D) commenced spawning on 16th September. If there was a direct linear relationship between the timing of the reduction in photoperiod and the onset of spawning, it would be expected that the fish in Grp C subjected to a reduction in light on 1st May would commence spawning exactly a month earlier than those in Grp D. In fact the actual time of spawning of fish in Grp C was only advanced by 17 days compared to fish exposed to 6L:18D from 1st June. Similarly those fish subjected to a reduction in photoperiod on 1st April and 1st March would be expected to spawn 2 and 3 months respectively ahead of the fish in Grp D. However the observed advancements were only 1 and 1.5 months respectively. The relationship between the time of the reduction in photoperiod and the 'expected' and observed onset of spawning are displayed graphically in Figure 4.15. Compared to Grp D the observed advancement in the time of spawning in the other treatments is only approximately 50% of expected.

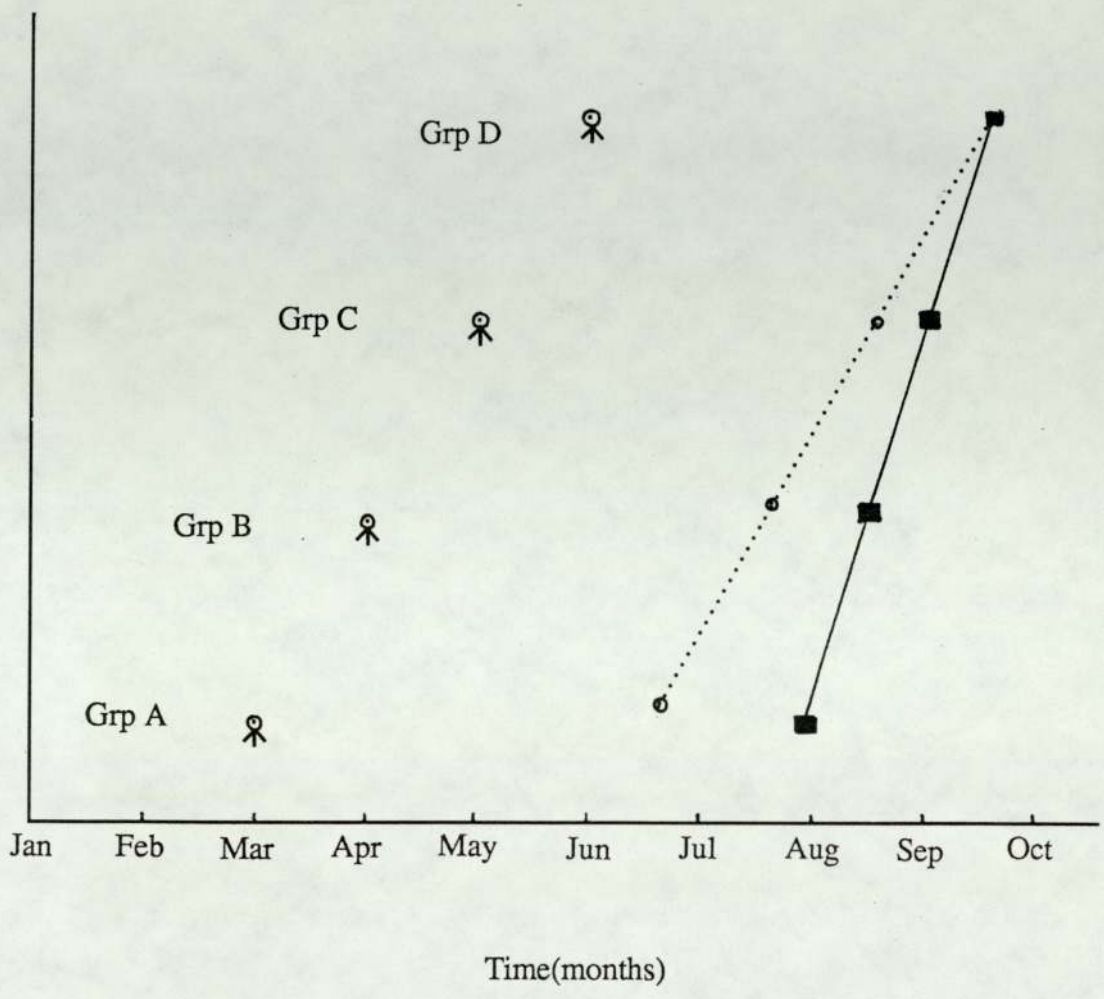



Fig. 4.15 Relationship between the observed ( ■ ) and 'expected' ( o ) time of onset of spawning of female rainbow trout in Groups A, B and C compared to Group D. The 'expected' time of spawning is based on the assumption that there is a linear relationship between the timing of the reduction in photoperiod ( ♀ ) and the timing of the onset of spawning. The vertical lines on the x-axis refer to the first day of the month as labelled.

These results are still compatible with the hypothesis of a reduction in photoperiod entraining an endogenous rhythm of maturation. However, the results indicate that the maturation process has a certain amount of inertia and there are limits to how far the time of spawning can be manipulated using changes in the photoperiod. The range of entrainment of circannual rhythms will be considered in detail in the discussion to this chapter.

From the results of Experiment 1 it was concluded that the abrupt reduction in photoperiod not only advanced the timing of maturation, but also served to synchronise the time of spawning of individual fish within a particular experimental group. The results from the present experiment confirm this proposition, the fish maintained on constant 18L:6D from 18th January spawned over a protracted period of approximately 10 weeks. In contrast, the four groups that were subjected to a reduction in photoperiod from 18L:6D to 6L:18D exhibited a more synchronised spawning profile. Once again these results support the hypothesis that the reduction in the photoperiod is serving to entrain an endogenous rhythm of maturation.

A further important finding of the present experiment is that there is a relationship between the timing of the reduction in photoperiod and the proportion of fish attaining maturation the following autumn (Figure 4.10). When the reduction from 18L:6D to 6L:18D was made on 1st March only 26% of the fish in that group (A) spawned, the rest remaining immature. In contrast over 90% of the fish spawned successfully when the reduction in photoperiod was made on 1st June (Grp D). Initially it was not possible to explain these results in terms of the hypothesis that photoperiod was merely serving to entrain an endogenous rhythm of maturation; they clearly showed that particular changes in daylength could dictate whether or not maturation occurred. However, retaining a small sample of spawning and non-spawning fish for a further 12 months under 6L:18D (Grp F) produced a result which provided additional support for the hypothesis that changes in the photoperiod serve to entrain an endogenous circannual oscillator. The results in Figure 4.11 illustrate that fish which had their initial spawning advanced to August-September 1985 proceeded to spawn again the following year with a circannual periodicity. Importantly, those fish which failed to mature in 1985 proceeded to show elevated serum calcium levels (Figure 4.13) and ovulated in 1986 at approximately the same time of the year as those fish spawning for a second time (Figure 4.11). It can be concluded that the abrupt reduction in the photoperiod during the first year of the experiment served to advance the phase of an endogenous circannual oscillator or 'clock' in both spawning and non-spawning fish. In spawning fish the phase advance in the clock was overtly expressed as an advance in the time of ovulation. In contrast, in non-spawning fish, although the circannual clock in each animal was

phase advanced they failed to mature during the first year of the experiment, only expressing the phase advance in the clock with an advanced spawning the following year. These results indicate that the endogenous circannual clock is an autonomous oscillator which is normally linked or coupled to the maturation controlling system, but under certain experimental conditions the two can be dissociated.

When the reduction in photoperiod was made in May or June a large percentage of the fish spawned the following autumn. In contrast, when subjected to a reduction in photoperiod in March the majority of the fish failed to mature during the first year of the experiment. There are at least two possible explanations for this behaviour:

(i) The reduction in photoperiod served to initiate the maturation process. When subjected to a reduction in the photoperiod in March most of the fish were incapable of maturing, possibly because the gonads and/or the hypothalamo-pituitary control system were not fully developed.

(ii) The maturation of the ovary commenced prior to 1st March, but when a reduction in photoperiod occurred on that date the developmental stage of the oocytes was not sufficiently advanced to be capable of responding. As a result the maturation process was arrested until the following year.

It is clear that these two explanations are not mutually exclusive because the response of the fish under both circumstances depends on the timing of the reduction in photoperiod in relation to the developmental stage of the oocytes. Dealing with the first explanation, it was suggested that when subjected to a long to short change in the photoperiod on 1st March most of the fish were still immature and incapable of responding. During the rest of March and the following months the fish in the other groups matured to a stage where they were physiologically competent to respond to the abrupt reduction in the photoperiod; thus a higher percentage of the fish in these groups spawned during the first year of the experiment. The fact that significant increases in serum oestradiol-17ß and total calcium within each group were only detected after the reduction in photoperiod tends to support the hypothesis that oocyte maturation only commenced after the reduction in photoperiod. However, it must be stressed that the detection of small increases in circulating hormones depends on the sensitivity of the respective assay techniques. There is evidence from other studies that oocyte maturation commences before changes in concentrations of serum components can be detected by assay methods similar to those used in this study (Schulz, 1984; Sumpter *et al*, 1984). The relationship between the timing of increases in oestradiol-17β, total calcium and the timing of

ovulation compared to the size of the mature oocytes also suggests that maturation commenced before the reduction in photoperiod. In general, in Grps A-D there is 2.5-3 months between the detection of significant increases in oestradiol-17ß and total calcium, and the onset of spawning. Therefore, it might be expected that the fish in these groups would all produce oocytes of approximately the same size. However Figure 4.14 shows that there is a clear relationship between oocyte diameter and the timing of ovulation. Because egg size also varies according to fish size (Springate, 1985), which was an uncontrolled variable in this experiment, it is difficult to draw any firm conclusions from the oocyte diameter data. Despite this reservation, the relationship between egg size and the timing of spawning can be better explained if it is assumed that oocyte maturation began at the same time of the year in all experimental groups, with reductions in the photoperiod serving to advance a process that has already commenced.

The above argument returns to the problem of explaining why many of the fish subjected to a reduction in photoperiod in March failed to mature. Explanation (ii) would contend that maturation of the oocytes had already commenced prior to 1st March, but the reduction in the photoperiod on that date was in some way inappropriate to the developmental stage of the oocytes, thus resulting in the fish failing to spawn that year. From studies on virgin female rainbow trout of the same age as fish used in this experiment, it has been concluded that oocyte maturation commences at least 12 months before ovulation (Schulz, 1984; Sumpter, 1984; Sumpter *et al*, 1984). The fish used in this experiment spawn in early December under natural conditions, therefore it is probable that oocyte maturation had indeed commenced before the reduction in photoperiod in March. The absence of any detectable increases in oestradiol-17ß and total calcium before the reduction in photoperiod was probably due to the sensitivity of the assay methods; using histological methods Sumpter *et al*(1984) showed that the initial stages of oocyte development commenced before any gross increases in vitellogenin or the reproductive hormones. Unfortunately it was not possible to sacrifice fish for histological investigations in the present experiment.

If it is assumed that maturation of the oocytes had commenced before or soon after the start of the experiment in January, the question that must be addressed is why does a reduction in photoperiod in March result in most of the fish remaining immature that year? Any explanation of this observation must be reconcilable with the advancement of the time of maturation of the remaining experimental fish. The model described below satisfies both conditions. It is proposed that the system controlling oocyte maturation in these experimental fish consists of two main components; the first is an endogenous circannual clock capable of being entrained by changes in the photoperiod, the second is an 'allowed zone' or 'gate' through which the fish can 'pass'

and go on to full maturation providing that the animal has reached a certain threshold stage of development. Entrainable endogenous oscillators have already been introduced; the concept of a 'gate' requires further explanation. It is proposed that the gate is a period of time closely associated with the phase of the endogenous circannual clock. The gate has two positions, open and closed. When the 'hand' of the endogenous circannual clock is coincident with the gate, it is open. For the rest of the circannual cycle when the 'hand' of clock is not coincident, then the gate is in the closed position (Figure 4.16).

It is suggested that this control mechanism determines whether a previously immature rainbow trout will go on to full maturation and spawning in a particular season, or remain immature for another year. If, for example an individual fish reaches a certain 'threshold' stage of development or size when the gate is open, then maturation continues and ovulation occurs. On the other hand, if the fish have not reached this threshold stage of development when the gate is open, then further maturation cannot occur. The oocytes of these fish do not develop until the following year when the 'hand' of the circannual clock again coincides with the gate; at this point they pass through the gate and undergo full maturation. It is proposed that the endogenous circannual clock not only determines when maturation can commence but also continuously modulates the development of the oocytes and controls the timing of ovulation. Under natural conditions the clock is entrained to the annual photocycle so that maturation and spawning occur at a time of year when the resulting offspring have an optimum chance of survival. Subjecting fish to artificial photoperiod regimes results in phase shifts of the circannual timing mechanism which alters the internal temporal relationship between the developmental stage of the gonads and the 'hand' of the clock. It is suggested that these phase shifts account for the results observed in the present experiment. In Grp A, sometime before the reduction in photoperiod on 1st March the hand of the circannual clock began to coincide with the gate. At this time only a small percentage of the fish had reached the threshold stage of development and they passed through the gate and on to full maturation (Figure 4.17a). On the 1st March the fish in Grp A were subjected to an abrupt reduction in the photoperiod, in accordance with the model this phase advanced the circannual clock and consequently brought forward the timing of ovulation of those fish that had already passed through the gate (Figure 4.17b). After 1st March the clock was no longer coincident with the gate and the remaining fish in this group (A) failed to mature that year. Subsequently, these fish developed to the threshold size and proceeded to mature the following year when the phase advanced clock was again coincident with the gate (Figure 4.11). The fish in Grp B had until 1st April to reach the threshold stage of development before the reduction in photoperiod phase advanced the

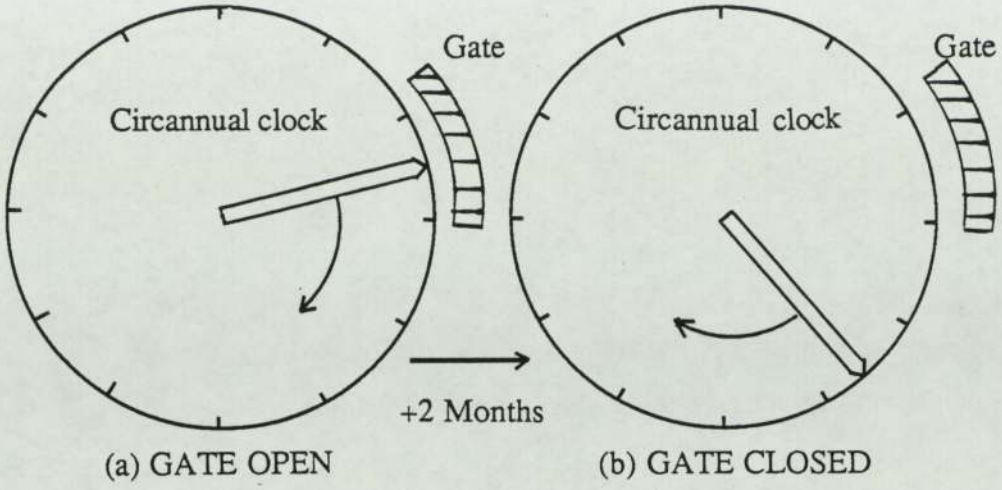



Fig. 4.16. Representation of the concept of a gating mechanism associated with an endogenous circannual clock. The gate is open when the 'hand' of the clock is coincident with the gate(a). For most of the circannual cycle the gate and the 'hand' are not coincident and therefore the gate is closed.

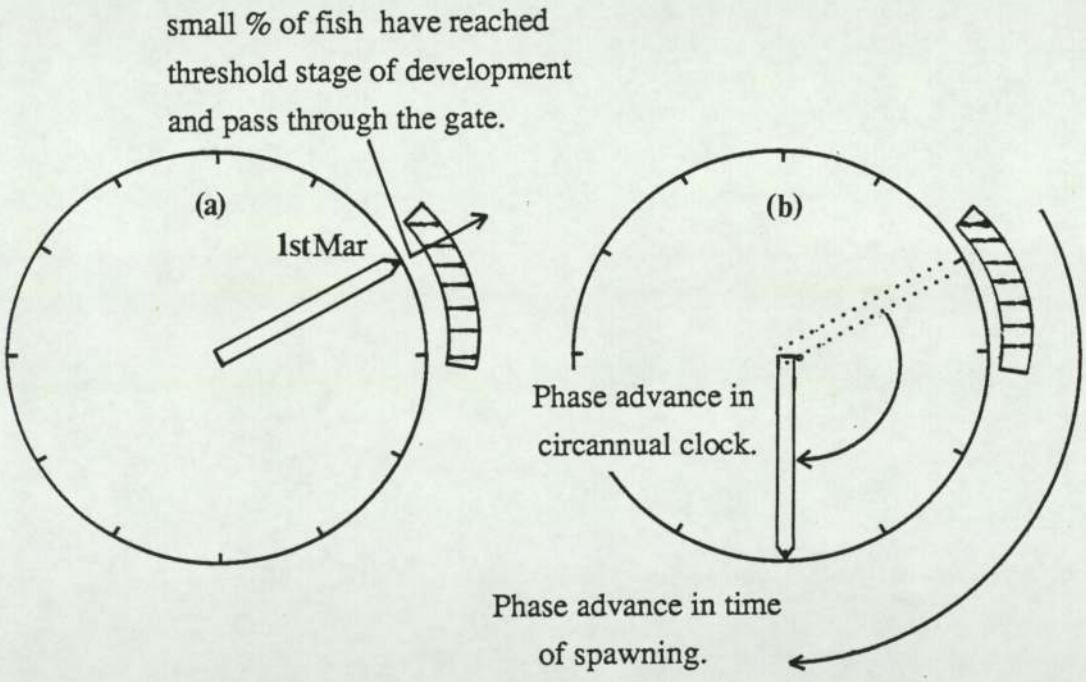



Fig. 4.17. Representation of the hypothesis of a circannual clock entrained by changes in photoperiod controlling the opening and closing of a gating mechanism. The gating mechanism allows fish which have reached a threshold stage of development to mature that particular season. (a): Prior to the reduction in photoperiod on 1st March the 'hand' of the clock is coincident with gate and the small % of fish which have reached threshold stage of development pass through the open gate. (b): The reduction in photoperiod on 1st March causes a phase advance in both the circannual clock and the time of spawning of those fish that have past through the gate. The 'hand' is no longer coincident with the gate (gate closed) therefore no additional fish are allowed to mature that particular year.

clock beyond the coincidence position. Because of the additional month of development over half the fish in this group passed through the gate and matured that year. Similarly fish in Grp C had until 1st May to reach the threshold stage and accordingly 77% of the individuals in this group spawned during the first year of the experiment. Over 90% of the fish in Grp D spawned successfully indicating that nearly all the fish had reached the threshold stage before the reduction in photoperiod on 1st June. Overall these results indicate that the circannual clock is coincident with the gate from just before 1st March to around the beginning of June. Any fish attaining the threshold stage of development during this period proceeded to spawn that year. From the present results it is not possible to determine what constitutes the 'threshold'; it could be the actual physical size of the fish, or possibly some step in the hypothalamo-pituitary-gonadal axis which has to be fully developed before maturation can occur. Gating mechanisms are given further attention in the main discussion to this chapter.

It must be re-emphasised that to fit in with the observed results the reduction in photoperiod not only advanced the endogenous clock but similarly advanced the timing of maturation of those fish which had passed through the gate (Figure 4.17b). It is suggested that ovulation occurs at a specific time in the oscillation of the circannual clock. As a result of the advancement in the phase of clock, the animal must complete the development of the oocytes in a shorter time than is normally available. This results in the production of smaller oocytes, as shown in Figure 4.14. Although not fully quantified the smaller eggs appeared to be equally viable in terms of % fertilisation as the larger oocytes. The results indicate that the extent to which spawning is advanced is dependent on when the reduction in photoperiod is made. The fish maintained on constant 18L:6D (Grp E) commenced spawning on 10th October; the fish that were subjected to a reduction in photoperiod on 1st March commenced spawning on 31st July, this represents an advance of 71 days compared to Grp E. When the reduction was made on 1st April, 1st May and 1st June, spawning commenced on 13th August, 30th August and 16th September respectively. Compared to the fish on constant 18L:6D these represent advances in the time of spawning of 58, 41 and 28 days respectively. These advances can be envisaged as phase advances in the endogenous circannual clock as shown in Figure 4.18. It is proposed that the extent to which the circannual clock and time of spawning is phase advanced is dependent on when the reduction in photoperiod occurs. It is possible that this is determined by the temporal relationship between the timing of the reduction in photoperiod and the stage of development of the oocytes; the more advanced the oocytes the less will a reduction in photoperiod advance the timing of spawning. Before proceeding further the main conclusions and hypotheses developed from Experiment 2 are summarised:

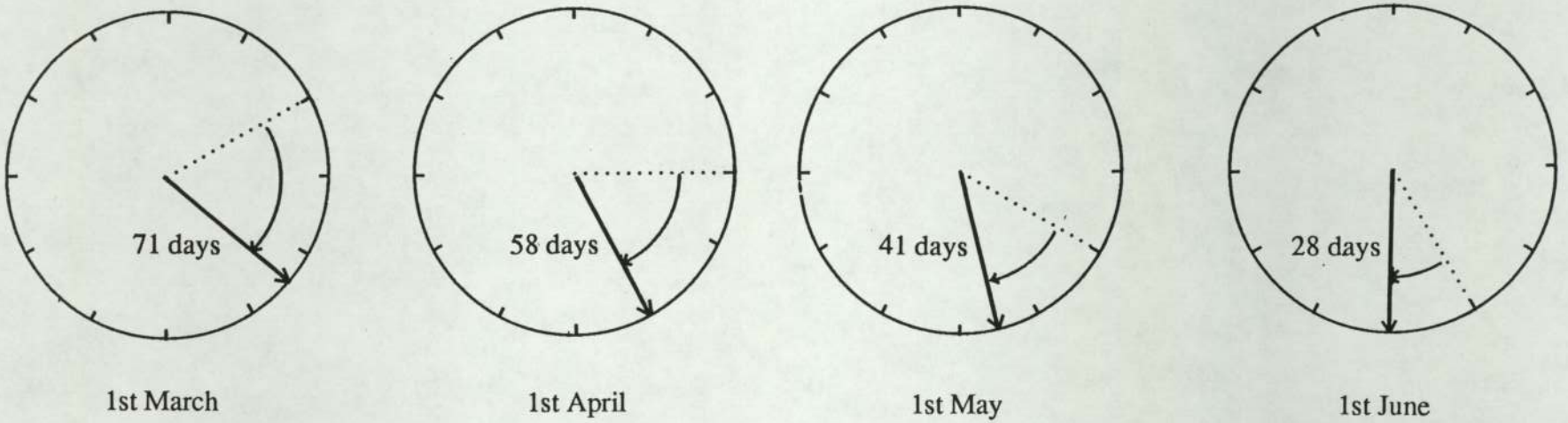



Fig. 4.18 Alteration in the timing of maturation of female rainbow trout due to a reduction in photoperiod from 18L:6D→6L:18D at the dates shown relative to fish maintained on 18L:6D constant(Group E), depicted as phase advancements in an endogenous circannual clock. The extent to which the timing of maturation is advanced is dependent on the timing of the reduction in photoperiod relative to the phase or position of the clock.

## Summary

(i) The results confirm the conclusions from Experiment 1 that an abrupt reduction in the photoperiod phase advances an endogenous circannual rhythm of maturation.

(ii) It is proposed that the fish have to reach a threshold stage of development before they are competent to mature.

(iii) Once the threshold stage of development has been attained, maturation can only proceed through to ovulation when an endogenous circannual clock is coincident with a 'gate'. The results suggest that this gate is open for approximately 3 months. Fish that fail to reach the threshold stage of development while the gate is open do not mature that season.

(iv) Once fish have passed through the gate, abrupt reductions in the photoperiod will phase advance the time of spawning. The extent of the advance in spawning is dependent on when the reduction in photoperiod occurs in relation to the developmental stage of the oocytes.

(v) In fish that fail to reach the threshold stage of development when the gate is open and do not mature that season, the circannual clock is still phase advanced by a reduction in photoperiod. This phase advance in the internal clock is overtly expressed approximately one year later by an advance in the time of spawning similar to those fish which matured during both the first and second years of the experiment.

(vi) The observation that the internal circannual timing mechanism can be dissociated from the neuroendocrine mechanisms which are responsible for the control of reproduction indicate that the 'clock' can be considered as an autonomous unit.

This model for the effects of photoperiod on the control of maturation has been developed exclusively from the experimental results. Relating the model to the results of other studies on rainbow trout reproduction and other work on the entrainment of endogenous clocks will be covered in the discussion to this chapter.

It is clear from the results of this experiment that the reduction in photoperiod at different times had an important effect on the timing of maturation. It is quite possible

that moving fish from ambient photoperiod into 18L:6D at the start of the experiment had an equally important effect on the timing of maturation, however the fact that all groups were placed on this photoperiod at the same time meant that any effect would not be apparent. It is conceivable that this abrupt increase in photoperiod induced a phase shift in the timing of maturation, or that the 18L:6D photoperiod *per se* had an effect on the maturation process. An attempt to answer these questions is made in the following experiment (Expt 3).

## 4.4 Experiment 3: The effect of a range of constant photoperiods followed by a reduction to a shorter constant photoperiod on the timing of maturation in the female rainbow trout.

The results from Experiments 1 and 2 have supported the hypothesis that maintaining rainbow trout on a constant long photoperiod (18L:6D) from mid-January for at least 6 weeks followed by a direct reduction to a shorter photoperiod can phase advance an endogenous circannual clock. This phase advance can be expressed as an advance in the timing of maturation if the reduction in photoperiod occurs after the fish have attained a certain threshold stage of development. If the reduction in photoperiod occurs before the trout have reached this threshold stage then the circannual clock is phase advanced but the fish remain immature that season.

Experiments 1 and 2 both adopted 18L:6D as the 'long' photoperiod. It is possible that this photoperiod *per se*, or the abrupt increase from ambient daylength to 18L:6D at the start of the experiment in January had an effect on the timing of maturation but because of experimental design, failed to be detected. In order to investigate this, starting in mid-January the present experiment maintained 3 groups of immature 2 year old female rainbow trout on 10L:14D, 14L:10D and 18L:6D respectively. They were held on this photoperiod until 8th May when the light was abruptly reduced to 6L:18D for the remainder of the experiment. This date for the reduction in photoperiod was chosen for 2 reasons: (a) the results from the previous two experiments indicated that maintaining fish on a long photoperiod until this time resulted in a high percentage of experimental animals maturing that season and thus would not be the limiting factor in the control of spawning, (b) the timing of the changes in photoperiod were identical to those in Experiment 1 and so allowed a comparison to be made between the two sets of results.

From the results of Experiments 1 and 2 it was concluded that the timing of the reduction in photoperiod is more important than the magnitude of the change in day length in advancing the time of spawning. However, this conclusion was only made from experiments where the photoperiod was reduced from a 'long' 18L:6D to a shorter photoperiod ($\leq$ 14L:10D). As a test of this hypothesis, in addition to the 3 photo-regimes already described, the current experiment included a group of fish maintained on 6L:18D from mid-January to 8th May followed by an abrupt reduction to 2L:22D. If the timing of the reduction in photoperiod is the most important 'cue' then the fish in this group would perhaps be expected to exhibit a similar advancement in the time of spawning to other groups in this experiment.

In Experiment 2 a photoperiod regime of 18L:6D from mid-January to 1st March followed by 6L:18D resulted in only 26% of the fish spawning that season. It was hypothesised that the low numbers of fish attaining maturation was due to the reduction from 18L:6D to 6L:18D occurring just after the 'hand' of the circannual clock had coincided with a 'gate', phase advancing the internal clock past the coincidence position before most of the fish in that group had reached the threshold stage of development. From these results it was concluded that it was the temporal position of the 18L:6D photoperiod in relation to the stage of development rather than its duration which determined the proportion of fish attaining maturity that season. To test this hypothesis, in the current experiment a group of fish were maintained on 18L:6D for a period of 6 weeks, identical to Group A in Experiment 2, but shifted by approximately two months to occur between late March and 8th May. Prior to, and following this period of 18L:6D the fish were maintained under 6L:18D.

Materials and Methods (Expt 3)

Five groups of 20-25 virgin female 2 year old rainbow trout raised under ambient photoperiod (Lat 52° 30'N) and water temperature (8.5-9.0°C) with a natural spawning time starting in early December were maintained from 17th January (1986) in lightproof 1200 litre tanks under the following photoperiods:

Group A - 18L:6D until 8th May followed by 6L:18D until spawning
Group B - 14L:10D until 8th May followed by 6L:18D until spawning
Group C - 10L:14D until 8th May followed by 6L:18D until spawning
Group D - 6L:18D until 8th May followed by 2L:22D until spawning
Group E - 6L:18D until 26th March, then 18L:6D until 8th May followed by 6L:18D until spawning.

The photoperiod in each tank was timed by an electronic clock controlling a 40W bulb providing 25 lux at the water surface. Water temperature was 8.5-9.0°C. Fish were fed 0.5% body weight $day^{-1}$ with a commercial trout diet. All fish were checked at monthly intervals to assess their state of maturity, and fortnightly once spawning had commenced. Blood samples were taken at approximately monthly intervals from a random sample of animals in each group. After centrifugation the serum was stored at -20°C. Because this experiment was performed during the final year of the project there was insufficient time to analyse the serum samples. However, it is clear from Experiments 1 and 2 that the spawning data alone serves as a satisfactory indicator of the effects of the respective photoperiod regimes on the timing of maturation. The

differences in spawning time between the groups were compared by Student's t-test or by d and treating it as t if the variances were statistically dissimilar. For an account of these materials and methods see Chapter 2.

## Results (Expt 3)

### Spawning

The time of spawning of individual fish in the respective groups is illustrated in Figure 4.19. Where the photoperiod was reduced to 6L:18D on 8th May from 18L:6D (Grp A), 14L:10D (Grp B) and 10L:14D (Grp C), spawning commenced on 23rd August, 3rd September and 2nd October respectively. In Grp A an estimated 86% of the female fish matured, all spawning within a period of 9 weeks from 23rd August. In Grp B, 72% of the fish matured within a period of 8 weeks, and in Grp C 66% of the fish attained maturity, also spawning over a period of 8 weeks. In Grp D where the photoperiod was reduced from 6L:18D to 2L:22D on 8th May an estimated 75% of the fish spawned, these within a period of 11 weeks between 2nd December 1986 and 19th February 1987. In Grp E where the fish were maintained on 6L:18D apart from a period of 18L:6D between 26th March and 8th May, 70% of the fish matured, commencing spawning on 18th September with the remainder of the trout in this group ovulating within a 6 week period.

Statistical analysis showed that the timing of spawning Grp A was not significantly different to Grp B but was advanced compared to Grp C ($P \leq 0.001$) and Grp E ($P \leq 0.01$). Grps B and E were both significantly advanced ($P \leq 0.001$) compared to Grp C. Grps A, B, C and E were all significantly advanced ($P \leq 0.001$) compared to Grp D.

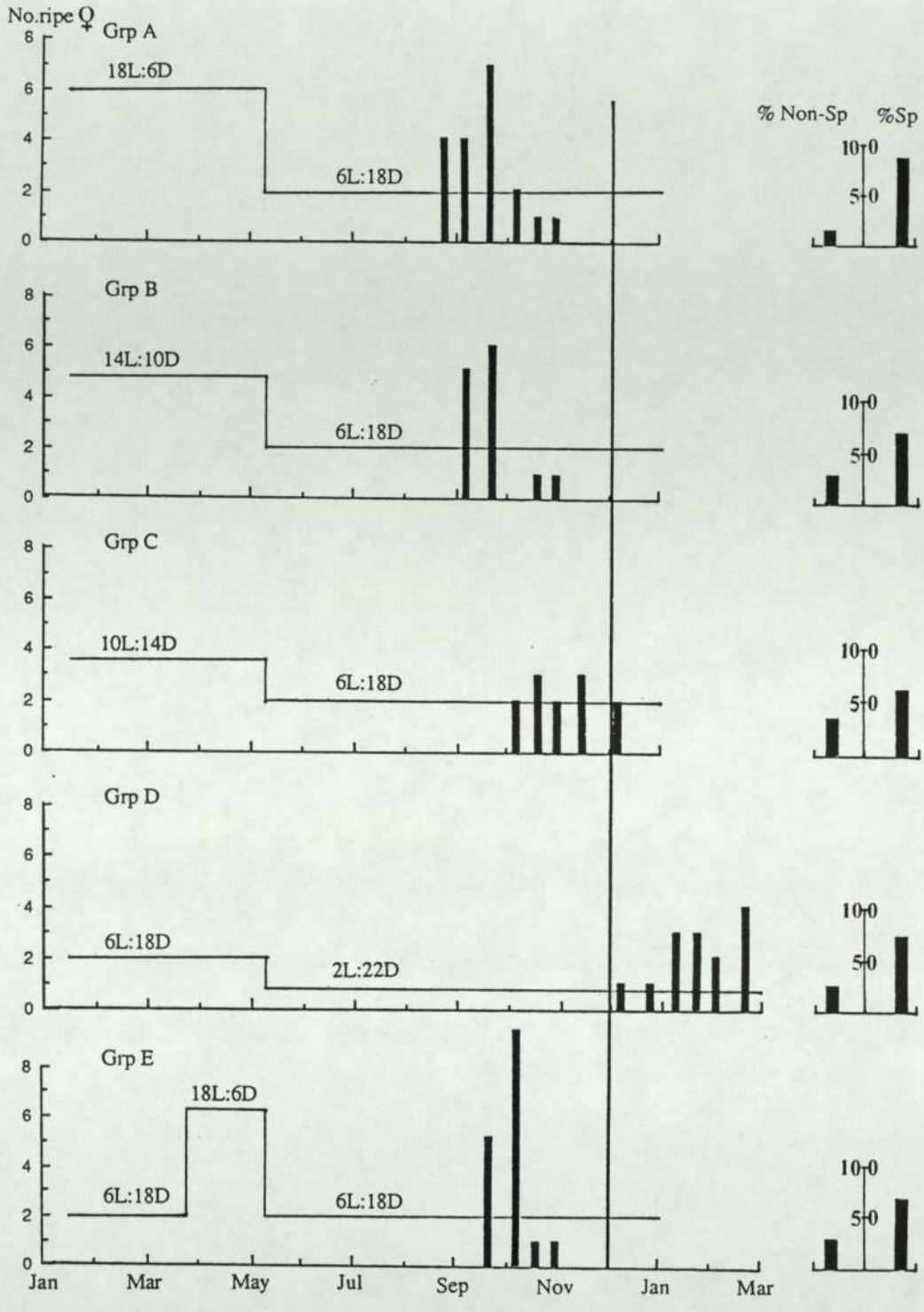



Fig. 4.19. The effect of five photoperiod regimes (Groups A-E) on the timing of maturation of female rainbow trout. Histograms illustrate the number of mature females(No. ripe ♀) at each sampling time. The percentage of females attaining maturation in each group are shown on the histograms at the right hand side of the figure. The photoperiod regime in each treatment is indicated by the horizontal lines. The vertical line running through all five histograms indicates the timing of the onset of spawning of rainbow trout of the same stock maintained under natural photoperiod. The vertical lines on the x-axis refer to the first day of the month as labelled.

Conclusions (Expt 3)

It can be concluded that 18L:6D (Grp A), 14L:10D (Grp B) and 10L:14D (Grp C) from mid-January are all perceived by the rainbow trout as a 'long' photoperiod and the subsequent reduction to 6L:18D on 8th May resulted in an advance in the timing of spawning of at least 2 months compared to fish under ambient conditions. These results support the hypothesis that the timing of the change in photoperiod provides an important cue in entraining the endogenous rhythm of maturation. However, it is clear that the timing of maturation in these three groups was not identical, Grp A commenced spawning on 23rd August, Grp B on 3rd September and Grp C on 2nd October. Similar differences in the timing of spawning between groups were also recorded in Expt 1 suggesting that the magnitude of the change in photoperiod has a supplementary effect on the timing of maturation. The differences in the timing of maturation of fish in Grps A, B and C are no doubt partly due to the reduction in photoperiod of either 12, 8 or 4 hrs respectively on the 8th May. However, consideration must also be given to the changes in daylength experienced by the fish at the start of the experiment in January, it is equally possible that the magnitude of the increase in photoperiod at this time of the year also had a effect on the timing of maturation. From this experiment alone it is not possible to quantify the individual effect of the magnitude of the change in photoperiod in January and May respectively on the timing of spawning; however a comparison of the results from Expt 1 and Expt 3 (see Discussion) indicate that they are of equal consequence. Certainly it is clear that the direction of the change in photoperiod at the start of the experiment is important in influencing the time of spawning. The ambient daylength at a latitude of 52° 30'N in mid-January is approximately 8.5 hrs, thus the fish in Grps A, B and C were all subjected to an abrupt increase in photoperiod, whereas in Grp D it was reduced to 6L:18D. The fact that Grps A, B and C commenced spawning within a six-week period starting in late-August compared to at least 2 months later in Grp D, support the conclusion from Expt 1 that an abrupt increase or decrease in photoperiod in January results in a phase advance or a delay respectively in the time of spawning. In Experiment 1 fish maintained under continuous 6L:18D from mid-January commenced spawning in March the following spring. In contrast, in the present experiment reducing the photoperiod from 6L:18D to 2L:22D (Grp D) on 8th May resulted in spawning commencing in early December and being completed by mid-February. It is suggested that this reduction in photoperiod had a phase advance effect on the timing of maturation, however it is not possible to make a firm conclusion because due to the limited experimental facilities it was not possible to run a continuous 6L:18D 'control' treatment. Despite this, the overall conclusion is that the response of

the rainbow trout to a particular photoperiod appears to depend on the previous daylength experienced by the fish. In Grps A, B and C, 6L:18D was interpreted as a 'short' photoperiod because they were previously maintained under a daylength greater than 10L:14D. In contrast, in Grp D 6L:18D was interpreted as a 'long' photoperiod because it was subsequently reduced to 2L:22D. The timing of gonadal maturation in rainbow trout and other species of vertebrates in relation to photoperiodic 'history' will be covered in more detail in the discussion to this chapter.

Finally, the results from Grp E show that a 6 week period of 18L:6D between late March and early May resulted in approximately 70% of the fish maturing, commencing spawning in late September. In contrast in Expt 2 where fish were maintained under 18L:6D for an identical duration, but between mid-January and 1st March (Grp A), only 26% of the animals matured. It was proposed that this low response was due to the reduction in photoperiod occurring before the majority of the trout had reached a threshold stage of development, phase advancing the endogenous clock so that the gate was in the closed position preventing maturation until the following year. In the present experiment it is proposed that shifting the period of 18L:6D to a position 9 weeks later in the spring allowed more fish to attain the threshold stage of development, pass through the gate and respond to the subsequent reduction in photoperiod on 8th May with a phase advance in the timing of spawning. It can be concluded that the results from Grp E support the hypothesis developed from Expt 2 that the duration of the period of long days is not crucial in determining the proportion of fish spawning, but rather the position they occur in relation to the phase of the maturation cycle.

## Summary

(i) The results support the hypothesis that changes in the length of the photoperiod serve to entrain an endogenous rhythm of maturation. The response to a particular photoperiod depends on the previous photoperiod experienced by the fish. For example, 6L:18D can be perceived as a 'long' photoperiod if rainbow trout are subsequently maintained under 2L:22D, or a 'short' photoperiod if they are previously under 10L:14D or greater daylength.

(ii) The magnitude of the change in daylength has a minor, supplementary effect on the timing of maturation; the greater the change in photoperiod the greater the phase shift.

(iii) The duration of the period of long days is not crucial in determining the proportion of fish maturing but rather the position they occur in relation to the phase of the maturation cycle.

## 4.5 Experiment 4: The effect of continuous light (LL) on the periodicity of the maturation cycle in the female rainbow trout.

Under natural environmental conditions rainbow trout characteristically spawn only once a year. However, when maintained under a constant photoperiod greater than 16L:8D rainbow trout proceed to spawn approximately every 6 months (Chapter 3, Bromage *et al*, 1984; Scott *et al*, 1984). These studies have indicated that in order to spawn again approximately 6 months later, fish raised under ambient conditions must be subjected to a long photoperiod from around the time of the natural winter ovulation. It has been suggested that a combination of the high circulating levels of gonadotropin that occur at ovulation and the long photoperiod results in a new batch of primary oocytes being immediately recruited into vitellogenesis (Scott *et al*, 1984). Under natural conditions these primary oocytes would not commence vitellogenesis until the spring (see Chapter 1). Subjecting rainbow trout to a long photoperiod a month after the winter ovulation will only advance the timing of the proceeding spawning by a few weeks compared to controls (Bromage and Whitehead, 1980; Bromage *et al*, 1984) indicating that there is only a limited time interval when the fish will respond to a long photoperiod with a spawning 6 months later.

As part of the study on the effects of photoperiod on the timing of maturation it was considered relevant to investigate this phenomenon of a 6 month spawning cycle more closely. Although it has been established that rainbow trout must be subjected to a long photoperiod at some time around the time of the natural winter ovulation to evoke another spawning six months later, this effect has only been superficially described. The aim of the current experiment was to quantify the relationship between the timing of the winter ovulation, long photoperiods and the occurrence of a 6 month spawning cycle. The experimental protocol involved groups of female rainbow trout approaching their first spawning being maintained under continuous light (LL) for a range of time periods and recording the periodicity of spawning. In addition to this main experiment a preliminary study involved maintaining a small group of rainbow trout on 16L:8D from just prior to ovulation. In addition to recording the timing of spawning, fish in the preliminary experiment were blood sampled at monthly intervals and the serum subsequently assayed for testosterone to give an indication of the stage of maturation.

### Materials and Methods (Expt 4)

(a) Preliminary experiment

A total of 7 virgin female rainbow trout approaching their first spawning were maintained in a lightproof 1200 litre tank from 24th November 1983 under a photoperiod

of 16L:8D. The tank was illuminated by a 40W bulb providing 30 lux at the water surface. Water temperature ranged from 11°C (winter) to 18°C (summer). Fish were fed 0.5% body weight day$^{-1}$ with a commercial trout diet. Each fish was identified with a numbered tag attached below the dorsal fin. Fish were checked for maturity at approximately two week intervals and in addition were blood sampled and the serum subsequently assayed for testosterone (see Chapter 2 for details).

(b) Main experiment

Approximately 300 virgin female 2 year old rainbow trout approaching their first spawning were selected from a stock of fish which spawn naturally in December and put into two holding tanks (1 and 2). Each tank was divided by netted screens into 6 sections with approximately 25 fish in each section. Tank 1 was maintained under an ambient photoperiod (Lat 51° N) and starting on 4th September 1985 Tank 2 was maintained under constant light (LL). Tank 2 was illuminated by a double filament 5 ft fluorescent strip suspended 1.5 metres above the tank providing between 19 and 74 lux at the water surface depending on the particular section (see results). By exchanging groups of fish between the constant light and ambient tanks at a variety of time intervals the following 12 photoperiod regimes were achieved:

| Grp | |
|---|---|
| 1 | LL from 4th September until 1st October then ambient photoperiod |
| 2 | LL from 4th September until 1st November then ambient photoperiod |
| 3 | LL from 4th September until 3rd December then ambient photoperiod |
| 4 | LL from 4th September until 9th January then ambient photoperiod |
| 5 | LL from 4th September until 6th February then ambient photoperiod |
| 6 | LL from 4th September until the end of the experiment (4.9.86) |
| 7 | Ambient photoperiod until 1st October then constant light (LL) |
| 8 | Ambient photoperiod until 1st November then constant light (LL) |
| 9 | Ambient photoperiod until 3rd December then constant light (LL) |
| 10 | Ambient photoperiod until 9th January then constant light (LL) |
| 11 | Ambient photoperiod until 6th February then constant light (LL) |
| 12 | Ambient photoperiod for entire experiment (control) |

Water temperature ranged between 7°C (winter) and 15.3°C (summer). Fish were fed 0.5% body weight day$^{-1}$ with a commercial trout diet. Each fish was identified by means of a numbered tag attached below the dorsal fin. Unfortunately during the course of the experiment most fish lost their tags as a result of them snagging on the net

partitions. Fish were checked for maturity at approximately monthly intervals during the winter spawning and at two week intervals during the spawning period the following summer. Spawning was said to have occurred when ripe eggs were expelled with gentle hand pressure on the abdomen, i.e. stripping. The eggs from a sample of fish from both the winter and summer spawnings were collected and the total egg volume, mean oocyte diameter together with post-stripped body weight were recorded from each individual. From these data total and relative fecundities were calculated. Student's t-test was used to compare means or by using d and treating it as t if the variances were statistically dissimilar (for further details of these materials and methods, see Chapter 2).

## Results (Expt 4)

(a) Preliminary experiment

Fish numbers 442, 429 and 440 were all spawned on 2nd December and again on 11th May, 30th May and 27th June respectively. Figure 4.20 shows that the serum testosterone levels of these fish were at basal levels (< 10 ng $ml^{-1}$) when they were first blood sampled on 9th December. The testosterone levels in all 3 fish rose steadily from January to March before increasing rapidly to reach peak levels of over 100 ng $ml^{-1}$ just prior to ovulation after which they returned towards basal. A peak of serum testosterone was not observed in fish 442 because it was not blood sampled close enough to the time of ovulation. Fish numbers 426 and 439 spawned on 2nd December, 435 on 9th December and 428 on the 16th December. None of these fish spawned the succeeding summer but did mature the following winter. Figure 4.21 shows that serum testosterone levels were all above 10 ng $ml^{-1}$ in early December but did not return to basal values until early February. Small increases of approximately 10 ng $ml^{-1}$ were detected in May which returned to basal before increasing rapidly in November prior to ovulation.

(b) Main experiment

Winter spawning: The fish in all 12 groups spawned for the first time between 1st November and 6th February (Figure 4.22a,b). Grps 3 and 7 ($P \leq 0.01$) and Grp 4 ($P \leq 0.05$) spawned significantly earlier compared to fish on an ambient photoperiod (Grp 12). There was no significant difference in the timing of spawning of any other groups compared to Grp 12.

Summer spawning: The spawning profiles of fish in Grps 1-12 are illustrated in Figures 4.22a and 4.22b.

Grp 1: No fish in this group spawned in the summer following the winter ovulation.

Grp 2: Two fish, 9.0% of the group spawned again in the summer.

Grp 3: Only one fish (4%) of the group spawned again in the summer.

Grp 4: 10 fish (37%) proceeded to spawn again in the summer between 29th May and 26th June. 5 fish in this group which had retained their identity tags were previously spawned on 3rd December.

Grp 5: 10 fish (45%) spawned in the summer, 9 fish between 29th May and 12th June and 1 fish on 7th August. Of the 7 fish with tags, 6 spawned previously on 3rd December and 1 on 9th January (this fish spawned again on 7th August).

Grp 6: 10 fish (50%) spawned again in the summer between 29th May and 20th August. Five fish with tags intact all previously spawned on 3rd December.

Grp 7: 16 fish (84%) proceeded to spawn again in the summer between 29th May and 20th August. The four fish with identity tags intact all previously spawned on 3rd December.

Grp 8: 13 fish (54%) spawned again in the summer between 29th May and 4th September. 7 out of the 9 fish with tags spawned previously on 3rd December, the other 2 on 9th January.

Grp 9: 4 of the remaining 11 fish (36%) spawned again between 12th June and 20th August. The two fish with tags previously spawned on 3rd December.

Grp 10: 5 fish (22%) spawned in the summer between 10th July and 4th September. All three fish with tags spawned previously on 3rd December.

Grp 11: 3 fish (14%) spawned again in the summer between 2nd May and 4th September.

Grp 12: 2 fish (8%) spawned again in the summer, both on 12th June. Both of these fish were found to be ripe on 9th January.

With the exception of Grp 4 which had a significantly smaller variance ($P \leq 0.05$; F-test), there was no significant difference in the distribution of the spawning profiles of the other groups. Apart from Grp 1 all the other groups contained at least one fish which spawned again in the summer. Statistical analyses were only performed on those groups with over 20% of the proportion spawning for a second time in the summer. Spawning in Grp 10 was significantly delayed compared to Grp 5 ($P \leq 0.001$) and Grps 4, 6 and 7 ($P \leq 0.01$). Spawning in Grp 5 was significantly advanced compared to Grp 8 ($P \leq 0.001$) and Grps 9 and 10 ($P \leq 0.05$). Grp 6 spawned significantly earlier than Grp 8 ($P \leq 0.05$). Spawning in Grp 4 was significantly advanced compared to Grp 8 ($P \leq 0.001$), Grps 7 and 10 ($P \leq 0.01$) and Grps 6 and 9 ($P \leq 0.05$).

Fish weight, egg size and fecundity data

A random sample of 23 fish were taken from those fish which were found to be ripe on 3rd December. The mean (± standard error mean) body weight of these fish was 1.188± 0.069 Kg; total fecundity 2689 ± 111 eggs $fish^{-1}$, relative fecundity of 2432 ± 174 eggs $kg^{-1}$ and oocyte diameter 4.654 ± 0.063mm. The same measurements were made on 53 fish which spawned again in the summer. Significant increases were detected in body weight (1.688 ± 0.067 Kg, $P \leq 0.001$) and total fecundity (3251 ± 136 eggs $fish^{-1}$, $P \leq 0.01$) compared to the spawning the previous winter. Significant decreases were detected in the mean oocyte diameter (4.351 ± 0.057 mm, $P \leq 0.001$) and relative fecundity (2025 ± 83 eggs $kg^{-1}$, $P \leq 0.05$).

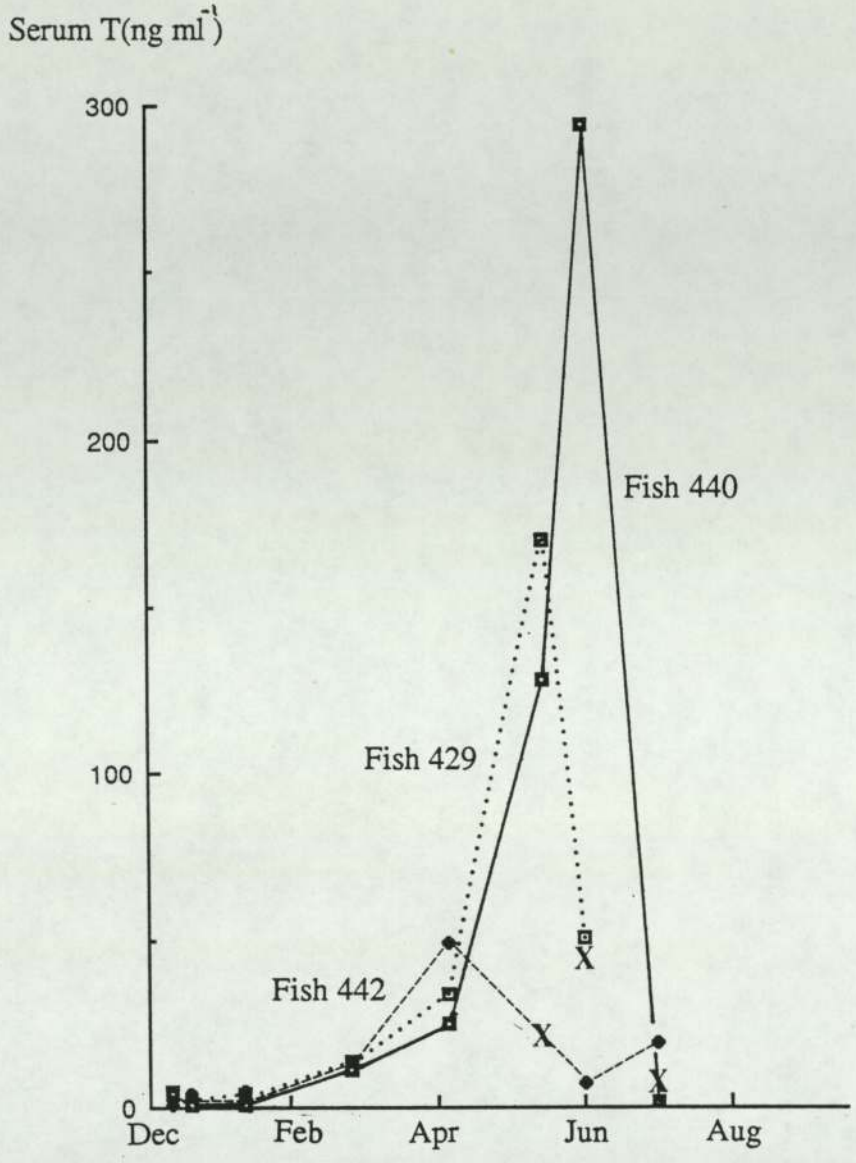



Fig. 4.20. Sequential changes in the levels of serum testosterone (T, ng $ml^{-1}$) of individual female rainbow trout maintained under constant 16L:8D from 24th November. All three fish spawned on 2nd December and again in the following summer at the dates shown(X). The vertical lines on the x-axis refer to the first day of the month as labelled.

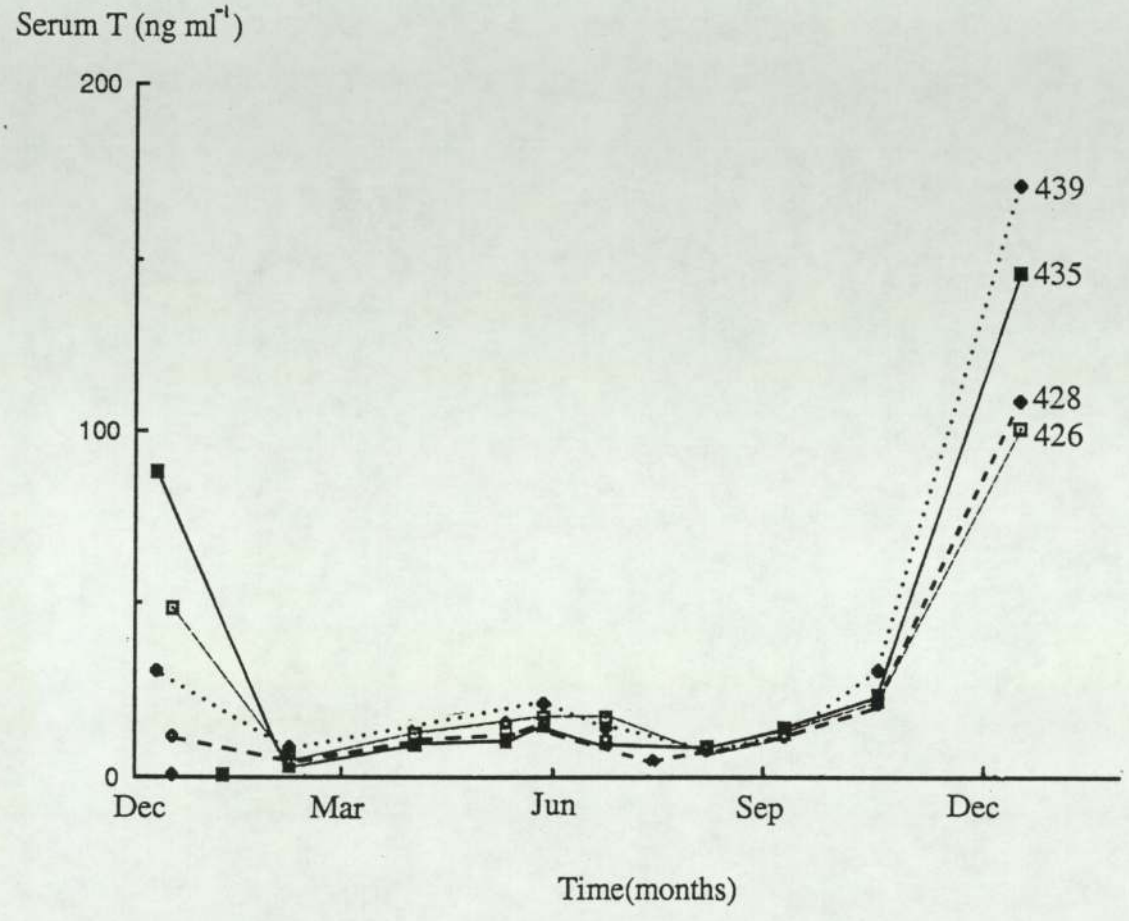



Fig. 4.21. Sequential changes in the levels of serum testosterone (T, ng ml$^{-1}$) of individual female rainbow trout maintained under constant 16L:8D from 24th November. All four fish spawned in the winter between 2nd-16th December but failed to spawn again the following summer , maturing instead the proceeding winter as indicated by the elevated testosterone levels. The vertical lines on the x-axis refer to the first day of the month as labelled.

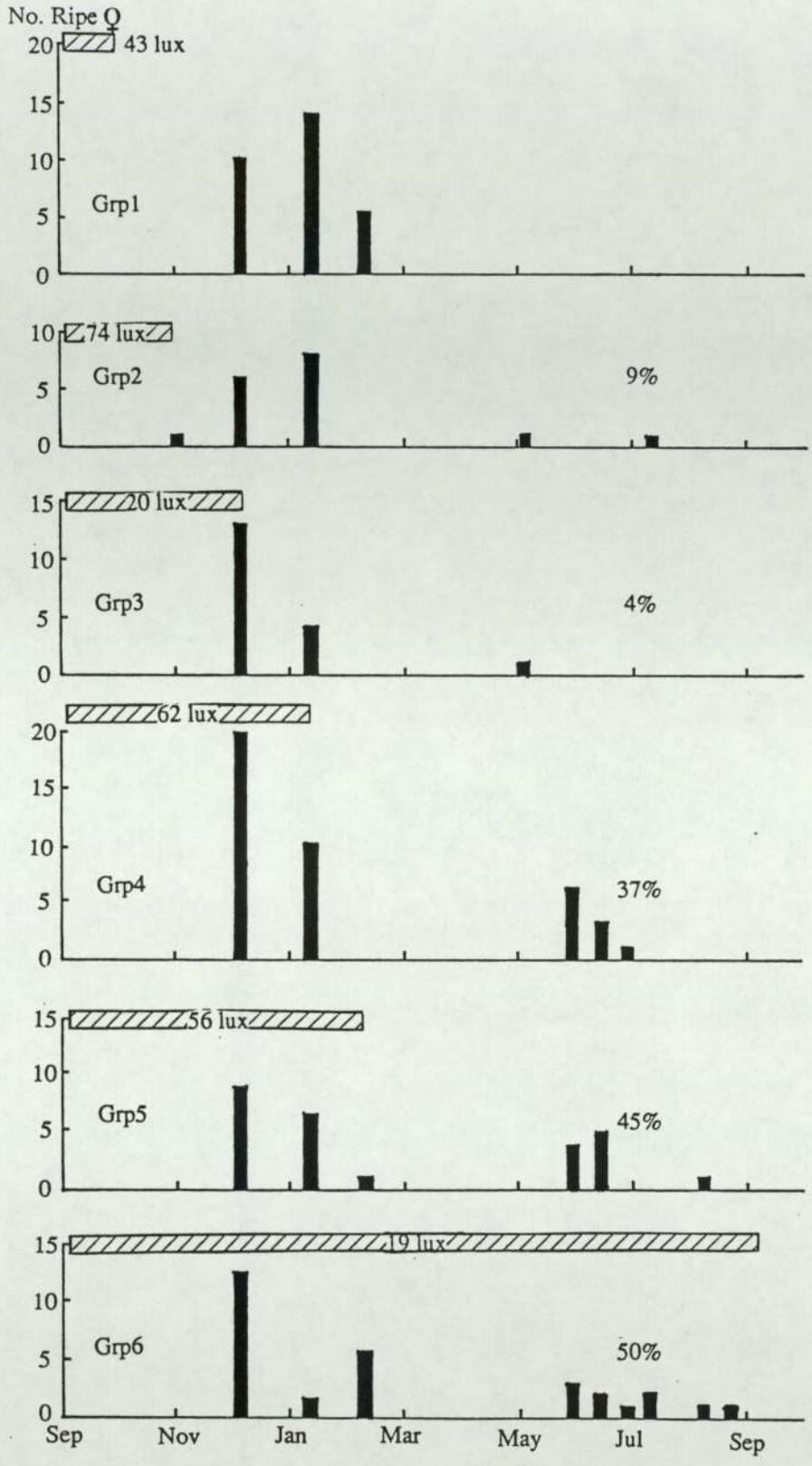



Fig. 4.22a. The effect of six photoperiod regimes (Groups 1-6) on the timing of maturation in female rainbow trout. Histograms illustrate the number of ripe females (No. ripe ♀) at each sampling time. The horizontal bars represent the period of time the fish in each treatment were maintained under constant light (LL) and the light intensity (lux). The number above the summer spawning histograms gives the percentage of fish in each group which spawned again in the summer. The vertical lines on the x-axis refer to the first day of the month as labelled.

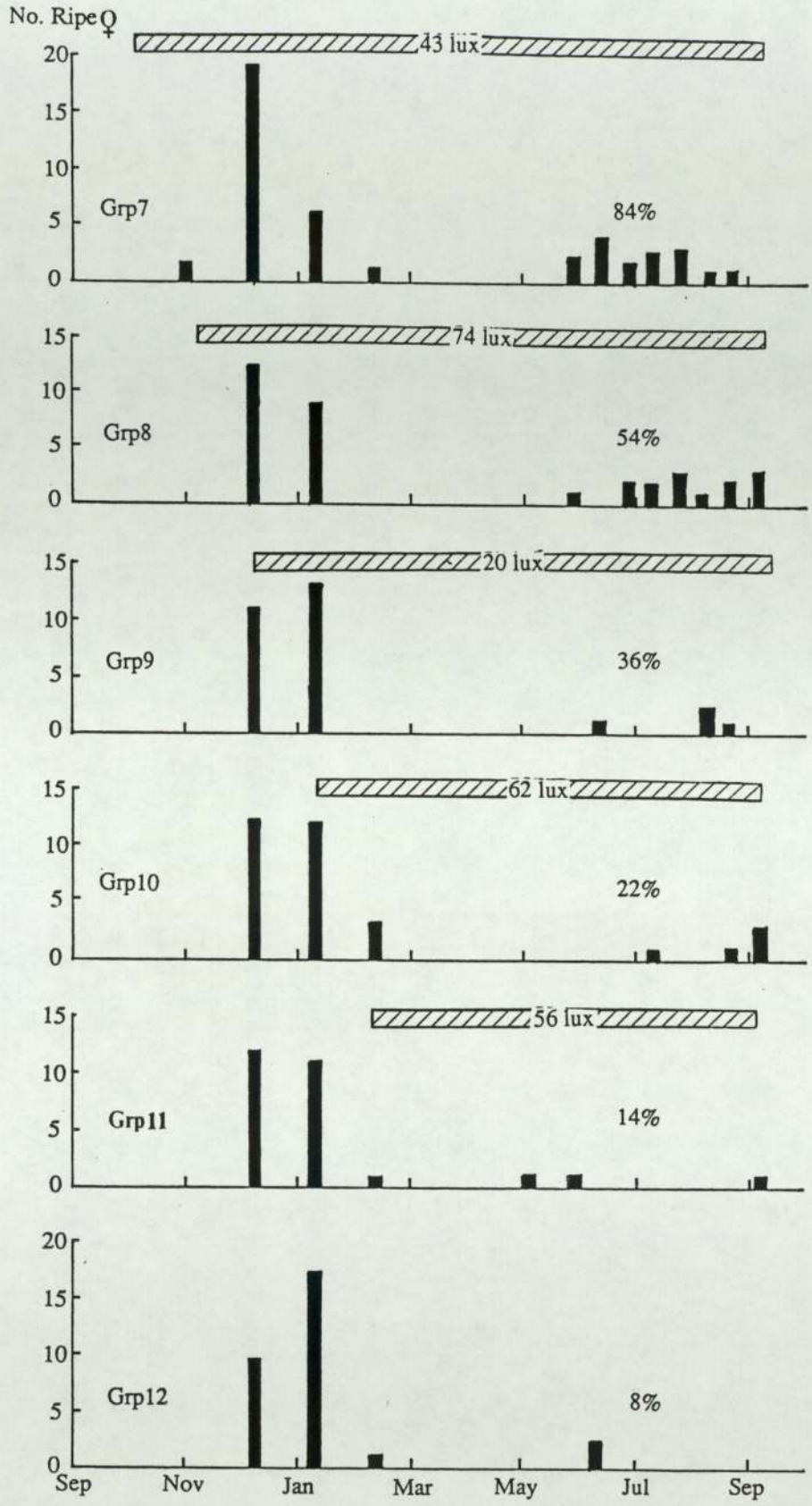



Fig. 4.22b. The effect of six photoperiod regimes (Groups 7-12) on the timing of maturation of female rainbow trout. Histograms illustrate the number of mature females (No. ripe♀) at each sampling time. The horizontal bars represent the period of time the fish in each treatment were maintained under constant light(LL) and the light intensity(lux). The number above the summer spawning histograms gives the percentage of fish in each group which spawned again in the summer. The vertical lines on the x-axis refer to the first day of the month as labelled.

Conclusions (Expt 4)

The results indicate that the timing of exposure to a long photoperiod in relation to the winter ovulation is critical if a further spawning approximately 6 months later is to be induced. This finding confirms the conclusions drawn from earlier studies (Bromage *et al*, 1984; Scott *et al*, 1984).

Continuous light (LL) prior to the winter ovulation had little effect in evoking a 6 month spawning cycle; Grps 1-3 only received LL during this period and as a result only a few fish spawned again in the summer. In Grp 3, after being maintained under LL for 3 months fish were returned to an ambient photoperiod on 3rd December on which date 13 animals were found to be ripe. All these fish ovulated under a long photoperiod and yet only one proceeded to spawn again in the summer. This suggests that LL at the time of, or even just after ovulation is not effective in stimulating spawning to occur the following summer. In contrast in Grp 4 where the long photoperiod was extended until 9th January, 37% of the fish proceeded to spawn again in the summer. 6 out of 7 of these fish which had retained their identity tags were previously ripe on 3rd December, supporting the conclusion that a long photoperiod up to 1 month post ovulation is necessary to evoke an additional spawning the following summer. In Grps 5 and 6, LL was extended to 9th February and for the entire experiment respectively; under both these regimes approximately 50% of the fish spawned again in the summer. The results from Grp 6 show that even under permanent LL not all the fish are physiologically competent to mature again immediately after the winter ovulation. 11 of the 13 fish in Grps 5 and 6 which retained their tags and spawned again in the summer were ripe on 3rd December, this indicates that the earlier maturing fish may have a better capacity of spawning again the following summer.

A further conclusion that can be drawn from the results from Grps 1-6 is that once a long photoperiod has initiated the maturation of a batch of oocytes it is not necessary to maintain them on LL, for returning them to ambient conditions does not prevent them spawning again in the summer (see Grps 4,5). The results from Grps 7-11 which were maintained under the reciprocal photoperiod regimes to Grps 1-5 broadly support the conclusion that subjecting fish to a long photoperiod during the immediate post-ovulatory period is important in causing them to spawn again in the summer. A long photoperiod from 9th February (Grp 11) onwards only resulted in a small percentage of the fish spawning again suggesting that this may be too late after the winter ovulation to induce the early maturation of a further batch of oocytes. The earlier in the year the groups were exposed to a long photoperiod the greater the percentage of the fish spawned the following summer. In Grp 10 the long photoperiod commenced from early January and resulted in 22% of the fish in this group spawned again in the summer.

Where fish were exposed to LL from the 3rd December (Grp 9), 1st November (Grp 8) and 1st October (Grp 7), 36, 54 and 84% respectively of the fish spawned again in the following summer. Similar to Grps 1-6, the results from Grps 7-11 indicate that the majority of the fish that spawn in the summer are the early spawning fish in the preceding winter: In Grp 7 all 4 fish which spawned in the summer and retained their identity tags were previously ripe on 3rd December. In Grp 8, 7 of the 9 summer spawning fish with tags were previously found to be mature on 3rd December, the other two on 9th January. In Grp 9, LL commenced on 3rd December and only resulted in 4 fish (36%) spawning in the summer, of these the two fish with tags were previously ripe on 3rd December. In Grp 10 the long photoperiod commenced from early January; interestingly the 3 fish with tags which spawned in the summer were all previously found to be mature on 3rd December. This result from Grp 10 indicates that some fish will spawn again in the summer even if there is at least a month between the winter ovulation and the onset of a long photoperiod.

To summarise, in all 12 groups a total of 76 (out of 262 remaining fish) spawned in the summer. Of the 43 fish which retained their tags, 34 spawned previously on 3rd December and only 9 on 9th January (Ratio = 3.8:1) which indicates that in general only the early winter spawners are competent to respond to the long photoperiod and spawn again the following summer. This result is not accountable to the distribution of the winter spawning as 77 fish spawned on 3rd December and 68 on 9th January (Ratio = 1.13:1). The results from the preliminary experiment also support the conclusion that fish which spawned early in the winter tend to be the ones which spawn again in the summer. In the three fish where serum testosterone levels had returned to basal by mid-December after ovulation, all spawned again in the summer (Figure 4.20). In contrast, the four other fish which ovulated later in the winter, as indicated by the fact that the testosterone levels were still elevated in December, did not respond to the long photoperiod and failed to spawn again in the summer (Figure 4.21). The possible mechanism determining whether or not a fish spawns again in the summer will be discussed presently.

In addition to long photoperiods after the winter maturation, the results from Grps 7 and 8 suggest that LL before ovulation may also have some importance in determining whether a fish will spawn again 6 months hence. In Grp 8, LL commenced from 1st November and resulted in 54% of the fish spawning again in the summer; in Grp 7 where LL commenced a month earlier on 1st October 84% of the trout matured again. This result indicates that a long photoperiod during October is important to a certain proportion of the fish in stimulating a 6 month spawning cycle. Unfortunately there are inconsistencies in the results; in Grp 6 for example only 50% of the fish

spawned in the summer even though they were maintained under LL from 1st September. A possible explanation for this difference is that the light intensity at the water surface in Grp 6 was only 19 lux whereas in Grp 7 it ws 43 lux. The light intensity was dependent on the distance of the light source from the respective sections, the positions of which were allocated randomly. Ideally the light intensity above each section should have been the same but this was not possible because the study was performed on a commercial trout farm which meant there were some unavoidable limitations to experimental design and construction.

The majority of fish in this study spawned for the first time between early December and early January. This spawning profile corresponds well with the general observation that the spawning of a strain of rainbow trout under natural conditions is confined to approximately 6 weeks (Kato, 1973). This experimental result supports the earlier conclusion that the long photoperiod regimes had little effect on the timing of the winter spawning. In contrast, the subsequent summer spawning of fish in Grps 6-10 were spread over a period of 3 months. This desynchronisation of spawning time of fish maintained under a constant long photoperiod has been observed in previous studies (Experiments 1, 2 this chapter; Chapter 3; Bromage *et al*, 1984). As a specific example, 7 fish in Grp 8 which retained their tags were synchronised in their winter maturation, all spawned on 3rd December. However when these particular animals matured again in the summer 1 fish spawned on 29th May, 2 fish on 26th June, 1 on 10th July, 1 on 7th August and 2 on 4th September. It is proposed that this desynchronisation of spawning is due to the 'free-running' of an endogenous circannual timing mechanism which ultimately controls maturation. The conclusions to the previous experiments described in this chapter have attempted to explain the effects of photoperiod on the timing of maturation in terms of the entrainment of this endogenous circannual oscillator. Accordingly the remainder of this section will consider whether it is possible to reconcile the results from the present experiment in terms of entrainment.

In contrast to the desynchronised summer spawning of fish in Grps 6-10, the fish in Grps 4 and 5 mainly spawned within a four week period commencing on the 29th May. Unlike Grps 6-10 which were maintained on LL until the end of the experiment, Grps 4 and 5 were maintained on a long photoperiod until the 9th January and 6th February respectively and then returned to an ambient photoperiod. It is suggested that this abrupt reduction in photoperiod had the effect of synchronising the maturation rhythms of the individual fish within each group similar to the results in the previous three experiments.

It has been established that a reduction in the photoperiod not only synchronises but also entrains the timing of maturation by causing a phase-shift of the underlying circannual oscillator (Experiments 1-3). In support of this hypothesis the timing of spawning in Grps 4 and 5 were both significantly advanced compared to Grps 8, 9, 10; in addition Grp 4 was also advanced compared to Grps 6 and 7. Thus the results indicate that once a summer spawning has been initiated by the stimulatory effect of a long photoperiod the maturation process behaves according to entrainment theory. The difference in interpreting the results from this experiment compared to Experiments 1-3 is that the underlying oscillator appears to exhibit a periodicity of approximately six months. Alternatively it could be argued that LL immediately after the winter ovulation had the effect of phase advancing the circannual rhythm of maturation by 6 months. However, it is established that rainbow trout maintained under a constant long photoperiod for an extended period of time exhibit a self sustained rhythm of maturation with a periodicity of approximately 6 months (Chapter 3). The fact that the rhythm appears to be 'free-running' indicates that the fish are perceiving the environmental conditions as being constant. For this reason it is suggested that a hypothesis of a long photoperiod causing repeated 6 month phase advances in the time of spawning is not valid. A preferred hypothesis to explain the 6 month periodicity of spawning is that involving multiple circannual oscillators as described in Chapter 3. The crux of the argument is that under ambient conditions the two oscillators are in phase with each other and the fish only spawn once per year; under conditions of a long photoperiod the two circannual oscillators 'split' and assume an approximately $180^{o}$ phase difference between each other. If both oscillators were capable of controlling maturation then spawning would be observed at approximately twice the natural frequency. The attraction of this hypothesis is that not only can it explain how a 6 month spawning could occur, but it can also account for the observed pattern of summer spawning which behaved as if being controlled by a free-running circannual oscillator capable of entrainment.

Intuitively the finite time taken to grow primary oocytes to the fully mature stage must have some bearing on the periodicity between winter and summer spawning. However, the results show that the summer spawning fish had significantly smaller eggs compared to the previous winter batch of eggs indicating that oocyte size *per se* does not dictate when ovulation occurs. In addition there was no correlation between mature total ovary volume and the weight of the fish indicating that relative gonad size does not dictate the time of ovulation. As concluded from the previous experiments in this chapter, it is proposed that an endogenous timing mechanism possibly located in the higher neural centres has a crucial role in controlling the whole process of maturation

possibly by modulating the release of the hormones of the hypothalamo-pituitary axis.

Although the role of the ovary in controlling the timing of ovulation is uncertain, the results suggest that the physiological state of the gonads may determine whether or not a fish will respond to a long photoperiod. Scott *et al* (1984) suggested that the combination of a long photoperiod and the high circulating levels of gonadotropin that occur around the time ovulation result in another batch of primary oocytes being recruited into vitellogenesis. The results from this experiment indicate that the actual mechanism is more complex; in general the fish which spawned later in the winter failed to mature again in 6 months despite receiving long photoperiods post-ovulation. The majority of fish which did mature again in the summer were those which were spawned in early December. There are parallels between these results and those from Experiment 2 which indicated some form of internal gating mechanism was involved in determining whether a fish would mature that season. It appears that there is only a specific period of time when the fish will respond to a long photoperiod and spawn again in the summer. In an attempt to define this gate more precisely a current experiment is investigating the effects of 2 month periods of LL at different times of the year on the timing of maturation. The results to this experiment will be reported elsewhere.

## Summary

(i) A long photoperiod after the winter spawning can initiate the maturation of oocytes which are ovulated the following summer.

(ii) The early spawning fish in the winter will respond to a long photoperiod, but those spawning later do not mature again in the summer despite receiving a long photoperiod post-ovulation.

(iii) Once a batch of oocytes have been recruited into maturation it is not necessary to maintain the fish on a long photoperiod for them to spawn successfully the following summer.

(iv) The results support the hypothesis that maturation in rainbow trout is controlled by an endogenous circannual clock (or clocks) which is capable of being entrained by changes in the photoperiod.

## 4.6 GENERAL DISCUSSION

The most important conclusion to be drawn from the experiments described in this chapter is that abrupt changes in the length of the photoperiod have significant effects on the timing of maturation in the female rainbow trout. In general the results support the hypothesis that changes in photoperiod serve to entrain an endogenous circannual oscillator or clock which is involved in controlling maturation. This hypothesis will be discussed in relation to present and previous research on salmonid reproduction and with the control of seasonal events in other organisms.

For endogenous timing mechanisms to be of any adaptive value they must be capable of being entrained to local time. This entrainment results in the physiological and behavioural functions remaining in synchrony with the periodic changes in the environment and ensures that they occur at times which optimise the chances of survival of the organism. Most of the research and theories on entrainment of endogenous clocks has been confined to oscillators with a circadian periodicity. It is proposed that there are analogies between the entrainment theory of circadian clocks and the behaviour of the timing of maturation in the rainbow trout to changes in photoperiod. Drawing on these similarities assist in gaining an understanding of how the photoperiodic control of the timing of maturation in this fish is achieved. In many species the 24 hr cycle of light intensity serves as an important entraining agent to circadian rhythms. A commonly used experimental protocol to investigate this entrainment is to record how the free-running rhythm under constant darkness (DD) responds to short pulses (~ 15 minutes) of light (Pittendrigh, 1981; Daan, 1982). As shown in Figure 4.2.3 the rhythm can be advanced or delayed depending on which phase of the cycle is illuminated. Under natural conditions these corrective phase shifts ensure that the circadian rhythm remains entrained to the 24 hr light-dark cycle. Analogously it is suggested that changes in the length of the photoperiod serve as entrainment cues to the endogenous circannual clock controlling the maturation cycle of the rainbow trout. Under natural conditions the annual cycle of daylength has an important role synchronising the rhythm to 12 months, but under constant environmental conditions the rainbow trout exhibits a free-running endogenously driven circannual rhythm of maturation (Chapter 3). The results presented in this chapter indicate that abrupt changes in the length of the photoperiod serve to phase shift this endogenous rhythm. Whether the rhythm is phase advanced or delayed depends on two major factors, the direction of the change in photoperiod and the timing of the change relative to the position or phase of the internal clock.

In general, in Experiments 1, 2 and 3 an abrupt reduction from a 'long' to a shorter photoperiod resulted in an advance in the timing of maturation. Similar photoperiod-induced changes in the timing of maturation of salmonids have been observed by other groups (Henderson, 1963; Shiraishi and Fukuda, 1966; Eriksson and Lundqvist, 1980; Bromage *et al*, 1984; Takashima and Yamada, 1984). It is suggested that this advance in the timing of spawning is due to the reduction in photoperiod occurring earlier than expected, the trout perceiving the internal circannual clock as running 'behind-time' and initiating a corrective phase advance. In Experiment 2 where groups of fish were subjected to reduction in photoperiod at different times, the later in the year the reduction occurred the smaller was the phase advance (Figure 4.18). This can be interpreted as being due to the reduction in photoperiod occurring progressively closer to the expected reduction in daylength normally experienced in autumn, thus resulting in a smaller corrective phase advance in the circannual rhythm. In a previous study on rainbow trout where a reduction from 18L:6D to 6L:18D in May and July respectively resulted in an advance in the timing of maturation compared to fish on constant 18L:6D, a similar reduction in photoperiod in early October resulted in a delay in spawning (Bromage *et al*, 1984). At the time these paradoxical results of an advance and a delay from an identical stimulus were difficult to explain. However, if the response to a particular change in photoperiod is plotted against the timing of the reduction there is a clear relationship between these two parameters (Figure 4.23). The phase-response 'curve' shown in Figure 4.23 is similar to the response of a circadian rhythm to pulses of light and can best be explained in terms of changes in photoperiod entraining an endogenous circannual rhythm. Because the phase changes shown in Figure 4.23 are compared to fish maintained on 18L:6D throughout the experiment, they can be directly attributed to the reduction from 18L:6D to 6L:18D. However, it is evident that the increase from an ambient to a long photoperiod at the start of the experiments in January or February would also have had the effect of entraining the endogenous circannual rhythm. It has been established that abrupt increases in photoperiod at this time of year results in an advance in the timing of maturation of 1-2 months compared to fish on an ambient photoperiod (Expt 1; Expt 2; Bromage *et al*, 1984; Takashima and Yamada, 1984). This effect can be explained in terms of the animals receiving long days earlier than expected and respond with a corrective phase advance in the circannual clock. Thus, the advance in the time of spawning of fish in Experiments 1, 2 and 3 was due to the combined effect of an increase in the photoperiod in January, followed by a reduction later in the experiment.

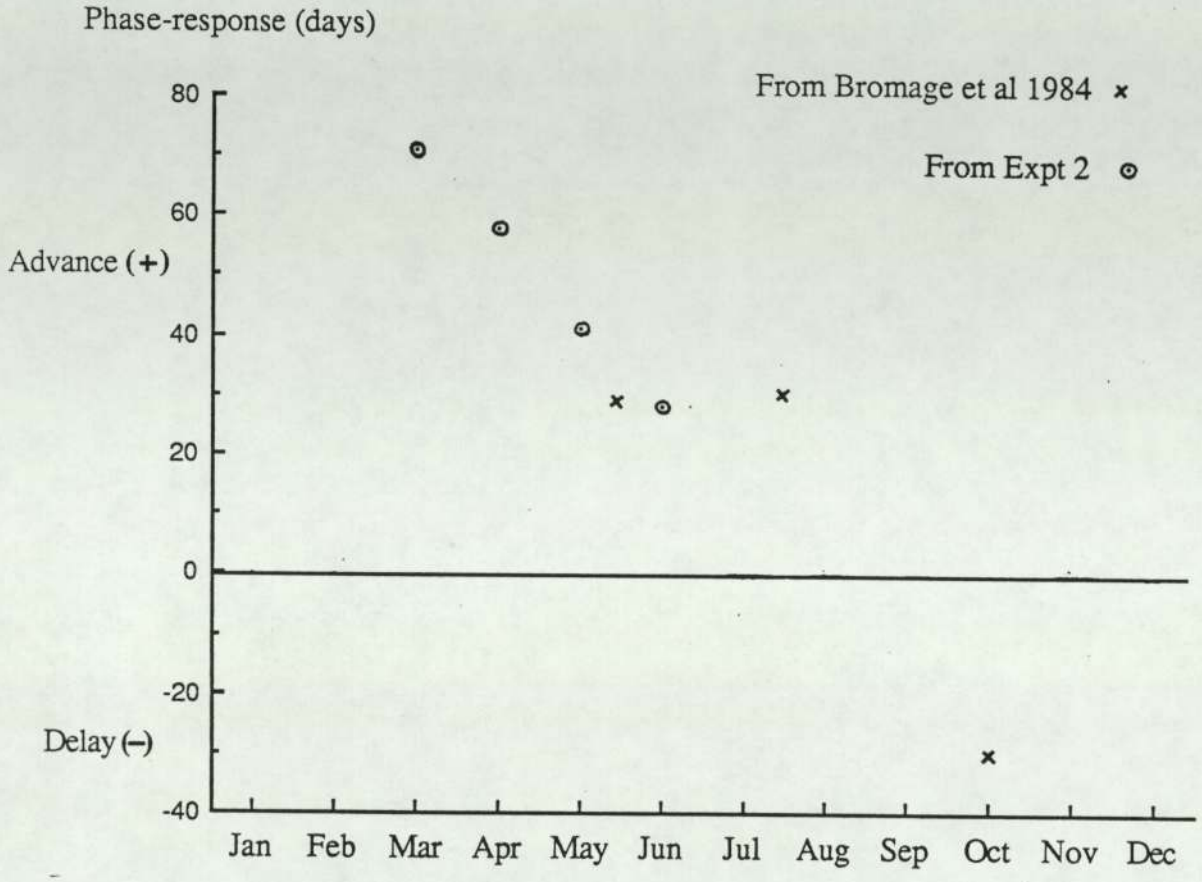



Fig 4.23 Phase-response 'curve' for the effect of an abrupt reduction from 18L:6D to 6L:18D at different times of the year on the alteration in the timing of maturation of female rainbow trout. The advance(+) or delay(-) in the timing of spawning (days) is calculated relative to fish maintained on constant 18L:6D. The vertical line on the x-axis refers to the first day of the month as labelled.

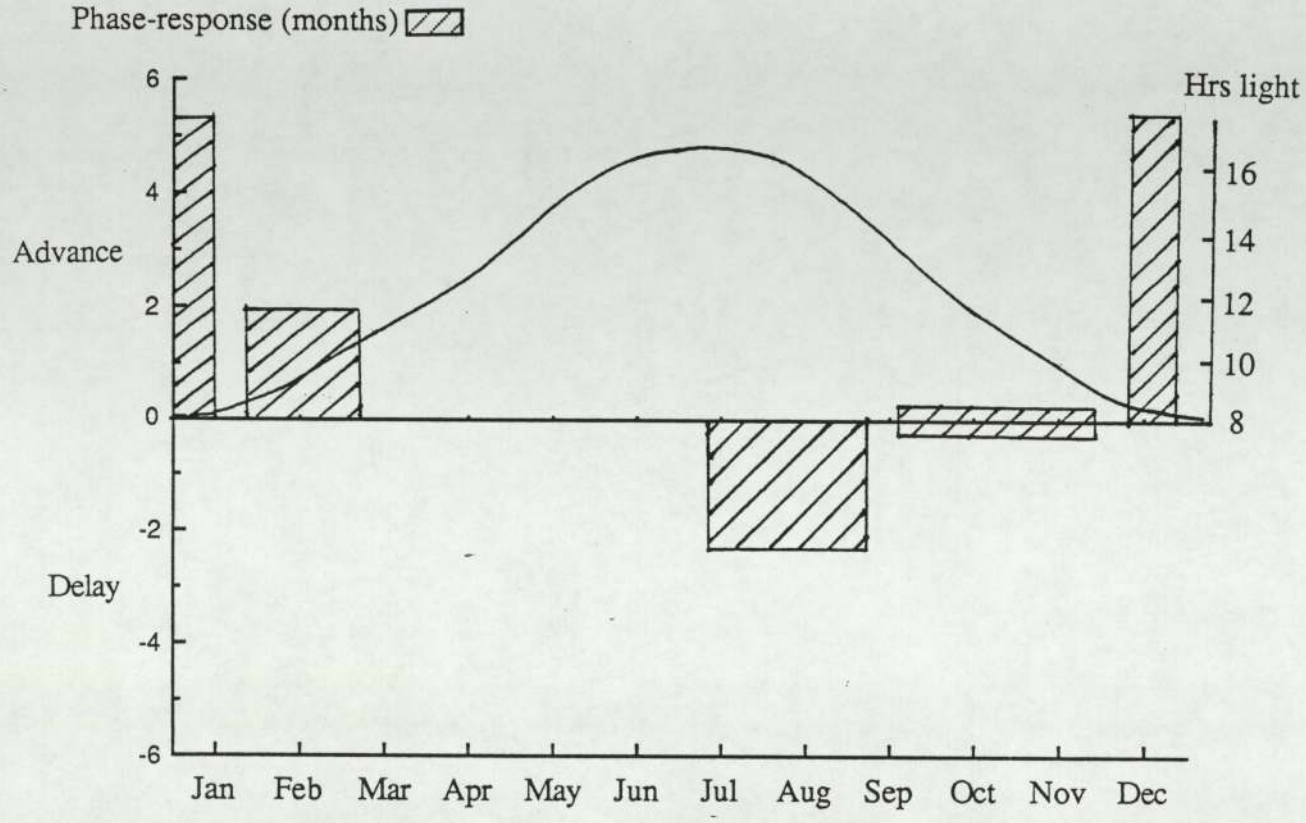



Fig 4.24 Phase-response 'curve' for the effect of an abrupt change from ambient photoperiod to a long photoperiod ($\geqslant$ 16L:8D) at different times of the year on the alteration in the timing of maturation of a naturally December spawning strain of rainbow trout. Data derived from several experiments (see text for details). Also depicted is the annual change in daylength (Lat $52^{o}$N). The vertical lines on the x-axis refer to the first day of the month as labelled.

An abrupt change from an ambient to a long photoperiod can also have phase shift effects on the timing of maturation similar to the reduction from a long to a short photoperiod described above. In accordance with the entrainment hypothesis the direction and magnitude of the shift is dictated by the timing of the change in photoperiod; whereas a change from ambient to a long photoperiod in January or February resulted in an advance in the time of spawning (see above), a similar change between June and mid-August resulted in a delay in the timing of maturation of between 3 weeks and 4 months depending on the species and strain of salmonid being studied (Allison, 1951; Shiraishi and Fukuda, 1966; Eriksson and Lundqvist, 1980; Takashima and Yamada, 1984). Again this effect can be interpreted as being due to a corrective phase delay in the circannual rhythm in response to receiving long days after they had been expected to decrease. It is interesting to note that maintaining rainbow trout on a long photoperiod from early September onwards (Expt 4) failed to delay the timing of the winter spawning compared to fish maintained on an ambient photoperiod. However, a long photoperiod around the time of the winter ovulation resulted in a proportion of the fish spawning again approximately 6 months later (Expt 4; Bromage *et al*, 1984; Scott *et al*, 1984). If kept under these conditions for a long period of time rainbow trout proceed to exhibit a free-running semi-circannual rhythm of maturation indicating that the animals perceive the photoperiod as 'constant' (see Chapter 3). Although the reason for this semi-circannual rhythm of spawning is unclear, it is suggested that the initial effect of a switch from an ambient to a long photoperiod around the time of the winter ovulation is a 6 month phase advance in the time of the succeeding spawning. It is clear that there is only a relative small time interval when this effect will occur because fish put onto a long photoperiod 1-2 months after the winter ovulation exhibit only a small advance in the timing of the next spawning (see above). To summarise, an abrupt switch from an ambient to a long photoperiod ($\geq$ 16L:8D) can advance or delay maturation depending on the timing of the change. Similar to the effects of a reduction from a constant long to a constant shorter photoperiod discussed earlier, the results can be interpreted as the change in photoperiod causing a phase advance or delay of a circannual clock involved in the control of maturation. Although these are not a comprehensive set of data, it is possible to represent the effects of an ambient to long change in the photoperiod in the form of a phase-response curve (Figure 4.24).

In addition to changes from ambient to a long photoperiod, the effect of a change from ambient to a shorter constant photoperiod has also been investigated. Maintaining autumn-winter spawning salmonids from January-February on 6L:18D resulted in a delay in the timing of maturation of 2-3 months compared to fish on an ambient photoperiod (Expt 1; Bromage *et al*, 1984; Takashima and Yamada, 1984). In

contrast, maintaining salmonids on a natural photocycle until summer followed by an abrupt reduction to a short photoperiod resulted in an advancement in the timing of maturation compared to controls (Shiraishi and Fukuda, 1966; Eriksson and Lundqvist, 1980; Lundqvist, 1980; Takashima and Yamada, 1984). Once again, these effects can be interpreted in terms of an endogenous circannual clock making corrective phase changes in response to the entraining influence of alterations in the length of the photoperiod. Unfortunately for this particular change in photoperiod there is insufficient data to construct a phase-response graph.

The review of seasonal reproduction in vertebrates (Chapter 1) illustrated that both endogenous long-term timing mechanisms and exogenous cues such as alterations in photoperiod and temperature co-operate in controlling the timing of maturation in a range of species (e.g. Teleosts: Sundararaj and Vasal, 1976; Baggerman, 1980; Sundararaj *et al*, 1982. Reptiles: Licht, 1972a, 1984; Cuellar, 1981. Birds: Gwinner, 1971, 1977; Berthold, 1979; Chandola *et al*, 1983; Sharp, 1984. Mammals: Lincoln, 1978; Reiter, 1980; Almeida and Lincoln, 1984). Although the concept of environmental cues entraining long-term endogenous rhythms is established, quantitative analysis of this phenomenon has only been the subject of a small number of studies. Only Gwinner (1971) working on birds has previously proposed that abrupt changes in photoperiod alter the timing of seasonal events by phase shifting an underlying circannual rhythm. He presented evidence which indicated than an endogenous circannual rhythm exhibited a periodically changing sensitivity to the stimulus provided by a zeitgeber analogous to the entrainment behaviour of circadian oscillators (see Introduction). Interestingly, research on the effects of abrupt changes in photoperiod on the onset of puberty in lambs (*Ovis aries*) also suggests the entrainment of an endogenous circannual oscillator, although this has not been considered by the authors concerned (review: Foster *et al*, 1986). The onset of puberty in lambs maintained on 9L:15D from March was delayed several weeks compared to controls (Yellon and Foster, 1985), this is similar to the delaying effect of a short photoperiod from January-February on the time of spawning in rainbow trout (Expt 1; Bromage *et al*, 1984). In contrast an advance in the onset of puberty in lambs occurred when the 9L:15D photo-regime was interrupted with a period of long days (15L:9D) before the end of July (Yellon and Foster, 1985). Again this is similar to the response of rainbow trout to such photoperiods (Expt 2; Expt 3) suggesting that similar mechanisms may be involved in the control of seasonal maturation in these two vertebrates. Significantly the 15L:9D photoperiod had to be maintained for as little as 7 days to produce the advance in the timing of puberty in the lambs (Yellon and Foster, 1985). Experiments on the rainbow trout have always adopted at least a 6 week duration of long daylengths; it

would be interesting in future studies to test whether much shorter durations were equally capable of entraining the endogenous rhythm of maturation. A prospective advantage in using shorter 'pulses' of a particular photoperiod is that it would serve as a more precise means of investigating the phase-response characteristics of the endogenous circannual rhythm of maturation.

In addition to experiments adopting abrupt changes in photoperiod, support for the hypothesis that photoperiod entrains an internal circannual clock in the rainbow trout also comes from experiments where groups of fish were maintained under photocycles with periodicities of 6, 9, 12 (Whitehead *et al*, 1978) and 18 months respectively (Bromage *et al*, 1984). The results to these experiments are summarised in Figure 4.25. If the photocycle is divided into 360$^{o}$ then under the 12 month regime the fish spawned approximately 30$^{o}$ after the shortest day. Maintained under the and 9 and 6 month photocycles, the time of spawning phase lagged 47$^{o}$ and 130$^{o}$ after the shortest day respectively. In contrast, under the 18 month photocycle the time of spawning occurred approximately 42$^{o}$ before the shortest day. This altering phase relationship between the driving zeitgeber and the endogenous rhythm is similar to that described in the entrainment of circannual cycles of antler growth in sika deer (*Cervus nippon;* Figure 4.5; Goss *et al*, 1974) and gonadal maturation in birds (Gwinner, 1977; Berthold, 1979) by compressed photocycles and conforms with entrainment theory developed primarily from studies on circadian systems (see Introduction). This data adds support to the hypothesis that the timing of maturation is controlled by an endogenous circannual oscillator capable of being entrained by changes in photoperiod.

It is clear from the results that the alteration in the timing of spawning depends on both the timing and the direction of the change in photoperiod. The absolute length of the photoperiod does not appear to have a significant effect on the response of the rainbow trout. A more important influence on the timing of maturation is the difference between the 'present' photoperiod and the photoperiod experienced previously. In Experiment 1 an abrupt reduction from 18L:6D to 14L:10D caused a similar (but not the same, see below) advancement in the timing of spawning as a reduction to 10L:14D or 6L:18D; all three treatments were interpreted by the fish as a 'long' to 'short' switch in daylength. Similarly in Experiment 3, reductions from 14L:10D and 10L:14D respectively to 6L:18D were also interpreted as 'long' to 'short' switches resulting in an advancement in the timing of maturation. Thus, particular photoperiods, in this case 14L:10D and 10L:14D can be interpreted as 'long' or 'short' depending on the length of the preceding or succeeding photoperiod. Although proper controls were not included in this experiment (Expt 3), an abrupt reduction from 6L:18D to 2L:22D also appeared to cause a phase advance compared to the established response of rainbow trout on 6L:18D

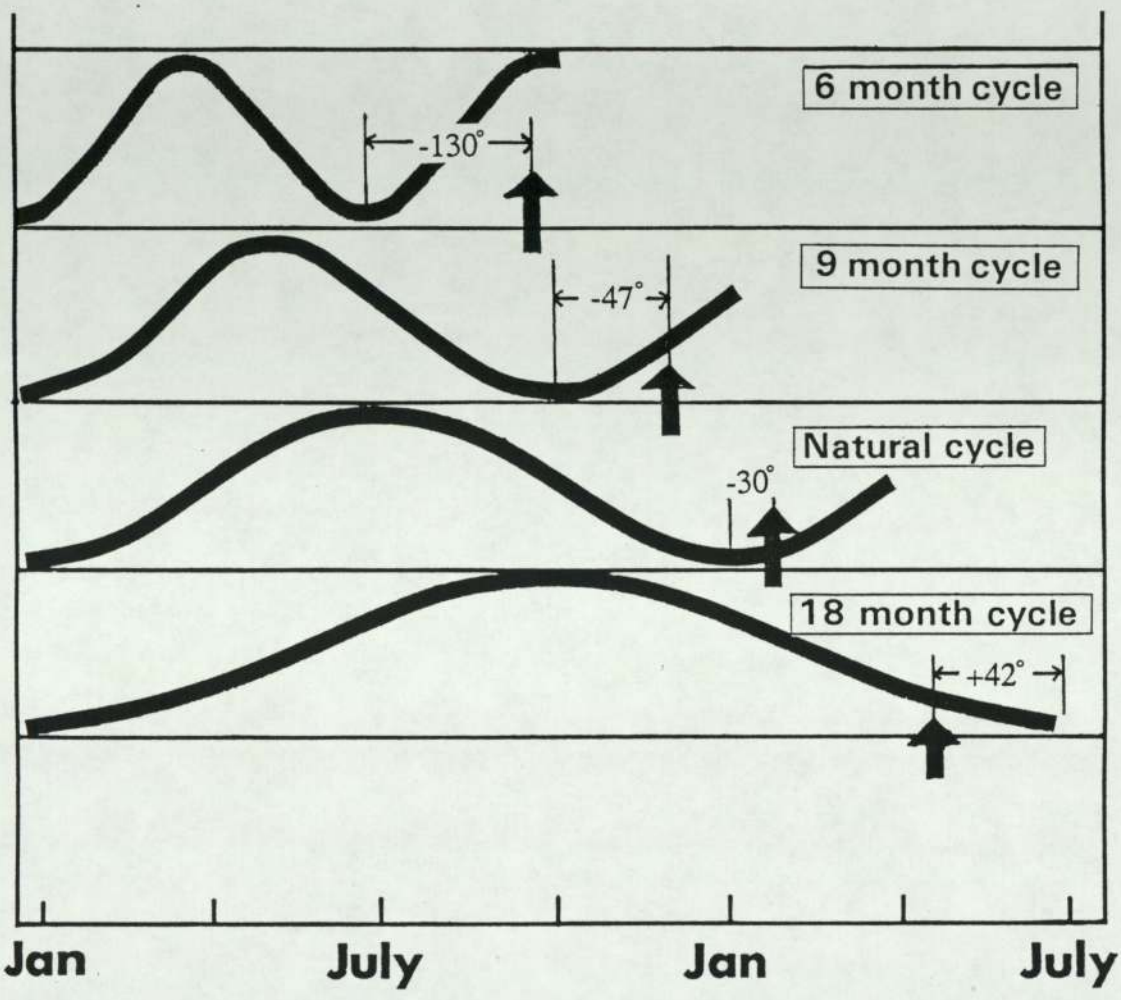



Fig.4.25. The effect of photoperiod cycles with periodicities of 6, 9, 12, and 18 months respectively on the timing of spawning (⬆) of female rainbow trout. The alteration in the phase relationship between the time of spawning and the photoperiod supports the hypothesis that changes in daylength entrain an endogenous rhythm of maturation. The vertical lines on the x-axis refer to the first day of the month as labelled.

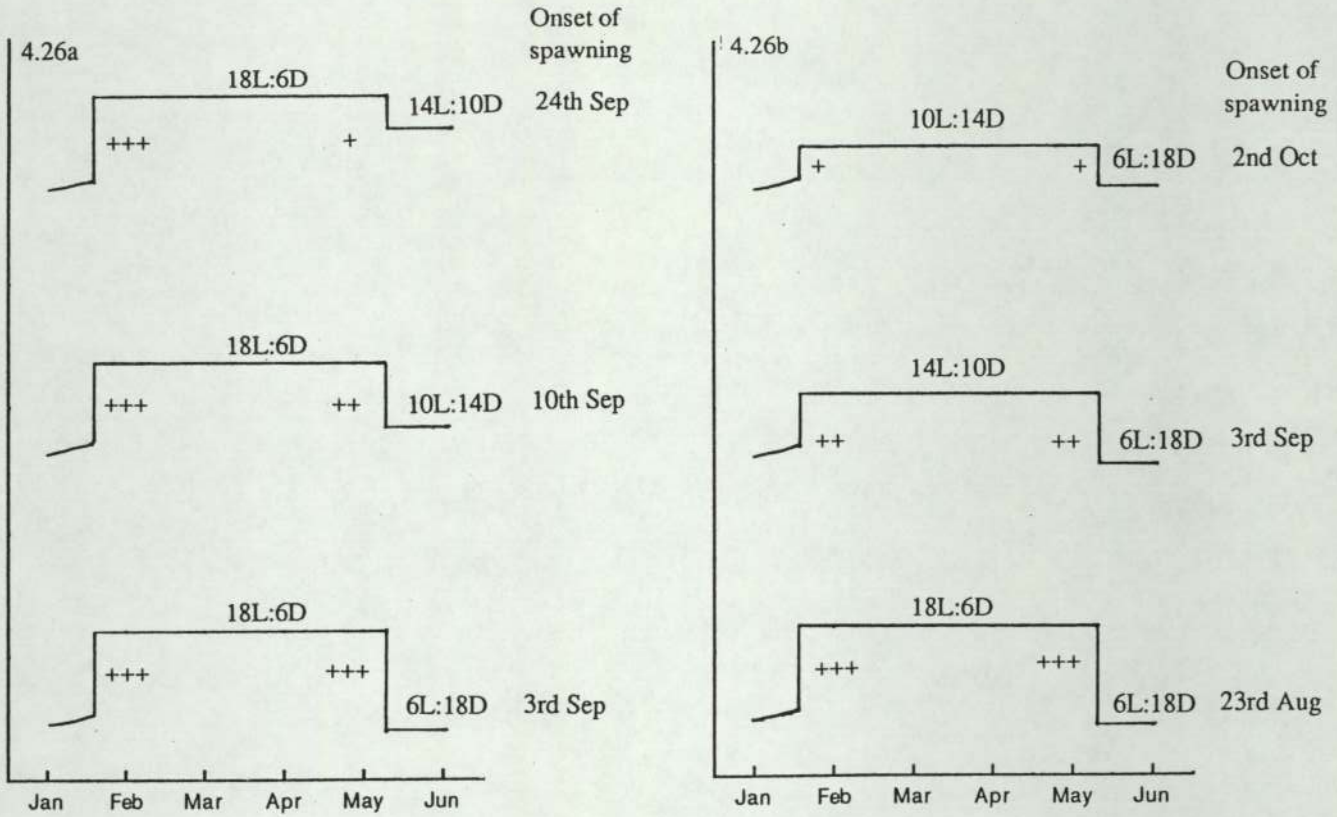



Fig. 4.26. The effect of the magnitude of the change in photoperiod on the timing of maturation in female rainbow trout. Graphs show the alteration in the photoperiod regimes in Expt 1(4.26a) and Expt 3 (4.26b), with the '+' signs emphasising the magnitude of the change in daylength. The figure illustrates that the magnitude of the change in photoperiod has a small supplementary effect on the alteration in the timing of maturation. The vertical lines on the x-axis refer to the first day of the month as labelled.

(Expt 1; Bromage *et al*, 1984) indicating that this photo-regime was also interpreted as a long to short shift in daylength. Photoperiodic 'history' has also been found to be important in determining gonadal development in the Japanese quail (*Coturnix coturnix*; Robinson and Follett, 1982) and some small mammals (Hoffman, 1984; Horton, 1984; Hastings *et al*, 1986). In the Japanese quail 13L:11D can be interpreted by the bird as 'long' or 'short' depending on whether the previous photoperiod was 8L:16D or 16L:8D respectively (Robinson and Follett, 1982). Despite the apparent similarity between the results from the present study on the rainbow trout and these experiments on higher vertebrates, they may only be analogous. The quoted experiments on birds and mammals all involved photoperiods that were close to the critical daylength suggesting that photoperiodic history caused some small alteration in its length. For example the Syrian hamster (*Mesocricetus auratus*) whose critical daylength is 12.5 hrs (Gaston and Menaker, 1967; Elliott, 1976), it was observed that 12L:12D could be interpreted as 'long' or 'short' depending on photoperiodic history (Hastings *et al*, 1986); no evidence has yet been provided to indicate that photoperiods of a duration several hours different to the critical daylength can have a similar dual effect. In contrast, in the rainbow trout there does not appear to be a critical photoperiod for gonadal maturation (Chapter 3) and daylengths ranging from 14L:10D to 6L:18D can be interpreted as long or short depending on the photo-regime. Although superficially the mechanisms appear to be different, the possibility remains that abrupt changes in the photoperiod are causing phase changes in an underlying circannual timing mechanism. This internal clock may then control maturation directly as appears to be the case in the rainbow trout, or possibly controls the position of a critical daylength as suggested by the studies on the Japanese quail (Robinson and Follett, 1982) hamster (Hoffman, 1984; Hastings *et al*, 1986) and proposed by Baggerman (1980) to occur in the stickleback (*Gasterosteus aculeatus*).

In addition to the timing and the direction of the change in photoperiod having a significant effect on the timing of spawning, in the rainbow trout, the magnitude of the change in photoperiod also had a small supplementary influence on the phase response. This effect which was observed in Experiment 1 and confirmed in Experiment 3 can be explained by referring to Figure 4.26. In Experiment 1, as all 3 groups were subjected to the same increase in photoperiod in January, the difference in the timing of the onset of spawning can probably be attributed to the magnitude of the change in photoperiod in May (Figure 4.26a). In Experiment 3 there were differences in the magnitude of the changes in photoperiod in the respective groups both in January and in May (Figure 4.26b); this had the effect of increasing the spread in the timing of onset of spawning from 21 days in Experiment 1 to 40 days. Thus, although the rainbow trout appears to

respond primarily to the direction of the change in photoperiod, the fact that they can detect changes in magnitude indicates that they possess a precise mechanism for measuring absolute daylength. The experiments described in detail in Chapter 3 suggested that circadian clocks were involved in measuring photoperiod, therefore in attempting to develop a model for the control of maturation in the rainbow trout these results support the hypothesis that there is a close interrelationship between both circannual and circadian oscillators. How these two timing mechanisms might interact has been discussed in the previous chapter.

The results from Experiment 2 indicated that some form of gating mechanism was involved in controlling the maturation of previously immature rainbow trout. To reiterate the hypothesis it is proposed that the gate is only open for a finite period of time (several months) each year when the internal circannual clock is in a certain phase of its cycle. Any virgin rainbow trout reaching a threshold stage of development when the gate is open would proceed to full maturation and ovulation that season. In contrast, slow developing fish only attaining the threshold stage after the gate has closed do not spawn that season, they have to wait another year until the internal circannual clock is again in the 'gate open' position before proceeding onto full maturation. Phase advancing the circannual clock by appropriate changes in the photoperiod had the effect of causing the gate to open earlier than under control conditions, this resulted in only a small percentage of fish reaching the necessary threshold stage of development before the gate closed. Those fish failing to mature in the first year of the experiment overtly expressed the phase shift of the circannual clock the following season with the time of spawning advanced by approximately 3 months. The concept of gated rhythms is well established in circadian oscillator research where there is good evidence that they are involved in controlling certain developmental stages of numerous species of insect (Pittendrigh, 1966; Truman, 1972; Saunders, 1976). The evidence for gated circannual rhythms is sparse but they may be more widespread than presently thought. Blake (1959) found that populations of the insect *Anthrenus verbasci* maintained under constant conditions exhibited a circannual rhythm in development which produced 'pulses' of pupation and adult emergence after one, two or three years. Although the periodicity of the rhythm was temperature compensated, temperature controlled the proportion of the population utilizing each annual gate. At 25$^{o}$ and 22.5$^{o}$C for example, all the larvae completed development in one year and emerged at the time of the 1st gate. At 17.5$^{o}$C some of the larvae, presumably the faster developers, were able to utilize the 1st gate whereas the slower developing members of the population emerged at the time of the 2nd gate approximately one year later (Figure 4.27). A similar gated circannual rhythm mechanism appears to be involved in the control of the timing of reproduction in

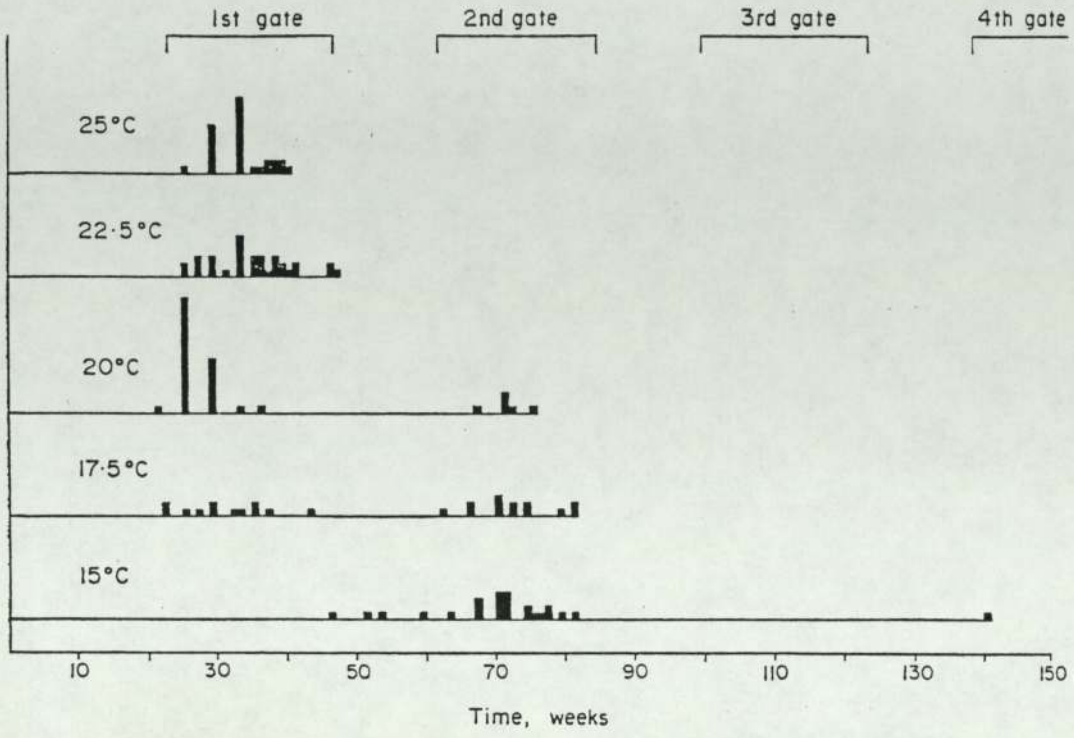



Fig. 4.27. The circannual rhythm of pupation in mixed-age populations of the beetle *Anthrenus verbasci* maintained under five constant temperature regimes (photoperiod and humidity also constant). The histograms illustrate the time of pupation of individual beetles. Note that the larvae at higher temperatures are able to utilize the first gate; at lower temperatures an increasing proportion of them are required to wait until the second gate. (After Blake,1959. From Saunders, 1977).

the marine invertebrate *Nereis diversicolor* (Olive and Garwood, 1983; Olive, 1984). The age of sexual maturity of *N.diversicolor* is not genetically fixed but occurs at 1, 2 or 3 years depending on the environmental conditions experienced by the worm. Like the beetle described above, temperature can influence the proportion of *N.diversicolor* reproducing at each of the respective yearly gates. Amongst the vertebrates there is evidence that some form of endogenous gating mechanism is involved in controlling the timing of the onset of puberty in lambs (*Ovis aries*; review: Foster *et al*, 1986). Winter and spring born lambs attain puberty the following autumn at 25-35 weeks of age, respectively (Foster and Ryan, 1979). Births later in the year lead to an even younger age at puberty as they also become mature in the autumn (Mallampati *et al*, 1971; Fitzgerald and Butler, 1982). However lambs born in later summer fail to attain puberty that autumn but remain immature until fall the following year (Hammond, 1944; Mallampati *et al*, 1971; Foster, 1981). Experiments have shown that inhibiting the growth rate of lambs by reducing the food supply can delay the onset of puberty. Returning lambs to an ample food supply during autumn and early winter resulted in oestrus cycles commencing within a few weeks. However, when returned onto a full ration in late winter, although rapid growth occurred, the sheep remained immature until the onset of the natural breeding season the following year (Foster *et al*, 1985). Thus it appears that lambs have to reach a threshold size before they are competent to mature, and then oestrus cycles can only occur during a specific period of year. Similar to the rainbow trout (Expt 2), as the timing of puberty in the lamb can be entrained by changes in the annual photocycle (Foster and Ryan, 1981; Yellon and Foster, 1985), the results suggest that some form of endogenous circannual clock with a closely associated gating mechanism may be involved in controlling the onset of oestus cycles in this mammal.

From the present studies on the rainbow trout it is not possible to ascertain what part(s) or step(s) of the neuroendocrine-gonadal axis is the limiting factor in determining whether an individual fish matures in a particular season or waits until the following year. However there are some parallels between this maturation strategy and the much studied phenomenon of smoltification which occurs in Atlantic salmon (*Salmo salar*) and some species of Pacific salmon (Hoar, 1976). Briefly, smoltification is a process where salmon parr undergo the transition from a freshwater to a marine mode of life before migrating out to sea. There are many physiological and morphological changes associated with this process, and it can occur a minimum of 1 year from first feeding while smaller fish require an additional year before becoming smolts (Thorpe *et al*, 1980, 1982; Saunders *et al*, 1982; Kristinsson *et al*, 1985). Thus smoltification also appears to be gated event, only occurring at a particular time of year with fish having to reach a threshold size before undergoing the process. As far as the sexual maturation of

rainbow trout is concerned it is established that commercially farmed fish fed on a high ration diet will spawn as 2 year olds whereas they normally do not produce eggs until the following year. The size of the fish may be an important factor in determining the timing of maturation, it is unfortunate that the growth of the experimental animals was not quantified in the present study. Under natural conditions a regulatory gating mechanism would be of significant adaptive importance; not only would it ensure that the initial maturation cycle proceeded at the 'correct' time of the year, but would also prevent small or underdeveloped fish from undergoing the process of developing a batch of oocytes before they were capable of surviving this energetically costly process. It must be emphasised that this gating hypothesis has been developed from experiments on virgin female rainbow trout and therefore only applies to the control of the onset of the initial maturation cycle. It is evident that subsequent cycles of ovarian growth are also synchronised to the annual changes in the environment but it is proposed that they are not gated, rather that there is close integration between the final maturation of one batch of eggs and the recruitment of another batch of primary oocytes into vitellogenesis (Scott and Sumpter, 1983).

In conclusion, the results described in this chapter add support to the hypothesis that the timing of maturation in rainbow trout is controlled by an endogenous circannual timing mechanism. The entrainment behaviour of the timing of maturation by changes in photoperiod is analogous to the phase-response characteristics of circadian oscillators and indicates that the circannual clock in this species can be considered as an oscillator in the mechanistic sense. The direction and the timing of the change in photoperiod are effective zeitgebers with the magnitude of the change having a supplementary effect on the synchronization of spawning. Although this study has only investigated the effect of photoperiod on the timing of maturation, it is entirely possible that under natural conditions other environmental factors also act as zeitgebers, all contributing to ensure that reproduction occurs at the appropriate time of year for the survival of the offspring. In addition to the basic hypothesis of an entrainable circannual clock controlling the timing of maturation the results provide evidence that some form of gating mechanism is involved in the process. It is proposed that this gate only allows fish that have reached a certain threshold stage of development to commence the energetically costly process of developing batch of oocytes to maturity.

Having discussed the important role of daylength in the control of seasonal breeding in rainbow trout and many other vertebrates, the following chapter investigates the physiological mechanisms involved in converting photic information into a humoral signal which then mediates in the control of gonadal maturation.

# CHAPTER FIVE

# THE ROLE OF MELATONIN AND THE PINEAL GLAND IN THE CONTROL OF GONADAL MATURATION IN THE FEMALE RAINBOW TROUT

## 5.1 INTRODUCTION

The experiments described in the previous two chapters provided good evidence that there is an endogenous circannual rhythm of gonadal maturation in the rainbow trout which can be entrained by changes in daylength. Based on research from a wide variety of vertebrates this chapter investigates how rainbow trout perceive photoperiod and how photic information is integrated and converted into a hormonal signal.

Most animals possess a complex apparatus which converts external environmental information into a neuroendocrine signal which can be integrated into the control of functions such as gonadal maturation. Light by virtue of its reliable daily and seasonal variation has been adopted by many animals as a synchroniser or zeitgeber for metabolic events that require temporal organisation (see previous chapters). The sum total of photoreceptors and neural centres that subserve this integrative function form a portion of the optic system which is distinct from the apparatus for vision and optic reflexes (Scharrer, 1964). In certain mammals where photoperiod is important in the control of seasonal reproduction rhythms, the pineal gland and its hormone melatonin have been shown to have a vital role in the transduction of photic information into a hormonal signal (reviews: Karsch *et al*, 1984; Tamarkin *et al*, 1985). Research on the form and function of the vertebrate pineal gland described below provides the background to the experimental work which investigated the role of melatonin in the control of maturation in the rainbow trout.

The pineal complex is present in all classes of vertebrates from lampreys upwards but has undergone profound evolutionary changes leading to variations in both its sensory and secretory functions (Hamasaki and Eder, 1977). Oksche (1971) and Collin (1971) elucidated the gradual transformation of the pineal from mainly a photoreceptor in lower vertebrates to having secretory function in mammals. Despite these differences, the development of the pineal from a single cell line (Figure 5.1; Collin and Oksche, 1981) suggests that the following review of the function of this structure in the various classes of vertebrates may assist in elucidating its role in the rainbow trout.

In teleosts the pineal consists of an end vesicle located just beneath the skull with a stalk connecting it to the roof of the diencephalon (Figure 5.1). Von Frisch (1911) and Scharrer (1928) both proposed that the pineal in the minnow (*Phoxinus laevis*) was a principal site of extra-ocular photoreception. This conclusion was drawn from studies where blinded minnows continued to exhibit a skin colour change in response to light. Young (1935) added support to this theory by showing that

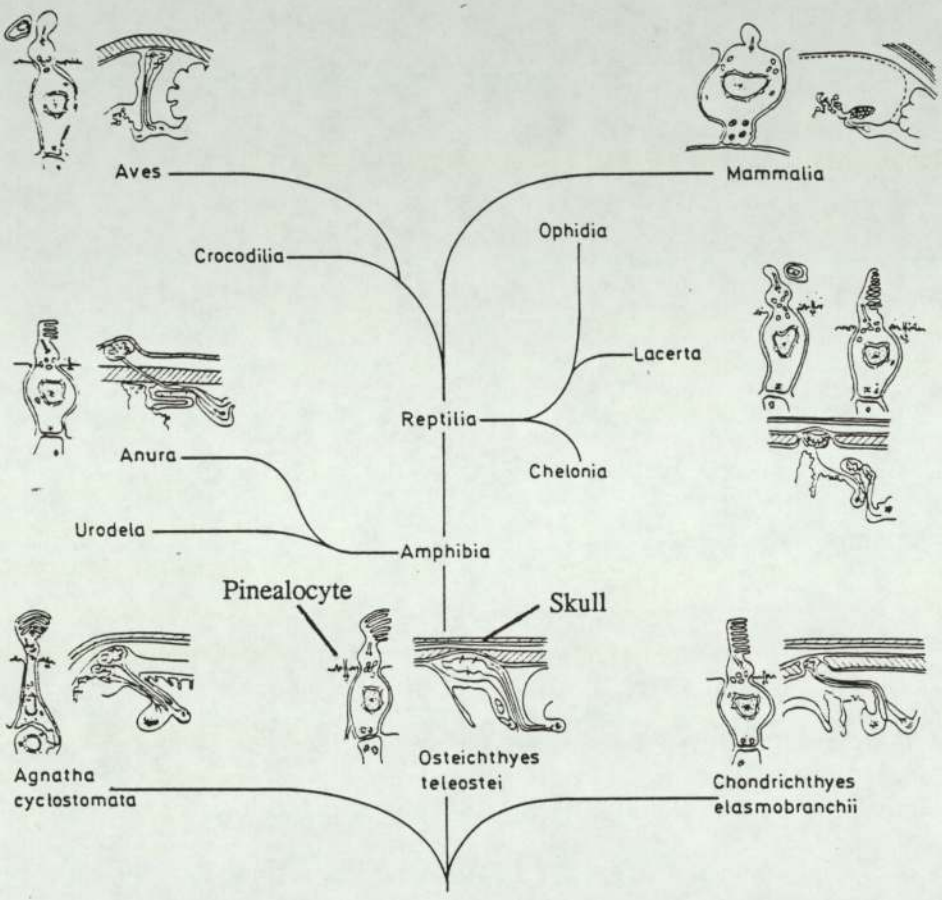



Fig. 5.1 Schematic representation of the morphological changes undergone by the pineal system over the course of evolution. (From Hamasaki and Eder, 1977).

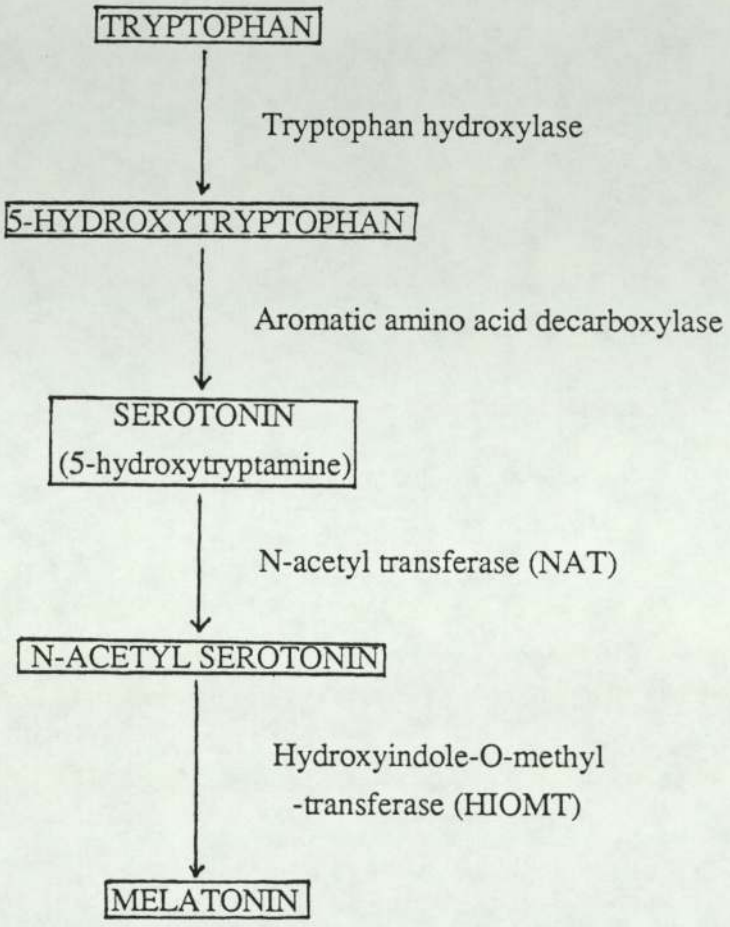



Fig. 5.2 The biosynthetic pathway of the production of melatonin from the amino-acid tryptophan. (For details see Wurtman *et al* ,1968 ).

removal of the pineal complex in larval lampreys (*Lampetra planeri*) abolished the skin pallor response which normally followed transfer of these animals from light to dark. Similarly pioneer studies by McCord and Allen (1917) suggested that the pineal had a role in the control of skin colouration of amphibians, as tadpoles (*Rana pipiens*) became pale when they were fed a bovine pineal extract. Although investigations into the involvement of the pineal in the control of skin colouration have been largely overtaken in recent years by studies on the pineal and reproduction, this paling effect provided the basis of a *Rana pipiens* skin bioassay used by Lerner *et al* (1958) in the isolation and identification of the indoleamine N-acetyl-5-methoxytryptamine or melatonin. Subsequent to this discovery of the structure of a potential pineal hormone there was a rapid expansion in the research on the physiological role of this organ and its metabolic products. Melatonin has since been identified in the pineal bodies of all vertebrates and also in extra-pineal sites of some species (Ralph, 1981).

A characteristic feature of melatonin synthesis (Figure 5.2) in vertebrates is that it exhibits a marked diurnal variation with pineal and blood levels of melatonin being higher at night than during the day (Fish: Gern *et al*, 1978a. Reptiles: Firth *et al*, 1979. Birds: Underwood *et al*, 1984. Mammals: Rollag and Niswender, 1976; Kennaway *et al*, 1977). The rhythm in melatonin levels is thought to reflect the higher night-time activity levels of the enzyme N-acetyltransferase which is believed to be the rate limiting step in the biosynthetic pathway (Binkley, 1981). The fact that the length of the photoperiod has an important role in controlling physiological and behavioural functions in many vertebrates (see previous chapters) and the observation that melatonin levels are elevated for the duration of the night, led to the development of the concept of the pineal gland functioning as a neuroendocrine transducer converting photic information into a daily hormonal rhythm (Axelrod, 1974). Although the present study concentrates on the role of the pineal in the control of reproduction, it should be stressed that the transduction of photoperiodic stimuli into a rhythm of melatonin may well influence many behavioural and metabolic functions.

In teleosts ultrastructural studies have identified photoreceptor cells in the pineal gland which also appear to have a secretory capacity (review: McNulty, 1984). Electrophysiological studies on rainbow trout and other species have established that the pineal is directly photosensitive (Dodt, 1963; Hanyu and Niwa, 1970) with increased neural activity occurring during darkness (Morita, 1966). In addition, the putative pineal hormone melatonin has been isolated from the pineal gland of Pacific salmon (*Oncorhynchus tshawytscha*; Fenwick, 1970a) and elevated blood levels have been recorded during darkness in the rainbow trout (Gern *et al*, 1978a). Thus the

teleost pineal appears to have the essential components of a transducer capable of converting photic information into a humoral signal.

There is considerable evidence indicating that the pineal organ and melatonin have a role in the control of gonadal maturation in teleosts although the mode of action remains unclear (reviews: Ralph, 1978, 1983; de Vlaming and Olcese, 1981). The data suggest that the pineal organ can be anti- or pro-gonadal depending on the time of year the experiments are performed. Pinealectomy of goldfish (*Carassius auratus*; Fenwick, 1970a; 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 decrease in the gonado-somatic 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, 1970b; 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, 1980) or pituitary and plasma levels of gonadotropin (Vodicnik *et al*, 1978). From these paradoxical results it has been difficult to develop a cogent hypothesis for the role of the pineal in the control of teleost maturation. However, whatever the role, there is some evidence to suggest that pinealectomy affects gonadal development in fish by altering the secretions from the hypothalamo-pituitary axis (de Vlaming and Vodicnik, 1977; Vodicnik *et al*, 1978; Olcese *et al*, 1981; Hontela and Peter, 1980).

Investigations into the role of melatonin in teleost reproduction have tended to involve observing the effects on gonadal development of interperitoneal (IP) injections of the hormone. In general melatonin causes gonadal regression. Thus daily IP injections of 20 μg melatonin into goldfish (*Carassius auratus* ) prevented an increase in the GSI and the activity of pituitary gonadotrophs normally observed when the fish are maintained under 16L:8D (Fenwick, 1970a). The daily injection of melatonin (≤ 250 μg) into fish undergoing gonadal recrudescence has also reduced the GSI in a number of species including *Oryzias latipes* (Urasaki, 1972) *Fundulus similis* (de Vlaming *et al*, 1974), *Liza ramada* (Sagi and Abraham, 1984), *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).

Although many of the studies on the effects of melatonin on reproduction tend to discuss the pineal gland as being the major site of melatonin synthesis it is now clear that melatonin is also produced in the retina of rainbow trout (Gern *et al*, 1978b) and other unidentified non-pineal, non-retinal areas (Gern *et al*, 1978c; Ralph, 1981). These observations may explain why pinealectomy experiments in fish produced paradoxical results, as the operation possibly failed to eliminate all the sources of melatonin production.

The role of melatonin and the pineal in the control of reproduction in other classes of lower vertebrates is also uncertain. In amphibians, temperature rather than photoperiod is important in controlling the timing of maturation (Chapter 1), therefore it might be expected that the photosensitive pineal (Dodt and Heerd, 1962; Donley, 1975) would not have a role in this process. However, there is evidence to indicate that the amphibian pineal complex has an important thermoregulatory function in monitoring the exposure to sunlight (Ralph *et al*, 1979). Thus, it remains a possibility that the pineal of amphibians acts as a transducer of environmental information. Melatonin has been identified in the anuran pineal (van der Veerdonk, 1967) and exhibited a diel rhythm in the blood of *Ambystoma tigrinum* which was abolished by pinealectomy (Gern and Norris, 1979). As discussed above, melatonin appears to have a physiological role as a dermal melanophore-contracting agent in larval amphibians (Bagnara, 1960). Studies on amphibian reproduction have shown that injection of melatonin is inhibitory to gonadal development (O'Connor, 1969; de Vlaming *et al*, 1974; Delgado *et al*, 1983) similar to the effect on teleosts.

The pineal structure in reptiles is very diverse; for example in crocodilians the pineal complex is absent (Hamasaki and Eder, 1977) whereas in many lizards the photosensitive capabilities are highly developed to form the 'third eye' (Eakin, 1973). In snakes and turtles it has lost nearly all photosensory elements to become a secretory structure similar to the mammalian pineal organ receiving photic information indirectly from the lateral eyes (Hamasaki and Eder, 1977). The reptilian pineal has been implicated in the control of many functions including locomotor activity, thermal sensitivity, metabolic rate and gonadal development. All of these effects are dependent on the external environment and it appears that the pineal is involved in transducing this information into a humoral signal. Detailed studies on circadian locomotor rhythms in lizards has shown that melatonin can function as an important entraining agent (Underwood, 1979, 1986). The diel pattern of circulating melatonin in reptiles is influenced by photoperiod and temperature (Firth *et al*, 1979; Vivien-Roels, 1981; 1985) suggesting that the effects of these two environmental factors on metabolism may in part be mediated by melatonin secreted by the pineal.

As photoperiod and temperature are important in determining the timing of maturation in reptiles, and gonadal development is affected by pinealectomy (Levey, 1973; Haldar and Thapliyal, 1977; Thapliyal and Haldar, 1979) and melatonin treatment (Levey, 1973; Packard and Packard, 1977), it is reasonable to suggest that melatonin is serving as a transducing agent for the environmental control of reproduction. However, in reptiles this hypothesis remains to be fully tested. As a note of caution, in birds the pineal and melatonin does not appear to have a physiological role in the control of reproduction:

The avian pineal complex is not directly photosensitive and the pinealocytes are considered to mainly have a secretory function (Hamasaki and Eder, 1977). As in other vertebrates the melatonin content of the pineal, blood and other tissues of birds varies in a rhythmic manner with increased amounts present during the night compared to the day (Ralph, 1976). The pineal and the retina are important sites of melatonin production in birds (Ralph, 1981; Underwood *et al*, 1984) with secretion appearing to be driven by a circadian clock since rhythms persist *in vitro* under constant conditions (Binkley, 1979; Takahashi *et al*, 1980; Binkley *et al*, 1981). The importance of the pineal gland as a circadian pacemaker was first realised by Gaston and Menaker (1968). They observed that the circadian perch hopping activity of sparrows (*Passer domesticus*) was abolished by pinealectomy. Furthermore, pinealectomised recipients regained their rhythmicity when pineal glands from donor sparrows were placed in the anterior chamber of the eye (Zimmerman and Menaker, 1979). Exogenously-administered melatonin is also an effective entraining agent to circadian locomotor rhythms in sparrows (Turek *et al*, 1976) and starlings (*Sturnus vulgaris*; Gwinner and Benzinger, 1978) implicating this hormone in the entrainment caused by the pineal. It should be noted that pinealectomy of galliform species has no effect on free-running locomotor rhythms (MacBride, 1973; Simpson and Follett, 1981) indicating that it is not possible to make generalisations about the role of the pineal in birds.

There have been numerous investigations into the possible role of the pineal in avian reproduction. The fact that the reproductive activity of birds is remarkably responsive to daylength (Chapters 1 and 4) and the pineal is responsive to light in terms of melatonin production suggested that this structure is responsible for the transduction of photic information for the reproductive system. However, the supporting evidence for such a role for the pineal gland in birds is weak. Pinealectomy caused no changes in the reproductive function of various passerine species (Hamner and Barfield, 1970; Donham and Wilson, 1970; Menaker *et al*, 1970) the Japanese quail (*Coturnix coturnix*; Homma and Sakakibara, 1971; Homma

*et al*, 1972) the chicken (*Gallus domesticus*; Harrison, 1972) and had only transitory effects on the duck (*Anas platyrhynchos*: Cardinali *et al*, 1971). However, in contrast pinealectomy of weaver birds (*Ploceus phillipinus*) resulted in sexual precocity and made the bird responsive to short photoperiods which are not normally stimulatory (Balasubramanian and Saxena, 1973).

Some experiments involving the administration of melatonin have indicated a role for this molecule or its biochemical derivatives in avian reproductive function. In a series of experiments on chickens (*Gallus domesticus*), Balemans and associates showed that melatonin was inhibitory to gonadal development whereas 5-methoxy-tryptophol (a derivative of 5-hydroxytryptophan) had a stimulatory effects (Balemans *et al*, 1977a,b). However because of poor experimental design it is difficult to draw firm conclusions from these results. Other experiments have also suggested that melatonin has a role in the control of gonadal development in birds, but again the evidence is not convincing (Sackman, 1977; Wright and Preslock, 1975).

In contrast to birds, the pineal and melatonin have an important role in controlling the timing of gonadal maturation in some seasonally-breeding mammals. The mammalian pineal is not directly photosensitive but receives information concerning daylength via the lateral eyes (Moore, 1979). As a result, like all other vertebrates mammals exhibit a daily rhythm in pineal and circulating levels of melatonin which are elevated during the hours of darkness compared to daytime (Kennaway *et al*, 1977; Bittman *et al*, 1983; Goldman and Darrow, 1983). Convincing evidence that the pineal is important in the control of maturation in mammals was first provided by R.A. Hoffman and colleagues in the mid-1960's working on the Syrian hamster (*Mesocricetus auratus*). The annual cycle of maturation in this animal is strongly influenced by daylength (Chapter 1); pinealectomy of male and female hamsters prevented the gonadal collapse that occurs in response to short photoperiods (Hoffman and Reiter, 1965, 1966). Following this work there has been a great deal of research on the role of the pineal and its secretory products in the control of reproduction in mammals (reviews: Reiter, 1980, 1981; Hoffman, 1981). However, despite this research until the 1980's the role of the pineal in the control of maturation remained uncertain. Experiments involving the administration of the pineal hormone melatonin produced paradoxical results with it having both pro- and anti-gonadal effects (Turek *et al*, 1975b; Reiter, 1978). A significant breakthrough was made when it was found that the timing of melatonin administration in relation to the photoperiod influenced the response of the pituitary-gonadal axis. Injections of melatonin into Syrian hamsters early in the light phase had no effect on reproduction whereas the same dose later in the day caused

gonadal atrophy (Tamarkin *et al*, 1976). Subsequently it was established that the duration of elevated melatonin levels was a crucial factor in determining the development of the gonads. The gonadal regression in the Djungarian hamster (*Phodopus sungorus*) exposed to short days was prevented if the animals were pinealectomised; however, if pinealectomised animals were infused with 10μg melatonin over a period of 12 hrs then gonadal regression ensued (Carter and Goldman, 1983a). In contrast, gonadal regression did not occur if pinealectomised hamsters were only infused with melatonin for 4-6 hrs (Carter and Goldman, 1983a). Furthermore, the stimulation of gonadal development in intact hamsters by long days, prevented by pinealectomy,was restored if pinealectomised animals were infused with melatonin (rate = 0.83ng $hr^{-1}$) for 4-6 hrs, whereas a longer infusion period (8-12 hrs) did not stimulate gonadal development (Carter and Goldman, 1983b). This work established the hypothesis that the pineal gland mediates in the control of maturation by converting the photoperiodic signal into a diel rhythm of circulating melatonin, with the duration of elevation of this hormone determining the response of the hypothalamo-gonadal axis. Strong support for this hypothesis came from a series of experiments on the gonadal response of the sheep (*Ovis aries*) to photoperiod and melatonin treatments (review: Karsch *et al*, 1984). The stimulatory effect of a short photoperiod (8L:16D) on gonadal development, abolished by pinealectomy, was restored by the infusion of melatonin over a 16 hr period, which mimicked the effect of a long night length of 8L:16D (Bittman *et al*, 1983). Similarly an inhibitory long photoperiod (short night length) was mimicked by infusing pinealectomised ewes with melatonin for 8 hrs per 24 hr period (Bittman and Karsch, 1984). The importance of the night-time melatonin elevation in mediating the reproductive response is such that pinealectomised ewes maintained under inhibitory long photoperiods could be induced to mature by infusing them with a short day profile of melatonin (16 hrs duration) for 90 days (Yellon *et al*, 1985).

In addition to infusion experiments where the duration of elevated melatonin levels can be carefully controlled, numerous studies have investigated the role of this hormone in seasonal reproduction by fitting animals with sub-cutaneous slow-release melatonin implants or by administering melatonin orally. In spite of any endogenous melatonin secretion these methods of administration produce continuously-elevated blood melatonin levels which are perceived by the animal as a long night (short photoperiod). Thus melatonin implants placed into sheep during mid-summer resulted in an advancement in the onset of breeding corresponding to that produced by exposure to artificial short photoperiods (Lincoln and Ebling, 1985; Nowak and Rodway, 1985; English *et al*, 1986). Similarly melatonin implants induced early

rutting behaviour and gonadal maturation in red deer (*Cervus elaphus*; Lincoln *et al*, 1984) which like sheep breed in the autumn as a result of the stimulatory effect of the shortening daylength. Oral administration of melatonin from around mid-summer was equally effective as slow-release implants in advancing the timing of maturation in sheep (English *et al*, 1986), and antler growth, rutting behaviour and winter moult in deer (Bubenik, 1983). Melatonin implants also mimicked the effect of short photoperiods in the Djungarian hamster (*Phodopus sungorus*: Hoffman, 1973), the white-footed mouse (*Peromyscus leucopus;* Lynch and Epstein, 1976) and the weasel (*Mustela erminea*; Rust and Meyer, 1969). In these long-day breeders the implants caused gonadal regression and the growth of the winter pelage. It can be concluded that in certain species of mammal where daylength is important in controlling the timing of reproduction, the pineal gland transduces photic information into a daily rhythm of melatonin which in turn affects the response of the neuroendocrine system. The fact that this hormone can cause behavioural and pelage growth changes indicates that the melatonin rhythm produced by the pineal gland not only controls gonadal maturation but may co-ordinate the timing of all seasonal events.

This review has illustrated that the pineal gland and its hormone melatonin are involved in the control of seasonal reproduction in a wide range of vertebrates, with the possible exception being the birds. In research on lower vertebrates the precise role of the pineal and melatonin in the control of maturation is unclear. However, recent work on mammals described above has led to the development of the hypothesis that the pineal transduces photic information into a daily rhythm of melatonin which mediates in the control of gonadal development. As the pineal gland of fish and other lower vertebrates also have the essential photosensory and secretory components to function as a transducer of photic information, it is proposed that melatonin may have a similar role to mammals in mediating in the control of physiological events that are entrained by photoperiod. The present study on the rainbow trout has provided convincing evidence that the annual cycle of reproduction is entrained by changes in photoperiod, therefore it is hypothesised that the pineal of this fish converts photic information into a daily rhythm of melatonin which modulates the activity of the hypothalamo-pituitary-gonadal axis. This chapter describes some experiments designed to test this hypothesis.

## 5.2 Experiment 1: Serum melatonin profiles in rainbow trout maintained under long (16L:8D) and short (8L:16D) photoperiods

It has been established that changes in photoperiod are important in controlling the timing of maturation in the rainbow trout (Chapter 4). Melatonin levels in rainbow trout are elevated during darkness (Gern *et al*, 1978a) and this hormone appears to be involved in the control of maturation in some species of teleost (de Vlaming and Olcese, 1981). It is hypothesised that the rainbow trout converts photic information into a diel rhythm of melatonin which modulates the response of the neuroendocrine-gonadal axis, similar to certain seasonally-breeding mammals. In the sheep for example, circulating melatonin levels remain consistently low during daylight (< 50 pg $ml^{-1}$) but increase rapidly at the onset of darkness and remain elevated (> 100 pg $ml^{-1}$) until 'dawn' when they return to a low level. The duration of elevated melatonin levels not only provides the sheep with information of the length of the photoperiod but directly drives the reproductive response (Bittman *et al*, 1983; Karsch *et al*, 1984).

As an initial experiment on the rainbow trout it was considered important to investigate whether the levels of melatonin reflect the environmental photoperiod. To test this, two groups of rainbow trout were maintained under 16L:8D and 8L:16D respectively. The fish were blood sampled at regular intervals over a 24 hr period and the serum subsequently assayed for melatonin.

### Materials and Methods (Expt 1)

Two groups (A,B) of twenty, 2 year old rainbow trout of mixed sex selected from a stock of fish raised under ambient conditions (Lat 52° 30'N) with a December spawning time, were maintained from 26th April 1985 in identical lightproof 1200 litre tanks. The fish in Grp A and Grp B were maintained under 8L:16D and 16L:8D respectively. The photoperiod in each tank was controlled by an electronic clock controlling a 40W bulb providing 30 lux at the water surface. Water temperature was 11°C. Fish were fed 0.5% body weight $day^{-1}$ with a commercial trout diet. Following an acclimation period of 12 days, fish in both groups were blood sampled at five time intervals over a 24 hr period. In each group a total of 4 fish were blood sampled at each of the five time intervals. After being sampled the fish were not returned to the experimental tank, thus ensuring that animals were only blood sampled once during the course of the experiment. A dim infra-red lamp was used to facilitate blood sampling during the hours of darkness. Although rainbow trout are relatively insensitive to light of this wavelength (Douglas, 1980) to ensure that the

sample fish were not perceiving any light the eye and pineal areas were covered with a cloth during night-time blood sampling. The blood samples from each fish were allowed to clot and after centrifugation, the serum stored at -20°C for subsequent assay of melatonin. Details of the melatonin radioimmunoassay procedure and other materials and methods are given in Chapter 2.

Results (Expt 1)

The results displayed in Table 5.1 and Figures 5.3a and 5.3b show that the serum levels of melatonin in the rainbow trout are elevated during the dark period.

| 8L:16D (Group A) | | | | | |
|---|---|---|---|---|---|
| | Light | Dark | | | Light |
| Sample | 1 | 2 | 3 | 4 | 5 |
| Serum melatonin pg $ml^{-1}$ ($\bar{x}$± sem) | 35±7 (n=2) | 150±14 (n=4) | 253±59 (n=4) | 165±19 (n=4) | 66±7 (n=3) |

| 16L:8D (Group B) | | | | | |
|---|---|---|---|---|---|
| | Light | | Dark | | Light |
| Sample | 1 | 2 | 3 | 4 | 5 |
| Serum melatonin pg $ml^{-1}$ ($\bar{x}$±sem) | 76±9 (n=4) | 70±4 (n=4) | 194±35 (n=4) | 315±50 (n=4) | 100±24 (n=3) |

Table 5.1 Serum melatonin levels in rainbow trout maintained under 8L:16D (Grp A) and 16L:8D (Grp B).

In Grp A the low serum levels of melatonin during the light period (< 100 $pgml^{-1}$) were elevated during the night to over 150 pg $ml^{-1}$ with a peak level of approximately 250 pg $ml^{-1}$. Similarly in Grp B the daytime serum levels of melatonin were in general less than 100 pg $ml^{-1}$ being elevated during darkness to over 150 pg $ml^{-1}$ with a peak level of over 300 pg $ml^{-1}$. In both Grp A and Grp B melatonin levels were significantly higher ($P < 0.05$, Student's t-test) during the dark compared to the light period.

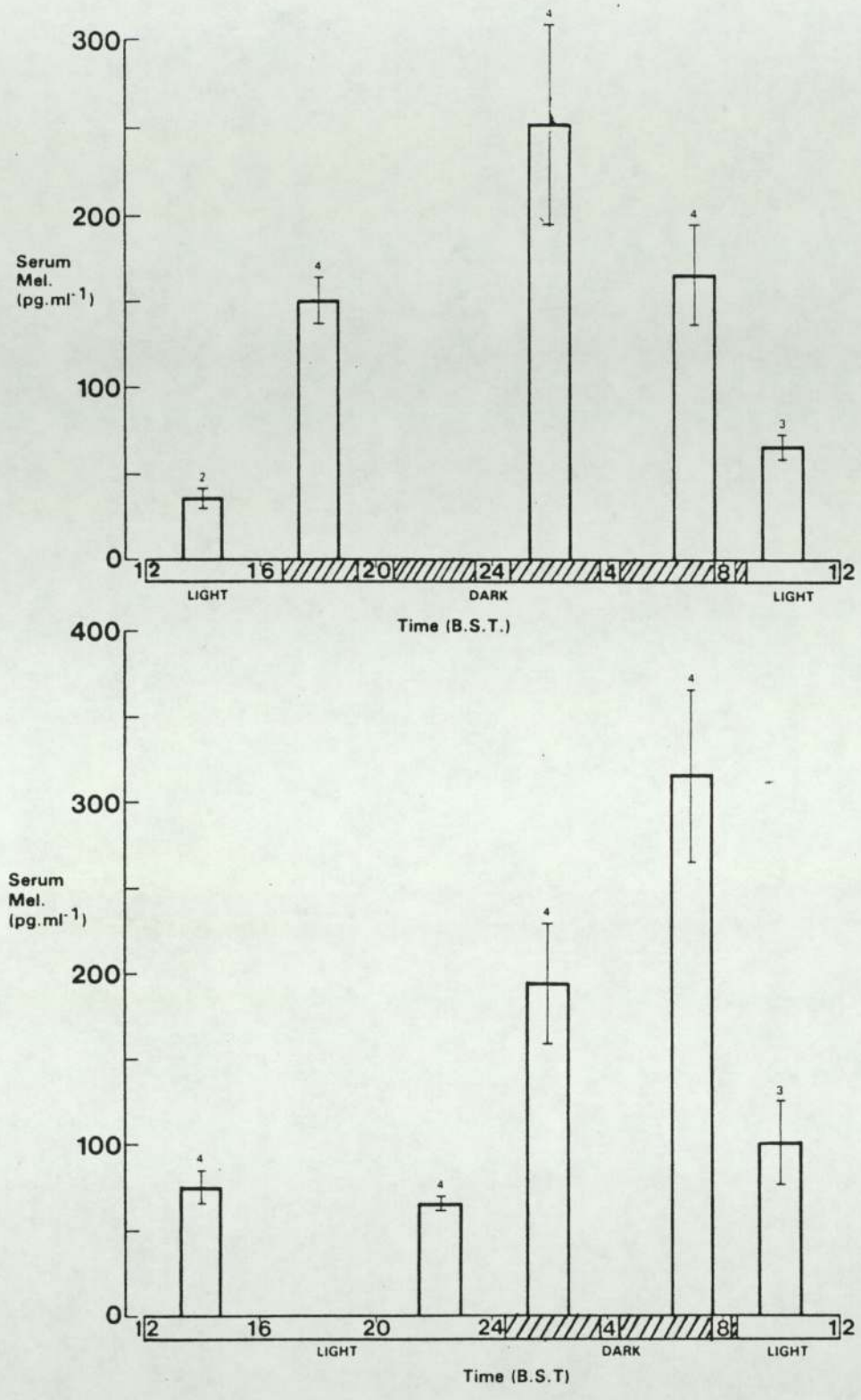



Fig. 5.3a, b. Histograms illustrating mean serum levels of serum melatonin (Mel, pg $ml^{-1}$) in rainbow trout maintained under (a) 8L:16D, and (b) 16L:8D. The integer above each bar indicates the number of fish sampled (n), with the vertical lines representing $\pm$ one standard error of the mean. The cross-hatched area indicates the period of darkness.

Conclusions (Expt 1)

The experimental results clearly show that serum melatonin levels in the rainbow trout are elevated during the period of darkness of a 24hr photoperiod cycle. The levels recorded in the present experiment are of the same order as reported in previous study on the rainbow trout in which day and night time levels of 81.2±19.2 pg $ml^{-1}$ (n=8) and 152.6±15.8 pg $ml^{-1}$ (n=8) were recorded respectively (Gern *et al*, 1978a). This similarity in levels using radioimmunoassay (RIA) procedures incorporating an antibody from different sources tends to confirm that the unvalidated RIA used in the present study was specifically detecting melatonin. However, as only a small number of fish were used in this study, and recurring problems with the RIA prevented any repeat of this experiment, the following conclusions drawn from the results must be treated with caution.

Overall the results support the hypothesis that photic information is converted into a diel rhythm of circulating melatonin which could potentially mediate in the timing of physiological responses that are entrained by photoperiod, similar to other higher vertebrates (see Introduction). Despite exhibiting a marked daily rhythm, it is clear that the elevated serum levels of melatonin during the dark period did not remain at a constant concentration but showed intra- and inter-treatment variations. The amplitude of the nocturnal rise in melatonin was greater, and occurred comparatively later in the night in the long photoperiod treatment (Grp B) compared to rainbow trout maintained under short days (Grp A). This observation is similar to the effect of long and short photoperiods on the diel melatonin profiles in the Japanese quail (*Coturnix coturnix*; Cockrem and Follett, 1985) and sheep (*Ovis aries*; Lincoln *et al*, 1982). From this preliminary experiment it is not possible to draw conclusions as to the significance of the effect of photoperiod on the amplitude and timing of the change in serum melatonin levels in the rainbow trout. However, recent work on mammals has provided good evidence that in addition to photoperiod, endogenous circadian oscillators are also involved in the control of melatonin production and its effect on gonadal maturation (Earl *et al*, 1985; Stetson *et al*, 1986). The endogenous rhythm of melatonin production in birds is well established (see Introduction) and Cockrem and Follett (1985) cited the fall in pineal melatonin levels in Japanese quail before 'lights on' as evidence of this circadian involvement. The serum melatonin levels of the trout maintained under 8L:16D also began to decrease before the termination of the dark period. This result together with the effect of photoperiod on the amplitude and timing of the peak serum concentrations show that melatonin rhythms in the rainbow trout are not merely due to regular removal of the inhibitory influence of light, but suggest a more complex mechanism. The possible involvement of endogenous oscillators in the control of melatonin production and action will be covered in greater detail in the discussion to this chapter.

## 5.3 Experiment 2: The administration of melatonin to rainbow trout and the effect on the timing of gonadal maturation and skin colouration

The results from Experiment 1 provided good evidence that serum melatonin levels in the rainbow trout are elevated for the duration of the dark period. They support the hypothesis that information concerning the environmental daylength is transduced into a diel rhythm of melatonin which could potentially mediate the effects of photoperiod on physiological processes. As photoperiod has an important role in controlling the timing of gonadal maturation in the rainbow trout (Chapter 4), it can be speculated that this effect is mediated by the diel rhythm of melatonin. Unfortunately the role of melatonin in the control of reproduction of salmonids has never been investigated and although melatonin has been implicated in the control of gonadal maturation in some cyprinid species, the mechanism of action is not understood (see Introduction). The aim of the present experiment was to administer melatonin to rainbow trout and record its effect on the timing of maturation.

Experimental procedures used to elucidate the role of melatonin in the control of maturation in mammals have employed relatively sophisticated timed infusion techniques where the duration of the daily rhythm of the hormone can be carefully controlled (Bittman *et al*, 1983; Carter and Goldman, 1983a,b). Unfortunately it was not feasible to use this method of melatonin administration on free-swimming rainbow trout. As described in the introduction to this chapter, other studies on mammals have administered melatonin orally or by fitting animals with slow release melatonin implants. These methods resulted in elevated melatonin levels which were perceived by the animal as a short day and accordingly advanced the timing of breeding in deer (*Cervus elaphus*; Bubenik, 1983; Lincoln *et al*, 1984) and sheep (*Ovis aries*; English *et al*, 1986), but caused gonadal regression in long-day breeding species such as the Djungarian hamster (*Phodopus sungorus*; Hoffman, 1973) and the weasel (*Mustela erminea*; Rust and Meyer, 1969). Photoperiod experiments on the rainbow trout have established that an abrupt reduction from a long to a short daylength can cause an advance in the timing of maturation (Chapter 4). Therefore it was hypothesised that the administration of melatonin would mimic the effect of a long to short reduction in photoperiod and similarly cause an advance in the timing of spawning compared to controls. Thus, the experiments described below investigate the effect of melatonin on the timing of maturation in the rainbow trout. Two methods of melatonin administration are employed: (a) orally by addition to the food, (b) via silastic implants.

## Materials and Methods (Expt 2)

(a) Oral administration experiment

Two groups of 4 and one group of five (Grp C) individually tagged female rainbow trout thought to be approaching their winter spawning were maintained from 29th October 1984 until November 1985 in separate 700 litre tanks with a recirculating water supply. Water temperature was constant 10°C. Each tank was maintained under continuous light (LL) provided by a 3 watt fluorescent strip approximately 1 metre above the water surface. This photoperiod regime was used because LL at this time of year does not advance the timing of maturation (Chapter 4), therefore any advancement effect caused by the melatonin treatments would be accentuated. The 3 groups of fish were fed a standard commercial diet dosed with melatonin and a control; the treatments were as follows:

| | |
|---|---|
| Group A | 100 μg melatonin per kg body weight $day^{-1}$ |
| Group B | 200 μg melatonin per kg body weight $day^{-1}$ |
| Group C | Control |

At approximately fortnightly intervals the fish were checked to assess their state of maturity, weighed to re-calculate the feeding rates and a blood sample was taken from each animal. After centrifugation the serum was stored at -20°C and subsequently assayed for melatonin. Details of these general materials and methods are described in Chapter 2. Details of the preparation of the melatonin dosed food and the feeding regime are given below.

The melatonin dosed food was prepared by spraying an ethanolic solution of the indoleamine onto commercial trout pellets. As no previous investigation had administered melatonin to rainbow trout, the daily dose rate was based on previous research on fish and other lower vertebrates where melatonin had an effect on gonadal maturation (see Introduction for references). Physiological effects in fish have been observed with dose rates in the order of 20-200 μg $day^{-1}$. In the present experiment it was decided to adopt two dose rates, 100 and 200 μg melatonin per kg body weight $day^{-1}$. It was estimated that the food consumption rate of the fish was 1% of body weight $day^{-1}$, a level within the satiation limits of adult rainbow trout (B.P. Nutrition Ltd., Witham, U.K.). Based on this estimation, the diet for Group A was prepared by dissolving 10 mg melatonin in a small volume of ethanol (EtOH), made up to 200 ml with EtOH and then sprayed evenly over 1 kg of trout food pellets (No. 6 size) spread as a monolayer in a fume cupboard. With the extractor fan switched 'on' the dosed food was allowed at least 24 hrs for the ethanol solvent to evaporate before

feeding to the fish. The food for Grps B and C were prepared in the same manner except 200 mg and zero mg of melatonin were used in their preparation respectively. In an attempt to produce melatonin levels in the fish that were consistently elevated, and to help ensure that the fish ate all the food that was offered to them, the daily ration was spread over 4 feeding times at 6 hr intervals. The timing and administration of food in each tank was controlled by an automatic electronic feeder (G.D. Gearing Electronics Ltd., Isle of Skye, Scotland).

(b) Silastic implant experiment

Three groups each consisting of 4 individually tagged female rainbow trout were maintained together from 9th July 1985 until mid-December 1985 in a 1200 litre tank with a recirculating water supply under an ambient photoperiod. Water temperature ranged from 18°C (July) to 11°C (December). The mean (± sem) weight of the fish was 1.08±0.10 kg. On 10th September 1985 a silastic implant was inserted into the interperitoneal cavity of each fish. The groups were as follows:

| | |
|---|---|
| Group A | 'High dose' melatonin implant |
| Group B | 'Low dose' melatonin implant |
| Group C | Sham control |

The high dose implants (Grp A) were made by dissolving 50 mg melatonin in 1 ml ethanol. This solution was throughly mixed with 4 ml Silastic 3110 RTV monomer and 1 ml catalyst (Dow Corning Ltd, Reading, U.K.). Using a syringe this mixture was extruded into gelatin moulds (Agar Aids Ltd., Stansted, U.K.), with an approximate volume of 0.25ml. It was estimated that each high dose implant contained 2.5mg melatonin. The 'low dose' and sham control implants were prepared in an identical manner except 10 mg and zero mg melatonin were used respectively. After allowing 24 hrs for the Silastic to harden, the gelatin moulds were dissolved in warm water and the implants were stored at -20°C until use. To install the implant each fish was anaesthetised and a 1cm lateral incision was made in the body wall just above the pelvic fin. The pellet shaped implant was then pushed into the interperitoneal cavity. A small amount of antiseptic denture cement (Cicaterine containing antibiotics, kindly donated by Ministry of Agriculture, Fisheries and Food, Lowestoft) was used to help seal the incision which generally healed within two weeks. At approximately 1-2 week intervals for 3 months post-implantation each fish was blood sampled and checked to assess their state of maturity. After centrifugation the serum was stored at -20°C and subsequently assayed for melatonin.

The details of the assay procedure are described in Chapter 2.

## Results (Expt 2)

(a) Oral administration experiment

Spawning: The times of spawning of individual fish are illustrated in Figure 5.4. In general, the spawning profiles showed no consistent pattern. In Grp A fish No. 363 was found to be ripe on 11.2.85 and again on 10.9.85, fish No. 360 also matured twice during the experiment firstly on 22.1.85 and again on 10.9.85. Fish No 364 spawned only once on 4.4.85; fish No. 362 remained immature throughout the experiment. In Grp B fish Nos 349 and 351 both spawned on 28.11.84; fish 351 subsequently died the following January and fish 349 remained immature until the end of the experiment. Fish No. 352 spawned on 11.2.85 and fish No. 350 remained immature throughout the experiment. In Grp C all five fish spawned once during the course of the experiment: fish Nos. 357, 358 and 353 spawned on 20.12.84, 22.1.85 and 11.2.85 respectively. Fish Nos. 225 and 356 spawned on 15.5.85 and 3.7.85 respectively.

Melatonin: In all three groups there were large intra- and inter-individual variations in the measured serum melatonin concentrations with levels ranging from less than 100 pg $ml^{-1}$ to over 500 pg $ml^{-1}$ being recorded with no consistent pattern. It is possible that these variations were real but it was suspected that they were partly due to unresolved problems with the radioimmunoassay procedure (see Conclusions). The overall mean (± sem) serum melatonin concentrations for Grps A, B and C were 215±39 pg $ml^{-1}$ (n=26), 340±57 pg $ml^{-1}$ (n=19) and 191±40 pg $ml^{-1}$ (n=31) respectively. Grp B being significantly elevated compared to control Grp C ($P \leq 0.05$, Student's t-test).

Skin colouration: After two weeks of the experiment it became apparent that the high melatonin treatment (Grp B) was causing the skin of the rainbow trout to become pale. Although not quantified the paling effect can be clearly seen in Plates 1 and 2.

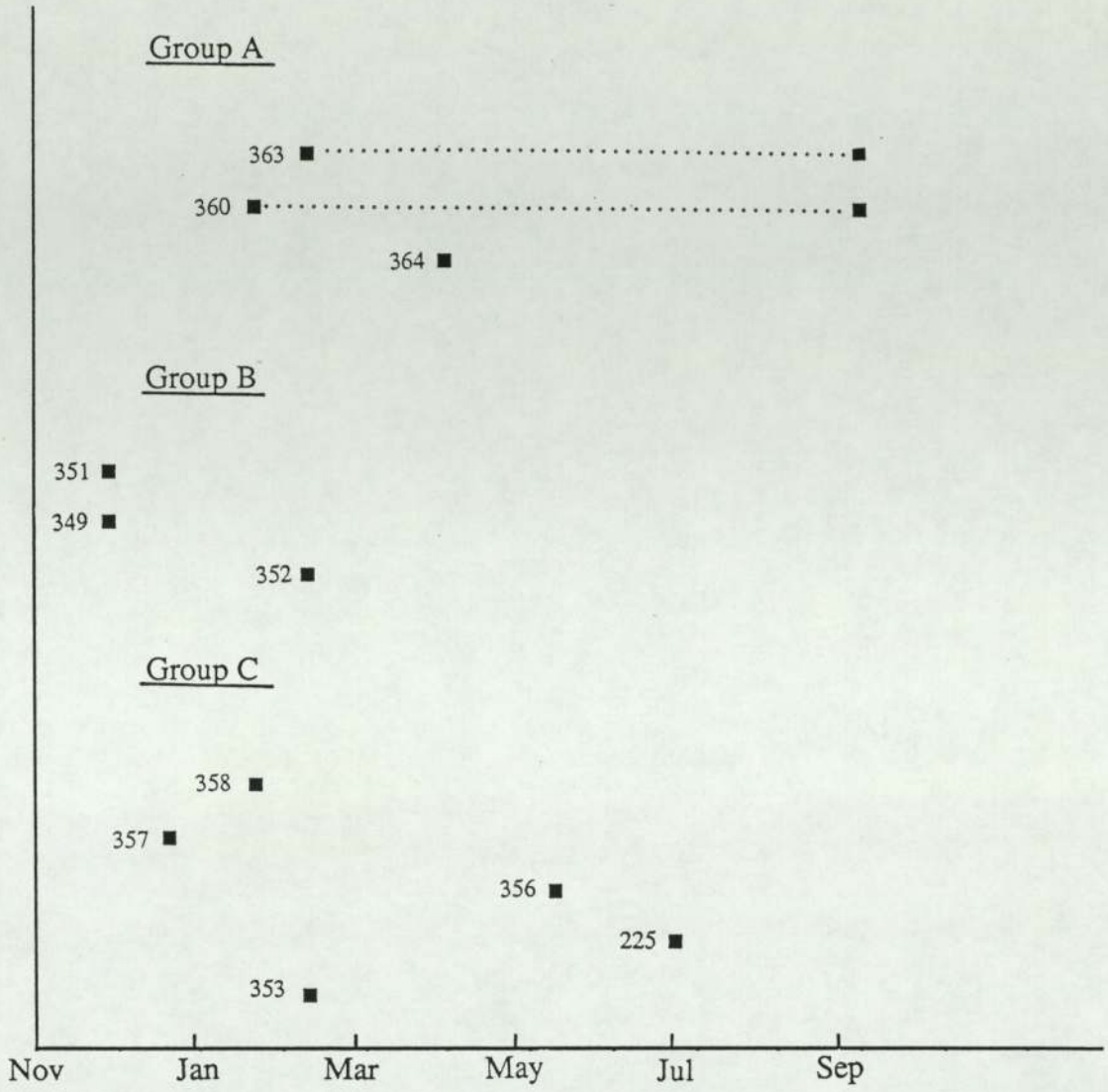



Fig. 5.4 The effect of the oral administration of melatonin on the timing of spawning( ■ ) of individual female rainbow trout. Figure illustrates the timing of spawning of fish in Group A( High dose melatonin), Group B(Low dose melatonin) and Group C(Control). The vertical lines on the x-axis refer to the first day of the month as labelled.


Plates 1, 2. The effect of orally administered melatonin (High dose, Grp B ) on the skin colouration in rainbow trout. The plates clearly show that the melatonin treated fish were paler than the respective controls.

(b) Silastic implant experiment

Spawning: One fish from Grp A and Grp C respectively were spawned on 10.12.85, none of the other experimental fish ovulated during the course of the experiment.

Melatonin: Fish in all three groups were individually blood sampled at seven time intervals up to 57 days post-implantation. The mean (±sem) recorded serum melatonin concentration in each group at each sample time is shown in Figure 5.5. The sample size is less than 4 in most cases because one fish in Grp A and one in Grp C died within two weeks of the the implantation operation. In addition it was sometimes impossible to extract enough blood from an individual fish to assay. With the exception of the 9-day post-implantation sample the mean serum melatonin levels in Grp A were elevated above 150 $pgml^{-1}$ for the first 9 days post-implantation after which they remained below this level. In the control Grp C, with the exception of one sample, the mean serum melatonin levels remained consistently below 100 pg $ml^{-1}$. The overall mean (±sem) serum melatonin concentrations for Grp A of 159±27 pg $ml^{-1}$ (n=21) was significantly higher (Student's t-test) than the comparable figure for Grp B (76±13 pg $ml^{-1}$, n=25; $P \leq 0.01$) and control Grp C (46±12 pg $ml^{-1}$, n=21; $P \leq 0.001$).

Skin colouration: The melatonin implants caused no apparent change in the skin colouration compared to the control group.

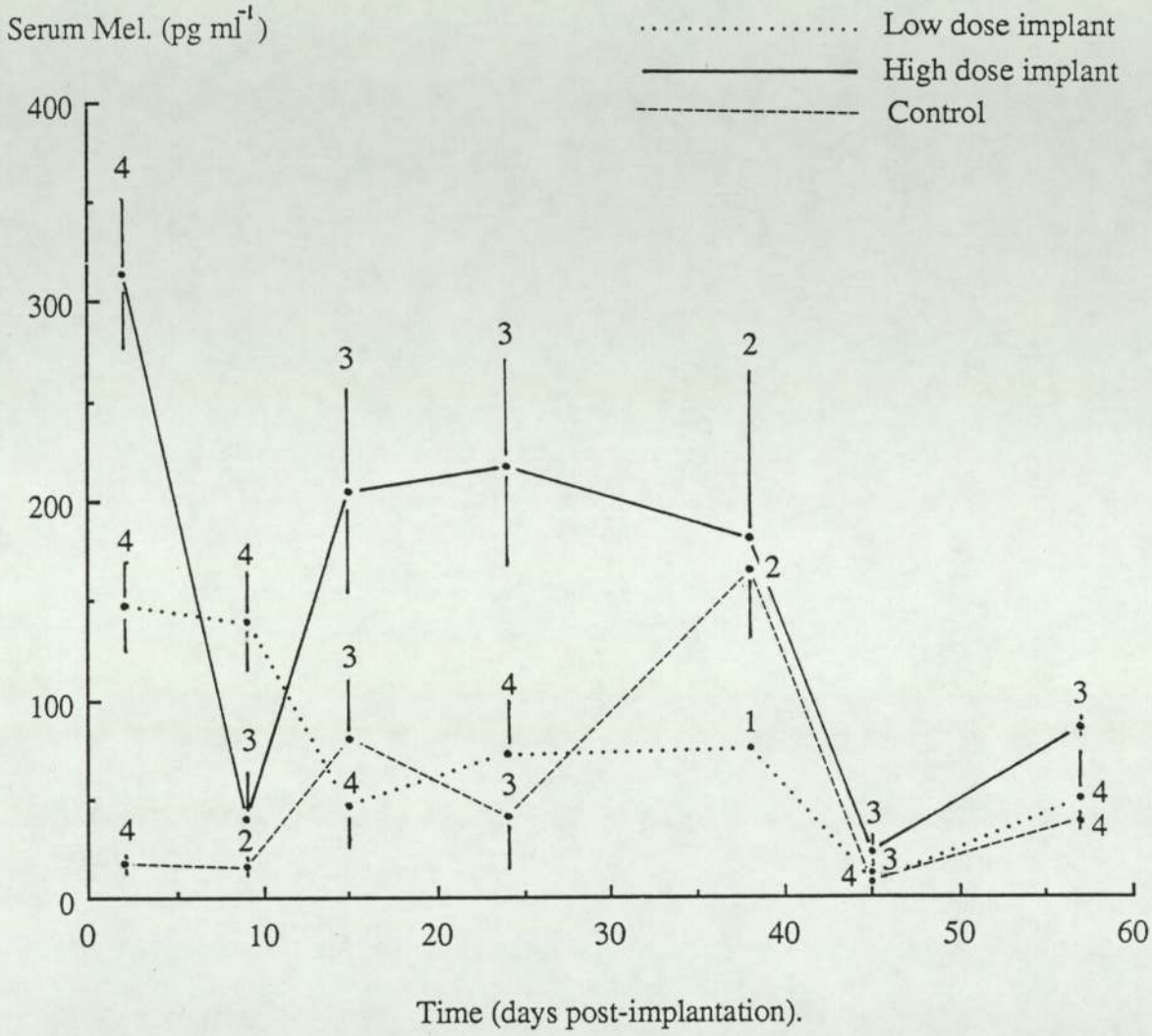



Fig. 5.5 The effect of melatonin implants (High dose, Low dose and Control) on the mean serum levels of melatonin in female rainbow trout. The integer above each coordinate indicates the number of fish sampled(n) with the vertical lines representing ± one standard error of the mean. Implants were inserted into the fish on Day 0.

Conclusions (Expt 2)

The experiments showed that interperitoneal Silastic implants and incorporation into the food were both viable methods for the administration of melatonin to rainbow trout. Both methods produced an elevation in serum melatonin levels. In addition, the high dose treatment in the feeding experiment caused the skin of the fish to go pale in agreement with the results of previous melatonin administration experiments on rainbow trout (Hafeez, 1970) and other lower vertebrates (Bagnara, 1960).

The main aim of the experiments was to test the hypothesis that the elevated circulating melatonin levels in the fish would mimic the effect of a short photoperiod and result in an advance in the timing of spawning. Unfortunately in the feeding experiment there was so much variation in the timing of maturation of fish within each experimental treatment that it is impossible to draw any conclusions regarding the role of melatonin in the control of rainbow trout reproduction. It is suggested that the inconclusive results were at least in part due to the fish selected for the experiment. At the start of the experiment it was not possible to obtain a homogenous batch of rainbow trout that had been raised under identical conditions. Consequently the fish that were used were selected from a holding stock of mixed and unknown origins. The Silastic implant experiment also failed to indicate whether melatonin had an effect on the timing of maturation as only two fish spawned during this study. At the start of the experiment in July all the fish selected were in good condition and appeared as if they would spawn successfully the following winter, it is unfortunate that most of them remained immature. It is not thought that the operation *per se* had any effect on maturation as the implants were small (1cm x 0.5cm x 0.5cm) and post-operatively the wound quickly healed and the fish soon exhibited normal feeding and swimming behaviour. Thus the basic experimental protocol in both feed and implant experiments appeared to be satisfactory. It is suggested that these experiments should be repeated with fish that have already spawned once, as they could be relied on to mature successfully. In addition, in future experiments it may be worthwhile increasing the amounts of melatonin in the Silastic implants. The 'high dose' implants only elevated serum melatonin levels for approximately 40 days before they became exhausted. If elevated melatonin levels do mimic short days then they may have to be maintained for longer than 40 days to cause an advance in the timing of maturation. Preliminary experiments suggest that there may be problems in incorporating increased amounts of melatonin into the existing implant size; because of this, forthcoming experiments in this laboratory are to use Silastic 'envelopes'

similar to those used in mammalian studies which can hold up to 1gm of crystalline melatonin (Lincoln and Ebling, 1985; English *et al*, 1986).

The advantage of the implant over the feeding method was that it ensured that each individual fish received a consistent dose of melatonin. From observations of the fish at feeding time in the oral administration experiment it was clear that the dominant fish in the tank were consuming more than their calculated quota of dosed food. In addition, the food often remained floating on the surface of the water for several minutes before being consumed by the fish; no doubt this resulted in some diffusion of melatonin into the water. Despite this it was clear that administration of melatonin via the food did cause an elevation in blood levels of this hormone. In conclusion these results suggest that endogenous melatonin may have a physiological role in controlling diurnal skin colouration changes in the rainbow trout; whether it also has a role in the control of gonadal maturation remains to be determined.

## 5.4 General Discussion

The present study on the rainbow trout showed that similar to other vertebrates this species exhibits a clear diel rhythm in blood levels of melatonin with higher levels occurring during the dark (Kennaway *et al*, 1977; Firth *et al*, 1979; Binkley, 1981; Underwood *et al*, 1984). There is good evidence that the pineal gland of the rainbow trout is primarily responsible for directly transducing photic information into a daily rhythm of melatonin as pinealectomy results in a lowering of night-time melatonin levels (Gern *et al*, 1978c). However, pinealectomy does not affect day time melatonin levels (Gern *et al*, 1978c) suggesting that non-pineal sources such as the retina (Gern *et al*, 1978b) contribute to the daily circulating rhythm, as has been shown in the Japanese quail (*Coturnix coturnix*; Underwood, 1984).

It was hypothesised in the introduction to this chapter that the daily rhythm of melatonin mediated the effects of photoperiod on the timing of maturation in the rainbow trout similar to some species of mammals. Although there is evidence that the pineal and melatonin are involved in the control of maturation in some cyprinids (review: deVlaming and Olcese, 1981), the fact that the present experiments failed to produce any conclusive results means that the question of whether melatonin has a role in the control of reproduction in the rainbow trout remains to be answered. However, the observation made in the present and a previous study (Hafeez, 1970) that melatonin causes the skin of rainbow trout to go pale may be significant. Gern (1981) hypothesised that the primitive function of melatonin is in dark adaptation, the photoreceptor cells of primitive vertebrates producing melatonin during darkness contributed a daily regular pulse of circulating melatonin which, as vertebrates evolved, came to be utilized in phasing temporal events such as gonadal maturation. The rainbow trout is a more primitive form of vertebrate compared to cyprinids (Young, 1981) and therefore the possibility remains that melatonin does not have a functional role in the control of gonadal development in this species. Despite this, the fact that photoperiod has an important effect on the timing of maturation in the rainbow trout, and that melatonin exhibits a diel rhythm does suggest that the pineal and its hormone may well be involved in the transduction of photic information into a humoral signal, which in turn could mediate in the control of the hypothalamo-pituitary-gonadal axis.

There is evidence of a close interrelationship between the pineal gland and endogenous rhythms in a range of vertebrates including fish (Kavaliers, 1979), amphibians (Demain and Taylor, 1977), lizards (Underwood, 1977), birds (Binkley, 1979) and mammals (Karsch *et al*, 1984). The present study has provided good

evidence that endogenous circadian and circannual oscillators are involved in the control of gonadal maturation (Chapters 3 & 4). The finding in Experiment 1 that the profile of the diel rhythm of melatonin in the rainbow trout does not merely follow the regular removal of the inhibitory influence of light has also been observed in other vertebrates (Goldman *et al*, 1982; Lincoln *et al*, 1982; Cockrem and Follett, 1985), and suggests an endogenous timing mechanism is involved in controlling the blood levels of this hormone This suggestion is supported by the presence of free-running rhythms of melatonin synthesis in a number of species maintained under constant darkness (Binkley, 1981). In addition, although work on sheep (Bittman *et al*, 1983) and some rodents (Carter and Goldman, 1983a,b) provided evidence that the duration of the elevation of melatonin dictated the reproductive response, the hypothesis failed to provide a full explanation for the photoperiodic control of reproduction. For example a large number of previous studies using such techniques as skeleton and resonance regimes had provided good evidence that an internal circadian clock was involved in the measurement of photoperiod (Chapter 3). At the time it was not immediately obvious how these mechanisms interacted with melatonin rhythms. However, more recent studies on mammals have provided evidence that in fact there is a close interrelationship between circadian clocks, the daily melatonin rhythm and the response of the gonadal maturation system. Exposing sheep (*Ovis aries*) to skeleton photoperiod regimes has shown that a 1 hr pulse of light during a prolonged night is interpreted as a dawn signal and the circulating melatonin levels remain low even when the lights are switched off (Brinklow *et al*, 1984; Earl *et al*, 1985). In addition to eliciting a short night (long day) melatonin profile, skeleton photoperiods also induced other changes in the endocrine system characteristic of sheep maintained under a long photoperiod (Brinklow *et al*, 1984; Earl *et al*, 1985). From these experiments it has been suggested that the secretion of melatonin and the resulting diel profile is controlled by two circadian clocks, one oscillator coupled to dusk is thought to control the evening rise in melatonin and another oscillator controlling the morning fall (Earl *et al*, 1985). This theory, which is basically a development of Pittendrigh's internal coincidence model for daylength measurement (Chapter 3) is attractive in that it can help to explain the shape of the diel rhythms of circulating melatonin observed in the present study on rainbow trout and also in work on other vertebrates (e.g. Goldman *et al*, 1982; Lincoln *et al*, 1982; Cockrem and Follett, 1985). A close interaction of two oscillators could explain the longer duration of elevated melatonin levels during a short photoperiod and also account for the increase in amplitude of the melatonin rhythm observed during long photoperiods. These speculations may not prove to be correct, however it is significant that

oscillator theories developed from studies where animals have been treated as 'black boxes' are now being applied to physiological investigations. No doubt in the future this will lead to a better understanding of the precise role of endogenous clocks in co-ordinating temporal events such as seasonal reproduction.

In addition to the possibility that the circulating rhythms of melatonin in the rainbow trout are controlled by endogenous circadian oscillators entrained by photoperiod, there is evidence from mammalian studies that tissues exhibit a daily rhythm of sensitivity to melatonin. The response of the reproductive system of hamsters to injections of melatonin depended on the timing of administration in relation to the phase of the circadian system. In animals maintained under long days there was a brief period before lights 'on' and a 5 hr period throughout the late afternoon when melatonin injections caused gonadal regression (Stetson and Tay, 1983; Stetson *et al*, 1986). The fact that these periods of sensitivity are of unequal duration indicates that the effect of injected melatonin is not merely due to an extension of the duration of the elevated circulating levels thus mimicking a short-day profile. These results have led to the development of the concept that a physiological response to melatonin only occurs when firstly the hormone is available to react with its target tissues, and secondly when the target tissues are sensitive to the hormone (Stetson *et al*, 1986). The timing of both of these processes appear to be controlled by endogenous circadian oscillators which are entrainable by photoperiod. Similar to the work on melatonin rhythms in sheep, research on hamster species is beginning to recognise that the internal coincidence of endogenous rhythms, as originally proposed by Pittendrigh (Chapter 3), may be of central importance in the control of gonadal maturation (Stetson *et al*, 1986). It is tempting to speculate that the daily rhythm of circulating melatonin in the rainbow trout has to be in a particular phase relationship with target tissues to elicit gonadal development. Certainly the evidence presented in Chapters 3 and 4 of the involvement of endogenous oscillators in the control of gonadal maturation, an indication of some form of gating mechanism and the lack of support for Bunning's external coincidence hypothesis, all indicate that a system for controlling gonadal development based on internal coincidence of endogenous oscillators is a more likely hypothesis. Unfortunately, whereas recent support for the internal coincidence hypothesis in mammals has culminated from years of research on the pineal and melatonin, comparable research on the rainbow trout and other lower vertebrates are at a very preliminary stage. The majority of studies on the involvement of the pineal in endogenous rhythmicity in fish have been on circadian locomotor rhythms (Beitinger, 1975; Kavaliers, 1979). Pinealectomy of lake chub (*Couesius plumbeus*) failed to abolish the circadian locomotor rhythm but caused a

subtle change in its periodicity (Kavaliers, 1979). It can be concluded from these results that in the chub the pineal is not a central pacemaker, as in some birds (Binkley, 1979), but it does have a role in the organisation of circadian rhythms. This view is supported by experiments on the white sucker (*Catostomus commersoni*) where pinealectomy resulted in the splitting of a circadian activity rhythm into two free-running components (Kavaliers, 1979). Earlier (Chapter 3) it was recognised that the phenomenon of splitting was good evidence for the existence of two loosely-coupled circadian oscillators. Pittendrigh and Daan (1976) proposed that the phase relationship between two such circadian oscillators could control the timing of seasonally-occurring phenomena such as annual breeding with one clock entrained to dawn and the other to dusk. Although this hypothesis has yet to be tested, the fact that the phase-relationship of circadian rhythms in the white sucker appear to be controlled by the pineal suggests that this structure may have a role in the control of the timing of seasonal events. This proposition that the teleost pineal is somehow involved in integrating circadian rhythms with longer term, possibly annual events, is supported by the observation that the effect of pinealectomy on activity rhythms depends on the time of year of the operation; pinealectomy of lake chub (*Couesius plumbeus*) in winter results in a shortening of the free-running periodicity, whereas in summer the removal of the pineal causes the period of the activity rhythm to lengthen (Kavaliers, 1979). In addition, burbot (*Lota lota*; Kavaliers, 1980), lake chub (Kavaliers, 1978) and dace (*Rhinichtys cataractae*; Kavaliers, 1981) have all been shown to exhibit an annual variation in the periodicity of circadian locomotor rhythms. These results suggest that annual or circannual rhythms could be evolved from circadian rhythms. This hypothesis known as frequency demultiplication (Gwinner, 1973; Mrosovsky, 1978) has already been discussed in Chapter 3 in relation to the generation of circannual rhythms of gonadal maturation in the rainbow trout. Although support for this hypothesis in the rainbow trout was poor, it can be speculated from these locomotor rhythm data that the pineal gland has an important role in controlling the seasonal variation in periodicity of circadian rhythms. As there is good evidence that the pineal gland functions as a transducer of photic information it is evident that under natural conditions the secretion of melatonin from the pineal could keep the endogenous rhythms of the fish entrained to the daily and annual changes in the photoperiod.

To summarise, the work described in this chapter showed that serum melatonin levels in the rainbow trout are elevated approximately for the duration of the dark period of a 24 hr photocycle. The results support the hypothesis that photic information is transduced by the pineal gland into a rhythm of melatonin which could

potentially mediate in the timing of physiological responses that are entrained by photoperiod. Furthermore the results suggest that endogenous control mechanisms, possibly circadian pacemakers interact with the photoperiod in controlling the profile of the melatonin rhythm. The hypothesis that the pineal gland in fish integrates external environmental information with internal rhythms is supported by work on locomotor rhythms which has indicated that this structure has an important role in the organisation of circadian and possibly circannual endogenous rhythms. Attempts in the present study to test the hypothesis that melatonin has a role in mediating the effects of photoperiod on the timing of reproduction in rainbow trout did not provide any conclusive results although the methods of melatonin administration and experimental design were considered adequate. It is proposed that a repeat of these experiments in future studies using experimental animals with a predictable spawning time would provide a better indication as to the role of melatonin in the control of reproduction in the rainbow trout.

# CHAPTER SIX
# GENERAL CONCLUSIONS

The observations made in the present study support the hypothesis that the annual cycle of reproduction in the female rainbow trout is controlled by an endogenous circannual timing mechanism(s) capable of being entrained by changes in the daylength. Previously it was considered that the rainbow trout had a requirement for photoperiods of a specific length at different stages of gonadal development. However, rather than photoperiod having a direct 'driving' influence in the control of seasonal breeding in the rainbow trout, the results indicate that daylength has a synchronising or zeitgeber role in the entrainment of an endogenous rhythm of maturation.

Female rainbow trout maintained under constant 6L:18D and constant temperature (8.5-9.0°C) for up to 4 years exhibited a self-sustained, free-running rhythm of ovarian maturation, where the periodicity in individual animals ranged from 11-15 months. These observations satisfied the criteria that gonadal development is controlled by an endogenous circannual timing mechanism. Under conditions of constant 18L:6D or constant light (LL) the periodicity of the maturation rhythm was 5.5-6 months. Why the maturation rhythm should have expressed different periodicities under different constant photoperiod regimes is unclear, although it may be due to an uncoupling or 'splitting' of multiple circannual timing mechanisms all associated with the control of gonadal development. How the endogenous long-term rhythms are generated is unknown. However, evidence is presented that the internal clock can be dissociated from the neuroendocrine systems controlling gonadal development indicating that the timing mechanism may be considered as an autonomous unit.

Under natural conditions seasonal breeding in the rainbow trout is synchronised to the annual changes in the environment ensuring that reproduction occurs at a time of year when the chances of survival are at an optimum. As with many other vertebrates indigenous to higher latitudes it is generally accepted that the annual cycle of daylength has an important influence on the timing of maturation in salmonids. The current investigations into the effect of daylength on the timing of spawning in the rainbow trout provided good evidence that changes in the length of photoperiod serve as a zeitgeber which entrains the circannual clock associated with the control of maturation. Abrupt changes in the length of the photoperiod caused phase-shifts in the circannual clock which resulted in alterations in the timing of maturation and associated changes in blood levels of oestradiol-17ß, testosterone and total calcium compared to controls. The direction of change in photoperiod (increasing or decreasing) and the timing of the change relative to the phase of the endogenous rhythm appear to be the most important factors in determining the extent

to which spawning is advanced or delayed. The magnitude of the change in photoperiod exerts only a minor supplementary effect on the alteration in the timing of maturation. However, in the entrainment of the maturation cycle by changes in daylength, the length of the photoperiod *per se* does not appear to be an important factor: Particular photoperiods can be interpreted by the rainbow trout as 'long' or 'short' depending on the length of the preceding or succeeding photoperiod. These observations emphasise the importance of photoperiodic 'history' in the interpretation of daylength change on the timing of seasonal reproduction cycles. Overall, the alteration in the timing of maturation in rainbow trout by specific changes in photoperiod could be described in terms of a phase-response curve analogous to the entrainment behaviour of circadian oscillators, thus adding additional support to the hypothesis that the underlying circannual clock can be considered as an oscillator in the mechanistic sense.

The results provided evidence of a gating mechanism being closely associated with the endogenous circannual clock, the proposed function of which is to ensure that only fish that have reached an appropriate developmental stage are allowed to undertake the energetically demanding process of ovarian development. The gate only appears to be 'open' for a finite period of time (several months) in each year when the internal clock is going through a specific phase of its circannual cycle; for the rest of the cycle it is closed. It is proposed that any virgin rainbow trout reaching the threshold stage of development when the gate is open proceed to spawn that particular season. In contrast, slower developing fish which only attain the threshold after the gate has closed do not spawn in that season but must wait until the following year when the internal clock is again in the 'gate-open' phase of its cycle. Abrupt changes in the photoperiod caused phase-shifts in the circannual clock which altered the timing of the opening and closing of the gate and in turn altered the percentage of the population of experimental fish which were able to mature in a particular year.

The present study showed that in the rainbow trout blood levels of the pineal hormone melatonin exhibit a daily rhythm corresponding to the environmental photoperiod, with higher levels during the hours of darkness. These results support the hypotheses that the pineal gland is involved in transducing photic information into a humoral signal. Skeleton and resonance photoperiod experiments indicate that circadian rather than hourglass mechanisms are involved in perceiving daylength. It is possible that measurement of photoperiod is effected by an interaction between the daily rhythm in circulating melatonin levels and endogenous circadian oscillators, as has been suggested by work on higher vertebrates. Quite how, or if, circadian oscillators interact with or generate circannual rhythms remains to be elucidated

although evidence is presented that frequency demultiplication is not a means by which long-term rhythms are produced.

The General Introduction (Chapter 1) to this thesis emphasised that in the study of the control of seasonal breeding in any particular animal it is important to consider the contribution of exogenous factors such as photoperiod, and also the involvement of endogenous rhythms in this process. Previous investigations on the control of the annual reproduction cycle in salmonids largely concentrated on exogenous factors and reached the general conclusion that daylength had an important role in determining the timing of spawning. These findings led to the development of the hypothesis of photoperiod having an inductive or driving role in the control of maturation. However, the fact that the present study clearly showed that the rainbow trout exhibits an endogenous rhythm of maturation meant that the role of photoperiod in the control of seasonal breeding in this fish had to be reassessed; this resulted in the entrainment hypothesis described herein. A comparison of the control of annual reproduction in other vertebrates with the model developed in the present study on the rainbow trout indicates that long-term endogenous rhythms of maturation and their entrainment by environmental factors appear to be a widespread phenomena amongst seasonal breeders in other phyletic groups.

# APPENDIX

## RESEARCH PUBLICATIONS

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Kugler Publications, Amsterdam/Berkeley

# Photoperiodic mechanisms and rhythms of reproduction in the female rainbow trout

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Keywords: daylength, trout, reproduction, circannual, rhythms, resonance, skeleton, photoperiod, endocrine

**Abstract**

The present work investigates the importance of circadian and circannual rhythms in the photoperiodic control of reproduction in the rainbow trout. Maintenance of groups of 20–30 female trout under continuous light (LL), constant long (18L:6D) or short (6L:18D) days and conditions of constant temperature (8.5–9.0°C) and feeding rates (0.5% body weight, $day^{-1}$), starting in February, produced markedly different spawning periodicities during the first and subsequent years of treatment. At the end of the first year, spawning was advanced by up to 2 months in the fish under LL and 18L:6D and delayed by up to 5 months in those under 6L:18D when compared with the December spawning of control fish under ambient light. Continued exposure of the fish to the same photoperiod regimes produced cycles of spawning and peak levels of testosterone, oestradiol-17$\beta$ and calcium (as an index of vitellogenin) at intervals of 150–170 days under LL and 18L:6D and 320–420 days under 6L:18D. The functional importance of these spawning cycles and their relationship to circannual rhythms and the control of reproduction is discussed.

Exposure of fish to skeleton (6L:4D:2L:12D, 6L:6D:2L:10D and 6L:8D:2L:8D) and resonance (6L:42D, 6L:48D and 6L:54D) procedures produced ranges of spawning times up to two months in advance of control fish. Results with the resonance regimes, where fish received only a half the light-dark cycles and a quarter of the total daylight hours of those on ambient light cycles show that trout do not measure daylength and time by counting daily cycles or by hour-glass mechanisms. Collectively, these data support the proposition that rhythmic processes of photosensitivity are involved in the modulation of reproductive development in the rainbow trout and that circadian and circannual rhythms cooperate in the timing and entrainment of this cycle.

## Introduction

It is now well established that the reproduction of the rainbow trout and probably all salmonid fish is initiated and subsequently modulated by photoperiodic cues derived from the seasonally-changing cycle of daylength (Whitehead *et al.* 1978; Bromage *et al.* 1982a, b). Ovarian recrudescence and early vitellogenesis would appear to be stimulated by long or increasing daylength whereas the synchronisation of the later stages of oocyte maturation and ovulation is dependent on a decreasing or short photoperiod (Henderson 1963; Whitehead and Bromage 1980; Bromage *et al.* 1984; Elliott

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*et al.* 1984). Despite our understanding of this control and our ability to modify the time of spawning both in the laboratory and for the production of all-year-round supplies of eggs for commercial farms (Bromage 1982; Bromage and Duston 1986; Bromage *et al.* 1984), we have little knowledge of the mechanisms which fish use to measure daylength and hence register time and season.

Work on other photoperiodic organisms suggests that they use either rhythmic (Bünning 1936, 1960; Pittendrigh and Minis 1964) or hour-glass mechanisms (Lees 1973) to measure changes in daylength. The former model proposes that an endogenous clock(s) or oscillator(s) provides a rhythm of variable sensitivity to light and that illumination of specific phases of this rhythm by natural seasonal changes in daylength elicits photoperiodic induction. Although Danilevsky *et al.* (1970) and Pittendrigh (1972) have both suggested that light may only be involved in the entrainment of multi-oscillator systems, and not directly with photoperiodic induction (i.e. internal coincidence model), the basic concept of endogenous rhythmicity remains. In contrast, mechanisms involving hour-glass or interval timers lack rhythmicity and are thought to depend on the progressive accumulation with increasing day or night length of a reaction-product which, on reaching a critical or threshold concentration, then brings about the photoperiodic response.

Several experimental procedures have been developed, specifically to differentiate hour-glass from rhythmic or circadian mechanisms of time measurement, these include skeleton and resonance procedures, T-experiments and constant light regimes (see Lees 1973; Follett 1981, 1984 for discussions). Use of these methods has shown that the majority of organisms so far investigated depend upon endogenous rhythms of photosensitivity for time measurement, with only a few insects (Lees 1973; Saunders 1981) and a lizard (Underwood 1981) clearly relying on hour-glass mechanisms. Only three studies have specifically addressed the question of daylength measurement and the control of reproduction in fish. Baggerman (1972) was the first to suggest that circadian rhythms of photosensitivity were involved in this group in her study of the control of spawning in the stickleback. Similar proposals were also made by Sundararaj and Vasal (1976) and Chan (1976) working with the catfish and medaka respectively. However, firm conclusions regarding the involvement of circadian processes in fish cannot be made from these studies because all three rely solely on data drawn from skeleton light regimes and it has been suggested that the results of such experiments may be subject to differing interpretation (Follett 1981). To date no work has been carried out on salmonids and neither resonance procedures nor T-experiments have been used in any teleost study.

Even if circadian processes are shown to be widely-involved in the primary responses of fish to light, questions regarding the timing and control of annual cycles remain to be explained. Under ambient conditions these cycles are closely entrained by the natural seasonal alterations in daylength. Similar patterns of entrainment can also be achieved under accelerated seasonal light cycles (Goss 1969; Bromage *et al.* 1982a; Elliott *et al.* 1984; Gwinner 1981), but not under constant photoperiod regimes. This suggests that the seasonally-changing daylength normally provides a sequence of photoperiodic cues (i.e. different daylengths) all of which are necessary for reproductive development. However, it is not clear how this process is brought about.

There is evidence for some birds (e.g. the Japanese quail), in which the involvement of circadian rhythms in the control of reproduction has been firmly established, that there are seasonal alterations in the 'critical daylength' required for gonadal recrudescence (Robinson and Follett 1982). It has also been suggested that seasonal variations in photosensitivity occur in birds (Gwinner 1973) and fish (Baggerman 1980) and that these are necessary for the normalisation of the reproductive cycle in several mammals (Elliott 1981; Herbert 1981).

Possibly, these alterations in photosensitivity represent the controlling influences of a yearly or circannual clock. Evidence for circannual rhythms has begun to appear over the past 10–15 years (Gwinner 1973, 1981) and although the most convincing data relate to birds (Berthold *et al.* 1972; Berthold 1974; Gwinner, 1973, 1981) and mammals

(Goss 1969; Pengelley and Asmundson 1974), a few studies suggest that circannual rhythms are also present in fish. Of these the clearest evidence of a circannual rhythm is provided by Sundararaj *et al.* (1973) in their study of ovarian weight changes in catfish maintained under continuous light for period of 33 months. Other studies on fish by Eriksson and Lundqvist (1982), Whitehead *et al.* (1978) and Bromage *et al.* (1984), although strongly suggestive of endogenous rhythms, are less convincing primarily because the cycles were not followed for sufficiently long periods of time.

This shortcoming is obviated in the present work which uses constant photoperiods and skeleton and resonance light procedures to investigate the possible involvement of circannual and circadian processes in the photoperiodic control of reproduction in the rainbow trout.

## Materials and methods

Groups of 20–30 one or two year-old female rainbow trout, *Salmo gairdneri*, with a natural spawning time in December, were maintained in 1.6 m diameter lightproof tanks, each illuminated with a 40W bulb providing 25 Lux at the water surface. Daylength was controlled by either conventional 24 hr time clocks or electronic devices accurate to within 1s of light day$^{-1}$. Three experimental series were conducted and each began in February. The procedures were as follows:

### *1. Constant light regimes*

Over a 2–4 year period, 3 groups of 30 two year-old fish, maintained in separate tanks, were subjected to short days (6L:18D), i.e. 6 h Light:18 h Dark, continuous light (LL) or long days (18L:6D). For each tank of fish the reproductive cycles were followed for at least three successive spawning periods.

### *2. Skeleton photoperiods*

Over a 2 year period, groups of up to 25 one year-old fish were exposed in separate tanks to one of the following photoperiod regimes: 6L:18D, 6L:4D: 2L:12D, 6L:6D:2L:10D or 6L:8D:2L:8D.

### *3. Resonance procedures*

Over a 2 year period, groups of 20 two year-old fish were exposed in separate tanks to one of the following photoperiod regime: 6L:18D, 18L:6D, 6L:42D, 6L:48D or 6L:54D. The total number of hours of light and dark and the number of-light dark cycles offered during the year by each of these regimes and the natural cycle are compared in Table 1.

Under each of the three series of experiments appropriate controls were maintained under ambient light conditions. Water temperature was a constant 8.5–9°C and fish were fed throughout at 0.5% body weight day$^{-1}$ with a commercial trout diet. The fish under the resonance procedures received equivalent amounts of food but this was only given

*Table 1.* Total numbers of light-dark cycles and the daylight hours under various photoperiod regimes

| *Photoperiod regime* | *No. light-dark cycles* | *Hr. light year*$^{-1}$ | Hr. dark year$^{-1}$ |
|---|---|---|---|
| Natural cycle | 365 | 4380 | 4380 |
| 18L:6D (Long day) | 365 | 6570 | 2190 |
| 6L:18D (Short day) | 365 | 2190 | 6570 |
| 6L:42D | 183 | 1095 | 7665 |
| 6L:48D | 162 | 973 | 7787 |
| 6L:54D | 146 | 876 | 7884 |

while the lights were on. Small numbers of male fish were also stocked in each tank to provide sperm for fertilization. All fish were identified with small numbered plastic discs attached through the dorsal fin. At approximately monthly intervals a number of female fish from each tank were anaesthetised with 2-phenoxyethanol (1:20,000) and blood samples taken from the Cuverian sinus for measurement of serum levels of testosterone, oestradiol-17$\beta$ and calcium (as an index of vitellogenin) using methods already described (Bromage *et al.* 1982a, b; Elliott *et al.* 1984). Sampling was always performed 3-4 hours after 'lights-on', LL treatment excepted. As the time of expected spawning approached fish were examined more frequently for the presence of ripe eggs (Bromage *et al.* 1982b). Mortalities over the course of the three experimental series were approximately 5% of total. Differences between means were tested statistically by a student t-test or by using d and treating it at t if the variances were dissimilar (Bailey 1959).

**Results**

The results are described under the headings of the three experimental series as follows:

*1. Constant light regimes*

During the first year's exposure to LL (continuous light) and 18L:6D (long days) spawning occurred in October and November up to 2 months in advance of control fish under ambient lighting and up to 7 months before some of the fish under 6L:18D (short days). Continued exposure of the same fish to these constant light regimes over the next two reproductive cycles produced different results from those of the first year's photoperiod treatments. Under both LL and 18L:6D spawning was repeated at intervals of approximately 160 days (Fig. 1a & b) although as the experiment progressed the loss of tags made it difficult to provide reliable informa-

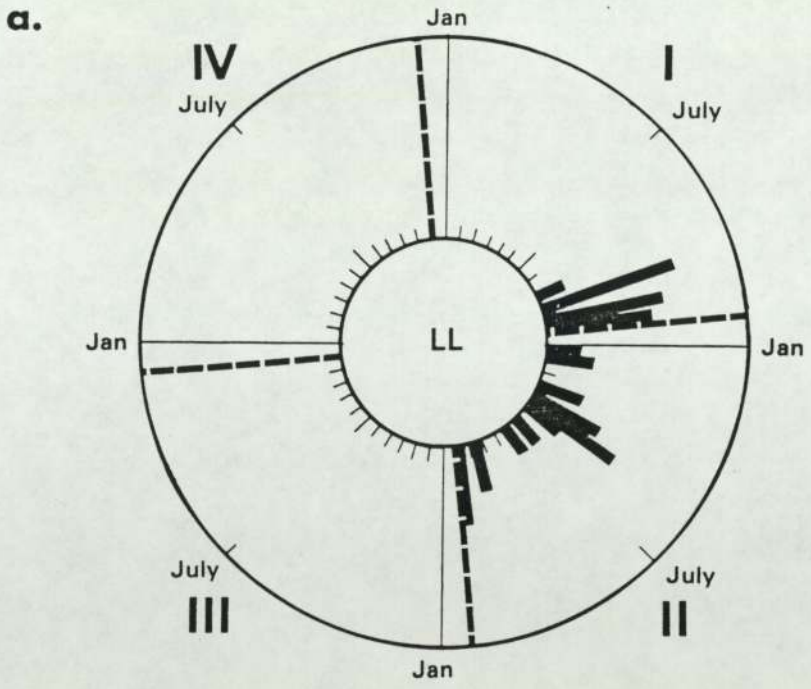
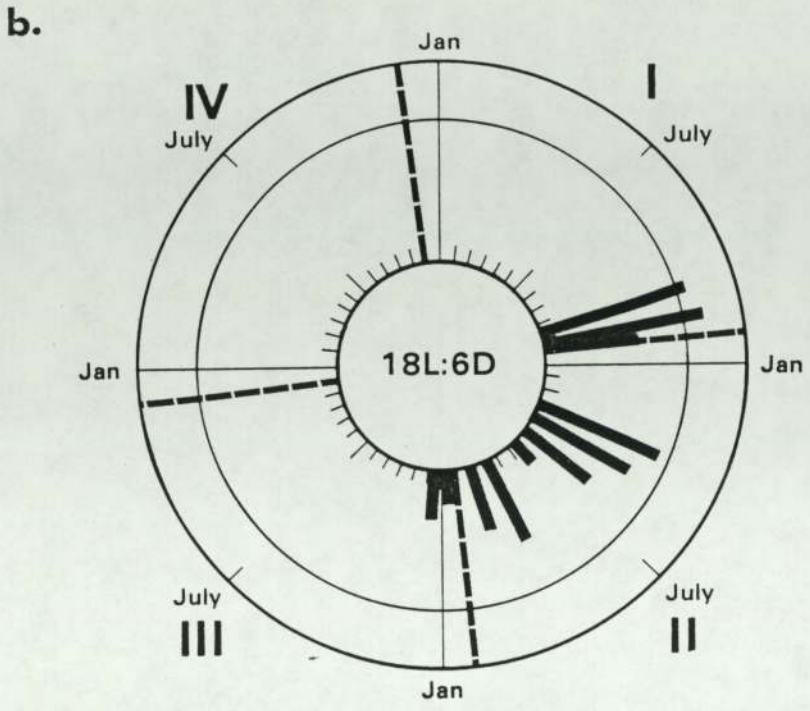



*Fig. 1a, b & c.* Effects of continuous light (1a), long days (1b) and short days (1c) on the periodicity of spawning of female rainbow trout over a 2–4 year period. Each scale point on the inner rings represents one month. The bars show the number of spawning females with a bar extending from the inner to the outermost rings being equivalent to 10 fish. The natural spawning of this strain began at the start of Dec (dotted line) and lasted for one month.


b.
Jan
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July
18L:6D

*Fig. 1b.*

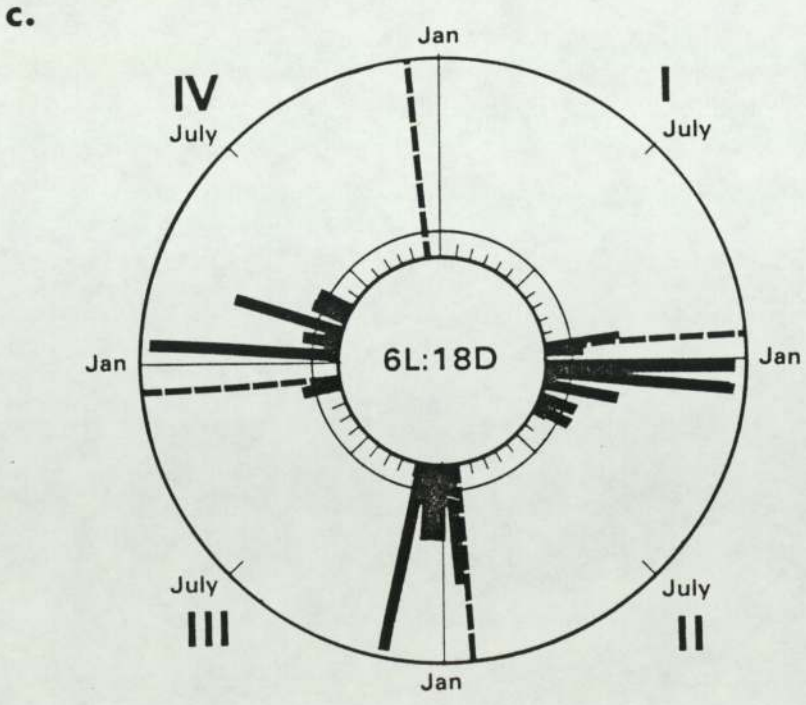
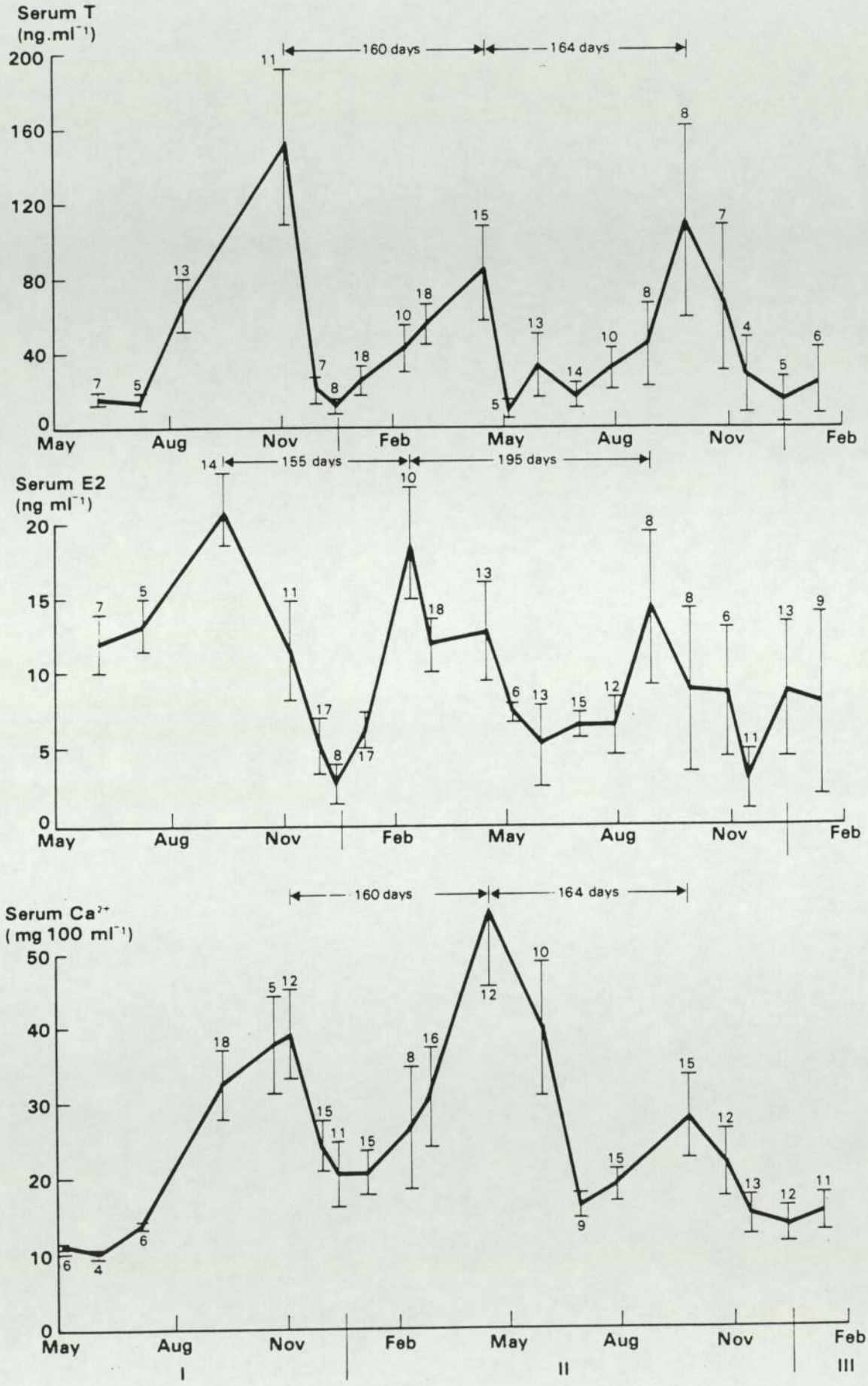

c.
Jan
I
July
Jan
II
July
Jan
III
July
Jan
IV
July
6L:18D

*Fig. 1c.*


Serum T
(ng.ml⁻¹)
160 days
164 days
Serum E2
(ng ml⁻¹)
155 days
195 days
Serum Ca²⁺
(mg 100 ml⁻¹)
160 days
164 days
May
Aug
Nov
Feb
May
Aug
Nov
Feb
I
II
III

tion on the period length between the successive spawnings of each individual fish. Much more convincing data were provided by the serum analyses of testosterone, oestradiol-17$\beta$ and calcium. Here the spawning periodicities were reflected by the distances between the peak heights of the three parameters measured. Under both LL (Fig. 2) and 18L:6D (Fig. 3), periodicities of approximately 150–170 days are clearly shown; in each case the differences between the mean peak heights and the lowest value between peaks were significantly different (with p at least $< 0.05$). Furthermore, similar period lengths were observed over 3 or 4 successive cycles. Some data are available for the multiple spawning of individual fish under LL and 18L:6D and a typical example is shown in Fig. 4a. Under both photoperiod regimes testosterone, oestradiol-17$\beta$ and calcium levels all peaked before spawning and had begun to fall before ripe eggs were stripped from the fish. Within a month of spawning testosterone and oestradiol levels had begun to increase and this was followed a few weeks later by increases in serum calcium.

Under 6L:18D, most fish spawned in February or March, delays of up to 5 and 7 months when compared with fish maintained in ambient light and 18L:6D respectively. Subsequent exposure of the same fish over two further years produced peaks of spawning in the same months (Feb/Mar) in each case i.e. at approximately one year intervals after the first delayed spawning (Fib. 1c). The mean spawning interval between the first and second spawnings under short days was 364 ± 6 days (mean ± s.e.m.) and between the second and third spawnings, 366 ± 9 days. Spawning intervals for individual fish ranged from 320 to 420 days. Similar periodicities were also provided by the serum analyses, with the testosterone and calcium peaks occuring just before spawning and those of oestradiol-17$\beta$ a little earlier (Fig. 5). In each case the differences between the mean peak heights and the lowest value between peaks were significantly different ($p < 0.005$). Again there was considerable variability in the timing of the hormonal and calcium changes in the successive spawning of individual fish under this photoperiod regime and Fig. 4b, c & d show examples of this variation. Although delayed by 3–4 months when compared with control fish, the period lengths of the fish under 6L:18D were not significantly different from the one year periodicity of control cycles.

*2. Skeleton photoperiods*

None of the 1+ year-old fish in this experiment spawned at the end of the first year of treatment. Subsequently, all the fish matured during the next cycle (Fig. 6). The spawning of the fish subjected to 6L:6D:2L:10D began in December with a mean time for this experimental group, significantly in advance of the fish under 6L:18D ($P < 0.025$), 6L:4D:2L:12D ($P < 0.05$) and 6L:8D:2L:8D ($P < 0.001$). The 6L:8D:2L:8D fish were also delayed in comparison with the 6L:4D:2L:12D treatment ($P < 0.01$).

*3. Resonance procedure*

During the first treatment cycle the fish under 6L:42D, 6L:48D and 6L:54D commenced spawning in late October/early November, significantly in advance of the fish under 6L:18D ($P < 0.001$) where spawning began in December and peaked between mid-January and early February (Fig. 7). The fish under 6L:48D and 6L:54D began spawning at the same time as a parallel group under 18L:6D. The second spawning of the fish, maintained under 6L:18D for a year, occurred almost exactly one year later again with a median time of early February. The 6L:54D regime produced a

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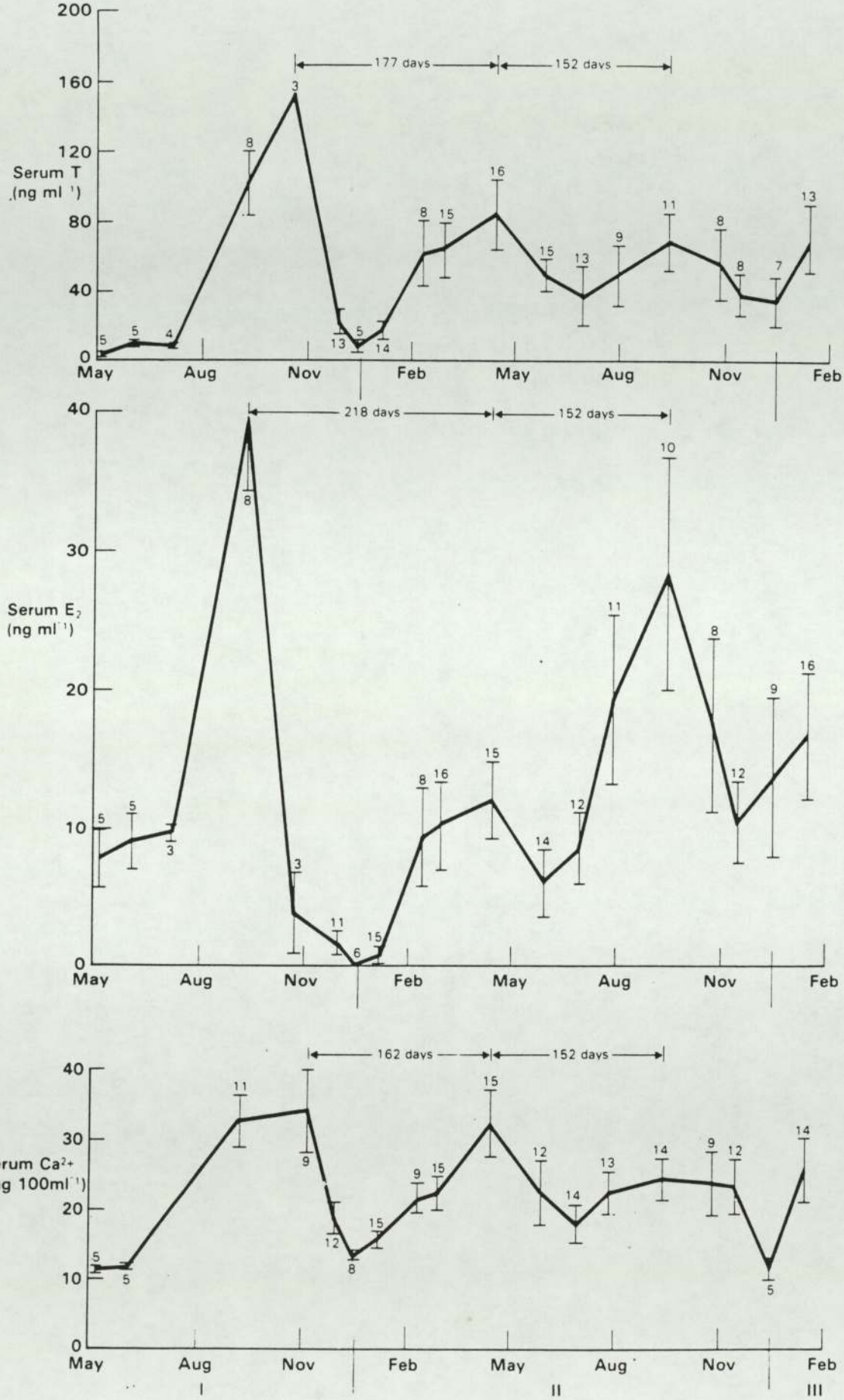
*Fig. 2.* Effects of continuous light (LL) on the mean (± SEM) serum levels of testosterone (T), oestradiol 17B ($E_2$) and calcium ($Ca^{2+}$) over a 2–3 year period. Period lengths between the peak heights of these three components are arrowed and the time interval indicated. Vertical bars are the standard errors of the mean with n indicated above for each.


Serum T
(ng ml⁻¹)
177 days
152 days
May
Aug
Nov
Feb
May
Aug
Nov
Feb
Serum E2
(ng ml⁻¹)
218 days
152 days
Serum Ca2+
(mg 100ml⁻¹)
162 days
152 days
I
II
III

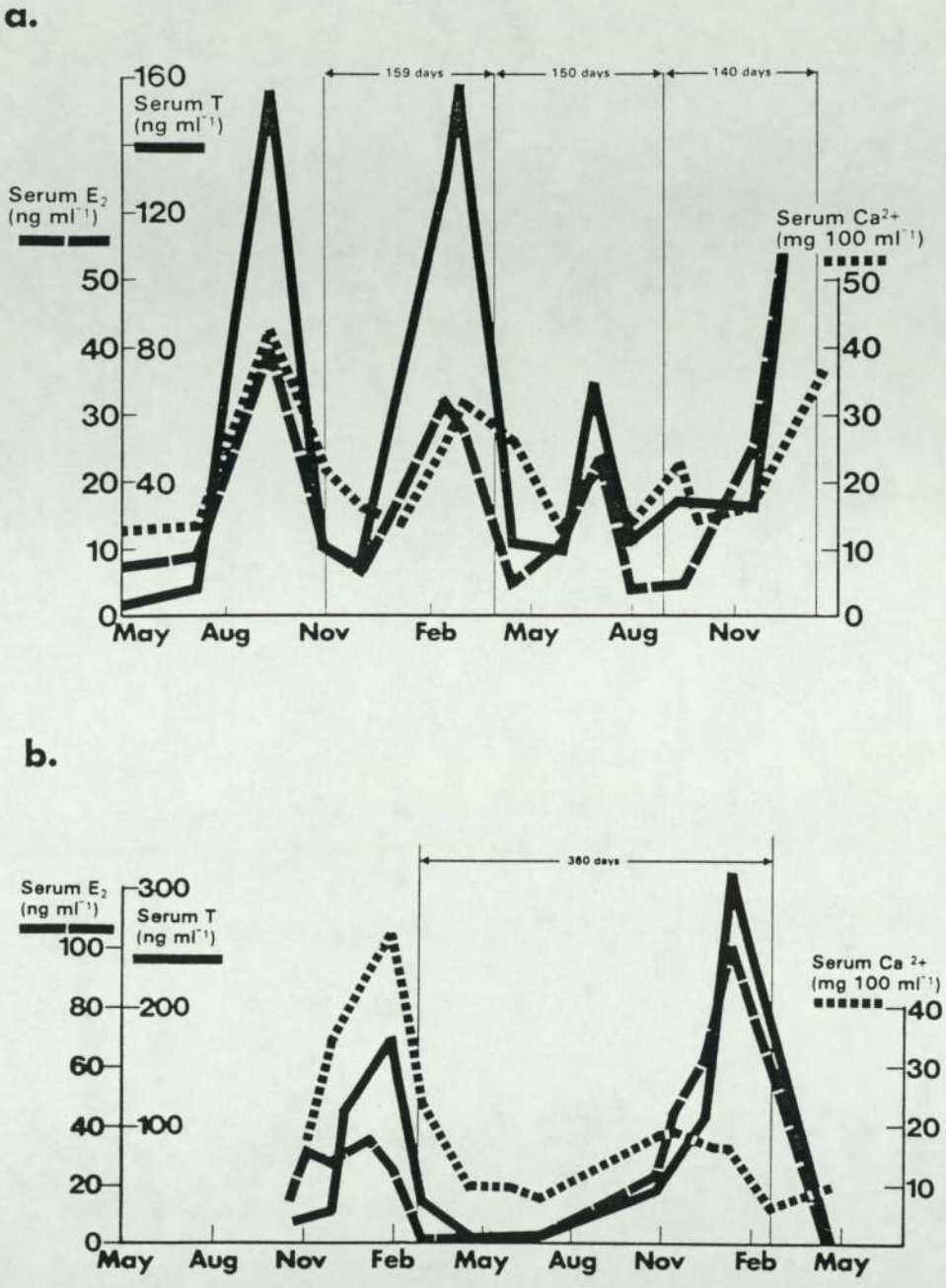



*Fig. 4a, b, c, & d.* Effects of continuous light (LL) and constant long (18L:6D) or short days (6L:18D) on the serum levels of testosterone (T), oestradiol 17$\beta$ (E2) on calcium ($Ca^{2+}$) and the timing of spawning (vertical lines) in individual female rainbow trout: 4a (LL); 4b, 4c, and 4d (6L:18D). The period-lengths between successive spawnings are shown (between arrows).

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*Fig. 3.* Effects of constant long days (18L:6D) on the mean (± SEM) serum levels of testosterone (T), oestradiol 17$\beta$ ($E_2$) and calcium ($Ca^{2+}$) over a 2–3 year period. Period lengths between the peak heights of these three components are arrowed and the time intervals indicated. Vertical bars are the standard errors of the mean with n indicated above for each.

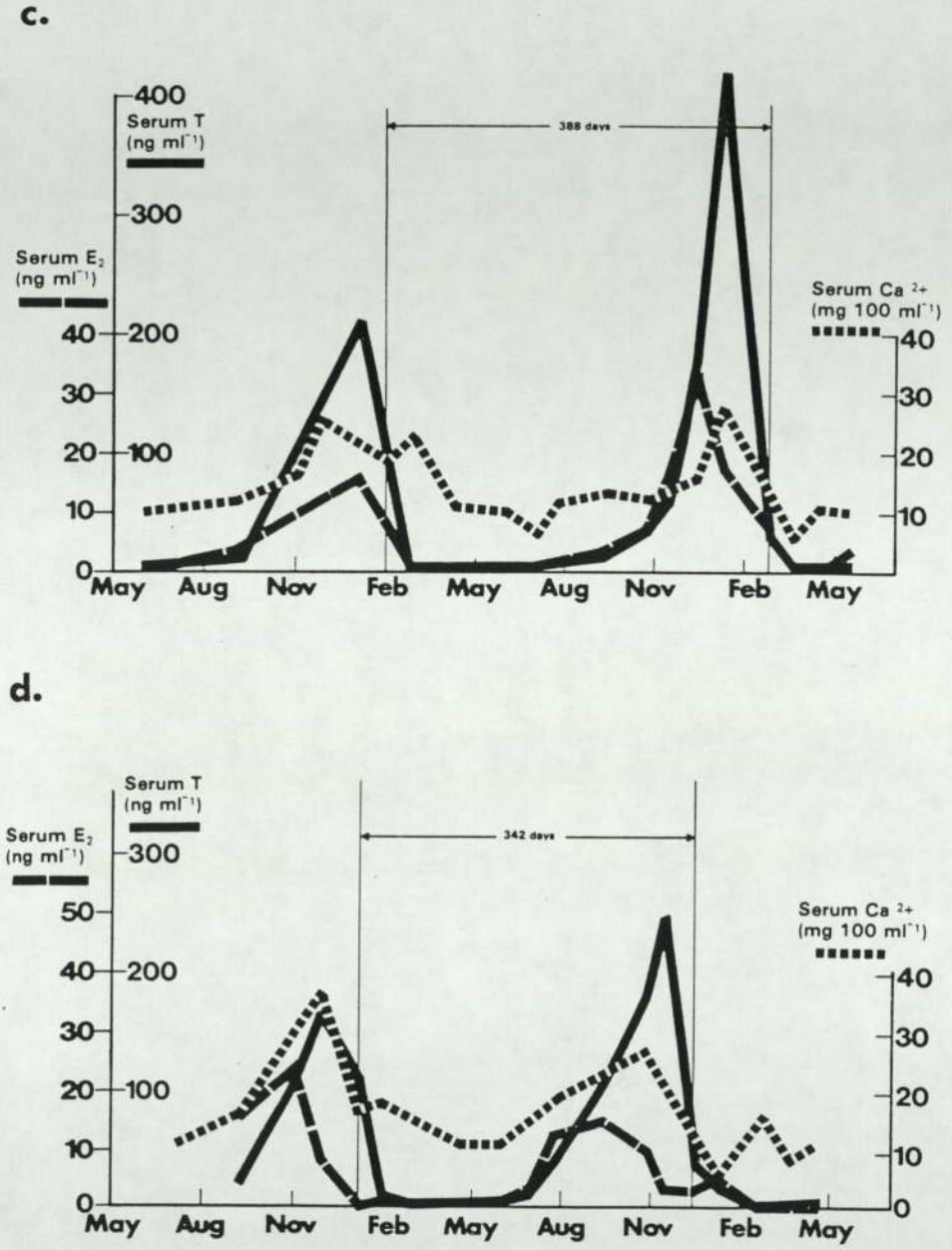
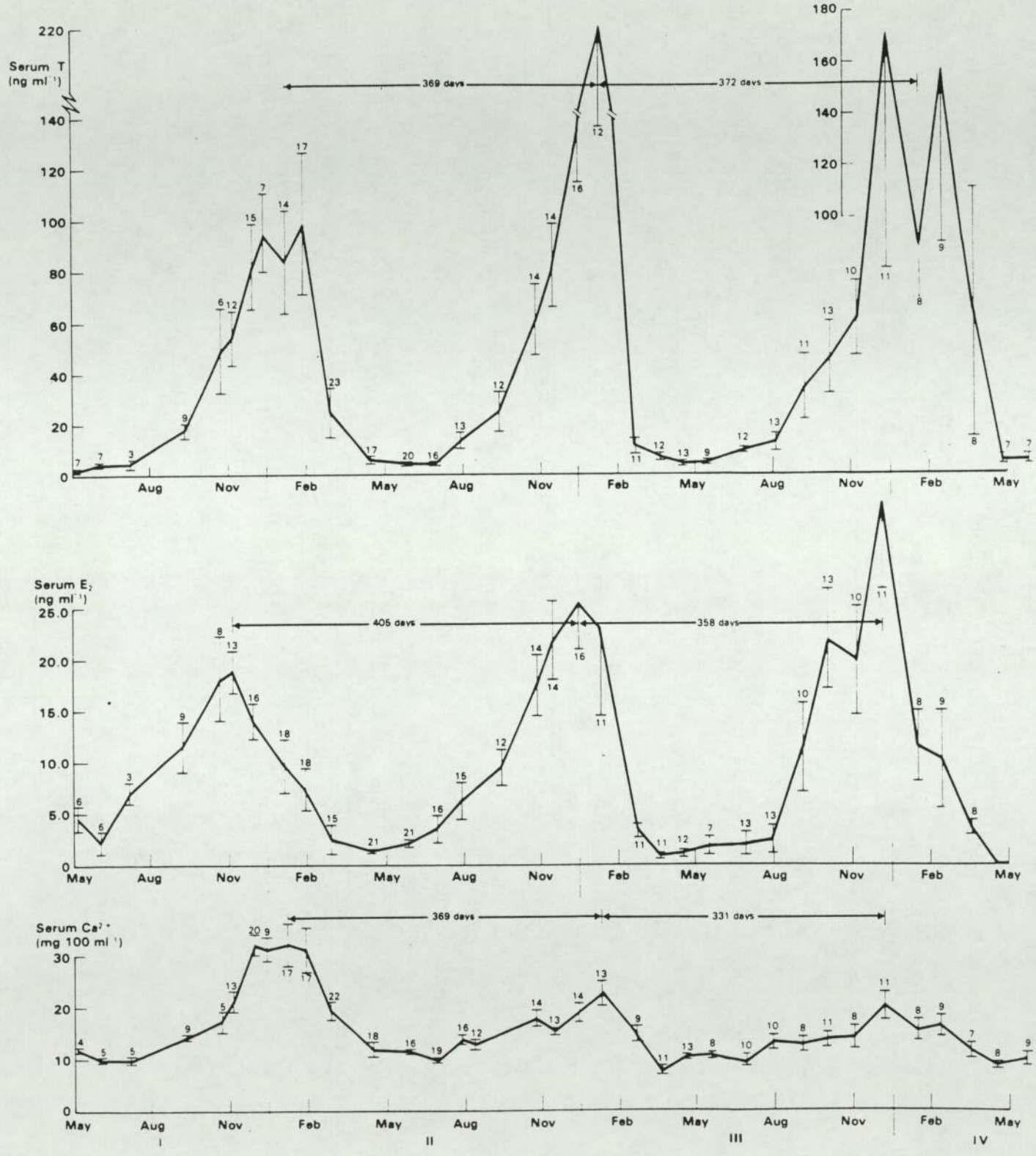



*Fig. 4c, d.*

*Fig. 5.* Effects of constant short days (6L:18D) on the mean (± SEM) serum levels of testosterone (T), oestradiol 17$\beta$ (E2) and calcium over a 3 year period. Period-lengths between the peak heights of these three components are arrowed and the time intervals indicated. Vertical bars are the standard errors of the mean with n indicated above for each.


Serum T
(ng ml⁻¹)
369 days
372 days
Serum E₂
(ng ml⁻¹)
405 days
358 days
Serum Ca²⁺
(mg 100 ml⁻¹)
369 days
331 days
May
Aug
Nov
Feb
May
Aug
Nov
Feb
May
Aug
Nov
Feb
May
I
II
III
IV

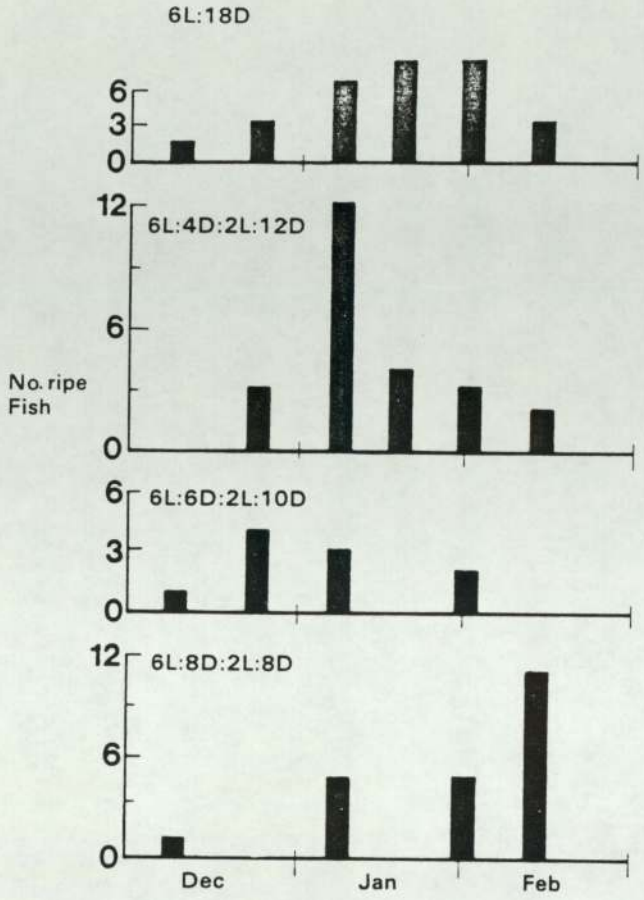



*Fig. 6.* Effects of skeleton photoperiods (6L:4D:2L:12D, 6L:6D:2L:10D and 6L:8D:2L:8D) and constant 6L:18D on the spawning of female rainbow trout. The vertical axis shows the numbers of ripe fish and the horizontal axis the date on which they were stripped.

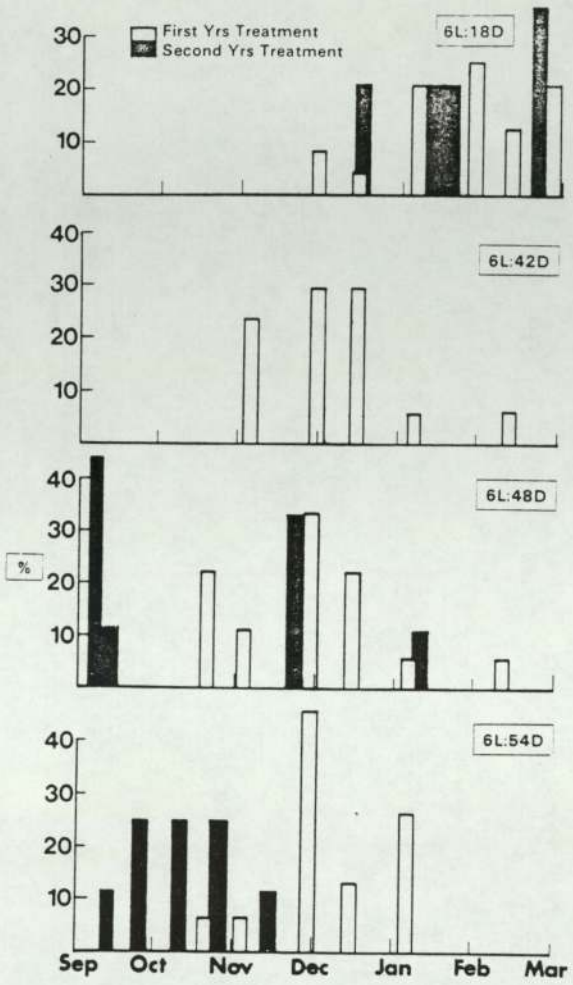



*Fig. 7.* Effects of resonance photoperiods (6L:42D, 6L:48D and 6L:54D) and constant 6L:18D on the spawning of female rainbow trout over a 2 year period. The vertical axis shows the percentage of ripe fish (n = 20 each group) and the horizontal axis the date on which they were stripped. The open histograms are first year's spawning under these photoperiods and the filled ones the second year's. The natural spawning time of this stock is Dec. The 6L:42D treatment was discontinued before the end of the second reproductive cycle.

second spawning peak in mid-October approximately 10 months after the first. The 6L:48D photoperiod produced an erratic spawning period which extended from mid-August until the following January. Unfortunately, persistent malfunction with the 6L:42D electronic timer caused this treatment to be prematurely terminated in mid-summer of the second year.

## Discussion

The present results provide evidence for the involvement of both circadian and circannual processes in the timing and control of reproductive development in the rainbow trout.

Use of both skeleton and resonance light procedures suggests that the differential effects of long and short days on the timing of spawning (Henderson 1963; Whitehead and Bromage 1980; Bromage *et al.* 1984) are mediated by rhythmic rather than hour-glass mechanisms. A skeleton photoperiod given in the form of a 6h main photoperiod followed by a 2h light 'pulse', 6 hours later (6L:6D:2L:10D),

advanced maturation when compared with the other skeleton regimes and also with the fish on light regimes where the 6–8h of light constituted a single photoperiod i.e. a short day. These results suggest that the positioning of the light periods of a skeleton within the 24h light-dark cycle is more important than the total hours of light or dark received. Similar results have been used as evidence of circadian rhythmicity in other photoperiodic organisms (Hamner 1964; Follett 1981). Daily phases of photosensitivity have also been reported in the control of reproduction in the stickleback (Baggerman 1972), the catfish (Sundararaj and Vasal 1976) and the medaka (Chan 1976). In each case the highest levels of maturation were experienced in the groups of fish exposed to skeleton regimes with the minor light pulse 16-18h after the presumptive dawn; this compares with the 12–14h point of maximum stimulation in this investigation. However, in all these studies and also the current one some maturation occurred with the minor photoperiod at other skeleton positions. Possibly, the range of responses recorded under such regimes relate more to the different ways in which dawn and dusk are read from the two photoperiods in skeleton regimes (see Follett 1981), than to any major difference in timing of the photosensitive phase.

In isolation, skeleton procedures are only able to provide supportive evidence of circadian rhythmicity. Ideally, they should be used alongside T-experiments or resonance procedures which are now generally acknowledged to be the most powerful experimental techniques for distinguishing rhythmic from hour-glass mechanisms (Hamner 1964; Lees 1973; Saunders 1981; Follett 1984). In the present study three resonance procedures were used and each of these advanced the timing of spawning when compared with fish under ambient conditions or short days but with only modest differences in spawning time between the procedures. Although spawning would have been expected to be advanced under 6L:48D and 6L:54D and delayed under 6L:42D, as reported by Hamner (1964) in his study of house finches, the same author showed in a later study of the same species that some testicular development also occurred even under supposedly non-stimulatory regimes (Hamner and Enright 1967). One of the difficulties of studies of photoperiodism and spawning in fish is that they do not appear to exhibit "all-or-nothing" responses to alterations in light conditions. Unlike many birds which remain immature under short days (Follett 1981, 1984) maturation in the trout and also the sheep (Howles *et al.* 1982; Robinson *et al.* 1985) is never completely arrested by non-stimulatory light regimes. Whether this is due to the presence of underlying circannual rhythms will be considered later.

Although there were few differences in spawning times between the different groups of fish under the three resonance procedures, results from this experiment strongly indicate that photoperiodic induction is not achieved by measurement of the total length of the light period nor by summation of the numbers of light-dark cycles. Had either mechanism been in operation, then the resonance procedures, which had only an eighth of the total hours of light and half the number of daily cycles of illumination of long day regimes (Table 1), would have been expected to delay spawning. Clearly, neither mechanisms were being used, as the fish under the resonance procedures spawned at similar times to those on long days and up to 2 and 7 months in advance of fish under ambient conditions and constant short days respectively.

Collectively, these data indicate that the photoperiodic response of the trout is mediated by rhythmic rather than hour-glass mechanisms. However, spawning is a yearly rather than a daily event and proper coordination of this process must involve some form of longer-term control. Under ambient lighting the annual cycle of reproduction of the trout is entrained by the seasonally-changing alterations in daylength (Bromage *et al.* 1982a) and it has been suggested that this entrainment may be dependent on seasonal variations in photosensitivity possibly involving a circannual clock (Gwinner 1973, 1981).

Gwinner (1981) proposed that circannual rhythms improve the ability of organisms, living in seasonally-variable environments, to time their seasonal activities. Under constant conditions these rhythms, if truly endogenous, would be expected to free-run with a periodicity approximating to, but

significantly different from a year. Circannual rhythms should also be capable of entrainment by yearly light cycles and should show temperature compensation. More importantly to be fully accepted as circannual these characteristics should be demonstrable over at least two cycles (see Gwinner 1981 for discussion). Not surprisingly, few examples are able to fully satisfy these exacting criteria. Birds and mammals predominate in the list of species in which circannual rhythms have been firmly established with only a few reports of such cycles in fish. Sundararaj *et al.* (1973) described circannual rhythms in ovarian weights of catfish maintained under DD and LL for 33 months. Only the rhythms under LL were said to be significantly different from the natural cycle although the reference, because it is an abstract, includes few details of the experimental results.

Other studies in fish have been less convincing, primarily because the cycles have not been followed for sufficiently long periods of time. Thus Eriksson and Lundqvist (1982) described 10 month cycles of growth and smoltification in a 14 month study of Atlantic salmon maintained under constant temperature and a 12L:12D light regime. Using the same 12L:12D photoperiod and also constant water temperatures and feed levels, Whitehead *et al.* (1978) reported a period length of 49 weeks in the time of spawning of rainbow trout. Further studies from this laboratory showed that exposure of trout to constant long or short days produces spawning times whose period-lengths are similar to but significantly different from the one year length of natural cycles (Whitehead and Bromage 1980; Bromage *et al.* 1984). Unfortunately, none of these may be fully considered as circannual because, the experiments were discontinued soon after the completion of the first spawning cycle.

Longer-term exposures to constant conditions have been investigated in the present work. Considering first the results under LL and 18L:6D; at the end of the first year of treatment, the fish in both regimes spawned 2 months earlier than the controls. Similar results have been reported in other studies of salmonids (Whitehead and Bromage 1980; Bromage *et al.* 1982b, 1984; Takashima and Yamada 1984). However, continued exposure of the fish to the same photoperiod regimes produced further spawnings at 5–6 month intervals. This periodicity of spawning was also confirmed by the cyclical alterations in serum levels of testosterone, oestradiol-17$\beta$ and egg protein (calcium). The timing of these hormonal and calcium changes also showed similar relationships to spawning as those seen in other studies of trout where spawning times were altered using modified seasonal and constant light regimes (Bromage *et al.* 1982a, b, 1984). Following the same fish over 4 successive reproductive cycles showed that the intervals between successive spawnings and between the peaks in serum levels of the two hormones and egg protein were 5–6 months on each occasion.

Spawning at intervals of 4–6 month intervals has also been reported with long-term exposures to long days in the stickleback (Baggerman 1972) and in other studies of trout (Bromage *et al.* 1984; Scott *et al.* 1984). Recently, two independent reports have also been made of strains of rainbow trout which naturally spawn twice each year (Lou *et al.* 1984; H.L. Kincaid 1983, personal communication). However, the relationship between this periodicity and photoperiodic induction by the seasonally-changing ambient light cycle remains obscure. Only Poston and Livingstone 1971), continuing the trial begun by Pyle (1969), have investigated the effects of LL over more than one reproductive cycle. Their 2–3 year study of the brook trout showed some evidence of an additional spawning in male fish during the second year of treatment although firm conclusions regarding the endogenous nature of this cycle could not be made because the investigation was not continued for further spawnings.

Twice-yearly phases of gonadal activity have also been reported in other vertebrate groups. Thus, Benoit *et al.* (1956) described both Spring and Autumnal testicular development in ducks maintained for 4 years under LL and DD. It was suggested that this represented an endogenous rhythm although similar biphasic patterns of gonadal activity occur in ducks and other birds maintained under ambient conditions (Benoit *et al.* 1956; Robinson and Follett 1982; Follett 1984) and also in birds under constant photoperiods (Berthold 1974). Reproductive periodicities of 5 months have also

been reported in lizards maintained on constant long and short photoperiods (Cuellar and Cuellar 1977) again with the suggestion that these were circannual rhythms. Whether these 5–6 months rhythms, and the cycles of similar length reported here after exposure of fish to LL and 18L:6D, should be described as circannual remains controversial. The period length between spawnings under these regimes is outside the range of 7–15 months which has been suggested as the widest acceptable limits for a circannual rhythm (Gwinner 1981). However, the 6 month rhythms were expressed under two different constant photoperiods and their period length maintained over four reproductive cycles. The loss in synchrony of spawning between the different individuals in each of the LL and 18L:6D regimes is also suggestive of "a free-running" rhythm. Possibly, the accepted period limits of circannual cycles should be wider for even the study of Goss (1969) on antler growth in sika deer, which is often cited as a model example of a circannual rhythm, also includes animals with two antler growth cycles per year.

Whereas exposure of trout to continuous and long daylengths initially produced an advancement in maturation and then a spawning rhythm of 5–6 months duration, markedly different results were achieved under constant short days. As a result of the first year's treatment, spawning occurred in February and March, delays of up to 5 months when compared with fish under ambient conditions. Continued exposure of the fish to short days for a further two years, however, produced spawning in February and March in each of the following years (i.e. with a periodicity which approximated to a year if timed from the first spawning under photoperiod treatment). Although the mean period-length of these cycles was not observed to be different from one year, possibly because fish were only examined once a month for ripeness, the periodicity of this rhythm and its variable length in different fish are indicative of a free-running circannual rhythm. The presence of both "semi-circannual" and circannual rhythms in the same species may merely be a reflection of the limited range of constant conditions under which classical circannual rhythms may be fully expressed. Possibly further work using constant photoperiods of different length may be more revealing.

In summary, it would appear that both circadian and longer-term rhythmic mechanisms are involved in the photoperiodic control of salmonid reproduction although, at present, it is not clear how the integration of these two patterns of rhythmicity is achieved. One must conclude from the results of the resonance experiments that calendar time is not derived from circadian time by counts of light-dark cycles i.e. by frequency demultiplication (see Gwinner, 1981). It would, thus, seem probable that the longer-term rhythms involve a seasonal alteration either, in position of the phase of photosensitivity of a circadian rhythm or oscillator, or in the phase relations of a multi-oscillator system. Seasonal changes in daylength, either by their illumination of sensitive phases, or by their effects on the entrainment of different oscillators, would then allow photoperiodic induction and the modulation and control of maturation and spawning.

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# Constant Photoperiod Regimes and the Entrainment of the Annual Cycle of Reproduction in the Female Rainbow Trout (*Salmo gairdneri*)

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The effect of constant photoperiod regimes on the timing of maturation and the associated changes in serum oestradiol-17β ($E_2$), testosterone (T), and total calcium ($Ca^{2+}$, as an index of vitellogenin) were investigated in virgin female rainbow trout (*Salmo gairdneri*). From mid-January four groups of fish were maintained on constant long days (18L:6D) and one group on constant short days (6L:18D; group A). On May 8 the photoperiod of three of the groups on long days was abruptly reduced from 18L:6D to 6L:18D (group B), to 10L:14D (group C), or to 14L:10D (group D); Group E remained on 18L:6D throughout the experiment. The reduction in photoperiod per se appeared more important than the magnitude of the reduction in the advancement of the time of spawning with the first fish in groups B, C, and D spawning on September 3, 13, and 24, respectively; spawning of the remaining fish in each group was spread over 6-week periods. In contrast, spawning in group E began on October 24 and was spread over a period of 12 weeks. Spawning in group A was delayed until the following March. Although a long photoperiod earlier in the year was clearly necessary for the advancement of spawning, as evidenced by the delay in maturation under constant short days, no clear increases in $E_2$, T, and $Ca^{2+}$ were detected until after the reduction in photoperiod. Thereafter, the sequence of changes in serum $E_2$, T, and $Ca^{2+}$ levels was similar in all groups although the timings of these alterations were modified in relation to the time of spawning. Thus, peak levels of the three serum parameters were attained earliest in the year by fish in group B followed by those in groups C, D, E, and A, respectively. Collectively, these data support the hypothesis that photoperiod entrains an endogenous rhythm of maturation rather than having a direct, driving influence on the control of reproduction. 

Reproduction of salmonids is characteristically an annual event with gonadal growth commencing in the spring in preparation for spawning in the autumn or winter months. As with many other vertebrates indigenous to higher latitudes, the annual photocycle has an important influence on the timing of maturation of salmonids (Corson, 1955; Whitehead *et al.*, 1978; MacQuarrie *et al.*, 1979; Bromage *et al.*, 1982a) with temperature probably having only a supplementary role (Henderson, 1963; de Vlaming, 1972). However, the seasonal change in photoperiod is not essential for successful maturation as rainbow trout maintained under constant long or short days from early spring spawn 2–3 months in advance and 2–3 months after fish on an ambient photoperiod, respectively (Bromage *et al.*, 1984). In teleosts the influences of photoperiod on reproduction are mediated by the hormones of the hypothalamic–gonadotropic–gonadal axis with modifications in photoperiod producing clear differences in the patterns of secretion of these hormones (Billard *et al.*, 1978; Whitehead *et al.*, 1978; Bromage *et al.*, 1982a; Elliott *et al.*, 1984).

The photoperiod most stimulatory to gonadal development is a long day (≥16 hr light $day^{-1}$) or a naturally increasing daylength until the spring or early summer followed by a constant short day (≤8 hr light $day^{-1}$). These types of photoperiod regime



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can advance maturation in salmonids by 3–4 months (Henderson, 1963; Shiraishi and Fukuda, 1966; Bromage *et al.*, 1984; Takashima and Yamada, 1984). Experimental results of this kind have led to the hypothesis that rainbow trout and possibly all salmonids have a requirement for different lengths of photoperiod at different stages of the annual reproductive cycle (Whitehead and Bromage, 1980; Bromage *et al.*, 1982b, 1984; Scott and Sumpter, 1983a). In a recent study, rainbow trout maintained under constant photoperiod conditions for periods up to 4 years exhibited a free-running circannual rhythm of reproduction (Duston and Bromage, 1986). Endogenously driven rhythms of this kind have been observed in two other fish (Sundararaj *et al.*, 1982; Baggerman, 1980) and a variety of other vertebrates (Gwinner, 1981; Farner, 1985). Rather than photoperiod directly initiating and modulating the spawning cycle of rainbow trout it may serve only to entrain an endogenous oscillator which ultimately controls maturation.

In an attempt to resolve whether light drives or entrains the maturation cycle the present experiment studies the effects of a constant long photoperiod followed by direct reductions to a range of shorter photoperiods on the timing of maturation of female rainbow trout. In addition to observations on the timing of ovulation, regular measurements of the circulating levels of oestradiol and testosterone and vitellogenin (as calcium) were made to provide information concerning the differences in timing of ovarian recrudescence and the rates of maturation induced by the different photoperiod regimes. If maturation is controlled by the length of photoperiod one would expect there to be a significant difference in the rates of maturation and the times of spawning between the groups. Alternatively, if the direct reduction from a long to a shorter photoperiod acts only as an entraining cue for a circannual rhythm, then the fish in each of the groups would probably be expected to attain maturation at similar times.

## MATERIALS AND METHODS

Five groups of 30 virgin female 2-year-old rainbow trout, raised under ambient photoperiod (51°N) and a constant 8.5–9.0° temperature with a natural spawning time starting in early December, were maintained from January 19 in lightproof 1200-litre tanks under the following photoperiods:

Group A—6L:18D constant (short day);
Group B—18L:6D until May 8 followed by 6L:18D until spawning;
Group C—18L:6D until May 8 followed by 10L:14D until spawning;
Group D—18L:6D until May 8 followed by 14L:10D until spawning; and
Group E—18L:6D constant (long day).

Water temperature was 8.5–9.0°. Light intensity at the water surface was 25 lux. Fish were fed 0.5% body wt $day^{-1}$ with a commercial trout diet. All fish were checked at monthly intervals to assess their state of maturity, and fortnightly once spawning had commenced. Blood samples were taken at approximately monthly intervals from a random sample of animals in each group. After centrifugation the serum was stored at −20° until analysis for hormones and vitellogenin (as calcium). The eggs from mature fish were collected and after fertilisation and water-hardening their diameters were measured.

### *Calcium (Vitellogenin) Measurement*

Total serum calcium levels vary concomitantly with the amount of vitellogenin in the serum (Elliott *et al.*, 1984). Levels were assayed using a Corning (Model 940) calcium analyser: the inter- and intraassay coefficients of variation were 1.27 and 0.02%, respectively.

### *Steroid Radioimmunoassays*

*Procedure.* Steroid hormones were extracted by adding 2 ml ethyl acetate to 100-μl serum samples, mixed for 2 hr, and then centrifuged (2000 rpm, 10 min). This method extracts 90–100% of the steroid from the serum and therefore a recovery step was not included in the routine assay procedure. Extracted aliquots (100 μl for the oestradiol-17β (E2) assay and 50 μl for the testosterone (T) assay) were transferred to glass assay tubes in duplicate with a 0- to 1000-pg range of standards. All tubes were then dried in a vacuum oven at not more than 40°. Then 100 μl of $^{3}H$-labeled steroid (~20.000 cpm; Amersham) and 100 μl of the specific antiserum (Steranti Research Ltd., St. Albans, Herts), each made up in 0.1 *M* phosphate, were added to each tube, vortex mixed, and allowed to equilibrate overnight (4°). A chilled dextran-coated

charcoal suspension (500 μl) was added to each tube, which was incubated for 10 min (4°) and then centrifuged at 2000 rpm for 10 min. Then 400 μl of the supernatent was transferred to a glass vial containing 9 ml of scintillation fluid (Optiphase Safe, LKB) and counted for 5 min (Packard Tri-Carb 2660 scintillation counter).

*Sensitivity*. For both the E2 and T assays the smallest amount of steroid statistically distinguishable from zero was 8 pg per tube.

*Accuracy*. Known amounts of unlabeled hormone were added to serum samples overnight (4°) and then assayed using the above procedure. The correlation between added and detected indicated that both oestradiol 17β ($r^2 = 0.998$, $P \leq 0.001$) and testosterone ($r^2 = 0.987$, $P \leq 0.001$) assays were highly accurate.

*Specificity*. The specificities of the E2 and T antisera were assessed by determining their cross reactivities with a number of structurally similar steroids. Cross reactivity is expressed as the diminution in the proportion of bound label produced by 100 pg of steroid relative to that of 100 pg of oestradiol-17β or testosterone depending on the antiserum being analysed (Table 1).

*Precision*. The inter- and intraassay coefficients of variation were 9.5 and 6.2% (oestradiol-17β), and 6.6 and 5.5% (testosterone), respectively.

*Statistical Analyses*. One-way analysis of variance (ANOVA) was used to study the statistical relationship between the profiles. Because many of the monthly samples were of unequal size the means were compared using the equation

$$t \text{ value} = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{s^2\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

where $n_1$ and $n_2$ are the number of observations in each sample and $\bar{x}_1$ and $\bar{x}_2$ the respective mean values.

TABLE 1
CROSS REACTIVITY OF OESTRADIOL 17β AND TESTOSTERONE ANTISERA WITH A NUMBER OF STRUCTURALLY SIMILAR STEROIDS

| | Oestradiol 17β antiserum (%) | Testosterone antiserum (%) |
|---|---|---|
| Oestradiol-17β | 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-20β-dihydroprogesterone | 1.0 | 1.66 |
| 17α-hydroxyprogesterone | 1.0 | 1.0 |
| Pregnenolone | 1.0 | 3.3 |
| Cortisol | 1.0 | 1.0 |

$s^2$ equals the error mean square value derived from the ANOVA table. Full details are given in Snedecor and Cochran (1967). Differences in spawning time between the groups were tested by a Student's $t$ test or by using $d$ and treating it as $t$ if the variances were statistically dissimilar (Bailey, 1959).

## RESULTS

The effects of the photoperiod treatments on the timing of spawning and the serum levels of oestradiol-17β, testosterone, and vitellogenin (as calcium) are described below.

*Spawning*. The time of spawning of individual fish is illustrated in Fig. 1. In the groups in which the photoperiod was reduced from 18L:6D to 6L:18D (group B), to 10L:14D (group C), and to 14L:10D (group D) on May 8, the first fish commenced spawning on September 3, 13, and 24, respectively. Subsequently, the remaining fish in each of the groups spawned within 6-week periods. Unfortunately, only four fish from group D survived to spawning as a result of an interruption of the water supply to this tank on September 20; examination of the ovaries of the dead fish indicated that they all were approximately 2 weeks from ovulation. Under the constant 18L:6D regime (group E), the first fish spawned on October 24 but the last fish in this group did not spawn until 12 weeks later. Fish in group A (6L:18D) commenced spawning on March 1, the following spring, approximately 5 months after those in group B. Excluding group D, the differences in the mean spawning times between groups were statistically significant (group B vs group C, $P \leq 0.025$; for the remainder, $P \leq 0.001$).

*Oestradiol-17β*. Between the start of the experiment and the reductions in photoperiod on May 8 there were no statistically significant increases in serum oestradiol-17β levels in any of the groups (Fig. 2). On May 15 groups B (18L:6D → 6L:18D) and C (18L:6D → 10L:14D) had significantly higher ($P \leq 0.001$) mean oestradiol-17β

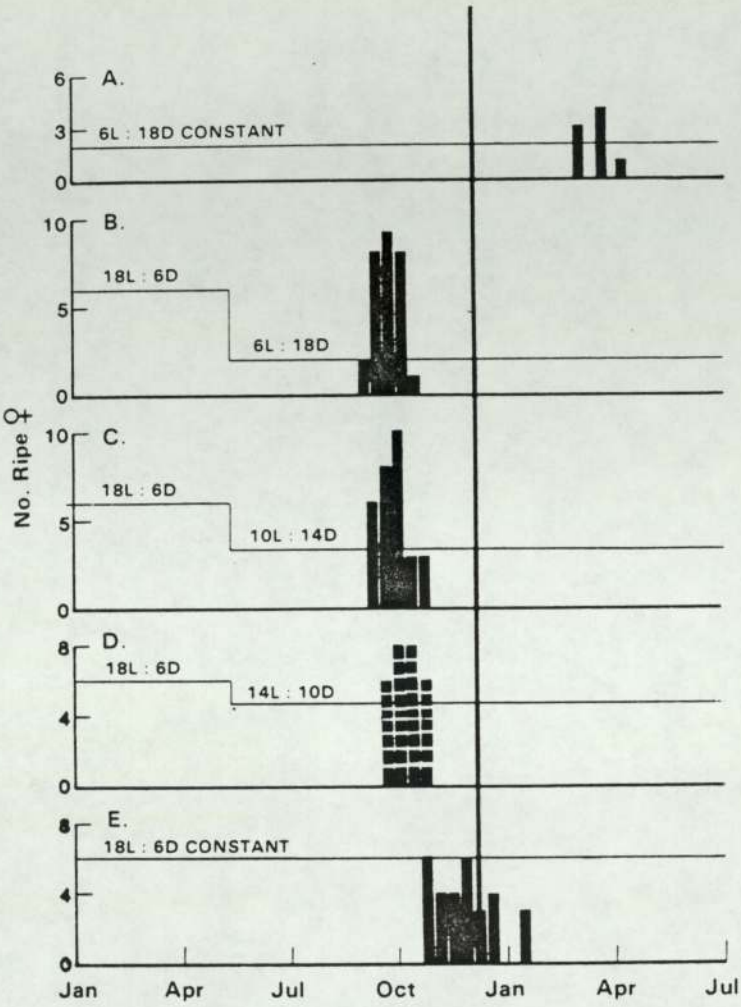


FIG. 1. The effect of the five photoperiod regimes (groups A–E) on the timing of maturation of female rainbow trout. Histograms illustrate the number of mature females (No. ripe ♀) at each sampling time. The broken histograms in group D represent an estimate of the spawning time (see Results). The vertical line running through all five histograms indicates the onset of spawning of rainbow trout of the same stock maintained under natural photoperiod. The photoperiod regime in each treatment is indicated by the horizontal lines. The vertical lines on the *x* axis refer to the first day of the month as labeled.

levels than the other three groups. Between the May and June samples there were significant increases in the mean oestradiol-17β levels of groups B ($P \leq 0.001$), C ($P \leq 0.01$), *D* (18L:6D → 14L:10D; $P \leq 0.001$), and E (18L:6D constant, $P < 0.05$). Groups B, C, and D exhibited similar rates of increase in oestradiol-17β titres reaching mean peak levels of 30–40 ng $ml^{-1}$ in late July, early September, and mid-September (estimate), respectively. By comparison the rate of increase of oestradiol-17β in group E was slower and maximal levels were not reached until late September. The oestradiol-17β levels of fish under constant 6L:18D (group A) remained at basal values until significant increases ($P \leq 0.05$) were detected between the September and Oc-

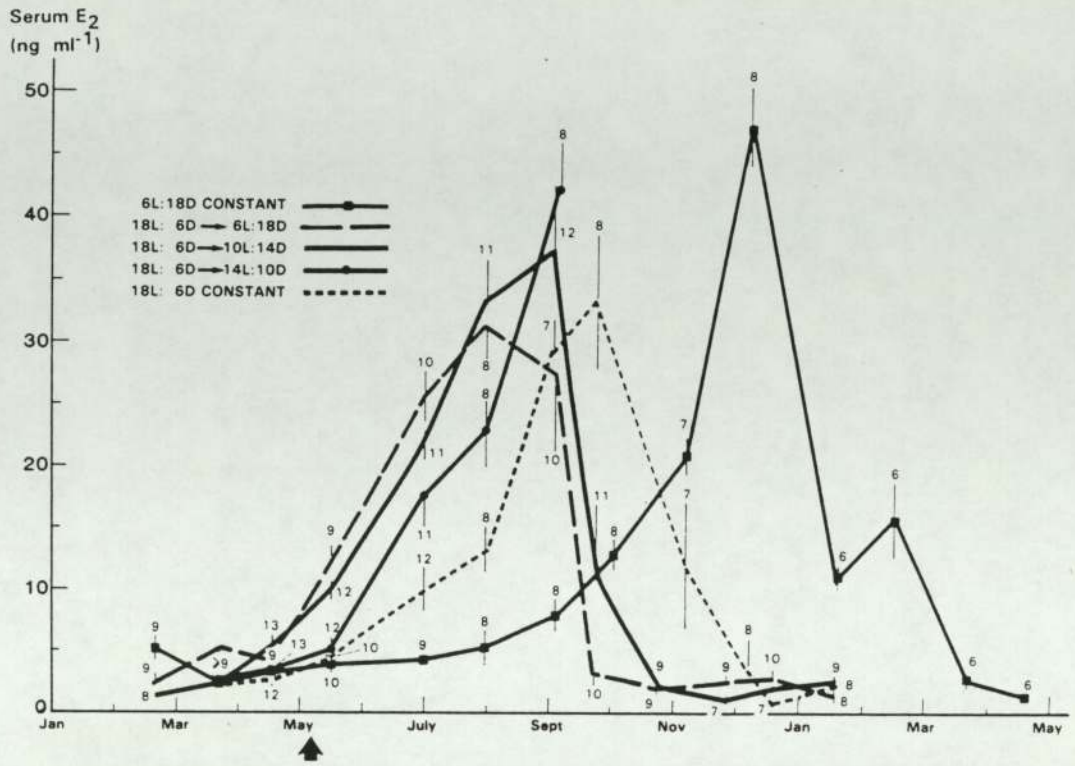


FIG. 2. The sequential changes in the mean levels of serum oestradiol-17β ($E_2$, ng $ml^{-1}$) in female rainbow trout maintained under the five photoperiod regimes: 6L:18D constant (group A), 18L:6D → 6L:18D (group B), 18L:6D → 10L:14D (group C), 18L:6D → 14L:10D (group D), and 18L:6D constant (group E). The integer above each coordinate indicates the number of fish sampled ($n$) with the vertical bars representing ± one standard error of the mean. The arrow (↑) indicates the time of the reduction in photoperiod (groups B, C, and D only). The vertical lines on the $x$ axis refer to the first day of the month as labeled.

tober samples. Thereafter, the oestradiol-17β levels increased rapidly to reach a peak of over 45 ng $ml^{-1}$ in early December. This level was significantly higher than the oestradiol-17β peak in group B ($P \leq 0.05$) but was not different from the corresponding values of the other groups. In all groups the oestradiol-17β levels returned to basal values within 3 months of attaining their highest levels.

*Testosterone*. Serum testosterone levels remained basal in all groups until the middle of May (Fig. 3). By late June the testosterone levels of group B were significantly higher ($P \leq 0.05$) than all the other treatments. By late June testosterone levels in groups C and D were significantly higher ($P \leq 0.01$) than in group A, and those in group C were significantly higher ($P \leq 0.01$) than in group E. Testosterone levels in groups B and C increased at similar rates and both reached peak levels of 250–300 ng $ml^{-1}$ in early September. The testosterone levels of fish in group D exhibited a similar profile to those in groups B and C but it was delayed by approximately 2–3 weeks. The constant 18L:6D treatment did not produce a significant increase in testosterone until August ($P \leq 0.05$) with peak levels of 250–300 ng $ml^{-1}$ being attained in early November. The testosterone levels of fish in group A (6L:18D), which remained basal until October, were significantly increased ($P \leq 0.001$) between the

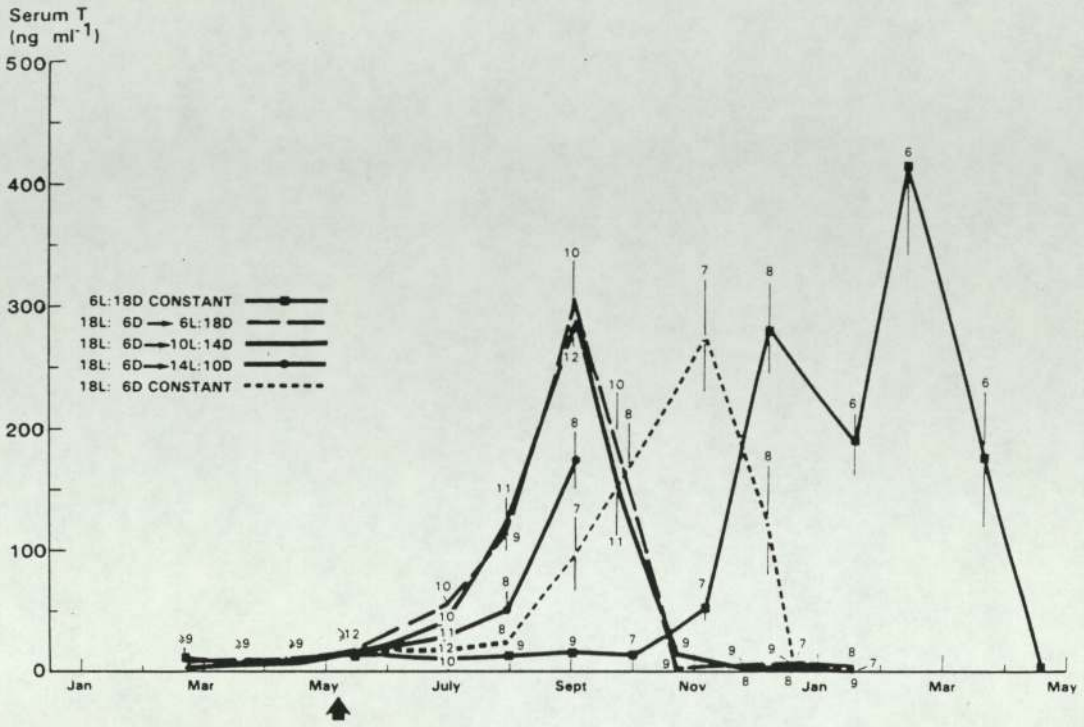


FIG. 3. The sequential changes in the mean levels of serum testosterone (T, ng ml$^{-1}$) in female rainbow trout maintained under the five photoperiod regimes: 6L:18D constant (group A), 18L:6D → 6L:18D (group B), 18L:6D → 10L:14D (group C), 18L:6D → 14L:10D (group D), and 18L:6D constant (group E). The integer above each coordinate indicates the number of fish sampled ($n$) with the vertical bars representing ± one standard error of the mean. The arrow ( ↑ ) indicates the time of reduction in photoperiod (groups B, C, and D only). The vertical lines on the $x$ axis refer to the first day of the month as labeled.

November and December samples and reached peak levels of over 400 ng ml$^{-1}$ in early February. There was no significant difference in the peak testosterone levels attained by the five different groups which in each case was reached just prior to ovulation before returning to basal values within 2 months.

*Total serum calcium.* The calcium levels in all groups remained at basal values (~10 mg 100 ml$^{-1}$ serum) until the middle of May (Fig. 4). Groups B and C showed significant increases ($P \leq 0.05$) in calcium levels between the May and July samples. These two groups exhibited almost identical profiles of total calcium levels, both reaching peak levels of approximately 35 mg 100 ml$^{-1}$ in late September before falling to basal levels in December. The calcium levels of group D increased significantly ($P \leq 0.05$) between May and July although the rate of increase was slower than in groups B and C with the levels in early September being only half those of groups B and C. The calcium levels of fish held on a constant long day (group E) became significantly elevated above basal during August ($P \leq 0.05$) to reach a mean peak level of 29 mg 100 ml$^{-1}$ in December. The calcium levels of the fish maintained on constant short days (group A) remained at basal levels until October when they rose significantly ($P \leq 0.01$) to a level of approximately 20 mg 100 ml$^{-1}$. This concentration, which was significantly lower ($P \leq 0.05$) than the peak $Ca^{2+}$ level in groups B

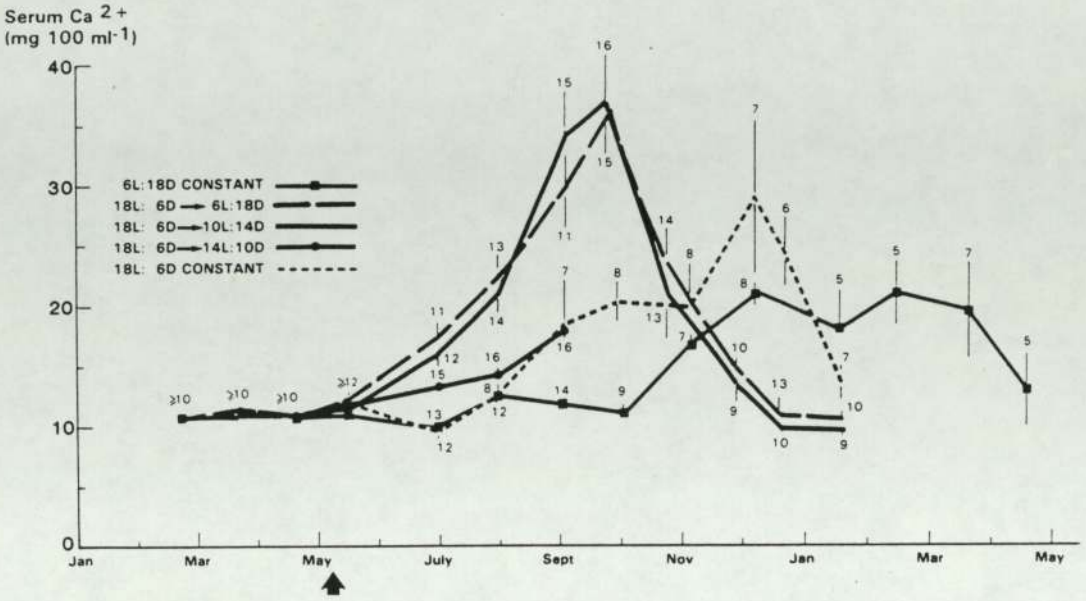


FIG. 4. The sequential changes in the total serum calcium ($Ca^{2+}$, mg 100 $ml^{-1}$) in female rainbow trout maintained under the five photoperiod regimes: 6L:18D constant (group A), 18L:6D → 6L:18D (group B), 18L:6D → 10L:14D (group C), 18L:6D → 14L:10D (group D), and 18L:6D constant (group E). The integer above each coordinate indicates the number of fish sampled ($n$) with the vertical bars representing ± one standard error of the mean. The arrow (↑) indicates the time of reduction in photoperiod (groups B, C, and D only). The vertical lines on the $x$ axis refer to the first day of the month as labeled.

and C, was maintained for the months of November through to April before falling toward basal.

## DISCUSSION

The results clearly show that the abrupt reduction in photoperiod provided an important cue in the advancement of the time of spawning in the rainbow trout. Similar photoperiod-induced changes in the timing of maturation of salmonids have been observed by other groups (Henderson, 1963; Shiraishi and Fukuda, 1966; Bromage *et al.*, 1984; Takashima and Yamada, 1984). The present work also shows that the magnitude of the reduction in photoperiod had only a minor or supplementary role in modifying the time of spawning of the rainbow trout. Similar results have been observed in the masu salmon (Takashima and Yamada, 1984), suggesting that all salmonids may respond in a similar way to changes in daylength. The reduction in photoperiod had the additional effect of synchronising the time of spawning of individual animals within each treatment, so that the period of time between the spawning of the first and last fish was only 6 weeks; this is similar to rainbow trout maintained under ambient conditions (Kato, 1973). In contrast, the corresponding spawning period of the fish under constant long days was 12 weeks.

In general the effects of photoperiod on the times of spawning are confirmed by the changes in the levels of serum oestradiol-17β, testosterone and total calcium. Within a week of the reduction in photoperiod on May 8 increases were detected in serum oestradiol-17β levels. Figure 2 shows clearly that the increases in the oestradiol levels were more pronounced the greater the reduction in photoperiod. By late June a clear gradation in the values of serum oestradiol-17β was established between the

five experimental groups. The alterations in levels of oestradiol-17β were followed by increases in testosterone and total calcium. Over the course of maturation the relationship between the changes in these three parameters within each group was similar to those observed in previous studies (Scott *et al.*, 1980; Scott and Sumpter, 1983b; Elliott *et al.*, 1984). However, there were differences between the experimental groups in the timing of these changes which resulted in the different spawning times. The oestradiol-17β and testosterone levels in each group reached peak levels approximately 1 month and 1 week before ovulation, respectively. The slightly broader and flatter profiles of the serum components of the fish under constant 18L:6D treatment were almost certainly due to the desynchronisation of maturation and spawning of individual animals in this group rather than to any effect of photoperiod on serum hormone levels. Total calcium levels peaked around the time of spawning but did not return to basal values until approximately 3 months after ovulation. The group that spawned last (group A) had a significantly higher mean oestradiol-17β peak ($P \leq 0.05$) and a significantly lower mean total calcium peak ($P \leq 0.05$) compared with the September spawning group (B). Similar observations have also been made by Elliott *et al.*, (1984) and it is suggested that these differences may be related to the increase in egg size which invariably occurs when the time of spawning is delayed. The fish spawning in September and October had oocyte diameters that were at least 0.5 mm smaller than those produced by fish spawning 5 months later in the following March. The relationship between the time of spawning and differences in egg size will be reported more fully elsewhere.

Although experimental changes in daylength can significantly alter the timing of maturation, the ability of the trout in groups A and E to spawn successfully under constant short and long days, respectively, indicates that changes in the environmental photoperiod are not essential for maturation. This observation confirms the findings of a recent study of Duston and Bromage (1986) in which a group of female rainbow trout maintained under constant photoperiod and temperature for over 4 years exhibited an undamped free-running rhythm of maturation. Consequently, it is likely that the annual cycle of maturation and spawning in the rainbow trout is controlled by endogenous circannual mechanisms. There is evidence that circannual systems are also involved in the control of reproduction of the catfish (Sundararaj *et al.*, 1982) and a number of other vertebrates including the golden-mantled ground squirrel (Licht *et al.*, 1982), the sika deer (Goss, 1969a), the sheep (Howles *et al.*, 1982; Robinson *et al.*, 1985), a lizard (Cuellar, 1981), and several species of birds (Farner, 1985). An important feature of circannual rhythms in certain species is their ability to be entrained by the seasonal changes in photoperiod. Under natural conditions the annual photocycle synchronises the maturation cycle with the seasonal changes in the environment, thus ensuring that offspring are produced at a time of year when their chances of survival are at an optimum. The entraining influence of photoperiod is such that the rhythms of maturation of certain species can be synchronised to photocycles with period lengths shorter or longer than 1 year (Goss, 1969b; Gwinner, 1977). Several studies have shown that such entrainment also occurs in salmonid fish (Wagner, 1974; Whitehead *et al.*, 1978; MacQuarrie, 1979; Bromage *et al.*, 1982a; Bromage and Duston, 1986). Generally, alterations in the period length of the entraining photoperiod lead to changes in the phase relationship between the light cycle and the rhythm of maturation. This behaviour is in agreement with general oscillator theory (Aschoff, 1960, 1980; Aschoff and Pohl, 1978), and although these ideas have been developed

primarily from studies on circadian systems, they support the proposition that maturation and spawning in the rainbow trout are controlled by an endogenous circannual oscillator(s).

In addition to entrainment by seasonal changes in daylength an important finding of the present study is that the phase of the rhythm of maturation can be advanced by an abrupt reduction from a constant long to a shorter photoperiod. Abrupt changes between constant long and short photoperiods have also been shown to entrain the reproductive cycles of other seasonal-breeding vertebrates (Wolfson, 1959; Lincoln, 1978). The similarity in the times of spawning among the three treatment groups which had received constant long and then different lengths of short days indicates that the reduction in the photoperiod appears to be more important than the magnitude of the reduction in advancing the phase of the rhythm and hence the time of spawning. In contrast, exposure of rainbow trout to a short followed by a long photoperiod has been shown to delay maturation (Bromage *et al.*, 1984; Bromage and Duston, 1986), presumably by a phase delay of the rhythm. Collectively, these results indicate that it is the direction of the change in photoperiod which dictates the nature of the phase response.

Previous studies have indicated the involvement of circadian as well as circannual processes in the entrainment of reproduction in the rainbow trout (Duston and Bromage, 1986) and other fish (Chan, 1976; Sundararaj and Vasal, 1976; Baggerman, 1980). Examination of the hormone profiles revealed that there there was a relationship between the magnitude of the reduction in photoperiod and the timing of the changes in serum oestradiol-17β, testosterone, and calcium, resulting in small differences in the times of spawning between the respective groups. It is possible that the differences in the times of spawning were due either to the magnitude of the reduction in photoperiod or to the lengths of short day experienced by groups B, C, and D differentially influencing a circadian rhythm of photosensitivity.

As well as altering the timing of maturation the abrupt reduction in photoperiod had the effect of synchronising the spawning of the individual animals in each treatment so that the spawning period for each of these groups was confined to 6 weeks. In contrast, the spawning period of the fish maintained under constant 18L:6D lasted for 12 weeks. A desynchronisation of spawning time was also reported by Bromage *et al.* (1984) in an earlier study of rainbow trout maintained under constant photoperiods. The similarity of these results to the behaviour of circannual rhythms in other vertebrates (Pengelley and Asmundson, 1974) suggests that this loss of synchrony is due to the free-running behaviour of the endogenous rhythm which controls maturation.

Previously, it had been suggested that rainbow trout are "long day" animals in that the presence of long days early in the maturation cycle is necessary to advance spawning (Whitehead and Bromage, 1980; Bromage *et al.*, 1982a). This proposition was supported by other studies on rainbow trout in which increases in the levels of circulating gonadotropic and steroid hormones and vitellogenin were detected under increasing or long daylengths (Bromage *et al.*, 1982b; Scott and Sumpter, 1983b; Elliott *et al.*, 1984; Sumpter *et al.*, 1984). In light of these results a surprising feature of the current work is the absence of significant increases in levels of oestradiol-17β, testosterone, or calcium under the constant long days which prevailed from January until early May. Notwithstanding the relative sensitivities of the assays, possibly this is a reflection of the time lag that is sometimes observed between an environmental cue and a measurable hormonal response. For example, in the Suffolk ewe delays of up to 50 days

have been reported between the time of exposure of animals to a stimulatory photoperiod and the consequent increases in circulating luteinizing hormone levels (Legan and Karsch, 1980). It is also possible that seasonally changing light cycles are more effective than constant photoperiods in entraining endogenous rhythms of maturation. This was also suggested by Clarke *et al.* (1978) in a study of Pacific salmon. However, Whitehead *et al.* (1980) and Lundqvist (1980) both showed that accelerated seasonal light cycles and constant photoperiod regimes produce similar advancements in the timing of spawning.

A more likely explanation for the failure of the long days from January to produce a more immediate hormonal change is that the circannual rhythm may show seasonal variation in responsiveness to photoperiodic entrainment. A periodically changing sensitivity to the stimulus provided by a zeitgeber is also a feature of circadian systems, where it is often described in the form of a phase response curve (Pittendrigh and Minis, 1964; Elliott, 1981). The difference in response of autumn and winter spawning strains of rainbow trout when subjected to constant long days in March (Scott and Sumpter, 1983a) and the circannual variation in the responsiveness of sticklebacks to specific photoperiods (Baggerman, 1980) may be reflections of this variation. Further work is currently in progress which aims to establish the presence of seasonal alterations in photosensitivity in the rainbow trout.

In conclusion the results presented in this paper support the hypothesis that photoperiod serves to entrain an endogenous rhythm of maturation rather than having a direct driving influence on reproduction of the rainbow trout. It is conceivable that rainbow trout and other vertebrates (Aschoff, 1980) use both endogenous circadian and circannual oscillators to maintain temporal orientation in relation to the Earth's rotation about its axis and also to its orbit around the Sun.

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