PHOTOPERIODIC CONTROL OF REPRODUCTION AND PATTERNS OF MELATONIN SECRETION IN THE RAINBOW TROUT, ONCORHYNCHUS MYKISS.

A thesis submitted to the University of Stirling
for the degree of Doctor of Philosophy

by

Clive Frederick Randall

Institute of Aquaculture, University of Stirling, Stirling, Scotland

February 1992

Declaration

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged the work described in this thesis has been conducted independently and has not been submitted for any other degree.

Signature of candidate: C. Randall

Signature of supervisor: N. Bromage

Date: 28/2/92

Abstract

Reproduction in salmonids is an annual event with spawning confined to a brief (typically 6 week) period each year. The reproductive cycle appears to be controlled by an endogenous circannual rhythm or 'clock' which, under natural conditions, is entrained by the seasonal changes in daylength. This thesis investigates the mechanisms by which photoperiod entrains the circannual clock which, it is proposed, controls maturation in the rainbow trout, Oncorhynchus mykiss.

Abrupt changes in photoperiod can either advance or delay spawning and the timing of changes in serum calcium, oestradiol-17β and testosterone which accompany maturation in the female rainbow trout. These effects can be interpreted as corrective phase advances or phase delays of a circannual clock. 'Long' photoperiods of between 12 and 22 hours applied in January, followed by shorter photoperiods of between 3.5 and 13.5 hours from May, were equally effective for the advancement of maturation in December-spawning female rainbow trout. Maturation was also advanced, though to a lesser extent, in fish which remained on typical winter photoperiods (8.5 or 10 hours), provided they received a decrease to an even shorter photoperiod prior to the summer solstice. In contrast, maturation was delayed in fish maintained under a constant winter photoperiod (8.5 hours), and these fish also exhibited a desynchronization of spawning times characteristic of endogenous circannual rhythms in free-run. Collectively, these results indicate that direction of change of daylength is the feature of the photoperiodic signal responsible for the entrainment of the endogenous circannual clock; the same photoperiod may be interpreted as 'long' or 'short' providing it is longer or shorter than that to which the fish have been previously exposed. The concept of a rigid 'critical' daylength for reproductive function is therefore untenable in the rainbow trout.

The timing of the increase to a 'long' photoperiod was also an important determinant of spawning time; maturation occurred in sequence in December-spawning female rainbow trout maintained on constant 'long' days from January and February, and in fish exposed to 'long' days from December, January and February, followed by 'short' days in May.

Maturation can also be advanced or delayed by exposing rainbow trout to short (≤2 months) periods of continuous light at different phases of the reproductive cycle. These effects can be described in the form of a partial phase-response curve. The proportion of fish responding to short periods of continuous light was dependent on both the duration of the light period, and, most importantly, its position in relation to the phase of the reproductive cycle. A high proportion (≥65%) of fish responded with an advance in spawning time only when the period of exposure to continuous light occurred close to the preceding natural breeding season. The minimum period of exposure capable of advancing maturation in a majority (≥60%) of rainbow trout was 1 month. In 3 consecutive experiments over 90% of female rainbow trout exposed to continuous light for 2 months from January to March spawned again in a 6-week period in July and August, approximately 5 months in advance of their natural spawning period. Exposure of rainbow trout to short periods of continuous light therefore provides a simple, cheap and predictable method for the production of out-of-season eggs on commercial fish farms.

Patterns of melatonin secretion in the rainbow trout accurately reflected the prevailing photoperiod, with levels elevated for the duration of darkness under both long (16L:8D or 18L:6D) and short (8L:16D or 6L:18D) daylengths. Distinct diurnal rhythms in circulating melatonin were also detected in the Atlantic salmon, Salmo salar, and Nile tilapia, Oreochromis niloticus. Melatonin production in the rainbow trout is not under endogenous circadian control; changes in melatonin levels always coincided with the light to dark or dark to light transitions, and the melatonin rhythm did not persist in constant darkness. Additionally, the melatonin rhythm immediately re-adjusted to the new photoperiod when rainbow trout were transferred from long (18L:6D) to short (6L:18D) days. These results indicate that melatonin production in the rainbow trout is a direct response to darkness. Although the seasonally-changing patterns of melatonin secretion clearly provide the rainbow trout with accurate information on both daily and calendar time the results of experiments designed to test the hypothesis that melatonin conveys photic information to the reproductive axis were inconclusive.

Acknowledgements

I am indebted firstly to my supervisor, Dr. Niall Bromage, for his advice and co-operation throughout the duration of this work. I would also like to thank all past and present staff and students with whom I have had the pleasure of working especially Jim Duston, John Jones, Briony Davies, Iain McEwan, and, in particular, Mark Thrush, who on several occasions helped with overnight sampling experiments which would otherwise have been impossible.

The initial research for this thesis was conducted at the University of Aston in Birmingham and I am grateful to Kevin Hughes for his assistance with maintenance of experimental fish during this period. I would also like to thank Niall Auchinachie and Willie Yeomans for maintenance of experimental fish during my fieldwork periods away from Stirling.

My thanks are extended to the proprietors and employees of Burwarton, Rainbow (Longbridge Deverill) and Avon Springs (Pewsey) trout farms, especially Nick Trotter, Chris Woods, John Symes and Bill Hope, for their co-operation and provision of experimental fish and facilities.

I would like to acknowledge that experiment 1 in chapter 3 was conducted in collaboration with Jim Duston and has been partially reported elsewhere (Duston, 1987). Experiments 5 and 6 in chapter 4 were collaborations with Mark Thrush and Iain McEwan respectively.

Finally, I would like to thank the Natural Environment Research Council for provision of a research studentship.

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CHAPTER ONE

GENERAL INTRODUCTION

The rainbow trout is indigenous to western North America but, with the exception of Antarctica, is now distributed world-wide (MacCrimmon, 1971). First described as Salmo gairdneri by Richardson in 1836 the rainbow trout is currently considered to be more closely related to the Pacific salmon (Oncorhynchus species) than to the Atlantic and Eurasian Salmo species (which include the Atlantic salmon, Salmo salar, and the brown trout, Salmo trutta), and was recently re-classified as Oncorhynchus mykiss (family: Salmonidae) by the American Fisheries Society (Kendall, 1988). Introduced into Europe in the late nineteenth century it is prized for both its sporting and eating qualities and is currently rivalled only by the Atlantic salmon as the most popular farmed fish in the United Kingdom.

1.1 Seasonal breeding

In common with many other organisms inhabiting temperate and polar latitudes the rainbow trout is a seasonal breeder. Described by Lincoln and Short (1980) as nature's contraceptive, seasonal breeding ensures that reproduction occurs at the time of year when the local environmental factors which dictate survival of both parents and offspring are optimal. These 'ultimate' factors (Baker, 1938) include temperature, rainfall and, most importantly, food availability. However, the time required for gonadal and embryonic development means that breeding cannot be initiated instantaneously when ultimate factors become optimal. Reproduction is therefore timed with reference to earlier 'proximate' environmental cues (Baker, 1938), which enable the organism to predict the time of year when ultimate factors will be most advantageous. The most reliable and 'noise-free' information on the time of year is provided by the seasonal changes in daylength which result from the tilted axis of the earth relative to the sun, and which become more pronounced as the distance from the equator increases (Figure 1.1). It is therefore not surprising that many organisms indigenous to middle and higher latitudes have evolved the ability to utilise daylength to time seasonal functions such as reproduction.

The importance of daylength in the timing of seasonal reproductive events was first demonstrated experimentally in a variety of plants by Garner and Allard (1920) who

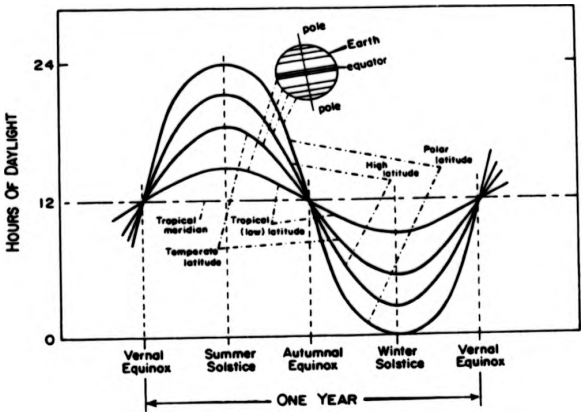


Figure 1.1: Seasonal changes in daylength at various latitudes. Note that the seasonal variation in daylength is slight at lower (tropical) latitudes and becomes more pronounced as the distance from the equator (north or south) increases. Daylength is constant at the tropical meridian (from Giese, 1976).

introduced the terms 'photoperiod' (daylength) and 'photoperiodism' (the physiological response of an organism to the seasonal changes in daylength). Subsequently, Marcovitch's (1924) work with aphids provided the first experimental evidence for photoperiodism in insects. Rowan's (1925, 1926) observations that artificially increased daylengths induced precocious gonadal maturation in the slate-coloured junco (snow bird), Junco hyemalis, whereas a shortening photoperiod caused gonadal regression, heralded the beginning of experimental work on photoperiodism in vertebrates. Similar phenomena were soon reported in mammals (Baker and Ranson, 1932; Bissonette, 1932), and Hoover and Hubbard (1937), working with the brook trout, Salvelinus fontinalis, were the first to report that artificial photoperiods could be used to manipulate the spawning time of fish. Although, with the possible exception of studies on birds, most subsequent investigations have concentrated on a few common laboratory animals and domesticated species of agricultural importance, it is now clear that the seasonally-changing photoperiod influences seasonal reproductive and other functions in many vertebrates inhabiting temperate and polar climates (reviewed by Hoffman, 1981).

In their early studies Garner and Allard (1920) realised that some species of plant were 'long-day' plants, only flowering when the daylength exceeded a certain species-specific daylength, whereas others were 'short-day' plants, flowering only when the daylength fell below a certain critical value. It was soon recognised that many insect and vertebrate species respond in a similar fashion. In many species indigenous to temperate climates the production of young is timed to coincide with the arrival of spring. Animals in which reproduction is a fairly rapid process, such as birds and small mammals (e.g. rodents), begin their breeding season in the spring of the same year as their offspring are born, concomitant with lengthening daylengths, and have therefore been classified as 'long-day' species. Conversely, mammals with longer gestation periods (5-6 months), such as sheep, Ovis aries, and red deer, Cervus elaphus, commence breeding during the shortening days of autumn and late winter, and have therefore been classified as 'short-day' species. The horse, Equus caballus, is also considered to be a 'long-day' species because it has a gestation period of nearly a year requiring it to mate under the

lengthening days of the previous spring. Thus, the period of time required for gonadal and embryonic development apparently determines the predictive cues utilised to time reproduction.

In many poikilotherms, including fish, temperature may also be an important proximate cue for the regulation of seasonal events (de Vlaming, 1972; Hoffman, 1981). In salmonids, however, the seasonally-changing photoperiod exerts the primary influence on reproductive timing (de Vlaming, 1972; Whitehead et al., 1978; Scott and Sumpter, 1983; Bye, 1984; Dodd and Sumpter, 1984; Lam and Munro, 1987; Bromage and Cumaranatunga, 1988). The principle strains of rainbow trout farmed in the United Kingdom spawn in late autumn or winter, and exposure to 'short' days in advance of the natural light cycle can advance maturation (e.g. Shiraishi and Fukuda, 1966). Hence, the species was originally classified as a 'short-day' animal (Breton and Billard, 1977; Billard et al., 1978a,b; Peter, 1981; Follett, 1982). Fertilisation in salmonids is external but gonadal development in preparation for the breeding season (spawning) may take a year or more to complete (Elliott et al., 1984; Sumpter, 1984; Sumpter et al., 1984). This fact, in combination with experiments demonstrating that 'long' days can stimulate reproduction in the rainbow trout (e.g. Whitehead and Bromage, 1980), supported the contention that the rainbow trout was a 'long-day' species (Bromage et al., 1982b; Elliott et al., 1984; Scott et al., 1984; Bromage and Duston, 1986). These concepts were unified into a composite hypothesis by several authors who suggested that the lengthening daylengths of late winter and spring initiate gametogenesis and the shortening daylengths of late summer and autumn accelerate the later stages of reproductive development (Bromage et al., 1982b; Scott and Sumpter, 1983; Bye, 1984; Elliott et al., 1984; Scott et al., 1984).

In the above scenario photoperiod is usually envisaged as providing a direct driving influence on reproductive activity, i.e. external environmental cues (exogenous factors) are considered to be of primary importance in the timing of reproduction. In recent years, however, there has been a growing awareness that, in many long-lived species, the seasonally-changing photoperiod does not directly induce reproductive activity, but rather

entrains (synchronizes) an endogenous circannual rhythm of reproductive function. Circannual rhythms in various functions, including reproductive activity, have so far been demonstrated (by persistence under constant environmental conditions) in approximately 40 organisms, principally birds and mammals (Gwinner, 1986). The first convincing experimental evidence that the seasonally-changing photoperiod can entrain a circannual rhythm was provided by Goss's (1969a,b) investigations on antler cycles in the sika deer, Cervus nippon. When exposed to constant daylengths of 8L:16D (8 hours of light and 16 hours of darkness), 16L:8D or continuous light (LL) these animals expressed an endogenous circannual rhythm of antler replacement with a frequency of approximately 10 months (Goss, 1969a). Under natural photoperiod conditions the antler cycle adopted a periodicity of 12 months, but could also be entrained by seasonal photocycles artificially compressed into 6, 4 and 3 months or expanded to 24 months; antler replacement reverted to a circannual periodicity on exposure to a seasonal photocycle compressed into 2 months suggesting that this frequency was beyond the range of entrainment (Goss, 1969b). Similarly, Gwinner (1977, 1986) reported that the circannual rhythms of testis growth and moult in the starling, Sturnus vulgaris, could be entrained by seasonal photocycles compressed into 8, 6, 4, 3 or 2.4 months; seasonal photocycles with periodicities of 2 months or less were again outwith the range of entrainment. Recent studies indicate that the annual reproductive cycle of the female rainbow trout is also under endogenous circannual control; fish maintained under a constant 6L:18D photoperiod, and constant temperature and food ration, exhibit a rhythm of gonadal maturation and spawning that is self-sustaining for at least three cycles, and free-runs with a periodicity that approximates, but can differ significantly from, one year (Duston and Bromage, 1986a, 1991). This rhythm can be entrained by seasonal photocycles compressed into 6 or 9 months (Whitehead et al., 1978) or expanded to 18 months (Bromage et al., 1984); a seasonal photocycle compressed into 3 months appears to be outside the range of entrainment for this species (Pohl et al., 1982). This suggests that (as in the sika deer and starling) under natural conditions the endogenous circannual 'clock' which ultimately controls reproduction in the rainbow trout is entrained to a periodicity of one year by the

seasonally-changing daylength.

1.2 Artificial manipulation of reproduction

Under ambient conditions the seasonally-changing daylength synchronizes the spawning time of a particular strain of rainbow trout to a 6-8 week period each year. This seasonality of egg production, of clear benefit to fish in the wild, places severe restrictions on the profitability of fish farming; year groups of fish all reach marketable size at the same time and farm facilities are either over or under utilised depending on the time of year. Since these problems can be avoided if eggs are produced throughout the year techniques designed to modify the time of spawning offer considerable commercial potential. Many of the experiments described in this thesis were conducted on commercial farms and were designed both to provide information on the mechanisms underlying the photoperiodic control of reproduction in the rainbow trout, and to meet the farmers' requirements for the development of commercial techniques for the production of 'out-of-season' eggs.

There are three main approaches to the modification of maturation and spawning time in salmonid fish: 1) Genetic selection, 2) Environmental manipulation, and 3) Hormonal manipulation.

The spawning time of a particular strain of rainbow trout is genetically determined (Kato, 1973; Busack and Gall, 1980). Wild rainbow trout are typically spring spawning fish (Behnke, 1979; Bromage and Cumaranatunga, 1988; Laird and Needham, 1988) but deliberate or unconscious selection over the last 100 years has produced a large number of different strains with spawning seasons in the U.K. ranging from August to April (Sumpter, 1984; Purdom, 1986; Lincoln, 1987). Many of these are commercially available and hence, in theory, a farmer could currently obtain eggs for 9 months of the year by stocking a variety of strains. Importing eggs from countries such as Australia and South Africa during the summer would complete the all-year round supply. In practice, however, it is difficult to obtain eggs in the United Kingdom outside of October to January (Bromage, personal communication). Moreover, the importation of strains from different

geographical locations presents two major problems. Firstly, there is a risk that disease will be transferred from one location to another, previously uninfected, site. Secondly, although the precise origins of most hatchery stocks are unknown (Bromage and Cumaranatunga, 1988), most established farms possess strains adapted to their particular environmental conditions (for example, water temperature, oxygen availability). Strains obtained from other locations may experience difficulty in adapting to their new environment, resulting in poor growth and mortalities. Farmers can, however, instigate a selection programme with their own stock. By retaining the earliest and latest spawners for future broodstock the spawning period can be extended with each generation (Kato, 1979; Busack and Gall, 1980). The principal advantage of this technique is that it imposes no artificial stresses, achieving an extension of spawning time by 'natural' methods. The main disadvantage of genetic manipulation is the long time period required for the results to become of economic consequence. Moreover, there are logistical problems in the long-term separation and identification of large numbers of different breeding stocks, and the spawning season may not even be stable after selection (Buss, 1982). For these reasons environmental and hormonal techniques for the manipulation of spawning time are generally preferred.

Daylength manipulation appears the most appropriate technique for the modification of spawning time since photoperiod resides at the highest level of the neuroendocrine pathway controlling reproduction (section 1.5; Figure 1.4) and can therefore influence all of the endocrine mechanisms involved in the initiation and subsequent regulation of gonadal development. It is therefore not surprising that this technique has so far proved to be the most successful, with a variety of photoperiod regimes capable of altering spawning time in the rainbow trout. For example, spawning can be advanced by exposing fish to seasonally-changing photoperiods compressed into periods of less than 12 months (e.g. Whitehead et al., 1978), 'long' (usually 16L:8D or 18L:6D) followed by 'short' (usually 8L:16D or 6L:18D) photoperiods in advance of the natural light cycle, either reduced gradually (e.g. Breton and Billard, 1977) or abruptly (e.g. Bromage et al., 1982), and constant 'long' photoperiods or continuous light applied from near the beginning of the

reproductive cycle (e.g. Bromage et al., 1984). Similarly, spawning can be delayed by exposure to seasonally changing photoperiods extended over periods greater than 12 months, to constant 'short' photoperiods during the first half of the reproductive cycle (e.g. Bromage et al., 1984), and to constant 'long' photoperiods or continuous light (LL) during the second half of the reproductive cycle (e.g. Shiraishi and Fukuda, 1966). These and other related studies are considered in more detail in a review of the literature pertaining to the photoperiodic control of reproduction in salmonid fish contained in the introduction to chapter 3 (section 3.1).

Although photoperiod is the primary environmental cue synchronizing reproduction in the rainbow trout, and probably all salmonids, temperature can also modify reproductive development and the timing of spawning (Goryczko, 1972; Titarev, 1975; Meiners-Gefken et al., 1988). In Finland, where water temperatures as low as 0.2°C are common in winter, certain strains of rainbow trout spawn in the spring when the water temperature exceeds 4°C (Nakari et al., 1988). Exposing these fish to compressed seasonal photocycles advanced reproductive development, but, despite the oocytes attaining their full size earlier than those of controls maintained under natural photoperiod, ovulation did not occur until the natural spawning time (Nakari et al., 1987,1988). Spawning was considerably advanced, however, in fish which were transferred into a constant water temperature of 10°C halfway through the experiment (Nakari et al., 1987). Conversely, Morrison and Smith (1986) found that spawning was delayed by approximately 3 months when winter-spawning rainbow trout, reared in constant temperature (10°C) spring water, were transferred to cold creek water about 3 months before the natural spawning time. Moreover, a recent study (Davies and Bromage, 1991) has shown that winter-spawning rainbow trout maintained on a constant 8-10°C borehole water supply commenced spawning 3 weeks earlier than fish supplied with river water of seasonally fluctuating temperature (2-17°C), irrespective of whether they were exposed to an ambient or seasonally advanced photoperiod. Tyler et al. (1987b) have shown that vitellogenin uptake into cultured ovarian follicles of rainbow trout is temperature dependent, with decreased incorporation at lower temperatures. Thus, temperature may

exert its effects on reproductive development directly rather than as an entraining agent.

The modification of spawning time by alteration of water temperature may be feasible for fish farms with a dual (spring and river) water supply but achieving artificial temperature changes in large volumes of water from a single source would clearly be uneconomic. Temperature can be more conveniently used to extend the availability of the fry by delaying the time of hatching of the eggs. Coldwater incubation (1-2°C), following incubation at normal temperatures for the first 10-12 days after fertilisation, has been shown to delay hatching by 50 days in rainbow trout (Bromage, 1982) and 100 days in brown trout (Maddock, 1974), with no reduction in egg quality.

The hormonal manipulation of reproduction provides a possible alternative to environmental methods. Current knowledge of the sequence of neuroendocrine events controlling reproduction in the female rainbow trout is reviewed in section 1.5. Clearly, simulation of the many hormonal changes which occur during the year or more required for ovarian development in the rainbow trout (Figures 1.5 and 1.7), even if possible, would be totally impractical in a farming environment. Moreover, our knowledge of the neuroendocrine events controlling reproduction in fish is far from complete (section 1.5), thus limiting our ability to manipulate their timing by administration of exogenous hormones. Nevertheless, intervention has been attempted at several levels of the hypothalamo-pituitary-gonadal axis in studies on a large variety of cultivated fish species (reviewed by Lam, 1982; Donaldson and Hunter, 1983; Crim et al., 1987; Billard, 1989a,b; Zohar, 1989). The most successful techniques have involved the elevation of GTH levels by administration of either crude fish pituitary extracts (usually derived from carp or salmon) or GnRH and its analogues (often in combination with dopamine antagonists). These techniques have been used to advance ovulation and spermiation, and to synchronize the maturation times of individual fish, in a number of salmonid species including the coho salmon, Oncorhynchus kisutch (Hunter et al., 1981; Donaldson et al., 1981; Fitzpatrick et al., 1984; Van Der Kraak et al., 1985), Atlantic salmon (Crim et al., 1983a; Crim and Glebe, 1984), lake trout, Salvelinus namaycush (Erdahl and McClain, 1987), and rainbow trout (Scott et al., 1982; Crim et al., 1983b; Billard et

al., 1984; Breton et al., 1990). However, the degree to which spawning can be advanced by such methods is limited to a maximum of about 1 month since ovulation can only be induced in fish possessing stage 7 oocytes (section 1.4; Bromage and Cumaranatunga, 1988); premature treatment produces a significant reduction in egg quality (Hunter et al., 1981; Crim and Glebe, 1984).

Attempts to manipulate earlier stages of the female reproductive cycle (i.e. initiate and maintain oogenesis/vitellogenesis) with hormonal treatments, and hence achieve greater alterations in the timing of maturation, have been largely unsuccessful (Billard et al., 1989a,b). However, comparable modifications in spawning time to those achieved using photoperiod treatments may be possible by the administration of a single hormone, melatonin. In many vertebrates the pineal gland converts photic information into a circadian rhythm of melatonin secretion, and, in certain seasonally-breeding mammals, the duration of the night-time increase in this hormone determines the reproductive response (reviewed by Bartness and Golding, 1989; Ebling and Foster, 1989; see sections 1.4 and 4.1 for more detailed discussions). If melatonin also conveys photoperiodic information to the reproductive axis in the rainbow trout it should be possible to mimic the effects of changes in photoperiod by imposing appropriately timed alterations in the patterns of melatonin secretion. For example, constant-release melatonin implants have been shown to mimic the effects of a switch from 'long' to 'short' days on reproductive timing in a number of seasonally-breeding mammals including red deer (Lincoln et al., 1984), sheep (Lincoln and Ebling, 1985; Nowak and Rodway, 1985; English et al., 1986; Poulton et al., 1987), goats, Capra hircus (Deveson et al., 1989) and silver foxes, Vulpes vulpes (Forsberg et al., 1990). The administration of constant-release implants containing melatonin may therefore provide a viable commercial alternative to photoperiod manipulation for the modification of spawning time in salmonid fish.

1.3 Aims of thesis

To summarise, the annual reproductive cycle of the female rainbow trout appears to be controlled by an endogenous circannual rhythm or 'clock', which is entrained by the

seasonal changes in daylength. In this respect 'long' (≥ 12 hours) and 'short' days have been considered the most important time cues during the early and later portions of the ovarian cycle respectively. This implies that exposure to photoperiods of specific length (i.e. 'critical' daylengths) is necessary for the entrainment of maturation and spawning in the rainbow trout. Recent work, however, suggests that neither absolute daylength, nor the magnitude of change in daylength, may be of paramount importance for the entrainment of the circannual clock controlling reproduction in the female rainbow trout (Duston and Bromage, 1987), and there is evidence that this may similarly apply to the masu salmon, Oncorhynchus masou (Takashima and Yamada, 1984). Studies in higher vertebrates have also challenged the concept of a rigid 'critical' daylength for particular photoperiodic reactions, instead emphasising the importance of the direction of change of daylength and previous photoperiodic experience (Robinson and Follett, 1982, Robinson and Karsch, 1987). The experiments described in chapter 3 of this thesis were designed to clarify the mechanisms by which photoperiod entrains the circannual clock controlling maturation in the female rainbow trout. Those presented in Section A examine the effects of a range of daylengths on reproductive timing in order to determine which feature(s) of the photoperiodic signal (absolute daylength, direction of change of daylength, magnitude of change in daylength) are important for the entrainment of the clock. In section B the reproductive response (advance or delay in the timing of maturation) to short (≤ 2 months) periods of LL applied at different phases of the annual reproductive cycle is investigated, with additional emphasis on the development of a commercially applicable method for the production of 'out-of-season' eggs without the need for blackout facilities.

As previously discussed, in several higher vertebrates the pineal gland transduces photoperiodic information into a circadian rhythm of melatonin secretion, the pattern of which determines the reproductive response. The rainbow trout also exhibits a diurnal rhythm in melatonin secretion but patterns of secretion under different photoperiod regimes have not been accurately defined, and there is no information available on the role of melatonin in salmonid reproduction. Chapter 4 therefore examines the role of melatonin in the transmission of photoperiodic information to the reproductive axis. Section A

defines patterns of melatonin secretion under various photoperiod regimes and investigates the nature of the mechanisms governing the generation of melatonin rhythms in the rainbow trout. In section B patterns of melatonin secretion are examined in two other commercially important fish, the Atlantic salmon and the Nile tilapia, Oreochromis niloticus. Finally, section C investigates the ability of constant-release melatonin implants to mimic the effects of changes in daylength on the timing of reproduction in the female rainbow trout.

1.4 The annual reproductive cycle of the female rainbow trout

This thesis is principally concerned with elucidating the mechanisms underlying the photoperiodic control of reproduction in the female rainbow trout and hence a brief description of the annual ovarian cycle is appropriate (for more detailed accounts see van den Hurk and Peute, 1979; Sumpter, 1984; Cumaranatunga, 1985; Scott, 1987; Bromage and Cumaranatunga, 1988).

The paired ovaries are suspended in the dorsal part of the body cavity and lie either side of the swimbladder. During maturation the ovaries increase in size from less than 1% to as much as 20% of total body weight. Each ovary is surrounded by a peritoneal membrane which forms a gonoduct through which mature eggs are ovulated into the body cavity prior to oviposition via the urogenital papilla. In the wild the female rainbow trout digs a redd (nest) in which she lays her eggs, but fish maintained in captivity appear to lack the appropriate, as yet unknown, environmental cues (possibly including a gravel substrate, social stimuli and pheromones) for oviposition and eggs have to be 'stripped' from the fish manually.

Rainbow trout may spawn for the first time at either 2 or 3 years of age depending on strain and environmental influences such as temperature and food availability, which determine size and growth rate. They produce a single batch of 2-3000 eggs each year but may spawn several times during their lifetime. At any one time at least two distinct populations of oocytes can be distinguished in their group-synchronous ovaries; one of primary oocytes, which are permanently available for recruitment into the later stages of egg development, and the other of synchronously developing secondary oocytes, destined to form that season's batch of eggs.

Sexual differentiation of primordial germ cells occurs at about 3 months of age leading to the formation of pre-meiotic cells known as oogonia. These cells soon start to proliferate by mitotic division, after which some are transformed into primary oocytes (oogenesis), marking the beginning of the first meiotic prophase. At this stage the oocytes become enveloped in a layer of follicle cells. A detailed histological examination enabled Cumaranatunga (Cumaranatunga, 1985; Bromage and Cumaranatunga, 1988) to

distinguish 7 interrelated stages of oocyte development in the rainbow trout ovary (Figure 1.2). The timing of these stages during ovarian development is illustrated in Figure 1.3. Stages 1-3 represent the primary growth phase (previtellogenesis) during which the oocytes increase in diameter from around 35 to about 350μm. By the end of stage 3 the follicle cells surrounding the oocytes have differentiated into a single granulosa and two thecal layers.

Stages 4-6 comprise the secondary growth phase during which the accumulation of yolk is primarily responsible for the massive increase in oocyte volume which occurs prior to ovulation. Vesicles observed during stage 4, previously thought to contain endogenously synthesised yolk (yolk synthesised within the oocyte), are pushed to the periphery when the accumulation of exogenously synthesised yolk begins in stage 5, and are now believed to be precursors of the cortical alveoli, which release their distinct glycoproteinaceous contents into the perivitelline space at fertilisation. Exogenous yolk consists of a high molecular weight (440,000) glycolipophosphoprotein named vitellogenin which is synthesised by the liver and released into the blood. During stages 5 and 6 (exogenous vitellogenesis) vitellogenin is sequestered by receptor-mediated endocytosis (Tyler et al., 1988) and enzymatically cleaved into a lipid-rich protein, lipovitellin (M.W. 390,000), and a phosphate-rich protein, phosvitin (M.W. 35,000) for storage. Exogenous vitellogenesis is first apparent 4-5 months before ovulation in fish maturing at 2 years of age and 8-9 months prior to spawning in fish maturing for either the first or second time at 3 years of age. During stage 6 the centrally located nucleus (germinal vesicle) starts migrating towards the periphery of the oocyte (the beginning of oocyte maturation) where the nuclear membrane disintegrates (germinal vesicle breakdown) and the first meiotic division is completed with the expulsion of one set of chromosomes (the first polar body). The resultant stage 7 oocytes may continue to sequester vitellogenin and water uptake may contribute an additional increase in size prior to ovulation (Riazi and Fremont, 1988), at which point the ripe eggs are expelled from their follicles into the body cavity, where they are bathed in ovarian fluid. By this stage the eggs have attained a diameter of about 5mm. Fertilisation occurs when a sperm

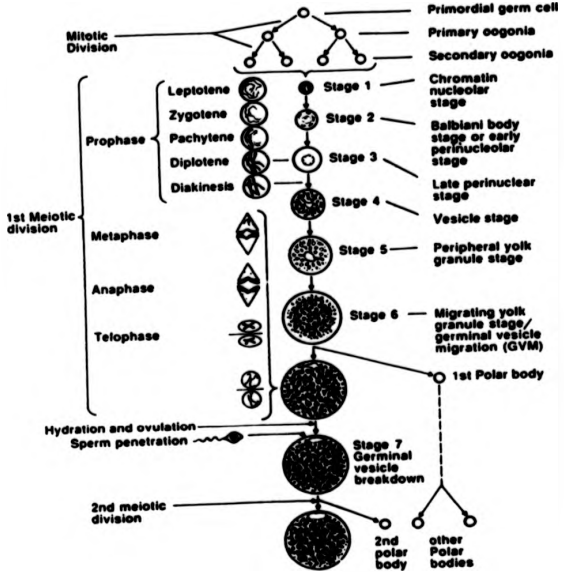


Figure 1.2: Schematic diagram illustrating the different stages of oocyte development which can be recognised during ovarian maturation in the rainbow trout (from Bromage and Cumaranatunga, 1988).

TIMING OF OVARIAN DEVELOPMENT

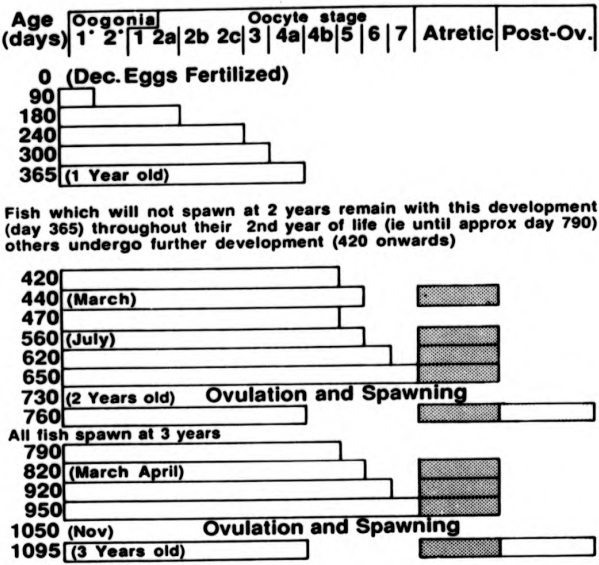


Figure 1.3: The timing of the different stages of ovarian development in the rainbow trout (from Bromage and Cumaranatunga, 1988).

penetrates the micropyle, an opening located adjacent to the ruptured germinal vesicle; meiosis resumes, the second polar body is eliminated and the male and female chromosome sets combine. After fertilisation water is absorbed into the perivitelline space (water-hardening) and the chorion becomes impervious to the entry of further water and solutes.

The total number of eggs ovulated is dependent on the degree of atresia (resorption of oocytes) occurring during ovarian development. Atresia is thought to occur at any stage of oocyte development but is considered to be of particular significance during exogenous vitellogenesis, during which a 75-fold reduction in oocyte numbers has been reported (Bromage and Cumaranatunga, 1988). However, Tyler et al. (1990) recently found no evidence for atresia during exogenous vitellogenesis. These differences may be explained by differences in the condition of the fish, for example their nutritional status, a factor known to affect the incidence of atresia (Springate et al., 1985).

Although the ovarian cycle has been described as a sequence of stages it should be noted that the phases of ovarian development may overlap and hence several stages may be observed in the ovary at the same time (Bromage and Cumaranatunga, 1988; Tyler et al., 1990). It is thought likely that the sequentially initiated physiological events (e.g. cortical alveoli formation, exogenous vitellogenesis) do not sequentially replace each other, but, once initiated, remain active throughout oocyte development (Wallace et al., 1987). Although ultimately determined by environmental cues, principally the seasonally-changing daylength, the direct coordination of these events is under multihormonal control and hence the following section reviews current knowledge of the principal components of the neuroendocrine system controlling ovarian development in salmonids, with emphasis placed on the rainbow trout whenever possible (Figure 1.4).

1.5 Neuroendocrine control of reproduction in female salmonids

The role of the pineal gland and melatonin in lower vertebrates is unclear, but there is some evidence, principally derived from cyprinids at present, that both are involved in some way in the timing of reproduction in teleost fish (reviewed by de Vlaming and Olcese, 1981). Melatonin was first identified in the pineal of a salmonid fish (chinook salmon, Oncorhynchus tshawytscha) by Fenwick (1970a). Subsequent studies have demonstrated diurnal rhythms in pineal (Burton and Gern, 1983) and circulating melatonin (Gern et al., 1978a,b; Owens et al., 1978) in the rainbow trout, with blood melatonin levels elevated at night for a period corresponding to the duration of darkness (Duston and Bromage, 1986b). This hormonal profile of seasonally changing daylength may act to synchronize a variety of bodily rhythms with the external environment, although there is currently no evidence that melatonin can influence reproduction in salmonid fish as it does in some higher vertebrates (discussed in detail in the introduction to chapter 4 (section 4.1)).

Although the precise site(s) of action of melatonin remain unknown, the brain, and in particular the hypothalamus, have been considered the most likely targets (Binkley, 1988). Experiments in which intrahypothalamic microimplants containing melatonin were used to provide a localised release of the hormone in the brains of rodents provide direct evidence that melatonin exerts its reproductive effects in the hypothalamus (Glass and Lynch, 1982; Hastings et al., 1988). This evidence is complemented by the identification of putative melatonin receptors in the hypothalamus of several higher vertebrates, although receptors have also been reported in other brain regions and peripheral sites such as the pituitary gland and gonads (reviewed by Morgan and Williams, 1989; Stankov and Reiter, 1990). Recent studies in the goldfish, Carassius auratus (Martinoli et al., 1991) and rainbow trout (Aggelopoulos and Demaine, 1990) indicate a widespread distribution of melatonin receptors in the teleost brain, including significant binding capacity in the hypothalamus. No melatonin receptor sites were detected in the pituitary gland (Martinoli et al., 1991). Melatonin may therefore act within the teleost hypothalamus to regulate secretion of gonadotropin releasing

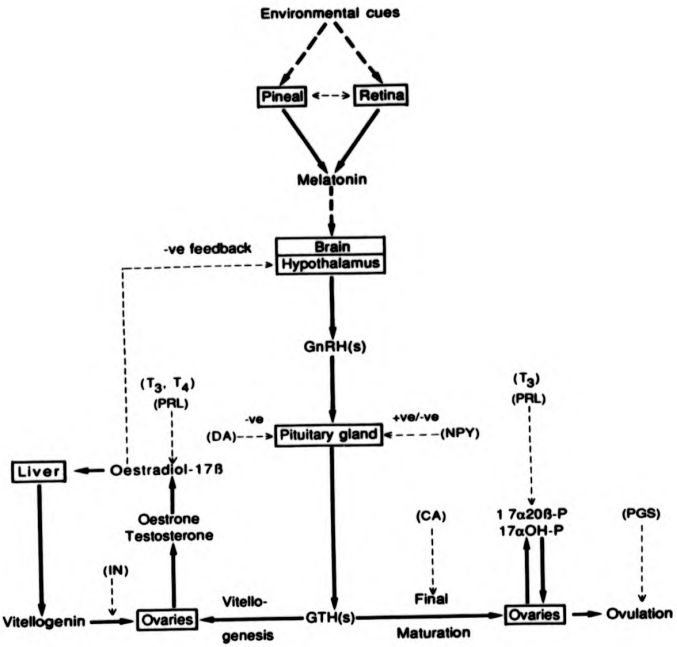


Figure 1.4: Principal components of the neuroendocrine system controlling reproduction in the female rainbow trout (GnRH, gonadotropin releasing hormone; GTH, gonadotropin; 17α20β-P,17α-hydroxy-20β-dihydroprogesterone;17αOH-P,17α-hydroxyprogesterone; PRL, prolactin; T_3, triiodothyronine; T_4, thyroxine; DA, dopamine; NPY, neuropeptide Y; CA, calcitonin; IN, insulin; PGS, prostaglandins). Pathways are stimulatory unless indicated.

hormone(s) (GnRH(s)), but this remains unproven.

Two forms of GnRH have recently been detected in the brain of the chum salmon Oncorhynchus keta. Salmon-I GnRH (s-GnRH-I) is a decapeptide which shows 80% sequence homology with mammalian GnRH (luteinizing hormone releasing hormone, LHRH), differing only in amino acids 7 and 8 (Sherwood et al., 1983). The primary structure of salmon-II GnRH (s-GnRH-II) is unknown, but its existence has been confirmed by chromatographic and cross-reactivity studies (Sherwood et al., 1987a, b), and it is thought to be identical to chicken-II GnRH (c-GnRH-II: Sherwood et al., 1984). At least seven other teleosts, including the rainbow trout, possess two forms of GnRH with the same chromatographic and immunological profiles as s-GnRH-I and s-GnRH-II (Sherwood et al., 1984; Sherwood, 1987b).

There is currently little information about changes in GnRH concentrations that may accompany sexual maturation in salmonids. Schafer et al. (1989) reported an increased immunoreactivity to GnRH (antisera raised against synthetic LHRH) in the brains of rainbow trout undergoing exogenous vitellogenesis compared to previtellogenic and vesicle stage (stage 4) fish. However, Okuzawa et al. (1990), using highly specific antisera, reported that s-GnRH-I concentrations were relatively low in the hypothalamus and other brain regions of mature rainbow trout compared to immature fish, whereas they were higher in the pituitary gland of mature than immature fish; c-GnRH-II concentrations were similar in mature and immature fish. These authors therefore suggested that more s-GnRH-I is transported from the brain to the pituitary gland as gonadal maturity progresses, but that, unlike s-GnRH-I, c-GnRH-II does not regulate gonadal maturation in the rainbow trout.

The primary function of GnRH(s) is stimulation of the synthesis and release of gonadotropin(s) (GTH(s)) from specific glycoprotein containing cells located in the pars distalis of the pituitary gland. A number of studies have provided evidence for this in salmonids. Both salmon and trout hypothalamic extracts stimulated GTH release in vitro from pituitaries of testosterone-primed juvenile rainbow trout (Crim and Evans, 1980; Crim et al., 1981a). Synthetic mammalian LHRH also stimulated GTH release from

rainbow trout pituitaries *in vitro* (Crim and Evans, 1980; Fahraeus-van Ree et al., 1983) and synthetic s-GnRH-I stimulated GTH release in a dose-related manner from isolated rainbow trout pituitary cells (Weil et al., 1986), although the minimum effective dose varied according to the stage of reproductive development (Weil and Marcuzzi, 1990). *In vivo* synthetic mammalian LHRH and s-GnRH-I and their analogues have proved effective in stimulating GTH release in a variety of salmonid fish including coho salmon (Van Der Kraak et al., 1983; Donaldson et al., 1984), Atlantic salmon (Crim et al., 1983a, 1988a, b; Crim, 1984), brown trout (Crim and Cluett, 1974; Crim et al., 1981) and rainbow trout (Weil et al., 1976; Crim et al., 1983b; Crim, 1984; Gielen and Goos, 1984; Crim et al., 1988a, b), although the response of the rainbow trout to GnRH *in vivo* also varies seasonally (Weil et al., 1978; Goos et al., 1982).

In addition to GnRHs there is now considerable evidence that a gonadotropin release inhibitory factor (GRIF) acts directly on the gonadotrophs to inhibit GnRH stimulated GTH release in teleosts (Peter 1982, 1983; Peter et al., 1986). The majority of studies have been conducted on the goldfish, in which the GRIF appears to be the catecholamine, dopamine. Chang and Peter (1983a) found that intra-peritoneal injections of dopamine and apomorphine, a dopamine agonist, caused a reduction in serum GTH levels in goldfish whereas pimozide, a dopamine antagonist, significantly increased serum GTH. Furthermore, dopamine and apomorphine blocked LHRH analogue (LHRH-a) induced GTH release whereas pimozide potentiated the effects of LHRH-a on GTH release and increased the occurrence of induced ovulation (Chang and Peter, 1982; Chang and Peter, 1983b). In the rainbow trout dopamine inhibited pituitary GTH release *in vitro* (Crim, 1981) and pimozide potentiated the effects of LHRH-a on GTH release *in vivo* (Billard et al., 1984). However, in contrast to many of the teleost species so far investigated, in which dopamine exerts such a strong inhibitory influence on GTH secretion that administration of LHRH-a alone (rather than in combination with a dopamine antagonist) is usually ineffective in inducing ovulation (Peter et al., 1989), ovulatory GTH levels can be attained in LHRH-a treated salmonids without using a dopamine antagonist (Donaldson and Hunter, 1983). Thus, as a GRIF, dopamine may play a relatively minor role in salmonids.

Although GTH preparations have been available for some time from salmon (Donaldson et al., 1972) and rainbow trout (Breton et al., 1976) elucidation of the role(s) of GTH(s) in reproductive physiology has been hindered by the lack of agreement on the number, identity and chemical structure of the GTH(s) in salmonid pituitaries. Some workers maintain that fish possess only a single GTH, which may, however, have several isoforms (reviewed by Fontaine and Dufour, 1987) while others favour the existence of two or more GTHs. Thus, Idler and co-workers (Idler, 1982; Idler and Ng, 1983; Ng and Idler, 1983) reported the isolation of two distinct GTHs from chum and chinook salmon pituitaries, using affinity chromatography on Concanavalin A-Sepharose. The carbohydrate poor fraction (designated Con AI), which is not adsorbed on the column, is thought to stimulate vitellogenin incorporation by the oocytes of a variety of fish including the rainbow trout and Atlantic salmon (Campbell, 1975; Idler, 1982; Ng and Idler, 1983; Idler and So, 1987). The carbohydrate rich fraction (designated Con AII), which is adsorbed on the column, has been reported to stimulate almost all ovarian activities but is considered to be principally involved with the induction of oestrogen, and hence vitellogenin, synthesis, and with final maturation and ovulation (Idler, 1982; Idler and Ng, 1983; Ng and Idler, 1983; Nagahama, 1987).

More recently, Kawauchi and colleagues, working with female chum salmon pituitaries, isolated and characterised two chemically distinct GTH(s) homologous to mammalian LH and FSH (Kawauchi et al., 1987; Itoh et al., 1988; Suzuki et al., 1988a, b, Kawauchi et al., 1989). These GTHs, designated GTH I and GTH II, were equally potent in stimulating gonadal growth in rainbow trout in vivo (Suzuki et al., 1988a) and oestadiol-17β production by mid-vitellogenic ovarian follicles of amago salmon, Oncorhynchus rhodurus, in vitro (Suzuki et al., 1987; Suzuki et al., 1988a, c). However, GTH II was more potent than GTH I in stimulating release of the maturation-inducing steroid 17α-hydroxy-20β-dihydroprogesterone (17α20β-P) by amago salmon post-vitellogenic ovarian follicles (Suzuki et al., 1987; Suzuki et al., 1988c).

The development of specific radioimmunoassays for GTH I and GTH II has revealed that GTH I is the predominant GTH in the pituitary and plasma during the early stages of

vitellogenesis in the rainbow trout, whereas GTH II predominates in mature rainbow trout and post-ovulatory amago and chum salmon (Kawauchi et al., 1987; Suzuki et al., 1987; Swanson et al., 1987; Suzuki et al., 1988d). Furthermore, a GnRH analogue, des-Gly^{10}[D-ALA^{6}]GnRH-a, significantly stimulated release of GTH I, but not GTH II, from pituitaries of rainbow trout at early vitellogenic stages: in contrast, pituitaries from reproductively mature trout released GTH II, but not GTH I, in response to GnRH-a (Kawauchi et al., 1987; Swanson et al., 1987). Thus, there appears to be differential synthesis and secretion of GTH I and GTH II at different stages of the salmonid reproductive cycle, possibly reflecting different biological roles. A similar study in coho salmon led Dickhoff and Swanson (1990) to propose the 'maturational surge hypothesis'. They suggest that GTH I regulates all aspects of reproductive development up to the time of spawning and is responsible for the production of steroids which causes accumulation of GTH II in the pituitary. Ovulation is then triggered by a surge in blood levels of GTH II at spawning time.

In conjunction these studies provide convincing evidence for the existence of at least two GTHs in salmonid fish. However, GTH I and GTH II are synthesised from two different cell types in salmonid pituitaries (Kawauchi et al., 1989; Nozaki et al., 1990a, b) and are homologous to mammalian LH and FSH (Itoh et al., 1988) while Con AI and Con AII may originate from a single gonadotroph (van Oordt and Peute, 1983) and only Con AII shows a high level of homology with LH and FSH (Idler and Ng, 1983). Thus, despite intensive research, the precise number and nature of salmonid GTHs is still far from clear. This creates problems in the interpretation of studies reporting changes in circulating GTH levels since it is impossible to know exactly what the available GTH radioimmunoassays developed in different laboratories actually measure (Dodd and Sumpter, 1984). The majority, however, appear to be directed primarily against the Con AII GTH (Peter, 1981; Dodd and Sumpter, 1984; Peter et al., 1986).

A number of studies in the rainbow trout have revealed a transient rise in blood GTH levels during the early stages of oocyte development followed by a much more pronounced increase during the period of final maturation and ovulation (Figure 1.5: Billard et al.,

1978; Bromage et al., 1982a, b; Zohar et al., 1982; Whitehead et al., 1983; Sumpter et al., 1984). Similar changes have been observed in the brown trout (Billard et al., 1978; Breton et al., 1983a). Other studies, however, have failed to detect an initial increase in GTH (Scott and Sumpter, 1983a; Sumpter and Scott, 1987). These discrepancies may be due to differences in assay specificity, as previously mentioned, or may reflect the pulsatile nature of GTH secretion in immature rainbow trout (Zohar et al., 1982; Zohar et al., 1986). It is worth emphasising, however, that studies utilising the homologous rainbow trout assay developed by Breton et al. (1978), rather than heterologous assays, have consistently demonstrated two phases of increased GTH levels during the ovarian cycle of the rainbow trout (Bromage and Cumaranatunga, 1988).

The initial elevation in blood levels of GTH(s) is thought to stimulate production of oestrogens, particularly oestradiol-17β and oestrone, by the ovaries (Idler and Campbell, 1980; Bromage et al., 1982a; Ng and Idler, 1983; Whitehead et al., 1983). Increasing levels of oestradiol-17β, which peak 2-3 months prior to ovulation (Figure 1.5), trigger the synthesis and secretion of vitellogenin by the liver (Lambert et al., 1978; Idler and Campbell, 1980; van Bohemen and Lambert, 1981; van Bohemen et al., 1982a; Whitehead et al., 1983). Oestrone, which is present in the blood before oestradiol-17β, is also capable of inducing vitellogenin synthesis, but is far less potent; van Bohemen et al. (1982b) suggest that it may serve to sensitise the liver hepatocytes to oestradiol-17β. Certainly, primary exposure to either oestrone or oestradiol-17β itself produces vitellogenin synthesis only after a lag but secondary exposure results in immediate synthesis of vitellogenin in the rainbow trout (Elliott et al., 1982; van Bohemen et al., 1982b; Lawless, 1987, cited in Lazier et al., 1987). Alternatively, since oestrone secretion precedes oestradiol-17β secretion, the presence of oestrone may simply be a reflection of its position in the stepwise synthesis of oestrogens by the ovary (Elliott et al., 1984; Bromage and Cumaranatunga, 1988).

Vitellogenin levels peak at ovulation (Figure 1.5), at which time it is the major blood protein (Sumpter, 1984). Calcium is an integral component of the vitellogenin complex and hence calcium levels rise concomitantly with those of vitellogenin (Figure1.5: Elliott

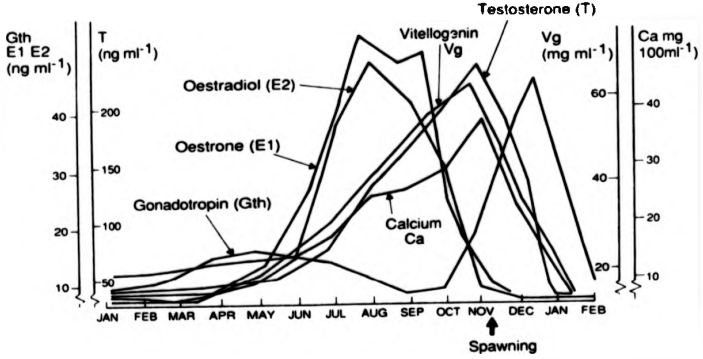


Figure 1.5: Composite graph illustrating the seasonal changes in serum levels of a variety of hormones associated with the annual cycle of reproduction in the female rainbow trout. The vertical lines on the x-axis refer to the first day of each month and the arrow (↑) denotes the timing of ovulation (from Bromage and Cumaranatunga, 1988).

et al., 1984). Serum calcium therefore provides a useful index of vitellogenin secretion.

The selective uptake of circulating vitellogenin by the developing oocytes may be stimulated and maintained by GTH(s) (Ng and Idler, 1983), but the situation is far from clear. Con AII GTH stimulated vitellogenin uptake by rainbow trout ovarian follicles *in vitro* (Breton and Derrien-Guinard, 1983; Tyler et al., 1987), but serum levels of this hormone remain low or undetectable during the most active period of vitellogenesis (Figure 1.5: see previous references). Idler and So (1987) have reported that Con AI GTH was elevated from 'endogenous' through exogenous vitellogenesis and stimulated ovarian uptake of vitellogenin in landlocked Atlantic salmon (Idler and So, 1987). Furthermore, administration of antibodies to Con AI GTH diminished vitellogenin uptake during 'endogenous' vitellogenesis and reduced normal gonadal growth during exogenous vitellogenesis (Idler and So, 1987). Thus, both Con AI and Con AII GTH may be involved in vitellogenin sequestration. The effects of GTH I and GTH II on vitellogenin incorporation have not been investigated. Again, further clarification awaits the elucidation of the number and nature of fish GTH(s).

Testosterone levels start to rise at about the same time as the oestrogens (Figure 1.5) but peak later, approximately a week before ovulation (Scott et al., 1983). The physiological role of high levels of testosterone in female salmonids is, however, unclear. One possibility is that testosterone has no primary function and is present in the circulation solely in its capacity as a precursor to oestradiol-17β. However, since far greater concentrations of testosterone have been recorded in female than in male rainbow trout (Scott et al., 1980), a functional role seems likely. Testosterone stimulates synthesis of GTH by the pituitaries of immature rainbow trout (Crim and Evans, 1979; Crim et al., 1981b; Gielen and Goos, 1984), but this positive steroid feedback, which appears to act at both the hypothalamic and pituitary levels (Goos, 1987), may be due to the conversion of testosterone to oestradiol-17β since only aromatisable androgens were effective in elevating pituitary GTH (Crim et al., 1981b). Lambert and van Bohemen (1980) have demonstrated the aromatisation of androgens to oestrogens by brain tissue from female rainbow trout and hence testosterone might be considered to be a target-organ

specific oestrogen (Scott and Sumpter, 1983b).

Testosterone may also be involved in the development of atresia. Cumaranatunga et al., (1985) found that treatment of female rainbow trout with testosterone during exogenous vitellogenesis produced widespread atresia. Whether this is a specific effect of testosterone or results from increased steroidal negative feedback on GTH secretion (which in turn might lead to a reduction in vitellogenin uptake by the developing oocytes) is unclear (see Bromage and Cumaranatunga, 1988).

Finet et al. (1988) have suggested that testosterone may inhibit the accumulation by oocytes of cyclic AMP (cAMP), which has been implicated in the regulation of maturational events associated with the actions of 17α20β-P (Figure 1.6: Nagahama et al., 1985; Jalabert and Finet, 1986; Nagahama and Yamashita, 1989). They proposed that the maintenance of low cAMP levels may be necessary to equilibrate the system before the final, irreversible, action of the maturational surge of 17α20β-P. Since testosterone levels remain above basal for approximately one month after ovulation (Scott et al., 1983) it has also been suggested that this hormone is involved with changes in the ovary in preparation for the next reproductive cycle (Bromage and Cumaranatunga, 1988).

Towards the end of the reproductive cycle, as oestrogen levels fall, there is a surge in circulating GTH(s) (Figure 1.5: Billard et al., 1978; Bromage et al., 1982a; Breton et al., 1983; Scott and Sumpter, 1983a; Scott et al., 1983; Whitehead et al., 1983; Zohar et al., 1986b; Sumpter and Scott, 1987). There is a highly significant negative correlation between levels of oestradiol-17β and GTH suggesting that the GTH surge occurs in response to a reduction in sex-steroid negative feedback (Scott et al., 1983; Whitehead et al., 1983: for a detailed discussion of steroid feedback mechanisms in fish see Goos, 1987). Increased GTH stimulates secretion of two further steroids by the ovaries, 17α-hydroxyprogesterone (17αOH-P) and 17α20β-P, resulting in final maturation and ovulation (Scott and Baynes, 1982; Scott et al., 1982; Goetz, 1983; Scott et al., 1983). These steroids rise concomitantly at or about the time of oocyte maturation in rainbow trout (Figure 1.7: Scott et al., 1983; Springate et al., 1984), but 17α20β-P is by far the most potent maturation-inducing steroid in salmonid fish (Goetz, 1983; Nagahama et

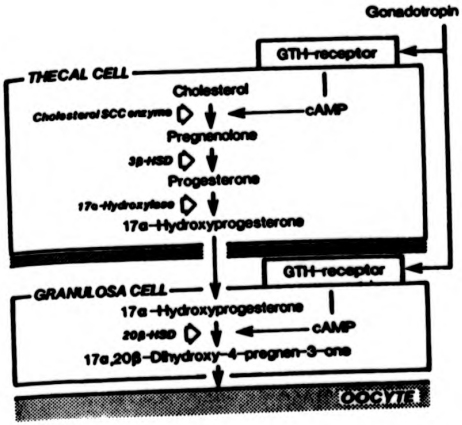


Figure 1.6: Two-cell type model for the production of 17α-hydroxy-20ß-dihydroprogesterone (17α,20ß-Dihydroxy-4-pregnen-3-one) by salmonid ovarian follicles (SCC, side-chain cleavage; 3ß-HSD, 3ß-hydroxysteroid dehydrogenase; 20ß-HSD, 20ß-hydroxysteroid dehydrogenase: from Nagahama and Yamashita, 1989).

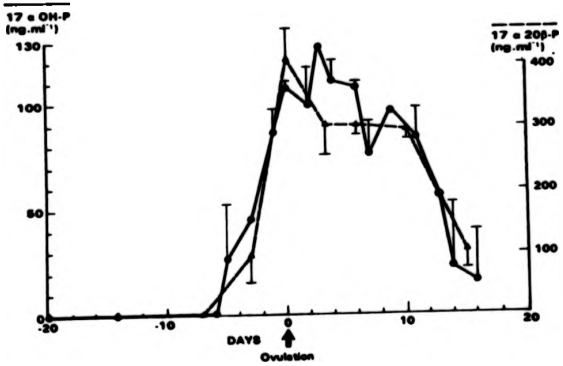


Figure 1.7: Changes in serum levels (mean ± 1SEM) of 17α-hydroxyprogesterone (17αOH-P) and 17α-hydroxy-20β-dihydroprogesterone (17α20β-P) during final maturation and ovulation in the rainbow trout (from Springate et al., 1984).

al., 1985; Nagahama, 1987; Scott and Canario, 1987). The principal function of 17αOH-P appears to be as the precursor of 17α20β-P; the thecal cell layer of the ovarian follicles produces 17αOH-P which traverses the basal lamina and is converted to 17α20β-P by the granulosa cell layer (Figure 1.6: Nagahama et al., 1985; Nagahama and Yamashita, 1989).

The foregoing account provides a brief overview of the principal components involved in the neuroendocrine control of reproduction in the female rainbow trout. Several of the parameters described (principally calcium, but also oestradiol-17β and testosterone) have been monitored in experiments presented in this thesis to provide an assessment of the rate of ovarian development under various photoperiod regimes. Although beyond the scope of this review the reader should be aware that a number of other substances have been implicated in the control of reproduction in salmonids (Figure 1.4). These include calcitonin (Watts et al., 1975; Haux et al., 1987; Fouchereau-Peron et al., 1990), insulin (Tyler et al., 1987a), neuropeptide Y (Breton et al., 1989, 1990), prolactin (Prunet et al., 1990), prostaglandins (Jalabert and Szollosi, 1975; Crim, 1981; Goetz et al., 1987), and the thyroid hormones, thyroxine and triiodothyronine (Cyr and Eales, 1988; Cyr et al., 1988; Dickhoff et al., 1989). Thus, our knowledge of the neuroendocrine events controlling salmonid reproduction is still far from complete.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

This chapter describes the materials and methods relevant to more than one experiment.

2.1 Experimental animals.

Domesticated stocks of female rainbow trout, Oncorhynchus mykiss (formerly Salmo gairdneri), were used for the majority of the experiments described in this thesis. Different experiments utilised fish of several different strains although, whenever possible, fish from the same source were used in related experiments. Detailed information regarding source, age and size of the fish is included in the methods section of each experiment.

2.1.1. Maintenance.

Fish were maintained in a variety of controlled environment aquarium systems appropriate to the experimental protocols:

Experiments 1-4 in Chapter 3 utilised 6 circular 1200 litre capacity glass fibre tanks (diameter 1.5m., water depth 0.6m.; Figure 2.1) enclosed in a wooden framework and lightproofed with industrial grade black polythene sheeting. The tanks were supplied with gravity fed spring water at a flow rate of approximately 50 litres per minute and a constant temperature of 7.5-8°C. Hinged doorways allowed access to individual tanks. Artificial light was supplied by 60W pearl tungsten filament light bulbs secured 1.5m. above the tanks and providing a light intensity of 25-30 lux (Lightmaster photometer; Evans Electroselenium Ltd., Halstead, Essex, U.K.) at the water surface. Daylength was controlled by 24 hour electronic time switches (Smith's Industries Ltd., London, U.K.). The tanks were covered with fine mesh bird netting to prevent fish escaping. Three similar tanks were used in experiments in Chapter 4. These received chlorine filtered mains water (Filtromat chlorine filters; Elga Ltd., High Wycombe, Bucks, U.K.) at a flow rate of approximately 20 litres per minute. Water temperature varied seasonally between 4 and 17°C. Hinged fibreglass lids covered in black polythene sheeting rendered the tanks lightproof. Artificial light was supplied by 60W pearl tungsten filament light bulbs

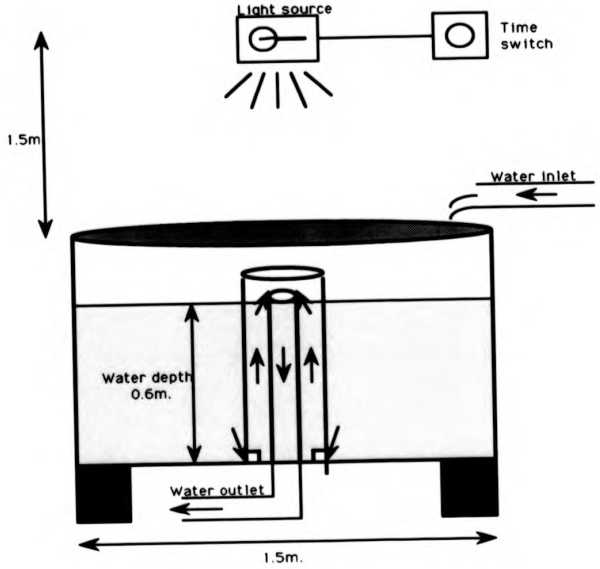


Figure 2.1: Diagram illustrating the main features of a 1200 litre capacity circular photoperiod tank (not to scale).

secured in waterproof bulkhead lamps (model EB.10; J. and G. Coughtrie Ltd., Glasgow, U.K.) 0.15m. above the water surface. These provided a light intensity of 2000 lux at the water surface directly beneath the lamps and 50-360 lux 0.5m. below the lamps (measured with water removed). Photoperiod was controlled by 24 hour digital electronic time switches fitted with batteries for protection against power cuts (Smith's Industries Ltd.).

Fish were maintained in 'oxyder' tanks in experiments 5-7 in Chapter 3. These are commercially available (Field, Stream and Covert Ltd., Meriden, U.K.) oblong-shaped fibreglass tanks (length 3.1m., width 1.65m., water depth 0.7m.) with a centrally located screen (Figure 2.2). The tanks were sub-divided into 8 sections with netted screens to allow different experimental groups to be segregated. Spring water was supplied at a rate of approximately 90 litres per minute and water circulation and oxygen levels were increased by a large submersible air pump. Water temperature varied seasonally between 7 and 18°C. Continuous illumination was supplied by cool white fluorescent tubes suspended 1m. above the tanks and providing a light intensity approximating 1000 lux over the majority of the water surface. Fine mesh bird netting prevented fish escaping.

Experiments 7 and 8 in Chapter 4 employed 2 rectangular fibreglass tanks (length 1.5m., width 0.9m., water depth 0.5m.) each with a separate supply of recirculating mains water maintained at a constant 12°C by an electric chiller unit (Grant Instruments Ltd., Cambridge, U.K.). Water was provided by each header tank at approximately 30 litres per minute and drained into a faecal trap and gravel filter prior to being pumped (pump model PV21; Beresford and Son Ltd., Birmingham, U.K.) back to the header tank (Figure 2.3). A ball-cock controlled top-up system compensated for water losses due to spillage and evaporation. Cool white fluorescent tubes secured to the ceiling 3m. above the tanks provided a light intensity of approximately 60 lux at the water surface. An on-line electronic time switch (MK Electric Ltd., Edmonton, London, U.K.) controlled the photoperiod for both tanks. Fine mesh bird netting prevented fish escaping.

At the beginning of each experiment the fish were weighed and thereafter fed daily with the recommended ration and pellet size of a commercially available trout diet

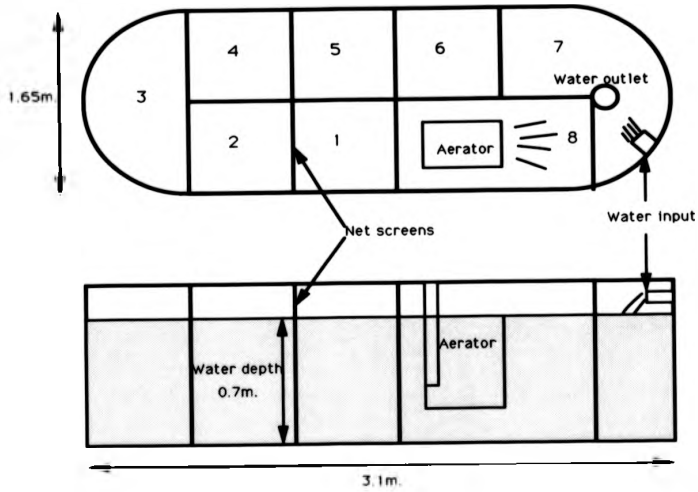


Figure 2.2: Diagram of an 'oxyder' fish holding tank divided into 8 sections by net screens to allow segregation of experimental groups (not to scale).

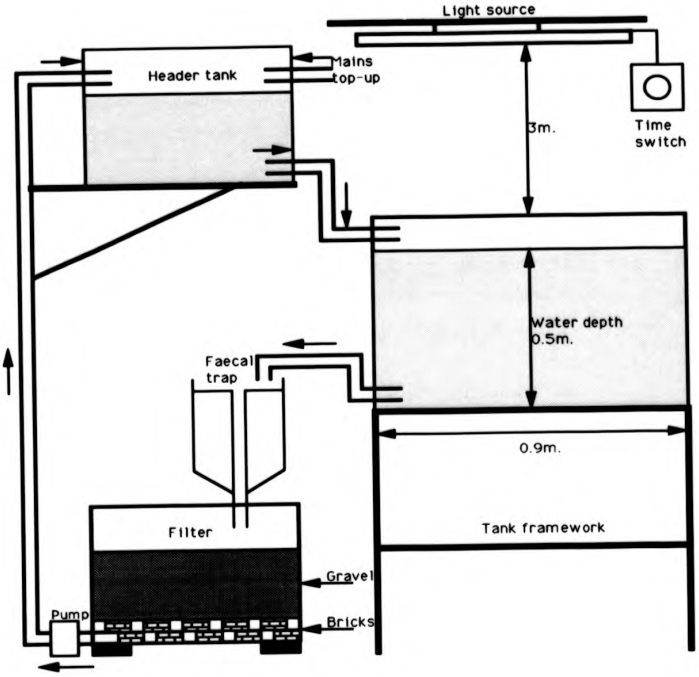


Figure 2.3: Diagram illustrating the main features of a rectangular fish holding tank with a recirculating water supply (not to scale).

(Mainstream; BP Nutrition U.K. Ltd., Witham, Essex, U.K.). A 2 ppm solution of malachite green (zinc free; Sigma Chemical Company Ltd., Poole, Dorset, U.K.) was occasionally used to prevent the spread of fungal infections. Fish were starved for 24-48 hours prior to any manipulative procedure.

2.1.2 Anaesthesia.

All manipulative procedures were conducted under anaesthesia to allow ease of handling and to minimise stress and scale damage. The preferred anaesthetic was 2-phenoxyethanol (Sigma Chemical Company Ltd.) at a concentration of 1:20,000 in water. Anaesthesia was generally induced within 1-2 minutes and fish placed in aerated water recovered within 5 minutes. Post-sampling mortalities were rare.

2.1.3 Identification.

When large numbers of fish required individual identification plastic numbered tags (Charles Neal Ltd., East Finchley, London, U.K.) were attached through the muscle at the base of the dorsal fin. If different year classes were maintained in the same tank different coloured tags were used to provide rapid identification of the age of a fish. The tags were secured by punching plastic pins through the twin holes in the tag and then through the body muscle using a tagging gun (Kimbal Systems Ltd., Leicster, U.K.) more commonly used by the clothing industry for attaching labels. Some tag losses were experienced during initial experiments due to the plastic pins pulling out through the muscle layer after entanglement with the net. This was remedied by using a double tagging technique whereby one tag was placed on either side of the dorsal fin. Tags were also examined at each sampling time and any loose tags re-secured.

When small numbers of fish required individual identification or where fish needed to be separated on a group basis a Panjet (F.H. Wright, Dental MFG Company Ltd., West Dundee, U.K.) was used to mark fish on their ventral surface with alcian blue dye (1% w/v in water; Sigma Chemical Company Ltd.). The Panjet, which is commonly used in dentistry for the induction of local anaesthesia, fires a fine stream of dye under high

pressure which is able to penetrate the fish's skin. Varying the numbers and/or positions of the dye markings on each fish provided a code for identification. Markings remained visible for up to a year but usually required one renewal during the first 6 months.

2.1.4 Blood sampling.

Blood samples were usually taken via the Cuverian sinus of phenoxyethanol anaesthetised fish although occasionally broodstock were bled by cardiac puncture and juvenile fish via the caudal dorsal aorta. For broodstock the blood was withdrawn into 5ml serum monovettes (Sarstedt Ltd., Leicster, U.K.) fitted with 21G sterile hypodermic needles (Gillette U.K. Ltd., Middlesex, U.K.) whereas for juveniles blood was withdrawn into 2ml syringes (Terumo Europe N.V., Leuven, Belgium) rinsed with ammonium heparin (4mg/ml, 179.1 units/mg; Sigma Chemical Company Ltd.) and fitted with 19G needles (Gillette U.K. Ltd.). After removal of the needle heparinised blood was transferred into new polystyrene tubes (LP3; Luckhams Ltd., Burgess Hill, Sussex, U.K.) and kept on ice prior to centrifugation (MSE Chilspin; Fisons Scientific Equipment Ltd., Loughborough, Leics., U.K.) at 2500 rpm (4°C) for 15 minutes. Serum monovettes were spun similarly after allowing the blood to clot. The resulting serum or plasma was transferred into new polystyrene tubes (LP3; Luckhams Ltd.), stoppered and either assayed immediately or stored at -20°C (serum) or -70°C (plasma) for future analysis.

2.2 Assessment of spawning time.

Rainbow trout will mature and ovulate in captivity but will not spawn. Fish were therefore examined at approximately monthly intervals outside the expected spawning period and at 2-weekly intervals as the fish approached maturity. Ripe (mature) females were recognised by distension and softening of the abdomen and the extrusion of the urogenital papilla which becomes swollen and reddish in colour. Ripe males, required for fertilisation of the eggs, were distinguished by a darkening flesh colour and the development of a pronounced kype (extension of the lower jaw). Anaesthetised females were lifted from the water head upwards and then, if found to be ripe, the eggs expressed

from the abdominal cavity by exerting gentle downward pressure on the abdomen starting just behind the pectoral fins; this process is termed 'stripping'. The point at which eggs could be stripped from an individual fish was defined as the time of spawning for that fish.

2.3 Egg Measurements.

The eggs from individual fish were stripped into clean dry 1 litre plastic jugs and the fish then weighed. Milt obtained from a minimum of 3 fish was checked under a microscope for motility in ovarian fluid and added to the eggs at a concentration of approximately 1ml to 10,000 eggs (~1ml/litre). The eggs and milt were mixed gently by hand and allowed to stand for a minimum of 2 minutes. After fertilisation the eggs were gently washed with 2 or 3 changes of clean running water to remove excess milt and debris. The eggs were allowed to stand for a further 30-45 minutes during which time they absorbed water, a process termed 'water hardening'. When the eggs had fully expanded they became hard and firm and the following measurements could be carried out safely and accurately:

2.3.1 Egg diameter.

For each fish the mean egg diameter was estimated by counting (to the nearest half of an egg) the number of water hardened eggs aligned along a 120mm V-shaped plastic measuring groove. Mean egg diameter was then calculated as follows:

Mean egg diameter (mm) = 120/Number of eggs along groove.

The accuracy of this method has been validated by comparisons with individual egg diameter measurements made with callpers; there was no significant difference (Student's t-test) between the two methods (Springate, 1985).

2.3.2 Fecundity.

The water hardened eggs were separated from the water using a plastic sieve, carefully poured into a graduated beaker and the egg volume estimated to the nearest 10ml. The number of eggs produced by each female, the total fecundity, was then calculated from the following equations:

$Y = -0.283X + 5.41$

Total fecundity = (antilogY)(Z/1000)

where: $Y = \log_{10}$ (number of eggs per litre)

X = egg diameter (mm)

Z = egg volume (ml)

These equations were derived from those of von Bayer (1950; cited in Leitritz and Lewis, 1976) by Springate (1985) who found there was a highly significant correlation ($r = 0.998$, $P \leq 0.001$) between actual counts of egg numbers and 'von Bayer' determinations.

The number of eggs produced per kilogram of body weight, the relative fecundity, was calculated as follows:

Relative fecundity (eggs/kg) = total fecundity/post-stripped weight of fish (kg).

2.3.3 Gonadosomatic index.

The state of maturity of some fish was assessed by calculation of the gonadosomatic index (GSI) which expresses gonad weight as a percentage of body weight:

GSI = (gonad weight (kg)/body weight (kg)) x 100.

2.4 Determination of total serum calcium.

Total serum calcium provides a valid index of vitellogenin levels in the female rainbow trout (Elliott et al. 1984). Serum calcium was determined fluorometrically using a commercially available calcium analyser (model 940; Corning Scientific Instruments, Medfield, Massachusetts, U.S.A.).

Principle: calcein, a fluorescein derivative, forms an intensely fluorescent non-dissociated complex with calcium in an alkaline medium (potassium hyroxide); this fluorescence is quenched by chelating the calcium with the titrant ethylene glycol tetra-acetic acid (EGTA).

The following protocol was followed for routine analysis:

1. Warm up the instrument (15 minutes).
2. Fill the cuvette with 1N potassium hydroxide and add 100µl calcein indicator solution.
3. Add 100µl calcium standard solution (10mg%) to cuvette, check for fluorescence and press the 'titrate' button.
4. Add 20µl calcium standard, titrate and record result. Repeat. If the two standards agree within the limits of precision (4% for 20µl samples) press the 'calibrate' button.
5. Add 20µl aliquot of unknown serum sample, titrate and record result. Repeat as necessary.

Aliquots of pooled serum with a calcium content of approximately 30mg% were used for quality control. The intra-assay coefficient of variation (section 2.7.2) was 2.80% and the inter-assay coefficient of variation was 4.87%.

2.5 Steroid Radioimmunoassays.

Serum samples were analysed for oestradiol-17ß and testosterone according to the established methods of Duston and Bromage (1987).

2.5.1 Assay buffer.

The following buffer constituents were dissolved in distilled water over a magnetic stirrer/hotplate and made up to a final volume of 500ml:

Disodium hydrogen phosphate	8.88g
Sodium dihydrogen phosphate	5.82g
Sodium chloride	4.50g
Gelatine	0.50g
Sodium azide	0.03g

All chemicals were supplied by BDH Chemicals Ltd., Poole, Dorset, U.K. (Analar grade when available) except for sodium azide (Fisons Ltd., Scientific Equipment Division, Loughborough, U.K.). Phosphate buffer (pH 7.0) was stored at 4°C for up to a week.

2.5.2 Antisera.

Rabbit anti-oestradiol-17β antiserum (Steranti research Ltd., St. Albans, Herts., U.K.) was raised against 17β-oestradiol-6-(CMO)-BSA prepared by o-carboxy-methyl-oxime formation at the steroid '6' position and coupling to bovine serum albumin (BSA). Rabbit anti-testosterone antiserum (Steranti research Ltd.) was raised against testosterone-3-(CMO)-BSA prepared by o-carboxy-methyl-oxime formation at the steroid '3' position and coupling to BSA. Cross reaction data are presented in Table 2.1. The freeze-dried antiserum was reconstituted with 1ml of assay buffer and transferred in 100µl aliquots to polystyrene tubes (LP3; Luckhams Ltd.) which were stored at -20°C until required. The working solution was prepared by diluting one 100µl aliquot to 10ml with assay buffer (sufficient for 100 tubes).

2.5.3 Radiolabel.

[2,4,6,7-^{3}H]oestradiol, specific activity 85-110 Ci/mmol, and [1,2,6,7-^{3}H] testosterone, specific activity 80-105 Ci/mmol, were obtained in 250µCi quantities from Amersham International Ltd., Amersham, Bucks, U.K. An intermediate solution was prepared by diluting 10µl of the stock label to 2ml with absolute ethanol. An aliquot of the intermediate solution was dried down under nitrogen and reconstituted in assay buffer to prepare a working solution of approximately 20,000 dpm/100µl, the concentration recommended by Abraham (1974).

2.5.4 Standards.

A stock standard solution of 100ng/ml was prepared by dissolving 1µg dry oestradiol-17β or testosterone (both from Steranti research Ltd.) in 10ml absolute ethanol. This solution was stored at -20°C. The working solution (10ng/ml) was prepared freshly for each assay by diluting 100µl stock standard to 1ml with absolute ethanol. Serial dilution of 100µl aliquots of the working solution provided standards of 7.8 to 1000 pg/tube for the standard curve.

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

Steroid	Cross-reactivity (%)	
	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-hydroxyprogesterone	1.0	1.0
17α-hydroxy-20β-dihydroprogesterone	1.0	1.66
Pregnenolone	1.0	3.3
Cortisol	1.0	1.0

Cross-reactivity is expressed as the diminution in the proportion of bound radiolabel produced by 100pg of steroid relative to that produced by either oestradiol-17β or testosterone (from Duston and Bromage, 1987).

2.5.5 Dextran-coated charcoal.

One 'Separex' dextran-coated charcoal tablet (Steranti research Ltd.) was dissolved in 50ml of assay buffer and stirred continuously on ice for 30 minutes before use.

2.5.6 Method.

Duplicate standards and samples were assayed according to the following protocol:

A) Extraction.

1. Add 100µl of each serum sample to separate polypropylene tubes (LP4; Luckhams Ltd.).
2. Add 2ml ethyl acetate (Analar; BDH Chemicals Ltd.) to each tube and tightly stopper.
3. Attach tubes to a rotary mixer for 1 hour.
4. Centrifuge at 1500 rpm (4°C) for 10 minutes.

At this stage extracts may be stored at 4°C if desired.

N.B. Ethyl acetate extracts 90-100% of oestradiol-17ß and testosterone from the serum and hence a recovery step was not included in the routine assay procedure.

B) Assay.

5. Transfer 100µl (oestradiol-17ß) or 50µl (testosterone) of each extract to rimless soda glass assay tubes (R.B. Radley and Company Ltd., Sawbridgeworth, Herts., U.K.).

N.B. A smaller volume of extract was generally used for the testosterone assay because of the comparatively high serum testosterone levels found in maturing female rainbow trout.

6. Prepare a series of dilutions of the standard hormone with absolute ethanol in glass assay tubes covering the range 0-1000 pg/100µl.
7. Dry down extracts and standards in a vacuum oven at less than 35°C.
8. Cool dry tubes to 4°C.
9. Add 100µl of anti-oestradiol-17ß or anti-testosterone antiserum to each tube.
10. Add 100µl of tritiated oestradiol-17ß or tritiated testosterone to each tube.
11. Vortex mix each tube for 10 seconds and incubate at 4°C overnight.
12. Add 0.5ml of dextran-coated charcoal to each tube (stirring during addition at 4°C), vortex mix and incubate at 4°C for 10 minutes.

13. Centrifuge at 2000 rpm (4°C) for 10 minutes.

14. Transfer 0.4ml of the supernatant (total volume 0.7ml) into glass scintillation vials (Canberra Packard, Pangbourne, Berks., U.K.) containing 9ml Optiphase 'safe' scintillation fluid (Pharmacia Ltd., Milton Keynes, Bucks., U.K.).

15. Vortex vials thoroughly and count the radioactivity for 5 minutes in a scintillation counter (Tri-Carb 2660/2000CA; Canberra Packard). Include 2 further vials containing 100µl tritiated hormone for estimation of the total radioactivity added to each tube and 1 vial containing scintillation fluid only for automatic subtraction of the background counts.

C) Calculations.

16. Multiply the mean total dpm added to each tube by 0.4/0.7 (to correct for the difference between the total reagent volume per tube and the volume of the supernatant counted).

17. Calculate the percentage binding of standards and samples relative to the corrected total counts (percentage binding = (standard or sample dpm/mean total dpm)x 100).

18. Plot the percentage binding of the standards against concentration on log-linear graph paper (Figure 2.4) and read the concentrations of the samples from the standard curve.

19. Multiply by 0.21 (oestradiol-17ß) or 0.42 (testosterone) to correct samples for volume of extract assayed (100 or 50µl from a total of 2.1ml; x 21 or 42), and volume of serum extracted (100µl; x 10) and to convert to ng/ml (x 1/1000).

The sensitivity of the assays, defined as the smallest quantity of oestradiol-17ß or testosterone statistically distinguishable from the zero standard, was 7.8pg/tube.

D) Quality control.

Aliquots of pooled serum containing approximately 8ng/ml oestradiol-17ß and 30ng/ml testosterone were used for quality control. For oestradiol-17ß, the intra-assay coefficient of variation (section 2.7.2) was 10.90% and the inter-assay coefficient of variation was 15.54%. For testosterone, the intra-assay coefficient of variation was 5.28% and the inter-assay coefficient of variation was 13.96%.

2.6 Melatonin radioimmunoassay.

Serum and plasma samples were analysed for melatonin by a direct radioimmunoassay adapted from that described by Fraser *et al.* (1983) for the measurement of melatonin in human plasma.

2.6.1 Assay buffer.

The following buffer constituents were dissolved in 150ml of freshly deionised water in a polystyrene specimen container (Sterilin Ltd., Hounslow, Middx., U.K.):

Tricine (N-Tris(hydroxymethyl)methylglycine)	2.688g
Sodium chloride	1.350g
Gelatine	0.150g

The container was placed in a water bath (~50°C) for 30 minutes to dissolve the gelatine. All chemicals (Analar when available) were supplied by BDH Chemicals Ltd. Tricine buffer (pH 5.5) was prepared freshly for each assay and maintained at 4°C prior to use.

2.6.2 Antiserum.

Sheep anti-melatonin antiserum (Guildhay Antisera Ltd./Stockgrand Ltd., Guildford, Surrey, U.K.) was raised against N-acetyl-5-methoxytryptophan conjugated through the side chain to bovine thyroglobulin. Two batches are available; batch number 704/6483 was used for all measurements described in this thesis. Comparative percentage cross-reactions (melatonin taken as 100) are 0.91 for N-acetyltryptamine, 0.33 for 6-hydroxymelatonin, 0.22 for N-acetyltryptophan and ≤ 0.06 for all other structurally related compounds (data supplied by Guildhay Antisera). Supplied freeze-dried the antiserum was reconstituted with 2ml of deionised water to provide an intermediate dilution of 1:10. Aliquots of 100µl were transferred into polystyrene tubes (LP3; Luckhams Ltd.) and stored at -20°C. The working solution was prepared by diluting one 100µl aliquot to 20ml with assay buffer. This provided sufficient reagent for 100 tubes with an initial dilution of 1:2000.

2.6.3 Radiolabel.

[O-methyl-^{3}H]melatonin, specific activity 70-85 Ci/mmol, was obtained in 250µCi quantities from Amersham International Ltd. An intermediate solution was prepared by diluting 20µl of the stock label to 2ml with absolute ethanol (AR grade; Fisons Ltd.). Storing the intermediate solution in polystyrene containers resulted in a decrease in measured radioactivity; glass containers were therefore used for storage of these solutions. The stock and intermediate solutions were stored at -20°C. The working solution was freshly prepared for each assay by further diluting the intermediate solution with assay buffer to give approximately 4000 dpm/100µl.

2.6.4 Standards.

A stock standard solution of 1mg/ml was prepared by dissolving 10mg melatonin (N-acetyl-5-methoxytryptamine; Sigma Chemical Company Ltd.) in 10ml absolute ethanol (Fisons Ltd.). This solution was stored at -20°C. The standards were freshly prepared for each assay as follows:

A) 100µl (1mg/ml) made up to 10ml with assay buffer (=10µg/ml)

B) 100µl (10µg/ml) made up to 10ml with assay buffer (=100ng/ml)

C) 100µl (100ng/ml) made up to 10ml with assay buffer (=1ng/ml)

D) 100µl (100ng/ml) made up to 5ml with assay buffer (=2ng/ml)

Serial dilution of 250µl aliquots of solution C with assay buffer provided standards in the range 3.9-250 pg/tube for the standard curve. Solution D allowed for the inclusion of a 500 pg standard if required.

2.6.5 Melatonin-free serum and plasma.

Melatonin-free serum or plasma was prepared by charcoal stripping of serum or plasma collected from fish during the photophase according to the following protocol:

1. Prepare a 10% w/v suspension of charcoal (activated, untreated; Sigma Chemical Company Ltd.) in serum or plasma in 30ml polystyrene 'universal' containers (Sterilin Ltd., Hounslow, Middx., U.K.).

2. Shake for 1 hour on ice in a shaking water bath (Camlab Ltd., Cambridge, U.K.).

3. Centrifuge at 1500 rpm (4°C) for 30 minutes.

4. Decant supernatant and resuspend in charcoal at 10% w/v.

5. Repeat steps 2 and 3.

6. Decant supernatant and centrifuge at 3000 rpm (4°C) for 15 minutes.

7. Decant supernatant and centrifuge at 20,000 rpm (30,000g; 4°C) for 30 minutes (L8-55M ultracentrifuge; Beckman Instruments, Inc., High Wycombe, Bucks., U.K.).

8. Filter supernatant through Millex-GV 0.22µm filter units (Millipore S.A., Molsheim, France).

9. Divide pooled serum or plasma into 6ml portions (sufficient for 1 standard curve) and store at -20°C in 7ml polystyrene 'Bijou' bottles (Sterilin Ltd.).

Before use each pool was checked against the previous pool to ensure that the serum or plasma was free of melatonin (percentage binding indistinguishable from that of the zero standard).

2.6.6 Method.

Duplicate standards and samples were assayed according to the following protocol:

A) Assay.

1. Prepare a series of dilutions of melatonin standard with assay buffer in polystyrene assay tubes (LP3; Luckhams Ltd.) covering the range 0-500pg/250µl.

2. Add 250µl of assay buffer to sample tubes and 450µl of assay buffer to a further 2 tubes to be used for estimation of non-specific binding.

3. Add 250µl of melatonin-free serum or plasma to standards and non-specific binding tubes and vortex mix.

4. Add 250µl aliquots of samples to sample tubes and vortex mix.

5. Add 200µl of antiserum to each tube, excepting the non-specific binding tubes, vortex mix and incubate at room temperature for 30 minutes.

6. Add 100µl of tritiated melatonin to each tube, vortex mix and incubate for 18 hours at 4°C.

7. Add 500µl dextran-coated charcoal (section 2.4.5) to each tube (stirring during addition at 4°C), vortex mix and incubate at 4°C for 15 minutes.

8. Centrifuge at 2000 rpm (4°C) for 15 minutes.

9. Transfer 1ml of the supernatant (total volume 1.3ml) into glass scintillation vials (Canberra Packard) containing 9ml Optiphase 'safe' scintillation fluid (Pharmacia Ltd.).

10. Vortex vials thoroughly and count the radioactivity for 10 minutes in a scintillation counter (Canberra Packard). Include 2 further vials containing 100µl tritiated melatonin for estimation of the total radioactivity added to each tube and 1 vial containing scintillation fluid only for automatic subtraction of background counts.

B) Calculations.

11. Multiply the mean total dpm by 1/1.3 (to correct for the difference between the total reagent volume per tube and the volume of the supernatant counted).

12. Subtract the mean non-specific binding dpm from that of the standards and samples.

13. Calculate the percentage binding of standards and samples relative to the corrected total counts (% binding= (standard or sample dpm/mean total dpm)x100).

14. Plot the percentage binding of the standards against concentration on log-linear graph paper (Figure 2.4) and read the concentrations of the samples from the standard curve.

15. Correct to pg/ml serum or plasma (multiply by 4 for 250µl samples).

The sensitivity of the assay, defined as the smallest quantity of melatonin statistically distinguishable from the zero standard, was 3.9-7.8pg/tube.

C) Quality control.

Aliquots of pooled serum taken from Atlantic salmon parr during the scotophase, and containing approximately 400 pg/ml of melatonin, were used for quality control. The intra-assay coefficient of variation (section 2.7.2) was 1.94% and the inter-assay coefficient of variation was 7.25%.

D) Validation.

Inhibition curves obtained from serial dilutions of pooled rainbow trout or Atlantic salmon serum collected during the scotophase were parallel to the standard curve (Figures 2.5 and 2.6). For both species analysis of covariance (calculated using an in-house

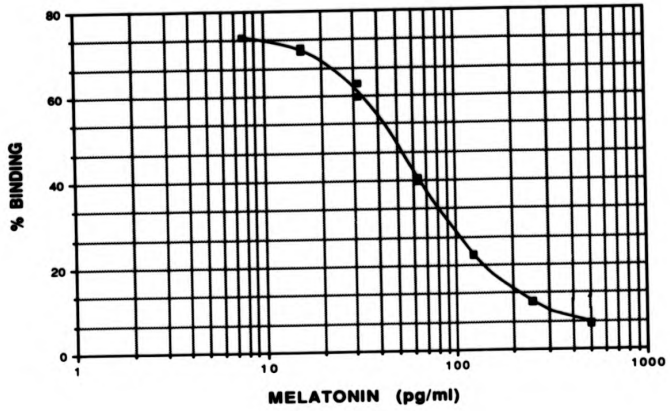


Figure 2.4: A typical standard curve from a radioimmunoassay (this example is from a melatonin assay performed in experiment 1 of chapter 4). The concentration of hormone in a sample is obtained by intersecting the standard curve at the point corresponding to the percentage binding (percentage of radiolabel bound to antibody) in the sample.

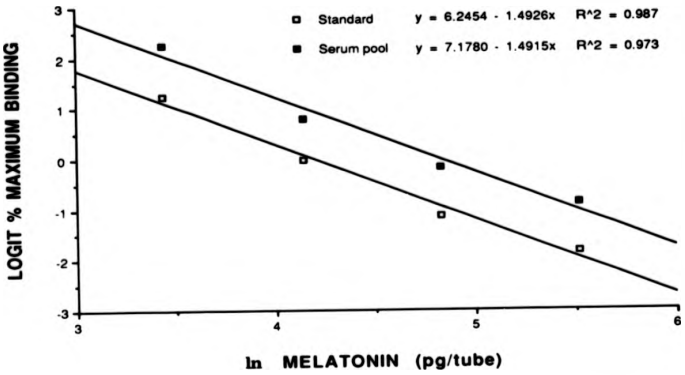


Figure 2.5: Parallelism of an inhibition curve obtained from a serial dilution (1:2) of 500µl aliquots of pooled rainbow trout serum (collected during darkness) with the melatonin assay standard curve. The two curves have been linearised using the logit transformation (Rodbard and Lewald, 1970): logit b = ln (b/100-b) where b is the proportion of radiolabel bound to antibody expressed as a percentage of that in the zero standard (% maximum binding). Each point represents the mean of duplicates. The scale on the x-axis denotes the natural log of the melatonin content in the standards.

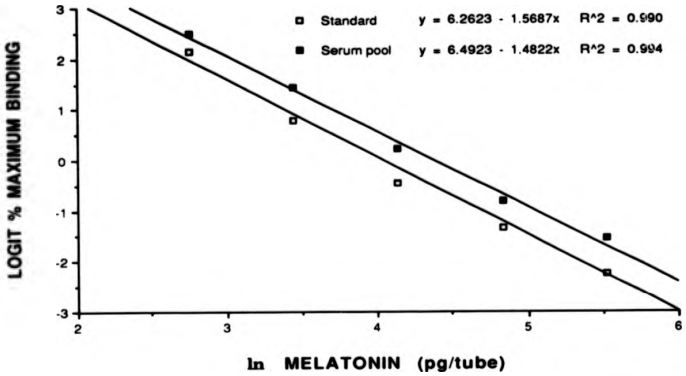


Figure 2.6: Parallelism of an inhibition curve obtained from a serial dilution (1:2) of 250 µl aliquots of pooled Atlantic salmon serum collected during darkness with the melatonin assay standard curve. The two curves have been linearised using the logit transformation (Rodbard and Lewald, 1970): logit b = ln (b/100-b) where b is the proportion of radiolabel bound to antibody expressed as a percentage of that in the zero standard (% maximum binding). Each point represents the mean of triplicates. The scale on the x-axis denotes the natural log of the melatonin content in the standards.

computer programme, courtesy of M. A. Thrush) revealed no significant difference ($P \geq 0.05$) between the slopes of the standard curve and the inhibition curve obtained from pooled serum. This indicates that the 'melatonin' detected in rainbow trout and Atlantic salmon serum was immunologically similar to the standard hormone.

Since plasma provides a greater sample volume than serum, melatonin was determined in plasma samples when experiments were conducted on juvenile fish. Prior to using plasma an experiment was performed to assess the effect of ammonium heparin on the melatonin assay. Blood samples (1.5-2ml) were taken from twelve 1-year old rainbow trout maintained under an 8L:16D artificial daylength during a period 1.5-2.5 hours after darkness onset. Approximately equal volumes of blood from each fish were dispensed into either empty polystyrene assay tubes (LP3; Luckhams Ltd.) or assay tubes containing 1 drop (approximately 10µl) ammonium heparin (4mg/ml, 179.1 units/mg; Sigma Chemical Company Ltd.). After centrifugation and storage at -20 °C (section 2.1.4) the nine un-haemolysed pairs of serum and plasma samples were assayed for melatonin as detailed above. There was a highly significant positive correlation ($r = 0.955$; $P \leq 0.001$) between melatonin concentrations determined in serum and those determined in plasma (Figure 2.7). There was no significant difference between mean serum melatonin (368 ± 56pg/ml) and mean plasma melatonin (352 ± 54pg/ml) concentrations (Student's t-test for matched pairs; Sokal and Rohlf, 1981), indicating that ammonium heparin does not interfere with the melatonin radioimmunoassay and that comparisons between experiments using serum samples and those utilising plasma samples are valid.

2.7 Statistical methods

Unless otherwise indicated detailed descriptions of the following statistical methods can be found in Snedecor and Cochrane (1980) and Sokal and Rohlf (1981).

2.7.1 Estimation of the mean

The arithmetic or sample mean, $\bar{x}$, provides the best estimate of the population mean, μ:

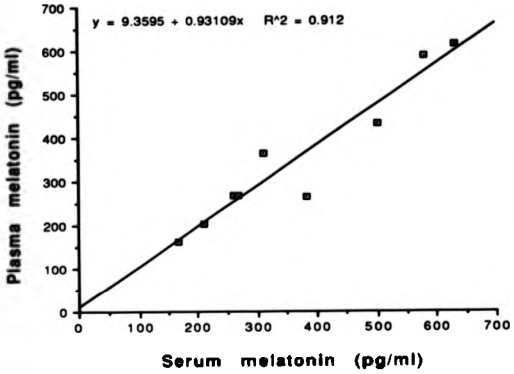


Figure 2.7: Correlation (r=0.955; P≤0.001) between the concentrations of melatonin detected by radioimmunoassay in heparinised (plasma) and non-heparinised (serum) aliquots of blood samples taken from rainbow trout during darkness.

$\bar{x} = \Sigma x/n$ where: n = number of observations

Σx = sum of the observations

Estimates of the sample mean are presented ± 1 standard error of the mean (± 1SEM):

SEM = $s/\sqrt{n}$ where: n = number of observations

s = sample standard deviation = $\sqrt{\dfrac{\Sigma x^2 - (\Sigma x)^2/n}{n-1}}$

2.7.2 Coefficient of variation

The coefficient of variation (C.V.) is a measure of relative variability and therefore allows the comparison of variation in populations with different means:

% C.V. = $(s \times 100)/\bar{x}$.

2.7.3 Homogeneity of variances

Homogeneity of variances was tested using the F-test, which tests for the departure of the variance ratio of two samples from unity:

$$F_s = \frac{\text{greater variance } (s_1^2)}{\text{lesser variance } (s_2^2)}$$ Degrees of freedom $v_1, v_2 = n_1 - 1, n_2 - 2$

Only the largest and smallest variances were compared when more than two samples were tested for homogeneity. If the calculated value for F_s was less than the tabulated value of F at P=0.05 (5%) it was concluded that the variances were homogenous. If F_s was greater than the tabulated value for F at P=0.05 it was concluded that the variances were heterogenous.

2.7.4 Comparison of two samples

If the variances of the two samples were homogenous (section 2.7.3), the means were compared using Student's t-test utilising a pooled estimate of the variance. If the variances were heterogenous the means were compared using Student's t-test utilising separate estimates of each variance and reduced degrees of freedom (see Parker, 1979 for details). The calculations for both tests were performed on a Hewlett-Packard mainframe computer using the 'Minitab' statistical package (Ryan et al., 1981). If the calculated value of t was greater than the tabulated value for t at P=0.05 (5%) or less the difference between means was concluded to be statistically significant.

2.7.5 Multiple comparisons

Providing the sample variances were homogenous (section 2.7.3), one-way analysis of variance (ANOVA) was used for the preliminary comparison of the means of three or more samples. The calculations were performed using the 'Minitab' statistical package. If a statistically significant (P≤0.05) variation between means was detected by ANOVA differences between pairs of means were tested for significance using the following equation:

$$t_s = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(s^2(1/n_1 + 1/n_2))}} \qquad \text{with } n_1 + n_2 - 2 \text{ degrees of freedom}$$

where: $\bar{x}_1$ and $\bar{x}_2$ = means of each of the two samples

n_1 and n_2 = number of obeservations in each of the two samples

s^2 = error mean square from the ANOVA

If the calculated value for t_s was greater than the tabulated value for t at P=0.05 (5%) or less the difference between means was concluded to be statistically significant.

If the sample variances were heterogenous (section 2.7.3), the Kruskal-Wallis test

was used for the preliminary comparison of three or more samples. The calculations were performed using the 'Minitab' statistical package. If a statistically significant difference ($P \leq 0.05$) was detected between groups by the Kruskal-Wallis test differences between pairs were tested for significance using Dunn's multiple comparisons procedure (Zar, 1984) as follows:

$$Q_{0.05,\,k} = \bar{R}_2 - \bar{R}_1 / SE$$

where: $\bar{R}_1$ and $\bar{R}_2$ = mean ranks of each of the two samples

(e.g. $\bar{R}_1$ = rank sum, R_1 / n_1)

$$SE = \text{standard error} = \sqrt{\left[\left(\frac{N(N+1)}{12} - \frac{\Sigma(t^3 - t)}{12(N-1)}\right)(1/n_1 + 1/n_2)\right]}$$

N = total number of observations in all (k) groups

t = number of ties for a given (tied) value

n_1 and n_2 = number of observations in each of the two samples

The calculations were performed using an in-house computer programme (courtesy of M. A. Thrush). If the calculated value for Q was greater than the tabulated value for Q at $P=0.05$ (5%), for a number of groups, k, the difference between groups was concluded to be statistically significant.

2.7.6 Comparison of proportions

The standard error of a proportion was calculated as follows:

$$S.E. = \sqrt{\frac{p(1-p)}{n-1}}$$

where: p = sample proportion

n = number of observations

Confidence limits were then calculated as ± 1.96 x S.E (95%) or ± 2.58 x S.E. (99%). If the 95% or 99% confidence limits of two proportions did not overlap it was concluded that they were significantly different at $P \leq 0.05$ or $P \leq 0.01$ respectively.

CHAPTER THREE

PHOTOPERIODIC CONTROL OF REPRODUCTION IN THE RAINBOW TROUT

3.1 Introduction

In common with the vast majority of organisms inhabiting temperate and polar latitudes, reproduction in salmonids is an annual event, with spawning confined to a brief (typically 6 week) period each year. This seasonality ensures that the fry emerge at a time when the local environmental factors which dictate their survival (ultimate factors), such as temperature and most importantly food availability, are at their most favourable. Breeding cannot be initiated instantaneously when ultimate factors become optimal, however, since gonadal development may take a year or more to complete (Scott and Sumpter, 1984; Bromage and Cumaranatunga, 1988). The initiation and modulation of reproductive development is therefore timed with reference to earlier predictive (proximate) environmental cues. In fish both temperature and photoperiod have been identified as important proximate cues but in salmonids the primary environmental influence on reproductive timing appears to be the seasonally changing daylength (de Vlaming, 1972; Scott and Sumpter, 1983; Bye, 1984; Dodd and Sumpter, 1984; Lam and Munro,1987; Bromage and Cumaranatunga, 1988).

The first recorded experiments on the modification of salmonid reproduction by light were conducted by Hoover and Hubbard (1937). They subjected brook trout (Salvelinus fontinalis), which had spawned once previously in December, to 1 additional hour of light per day during the first week of February, 2 additional hours in the second week, and so on, until the excess over the natural daylength reached 8 hours. The photoperiod was maintained at this level for 4 weeks after which it was rapidly decreased to approximately 7 hours by the time the fish spawned in August, 4 months in advance of their natural spawning period. This technique was subsequently used commercially and over a 4 year period groups of about 2000 females consistently spawned 3-4 months in advance of the natural stock (Hazard and Eddy, 1951). Similar compressed (accelerated) seasonal light cycles were used by Corson (1955), Nomura (1962) and Henderson (1963) to advance maturation in the brook trout. The latter author, however, found the method to be effective only in fish which had matured previously; virgin fish were apparently unresponsive.

Kingsbury (1952; cited by Henderson, 1963) and Buss (1960) used the same

approach to achieve comparable advances in the spawning time of brown trout (Salmo trutta). A large number of studies have also confirmed the effectiveness of compressed seasonal photocycles for the advancement of maturation in both virgin and previously matured rainbow trout, Oncorhynchus mykiss (Nomura, 1962; Goryczko, 1972; Kunesh et al., 1974; Whitehead et al., 1978, 1980; Buss, 1980, 1982; Bromage et al., 1982a; Pohl et al., 1982; Elliott et al., 1984; Bromage, 1987; Meiners-Gefken et al., 1987, 1988; Pohl-Branscheid and Holtz, 1990). Nakari et al. (1987, 1988) observed a similar acceleration of gonadal development in rainbow trout under compressed photocycles but spawning only occurred in fish transferred to water at 10°C; the very low ambient water temperature (0.4°C) experienced by other fish in these experiments inhibited ovulation (see section 1.2). Most of these studies utilised light cycles compressed into 6 or 9 month periods which usually advanced spawning time by about 12 and 6 weeks respectively. The sequences of hormonal changes associated with the reproductive cycle were also advanced consistent with the advances in spawning time. Conversely, subjecting rainbow trout to a seasonal light cycle expanded to 18 months delayed spawning by 3 months (Bromage et al., 1984). Interestingly, exposure of rainbow trout to a seasonal photocycle compressed into 3 months resulted in the desynchronization of maturation with spawning occurring over a 6 month period (Pohl et al., 1982). The possible significance of this observation will be discussed later.

MacQuarrie et al. (1978) adopted a slight variation of the same technique to induce off-season spawning of the coho salmon (Oncorhynchus kisutch), a Pacific salmonid which normally matures at 3 years of age. By compressing or expanding by 4 months a 13.7 month period encompassing the second year of development, and then rearing the fish under a simulated natural light cycle shifted out of phase to maintain the time differences attained, spawning was advanced by 3 months and delayed by 5 months respectively. Similarly, in pink salmon (Oncorhynchus gorbuscha), which normally mature at 2 years of age, compressing an 8.3 month portion of the first year of development by 3 months, or expanding it by 3 or 6 months, and subsequently maintaining these time differences under a phase-shifted seasonal photoperiod, achieved an advance of 2 months and delays of 4 and

7 months respectively (MacQuarrie et al., 1979). When these authors maintained the progeny of the 7 month delayed group under a 7 month phase-shifted seasonal photoperiod spawning occurred exactly 2 years later, 7 months after fish raised under natural daylength. Similarly, McCormick and Naiman (1984) delayed spawning by 3 months in brook trout by subjecting them to a simulated seasonal photocycle phase-shifted 3 months behind the natural cycle, and Elliott et al. (1984) advanced maturation by 6-7 months in rainbow trout exposed to an artificial seasonal light cycle 180° out of phase with the natural photoperiod. It is of interest to note that the first indications of the importance of daylength in salmonid reproduction were in fact observed many years before the first experimental studies when brown and rainbow trout were translocated from their native northern areas to sites in the southern hemisphere. These fish re-adjusted their maturation cycles so that spawning occurred 6 months out of phase (at precisely the same daylength in the seasonally changing photocycle) with corresponding stocks in the northern hemisphere (Bromage et al., 1989; Scott, 1990).

Compressed and phase-shifted seasonal photoperiods have also been used to modify maturation in a number of non-salmonid fish. Thus, sea bass (Dicentrarchus labrax), turbot (Scophthalmus maximus) and gilthead sea bream (Sparus aurata) maintained on a seasonal photocycle compressed into 10 months spawned, on average, 5 months ahead of the controls after 3 years (Girin and Devauchelle, 1978). Turbot subjected to compressed cycles of 9 and 6 months duration matured 3-4 and 5-6 months in advance of the natural spawning period respectively, whereas expanding the photocycle to 15 months caused a 2-3 month delay in spawning (Bye, 1987). Compressed 6 month photocycles also advanced spawning by 2-3 months in another marine flatfish, the dab (Limanda limanda), and a simulated seasonal photocycle phase-shifted 3 months ahead of the natural light cycle advanced maturation in the sole (Solea solea) by just over 2 months (Bye, 1987). Moreover, Worthington et al. (1982) found that accelerating the changes in daylength by twice the natural rate for just 10 weeks advanced spawning by 3-4 months in the roach (Rutilus rutilus), a freshwater cyprinid.

Although varying the period of the natural photocycle confirmed the importance of

daylength as a predictive cue for reproductive timing in salmonid fish it provided little information on the mechanisms involved. In this respect more abrupt changes in daylength and the substitution of different phases of the natural photocycle with constant 'long' or 'short' photoperiods has proved more informative.

A series of studies initiated in the late 1970's by French workers (reviewed by Billard, 1985) involved abrupt increases from the ambient daylength to 16L:8D (16 hours of light and 8 hours of dark per day) at various times between January and April. The photoperiod was then reduced in weekly increments reaching 8L:16D within 6 months. This advanced the maturation of rainbow trout by 2-6 months relative to their natural spawning time of late December; the earlier the regime commenced the greater was the advance in spawning time. These experiments were conducted in the laboratory at constant temperature but similar procedures were effective in advancing the spawning time of both rainbow and brown trout on commercial farms with naturally fluctuating temperatures (Breton et al., 1983b).

The stimulatory effect of decreasing photoperiods on gonadal maturation was consistent with the view that gametogenesis in salmonids occurred in the summer and autumn months (Billard and Breton, 1978; Billard et al., 1978; Billard, 1982). Additionally, elevated levels of GTH (Breton and Billard, 1977), vitellogenin (measured as calcium or phosphoprotein phosphorus) and oestradiol-17β (Whitehead et al., 1978) had been associated with decreasing photoperiods. Several authors therefore concluded that decreasing daylengths were the most important factor for the stimulation of gonadal development in salmonids, that is, they were 'short-day' animals (Breton and Billard, 1977; Billard and Breton, 1978; Billard et al., 1978; Peter, 1981; Follett, 1982). This hypothesis was supported by the apparent inhibitory effects of long daylengths on salmonid reproduction. Thus, spawning was delayed by 6 weeks when mid-autumn (October/November) spawning brook trout were maintained under a daylength artificially extended to 17 hours in August or September (Allison, 1951; Hazard and Eddy, 1951) and by 2-3 months in fish exposed to 20L:4D from late April (Henderson, 1963). Combs et al. (1959) observed a 1 month delay in the maturation of early autumn

(September/October) spawning sockeye salmon (Oncorhynchus nerka) maintained under continuous light (LL) from late July and Shiraishi and Fukuda (1966) reported delays of 2-3 months in early autumn spawning sockeye and amago (Oncorhynchus rhodurus) salmon and late autumn (November/December) spawning strains of brook and rainbow trout subjected to 16L:8D or LL from early June. Similarly, Atlantic salmon (Salmo salar) exposed to 20L:4D from early August matured 6 weeks after the October spawning time of their counterparts under natural photoperiod (Eriksson and Lundquist, 1980; Lundquist, 1980). A slight delay (2-3 weeks) was also observed in early autumn spawning chinook salmon (Oncorhynchus tshawytscha) reared under 16L:8D for 3 months from the summer solstice (Johnson, 1984). The most extensive delay reported was for mid-autumn spawning masu salmon (Oncorhynchus masou) in which exposure to 18L:6D from close to the summer solstice delayed maturation by at least 5 months (Takashima and Yamada, 1984).

Further investigations on the rainbow trout indicated a variability in response according to the natural spawning time of the strain studied. Thus, constant 16L:8D or LL from the summer solstice, or 16L:8D gradually increased to LL over 6months, all caused a 2 month delay in maturation of a November spawning strain (Bourtier and Billard, 1984a,b). Fish which naturally spawn in December/January showed a 1 month delay in maturation when exposed to 18L:6D from June (Skarphedinsson et al., 1982). Whitehead and Bromage (1980) and Bromage et al. (1982b), however, reported that fish exposed to 16L:8D from the summer solstice spawned in January and February at approximately the same time as the controls.

A further example of the influence of natural spawning time on the response to long photoperiods was observed by Scott (unpublished; cited in Bye, 1984). He found that maintenance under an 18L:6D daylength from March delayed maturation in an autumn spawning (October/November) strain but advanced maturation in a winter (January) spawning strain. Thus, in agreement with the studies previously described, constant long days during the later stages of the reproductive cycle delayed maturation. Constant long days during the earlier part of the cycle, however, advanced maturation and spawning

time. Similar results have been achieved in a number of studies; exposure of late autumn and winter spawning strains of rainbow trout to 18L:6D or LL from January or February advanced spawning time by 6-10 weeks (Whitehead and Bromage, 1980; Bromage et al., 1982a, 1984; Duston and Bromage, 1986a, 1987, 1988). Furthermore, continued maintenance of these fish under constant long photoperiods produced subsequent spawnings at approximately 6 month intervals (Bromage et al., 1984; Bromage and Duston, 1986). Interestingly, 6 month cycles can be induced immediately if exposure to long days or LL occurs at or very close to spawning time (Skarphedinsson et al., 1982; Scott et al., 1984).

The discovery that long photoperiods were stimulatory when applied early in the reproductive cycle prompted several workers to re-label salmonids as 'long-day' animals (Bromage et al., 1982b; Elliott et al., 1984; Scott et al., 1984; Bromage and Duston, 1986). This assessment was supported by more detailed hormonal and histological studies. These revealed that the hormonal changes associated with the initiation of the second reproductive cycle in female rainbow trout begin at least 9 months prior to spawning (Scott and Sumpter, 1983). Moreover, the first signs of increased hormonal activity and oocyte development in virgin fish occur at least 1 year before ovulation (Sumpter, 1984; Sumpter et al., 1984). Thus, gonadal recrudescence begins much earlier than had previously been thought.

There are also marked variations in the effects of constant short days on reproduction. Exposing winter spawning rainbow trout to 6L:18D from shortly after the natural spawning period delayed maturation by between 1 and 5 months dependent on the time of introduction onto the short photoperiod and the natural spawning time of the strain used (Bromage et al., 1984; Duston and Bromage, 1986a, 1987). Fish subjected to constant short days during the middle third of the reproductive cycle (or slightly later), however, respond with an advance in maturation and spawning time. This was first demonstrated by Combs et al. (1959) who advanced the spawning time of early autumn spawning sockeye salmon by 2-3 weeks by abruptly reducing the photoperiod to either 5 or 13 hours less than ambient from mid-July. When Shiraishi and Fukuda (1966) subjected early autumn

spawning sockeye and amago salmon and late autumn spawning brook and rainbow trout to an abrupt decrease from ambient daylength to 8L:16D in early June spawning was advanced by 4-6 weeks with the greatest advances observed in the trout. Similarly, Whitehead and Bromage (1980) achieved a 3 month advance in the spawning time of winter spawning rainbow trout exposed to a sudden reduction from ambient photoperiod to 8L:16D at the summer solstice. Again it is clear, however, that the extent of the response is dependent on both the timing of the switch to the short photoperiod and the natural spawning time of the strain under investigation.

In contrast, Eriksson and Lundquist (1980) found that an abrupt change from natural daylength (17L:7D) to 7L:17D in early August had no effect on maturation of autumn spawning precocious male Atlantic salmon. However, the same authors reported a significant advance in spawning when the decrease was accomplished in gradual steps over 20, 40 or 80 days; the shorter the period of deceleration the greater the advance in spawning time. They suggested that the rate of change of photoperiod (differential effect) rather than the actual daylength (proportional effect) is of most importance in this species. A similar effect has not been demonstrated in other salmonid species. Thus, rainbow trout subjected to a seasonal light cycle compressed into 6 months spawned 3 months in advance of the controls; subsequent exposure of these fish to the first half of another 6 month photocycle in combination with an abrupt reduction to 8L:16D at the longest day caused a similar advance in spawning time (Whitehead et al., 1980). Similarly, when November spawning rainbow trout were subjected to 17L:7D from early February, followed by an abrupt reduction to 6L:18D in early May, the same 3 month advance in spawning time was observed as when the decrease was achieved in 3 weekly reductions of 3-4 hours each (Bromage et al., 1984). In each case an abrupt reduction to a constant short photoperiod was as effective in advancing spawning as a gradually changing photoperiod.

One of the most stimulatory photoperiod regimes is a combination of constant long photoperiods early in the reproductive cycle followed by an abrupt reduction to constant shorter photoperiods after 3-4 months, an example of which has just been described. In

general a much greater advance in spawning time (3-4 months) is achieved with this combination than with long photoperiods alone (1.5-2.5 months). As before, the extent of the response is determined by the timing of the changes in photoperiod and the natural spawning time of the strain used; advances in spawning time of between 6 weeks and 4 months have been reported for the rainbow trout (Whitehead and Bromage, 1980; Bromage et al., 1982b, 1984; Elliott et al., 1984; Duston and Bromage, 1987, 1988). Similarly, a detailed study by Takashima and Yamada (1984) on the effects of constant long photoperiods (18L:6D or LL) followed by constant short photoperiods (6L:18D) found that spawning of masu salmon could be advanced by 2-4 months according to the time of exposure. The mechanisms underlying this response will be considered later in the chapter.

Constant photoperiod regimes have also been used to modify maturation in non-salmonid fish. The following account concentrates on those species in which photoperiod appears to be the primary environmental cue. The response of the sea bass to photoperiod manipulation is similar in many respects to that of salmonids although temperature also appears important in this species (Carillo et al., 1989a; reviewed by Carillo et al., 1989b). Sea bass in south eastern Spain spawn between January and March. Exposure to 1 month of long days (15L:9D) between April and August in an otherwise short day (9L:15D) regime advanced spawning by between 2 weeks (August) and 9 weeks(April). Fish subjected to 1 month of long days in September, or to 15L:9D from the summer solstice reduced gradually to 9L:15D by the end of February, were delayed in their maturation by 1 month and 3 months respectively. Thus, as in salmonids, long days early in the reproductive cycle appear to stimulate gametogenesis whereas long days later in the cycle delay gonadal development. The North Sea dab, which spawns naturally in March, also showed delayed ovarian development when exposed to constant long days (16L:8D or 20L:4D) from early August, and ovarian development (but not ovulation) was advanced when the photoperiod was reduced from ambient to constant short days (4L:20D or 8L:16D) from July or August (Bye, 1987). Turbot in the North Sea typically spawn between May and July. Extending the period of long days which naturally occur at spawning

time by 2 months delayed the subsequent spawning by 2 months in these fish, whereas 2 months of short days immediately after spawning followed by constant long days advanced spawning by several months (Bye, 1984). It has therefore been suggested that gametogenesis in turbot is initiated by short winter days and accelerated by the increasing daylengths following the winter solstice (Bye 1984, 1987). In the stickleback (Gasterosteus aculeatus), which spawns from April-July, the initial stages of gametogenesis appear to be independent of photoperiod and temperature. However, full maturity can be advanced by up to 4 months by exposure to 16L:8D from November, at which time the fish remain unresponsive to short days: as the natural spawning time approaches the fish gradually become more responsive to short photoperiods until by February and March all are capable of attaining maturity, even under 8L:16D (Baggerman, 1980). If an 8L:16D photoperiod is continued after spawning, however, maturation will not occur again (Baggerman, 1980) suggesting an obligate requirement for long days.

The foregoing account has reviewed the effects of the various experimental photoperiod regimes used to investigate the control of reproduction in salmonids and a few non-salmonid species. Procedures which have been found to either advance or delay maturation in salmonids are illustrated in Figures 3.1 and 3.2 respectively. To summarise: 1) long photoperiods applied early in the reproductive cycle advance spawning but maturation is delayed by the same photoperiods applied during the later stages of development. 2) short photoperiods applied from shortly after the natural spawning time delay subsequent maturation but short photoperiods applied during the middle third or so of the reproductive cycle advance spawning. The principle hypothesis arising from these observations was that the lengthening daylengths of late winter and spring initiate gametogenesis whereas the shortening daylengths of late summer and autumn accelerate the later stages of reproductive development (Scott and Sumpter, 1983b; Bye, 1984; Scott et al., 1984).

More recently, however, attention has focussed on the possibility that, rather than actively driving (inducing) reproductive development, photoperiod may exert its effects

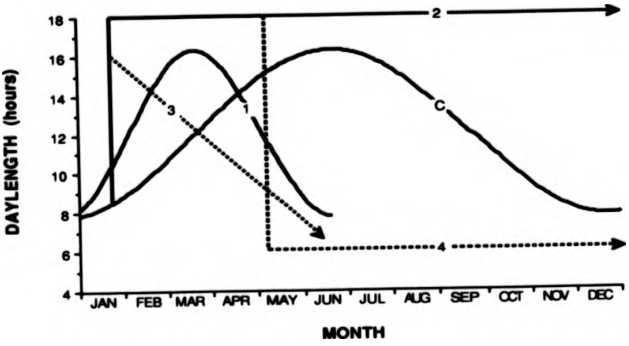


Figure 3.1: Photoperiod regimes used to advance maturation in salmonids. 1) Compressed seasonal photocycle. 2) Constant 'long' photoperiod. 3) Advanced decreasing photoperiod. 4) 'Long' days followed by 'short' days. C) Control; natural seasonal photoperiod. Adapted from Billard (1985).

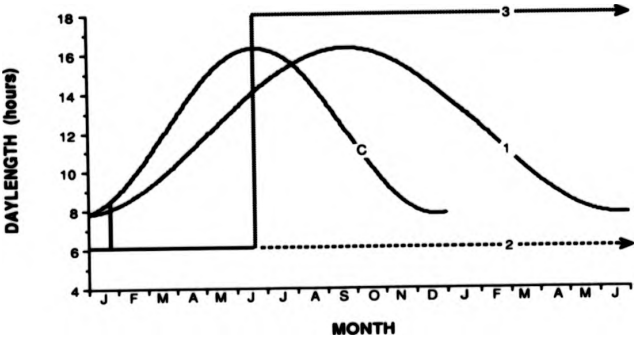


Figure 3.2: Photoperiod regimes used to delay maturation in salmonids. 1) Expanded seasonal photocycle. 2) Constant 'short' photoperiod. 3) Constant 'short' photoperiod, or ambient daylength, followed by constant 'long' photoperiod. C) Control; natural seasonal photoperiod.

by entraining (synchronizing) an endogenous circannual rhythm of maturation. The essential principles of entrainment are illustrated in Figure 3.3. They are derived from the general oscillator model developed by Aschoff and Pittendrigh for the analysis of circadian systems, in which the behaviour of biological rhythms is equated to that of physical oscillators (for more detailed accounts see Aschoff, 1981; Pittendrigh, 1981a). Under constant environmental conditions endogenous biological rhythms (internal 'clocks' or 'oscillators') free-run revealing their natural, self-sustained, periodicity (Figure 3.3a). For a circannual system this will be close to, but significantly different from, 1 year. Under natural conditions, however, endogenous rhythms do not free-run but are entrained to periodic changes in the environment, such as the seasonal changes in photoperiod. The endogenous rhythm is thereby modified, such that it adopts the same periodicity as the entraining agent, or zeitgeber ('time-giver': German; Aschoff,1960), and also adopts a particular phase relationship to it (Figure 3.3b). A prerequisite for entrainment is that the endogenous rhythm possesses a periodically changing sensitivity to the stimulus provided by a particular time cue, such that the cue causes adjustments (displacements along the time axis: phase shifts) of different magnitude and sign depending on the phase at which the rhythm is perturbed. Thus, an appropriate environmental cue may cause the rhythm to adopt a new phase either in advance of (Figure 3.3c), or behind (Figure 3.3d) the previous phase, or may have no effect at all, according to when it is applied. This property is clearly an essential component of the entrainment process since an environmental signal which had the same effect at all times would not be a useful timing cue.

To establish that an annual cycle is an endogenous circannual rhythm a number of criteria should be satisfied: 1) under constant environmental conditions the rhythm should free-run with a periodicity approximating to, but significantly different from, 1 year, and this should be demonstrable over at least 2 complete cycles. 2) the period of the rhythm should be relatively independent of temperature, that is, temperature compensated. 3) the rhythm should be capable of entrainment by a yearly zeitgeber so that it assumes a period of exactly 12 months. 4) the rhythm should possess a differential

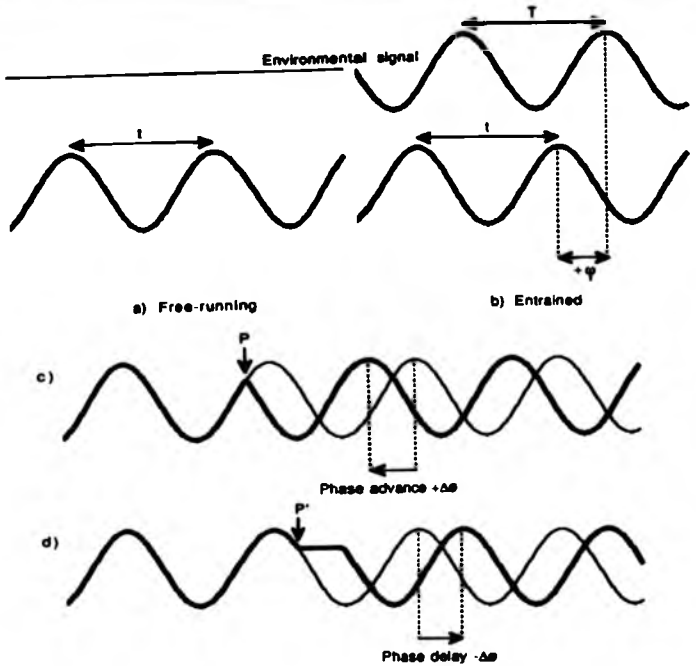


Figure 3.3: Schematic illustration of the principles of entrainment. a) An endogenous rhythm free-runs under constant environmental conditions revealing its natural period (t). b) An endogenous rhythm is entrained by a periodic environmental signal, with a period length T, such that t is now equal to T. Note that in this illustration t in free-run is less than T and hence the entrained rhythm phase leads (+Ψ) the environmental cycle. c) An endogenous rhythm in free-run is subjected to a perturbation P, causing an advance phase-shift (+Δø). d) An endogenous rhythm in free-run is subjected to a perturbation P_1, causing a delay phase-shift (-Δø). Adapted from Aschoff, 1981 (a and b) and Saunders, 1977 (c and d).

sensitivity to the phase-shifting effects of the zeitgeber according to the phase of the rhythm perturbed (Saunders, 1977). It is not surprising that all these conditions are seldom met and in practice most emphasis is usually placed on the first criterion.

Although endogenous annual rhythms had been implicated in a number of studies dating back as far as the early 18th century (reviewed by Gwinner, 1986) the first convincing experimental evidence for their existence came from the studies of Pengelley and Fisher (1957, 1963). They demonstrated clear circannual rhythms of hibernation and the associated parameters of body weight and food consumption in golden-mantled ground squirrels (Spermophilus lateralis) maintained under 12L:12D and constant temperature for 23 months. The period of the annual hibernation cycle under these conditions was about 10 months over 2 cycles and was later shown to persist for at least 5 cycles (Pengelley and Asmundson, 1969). Circannual rhythms persisting under constant environmental conditions for at least 2 cycles with periods deviating significantly from 1 year have since been demonstrated in approximately 40 organisms and less conclusive evidence exists for considerably more (Gwinner, 1986).

The first clear evidence of an endogenous circannual rhythm in fish was provided by Sundararaj and colleagues (Sundararaj et al., 1973, 1976, 1982; Sundararaj, 1978). They found that Asian catfish (Heteropneustes fossilis) maintained under DD or LL and constant temperature for 34 months showed a circannual rhythm of ovarian weights comparable to that seen in fish under natural conditions but, in LL at least, the rhythm had a period length significantly different from 1 year. Circannual rhythms in reproductive activity and behavioural thermoregulation have since been shown to persist for at least 2 cycles in the stickleback (Baggerman, 1980) and the white sucker, Catostomus commersoni (Kavaliers, 1982) respectively. There is some evidence suggesting that circannual rhythmicity may also participate in the reproductive activity of a number of marine fish. Thus, dabs maintained under 12L:12D and constant temperature for 18 months underwent a similar sequence of changing gonad weight and spawning time as fish under a simulated natural cycle or in the wild (Htun Han, 1975; cited in Bye, 1984, 1987). Turbot exposed to 16L:8D and restricted (9-18°C) but not constant temperature

for over 2 years spawned at 6-8 month intervals and exhibited a marked desynchronization of spawning time between individual fish (Bye, 1987). Sole subjected to 18L:6D and restricted (11-15°C) temperature for 3 years also became desynchronized with spawning eventually occurring over a 6 month period, four times longer than normal (Bye, 1987). Such desynchronization is suggestive of individuals free-running on different duration circannual cycles such that they gradually drift out of phase with each other. In the sea bass Carillo et al. (1989b) have reported circannual rhythms in reproductive activity over 2 or 3 cycles under constant 15L:9D and 9L:15D respectively but temperature was not controlled. Under both photoperiods, however, the period length of the rhythm was significantly different from 1 year.

The rather equivocal results of Poston and Livingstone (1971), continuing the work of Pyle (1969), provided some initial evidence for circannual rhythmicity in salmonids. Brook trout were maintained from the age of 5 months under LL, DD or simulated natural photoperiod and constant temperature. At 2 years of age all the fish matured at about the same time except for the males in LL which spawned 8 weeks early. These fish subsequently spawned again 38 weeks later while male fish under a simulated natural photoperiod maintained a spawning periodicity of 1 year and spawning was delayed by 1 month in DD. Spawning in females was delayed by 1 month in LL and 2 months in DD in the second reproductive cycle suggesting that the period of any endogenous rhythm may differ in males and females. Eriksson and Lundquist (1982) reported 10 month cycles of growth and smoltification in Atlantic salmon reared under 12L:12D and constant temperature for 14 months. In the rainbow trout Whitehead et al. (1978) observed that fish maintained under 12L:12D and constant temperature spawned at approximately the same time as the controls and that changes in the serum components associated with reproduction occurred just before those under the normal yearly cycle. A subsequent study found that rainbow trout maintained under similar conditions matured after 49 rather than 52 weeks (Bromage et al., 1982b). Further studies utilising a range of other constant photoperiods (6L:18D,18L:6D,18L:6D, LL) over the majority of the reproductive cycle indicated that spawning always occurs eventually but is often desynchronized and usually has a period

length different from 1 year (Whitehead and Bromage, 1980; Bromage et al., 1982b, 1984; Skarphedinsson, 1982; Scott et al.,1984). Unfortunately, these experiments, though suggestive of endogenous rhythmicity, are not entirely convincing since the rhythms were followed for only 1 cycle or showed period lengths atypical for a circannual rhythm.

A further indication of circannual rhythmicity is implicit in the results of previously described studies which used compressed and expanded seasonal light cycles. Under the various experimental regimes spawning did not occur at the same phase of the seasonal cycle as under natural daylength. This suggests that photoperiod does not directly induce reproductive development. Figure 3.4 shows the data of Whitehead et al. (1978) and Bromage et al. (1984) plotted to show changes in the phase-angle (Ψ: see Figure 3.3) of spawning time under seasonally changing photocycles of varying periodicity. The time of the shortest photoperiod ('winter solstice') was used as the phase reference point in each case and the photocycle was divided into 360°. It is clear that the spawning phase of the reproductive cycle occurs at a progressively later phase of the photocycle as the period of the photocycle is decreased. This decrease in phase-angle difference with decreasing zeitgeber period, which is well established for entrained circadian systems (Aschoff, 1978, 1981), has been found in all studies in which circannual rhythms have been entrained to zeitgeber cycles of varying period (Gwinner, 1981).

The most compelling evidence for the existence of endogenous circannual rhythms in salmonids has recently been obtained by Duston and Bromage (1986a). They maintained female rainbow trout under constant LL, 18L:6D or 6L:18D and constant temperature for at least 3 successive spawning periods. Under constant long photoperiods spawning was advanced by 2 months during the first year of treatment and then occurred at approximately 6 month intervals over 4 successive cycles. Under constant short days spawning was initially delayed by up to 5 months and then occurred at approximately 1 year intervals over 2 successive cycles, maintaining the time difference relative to the controls. The spawning time of individual fish became desynchronized under all three regimes. These results are strongly indicative of free-running endogenous rhythms of

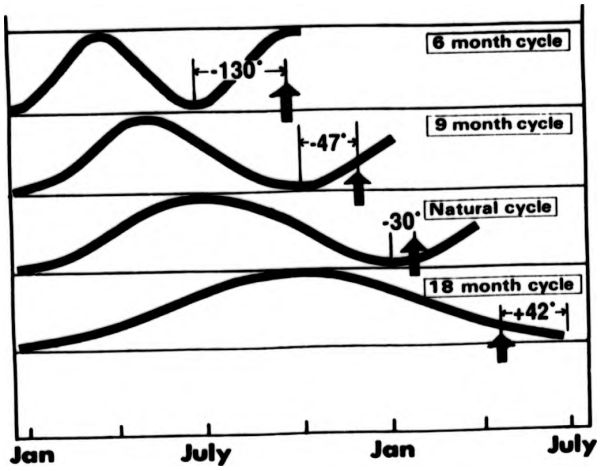


Figure 3.4: Effects of compressed and expanded seasonal light cycles on the timing of spawning in rainbow trout. The time of spawning under each regime is indicated by an arrow. Each photocycle is divided into 360° and the phase-angle differences are calculated relative to the winter solstice. Under the experimental photocycles spawning does not occur at the same phase of the seasonal cycle as under natural daylength. Note also that spawning occurs at a progressively later phase of the photocycle as the period of the photocycle is decreased, i.e. there is a decrease in phase-angle difference with decreasing zeitgeber period. From Bromage et al. (1989).

reproduction, although, as in some previous studies (Bromage et al., 1984; Scott et al., 1984), the period length under LL was atypical for a circannual rhythm.

With the demonstration of a circannual clock it became apparent that many of the experimental results detailed previously could be explained in terms of the entrainment of the clock by photoperiod acting as a zeitgeber. Thus, the advance in spawning time caused by a reduction from a constant long to a constant short photoperiod could be interpreted as a phase advance of the endogenous clock (Duston and Bromage, 1987, 1988). The ability to cause such phase-shifts is an obligate requirement of a zeitgeber of an endogenous rhythm. In this chapter the probable existence of an endogenous circannual rhythm controlling reproduction in the female rainbow trout provides the foundation for experiments designed to clarify the mechanisms by which photoperiod entrains this clock. Two series of experiments were conducted. Those described in Section A examine the effects of various 'long' to 'short' photoperiod regimes on reproductive timing and the entrainment of the clock. Section B similarly investigates the ability of short periods of LL to cause phases-shifts of the circannual clock with additional emphasis on the development of a simple, cheap and predictable method for the commercial production of 'out-of-season' eggs without the need for blackout facilities. As a corollary these experiments provide a further test of the hypothesis that maturation in the female rainbow trout is ultimately under endogenous control.

3.2 Section A: The Effects of 'Long' to 'Short' Changes in Photoperiod on the Entrainment of the Annual Cycle of Reproduction in the Female Rainbow Trout.

Gwinner (1986) states that four types of experiments can be performed to test whether an environmental variable is a zeitgeber of a circannual rhythmicity: 1) exposure of a free-running rhythm to an environmental cycle; the former should assume the period of the latter if it is an effective zeitgeber. 2) varying the period of the environmental cycle; the period of the endogenous rhythm should (within certain limits) follow changes in the period of the environmental cycle. 3) phase-shifting the environmental cycle; the endogenous rhythm should follow that phase-shift within a few cycles. 4) exposure of animals maintained under constant conditions to pulsatile or stepwise changes of an environmental variable; if it is a zeitgeber the pulse or step should produce a phase-shift, the magnitude and direction of which should depend on the phase of the circannual rhythm exposed to the stimulus (i.e a phase-response curve should be available). Experiments in the rainbow trout (detailed in section 3.1) have demonstrated the effectiveness of photoperiod as a zeitgeber for the annual reproductive cycle using procedures 2 (compressed and expanded seasonal photocycles), 3 (phase-shifting the seasonal photocycle by 180°) and 4 (abrupt changes in photoperiod). The experiments described in this section utilised procedure 4 to investigate the mechanisms by which photoperiod entrains the endogenous circannual clock which, it is proposed, controls reproduction in the female rainbow trout.

Recent work (Duston and Bromage, 1987, 1988) has indicated that an abrupt reduction from a constant 'long' photoperiod to a constant shorter photoperiod can advance maturation by 3-4 months in the female rainbow trout. These studies have emphasised the importance of an abrupt reduction in daylength as an entraining cue for the advancement of spawning time. However, all the experiments used 18L:6D as the 'long' photoperiod and hence any effects that this daylength *per se* may have had on spawning time were undetectable. Consequently, experiments 1, 3 and 4 were principally designed to discriminate the effects of changes in daylength from those of daylength *per se* (absolute

daylength). This question was initially addressed in experiment 1 by exposing fish to a variety of constant 'long' days (18L:6D, 14L:10D or 10L:14D) from mid-January (ambient daylength 8.5L:15.5D), followed by an abrupt reduction to a constant 'short' day (6L:18D) in early May. The range of 'long' days examined was extended in experiments 3 and 4 (10L:14D, 12L:12D, 14L:10D, 16L:8D, 18L:6D, 20L:4D and 22L:2D), in which the variability in the magnitude of the reduction in daylength inherent in experiment 1 (12, 8 and 4 hours) was negated by decreasing the photoperiod by 8.5 hours (which approximates to the difference in daylength between the summer and winter solstice at the latitude of the experiments) in all groups. Two additional groups of fish were included to test the hypothesis that maturation can be advanced even in fish which do not experience an increase in daylength in spring (i.e. remain on a winter photoperiod) provided they receive a decrease to an even shorter photoperiod prior to the summer solstice. Thus, fish were maintained on either 6L:18D (experiment 1) or 8.5L:15.5D (experiment 4) from late January, and then subjected to an abrupt decrease in photoperiod to 2L:22D (experiment 1) or 1.5L:22.5D (experiment 4) in early May.

This section also includes a preliminary investigation of the importance of the position (in relation to the phase of the reproductive cycle) and duration of exposure to a given photoperiod (more thoroughly examined in section B). In a recent study only 26% of female rainbow trout exposed to a 'long' photoperiod from mid-January to early March attained early maturity, whereas 77% of those subjected to 'long' days from mid-January to early May matured early (Duston and Bromage, 1988). This suggests that both the duration and the position of the 'long' photoperiod may be important determinants of the proportion of fish responding with an advance in maturation. In order to discriminate between these two possibilities a supplementary group of fish were exposed to a 'long' photoperiod from late March to early May in experiment 1. The importance of duration of exposure was further investigated in experiment 3 by comparison of two groups maintained under either constant 'short' days or exposed to 1 week of 'long' days in an otherwise 'short' day regime.

3.2.1 General methods.

All fish used in experiments 1-4 came from an established domesticated stock with a natural spawning period of November-December. At the start of each experiment fish were transferred from ambient daylength (latitude 52°30'N) to one of 6 circular photoperiod tanks (section 2.2.1; Figure 2.1). The tanks were supplied with constant temperature (7.5-8°C) spring water and tungsten filament light bulbs provided a light intensity of 25-30 lux at the water surface. Fish were individually tagged (section 2.1.3) and weighed upon transfer. When 2 groups of fish were maintained in the same tank they were dye marked (section 2.1.3) to facilitate group identification in the event of a tag loss.

The rate of maturation under the various photoperiod regimes was assessed by measuring serum calcium (as an index of vitellogenin; section 2.4) at approximately monthly intervals. As the fish approached maturity they were examined at 2-weekly intervals (section 2.2) and the spawning times of individual fish recorded for each group. Egg diameter, total egg volume and post-stripped body weight were measured for each fish and both total and relative fecundities calculated (section 2.3).

Unless otherwise stated spawning profiles and egg data were analysed by Student's t-test for homogenous variances (section 2.7.4) or one-way analysis of variance followed by a parametric multiple comparisons procedure (section 2.7.5). The proportions of fish attaining maturity were compared by calculation of confidence limits (section 2.7.6). Hormone data was analysed by Student's t-test for heterogenous variances (section 2.7.4) or the Kruskal-Wallis test followed by a non-parametric multiple comparisons procedure (section 2.7.5).

3.2.2 Experiment 1: The effects of a range of constant 'long' photoperiods followed by a reduction to constant shorter photoperiods on the timing of maturation in the female rainbow trout.

3.2.2.1 Protocol

Five groups of approximately 25 two-year old virgin female rainbow trout were transferred on January 17 from ambient daylength (8.5L:15.5D) to the following photoperiod regimes:

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

In addition to serum calcium, circulating oestradiol-17β and testosterone levels were measured (section 2.5) at approximately monthly intervals to provide an alternative estimate of maturation rate.

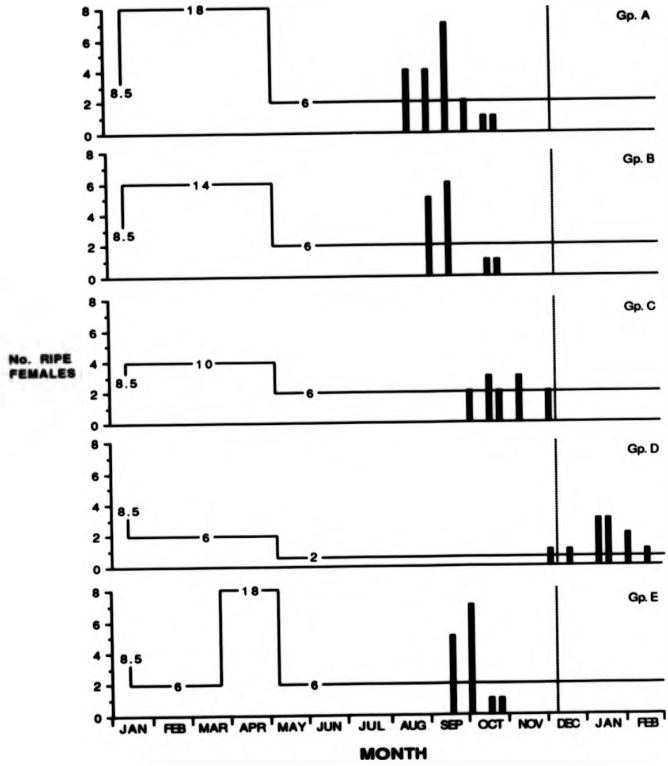
3.2.2.2 Results

Spawning

The spawning times of the individual fish in each group are illustrated in Figure 3.5. When the photoperiod was reduced to 6L:18D on May 8 from 18L:6D (Gp. A), 14L:10D (Gp. B) or 10L:14D (Gp. C) spawning commenced on August 23, September 3 and October 2 respectively, in each case considerably in advance of the natural spawning period. In Gps. A, B and C respectively 86, 72 and 66% of the fish attained maturity over periods of 10, 8 and 9 weeks.

Gp. D, which experienced a reduction in daylength from 6L:18D to 2L:22D in early May, commenced spawning on December 2, approximately 1 month after natural spawning

Figure 3.5 (opposite): The effects of five photoperiod regimes (Gps. A-E, experiment 1) on the timing of maturation in female rainbow trout. The histograms represent the number of ripe females on each sampling date. Line graphs indicate the photoperiod regime (hours of light/day) applied to each group. Dashed vertical lines indicate the mean spawning time of fish maintained under ambient daylength.


Gp. A
Gp. B
Gp. C
Gp. D
Gp. E
No. RIPE FEMALES
MONTH
JAN
FEB
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
NOV
DEC
JAN
FEB
8.5
18
6
14
10
2

began. During an 11 week period 75% of the fish in Gp. D attained maturity. On termination of the experiment on February 19 hormone measurements indicated that 2 of the 5 remaining immature fish in Gp. D would shortly have completed maturation had the experiment been allowed to continue.

Gp. E, which received 6 weeks of 'long' days from late March to early May in an otherwise 'short' day regime, commenced spawning on September 18, well in advance of the natural spawning period. During a 6 week period 70% of the fish in Gp. E attained maturity.

The differences in mean spawning times between groups were all significant at the $P \leq 0.001$ level with the exception of Gps. A vs E ($P \leq 0.05$), and Gps. A vs B and B vs E (N.S.). There were no significant differences in the proportions of fish attaining maturity.

Calcium

All groups exhibited significant changes ($P \leq 0.001$) in total serum calcium levels during the reproductive cycle. However, there were considerable differences in the timing of these changes (Figure 3.6), consistent with the differences in spawning time between groups. Thus, calcium levels in Gp. A began to increase in May, rose steeply through June to August, and peaked at about 61mg% in early September; these levels were significantly elevated ($P \leq 0.05$) compared to Gp. E in July, and Gp. D in July, August and September. In Gp. B serum calcium increased at a similar rate to Gp. A from May to August, but exhibited a lower and broader peak attaining a maximum of approximately 44mg% in August. Calcium levels in Gp. B were significantly higher ($P \leq 0.05$) than those in Gp. D during both July and August. In Gp. C the first substantial increase in calcium levels occurred between July and August, with a broad peak of about 33mg% extending from early September to late October; levels were significantly elevated ($P \leq 0.05$) at both October sampling points compared to those in Gp. D, and were also significantly higher ($P \leq 0.05$) in late October than those in Gp. A., which were approaching basal at this time. Serum calcium began to rise at about the same time in Gp. E as in Gp. C, but peaked higher, reaching approximately 51mg% in early October, at which time levels were significantly

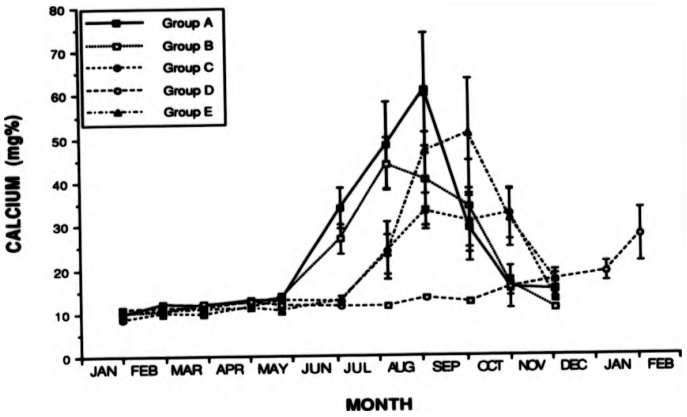


Figure 3.6: The effects of five photoperiod regimes (Gps. A-E, experiment 1) on the timing of changes in total serum calcium levels (mean ± 1SEM) during maturation in female rainbow trout (Gp. A, 18L:6D/6L:18D; n=5-12: Gp. B, 14L:10D/6L:18D; n=6-10: Gp. C, 10L:14D/6L:18D; n=5-12: Gp. D, 6L:18D/2L:22D; n=9-13: Gp. E, 6L:18D/18L:6D/6L:18D; n=9-15).

elevated (P≤0.05) compared to those in Gp. D. As calcium levels in Gps. A-D approached basal between late October and early December, levels in Gp. E began a gradual rise towards a maximum of about 27mg% at the last sampling point in early February.

Oestradiol-17β

All groups exhibited significant changes (P≤0.001, Gps. A, B and E; P≤0.01, Gp. C; P≤0.05, Gp. D) in serum oestradiol-17β levels during the reproductive cycle. However, there were considerable differences in the timing of these changes (Figure 3.7), consistent with the differences in spawning time between groups. Thus, oestradiol-17β levels in Gp. A began to increase in May, rose steeply through June and July, and peaked at about 25ng/ml in August; levels were significantly elevated (P≤0.05) compared to Gps. B, C and D in late May, Gp. C in early July, and Gp. D in August. Gp. B showed a sharp increase in oestradiol-17β levels between late May and July, reaching a peak of approximately 20ng/ml in August. A similar profile, with a peak of about 18ng/ml in August, was observed in Gp. E, although oestradiol-17β levels in September remained significantly elevated (P≤0.05) compared to those in Gp. B, which had returned to basal. Oestradiol-17β levels in Gp. C began to increase between July and August reaching a comparatively low peak of approximately 12ng/ml in September, at which point they were significantly higher (P≤0.05) than those in Gp. B. As oestradiol-17β levels approached basal in Gps. A-C and Gp. E, those in Gp. D rose sharply, attaining a level significantly higher (P≤0.05) than those in Gps. B, C and E by late October. Interestingly, oestradiol-17β levels in Gp. A began to increase again at the same time and were also significantly elevated (P≤0.05) compared to Gps. B, C and E by late October. Gp. D exhibited a more gradual increase in serum oestradiol-17β after October exhibiting a broad peak between December and the last sampling point in February, at which time levels had reached about 16ng/ml.

Testosterone

All groups exhibited significant changes (P≤0.001) in serum testosterone levels during the reproductive cycle. Differences in the timing of these changes were again

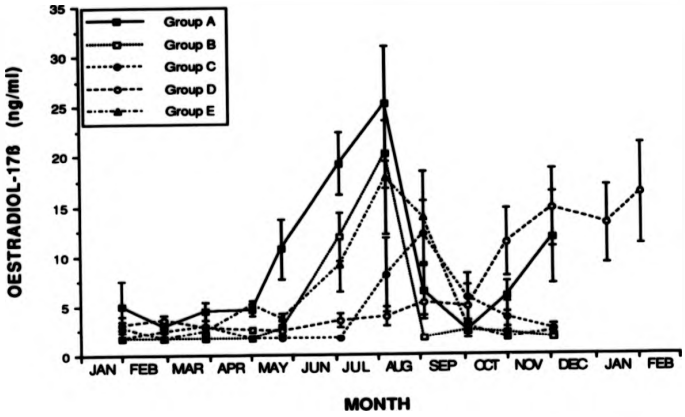


Figure 3.7: The effects of five photoperiod regimes (Gps. A-E, experiment 1) on the timing of changes in serum oestradiol-17β levels (mean ± 1SEM) during maturation in female rainbow trout (Gp. A, 18L:6D/6L:18D; n=5-12: Gp. B, 14L:10D/6L:18D; n=6-10: Gp. C, 10L:14D/6L:18D; n=4-12: Gp. D, 6L:18D/2L:22D; n=9-13: Gp. E, 6L:18D/18L:6D/6L:18D; n=9-15).

consistent with the differences in spawning time between groups (Figure 3.6), although, Gp. D apart, the differences between groups were less well defined than for calcium and oestradiol-17ß. Testosterone levels began to increase in Gps. B, C and E in May with the result that by late May levels were significantly elevated ($P \leq 0.05$) in Gps. B and E compared to Gp. D. Serum testosterone rose steeply in Gps. A, B and E between late May and early July, at which time levels were significantly higher ($P \leq 0.05$) in Gps. A and B than in Gp. D, and peaked at approximately 81ng/ml (Gp. A), 82ng/ml (Gp. B) and 106ng/ml (Gp. E) between August and September, before falling towards basal in October. Testosterone levels in Gp. A began to increase again between late October and December, in accordance with similar changes in oestradiol-17ß levels. In Gp. C testosterone levels increased slowly between May and July, rose steeply between July and August, and remained elevated through September and October to peak at about 104ng/ml in late October; levels were significantly elevated ($P \leq 0.05$) compared to Gp. A in early October and Gps. A and E in late October. Testosterone levels in Gp. D increased gradually from September onwards, were significantly higher ($P \leq 0.05$) than those in Gp. A by late October, and rose steeply between January and the last sampling point in February to reach a peak of about 129ng/ml.

Egg diameter and fecundity

Egg diameter and fecundity measurements are summarised in table 3.1. The most advanced spawning fish (Gps. A, B and E) produced significantly smaller eggs ($P \leq 0.001$) than the later spawners (Gps. C and D), but there were no significant differences in either total or relative fecundity.

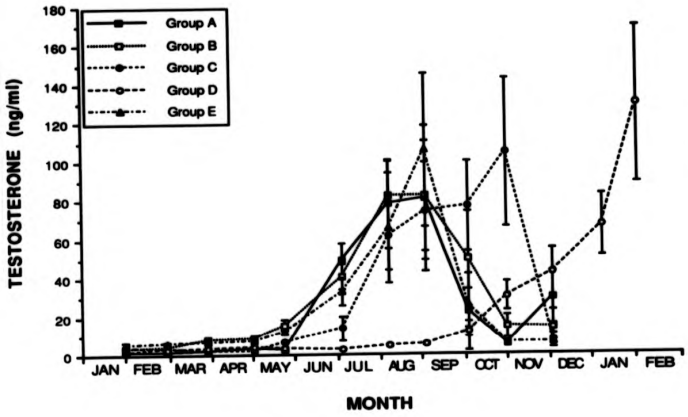


Figure 3.8: The effects of five photoperiod regimes (Gps. A-E, experiment 1) on the timing of changes in serum testosterone levels (mean ± 1SEM) during maturation in female rainbow trout (Gp. A, 18L:6D/6L:18D; n=5-12: Gp. B, 14L:10D/6L:18D; n=7-10: Gp. C, 10L:14D/6L:18D; n=5-12: Gp. D, 6L:18D/2L:22D; n=6-13: Gp. E, 6L:18D/18L:6D/6L:18D; n=6-14).

Table 3.1: Egg size and fecundity data from experiment 1.

Group	A	B	C	D	E
Spawning Period	Aug. 23-Oct. 31	Sept. 9-Oct. 31	Oct. 2-Dec. 2	Dec. 2-Feb. 19	Sept. 18-Oct. 31
% Maturation	86	72	66	75	70
Egg Diameter (mm.)	3.92 ± 0.08^{17}	3.86 ± 0.08^{14}	$4.33 \pm 0.03^{b\ 12}$	$4.51 \pm 0.05^{b\ 14}$	3.85 ± 0.09^{14}
Total Fecundity	2542 ± 126^{17}	2807 ± 232^{14}	2493 ± 140^{12}	2769 ± 68^{14}	2271 ± 192^{13}
Relative Fecundity[a]	2824 ± 180^{17}	3284 ± 200^{14}	2792 ± 168^{12}	2974 ± 166^{14}	2562 ± 192^{13}

All measurements are means (± 1SEM). The number of observations (n) per sample is indicated by the superscript on the right of each value.

a; number of eggs/kg.

b; significantly different ($P \leq 0.001$) from Gps. A, B and E.

3.2.3 Experiment 2: The effects of varying the time of an increase to a constant long photoperiod (18L:6D) on the timing of maturation in the female rainbow trout.

3.2.3.1 Protocol

Seven groups of female rainbow trout were transferred, over a period of 3 months, from ambient daylength to the following photoperiod regimes:

Group A - 18L:6D from December 2 until May 15 followed by 6L:18D until spawning.
Group B - 18L:6D from January 19 until May 15 followed by 6L:18D until spawning.
Group C - 18L:6D from December 23 until May 15 followed by 6L:18D until spawning.
Group D - 18L:6D from January 19 until May 15 followed by 6L:18D until spawning.
Group E - 18L:6D from January 19 until spawning.
Group F - 18L:6D from February 19 until May 15 followed by 6L:18D until spawning.
Group G - 18L:6D from February 19 until spawning.

Gps. A and B contained 19 and 18 fish respectively maintained in the same tank; these fish were distributed evenly from Gps. A, B, C and E which had spawned several months in advance of the natural spawning season after photoperiod treatment in experiment 1. Prior to transfer to 18L:6D they were maintained on 6L:18D. The remaining groups were comprised of 10 two-year old (2+) virgin fish and 15 three-year olds (3+) which had recently (prior to December 23) spawned naturally for the first time. These fish were maintained on ambient daylength until transfer.

3.2.3.2 Results

Spawning

The spawning times of the individual fish in each group are illustrated in Figures 3.9 (Gps. A and B) and 3.10 (Gps. C-G). In fish which had previously experienced an advance in spawning time in experiment 1 a reduction in photoperiod in May, following an increase to 18L:6D on December 2 (Gp. A) or January 19 (Gp. B), resulted in main spawning

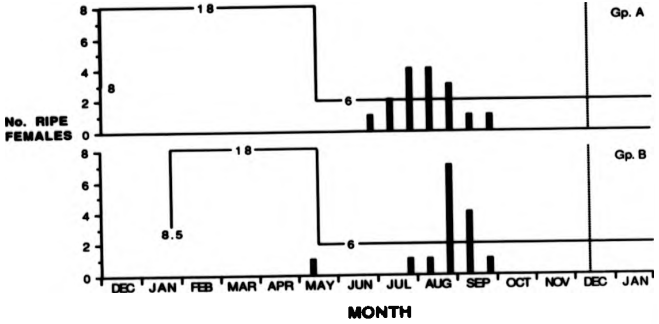


Figure 3.9: The effects of two photoperiod regimes (Gps. A and B, experiment 2) on the timing of maturation in female rainbow trout subjected to an advance in spawning time the previous year. The histograms represent the number of ripe females on each sampling date. Line graphs indicate the photoperiod regime (hours of light/day) applied to each group. Dashed vertical lines indicate the mean spawning time of fish maintained under ambient daylength.

periods commencing on June 29 and July 26 respectively. In Gp. A 100% of the fish attained maturity within a 12 week period and in Gp. B 94% of the fish matured over 8 weeks.

Groups of previously untreated fish, in which the reduction in photoperiod in May was preceded by an increase to a 'long' daylength on December 23 (Gp. C), January 19 (Gp. D) or February 19 (Gp. F), commenced spawning on August 26, August 26 and September 23 respectively. In Gps. C, D and F respectively 91, 84 and 67% of the fish attained maturity over periods of 6, 8 and 6 weeks.

Groups which received an increase in daylength only, on January 19 (Gp. E) and February 19 (Gp. G), commenced spawning on October 7 and November 5 respectively, considerably later than their counterparts in Gps. D and F. In Gp. E 86% of the fish spawned over a period of 10 weeks. Unfortunately, an interruption to the water supply resulted in the death of all the fish in Gp. G on December 2-3. Examination of mortalities on December 4 revealed that 2 had ovulated. The remaining 9 fish were assessed for maturity by visual inspection, measurement of oocyte diameter and ovary weight and calculation of the GSI (section 2.3.3). Seven of the fish appeared close to ovulation (GSI= 6.3-14.7) and would probably have matured by the next or subsequent visit (2-4 weeks). Two of the fish were assessed as immature (GSI= 0.25 and 0.57) and unlikely to spawn that year. On the basis of these estimates, which have been incorporated in Figure 3.10, 85% of the fish in Gp. G would have spawned over a period of 6-8 weeks.

The differences in mean spawning times between groups were all significant at the $P \leq 0.001$ level with the exception of Gps. A vs B and C vs D ($P \leq 0.01$), Gps. D vs F and E vs F ($P \leq 0.05$), and Gps. B vs C (N.S.), though it should be noted that the single fish which spawned in early May in Gp. B (Figure 3.9) and the single fish which spawned in early July in Gp. G (Figure 3.10) were excluded from the statistical analysis (i.e. were treated as 'outliers') to maintain homogeneity of variances. Although there was a tendency for 2+ rainbow trout to spawn later than 3+ fish the effect of age on spawning time was not significant ($P \geq 0.05$, two-way analysis of variance (Gp. G. excluded); 'Minitab' statistical package, Ryan et al., 1981). There were no significant differences between the

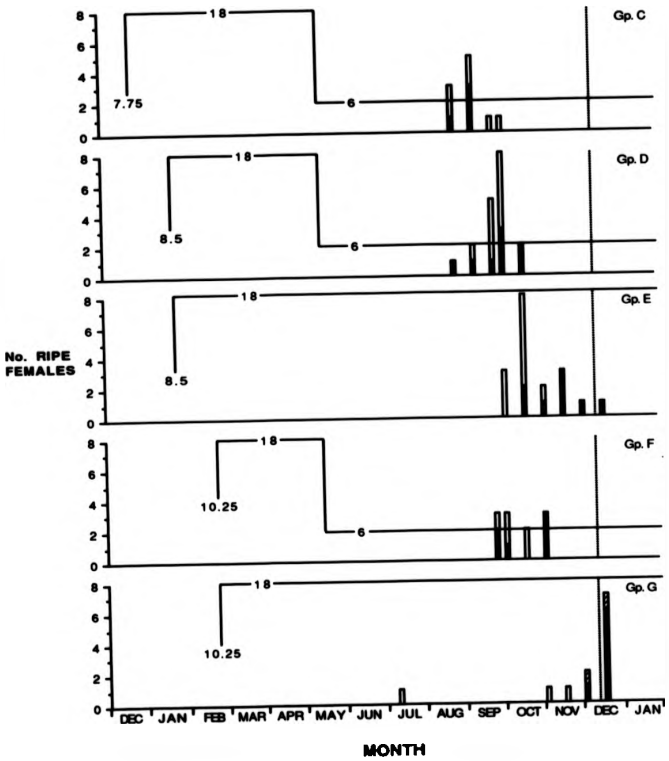
periods commencing on June 29 and July 28 respectively. In Gp. A 100% of the fish attained maturity within a 12 week period and in Gp. B 94% of the fish matured over 8 weeks.

Groups of previously untreated fish, in which the reduction in photoperiod in May was preceded by an increase to a 'long' daylength on December 23 (Gp. C), January 19 (Gp. D) or February 19 (Gp. F), commenced spawning on August 26, August 26 and September 23 respectively. In Gps. C, D and F respectively 91, 84 and 67% of the fish attained maturity over periods of 6, 8 and 6 weeks.

Groups which received an increase in daylength only, on January 19 (Gp. E) and February 19 (Gp. G), commenced spawning on October 7 and November 5 respectively, considerably later than their counterparts in Gps. D and F. In Gp. E 86% of the fish spawned over a period of 10 weeks. Unfortunately, an interruption to the water supply resulted in the death of all the fish in Gp. G on December 2-3. Examination of mortalities on December 4 revealed that 2 had ovulated. The remaining 9 fish were assessed for maturity by visual inspection, measurement of oocyte diameter and ovary weight and calculation of the GSI (section 2.3.3). Seven of the fish appeared close to ovulation (GSI= 6.3-14.7) and would probably have matured by the next or subsequent visit (2-4 weeks). Two of the fish were assessed as immature (GSI= 0.25 and 0.57) and unlikely to spawn that year. On the basis of these estimates, which have been incorporated in Figure 3.10, 85% of the fish in Gp. G would have spawned over a period of 6-8 weeks.

The differences in mean spawning times between groups were all significant at the $P \leq 0.001$ level with the exception of Gps. A vs B and C vs D ($P \leq 0.01$), Gps. D vs F and E vs F ($P \leq 0.05$), and Gps. B vs C (N.S.), though it should be noted that the single fish which spawned in early May in Gp. B (Figure 3.9) and the single fish which spawned in early July in Gp. G (Figure 3.10) were excluded from the statistical analysis (i.e. were treated as 'outliers') to maintain homogeneity of variances. Although there was a tendency for 2+ rainbow trout to spawn later than 3+ fish the effect of age on spawning time was not significant ($P \geq 0.05$, two-way analysis of variance (Gp. G. excluded); 'Minitab' statistical package, Ryan et al., 1981). There were no significant differences between the

Figure 3.10 (opposite): The effects of five photoperiod regimes (Gps. C-G, experiment 2) on the timing of maturation in female rainbow trout. The stacked histograms represent the number of ripe females on each sampling date; fish spawning for the first time are shown in black, those undergoing their second reproductive cycle in white. The hatched histograms in Gp. G represent an estimate of the spawning time. Line graphs indicate the photoperiod regime (hours of light/day) applied to each group. Dashed vertical lines indicate the mean spawning time of fish maintained under ambient daylength.


Gp. C
Gp. D
Gp. E
Gp. F
Gp. G
18
6
7.75
8.5
10.25
No. RIPE FEMALES
DEC JAN FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC JAN
MONTH

proportions of fish attaining maturity (either between or within groups).

Calcium

All groups exhibited significant changes ($P \leq 0.001$) in total serum calcium levels during the reproductive cycle. The timing of the changes in total serum calcium was similar in Gps. A and B, but calcium levels peaked (at about 33mg%) in July in Gp. A, approximately 1 month earlier than peak levels (approximately 58mg%) occurred in Gp. B (Figure 3.11), consistent with the difference in spawning time between the two groups. In August, calcium levels were significantly higher ($P \leq 0.001$) in Gp. A than Gp. B; there were no significant differences at other times points.

The timing of the changes in total serum calcium in Gps. C-G was similar in 2+ and 3+ fish, and there was no consistent relationship between age and the amplitude of the calcium profiles (Figures 3.12-3.16). However, there were considerable differences in the timing of these changes (Figure 3.17), consistent with the differences in spawning time between groups. Thus, calcium levels in Gp. C began to increase between May and June, rose steeply between June and July and peaked at about 60mg% in August; at the latter 3 time points levels were significantly higher ($P \leq 0.05$) in Gp. C than in Gps. E., F and G. Much shallower increases in serum calcium occurred in Gps. D-G between May and June, at which time levels were significantly higher ($P \leq 0.05$) in Gp. D than in Gp. G. In Gp. D levels rose steeply through July and August and peaked at approximately 58mg% in September; these levels were significantly elevated ($P \leq 0.05$) compared to Gp. E in July, Gp. F. in August, and Gp. G at all three time points. Serum calcium profiles were similar in Gps. E and F, exhibiting a more gradual increase than those observed in Gps. C and D, and attaining lower peaks of about 41mg% by September. Calcium levels in Gp. G remained slightly higher than basal values from June to August, after which they gradually increased, reaching approximately 35mg% by the last sampling point in November; at this time levels in Gp. G were significantly elevated ($P \leq 0.05$) compared to those in Gps. C and D, which were close to basal.

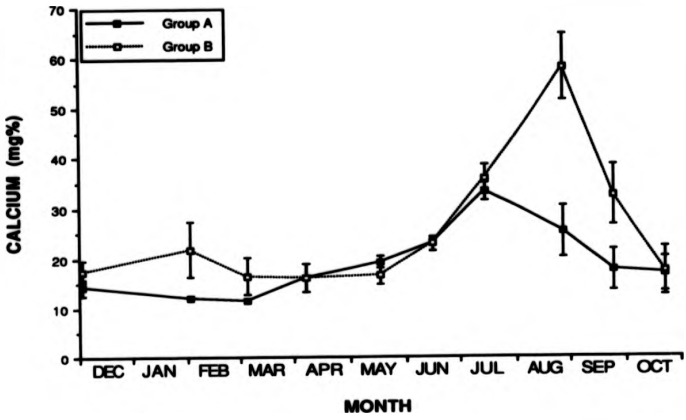


Figure 3.11: The effects of two photoperiod regimes (Gps. A and B, experiment 2) on the timing of changes in total serum calcium levels (mean ± 1SEM) during maturation in female rainbow trout (Gp. A, 18L:6D-Dec./6L:18D-May; n=13-19: Gp. B, 18L:6D-Jan./6L:18D-May; n=10-18).

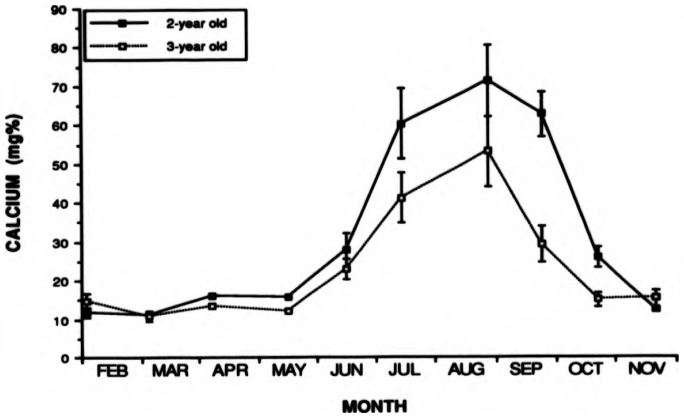


Figure 3.12: Comparison of the effects of a long photoperiod (18L:6D) from December, followed by a short photoperiod (6L:18D) from May (Gp. C, experiment 2), on the timing of changes in total serum calcium levels (mean ± 1SEM) during maturation in virgin fish (2-year olds; n=2-5) and fish undergoing their second reproductive cycle (3-year olds; n=5-11).

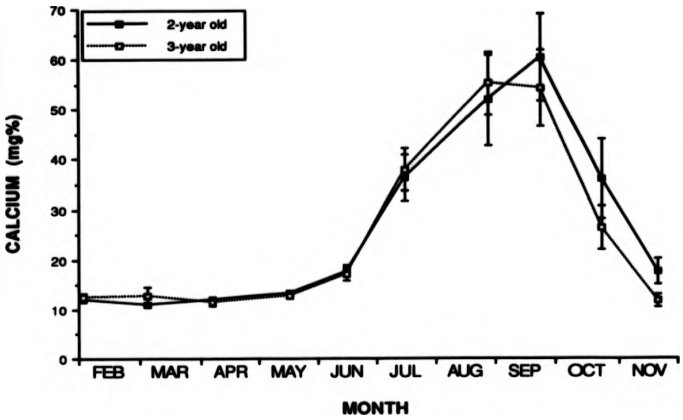


Figure 3.13: Comparison of the effects of a long photoperiod (18L:6D) from January, followed by a short photoperiod (6L:18D) from May (Gp. D, experiment 2), on the timing of changes in total serum calcium levels (mean ± 1SEM) during maturation in virgin fish (2-year olds; n=9-15) and fish undergoing their second reproductive cycle (3-year olds; n=7-14).

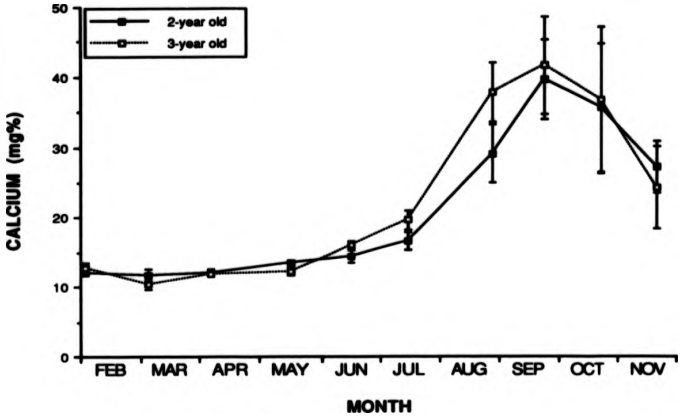


Figure 3.14: Comparison of the effects of a constant long photoperiod (18L:6D) from January (Gp. E, experiment 2) on the timing of changes in total serum calcium levels (mean ± 1SEM) during maturation in virgin fish (2-year olds; n=6-10) and fish undergoing their second reproductive cycle (3-year olds; n=6-15).

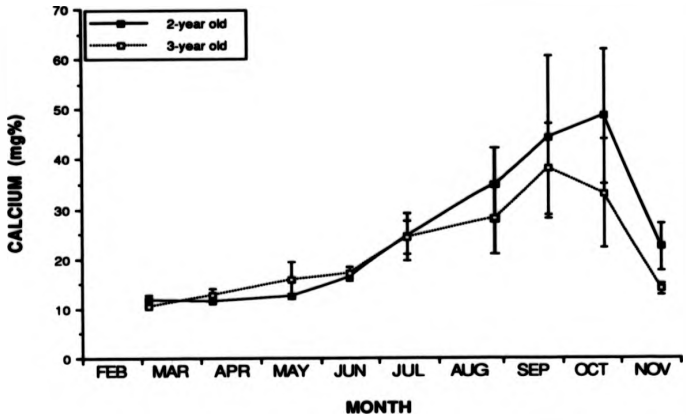


Figure 3.15: Comparison of the effects of a long photoperiod (16L:8D) from February, followed by a short photoperiod (6L:18D) from May (Gp. F, experiment 2), on the timing of changes in total serum calcium levels (mean ± 1SEM) during maturation in virgin fish (2-year olds; n=6-10) and fish undergoing their second reproductive cycle (3-year olds; n=7-13).

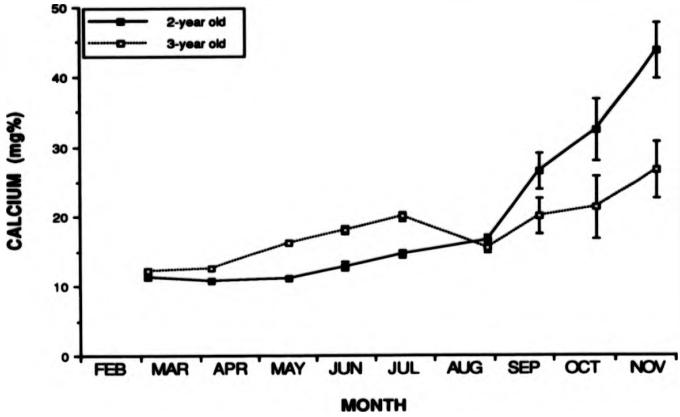


Figure 3.16: Comparison of the effects of a constant long photoperiod (18L:6D) from February (Gp. G, experiment 2) on the timing of changes in total serum calcium levels (mean ± 1SEM) during maturation in virgin fish (2-year olds; n=6-10) and fish undergoing their second reproductive cycle (3-year olds; n=4-14).

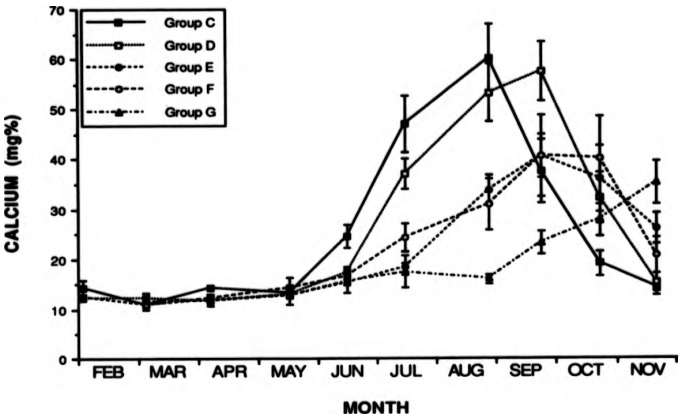


Figure 3.17: The effects of five photoperiod regimes (Gps. C-G, experiment 2) on the timing of changes in total serum calcium levels (mean ± 1SEM) during maturation in female rainbow trout; data combined from 2 and 3-year old fish (Gp. C, 18L:6D-Dec./6L:18D-May; n=6-16: Gp. D, 18L:6D-Jan./6L:18D-May; n=18-26: Gp. E, 18L:6D-Jan.; n=12-25: Gp. F, 18L:6D-Feb./6L:18D-May; n=13-22: Gp. G, 18L:6D-Feb.; n=12-24).

Egg diameter and fecundity

Egg diameter and fecundity measurements are summarised in table 3.2. The most advanced spawning 2+ fish (Gps. C and D) produced significantly smaller eggs ($P \leq 0.01$ and $P \leq 0.001$ respectively) than the later spawning fish of Gp. E, but there were no significant differences in egg diameter between groups in 3+ fish. There were no significant differences between groups in either total or relative fecundity. Two-way analysis of variance ('Minitab' statistical package, Ryan et al., 1981) revealed that both egg diameter and total fecundity were significantly higher ($P \leq 0.001$) in 3+ than 2+ fish, whereas relative fecundity was significantly lower ($P \leq 0.05$) in 3+ than 2+ fish.

Table 3.2: Egg size and fecundity data from experiment 2.

Group	Age	A	B	C	D	E	F	G
Spawning Period	n/a	June 29-Sept. 23	July 28-Oct. 7	Aug. 26-Oct. 7	Aug. 26-Oct. 21	Oct. 7-Dec. 18	Sept. 23-Nov. 5	Nov. 5-Dec. 18
% Maturation	2+	n/a	n/a	100	67	80	75	100
	3+	100	94	86	100	91	60	67
	combined	n/a	n/a	91	84	86	67	85 [a]
Egg Diameter (mm.)	2+	n/a	n/a	4.02 ± 0.27 [4]	3.98 ± 0.11 [8]	4.60 ± 0.07 [b 8]	4.27 ± 0.08 [6]	nd
	3+	4.63 ± 0.08 [16]	4.36 ± 0.11 [12]	5.02 ± 0.13 [6]	4.94 ± 0.10 [9]	4.97 ± 0.15 [10]	4.47 ± 0.16 [5]	5.11 ± 0.11 [2]
	combined	n/a	n/a	4.62 ± 0.21 [10]	4.49 ± 0.14 [17]	4.81 ± 0.10 [18]	4.36 ± 0.09 [11]	n/a
Total Fecundity	2+	n/a	n/a	4165 ± 255 [4]	3453 ± 245 [8]	3598 ± 390 [8]	2977 ± 597 [6]	nd
	3+	3381 ± 216 [16]	3266 ± 179 [12]	5538 ± 710 [6]	5490 ± 528 [9]	5910 ± 499 [7]	7247 ± 1138 [5]	6086 ± 2280 [2]
	combined	n/a	n/a	4989 ± 477 [10]	4531 ± 388 [17]	4677 ± 431 [15]	4918 ± 886 [11]	n/a
Relative Fecundity (no. eggs/kg)	2+	n/a	n/a	2119 ± 98 [4]	2679 ± 321 [8]	2486 ± 282 [7]	2129 ± 174 [6]	nd
	3+	2641 ± 160 [16]	2692 ± 183 [12]	1782 ± 260 [6]	1577 ± 128 [9]	1656 ± 137 [7]	2456 ± 647 [5]	1914 ± 407 [2]
	combined	n/a	n/a	1917 ± 164 [10]	2096 ± 211 [17]	2071 ± 190 [14]	2278 ± 295 [11]	n/a

All measurements are means (± 1SEM). The number of observations (n) per sample is indicated by the superscript on the right of each value.

a; estimate of percentage maturation. b; significantly different from Gps. C ($P \leq 0.01$) and D ($P \leq 0.001$).

n/a; not applicable. nd; not determined.

3.2.4 Experiment 3: The effects of a range of constant 'long' photoperiods followed by identical reductions to a range of constant shorter photoperiods on the timing of maturation in the female rainbow trout 1.

3.2.4.1 Protocol

Seven groups of 2-year old virgin female rainbow trout were transferred on January 19 from ambient daylength (8.5L:15.5D) to the following photoperiod regimes:

Group A - 22L:2D until May 6 followed by 13.5L:10.5D until spawning.
Group B - 20L:4D until May 6 followed by 11.5L:12.5D until spawning.
Group C - 18L:6D until May 6 followed by 9.5L:14.5D until spawning.
Group D - 16L:8D until May 6 followed by 7.5L:16.5D until spawning.
Group E - 14L:10D until May 6 followed by 5.5L:18.5D until spawning.
Group F - 8.5L:15.5D until spawning.
Group G - 8.5L:15.5D until April 29, 18L:6D until May 6, 8.5L:15.5D until spawning.

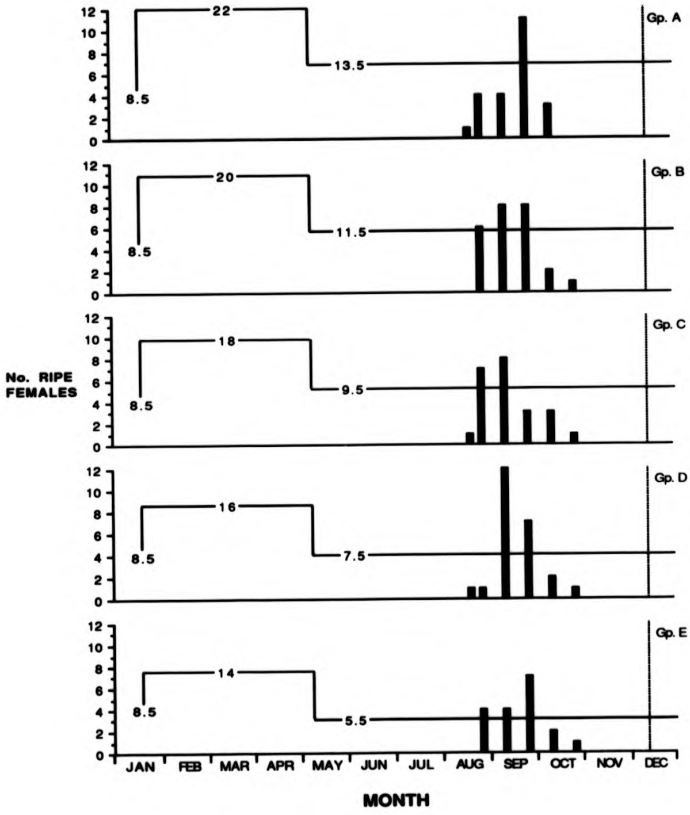
Gps. A-E each contained approximately 25 fish maintained in separate tanks. Gps. F and G each contained 15 fish maintained in the same tank except for a 1 week period from April 29 until May 6 when Gp. G were housed with Gp. C for exposure to 18L:6D.

3.2.4.2 Results

Spawning

The spawning times of the individual fish in each group are illustrated in Figures 3.18 (Gps. A-E) and 3.19 (Gps. F and G). When the photoperiod was reduced by 8.5 hours on May 6 from 22L:2D (Gp. A), 20L:4D (Gp. B), 18L:6D (Gp. C), 16L:8D (Gp. D) or 14L:10D (Gp. E) spawning commenced on August 17, August 31, August 17, August 17 and August 31 respectively, considerably in advance of the natural spawning period. The proportion of fish attaining maturity in Gps. A-E respectively was 92, 96, 100, 96 and 82% over periods of 8, 8, 10, 10 and 8 weeks.

Figure 3.18 (opposite): The effects of five photoperiod regimes (Gps. A-E, experiment 3) on the timing of maturation in female rainbow trout. The histograms represent the number of ripe females on each sampling date. Line graphs indicate the photoperiod regime (hours of light/day) applied to each group. Dashed vertical lines indicate the mean spawning time of fish maintained under ambient daylength.


No. RIPE FEMALES
Gp. A
22
13.5
8.5
Gp. B
20
11.5
8.5
Gp. C
18
9.5
8.5
Gp. D
16
7.5
8.5
Gp. E
14
5.5
8.5
0
2
4
6
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JAN
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MONTH

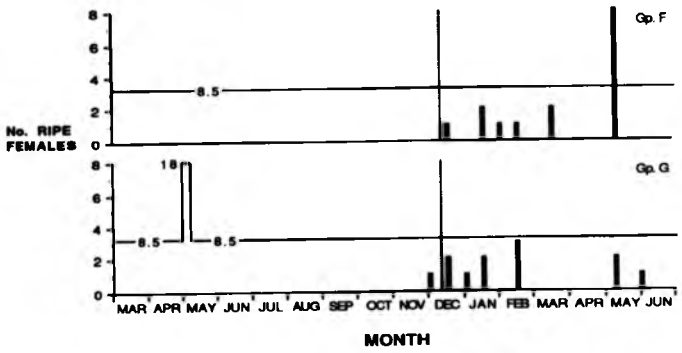


Figure 3.19: The effects of two photoperiod regimes (Gps. F and G, experiment 3) on the timing of maturation in female rainbow trout. The histograms represent the number of ripe females on each sampling date. Line graphs indicate the photoperiod regime (hours of light/day) applied to each group (note that exposure to a constant 8.5 hour photoperiod began in January). Dashed vertical lines indicate the mean spawning time of fish maintained under ambient daylength.

Gps. F ('short' days only) and G (one week of 'long' days within an otherwise 'short' day regime) commenced spawning on December 20 and December 6 respectively, approximately 1 month, and 2 weeks, after natural spawning began. Spawning was desynchronized in Gps. F and G relative to Gps. A-E; 100% of the fish in Gp. F attained maturity within a 20 week period and 92% of those in Gp. G matured over 26 weeks.

There were no significant differences between the mean spawning times of Gps. A-E or between the proportions of fish attaining maturity in each group. The variances of the spawning profiles of Gps. F and G were both significantly greater ($P \leq 0.001$; F-test) than those of Gps. A-E. Gps. F and G spawned significantly later ($P \leq 0.05$; nonparametric multiple comparisons procedure) than Gps. A-E. There was no significant difference between either the mean or median (Mann-Whitney U-test; 'Minitab' statistical package, Ryan et al., 1981) spawning times of Gps. F and G.

Calcium

All groups exhibited significant changes ($P \leq 0.001$) in total serum calcium levels during the reproductive cycle. The timing of the changes in calcium levels was virtually identical in Gps. A-E (Figure 3.20), consistent with the similarity in spawning times in these groups. Calcium levels in Gps. A-E began to rise between May and June, were significantly higher ($P \leq 0.05$) than basal values by July, and continued to rise steeply until peaking at approximately 70 mg% in August (Gp. C) or 45mg% (Gp. A), 61mg% (Gp. B), 54mg% (Gp. D) and 57mg% (Gp. E) in September. A rapid decline in serum calcium occurred in Gps. A-E between September and October, and levels were approaching basal by November. Significant differences in calcium levels between groups were only detected during the early part of the annual cycle; levels in Gp. B in April, and Gp. C in both April and June, were significantly elevated ($P \leq 0.05$) compared with those in Gp. E.

The timing of the changes in serum calcium was also virtually identical in Gps. F and G (Figure 3.21), again consistent with the similarity in spawning times in these groups. In both groups calcium levels first increased significantly ($P \leq 0.05$) above basal values between September and October, and had reached 27ng/ml and 25ng/ml respectively by

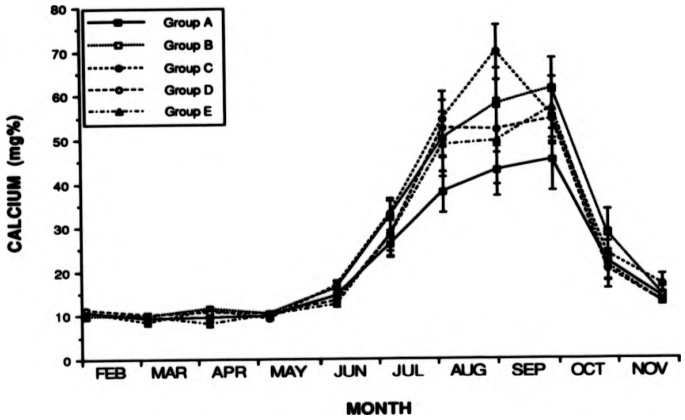


Figure 3.20: The effects of five photoperiod regimes (Gps. A-E, experiment 3) on the timing of changes in total serum calcium levels (mean ± 1SEM) during maturation in female rainbow trout (Gp. A, 22L:2D/13.5L:10.5D; n=10-12: Gp. B, 20L:4D/11.5L:12.5D; n=11-12: Gp. C, 18L:6D/9.5L:14.5D; n=10-12: Gp. D, 16L:8D/7.5L:16.5D; n=10-12: Gp. E, 14L:10D/5.5L:18.5D; n=10-13).

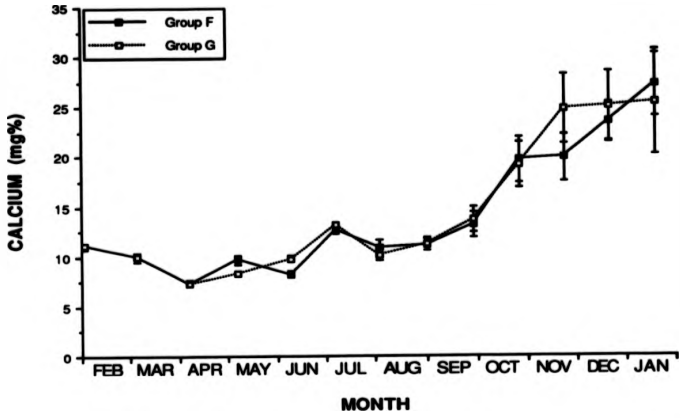


Figure 3.21: The effects of two photoperiod regimes (Gps. F and G, experiment 3) on the timing of changes in total serum calcium levels (mean ± 1SEM) during maturation in female rainbow trout (Gp. F, 8.5L:15.5D; n=8-12: Gp. G, 8.5L:15.5D/18L:6D-1wk/8.5L:15.5D; n=9-12).

the last sampling point in January. Significant differences in calcium levels between the two groups were only detected in May, when levels were significantly higher ($P \leq 0.05$) in Gp. F than in Gp. G, and in June, when levels were significantly higher ($P \leq 0.01$) in Gp. G than in Gp. F.

Egg diameter and fecundity

Egg diameter and fecundity measurements are summarised in table 3.3. The most advanced spawning fish (Gps. A-E) produced significantly smaller eggs ($P \leq 0.001$) than the later spawners (Gps. F and G). Mean egg diameter was also significantly greater in Gp. A than in Gps. D ($P \leq 0.01$) and E ($P \leq 0.05$). There were no significant differences in either total or relative fecundity.

Table 3.3: Egg size and fecundity data from experiment 3.

Group	A	B	C	D	E	F	G
Spawning Period	Aug. 17-Oct. 11	Aug. 31-Oct. 25	Aug. 17-Oct. 25	Aug. 17-Oct. 25	Aug. 31-Oct. 25	Dec. 20-May 9	Dec. 6-June 6
% Maturation	92	96	100	96	82	100	92
Egg Diameter (mm.)	4.26 ± 0.04[b] [23]	4.14 ± 0.06 [24]	4.13 ± 0.04 [22]	4.08 ± 0.04 [24]	4.09 ± 0.05 [18]	4.81 ± 0.05[c] [15]	4.69 ± 0.03[c] [11]
Total Fecundity	3389 ± 155 [23]	3578 ± 202 [24]	3458 ± 166 [22]	3298 ± 175 [24]	3610 ± 185 [18]	3828 ± 150 [15]	4142 ± 263 [11]
Relative Fecundity[a]	2431 ± 98 [23]	2531 ± 117 [24]	2419 ± 123 [22]	2549 ± 139 [24]	2748 ± 192 [18]	2339 ± 84 [14]	2948 ± 192 [9]

All measurements are means (± 1SEM). The number of observations (n) per sample is indicated by the superscript on the right of each value.

a; number of eggs/kg.

b; significantly different from Gps. D ($P \leq 0.01$) and E ($P \leq 0.05$).

c; significantly different ($P \leq 0.001$) from Gps. A-E.

3.2.5 Experiment 4: The effects of a range of constant 'long' photoperiods followed by identical reductions to a range of constant shorter photoperiods on the timing of maturation in the female rainbow trout II

3.2.5.1 Protocol

Six groups of female rainbow trout were transferred on January 17 from ambient daylength (8.5L:15.5D) to the following photoperiod regimes:

Group A - 22L:2D until May 9 followed by 13.5L:10.5D until spawning.
Group B - 18L:6D until May 9 followed by 9.5L:14.5D until spawning.
Group C - 14L:10D until May 9 followed by 5.5L:18.5D until spawning.
Group D - 12L:12D until May 9 followed by 3.5L:20.5D until spawning.
Group E - 10L:14D until May 9 followed by 1.5L:22.5D until spawning.
Group F - 8.5L:15.5D until May 9 followed by 1.5L:22.5D until spawning.

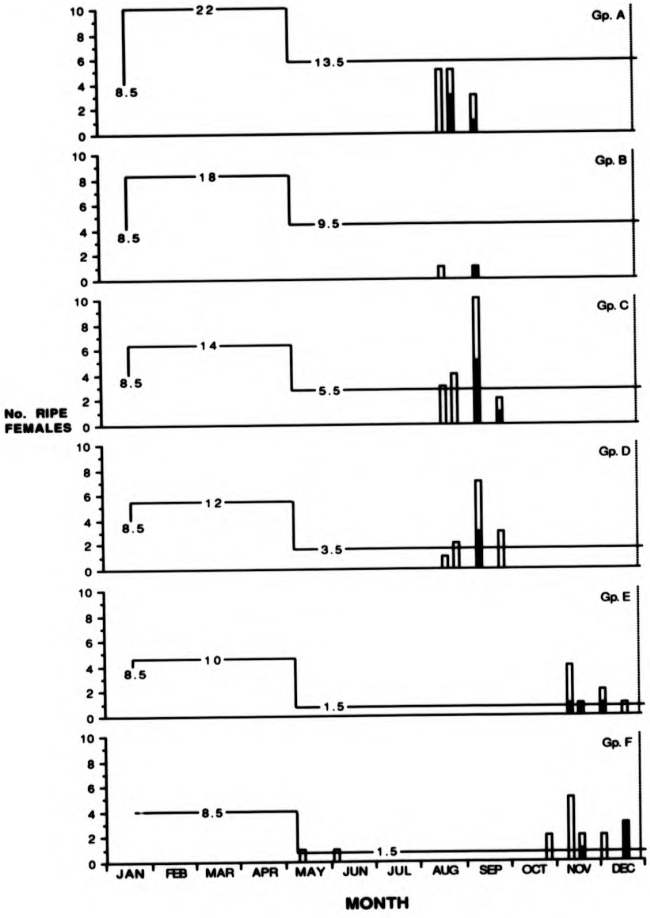
Each group comprised 10 two-year old virgin fish and 15 three-year olds which had recently spawned naturally for the first time. Blood samples for serum calcium analysis were taken exclusively from 3-year old fish.

3.2.5.2 Results

Spawning

The spawning times of the individual fish in each group are illustrated in Figure 3.22. When the photoperiod was reduced by 8.5 hours on May 9 from 22L:2D (Gp. A), 18L:6D (Gp. B), 14L:10D (Gp. C) and 12L:12D (Gp. D) spawning commenced in all groups on August 18, considerably in advance of the natural spawning period. Unfortunately, an interruption to the water supply on June 26 caused the death of all but 2 of the fish in Gp. B, which subsequently spawned on August 18 and September 14. No examination of mortalities was possible on this occasion. Further interruptions to the water supply between August 18 and 31 and, most seriously, on October 10, resulted in the death of the

Figure 3.22 (opposite): The effects of six photoperiod regimes (Gps. A-F, experiment 4) on the timing of maturation in female rainbow trout. The stacked histograms represent the number of ripe females on each sampling date; fish spawning for the first time are shown in black, those undergoing their second reproductive cycle in white. Line graphs indicate the photoperiod regime (hours of light/day) applied to each group. Dashed vertical lines indicate the mean spawning time of fish maintained under ambient daylength.


Gp. A
22
13.5
8.5
Gp. B
18
9.5
8.5
Gp. C
14
5.5
8.5
Gp. D
12
3.5
8.5
Gp. E
10
1.5
8.5
Gp. F
8.5
1.5
No. RIPE FEMALES
0
2
4
6
8
10
JAN
FEB
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
NOV
DEC
MONTH

majority of fish in Gps. A-D. Thus, although most of the fish in these groups had spawned by October 10, there is a possibility that the spawning profiles were slightly curtailed.

When the photoperiod was reduced to 1.5L:22.5D in early May from 10L:14D (Gp. E) or 8.5L:15.5D (Gp. F) spawning commenced on November 9 and October 24 respectively, in both cases in advance of the natural spawning period. The proportion of fish attaining maturity in Gps. A, C, D, E and F was estimated as 87, 79, 65, 62 and 71% over periods of 4, 6, 6, 6 and 8 weeks respectively.

There was only one significant difference (Gps. A vs D; $P \leq 0.05$) between the mean spawning times of Gps. A-D and no significant difference between those of Gps. E and F. There were, however, highly significant differences ($P \leq 0.001$) between the mean spawning times of both Gps. E and F when compared with Gps. A-D, though it should be noted that the two fish which spawned in early May and early June in Gp. F (Figure 3.22) were excluded from the statistical analysis (i.e. were treated as 'outliers') to maintain homogeneity of variances. There was a significant effect of age on spawning time ($P \leq 0.01$, two-way analysis of variance; 'Minitab' statistical package, Ryan et al., 1981); the mean spawning time of 3+ fish occurred approximately 2 weeks earlier than that of 2+ fish. Between group differences in the proportion of fish attaining maturity were not significant but there were significant differences between year classes within groups: the proportion of 3+ fish maturing was significantly greater than that of 2+ fish in Gps. C ($P \leq 0.01$), D ($P \leq 0.05$) and E ($P \leq 0.01$).

Calcium

All groups exhibited significant changes ($P \leq 0.001$) in total serum calcium levels during the reproductive cycle. However, there were considerable differences in the timing of these changes (Figure 3.23), consistent with the differences in spawning time between groups. The timing of the changes in calcium levels was virtually identical in Gps. A and C, consistent with the similarity in spawning times. Calcium levels in these groups increased slightly between March and May, were significantly higher ($P \leq 0.05$) than basal values by June, and continued to rise at a moderate rate until they peaked at approximately 33mg%

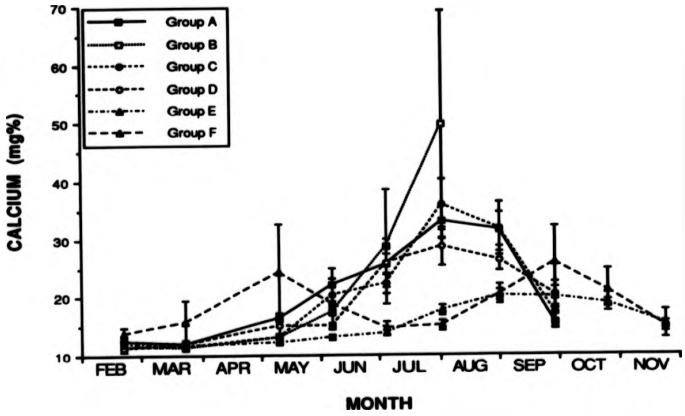


Figure 3.23: The effects of six photoperiod regimes (Gps. A-F, experiment 4) on the timing of changes in total serum calcium levels (mean ± 1SEM) during maturation in female rainbow trout (Gp. A, 22L:2D/13.5L:10.5D; n=6-10: Gp. B, 18L:6D/9.5L:14.5D; n=2-10: Gp. C, 14L:10D/5.5L:18.5D; n=9-11: Gp. D, 12L:12D/3.5L:20.5D; n=8-11: Gp. E, 10L:14D/1.5L:22.5D; n=5-10: Gp. F, 8.5L:15.5D/1.5L:22.5D; n=6-12).

(Gp. A) and 36mg% (Gp. C) in early August, prior to a decline towards basal values in September. Compared to Gp. E, levels were significantly elevated ($P \leq 0.05$) in Gp. A in May, and in Gps. A and C in June, July, early August and late August. Calcium levels in Gps. A and C were also significantly higher ($P \leq 0.05$) than those in Gp. F in July and early August and levels in Gp. A were significantly elevated ($P \leq 0.05$) compared to Gp. F in late August. The timing of the changes in calcium levels in Gp. B was comparable to that in Gps. A and C, consistent with the similarity in spawning times between the two surviving fish in Gp. B and those in Gps. A and C. Peaking at about 29mg% in early August, Gp. D exhibited a similar serum calcium profile to Gps. A and C, except that the first significant increase ($P \leq 0.05$) above basal values occurred approximately one month later, between June and July; calcium levels in Gp. D were significantly higher ($P \leq 0.05$) than those in Gp. E in July and Gp. F in early August.

Compared with Gps. A-D significant ($P \leq 0.05$) changes in serum calcium occurred 1-3 months later in Gps. F and G (Figure 3.23), consistent with the later spawning times in the latter groups. The first significant rise ($P \leq 0.05$) in calcium levels in Gp. E occurred between July and early August and extended into a broad peak lasting until late October and attaining a maximum of only 20mg% prior to a decrease in levels between October and November. Two early spawning fish in Gp. F (see Figure 3.22) caused a transient increase in mean serum calcium levels in May, but these two fish were not representative of the sample population and hence the difference between Gp. F and Gps. A-E was not significant at this time. The first significant increase ($P \leq 0.05$) in calcium levels in Gp. F occurred in August with levels gradually increasing to peak at about 26mg% in late September, before commencing a symmetrical decline in October and November.

Egg diameter and fecundity

Egg diameter and fecundity measurements are summarised in table 3.4. Eggs produced by 2+ fish were significantly smaller in Gps. A ($P \leq 0.01$), C ($P \leq 0.001$), D ($P < 0.01$) and E ($P \leq 0.05$) compared to Gp. F, and in Gp. C compared to Gp. E ($P \leq 0.05$), but there were no significant differences in egg diameter between groups in 3+ fish. There were no

Table 3.4: Egg size and fecundity data from experiment 4.

Group	Age	A	B	C	D	E	F
Spawning Period	n/a	Aug. 18-Sept. 14	Aug. 18-Sept. 14	Aug. 18-Sept. 28	Aug. 18-Sept. 28	Nov. 9-Dec. 20	Oct. 24-Dec. 20
% Maturation	2+	50	100 [a]	55[b]	30[c]	38[b]	50
	3+	100	100 [a]	100	91	100	85
	combined	87	100	79	65	62	71
Egg Diameter (mm.)	2+	4.05 ± 0.02 [4]	4.07 [1]	3.96 ± 0.11 [6]	4.16 ± 0.05 [3]	4.34 ± 0.05 [3]	4.71 ± 0.04 [4]
	3+	4.64 ± 0.04 [9]	4.53 [1]	4.61 ± 0.05 [13]	4.48 ± 0.05 [9]	4.73 ± 0.07 [4]	4.67 ± 0.08 [8]
	combined	4.46 ± 0.08[13]	4.30 ± 0.23[2]	4.40 ± 0.09[19]	4.40 ± 0.06[12]	4.56 ± 0.09[7]	4.68 ± 0.05[12]
Total Fecundity	2+	3030 ± 134 [4]	2268 [1]	3257 ± 240 [6]	2621 ± 277 [3]	2810 ± 27 [3]	3139 ± 431 [4]
	3+	4505 ± 295 [9]	3495 [1]	4497 ± 263 [13]	3948 ± 241 [9]	3760 ± 889 [4]	4400 ± 365 [8]
	combined	4051 ± 283[13]	2882 ± 613[2]	4105 ± 235[19]	3617 ± 255[12]	3353 ± 512[7]	3980 ± 325[12]
Relative Fecundity (no. eggs/kg)	2+	3033 ± 45 [4]	3041 [1]	3436 ± 487 [6]	3010 ± 424 [3]	3363 ± 281 [3]	nd
	3+	2498 ± 160 [9]	1859 [1]	2517 ± 175 [13]	2510 ± 227 [9]	2793 ± 596 [3]	2661 ± 250 [8]
	combined	2663 ± 131[13]	2450 ± 591[2]	2807 ± 212[19]	2635 ± 201[12]	3078 ± 321[6]	n/a

All measurements are means (± 1SEM). The number of observations (n) per sample is indicated by the superscript on the right of each value.
a; only 1 fish. b; significantly different from 3+ ($P \leq 0.01$). c; significantly different from 3+ ($P \leq 0.05$).
n/a; not applicable. nd; not determined.

significant differences between groups in either total or relative fecundity. Two-way analysis of variance ('Minitab' statistical package, Ryan et al., 1981) revealed that both egg diameter and total fecundity were significantly higher ($P \leq 0.001$) in 3+ than 2+ fish, whereas relative fecundity was significantly lower ($P \leq 0.01$) in 3+ than 2+ fish.

Summary of Results: Section A

1. Exposure of rainbow trout to a constant 'long' photoperiod early in the reproductive cycle, followed by a reduction to a shorter daylength after 3-4 months, advanced maturation by up to 4 months.

2. Maintenance of rainbow trout under a constant 'long' photoperiod from early in the reproductive cycle until spawning advanced maturation by 1-2 months.

3.'Long' photoperiods of between 12 and 22 hours, followed in each case by an 8.5 hour reduction in daylength, each caused a similar advance (3-4 months) in the time of spawning.

4. Maturation was advanced (1-2 months) in fish subjected to a 'long' day of only 10 hours, when followed by a reduction in daylength of 4 or 8.5 hours.

5. Maturation was advanced (2 months) even in fish which did not experience an increase in daylength in advance of the natural light cycle (i.e. remained on a winter photoperiod from January) providing they received a reduction to an even shorter photoperiod (1.5L:22.5D) in May.

6. Maturation was delayed and the spawning time of individuals became desynchronized in fish maintained under constant 8.5L:15.5D from January; a 1 week period of 16L:8D (late April to early May) within this 'short day' regime had no significant effect on the spawning profile.

7. Maturation was slightly delayed in fish maintained under 6L:18D from January, followed by a reduction to 2L:22D in May.

8. The timing of the increase to a 'long' photoperiod was an important determinant of the timing of maturation; spawning occurred in sequence in fish exposed to an increase in photoperiod in December, January and February, followed by a reduction in daylength in May, and in fish subjected to a constant 'long' photoperiod from January or February.

9. A 'long' to 'short' photoperiod regime further advanced (by 1-2 months) the maturation of fish which had experienced an advance in spawning time the previous year; the time of spawning was again dependent on the timing of the increase in photoperiod.

10. There were significant differences in the timing of the changes in serum levels of calcium, oestradiol-17β and testosterone, consistent with the differences in spawning time.

11. Virgin fish and fish undergoing their second reproductive cycle responded similarly to changes in photoperiod.

12. Egg diameter and total fecundity were significantly higher in 3+ than 2+ fish, whereas relative fecundity was significantly lower in 3+ than 2+ fish.

13. Egg size was related to spawning time; virgin fish attaining early maturity produced significantly smaller eggs than later spawners.

14. There were no significant differences in either total or relative fecundity between fish exposed to a variety of photoperiod regimes.

3.3 Section B: The Effects of Short Periods of Continuous Light (LL) on the Entrainment of the Annual Cycle of Reproduction in the Female Rainbow Trout.

Photoperiod regimes utilising LL are particularly attractive for the commercial production of out-of-season eggs because neither time control or blackout are required. However, long-term exposure to LL can produce erratic spawning and a considerable reduction in the proportion of fish attaining maturity (Bromage et al., 1984; Duston, 1987; Bromage and Cumaranatunga, 1988). The experiments described in this section assessed the ability of short (≤ 2 months) periods of LL to modify the timing of maturation in the rainbow trout.

A previous study in which female rainbow trout were subjected to relatively long periods of LL indicated that the timing of exposure to LL relative to the phase of the reproductive cycle could influence both the extent to which spawning was advanced and the proportion of fish attaining maturity (Duston, 1987). Experiment 5 therefore examined the ability of 2 month periods of LL, applied at various times within a 7 month period encompassing the natural spawning season, to advance maturation in female rainbow trout. The importance of duration of exposure to LL on the proportion of female rainbow trout responding with an advance in spawning time was subsequently investigated in experiment 6 by exposing fish to LL periods of 2 weeks, 1 month and 2 months. In experiment 7 a much larger number of female rainbow trout, more relevant to commercial production requirements, were subjected to the most effective photoperiod regime tested in experiments 5 and 6. Some commercial producers are reluctant to use photoperiod treatments because of the possibility that males and females may respond differently to the same regime. In this respect a differential effect of LL on the timing of reproduction in male and female fish has been reported for the brook trout (Poston and Livingstone, 1971). Consequently, the effects of LL on reproductive timing in male rainbow trout were also documented in the commercial trial.

To further spread the production of out-of-season eggs within a single reproductive cycle it is necessary to develop techniques to delay maturation. Exposure to 16L:8D or LL

from June until spawning delayed maturation of November/December spawning rainbow trout by 2-3 months (Shiraishi and Fukuda, 1966; Bourlier and Billard, 1984a,b), although the spawning times of individual fish became desynchronized (Boulier and Billard, 1984a,b). Similar results have been recorded for other salmonids exposed to constant 'long' photoperiods or LL after the summer solstice (see section 3.1). Experiment 8 therefore examined the ability of a 2 month period of LL applied after the summer solstice to delay maturation in female rainbow trout, again using fish numbers appropriate to commercial production requirements.

3.3.1 General Methods

All fish used in experiments 5-8 came from an established domesticated stock with a natural spawning period under ambient daylength (latitude 51°10'N) of November-January. When it was necessary to separate small groups of fish they were maintained indoors in oxyder tanks consisting of 8 sections separated by netted screens (section 2.1.1; Figure 2.2). The larger numbers of fish required for commercial trials were also housed indoors, but in tanks without screens. Natural light entered the buildings via plastic corrugated roofing and provided variable light intensity according to external environmental conditions: the minimum light intensity measured at the water surface of tanks maintained under ambient photoperiod (at about midday) was 6 lux and the maximum was 800 lux. Photoperiod tanks were exposed to continuous (24 hour) light provided by cool white fluorescent strips suspended approximately 1m. above the water surface. The light intensity at the water surface of the sectioned photoperiod tank ranged from 700-1000 lux. Duston (1987) suggested that differences in light intensity may cause variations in the proportion of fish maturing after photoperiod treatments. To avoid this complication only sections 1, 2, 4 and 5 of the LL tank (section 2.1.1; Figure 2.2) were used for maintenance of experimental fish since the light intensity at the water surface of these sections was virtually identical. Light intensity at the water surface of the 2 non-sectioned photoperiod tanks ranged from 100 lux at the far ends to 1500 lux directly beneath the fluorescent strips, but was 700-1200 lux over the majority of each tank; this area included the most densely populated parts of the tanks close to the air pumps. Water temperature varied seasonally consistent with variations in external environmental conditions; maximum and minimum temperatures obtained at sampling times are therefore reported separately for each experiment.

At the start of each experiment approximately 250 two-year old virgin female rainbow trout approaching their first natural spawning were randomly selected from the broodstock. As the fish approached maturity they were examined at 2-weekly intervals (section 2.2) and the spawning times of individual fish recorded. Individual fish were colour tagged (experiment 5) or dye marked (experiment 6) to allow subsequent

identification of the date on which they first spawned (section 2.1.3). When the experimental treatments were due to begin before the natural spawning time the fish were assigned to groups randomly; when treatments began after natural spawning each group received approximately equal numbers of fish from each spawning date. Groups of fish were assigned to tank sections in a random manner. The fish were subsequently checked at monthly intervals until they approached their second spawning period when they were again examined at 2-weekly intervals and the spawning times of individual fish recorded for each group. Egg diameter, total egg volume and post-stripped body weight were measured for samples of both first and second spawning fish and the total and relative fecundities calculated (section 2.3).

Unless otherwise stated spawning profiles and egg data were analysed by one-way analysis of variance followed by a parametric multiple comparisons procedure (section 2.7.5). The proportions of fish attaining maturity were compared by calculation of confidence limits (section 2.7.6). Hormone data was analysed by the Kruskal-Wallis test followed by a non-parametric multiple comparisons procedure (section 2.7.5).

3.3.2 Experiment 5: The effects of 2 month periods of LL on the timing of maturation in the female rainbow trout.

3.3.2.1 Protocol

Six groups of approximately 22 two-year old female rainbow trout were exposed to 2 month periods of LL by transferring them between tanks maintained under either ambient daylength or LL according to the following protocol:

Group A - LL from September 15 until November 17.
Group B - LL from October 15 until December 16.
Group C - LL from November 17 until January 21.
Group D - LL from December 16 until February 17.
Group E - LL from January 21 until March 25.
Group F - LL from February 17 until April 23.

At all other times the fish were maintained under ambient photoperiod. Two further groups of 22 and 44 fish respectively were maintained under ambient photoperiod only and LL only (from September 15) for the duration of the experiment. Water temperature varied seasonally from 7.5-13.5°C. As a corollary to this experiment an additional 70 fish were subjected to LL only (in tank sections 3, 6, 7 and 8; see section 3.3.1) with the aim of producing extra eggs for commercial use. The experiment was terminated on October 8.

3.3.2.2 Results

Spawning

The spawning times of the individual fish in each group are illustrated in Figures 3.24 (controls) and 3.25 (Gps. A-F). The fish in all 8 groups spawned for the first time between November 17 and January 21. Although some groups received LL prior to their first (winter) spawning there were no significant differences between the mean spawning

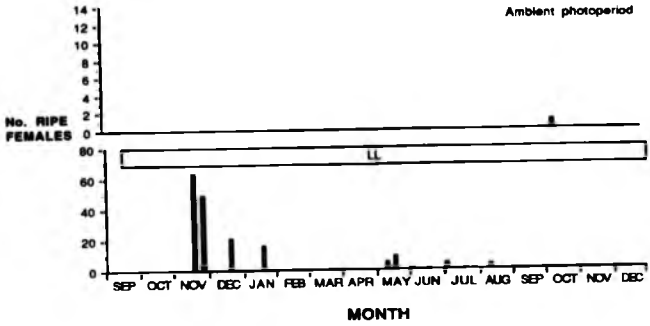


Figure 3.24: The timing of maturation in female rainbow trout maintained on natural seasonal photoperiod (upper graph) or continuous light (lower graph) throughout experiment 5. The histograms represent the number of ripe females on each sampling date (natural spawning time on the left). In the lower graph the main experimental group are shown in black, and additional fish included for commercial purposes in white. A rectangular box indicates the timing of the LL period in the lower graph.

times of any of the groups at this stage.

The timing of the second (summer) spawning period in each group was directly related to the timing of the light period, commencing 6-7 months after first exposure to LL (that is, 3-7 months in advance of the expected spawning time of the ambient photoperiod controls). Thus, Gps. A-F (LL from Sept.-Nov., Oct.-Dec. and so on) commenced spawning on April 23, April 23, June 2, June 17, July 16 and August 24 for periods of 3, 4, 10 (4/5 fish in 4 weeks), 8, 10 (17/18 fish in 6 weeks) and 6 weeks respectively. Unfortunately, a number of tag losses occurred precluding conclusions regarding the effect of the time of first spawning on the ability of an individual to spawn again in the summer in response to a particular light treatment. As might be expected, however, there was a general tendency for fish which spawned earliest initially to spawn early again in the summer.

Only one (8%) of the ambient photoperiod controls matured, spawning on October 8, the date on which the experiment was terminated; visual inspection indicated that none of the remaining fish in this group were close to maturity at this time. Additionally, 2-year old broodstock of the same strain maintained under ambient photoperiod subsequently spawned (for the first time) between December 11 and January 5. Fish maintained on LL for the duration of the experiment commenced spawning on May 11 for a period of 13 weeks. A similarly desynchronized spawning pattern was observed in the additional fish included for commercial purposes (Figure 3.24).

There were marked differences in the proportion of fish attaining early maturity in each group (Figure 3.26). Thus, a large majority of the fish (≥85%) spawned in groups exposed to LL from December-February (Gp. D) or January-March (Gp. E) but only a minority of the fish (≤33%) subjected to 2 months LL outside these periods (Gps. A, B, C and F) matured. Substantial mortalities made it difficult to calculate the exact proportion of fish attaining maturity under LL only but minimum and maximum estimates were 30 and 50% respectively.

The differences in the mean (summer) spawning times between groups were all significant at the $P \leq 0.001$ level with the exception of Gps. A vs D, A vs F, D vs E and E vs F

times of any of the groups at this stage.

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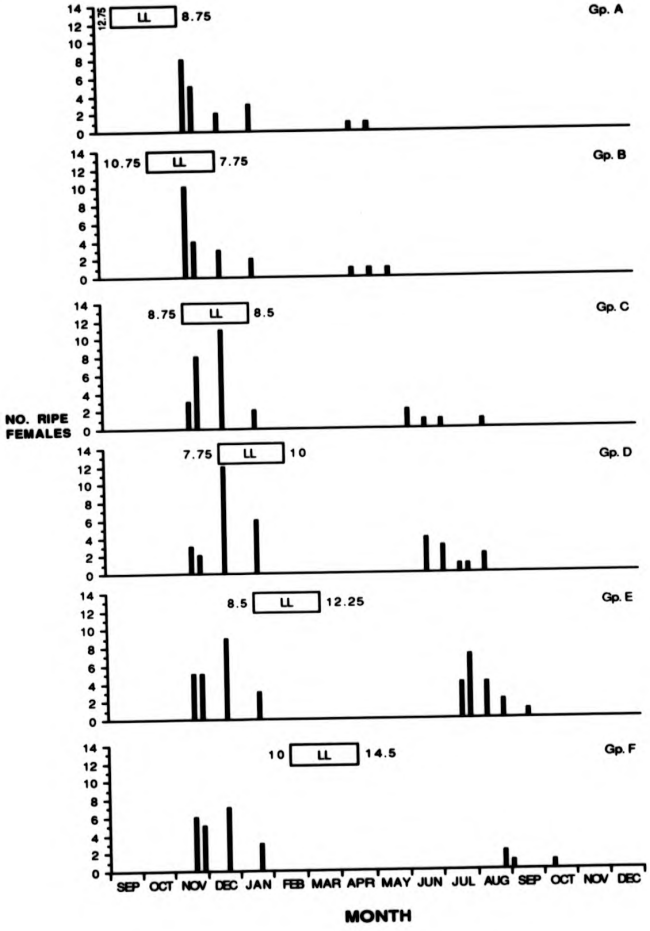
There were marked differences in the proportion of fish attaining early maturity in each group (Figure 3.26). Thus, a large majority of the fish (≥85%) spawned in groups exposed to LL from December-February (Gp. D) or January-March (Gp. E) but only a minority of the fish (≤33%) subjected to 2 months LL outside these periods (Gps. A, B, C and F) matured. Substantial mortalities made it difficult to calculate the exact proportion of fish attaining maturity under LL only but minimum and maximum estimates were 30 and 50% respectively.

The differences in the mean (summer) spawning times between groups were all significant at the $P<0.001$ level with the exception of Gps. A vs D, A vs F, D vs E and E vs F

Figure 3.25 (opposite): The effects of 2 month periods of continuous light (LL) on the timing of maturation in female rainbow trout (Gps. A-F, experiment 5). The histograms represent the number of ripe females on each sampling date (natural spawning time on the left). A rectangular box indicates the timing of the LL period for each group. Integers next to the LL periods indicate ambient daylength before and after exposure.


Gp. A
12.25
LL
8.75
Gp. B
10.75
LL
7.75
Gp. C
8.75
LL
8.5
Gp. D
7.75
LL
10
Gp. E
8.5
LL
12.25
Gp. F
10
LL
14.5
NO. RIPE FEMALES
0 2 4 6 8 10 12 14
SEP OCT NOV DEC JAN FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC
MONTH

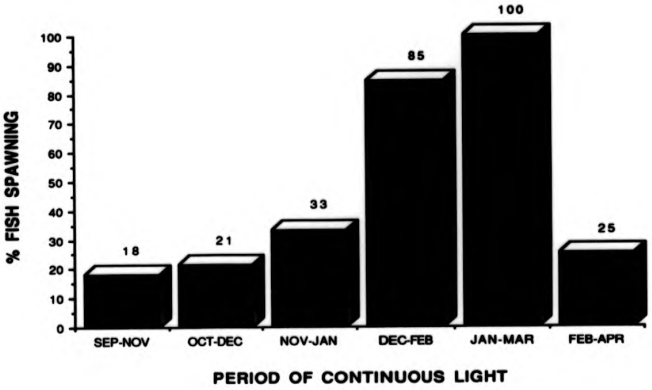


Figure 3.26: The proportion of female rainbow trout responding to 2 month periods of continuous light with an advance in the timing of maturation (experiment 5).

($P \leq 0.01$), A vs C and B vs C ($P \leq 0.05$), and C vs D (N.S.). Comparison of confidence limits showed that the proportion of fish attaining early maturity was significantly lower in Gps. A, B and F ($P \leq 0.01$), and in Gp. C ($P \leq 0.05$), than in Gps. D and E.

Egg diameter and fecundity

During the natural winter spawning season at the start of the experiment the mean egg diameter (mm.), total fecundity (number of eggs) and relative fecundity (number of eggs/kg) were 4.86 (±0.06; n=16), 4373 (±233; n=16) and 2180 (±117; n=16) respectively (all measurements made on December 16).

During the summer, mean egg diameter (mm.) in Gps. A-F was 3.87 (n=1), 4.07 (n=1), 4.29 (±0.08; n=2), 4.27 (±0.11; n=9), 4.73 (±0.05; n=17) and 4.78 (±0.08; n=4) respectively. Gp. D produced significantly smaller eggs than the later spawning fish of Gps. E ($P \leq 0.001$) and F ($P \leq 0.01$). Similarly, Gp. C eggs were significantly smaller ($P \leq 0.05$) than those of Gp. E. The mean egg diameter (mm.) of fish maintained under LL only was 4.73 (±0.07; n=10), which was significantly greater than that of Gps. C ($P \leq 0.05$) and D ($P \leq 0.001$). Eggs produced the previous winter were also significantly larger than those produced in the summer in Gps. C ($P \leq 0.01$) and D ($P \leq 0.001$).

Mean total fecundity (number of eggs) in Gps. A-F during the summer was 1858 (n=1), 2990 (n=1), 4731 (±231; n=2), 4848 (±406; n=9), 4096 (±246; n=17) and 4124 (±284; n=4) respectively. The total fecundity of fish maintained under LL only was 4265 (±280; n=10). Differences in total fecundity were not significant. Insufficient data was available for calculation of relative fecundities.

3.3.3. Experiment 6: The effect of varying the duration of exposure to LL on the proportion of female rainbow trout responding with an advance in spawning time.

3.3.3.1 Protocol

Six groups of approximately 26 two-year old female rainbow trout, all of which spawned for the first time in December, were exposed to a variety of periods of LL by transferring them between tanks maintained under either ambient daylength or LL according to the following protocol:

Group A - 2 weeks LL from January 25 until February 8.
Group B - 2 weeks LL from February 23 until March 9.
Group C - 2 weeks LL from March 24 until April 7.
Group D - 1 month LL from January 15 until February 15.
Group E - 1 month LL from February 15 until March 16.
Group F - 1 month LL from March 16 until April 15.
Group G - 2 months LL from January 15 until March 16.
Group H - 2 months LL from February 15 until April 15.

At all other times the fish were maintained under ambient photoperiod. A control group of 15 fish was exposed to LL only from January 15 until the experiment was terminated on January 2, the following year. Water temperature varied seasonally from 6-15.5°C.

3.3.3.2 Results

Spawning

The spawning times of the individual fish in each group are illustrated in Figures 3.27 (Gps. A-C), 3.28 (Gps. D-F) and 3.29 (Gps. G, H and LL only controls). The fish were stripped for the first time on December 11, December 23 and January 5 and were distributed equally between groups in an approximate ratio of 10:14:2 from each spawning date (Figures 3.27-3.29).

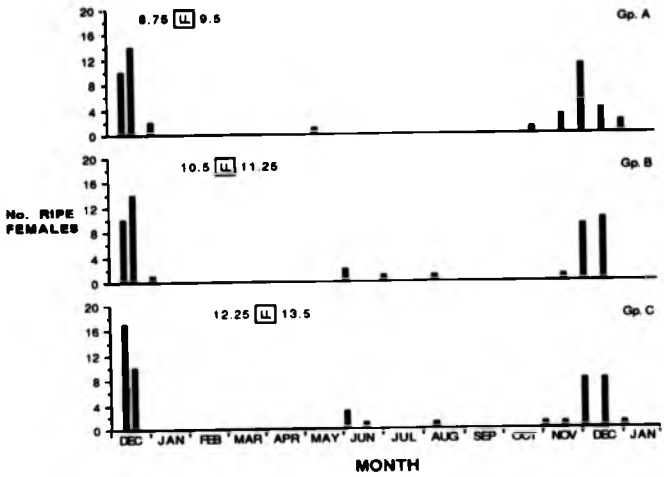


Figure 3.27: The effects of 2 week periods of continuous light (LL) on the timing of maturation in female rainbow trout (Gps. A-C, experiment 6). The histograms represent the number of ripe females on each sampling date (natural spawning time on the left). A rectangular box indicates the timing of the LL period for each group. Integers next to the LL periods indicate ambient daylength before and after exposure.

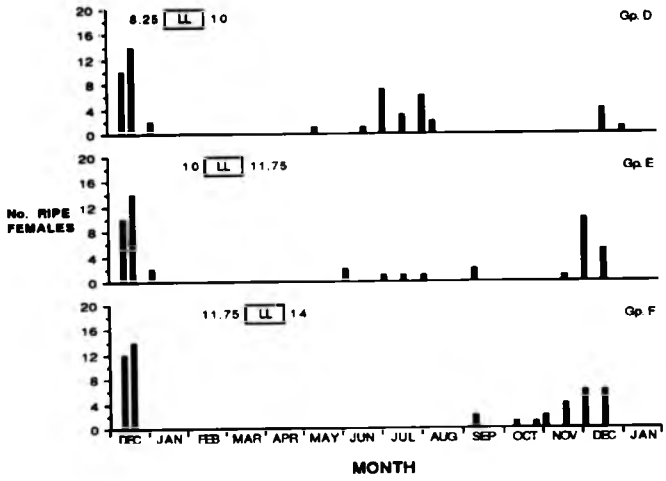


Figure 3.28: The effects of 1 month periods of continuous light (LL) on the timing of maturation in female rainbow trout (Gps. D-F, experiment 6). The histograms represent the number of ripe females on each sampling date (natural spawning time on the left). A rectangular box indicates the timing of the LL period for each group. Integers next to the LL periods indicate ambient daylength before and after exposure.

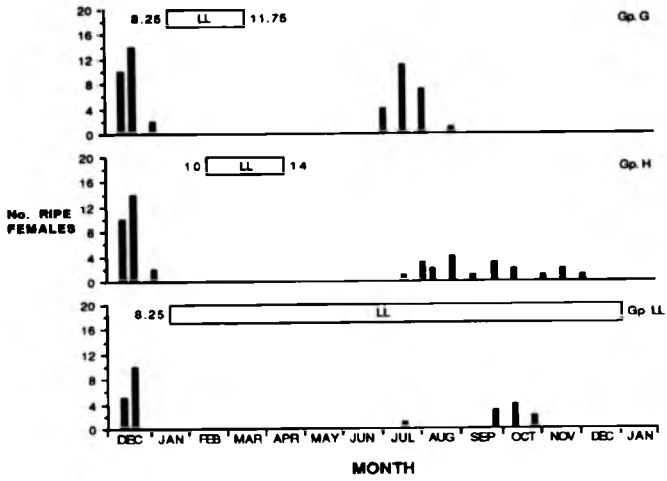


Figure 3.29: The effects of 2 month periods of continuous light (LL) on the timing of maturation in female rainbow trout (Gps. G and H, experiment 6; upper and middle graphs). The lower graph shows the timing of maturation in fish maintained on LL throughout the experiment. The histograms represent the number of ripe females on each sampling date (natural spawning time on the left). A rectangular box indicates the timing of the LL period for each group. Integers next to the LL periods indicate ambient daylength before and after exposure.

To enable comparison of the second (summer) spawning period with the results of experiment 5, which was terminated on October 8, all fish which spawned up to, and including, October 10, were deemed to have matured in advance of the natural spawning season. Gps. A-C, which experienced 2 week periods of LL (Jan.-Feb., Feb.-Mar. and Mar.-Apr.), commenced spawning on May 9 (1 fish only), June 6 and June 6 respectively (Figure 3.27). In the latter two groups spawning occurred over 4 (Gp. B) and 2 (Gp. C) week periods. Gps. D-F, which experienced 1 month periods of LL (Jan.-Feb., Feb.-Mar. and Mar.-Apr.), commenced spawning on May 9 (main spawning period began June 20), June 6 and September 12 for periods of 14 (19/20 in 8 weeks), 8 and 4 (at defined end of summer spawning period) weeks respectively (Figure 3.28). Gps. G and H, which experienced 2 month periods of LL (Jan.-Mar. and Feb.-Apr.), commenced spawning on July 4 and July 18 for periods of 8 (22/23 in 4 weeks) and 12 (at defined end of summer spawning period) weeks respectively (Figure 3.29). Of the 13 surviving fish maintained on LL for the duration of the experiment, 1 spawned on July 18 and 9 within a 4 week period commencing on September 26 (Figure 3.29); the remaining 3 fish failed to mature during the experimental period. Apart from one ill-conditioned fish in Gp. C, all surviving fish in Gps. A-H which did not mature for the second time on or before October 10 spawned during the period from October 24 to January 2, the majority during the peak of the natural spawning season in December (Figures 3.27 and 3.28).

The time of first spawning had no apparent effect on the ability of an individual to spawn again in the summer (of the fish attaining early maturity (i.e. on or before October 10) 37 had previously spawned on December 11, 39 on December 23 and 2 on January 5). However, as in experiment 5, there was a tendency for fish which had spawned earliest initially to spawn earliest at the subsequent spawning (of the 11 fish which spawned for the second time in May and June, 9 had previously spawned on December 11).

There were marked differences in the proportion of fish attaining early maturity (i.e. on or before October 10) in each group (Figure 3.30). Only a small proportion (≤20%) of fish exposed to 2 weeks LL (Gps. A-C) spawned again in the summer. Similarly, only a

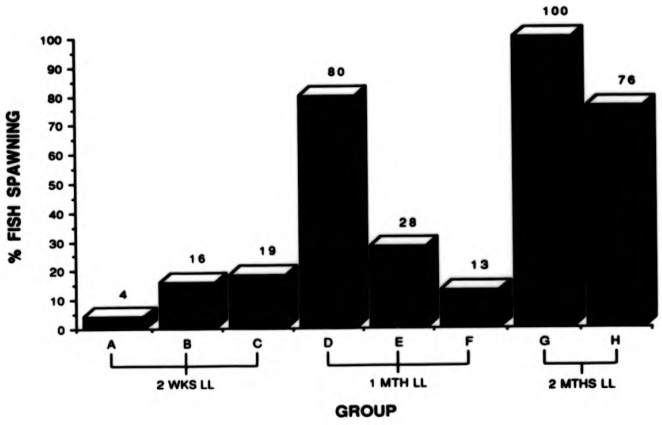


Figure 3.30: The proportion of female rainbow trout responding to either 2 week (Gps. A-C), 1 month (Gps. D-F) or 2 month (Gps. G and H) periods of continuous light with an advance in the timing of maturation in experiment 6 (LL from: Gp. A, Jan. 25-Feb. 8; Gp. B, Feb. 23-Mar. 9; Gp. C, Mar. 24-Apr. 7; Gp. D, Jan. 15-Feb. 15; Gp. E, Feb. 15-Mar. 16; Gp. F, Mar. 16-Apr. 15; Gp. G, Jan. 15-Mar. 16; Gp. H, Feb. 15- Apr. 15).

minority (≤28%) of fish subjected to 1 months LL from Feb.-Mar. (Gp. E) or Mar.-Apr. (Gp. F) attained early maturity. However, a high proportion (≥80%) of fish attained early maturity after exposure to LL for 1 month from Jan.-Feb. (Gp. D) or 2 months from Jan.-Mar. (Gp. G). A majority (76%) of the fish subjected to LL for 2 months from Feb.-Apr. (Gp. H) also matured early, but, in contrast to the highly synchronized spawning profiles of Gps. D and G, spawning occurred over an extended period in Gp. H.

Comparison of confidence limits showed that the proportion of fish attaining early maturity (i.e. on or before October 10) was significantly lower in Gps. A, B, C and F than in Gps. D, G and H ($P \leq 0.01$), and in Gp. E than in Gps. D ($P \leq 0.01$), G ($P \leq 0.01$) and H ($P \leq 0.05$). There were significant differences ($P \leq 0.05$; nonparametric multiple comparisons procedure) between the mean (summer) spawning times of Gps. C and F ($P \leq 0.05$) and between Gp. H and Gps. B ($P \leq 0.05$), C ($P \leq 0.01$), D ($P \leq 0.001$) and G ($P \leq 0.01$).

Egg diameter and fecundity

During the natural winter spawning season at the start of the experiment the mean egg diameter (mm.) and total fecundity (number of eggs) were 4.36 (±0.03; n=41) and 3246 (±96; n=41) respectively (measurements made on December 11 and 23; insufficient data for calculation of relative fecundity). The mean weight (gm.) was 1179 (±51; n=41: measurements made on January 5).

As some fish were found to be ripe on every sampling visit from May through to January this experiment provided a comprehensive test of the effect of spawning time on egg diameter and fecundity. There was a significant variation ($P \leq 0.001$) in mean egg diameter (mm.) over time (Figure 3.31), with values ranging from 3.71 (±0.23; n=2) in May to a peak of 4.99 (±0.17; n=4) in late October. There was also a significant variation ($P \leq 0.001$; Kruskal-Wallis test) in both total (Figure 3.32) and relative (Figure 3.33) fecundity over time in the abscence of a significant variation in mean weight (Figure 3.34).

Mean total fecundity (number of eggs) peaked at 5697 (±651; n=6) in mid-August

with a nadir of 3220 (±141; n=43) in early December; total fecundity in early December was significantly different from that in mid-July, early and mid-August, and mid-October, and total fecundity in mid-December was significantly different from that in mid-July and early August ($P \leq 0.05$; nonparametric multiple comparisons procedure). Mean relative fecundity (number of eggs/kg) peaked at 3282 (±259; n=5) in mid-September with a nadir of 1789 (±81; n=43) in early December; relative fecundity in early December was significantly different from that in early and mid-July, early August and mid-September, and relative fecundity in mid-December was significantly different from that in early August ($P \leq 0.05$; nonparametric multiple comparisons procedure). Mean weight (gm.) ranged from 1524 (±103; n=7) in early June to 2083 (±148; n=7) in mid-October.

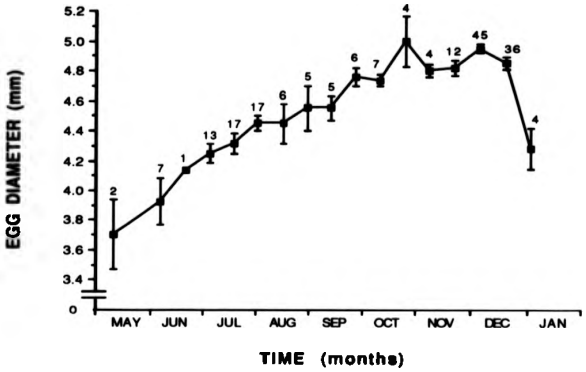


Figure 3.31: The relationship between egg size (mean ± 1SEM) and the timing of spawning in rainbow trout (pooled data from all groups in experiment 6). The number of fish examined on each sampling date is indicated above the error bars.

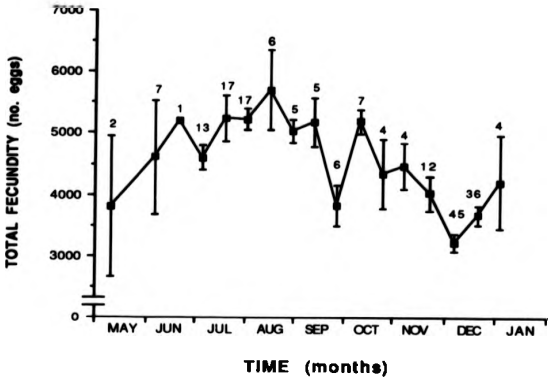


Figure 3.32: The relationship between total fecundity (mean ± 1SEM) and the timing of spawning in rainbow trout (pooled data from all groups in experiment 6). The number of fish examined on each sampling date is indicated above the error bars.

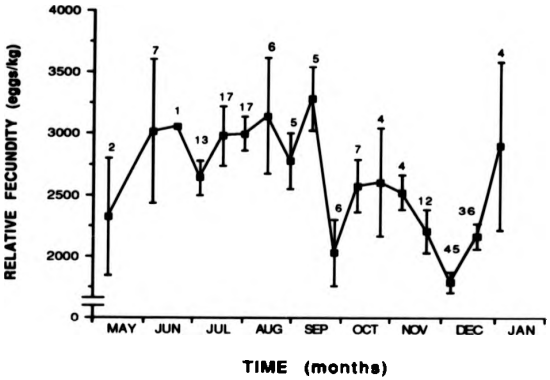


Figure 3.33: The relationship between relative fecundity (mean ± 1SEM) and the timing of spawning in rainbow trout (pooled data from all groups in experiment 6). The number of fish examined on each sampling date is indicated above the error bars.

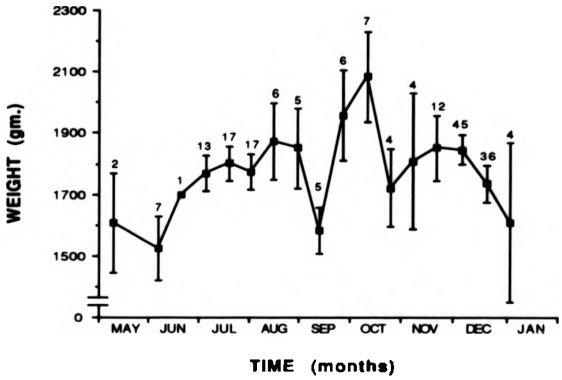


Figure 3.34: The relationship between fish weight (mean ± 1SEM) and the timing of spawning in female rainbow trout (pooled data from all groups in experiment 6). The number of fish examined on each sampling date is indicated above the error bars.

3.3.4 Experiment 7: The advancement of spawning in the rainbow trout by 2 months exposure to LL: a commercial trial

3.3.4.1 Protocol

By transferring fish between tanks maintained under ambient daylength or LL, 224 female broodstock, all of which spawned for the first time in December, were exposed to 2 months of LL from January 16 until March 15 (Group A). Male broodstock were also added to the tanks at a ratio of 1 male to 3 females. A further 25 females (Group B) and 40 males (Group C) were subjected to LL only from January 16 until the experiment was terminated on October 16. Another 40 males (Group D) were exposed to 2 months of LL from January 16 until March 20 in an otherwise ambient regime. Water temperature varied seasonally from 7-18°C.

The rate of maturation was assessed by measuring serum calcium (as an index of vitellogenin: section 2.4) levels at approximately monthly intervals. Male fish were stripped in a similar manner to females (section 2.2). When milt could be expelled from the urogenital papilla by applying gentle pressure to the abdomen a fish was said to be 'running'. The number of running and 'non-running' males was recorded at monthly intervals.

3.3.4.2 Results

Spawning

The spawning times of the fish in each group are illustrated in Figure 3.35. Apart from one fish, which spawned on May 8, spawning in females subjected to 2 months LL from January to March (Gp. A) commenced on July 3, with 96% (133) of the 139 surviving fish attaining early maturity, 92% within a 6-week period in July and August. In contrast, only 63% of females exposed to LL throughout the experiment (Gp. B) attained early maturity, spawning over an extended period commencing with a single fish on July 21. Milt was first available from male fish on June 5, approximately one month before spawning commenced in the females. At this time approximately 88% of the males

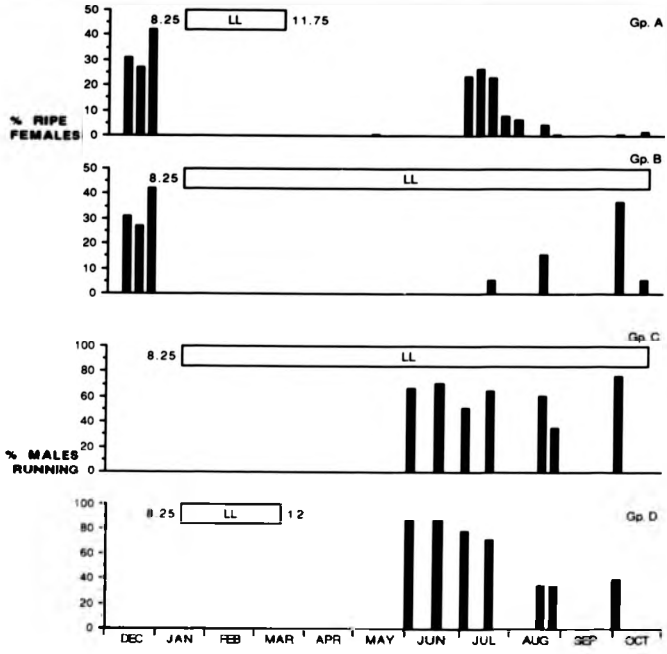


Figure 3.35: The advancement of spawning by exposure of female (Gp. A) and male (Gp. D) rainbow trout to 2 month periods of continuous light (LL) in a commercial scale trial (experiment 7). The timing of maturation in smaller numbers of female (Gp. B) and male (Gp. C) fish maintained under LL throughout the experiment is also shown. The histograms represent the percentage of ripe females or running males on each sampling date (natural spawning time of females shown on the left in upper graphs). A rectangular box indicates the timing of the LL period for each group. Integers next to the LL periods indicate ambient daylength before and after exposure.

subjected to 2 months LL from January to March (Gp. D), and 67% of those exposed to LL throughout the experiment (Gp. C), were running. Milt continued to be available from male fish throughout the spawning period of the females, with the percentage of males running remaining slightly higher in Gp. D than Gp. C until July, after which the situation was reversed.

Calcium

Changes in total serum calcium levels during the reproductive cycle were significant in both groups of female fish ($P \leq 0.001$, Gp. A; $P \leq 0.01$, Gp. B), but there were no significant changes in calcium during the reproductive cycles of the male fish (Gps. C and D). The calcium profiles of Gps. A and B differed in accordance with the differences in their spawning profiles (Figure 3.36). Calcium levels in Gp. A began to increase between March and May and continued to rise at a moderate rate from May to June before rising steeply to reach about 40mg% by the last sampling point in July; calcium levels in Gp. A were significantly higher than those in Gps. C and D in May, June and July. Calcium levels in Gp. B, at 13-14mg%, were consistently 2-3mg% higher than those in Gps. C and D (significant at $P \leq 0.05$ in May compared to Gp. C), but did not begin to increase above these levels until the last sampling point in July, at which time they were significantly elevated compared to Gps. C and D, having reached approximately 19mg%.

Egg diameter and fecundity

In the winter prior to photoperiod treatment the mean egg diameter (mm.), total fecundity (number of eggs) and relative fecundity (number of eggs/kg) were 4.37 (±0.09; n=19), 4062 (±226; n=19), and 3187 (±290; n=19) respectively (all measurements made on December 19).

In the summer spawning fish of Gp. A the mean egg diameter, total fecundity and relative fecundity were 4.04 (±0.04; n=37), 4909 (±273; n=34) and 2462 (±120; n=34) respectively (data combined from measurements taken on July 17 and 21, and August 2). Egg diameter and relative fecundity were both significantly lower ($P \leq 0.01$ and

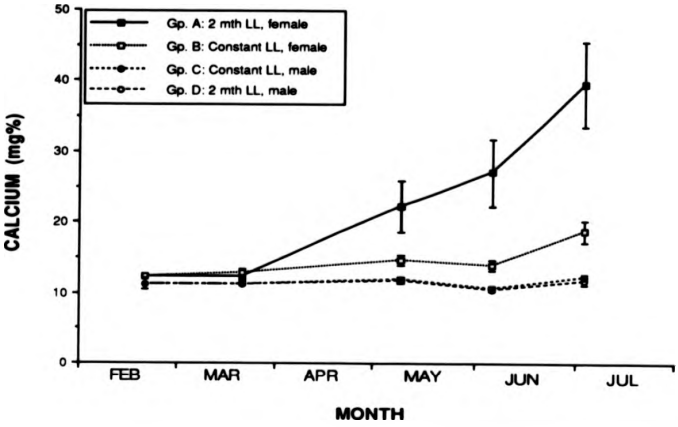


Figure 3.36: The effects of exposure to a 2 month period of continuous light (LL) or LL throughout the reproductive cycle (experiment 7) on the timing of changes in total serum calcium levels (mean ± 1SEM) during the maturation of female (Gps. A (n=12) and B (n=6-12)) and male (Gps. C (n=6-11) and D (n=6)) rainbow trout.

$P \leq 0.05$ respectively) in the summer than in the previous winter, whereas total fecundity was significantly higher ($P \leq 0.05$) in summer spawning fish. Insufficient data was available for analysis of egg diameter and fecundity in Gp. B.

3.3.5 Experiment 8: Delaying spawning in the rainbow trout by 2 months exposure to LL: a commercial trial

3.3.5.1 Protocol

By transferring fish between tanks maintained under ambient photoperiod or LL approximately 200 two-year old female rainbow trout, which had spawned for the first time the previous December (same year class as those in experiment 7), were exposed to 2 months of LL from July 21 until September 22. Male broodstock were also added to the tanks at a ratio of 1 male to 3 females. As the fish approached maturity the following year they were examined at 1-2 week intervals by farm workers who recorded the spawning times of individual fish and the number of fish which failed to mature. Egg diameter and fecundity measurements were recorded on February 5 and February 13. The experiment was terminated on April 26.

3.3.5.2 Results

Spawning

The spawning times of the fish are illustrated in Figure 3.37. Although 1 fish spawned on January 23 the main spawning period commenced on February 5. Over a period of about 12 weeks 125 of the 171 surviving fish (73%) attained maturity. Of these, approximately 43% spawned in February, 22% in March and 8 % in April. Visual inspection indicated that none of the remaining 27% were close to maturity when the experiment was terminated on April 26. It should be noted that 2-year old broodstock of the same strain maintained under ambient photoperiod spawned (for the first time) between January 6 and January 18, slightly later than usual, although it had not been possible to inspect these fish in December.

Egg diameter and fecundity

The mean egg diameter (mm), total fecundity (number of eggs), and relative fecundity (number of eggs/kg), calculated from fish which matured on February 5 and 13, was 4.60

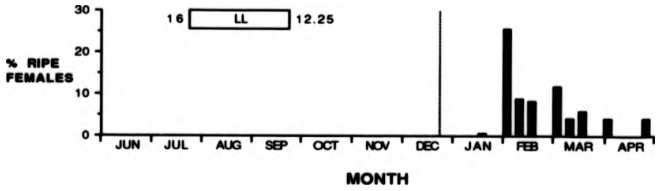


Figure 3.37: The delay in spawning time obtained by exposing female rainbow trout to a 2 month period of continuous light (LL) in a commercial scale trial (experiment 8). The histograms represent the percentage of ripe females on each sampling date. The dashed vertical line indicates the mean natural spawning time of the same year class the previous winter.

(±0.07; n=16), 3209 (±222; n=16) and 1713 (±151; n=16) respectively.

3.3.6 Phase-response curve

By combining the results of experiments 5-8 it is possible to construct a partial phase-response curve, analogous to those commonly used to monitor the response of circadian rhythms to environmental signals. The curve describes the changing magnitude of the phase-shifts (advance or delay in the timing of maturation) which occur in response to 2 month periods of LL applied at different phases of the circannual cycle (Figure 3.38). Each phase-shift is plotted against the mid-point of the LL period to which it relates. Since it was not always possible to maintain untreated fish of the same stock until the natural breeding season following the end of each experiment, the advance or delay (in mean spawning time) was calculated relative to the mean natural spawning time of the fish at the start of each experiment (i.e., it was assumed that, had no stimulus been applied, the fish would have spawned at a similar time the following year).

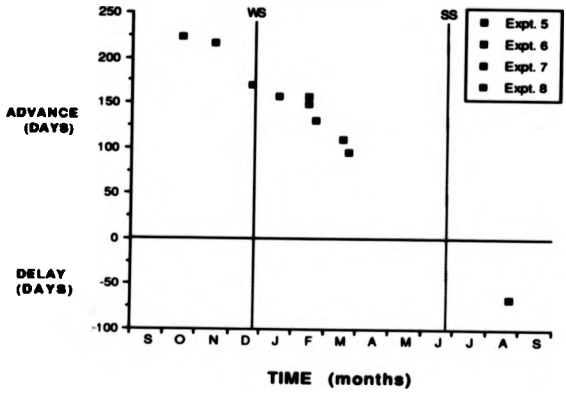


Figure 3.38: Partial phase-response curve illustrating the phase-shifts (advance or delay in spawning time) of an endogenous circannual rhythm caused by exposure to 2 month periods of continuous light at various phases of the reproductive cycle of the female rainbow trout (WS = winter solstice, SS = summer solstice). Phase-shifts are calculated relative to the mean natural spawning time of the fish at the start of each experiment (curve prepared by combining data from experiments 5-8).

Summary of Results: Section B

1. Exposure of rainbow trout to 2 month periods of LL at various times (mid-September to mid-April) close to the natural breeding season advanced subsequent maturation by 3-7 months.

2. The timing of the second (summer) spawning period was directly related to the timing of the light period, commencing 6-7 months after first exposure to LL.

3. The proportion of fish responding to LL with an advance in spawning time was dependent on the timing of the light period: a majority (≥85%) of the fish maintained under LL from December-February and January-March attained early maturity, but only a minority (≤33%) of fish subjected to LL outside these periods matured.

4. The minimum duration of exposure to LL required to advance maturation in a majority (≥80%) of the fish was 1 month (January to February); 2 weeks exposure to LL advanced spawning in less than 20% of the fish.

5. The majority of fish which failed to respond to short periods of LL with an advance in spawning time attained maturity during the natural breeding season.

6. In a commercial trial 133 out of 139 fish (96%) subjected to 2 months LL from January to March spawned again in the summer (principally July and August); milt was available from similarly treated males throughout this period.

7. Spawning occurred earlier and was more synchronized in fish exposed to short (1 or 2 month) periods of LL than in fish subjected to constant LL from the same date; these differences were reflected in the serum calcium profiles recorded under each photoperiod.

8. Exposure of rainbow trout to a 2 month period of LL from late July to late September delayed maturation until February to April (1-4 months).

9. Combining the results of experiments 5-8 allowed the effects of 2 month periods of LL on the entrainment of the circannual clock controlling reproduction to be described in the form of a phase-response curve.

10. Egg size was related to spawning time; fish attaining early maturity produced significantly smaller eggs than later spawners.

11. Both the total and relative fecundities of fish in which spawning was advanced to July and August by exposure to LL were significantly higher than those of fish which matured during the natural spawning period in December.

3.4 Discussion

3.4.1 Entrainment of the endogenous circannual clock by changes between 'long' and 'short' daylengths.

The results of experiments 1-4, which are summarised in Table 3.5, demonstrate that exposure to a constant 'long' photoperiod early in the reproductive cycle, followed by an abrupt reduction to a shorter daylength after 3-4 months, can advance the time of spawning in the rainbow trout by up to 4 months. This is in general agreement with previous studies on the rainbow trout (Whitehead and Bromage, 1980; Bromage et al., 1982b, 1984; Elliott et al., 1984; Duston and Bromage, 1987, 1988), and the masu salmon (Takashima and Yamada, 1984). If one accepts that maturation in the rainbow trout is ultimately under endogenous control (section 3.1), further evidence for which will be discussed later, it follows that any modification in spawning time can be interpreted as the overt expression of either a phase advance or a phase delay of the endogenous circannual clock. It is therefore proposed that the advances in spawning time observed in response to 'long' to 'short' photoperiod regimes in experiments 1-4 reflect phase advances of the endogenous clock. They do not, however, reflect single phase advances caused by exposure to 'long' days in advance of those experienced under a natural photoperiod. This point is well illustrated by the results of experiment 2 (Figure 3.10). Although spawning was advanced by 1-2 months in previously untreated fish maintained under constant 18L:6D from January or February (Gps. E and G), a 3-4 month advance was observed in fish which were additionally exposed to a reduction in photoperiod in May (Gps. D and F). Similar results have been reported in several previous investigations (Bromage et al., 1982; Duston and Bromage, 1987, 1988). The increase in daylength early in the reproductive cycle, and the subsequent decrease to a 'short' photoperiod in May, therefore caused two separate phase advances of the endogenous clock, i.e., the fish interpreted each change in photoperiod (premature arrival of 'long' or 'short' daylengths) as an indication that their clock was running 'slow' and compensated with a corrective forward adjustment.

Table 3.5: Summary of the effects of 'long' to 'short' photoperiod regimes (section A) on the time of spawning of female rainbow trout.

Expt./Gp.	Photoperiod Regime	Date of Increase	Date of Decrease	Main Spawning Period	Mean Natural Spawning time
1A [99]	18L:6D/6L:18D	Jan. 17	May 8	Aug. 23-Oct. 10	Dec. 7
1B [99]	14L:10D/6L:18D	Jan. 17	May 8	Sept. 3-Oct. 31	Dec. 7
1C [99]	10L:14D/6L:18D	Jan. 17	May 8	Oct. 2-Dec. 2	Dec. 7
1D [99]	6L:18D/2L:22D	None [a]	May 8	Dec. 2-Feb. 19	Dec. 7
1E [99]	6L:18D/18L:6D/6L:18D	Mar. 26	May 8	Sept. 18-Oct. 31	Dec. 7
2A [108, b]	18L:6D/6L:18D	Dec. 2	May 15	June 29-Sept. 23	Dec. 11
2B [108, b]	18L:6D/6L:18D	Jan. 19	May 15	July 28-Oct. 7	Dec. 11
2C [110]	18L:6D/6L:18D	Dec. 23	May 15	Aug. 26-Oct. 7	Dec. 11
2D [110]	18L:6D/6L:18D	Jan. 19	May 15	Aug. 26-Oct. 21	Dec. 11
2E [110]	18L:6D	Jan. 19	None	Oct. 7-Dec. 18	Dec. 11
2F [110]	18L:6D/6L:18D	Feb. 19	May 15	Sept. 23-Nov. 5	Dec. 11
2G [110]	18L:6D	Feb. 19	None	Nov. 5-Dec. 18 [c]	Dec. 11
3A [122]	22L:2D/13.5L:10.5D	Jan. 19	May 6	Aug. 17-Oct. 11	Dec. 11
3B [122]	20L:4D/11.5L:12.5D	Jan. 19	May 6	Aug. 31-Oct. 25	Dec. 11
3C [122]	18L:6D/9.5L:14.5D	Jan. 19	May 6	Aug. 17-Oct. 25	Dec. 11
3D [122]	16L:8D/7.5L:16.5D	Jan. 19	May 6	Aug. 17-Oct. 25	Dec. 11
3E [122]	14L:10D/5.5L:18.5D	Jan. 19	May 6	Aug. 31-Oct. 25	Dec. 11
3F [123]	8.5L:15.5D	None	None	Dec. 20-May 9	Dec. 11
3G [123]	As 3F + 1wk 18L:6D	April 29	May 6	Dec. 6-June 6	Dec. 11
4A [130]	22L:2D/13.5L:10.5D	Jan. 17	May 9	Aug. 18-Sept. 14	Dec. 27
4B [130]	18L:6D/9.5L:14.5D	Jan. 17	May 9	Aug. 18-Sept. 14 [d]	Dec. 27
4C [130]	14L:10D/5.5L:18.5D	Jan. 17	May 9	Aug. 18-Sept. 28	Dec. 27
4D [130]	12L:12D/3.5L:20.5D	Jan. 17	May 9	Aug. 18-Sept. 28	Dec. 27
4E [130]	10L:14D/1.5L:22.5D	Jan. 17	May 9	Nov. 9-Dec. 20	Dec. 27
4F [130]	8.5L:15.5D/1.5L:22.5D	Jan. 17	May 9	Oct. 24-Dec. 20	Dec. 27

Graphical representation of the results for each group can be found on the page indicated by the superscript.
a; photoperiod reduced from ambient (8.5L:15.5D) to 6L:18D on Jan. 17.
b; fish previously subjected to advancing photoperiods in experiment 1.
c; estimate of spawning period.
d; only two surviving fish.

Previous studies utilising 'long' to 'short' photoperiod regimes to investigate the entrainment of the annual reproductive cycle of the rainbow trout have emphasised the importance of the abrupt reduction in daylength as an entraining cue for the advancement of spawning time (Duston and Bromage, 1987, 1988). As these investigations used 18L:6D as the 'long' photoperiod in all their experiments they were unable to detect any effects that this daylength per se may have had on maturation. Experiments 1, 3 and 4 were principally designed to investigate the effects of varying the length of the (or magnitude of the increase to a) 'long' photoperiod in fish subjected to 'long' followed by 'short' daylengths in advance of the natural light cycle, in order to determine which feature(s) of the photoperiodic signal (absolute daylength, magnitude of change in daylength, direction of change of daylength) is important for the entrainment of reproduction.

In experiment 1 fish were exposed to an increase in photoperiod from 8.5L:15.5D (ambient daylength) in January to either 18L:6D (Gp. A), 14L:10D (Gp. B) or 10L:14D (Gp. C), reduced to 6L:18D in all groups in May. Spawning was considerably advanced in all 3 groups in comparison with the natural spawning period (Figure 3.5), even though the fish in Gp. C received a maximum daylength of only 10 hours, which would normally be regarded as a 'short' day. It is proposed that the circannual clock controlling maturation was advanced in each group firstly, by the increase in photoperiod in January and secondly, by the decrease in photoperiod in May. These results suggest that it was the increase in photoperiod that was important for the entrainment of the endogenous clock rather than the absolute daylength. However, although spawning was considerably advanced in all 3 groups the timing of maturation was not identical; the greater the length of the 'long' photoperiod, and hence the greater the magnitude of the changes in daylength in January and May, the greater was the advancement in spawning time (Figure 3.5). A similar relationship between the magnitude of the change in photoperiod and spawning time was observed by Duston and Bromage (1987) in fish exposed to a constant long photoperiod (18L:6D) followed by one of a range of shorter photoperiods (6L:18D, 10L:14D and 14L:10D). In their study spawning commenced in the group exposed to the

largest reduction in daylength 21 days before the group subjected to the smallest reduction. In experiment 1, however, the fish exposed to the largest changes in photoperiod (Gp. A) commenced spawning 40 days earlier than those subjected to the smallest changes. This suggests that the magnitude of the increase in photoperiod in January and the decrease in daylength in May caused approximately equal (though relatively minor) adjustments in the timing of maturation.

Experiments 3 and 4 further investigated the importance of absolute daylength by subjecting fish to a wide range of 'long' photoperiods varying from 10 to 22 hours; the differences in the magnitude of the May reduction in photoperiod inherent in the design of experiment 1 were eliminated by decreasing the daylength by 8.5 hours (approximating to the difference in daylength between the summer and winter solstices) in all groups. Spawning was advanced in all groups (Gps. A-E, experiment 3; Gps A-E, experiment 4) compared to fish maintained under ambient conditions (Figures 3.18 and 3.22). Apart from Gp. A vs Gp. D in experiment 4, there were no significant differences in spawning time between groups subjected to 'long' photoperiods of between 12 and 22 hours (12L:12D, 14L:10D, 16L:8D, 18L:6D, 20L:4D and 22L:2D), which represented increases from the ambient daylength in January of between 3.5 and 13.5 hours; the advances achieved in each experiment were virtually identical. Thus, a 'long' photoperiod of 12 hours (Gp. D, experiment 4) was as effective as daylengths of up to 22 hours (Gp. A, experiment 3) for the advancement of spawning. Moreover, maturation was independent of the length of the 'short' photoperiod, which ranged from 3.5 to 13.5 hours in groups exposed to 'long' photoperiods of between 12 and 22 hours. Similarly, Takashima and Yamada (1984) reported virtually identical advances in the timing of maturation in masu salmon exposed to 6, 8 or 12 hour photoperiods after maintenance on LL from December to April, although a reduction to only 18 hours was less effective. These results support the proposition that it is the change in photoperiod (increase or decrease), rather than the absolute daylength, or the magnitude of the change in photoperiod, which is most important for the entrainment of the endogenous clock controlling reproduction in the female rainbow trout.

Although advanced compared to fish maintained under ambient conditions, fish exposed to a 'long' photoperiod of only 10 hours (Gp. E, experiment 4) did not commence spawning until about 12 weeks after those subjected to daylengths of 12 hours or more (Figure 3.22). Analogous results were obtained in experiment 1 (Gp. C vs Gps. A and B; Figure 3.5), and it was suggested that such differences in spawning time may be attributable to the differences in the magnitude of the changes in photoperiod. In experiments 3 and 4, however, the magnitude of the decrease in photoperiod was constant in fish subjected to 'long' daylengths of between 10 and 22 hours, and there was no difference in the effects of increases in photoperiod varying between 3.5 and 13.5 hours. It is interesting therefore that fish subjected to a 1.5 hour increase in photoperiod in January (Gp. E, experiment 4) spawned nearly 3 months later than those exposed to only a 3.5 hour increase (Gp. D, experiment 4). Two possible explanations will be considered. Firstly, the rainbow trout may be able to discriminate between photoperiods of 10 and 12 hours (or between increases in photoperiod of 1.5 and 3.5 hours). This implies the existence of a 'critical' photoperiod for the initiation of reproductive development, and it is true to say that daylengths greater than 12 hours have generally been accepted as 'long' days and the spring equinox has been assumed to be the time at which gonadal recrudescence is initiated in the rainbow trout under natural conditions (Scott et al., 1984; Scott, 1990). However, the concept of a critical daylength is considered untenable for the rainbow trout for reasons which will become clear later. An alternative explanation is that the fish were unable to detect a single abrupt increase in daylength of only 1.5 hours and hence the advance in spawning time was due solely to the decrease in photoperiod in May. Certainly the 1-2 month advance achieved with a 'long' photoperiod of 10 hours in both experiment 4 (Gp. E) and experiment 1 (Gp. C) equates with that attributed to a single change in photoperiod (an increase) in experiment 2 and previous studies (Whitehead and Bromage, 1980; Bromage et al., 1982b, 1984; Duston and Bromage, 1986a, 1987, 1988). A note of caution regarding the results obtained for Gp. E in experiment 4; a 2-4 week interruption of the period of 'long' days may have occurred due to an electrical malfunction. This may explain why these fish, which received an 8.5 hour decrease in photoperiod in May, commenced

spawning more than 1 month later than fish which experienced a reduction of only 4 hours at this time (Gp. C, experiment 1), whereas the groups exposed to a 'long' photoperiod of 18 hours in the two experiments spawned at approximately the same time (cf. Figures 3.5 and 3.22).

Clearly, to distinguish the effects of an increase in photoperiod to 10 hours in January from that of a decrease in daylength in May it is necessary to repeat the experiment with a control group maintained on a constant 10L:14D photoperiod from January. However, further support for the conclusion that the advance in spawning time in groups exposed to 'long' days of 10 hours was due solely to the subsequent reduction in daylength in May is provided by the important observation that maturation can be advanced even in fish which do not experience any increase in daylength in advance of the natural light cycle. Thus, spawning was advanced by about 2 months relative to the natural spawning period in fish maintained from January on a daylength of only 8.5 hours (approximating to ambient daylength at the time) followed by a reduction to 1.5L:22.5D in May (Gp. F, experiment 4; Figures 3.22 and 3.39). This is similar to the advances obtained in fish exposed to 'long' days of 10 hours, followed by 'short' days of 6L:18D and 1.5L:22.5D in experiments 1 and 4 respectively. Conversely, spawning was delayed and occurred over an extended period in fish maintained under a constant 8.5L:15.5D photoperiod from January in experiment 3 (Gp.F; Figures 3.19 and 3.39), whereas the spawning times of other directly comparable groups in the two experiments (those subjected to 'long' photoperiods of 22, 18 and 14 hours) were virtually identical. This suggests that the reduction in photoperiod in May provided a cue which both advanced and synchronized maturation. In contrast, fish maintained on constant daylength exhibited a desynchronization of spawning times characteristic of a free-running circannual rhythm (discussed more thoroughly later).

If, as suggested, an increase in photoperiod of 1.5 hours in January was insufficient to advance spawning, whereas a decrease in daylength of 2.5 hours in January phase delayed the circannual clock, it would appear that the minimum abrupt change in daylength capable of phase-shifting the clock lies between 1.5 and 2.5 hours. Abrupt changes in photoperiod do not, of course, occur under natural conditions, in which the annual

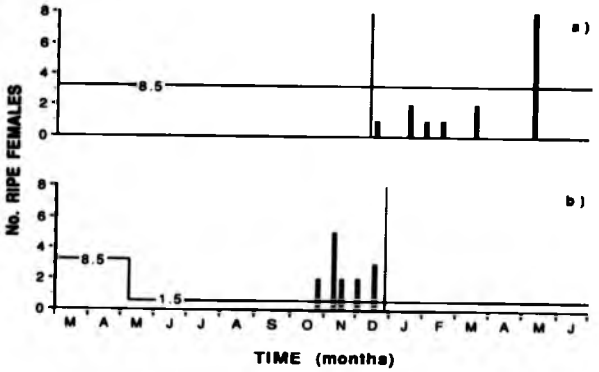


Figure 3.39: Comparison of the effects of exposure to a) constant 8.5L:15.5D (ambient daylength at the start of the experiment in January) or b) 8.5L:15.5D from mid-January to early May, followed by 1.5L:22.5D until spawning, on the spawning time of female rainbow trout (data from a) experiment 3, Gp. F and b) experiment 4, Gp. F). The dotted lines indicate the mean natural spawning time in each experiment.

reproductive cycle is entrained by the seasonally-changing daylength. However, the stability of the clock in response to small but abrupt (rather than gradual) changes in photoperiod may protect it from the effects of 'noise' inherent in the environmental signal, such as coloured water during flooding or extensive cloud cover which may otherwise be perceived as late dawn or early dusk and cause a phase-shift of the clock.

As previously noted, not all photoperiod regimes applied in experiments 1-4 advanced maturation. In experiment 1 Gp. D were exposed to 6L:18D from January, reduced to 2L:22D in May. These fish commenced spawning in early December, a slight delay when compared to the natural spawning period (Figure 3.5). Unfortunately, the limited facilities available precluded the inclusion of a group of fish maintained under a constant 6L:18D photoperiod as a control for Gp. D, and hence interpretation of the results for this group requires caution. However, fish of the same strain exposed to constant 6L:18D under identical conditions the previous year did not commence spawning until March (Duston and Bromage, 1987). Similar delays in spawning time have been reported by Bromage et al. (1984). Furthermore, when fish which had spawned late under 6L:18D were subjected to this daylength for an additional period spawning was not delayed further, but occurred at intervals of approximately 1 year (Duston and Bromage, 1988a). These data suggest that the considerable delay in spawning time initially observed under constant short photoperiods is caused by the reduction from ambient photoperiod (8.5L:15.5D) to 6L:18D. This conclusion is supported by the results of Gp. F in experiment 3. From January onwards this group were maintained on a constant 8.5L:15.5D photoperiod, which approximated to ambient daylength at the time. However, in contrast to Duston and Bromage (1987), who reported that fish maintained on constant 6L:18D from the same date (and under identical temperature, light intensity and feeding conditions) did not commence spawning until March the following year, the fish maintained under 8.5L:15.5D in this study commenced spawning in December (Figures 3.19 and 3.39). The marked desynchronization in spawning time between individuals maintained under constant 8.5L:15.5D (spawning occurred over a 6 month period) compared to those exposed to a reduction to 6L:18D in January and to 2L:22D in May (spawning occurred over a 3 month

period) suggests that at least one of these changes in photoperiod acted as a synchronizing cue. Moreover, as mentioned previously, when fish maintained on 8.5L:15.5D from January were subjected to a decrease in photoperiod to 1.5L:22.5D in May (Gp. F, experiment 4) they commenced spawning in October, approximately 2 months in advance of those maintained on the constant 'short' daylength (Figure 3.39). It is therefore proposed that the circannual clocks of the fish in Gp. D (experiment 1) were initially phase delayed by the reduction from ambient daylength to 6L:18D in January, and that spawning was only slightly delayed in these fish because they also received a phase advance when the photoperiod was further reduced to 2L:22D in May. Thus, the fish interpreted the decrease in photoperiod in January as an indication that their endogenous clock was running 'fast', and responded with a corrective backward adjustment, whereas the decrease in photoperiod in May was perceived as an indication that their clock was running 'slow' and hence they responded with a corrective forward adjustment.

3.4.2 Direction of change of daylength and photoperiodic history.

The principle conclusion to emerge from the results of experiments 1, 3 and 4 is that the direction of change of daylength is responsible for the entrainment of the endogenous circannual clock which controls reproduction in the female rainbow trout; daylength per se (absolute daylength), and the magnitude of change in daylength, are of little importance in the entrainment process. This implies that the rainbow trout reads daylengths comparatively, with reference to the preceding photoperiod, rather than absolutely. The response of the fish to a particular daylength therefore depends on the previous photoperiod(s) experienced, that is, their photoperiodic history. In experiments 3 and 4, for example, 14L:10D and 12L:12D were interpreted as 'long' days after an increase from 8.5L:15.5D in January, but similar photoperiods (13.5L:10.5D and 11.5L:12.5D) were perceived as 'short' days after a decrease from either 22L:2D or 20L:4D in May. Similarly, reductions in daylength from 6L:18D to 2L:22D (experiment 1), and from 8.5L:15.5D to 1.5L:22.5D (experiment 4), were both interpreted as decreases from a 'long' to a 'short' photoperiod. Clearly, any photoperiod may be perceived by the fish as

'long' or 'short' providing it is longer or shorter than that to which they have been previously exposed. The traditional concept of a rigid 'critical' daylength for reproductive function is therefore untenable in the rainbow trout.

The importance of photoperiodic history for salmonid fish was, in fact, recognised many years ago by Henderson (1963) from her work on the brook trout: 'the effect of a given photoperiod on one phase (of the reproductive cycle) can depend on the photoperiod to which the fish has been exposed during a previous phase'. The reproductive response to a particular daylength has also been shown to be dependent on recent photoperiodic history in a number of higher vertebrates including Japanese quail, (Coturnix coturnix japonica: Robinson and Follett, 1982), voles (Microtus montanus: Horton, 1984, 1985: Microtus pennsylvanicus: Lee and Zucker, 1988), hamsters (Phodopus sungorus: Hoffman, 1984, Stetson et al., 1986: Mesocricetus auratus: Hastings et al., 1986, Russk, 1988), rabbits (Oryctolagus cuniculus: Boyd, 1986) and sheep (Ovis aries: Robinson and Karsch, 1987). For example, LH concentrations increased when ewes were subjected to a decrease in daylength from 16L:8D to 13L:11D, but fell to undetectable levels in animals exposed to an increase in photoperiod from 10L:14D to 13L:11D (Robinson and Karsch, 1987). The sheep were therefore able to perceive 13L:11D as either a 'short' or a 'long' day, depending on the direction of change of daylength. In contrast, the ability of starlings, Sturnus vulgaris, to accurately measure (absolute) daylength is not affected by photoperiodic history and hence the gonadal response to a particular photoperiod is constant (Dawson, 1987). Thus, photoperiodic history is important for seasonal reproduction in a variety of species but may not be of universal significance.

3.4.3 Influence of the timing of a change in daylength on the entrainment of the endogenous circannual clock.

It is clear from experiment 2 that, in addition to the direction of change of daylength, the timing of each change in photoperiod, is important for the entrainment of the endogenous clock controlling maturation in the rainbow trout. Thus, spawning was advanced compared to the natural spawning period in all groups subjected to long

(16L:8D) followed by short (8L:16D) daylengths in advance of the natural light cycle. However, there were marked differences in spawning time between groups in accordance with the timing of the increase to a 'long' photoperiod (Figures 3.9 and 3.10). Spawning occurred in sequence in previously untreated fish exposed to an increase in photoperiod in December (Gp. C), January (Gp. D) and February (Gp. F), followed in each case by a decrease in daylength in May. Similarly treated fish, which had experienced a previous advance in spawning time in experiment 1, also attained maturity earlier when the increase in photoperiod occurred in December (Gp. A) than when it occurred in January (Gp. B). Moreover, spawning occurred earlier in fish exposed to a constant 'long' photoperiod (no reduction in May) from January (Gp. E) than in those subjected to the same photoperiod from February (Gp. G). The timing of the increase to a 'long' photoperiod is therefore an important determinant of spawning time.

The timing of an increase in photoperiod in advance of the natural light cycle also determines the timing of certain morphological and physiological changes associated with the process of smoltification (seawater adaptation) in Atlantic salmon. Thus, the development of salinity tolerance and reduction in condition factor associated with this major developmental change occurred in late February, late February, mid-March and mid-April respectively in salmon parr subjected to an increase from ambient daylength to 16L:8D on December 31, February 1, March 1 and April 1, in each case considerably advanced in comparison with the natural time of smoltification in late May (Duston and Saunders, 1990). Similarly, smoltification was advanced by 7, 5 and 3 weeks in Atlantic salmon parr transferred from 8L:16D to 16L:8D in late December, late January and late February (Thrush and Bromage, unpublished). Smoltification in Atlantic salmon is also thought to be under endogenous circannual control (Eriksson and Lundquist, 1982), suggesting that similar mechanisms are involved in the timing of reproduction and the timing of smoltification in salmonids.

Clearly, the differences in spawning time between the groups subjected to a constant 'long' photoperiod only (Gps. E and G) in experiment 2 can be attributed exclusively to the difference in the timing of exposure. In groups exposed to a 'long' to 'short' photoperiod

regime, however, the reduction in photoperiod to 6L:18D in May may also have had a differential phase-advancing effect on spawning time, dependent on the timing of the increase in photoperiod. If, as the results suggest, increases in photoperiod in December, January or February caused advance phase-shifts of different magnitude in accordance with their timing, then the reduction in photoperiod in May would be expected to perturb the circannual clock at a different phase in each group. This may explain why the difference in mean spawning time between the groups exposed to a January/May 'long' to 'short' switch (Gp. D) and a constant 'long' photoperiod from January (Gp. E) was only 32 days, whereas that between the groups subjected to a February/May 'long' to 'short' regime (Gp. F) and a constant 'long' daylength from February (Gp. G) was at least 55 days. Clearly, the difference in spawning time within each pair (Gp. D vs Gp. E; Gp. F vs Gp. G) can be attributed solely to the reduction in photoperiod in May. Thus, the decrease in photoperiod advanced spawning by 32 days in Gp. D (January increase) and by 55 days in Gp. F (February increase). It is proposed that this differential effect of 'short' days arose because exposure to an increase in photoperiod in January advanced the circannual clock to a greater extent than an increase in February, and hence the reduction in photoperiod in May occurred at an earlier (less advanced) phase of the circannual cycle after a February (Gp. F) than a January (Gp. D) increase. The earlier in the circannual cycle a reduction from a 'long' to a 'short' photoperiod occurs (up to a point), the greater the phase advance (Duston and Bromage, 1988). Thus, the decrease in daylength occurred at an earlier phase of the circannual cycle in Gp. F than in Gp. D and therefore caused a greater advance in spawning time (55 days compared to 32 days).

Experiment 2 also demonstrated that 'long' to 'short' photoperiod regimes are an effective means of further advancing the spawning time of fish in which maturation has been advanced by similar photoperiodic manipulations the previous year. Fish which had spawned several months in advance of the natural spawning season in experiment 1 (principally in September and October) were transferred to 6L:18D after spawning and then exposed to 18L:6D from either early December (Gp. A) or mid-January (Gp. B), followed in each case by a reduction in photoperiod to 6L:18D in May. Spawning commenced

at least 2 months (Gp. A) or 1 month (Gp. B) earlier in these fish than in the previous year (c.f. Figures 3.5 and 3.9), and was similarly advanced compared to the earliest spawning fish from other groups in the same experiment (c.f. Figures 3.9 and 3.10). As previously noted, however, maturation can be advanced by 3-4 months in previously untreated fish using 'long' to 'short' photoperiod regimes. It is proposed that such advances were not achieved in the previously manipulated fish because they were not exposed to the 'long' photoperiod until either 2-3 (Gp. A) or 3-4 (Gp. B) months after spawning. In this respect Gp. A is comparable to Gp. F (experiment 2), which was subjected to an increase in photoperiod about 2 months after the natural spawning period, and subsequently attained maturity approximately 2 months in advance of fish maintained under ambient conditions (Figure 3.10). It is therefore suggested that, had the increase in daylength occurred closer to the spawning time of the previously advanced stock (e.g late October/November), and the decrease in daylength also been brought forward accordingly (e.g to February/March), a further 3-4 month advance in the timing of maturation could have been achieved.

3.4.4 Effects of changes in daylength on the hormonal changes accompanying maturation.

The sequence of changes in serum levels of oestradiol-17β, testosterone and calcium (as an index of vitellogenin) in experiment 1 was the same as that observed in previous studies (Figure 1.5: Scott et al., 1980; Scott and Sumpter, 1983; Elliott et al., 1984; Duston and Bromage, 1987). However, there were often marked differences in the timing of these changes in accordance with the differences in spawning time between groups. Thus, steroid and calcium levels started to increase in Gp. A, which commenced spawning on August 23, well before those in Gp. C, which commenced spawning on October 2 (Figures 3.6, 3.7 and 3.8). Similarly, hormonal changes occurred much earlier in Gp. C than in Gp. D, which commenced spawning on December 2. As the sequence of endocrine changes accompanying maturation in the rainbow trout is well established (section 1.5; Figure 1.5) only a single hormonal parameter was used to monitor maturation rate in experiments 2-4. In each of these experiments the timing of the changes in serum calcium

levels was consistent with the timing of maturation. Thus, in experiment 2, calcium levels in fish exposed to a January/May 'long' to 'short' photoperiod regime (Gp. D) began to increase approximately 1 month before those in their counterparts subjected only to a constant 'long' photoperiod from January (Gp. E; Figure 3.17). Similarly, fish exposed to a February/May 'long' to 'short' photoperiod regime (Gp. F) exhibited marked increases in calcium levels about 2 months before a similar rise occurred in fish subjected only to a constant 'long' photoperiod from February (Gp. G; Figure 3.17). In both examples differences in the timing of changes in calcium levels accurately reflected the difference in spawning time between the groups. In experiment 3 the temporal changes in serum calcium were virtually identical in fish exposed to 'long' photoperiods of between 14 and 22 hours (Gps. A-E; Figure 3.20), as were those in fish subjected to constant 'short' days and 'short' days interspersed with one week of 'long' days (Gps. F and G; Figure 3.21). Concomitant with the differences in spawning time, however, marked increases in serum calcium were not apparent in Gps. F and G until about 3-4 months after they occurred in Gps. A-E. Similarly, in experiment 4, serum calcium levels increased 2-3 months later in fish exposed to 'long' days of 10 and 8.5 hours (Gps. E and F) than in those subjected to 'long' photoperiods of between 12 and 22 hours (Gps. A-D; Figure 3.23), again consistent with differences in spawning time. Additionally, the first significant ($P \leq 0.05$) increase in serum calcium in fish exposed to a 'long' daylength of 12 hours (Gp. D) occurred 1 month after that in fish subjected to a 'long' photoperiod of 22 hours (Gps. A), coincident with the slightly later spawning time in the former group. It is clear, therefore, that the modifications in spawning time achieved by manipulation of photoperiod in experiments 1-4 were mediated by changes in the timing of the endocrine events controlling reproduction (see section 1.5).

Considering the three directly comparable groups in experiment 1 (Gps. A, B and C), increases in serum levels of calcium and oestradiol-17β (but not testosterone) were more pronounced in the fish which experienced the greatest advances in spawning time. Accepting the possible misinterpretation that may arise because hormone levels were measured only at monthly intervals, peak levels of these two serum components in fish

which commenced spawning on August 23 (Gp. A) were approximately double those in fish which commenced spawning on September 19 (Gp. C; Figures 3.6 and 3.7). Although these differences were not statistically significant, a similar relationship between calcium (but not steroid) levels and spawning time was observed by Whitehead et al. (1978), who suggested it may reflect an increased rate of vitellogenesis in preparation for early spawning. Alternatively, lower blood calcium levels may result from an enhanced or more efficient uptake of vitellogenin by the developing oocytes. It is unlikely, however, that reduced oestradiol-17β levels would accompany such an effect.

Differences in peak hormone levels may also be related to the magnitude of the changes in photoperiod experienced by each group. Thus, the greater the magnitude of the changes in photoperiod in experiment 1 (e.g. a 9.5 hour increase followed by a 12 hour decrease in Gp. A, compared to a 1.5 hour increase followed by a 4 hour decrease in Gp. C) the greater were the serum calcium and oestradiol-17β levels attained. A similar effect was noted by Robinson and Karsch (1987) who found that peak levels of luteinizing hormone (LH) in sheep were much lower following a 3-hour decrease in daylength (approximately 3ng/ml) than after an 6-hour decrease in daylength (approximately 10ng/ml; Karsch et al., 1986). The minor adjustments in spawning time attributed to differences in the magnitude of the changes in photoperiod in experiment 1 may therefore be mediated through a differential effect on the endocrine events controlling ovarian development. In experiments 3 and 4, however, there was no consistent relationship between peak calcium levels and the magnitude of the increase in photoperiod (3.5-13.5 hours) in fish which attained maturity at the same time (Gps. A-E, experiment 3; Gps A-D, experiment 4: Figures 3.20 and 3.23), but peak calcium levels were considerably lower in fish which spawned within a similar period 2-3 months later (Gps. E and F, experiment 4: Figure 3.23). Although it should be noted that the total difference in magnitude of the photoperiodic changes experienced by these groups was less than in experiment 1, because the reduction in daylength was constant, these data support the hypothesis that differences in peak calcium levels are related to the time of spawning rather than to the magnitude of the changes in photoperiod (although these hypotheses are not mutually exclusive), indicative of an

increased rate of vitellogenesis in earlier spawning fish.

The peak in serum calcium and oestradiol-17β levels may also have been lower in some of the later spawning fish because the spawning period was extended, reflecting a desynchronization of spawning time between individual animals (discussed in more detail later). Thus, serum calcium and oestradiol-17β profiles were broader in fish which spawned over an 11 week period in experiment 1 (Gp. D) than in fish with a spawning period of 6-10 weeks (Gps. A, B, C and E; Figures 3.6 and 3.7). It should be noted that 11 weeks is probably an underestimate of the spawning period in Gp. D as calcium and sex steroid levels in 2 of the 5 remaining immature fish indicated that they would have completed maturation had the experiment not been terminated. Although it was not possible to blood sample fish in Gps. F and G in experiment 3 throughout their extended spawning periods (20 and 26 weeks respectively) it is clear that their serum calcium profiles were also extended (Figure 3.21).

3.4.5 Photoperiodic effects on reproduction in virgin and previously mature fish.

Henderson (1963) reported that gonadal maturity could be advanced by photoperiod manipulation in brook trout undergoing their second or third reproductive cycle, but not in those maturing for the first time. No such difference was apparent in experiment 2 of the present study in which virgin fish, and fish undergoing their second reproductive cycle, responded similarly to a variety of photoperiod regimes; although there was a tendency for 2+ fish to spawn later than 3+ fish (Figure 3.10) the difference was not significant, and there were no consistent differences between 2+ and 3+ fish with regard to serum calcium profiles (Figures 3.11-3.16) or the proportion of fish attaining maturity (Table 3.2). Moreover, virgin and previously mature fish in experiment 2 (Gp. D) attained maturity at approximately the same time as virgin fish exposed to a similar photoperiod regime in experiment 1 (Gp. A). This indicates that, for the rainbow trout, it is valid to make comparisons between experiments utilising either virgin fish or fish which have spawned previously. It should be noted, however, that there was a significant ($P \leq 0.01$) effect of age on spawning time in experiment 4, the mean spawning time of 2+

fish occurring about 2 weeks later than that of 3+ fish (Figure 3.22). Additionally, in several groups (Gps. C, D and E), significantly less ($P \leq 0.05$) virgin fish than previously mature fish attained maturity (Table 3.4). These effects may be related to the consistently low water flow rates imposed on the fish in experiment 4 (due to drought conditions), which may have had deleterious effects on factors such as oxygen availability, feeding and stress. Feeding and growth are suppressed by stress (Pickering, 1989, 1990) and fish size is considered to be a major determinant of maturation in salmonids (Alm, 1959; McCormick and Naiman, 1984). Artificial diet restriction suppresses growth of rainbow trout (Springate et al., 1985; Bromage and Jones, 1991), produces a slight delay in spawning time (Springate et al. , 1985), and causes a reduction in the proportion of fish attaining maturity (Scott, 1962; Springate et al., 1985; Bromage and Jones, 1991). In the most recent study only 35% of female rainbow trout fed a 'low' ration diet throughout the reproductive cycle attained maturity compared to 68% fed a 'high' ration diet (Bromage and Jones, 1991). Diet restriction has also been reported to decrease the proportions of brown trout (Bagenal, 1969) and Atlantic salmon attaining maturity (Thorpe, 1989). Unfortunately, fish which did not mature in experiment 4 were not weighed. However, the mean post-spawning weight of the virgin fish which did mature in experiment 4 was significantly less than that of their counterparts in experiment 2 (925g. vs 1517g.; $P \leq 0.001$, Student's t-test), which were similarly subjected to competition from older (larger) fish. There was no difference in the mean weight of the fish at the start of each experiment. This suggests that the poor environmental conditions experienced by the fish in experiment 4 suppressed growth, and, in virgin fish, which would be expected to have lower energy reserves than the larger, previously mature fish, may have inhibited reproduction (diverting energy resources to maintenance of body weight). In this respect, Thorpe (1986) has suggested that maturation of Atlantic salmon will proceed only if their rate of aquisition of surplus energy is above a genetically determined threshold level in the spring, and Dutil (1986) postulated that the 'decision' of Arctic char, Salvelinus alpinus, to mature is also dependent on the internal perception of energy reserves in the spring. Moreover, recent work (Carragher and Sumpter, 1990)

has indicated that stress affects all levels of the reproductive axis in salmonids, causing changes in secretion of GTH and sex steroids and suppressing gonadal growth.

3.4.6 The advancement of maturation by short periods of continuous light.

Photoperiod regimes utilising continuous light (LL), and hence requiring no blackout facilities, are potentially attractive for the commercial production of out-of-season eggs. A series of experiments, summarised in Table 3.6 (experiments 5-8), were therefore conducted to assess the ability of short periods of LL to modify the timing of maturation in rainbow trout maintained under naturally fluctuating water temperatures. Experiment 5 investigated the effects of exposure to 2 month periods of LL at a variety of times (between mid-September and mid-April) close to the natural spawning season (November-January). Although some fish were exposed to LL just prior to the natural spawning period (e.g. September-November) this did not affect the timing of their first (winter) spawning. The timing of the second (summer) spawning period in each group was directly related to the timing of the light period, commencing 6-7 months after first exposure to LL (i.e. 3-7 months in advance of the expected spawning time of the ambient controls; Figure 3.25). Similar effects were observed in response to 2 month periods of LL in experiments 6 and 7 (Figures 3.29 and 3.35). These results concur with those of experiment 2 and confirm that the timing of a change in photoperiod is an important determinant of spawning time. Moreover, they confirm the predominance of photoperiod over temperature as a zeitgeber for the annual reproductive cycle since the experiments were conducted under naturally fluctuating water temperatures; in each case spawning occurred at the peak of the seasonal temperature cycle rather than the nadir as occurs under natural conditions. Takashima and Yamada (1984) have also investigated the effects of short periods of LL on the timing of reproduction in salmonid fish. They reported that maturation was advanced by about 4 months in masu salmon exposed to LL from mid-December to mid-February (and 6L:18D thereafter), compared to a 2-3 month advance in fish subjected to LL from mid-February to mid-April or mid-December to mid- April. Clearly, the greater advance observed in the former group was caused by the earlier

Table 3.6: Summary of the effects of continuous light photoperiod regimes (section B) on the time of spawning of female rainbow trout.

Expt./Gp.	Time of Exposure	Duration of Exposure	Percentage Maturation [a]	Main Asesasonal Spawning Period	Mean Natural Spawning time [b]
5A [145]	Sept. 15-Nov. 17	2 months	18	Apr. 23-May 5	Dec. 12
5B [145]	Oct. 15-Dec. 16	2 months	21	Apr. 23-May 22	Dec. 12
5C [145]	Nov. 17-Jan. 21	2 months	33	June 2-Aug. 10	Dec. 12
5D [145]	Dec. 16-Feb. 17	2 months	85	June 17-Aug. 10	Dec. 12
5E [145]	Jan. 21-Mar. 25	2 months	100	July 16-Sept. 21	Dec. 12
5F [145]	Feb. 17-Apr. 23	2 months	25	Aug. 24-Oct. 8	Dec. 12
Amb. [143]	None	None	8	Oct. 8	Dec. 12
LL [143]	Sept. 15 onwards	All year	30-50 [c]	May 11-Aug. 10	Dec. 12
6A [149]	Jan. 25-Feb. 8	2 weeks	4	May 9	Dec. 19
6B [149]	Feb. 23-Mar. 9	2 weeks	16	June 6-Aug. 15	Dec. 19
6C [149]	Mar. 24-Apr. 7	2 weeks	19	June 6-Aug. 15	Dec. 19
6D [150]	Jan. 15-Feb. 15	1 month	80	May 9-Aug. 15	Dec. 19
6E [150]	Feb. 15-Mar. 16	1 month	28	June 6-Sept. 12	Dec. 19
6F [150]	Mar. 16-Apr. 15	1 month	13	Sept. 12-Oct. 10	Dec. 19
6G [151]	Jan. 15-Mar. 16	2 months	100	July 4-Aug 29	Dec. 19
6H [151]	Feb. 15-Apr. 15	2 months	76	July 18-Oct. 10	Dec. 19
LL [161]	Jan. 15 onwards	All year	62	July 18-Oct. 10	Dec. 19
7A (f) [161]	Jan. 16-Mar. 15	2 months	96	July 3-Aug. 30	Dec. 21
7B (f) [161]	Jan. 16 onwards	All year	63	July 21-Oct. 16	Dec. 21
7C (m) [161]	Jan. 16 onwards	All year	36-76 [d]	June 5-Oct. 2	nd
7D (m) [161]	Jan. 16-Mar. 20	2 months	34-88 [d]	June 5-Oct. 2	nd
8 [166]	July 21-Sept. 22	2 months	73	Jan. 23-Apr. 26	Dec. 21

Graphical representation of the results for each group can be found on the page indicated by the superscript.
a; percentage asesasonal maturation.
b; natural spawning time of the fish at the start of each experiment.
c; estimate of percentage maturation.
d; percentage of 'running' males on each sampling occasion.
f; females.
m; males.
nd; not determined.

reduction from LL to 6L:18D, verifying the importance of the timing of photoperiodic change demonstrated in the present work.

One feature of the summer spawning profiles recorded in experiment 5 is that the majority of the individuals in each group matured within a short period, generally less than 8 weeks. In contrast, fish maintained under constant LL from September in experiment 5 spawned over a 13 week period (Figure 3.24). Although this effect was not clear in fish maintained under the same photoperiod in experiment 6 this group contained only small numbers of fish by spawning time and received its last synchronizing cue (the increase to LL) at a later date (January). Moreover, a similar desynchronizing effect has been observed in fish maintained under constant LL in previous studies (Bourlier and Billard a,b, 1984; Bromage et al., 1984; Duston and Bromage, 1986a; Duston, 1987). These results suggest that the advances in spawning time achieved in experiments 5-7 were due to 2 synchronizing cues, as in experiments 1-4. Thus, both the increase from ambient photoperiod to LL and the decrease from LL to ambient daylength 2 (or less) months later phase advanced the circannual clock controlling reproduction (as discussed previously the decrease in photoperiod would be expected to have a differential effect in each group according to the phase to which the clock had been advanced by the preceding increase in photoperiod). This conclusion is supported by the observation that fish subjected to constant LL from September (experiment 5) or January (experiments 6 and 7) spawned later than their counterparts exposed to 2 months LL from September-November (experiment 5) or January-March (experiments 6 and 7).

It is notable that the advance in spawning time achieved with periods of LL was greater in experiments 5-7 than in fish subjected to comparable 'long' to 'short' photoperiod regimes in experiments 1-4. Thus, spawning commenced in early July following exposure to LL from January to March, and in late August after receiving 18L:6D from January to May. Although this could be attributed to a difference in responsiveness between strains both stocks had a similar natural spawning period with a peak in December. There are several alternative explanations, which, it should be emphasised, are not mutually exclusive. Firstly, fish exposed to LL from January to March experienced an increase in

photoperiod of about 16 hours followed by a decrease of approximately 14 hours, whereas those subjected to 18L:6D from January to May received an increase of 9.5 hours followed by a decrease of 12 hours. Although the results of experiment 1 suggested that large differences in the magnitude of the changes in daylength may cause minor adjustments in spawning time experiments 3 and 4 indicated that differences in magnitude totalling up to 12 hours had no effect on spawning time. Thus, it is unlikely that the difference in spawning time between the two treatments was due to the difference in magnitude of the photoperiodic change. A more likely explanation is that the reduction from LL to ambient daylength in March caused a greater advance in spawning time than a reduction from 18L:6D to 6L:18D in May. A previous study, using the same strain of rainbow trout maintained under identical conditions to those used in experiments 1-4, has demonstrated that, up to a point, the earlier the reduction in photoperiod occurs the greater the advance in spawning time; reductions on March 1 and May 1 advanced the commencement of spawning to late July and late August respectively (Duston and Bromage, 1988). Interestingly, however, only 26% of the fish exposed to a reduction in daylength in March attained maturity compared to 77% of those subjected to a reduction in May (Duston and Bromage, 1988). In contrast, virtually all of the fish attained maturity in the present study when exposed to a reduction from (a 2 month period of) LL to ambient photoperiod in March. This suggests that either the changes in photoperiod occur at a different phase of the circannual clock in the 2 strains or that LL affects the clock in a different manner to 'long' days. Another alternative is that the increasing temperatures experienced by the fish exposed to LL during the pre-spawning period may have accelerated gonadal development compared to fish subjected to a 'long' to 'short' switch and maintained under a constant temperature of 8°C. Certainly, the incorporation of vitellogenin into rainbow trout ovarian follicles in vitro rises with increasing temperature (Tyler et al., 1987b), and maintenance of rainbow trout in cold water delays spawning (Morrison and Smith, 1986). Although temperature is clearly not the predominant environmental cue controlling reproduction in the rainbow trout it may serve to 'fine tune' the time of spawning (section 1.2).

3.4.7 Influence of the timing of exposure to short periods of continuous light on the proportion of fish attaining maturity.

Although some fish from each group in experiment 5 responded to 2 month periods of LL with an advance in the timing of maturation there were marked differences in the proportion of fish attaining maturity depending on the position of the LL period in relation to the phase of the reproductive cycle. Thus, a majority of the fish (≥85%) exposed to 2 months LL from December-February and January-March attained maturity but only a minority (≤33%) subjected to LL outside these periods matured (Figure 3.26). Similarly, maturity was advanced in a high proportion (≥80%) of fish exposed to 1 month of LL from January-February in experiment 6, but only a few fish (≤28%) exposed to this light treatment at other times responded (Figure 3.30; Table 3.6). The reasons for this differential response to the same photoperiod are unclear. However, a similar variation in the proportion of female rainbow trout maturing following photoperiod treatment has recently been reported by Duston and Bromage (1988). They found that the incidence of sexual maturation following exposure to a 'long' to 'short' photoperiod regime was dependent on the timing of the reduction in daylength relative to the phase of the reproductive cycle. Thus, the proportion of fish attaining early maturity when an increase in photoperiod in January was followed by a reduction in daylength on the first day of March, April, May or June was 26, 52, 77 and 92% respectively. Clearly, however, the duration of exposure to the 'long' photoperiod was also a variable in this experiment, ranging from about 6 to 19 weeks. In this respect, Bromage et al. (1984) found that exposing fish previously maintained on 6L:18D to 6 weeks 18L:6D from early May to mid-June had no effect on spawning time. In these two studies, therefore, only a small minority or zero fish respectively, responded to 6 weeks exposure to a 'long' photoperiod with an advance in spawning time. In contrast, 70% of the fish attained early maturity when exposed to 18L:6D for 6 weeks from late March to early May in experiment 1 (Gp. E) of the present study, a similar percentage to those recorded for groups exposed to 'long' days for nearly 4 months in the same experiment. Thus, the timing of exposure to a 'long' photoperiod in relation to the phase of the reproductive cycle appears to be more

important than the duration in determining the proportion of fish attaining early maturity.

Interestingly, when fish which initially spawned 3-4 months early in Duston and Bromage's (1988) experiment (previous paragraph) were maintained on a constant 'short' day, along with their non-responding counterparts, both the previously mature and immature fish attained maturity approximately 1 year later, again 3-4 months in advance of the natural spawning period. Thus, the circannual clock of the non-responding fish was also phase advanced by the initial photoperiod treatment, but this advance was not overtly expressed (as an advance in spawning time) until the following year. These results lead the authors (Duston and Bromage, 1988) to propose the hypothesis that there is a period of time associated with a particular phase of the circannual clock which represents an allowed zone, or 'gate', through which the fish must pass if they are to mature that year. The concept of gating was originally developed by Pittendrigh (1966) to describe circadian rhythms in events which occur only once in the lifetime of an individual and hence are only detectable in mixed-age populations. This phenomenon has principally been studied in insects where events such as egg hatch, pupation and eclosion can occur only at the phase of the circadian cycle when the gate is open; an individual which has not completed the necessary developmental steps prior to the gate closing must wait until the gate re-opens the following day (Saunders, 1977; Brady, 1981). Duston and Bromage (1988) similarly speculated that in order for rainbow trout to pass through the proposed gate in a particular year they must reach a certain threshold stage of development before the circannual clock reaches the phase at which the gate is closed; fish which do not attain this threshold must wait for the gate to re-open the following year (in this model a progressively greater number of fish would have reached the threshold stage of development before the reductions in photoperiod in March, April, May or June advanced the phase of the circannual clock beyond the 'gate-open' phase). Thorpe (1986) suggested an analogous model of developmental regulation in the Atlantic salmon, whereby the 'decision' to mature or smolt depends on a metabolic assessment taken at seasonally critical times. Thus, if a fish perceives that its rate of aquisition of surplus energy is above a

genetically determined threshold during either the critical period for maturation in the spring, or the critical period for smolting in the late summer, the relevant developmental conversion will proceed. In contrast, under poor developmental conditions (e.g. low availability of food), growth opportunities during the critical periods will be reduced and hence the developmental conversions will be postponed until the following year. Duston and Bromage (1986) have suggested that a similar gating mechanism is involved in the timing of puberty in sheep as they will not mature below a certain threshold size and, even then, oestrous can only occur during a specific period of the year (Foster et al., 1985, 1986).

As previously mentioned, the concept of gating is generally applied to once in a lifetime events and, in rainbow trout, only the timing of puberty has previously been suggested to involve circannual gating mechanisms (Duston, 1987; Duston and Bromage, 1988). The rainbow trout may undergo as many as 5 reproductive cycles and it is open to debate whether the maturation and ovulation of a particular batch of oocytes should be considered a once in a lifetime event. More likely, it would be viewed as one expression of an annually-repeated cycle. Nevertheless, the involvement of endogenous rhythms in the control of once in a lifetime or repeated events, although qualitatively different, merely involves two aspects of the same phenomenon (Saunders, 1977; Brady, 1981); in each case the event can only occur at a particular phase of, for example, the circadian or circannual cycle. In this respect there may be types of annually-repeated events, the control of which, like some forms of daily-repeated behaviour (Brady, 1981), involves some form of gating mechanism. It is possible, therefore, to explain the differences in the proportion of fish responding to 2 (and 1) month periods of LL with an advance in spawning time in the current study by the involvement of a gating mechanism. Thus, only a minority of the fish exposed to LL from September-November, October-December and November-January in experiment 5 attained maturity because only a small proportion had reached the threshold stage of development before the circannual clock was advanced beyond the gate-closed position. In contrast, a majority of the fish subjected to LL from December-February and January-March had reached the threshold before the clock was advanced beyond the gate-closed position and hence proceeded to maturity. This hypothesis

does not, however, explain why only a minority of the fish subjected to LL from February-April in experiment 5 attained early maturity (a majority of the fish exposed to the same photoperiod attained early maturity in experiment 6, but the LL period occurred slightly earlier relative to the winter spawning and the summer spawning was desynchronized); all the fish would have been expected to have reached the threshold before the clock was phase advanced. The most likely explanation is that the increase in photoperiod to LL occurred close to the time at which the fish perceived an increase in photoperiod was occurring under natural conditions; that is, the 'long' (LL) photoperiod was not interpreted as arriving prematurely by the majority of the fish.

Additional evidence supporting the existence of gated circannual rhythms is provided by Blake's (1959) work on pupation and eclosion rhythms in the carpet beetle, Anthrenus verbasci. Similar mechanisms appear to be involved in the timing of reproduction in the ragworm, Nereis diversicolor (Olive and Garwood, 1983; Olive, 1984). In both species the proportion of the population passing through each of the annual gates appeared to be dependent on temperature. It is thought unlikely that temperature per se affected the proportion of fish responding to each period of LL (or passing through the 'gate') in the present investigation since in the study previously referred to, in which a similar phenomenon was observed (Duston and Bromage, 1988), the fish were maintained under constant temperature.

The application of the 'gating' hypothesis to the results of experiment 5 may be inappropriate. A much simpler interpretation of the results is that exposure to LL during the last few months of the first reproductive cycle had little effect on the subsequent reproductive cycle because the next batch of oocytes had not yet been recruited; only a few of the earliest spawning fish were able to respond to the LL period at this time. Scott et al. (1984) suggested that the 6 month advancement in spawning time observed in fish subjected to a contant long (16L:8D) photoperiod was related to the coincidence of the photoperiod treatment with the surge in GTH release which occurs during final oocyte maturation (migration of the nucleus and germinal vesicle breakdown) and the period immediately following ovulation. The maintenance of this surge in GTH levels following

ovulation may be important for the recruitment of the following batch of oocytes, and hence only oocytes which have been exposed to LL during or after this surge are able to mature early.

As previously mentioned, fish which did not respond to a 'long' to 'short' photoperiod regime with an advance in spawning time in a previous study, did not then spawn at the natural time when subsequently maintained under a constant 'short' photoperiod, but instead expressed the advance the following year (Duston and Bromage, 1988). When not exposed to LL the fish in experiment 5 were maintained under ambient photoperiod. Unfortunately, it was not possible to maintain fish which failed to attain early maturity in experiment 5 until the subsequent natural breeding season. However, it is evident from the results of experiment 6 that all of the fish which failed to respond to the LL period eventually spawned at the normal time (Figures 3.27 and 3.28). This suggests that either the LL period was ignored completely or (perhaps indicated by the slight desynchronization of spawning close to the natural time) that the fish which were physiologically incompetent to mature when their clock was advanced by exposure to LL re-entrained to the natural photocycle when returned to ambient daylength.

3.4.8 Influence of the duration of exposure to continuous light on the proportion of fish attaining maturity.

One feature of interest from both a physiological and commercial viewpoint is the duration of exposure to a 'long' photoperiod required to elicit a reproductive response. The discussions of experiment 1 (Gp. E) and experiment 5 concluded that the position of the 'long' photoperiod in relation to the phase of the reproductive cycle is more important than its duration in determining the proportion of fish attaining maturity. However, the duration of the 'long' photoperiod was 6 weeks in experiment 1 (Gp. E) and 2 months in experiment 5. Similarly, the minimum duration of exposure to 'long' days used in previous studies is 6 weeks (Bromage et al., 1984; Duston and Bromage, 1988). Experiment 6 therefore investigated the importance of duration of exposure to 'long' days by subjecting fish to either 2 weeks, 1month or 2 months LL during the period (shortly

after the preceding spawning season) when a majority of the fish responded to 2 months LL with an advance in spawning time in experiment 5. Although a few fish (≤19%) responded to only 2 weeks LL, spawning was advanced in a majority of fish (≥80%), and in a synchronized manner, only in those groups exposed to LL for 1 month from January to February or for 2 months from January to March (Figures 3.28 and 3.29). The minimum duration of exposure to LL required to advance spawning in a majority of the fish therefore lies between 2 weeks and 1 month. This conclusion is supported by the results of experiment 3 (Gp. G) in which fish were exposed to 1 week of 'long' days from late April to early May in an otherwise short day regime. This treatment had no significant effect on the timing of spawning compared with fish maintained on constant 'short' days (Gp. F; Figure 3.19), although it should again be noted that the timing of the exposure may have been more important than the duration in this respect. Certainly, even within the relatively short period during which the light treatments were applied in experiment 6 (January-April) the position of the LL periods in relation to the phase of the reproductive cycle determined the proportion of fish responding to 1 or 2 months exposure with an advance in spawning time.

Only two previous studies have investigated the effects of short periods of LL on reproduction in salmonid fish. Duston (1987) found that 1 months exposure to LL from early September to early October had no effect on subsequent spawnings of rainbow trout of the same strain as those used in the present study, and maintenance under LL from early September to early November resulted in only a small proportion of fish spawning the following summer, a similar result to the present study. Takashima and Yamada (1984) monitored changes in the mean GSI of male and female masu salmon exposed to either 4 months (mid-December to mid-April), 2 months (mid-February to mid-April), 1 month (mid-March to mid-April) or 10 days (mid-March to late March) of LL. They reported that gonadal development was enhanced in all groups compared to fish maintained under ambient photoperiod, but the maturation rate in fish exposed to only 10 days LL was much slower than in the other groups. These results are in broad agreement with those of the present study, and it is unfortunate that no details of the proportion of fish responding to

each treatment were provided. Recent studies in the sea bass have also demonstrated that 1 months exposure to 'long' days (15L:9D) is sufficient to advance or delay maturation, but other periods of exposure were not tested (Carrillo et al., 1989a,b). There appears to have been little similar work in other vertebrates although it is well known that as little as 1 day of exposure to a 'long' photoperiod is sufficient to stimulate reproductive development in some birds such as the Japanese quail (Follett, 1982) and Yellon and Foster (1985) reported that 1 week of 'long' (15L:9D) days was sufficient to ensure the normal onset of puberty in female sheep, although oestrus commenced slightly later than in animals exposed to 5 and 10 week blocks of 'long' days.

The preceding discussion indicated that the rainbow trout is insensitive to periods of LL of less than 1 month. Although the majority of fish did not respond to 2 weeks LL, however, a small proportion did. This suggests that, like some other vertebrates, fish are able to detect relatively short periods of 'long' days. The reason for the low percentage response to 2 week periods of LL may be inherent in the heterogenous nature of the population, rather than an inability to detect very short periods of LL per se. Since the natural spawning period of individuals within the population may vary by 6-8 weeks a period of LL lasting 2 weeks would be expected to impinge on the circannual clock of individual fish at different phases. Longer periods of LL, however, might be expected to perturb the clock at the phase(s) required to advance spawning in a much greater number of the fish; a 2 month period of LL would encompass the individual variation of the entire population and would therefore be expected to invoke a response in all the fish.

3.4.9 Commercial application of continuous light photoperiod regimes.

One of the primary a[illegible] experiments with LL was to develop a reliable method for the commercial p[illegible]eson rainbow trout eggs without the need for blackout facilities. In [illegible]s (5-7) over 90% of females exposed to a 2 month period of LL from January March spawned again in a 6 week period in July and August; spawning commenced on a similar date in each experiment (Figures 3.25, 3.29 and 3.35). Moreover, milt was available from similarly treated males throughout the

spawning period of the females (Figure 3.35). Exposure of rainbow trout to short periods of LL close to the natural spawning season therefore provides a simple, cheap and predictable method for the production of out-of-season eggs. In contrast, the proportion of fish attaining maturity is reduced following exposure to LL throughout the reproductive cycle and spawning occurs over an extended period (experiments 5-7: Bourlier and Billard, 1984a,b; Bromage et al., 1984; Duston, 1987; Bromage and Cumaranatunga, 1988). This point is corroborated by the results of an early commercial scale trial using the same strain of fish as in the present study, maintained under similar conditions (i.e. on the same farm), in which the fish farmer observed that spawning was desynchronized in rainbow trout exposed to LL throughout the reproductive cycle and estimated that only a third of these fish attained maturity (C. Woods, personal communication). From a practical viewpoint the method developed in this study provides several clear advantages over other methods for the photoperiodic manipulation of spawning time in that the expense and labour required for the construction and maintenance of blackout facilities is unnecessary and no re-programming of time switches is required. Additionally, the method worked well on a farm with naturally fluctuating water temperature, but herein lies a possible drawback of the technique. Ovulation occurred at the warmest time of the year when water temperatures reached 22°C (J. Symes, personal communication), although the highest temperature recorded on any sampling occasion was 18°C. Ovulated eggs remain viable in the abdominal cavity for a much shorter time at higher temperatures (Figure 3.40: Billard and Breton, 1977; Billard, 1985). This necessitates checking the fish for maturity at more frequent intervals than usual, which may lead to an increase in stress and incidence of disease. Although there were no obvious signs of such deleterious effects when fish were checked frequently in experiment 7, it is clearly preferable to maintain the fish in water of a lower (constant, if possible) temperature, at least during the spawning period (although care should be taken to avoid dramatic changes in water temperature).

In addition to advancing maturation, exposure to a short period of LL was able to delay spawning. Thus, spawning was delayed by approximately 2-3 months in fish exposed to a 2

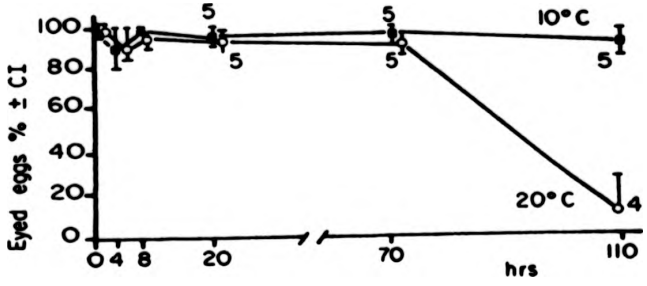


Figure 3.40: Changes in post-ovulatory egg viability in the abdominal cavity of female rainbow trout maintained at either 10 or 20 °C for 110 hours following ovulation. Fertilisation rate was assessed by measuring the percentage of eggs reaching the eyed stage (from Billard and Breton, 1977).

month period of LL from late July until late September (Figure 3.37), with 73% of the fish attaining maturity during the experimental period. A similar 2-3 month delay in maturation has previously been observed in November/December spawning strains of rainbow trout maintained under constant 16L:8D or LL from June (Shiraishi and Fukuda, 1966; Bourlier and Billard, 1984a,b). Maturation was also delayed by 1 month in a December/January spawning strain subjected to 18L:6D from the same time (Skarphedinsson, 1982), but not in similarly treated January/February spawning fish (Whitehead and Bromage, 1980; Bromage et al., 1982b). However, spawning was delayed by 3-4 months in a January/February spawning strain of rainbow trout maintained on constant 8L:16D from late February to late July and 18L:6D thereafter (Bromage et al., 1984). Exposure to constant long photoperiods or LL after the summer solstice has also been reported to delay spawning in brook trout (Allison, 1951; Hazard and Eddy, 1951; Henderson, 1963; Shiraishi and Fukuda, 1966), sockeye salmon (Combs et al., 1959; Shiraishi and Fukuda, 1966), amago salmon (Shiraishi and Fukuda, 1966), Atlantic salmon (Eriksson and Lundquist, 1980; Lundquist, 1980), chinook salmon (Johnson, 1984; Clarke, 1990), coho salmon (Clarke, 1990) and masu salmon (Takashima and Yamada, 1984). These studies have all subjected the fish to the photoperiod treatment throughout the experimental period (until close to spawning time), whereas the present study demonstrates that only a 2 month period of LL is necessary to delay spawning, provided it is applied at an appropriate phase of the reproductive cycle. Exposure to short periods of LL during the latter half of the reproductive cycle therefore provides an easily applicable technique to further spread the production of out-of-season eggs. In the present work the majority of the fish spawned in February, but spawning was slightly desynchronized, continuing through March and April. This may suggest that the increase to a long photoperiod after the summer solstice phase delayed the circannual clock controlling reproduction (clock perceived to be running 'fast'), but that the reduction in photoperiod occurred too late (too close to spawning time) to provide a synchronizing cue.

3.4.10 Egg size and fecundity.

Clearly, the commercial advantages conferred by the production of out-of-season eggs would be negated if the quality or number of the eggs produced was adversely affected. Egg quality is sometimes defined in terms of egg size and small eggs (≤4.75mm in diameter) may be unacceptable for sale (Springate and Bromage, 1984). The results of the present study demonstrate that egg size is related to the time of spawning; the greater the advance in spawning time the smaller the eggs produced. This is clear from the results of experiments 1-5, but is best demonstrated by the comprehensive set of data obtained in experiment 6 (Figure 3.31). In the latter experiment no significant change in mean fish weight occurred over the extended spawning period (Figure 3.34) allowing any effect of fish size on egg diameter to be discounted. Several previous studies have reported a similar relationship between egg size and spawning time (Nomura, 1962; Buss, 1980; Bromage et al., 1984; Duston and Bromage, 1988), possibly indicative of the shorter time available for vitellogenin incorporation into the developing oocytes of earlier spawning fish.

A pertinent question is 'Are small eggs really of a lower quality than large eggs?'. In this respect fertilisation rate, and growth and survival of the fry, may provide a more accurate index of egg quality. The percentage fertilisation (approximately 80%) of the small eggs produced in experiments 1-4 was similar to that of eggs obtained from fish during the natural spawning period (N. Trotter, personal communication; no data available for experiments 5-8). Springate et al. (1984) have shown that percentage fertilisation provides a reliable prediction of subsequent egg and fry survival. Pitman (1979) compared the survival rates of progeny reared from the relatively large eggs of 5-year old rainbow trout with those from the smaller eggs of 2-year old fish. He reported that a greater proportion of the large than the small eggs hatched and that subsequent growth and survival was greater in progeny derived from the larger eggs. A recent study conducted under carefully controlled commercial conditions confirmed that rainbow trout fry hatched from larger eggs are initially larger than those obtained from smaller eggs, but found that this size advantage had disappeared 4 weeks after first feeding and that survival rates to eyeing, hatch, swim-up and as 3-month fed fry were not correlated with egg size

(Springate and Bromage, 1985). Similarly, the initial size difference between Atlantic salmon fry derived from large and small eggs was lost during the first year of growth (Hayes and Armstrong, 1942; Thorpe et al., 1984). Thus, although larger eggs may confer an advantage for survival in the wild, under the relatively favourable conditions of the commercial hatchery egg size is probably not a major determinant of long-term growth and survival. Notwithstanding the possible commercial significance of reduced size on egg 'quality' perhaps the most important implication of studies which show that smaller eggs are produced when the timing of maturation is advanced is that egg size is less important than environmental factors in determining the time of ovulation.

In experiments 1-4, conducted at constant temperature, there were no significant differences between groups in either total fecundity (number of eggs) or relative fecundity (number of eggs/kg), although there was a tendency for the later spawning (and hence larger) fish to produce a larger number of eggs (e.g. Gps. F and G, experiment 3; Table 3.3). It is well established that larger (and older) rainbow trout have higher total fecundities than smaller (and younger) fish, although, because larger fish also produce larger eggs, they have lower relative fecundities (Springate and Bromage, 1984; Bromage et al., 1990). Experiment 6 provided a comprehensive test of the effect of spawning time on egg production. Interestingly, both the total and relative fecundities of fish in which spawning was advanced to July and August were significantly higher ($P \leq 0.05$) than those of fish which matured during the natural spawning period in December (Figures 3.32 and 3.33). The parallel changes in total and relative fecundity are explained by the previously mentioned observation that no significant change in the mean weight of the fish occurred over the period from May to January when data were collected. The lower fecundities recorded in the later spawning fish were associated with larger eggs, reflecting the 'trade-off' between egg size and number which occurs in rainbow trout (Springate and Bromage, 1984; Springate et al., 1985; Bromage et al., 1990). Thus, far from having deleterious effects on fecundity, the advancement of spawning by exposure to LL actually increased egg production.

The reason why the earlier spawning fish in experiment 6 should have a higher total

fecundity than those attaining maturity later is unclear. One possible reason is the increasing temperatures experienced by the summer-spawning fish during the pre-spawning period. Rates of food consumption are temperature dependent, a property reflected in the increase in weight of the fish between the start of the experiment in January and the commencement of spawning. In contrast, no further growth occurred during the latter half of the year as temperatures decreased, indicative of a reduction in food consumption. Food consumption is a major determinant of fecundity in fish, and a reduction in consumption is likely to produce a reduction in fecundity (Wootton, 1979). Thus, while the greater time available for the incorporation of vitellogenin into the eggs allowed for the production of larger eggs by the later spawning fish, in the absence of an increase in fish weight, and hence ovary volume (Springate and Bromage, 1984; Springate et al., 1985), the 'trade-off' between egg size and number resulted in the production of fewer eggs. An alternative explanation is that the combined effect of a greater blood vitellogenin concentration, due to increased temperature and an increased rate of vitellogenesis in preparation for early spawning, and the increased incorporation of vitellogenin into the developing oocytes which occurs (at least in vitro) at higher temperatures (Tyler et al., 1987b), was to reduce the level of atresia below that which occurs normally during ovarian development (section 1.4). Increased feeding at higher temperatures may also have decreased atresia in the earlier spawning fish since a reduced food intake, as would probably have occurred in the later spawning fish during the latter half of the year as temperatures decreased (and vitellogenesis increased), has been shown to be associated with an increase in the level of follicular atresia (Scott, 1962). In this respect, Bromage and Jones (1991) found that total fecundity was significantly reduced in female rainbow trout fed a 'low' ration diet compared to fish fed a 'high' ration diet during the middle third of the reproductive cycle. It should be noted, however, that reducing the ration over the last 3 months prior to spawning had no detrimental effects on fecundity (Bromage and Jones, 1991) and, although the fecundity of brown trout fed a restricted diet was also lower, and the eggs larger (measured as dry weight), than those of the same size (length) fed on high rations, these fecundity differences could not be accounted for by

differences in the level of atresia (Bagenal, 1969).

3.4.11 Further evidence for an endogenous circannual clock.

The preceding discussion has interpreted the experimental results in terms of the photoperiodic entrainment of an endogenous circannual clock controlling maturation. There is a considerable volume of evidence that such a clock exists (section 3.1), the strongest of which is the demonstration of free-running rhythms of reproduction in rainbow trout maintained under constant photoperiods for several years (Duston and Bromage, 1986a, 1991). Although the experiments conducted in the present study followed only one reproductive cycle they provided corroborative evidence for the hypothesis that maturation in the rainbow trout is ultimately under endogenous control. One feature of circannual rhythms is that, within a species, the free-running period may exhibit considerable inter-individual variation (Gwinner, 1986), causing the rhythms of individual animals to become desynchronized under constant conditions. The best demonstration of this characteristic in the present study is provided by the results of Gp. F in experiment 3 (Figures 3.19 and 3.39). These fish were maintained under a constant 8.5L:15.5D photoperiod, which approximated ambient daylength at the start of the experiment in January. Spawning subsequently occurred over an extended period of 20 weeks, whereas spawning was confined to 8-10 weeks in fish subjected to 'long' to 'short' photoperiod regimes in the same experiment (Gps. A-E; Figure 3.18). This indicates that, in the absence of photoperiodic cues, the circannual rhythms of individual fish were free-running with variable periodicity and hence became desynchronized. Similar effects have been observed in a number of other studies in which rainbow trout have been maintained under constant conditions (Bourlier and Billard, 1984a,b; Bromage et al., 1984; Duston and Bromage, 1986, 1987, 1988).

Another property of endogenous clocks, and a prerequisite for entrainment, is that they possess a differential sensitivity to the phase-shifting effects of the zeitgeber (Aschoff, 1965; Gwinner, 1973, 1986; Saunders, 1977; Brady, 1979; Daan, 1982). A particular time cue therefore causes phase-shifts of different magnitude and sign depending on the

phase at which the rhythm is perturbed (Figure 3.3c,d). This phenomenon has been most thoroughly investigated in circadian systems. Typically, individuals of a species are placed in constant darkness and exposed to a short light pulse (generally less than 1 hour) at different phases of their free-running circadian cycle; the magnitude and sign of the phase-shift (if any) caused by each light pulse is measured over subsequent cycles (Saunders, 1977; Brady, 1979; Daan, 1982). These phase-shifts can be plotted graphically against the time (phase) of the rhythm when the cue was applied to obtain a phase-response curve.

Phase-response curves have received much attention from those studying circadian rhythms because they provide an identifying profile of the underlying clock (Brady, 1979; Daan, 1982). If the entrainment behaviour of circannual rhythms is truly analogous to that of circadian systems it should also be possible to describe the entrainment of circannual clocks in the form of a phase-response curve. However, there have been few reports of phase-dependent phase-shifts of circannual rhythms in response to a zeitgeber stimulus. In the present work (experiment 2) the magnitude of the advance in spawning time caused by exposure to a long photoperiod (18L:6D) was dependent on its timing relative to the phase of the reproductive cycle; spawning occurred in sequence in fish subjected to an increase in photoperiod in December, January or February (Figure 3.10). Thus, the earlier the increase to a 'long' photoperiod occurs relative to that experienced under a natural seasonal photocycle (i.e. the slower the circannual clock is perceived to be running) the greater the corrective phase advance required to re-set the circannual clock. Although it is not possible to construct a phase-response curve from the limited set of data obtained in experiment 2 a number of previous studies on the rainbow trout have indicated that exposure to a 'long' photoperiod behind that experienced under a natural seasonal photocycle (i.e. clock perceived to be running 'fast') causes a phase delay of the circannual clock (Shiraishi and Fukuda, 1966; Skarphedinsson, 1982; Bourlier and Billard, 1984a,b). Furthermore, Bromage et al. (1984) reported that when January/February spawning fish were maintained on constant 6L:18D from February an increase to 18L:6D had no effect on spawning time when applied in early May, but delayed

maturation by 3-4 months when applied in late July. Clearly then, the reproductive response to a 'long' photoperiod is phase-dependent.

This property is amply demonstrated in the present study by the more comprehensive set of data obtained by exposing rainbow trout to 2 month periods of LL at different phases of the reproductive cycle (experiments 5-8). These data are presented in the form of a partial phase-response curve in Figure 3.38. It is not possible to calculate the extent of the individual phase-shifts attributable to either the increase from ambient photoperiod to LL or the subsequent decrease from LL back to ambient photoperiod for each light period. For the purpose of plotting a phase-response curve the time of exposure to LL was therefore taken as the mid-point of the 2 month light period. The phase-response curve shows that periods of LL applied in advance of a significant increase in daylength occurring under ambient conditions phase advanced maturation (clock perceived as running 'slow'); as in experiment 2 (previous paragraph), the more prematurely the arrival of a 'long' photoperiod occurred the greater was the advancement of the circannual clock. Conversely, a period of LL applied behind the natural occurrence of 'long' days phase delayed maturation (clock perceived as running 'fast'). It is suggested that 'dead zones' exist at the transition between the phases of the clock at which advance and delay phase-shifts occur; 2 month periods of LL applied at these times would have no effect on the phase of the circannual clock (clock perceived as 'accurate') and spawning would occur at the normal time. Similar dead zones are characteristic of circadian phase-response curves (Daan, 1982). From the present work one such dead zone would be expected to occur between about April and July, i.e. at the time when 'long' (or increasing) photoperiods occur naturally. A second dead zone is apparent close to the natural spawning time; although 2 month periods of LL applied from between September and spawning time may advance maturation in a minority of fish in the following reproductive cycle, they have no effect on the timing of spawning immediately following their application. Presumably, the circannual clock has become entrained by the naturally decreasing daylength at this stage and hence the fishes' perception of a 'long' photoperiod gradually switches from that which indicates their circannual clock is running 'fast' (delay phase-shift) to that which indicates their

clock is running 'slow' (advance phase-shift). To exclude the effect of the seasonally-changing daylength on the entrainment of the circannual clock it is obviously necessary to apply the light periods to fish maintained under constant conditions (e.g. 6L:18D).

In contrast to most phase-response curves for circadian systems, which are usually constructed by the application of light pulses to a free-running rhythm under constant conditions, the phase-response curve presented in the current work represents the effects of periods of LL applied within a seasonally-changing photoperiod and temperature regime. Temperature appears to have only a minor, direct effect on reproductive timing in salmonids (section 1.2), and the periodicity of the circannual clock would be expected to be temperature compensated (section 3.1), but it should be noted that a phase-response curve obtained from an organism in free-run may be different to that recorded in the entrained state (Aschoff, 1965). However, a similar, though less detailed, phase-response curve has been presented by Duston and Bromage (1988) to describe the effects (advance or delay) of a reduction from a constant long (18L:6D) to a constant short (6L:18D) photoperiod at different phases of the reproductive cycle; the circannual clock would therefore have been free-running when exposed to the reduction in daylength. As would be expected, the magnitude of the phase-shifts plotted in the latter study, which only related to a single change in photoperiod (a reduction), was about half that presented in the current work, in which, it is proposed, the total phase-shifts plotted represent the summation of two separate responses to the increase to, and decrease from, LL.

Phase-response curves for circannual rhythms cannot be expected to provide as accurate a picture of the underlying clock as those described for circadian systems because of the long time scale involved. Following a perturbation circadian rhythms typically pass through a series of transient cycles before a steady state phase-shift is attained (Saunders, 1977; Pittendrigh, 1981a). Since circannual rhythms may also undergo transient cycles during re-entrainment (Gwinner, 1986) phase-response curves obtained during a single cycle may not be fully representative of the phase-shifting effects of the zeitgeber stimulus on the clock. Notably, however, fish maintained on constant 6L:18D after a reduction from 18L:6D in March had advanced spawning by 3-4 months,

spawned again the next year with a circannual periodicity (Duston and Bromage, 1988). Similarly, fish in which spawning was initially delayed by 2-3 months following a reduction from ambient photoperiod to 6L:18D in February spawned with a circannual periodicity for the next two to three years when maintained on the same photoperiod (Duston and Bromage, 1986a, 1991). This suggests that the circannual rhythm attains a steady-state phase-shift almost immediately (within one cycle) in response to these stimuli.

There have been only a few reports of phase-dependent phase-shifts of circannual rhythms in response to a zeitgeber stimulus in other species. The first of these came from Gwinner (1971) who found that the subsequent moulting (postjuvenile or postnuptial) was delayed in willow warblers (Phylloscopus trochilus) transferred from ambient photoperiod to constant 18L:6D in spring and early summer, but was advanced when the transfer occurred in autumn. Transferring birds to constant 12L:12D at these times produced the opposite response. In a subsequent study Gwinner (1973) found that the testicular response of starlings to a 1 month period of either LL or constant darkness (DD) was dependent on the time of year at which the transfer from ambient photoperiod occurred. Thus, gonadal development was enhanced in starlings transferred to LL in late November and late February, inhibited in birds subjected to LL from late May and unaffected in animals exposed to LL from late August; the effects of DD were less striking but were approximately opposite to those of LL (Figure 3.41). Circannual rhythms in moulting, and in testicular size, have been demonstrated in willow warblers (Gwinner, 1971, 1973) and starlings (Schwab, 1971) respectively, and hence these results can be interpreted in terms of differential phase-shifts of a circannual clock (Gwinner, 1973, 1986).

Reproductive activity in sheep also appears to be under endogenous circannual control (Ducker et al., 1973; Howles et al., 1982; Almeida and Lincoln, 1984b; Karsch et al., 1989), and there is a growing awareness that the effects of photoperiodic change on the timing of reproduction in this species can also be interpreted in terms of phase-shifts of an endogenous clock (Malpaux et al., 1988, 1989; Jackson et al., 1989; Malpaux and

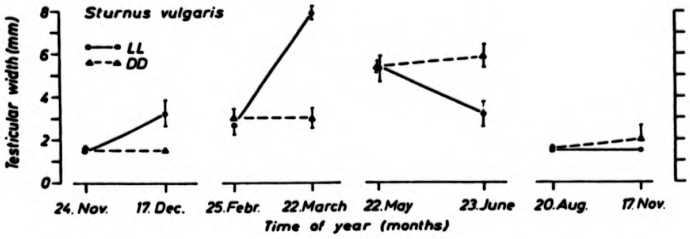


Figure 3.41: Changes in testicular width (mean ± 1SEM) in European starlings transferred to either constant darkness (DD) or continuous light (LL; mean light intensity of 0.7 lux) at four different times of the year. In each group testicular width was measured at the beginning of the experiment and approximately 1 month later (from Gwinner, 1986; after Gwinner, 1973).

Karsch, 1990; Wayne et al., 1990). The possibility that changes in daylength may have differential phase-resetting effects at different times of the year was recognised by Jackson et al. (1989) who subjected sheep to alternating 90 day periods of constant 8L:16D and 16L:8D, with transitions between the two photoperiods timed to occur at the spring and autumn equinoxes and the summer and winter solstices. They reported significant seasonal variations in both the interval between the onset of 'short' days and the commencement of oestrous (assessed by monitoring circulating progesterone levels), and the time from onset of 'long' days to the cessation of ovarian cycles, in each case consistent with the hypothesis of an endogenous circannual clock exhibiting a periodically changing sensitivity to the zeitgeber. Also consistent with this hypothesis are the results of Malpaux et al. (1989) who reported that increases in daylength in advance or behind those of the natural light cycle, respectively advanced and delayed the breeding season in sheep (assessed by monitoring circulating LH levels). One interpretation of these observations is that the sheep perceived the premature arrival of 'long' days as an indication that their endogenous clock was running 'slow' and therefore compensated with a corrective forward adjustment (phase advance), whereas the late appearance of 'long' days was perceived as an indication that their clock was running 'fast', initiating a corrective backward adjustment (phase delay). These responses are similar to those observed in the rainbow trout (e.g experiments 2, 5 and 8 in the present study) suggesting that similar mechanisms may time seasonal events in these species.

Further evidence supporting the existence of an endogenous circannual clock was provided by the results of experiments in which spawning was advanced in fish which received 'long' days of only 10 or 8.5 hours in January (experiment 1, Gp. C; Figure 3.5: experiment 4, Gps. E and F; Figure 3.22), provided this was followed by a reduction in photoperiod in May. These photoperiods would normally be regarded as 'short' daylengths, but were clearly perceived as being 'long' relative to the subsequent reduction. These results indicate that the daylength does not have to reach a 'critical' value for reproduction to be induced, and support the proposal that photoperiod does not directly drive reproductive function.

3.4.12 Circadian involvement in circannual mechanisms.

The experiments described in this chapter have elucidated which features of the photoperiodic signal are most important for the entrainment of the circannual clock controlling reproduction in the female rainbow trout, but they were not designed to investigate the mechanisms underlying the generation of the circannual rhythms themselves. Nevertheless, several aspects of the results may shed some light on hypotheses proposed to explain these phenomena. Given the long-term nature of circannual rhythms it has been suggested that they may be derived from rhythms with higher frequencies, especially circadian rhythms. The following brief review of the three principle hypotheses proposed to accomodate this theory concentrates on their possible application to the circannual mechanism underlying reproduction in the rainbow trout in light of evidence accrued from the present and previous studies on this species (for more detailed discussions of these hypotheses see Gwinner, 1973, 1981, 1986).

The first hypothesis states that circannual cycles are generated by frequency demultiplication of circadian rhythms, that is, subjective circadian days are counted with approximately 365 being perceived as equivalent to 1 year. An analogous mechanism is that by which an electric clock converts the 50 cycles per second frequency of the commercial electrical current to 1 cycle per 12 or 24 hours. This hypothesis predicts that the period of the circannual rhythm is dependent on the period of the circadian rhythm. However, the currently available data disagree with this prediction (Gwinner, 1981, 1986), apart from one study which showed a weak positive correlation between the periodicities of circadian locomotor activity and circannual moult in starlings (Gwinner, 1973, 1986). Other experimental evidence and theoretical considerations are also in conflict with the hypothesis for the species studied (Gwinner, 1986). Similarly, there is currently no evidence that frequency demultiplication is involved in the generation of the circannual rhythm of maturation in the rainbow trout, but several studies have provided evidence against the hypothesis. Using resonance procedures (6L:42D, 6L:48D and 6L:54D; discussed more thoroughly later), which exposed rainbow trout to 50% or less of the normal number of light-dark cycles per year, Duston and Bromage (1986a) observed

that female rainbow trout matured slightly in advance of those maintained under ambient conditions. Clearly, a delay in maturation would have been expected if the fish had been counting the number of light-dark cycles. Their study also demonstrated that the fish did not summate the total duration of daily light exposure since they received 25% or less hours of light per year under the resonance regimes than under a natural photoperiod. This is also apparent from the present work since fish which received 'long' days of 22 hours and 'short' days of 13.5 hours (Gp. A, experiments 3 and 4) matured at approximately the same time as fish subjected to 10 hours less light per day (Gp. D, experiment 4). Duston and Bromage (1986a) also found that the circannual periods of fish maintained under the same constant photoperiod (6L:18D) for 3 years ranged from 320 to 420 days, a variability which was also apparent after only 1 cycle in fish maintained under constant 8.5L:15.5D in experiment 4. To accomodate this large inter-individual variability in circannual period length in the frequency demultiplication hypothesis there would also have to be a large inter-individual variation in the number of light-dark cycles perceived as a year. Also inconsistent with the hypothesis are the observations that fish maintained for several years under constant 6L:18D and 18L:6D show markedly different spawning periodicities (about a year and 6 months respectively: Bromage et al., 1984; Scott et al., 1984; Duston and Bromage, 1986a), and that the reproductive cycle of the rainbow trout and other salmonids can be synchronized to artificial seasonal photocycles with periods considerably different from 1 year (section 3.1). Since the fish in such experiments were always exposed to daily light-dark cycles of 24 hours, which would have been expected to entrain any circadian rhythms to this period, the differences in spawning time under the different photoperiod regimes cannot easily be reconciled with a model in which circannual rhythms are derived by counting circadian days. It is therefore unlikely that the circannual rhythm of reproduction in the rainbow trout is generated by frequency demultiplication of circadian cycles.

The second hypothesis has been developed from the 'external coincidence' model for photoperiodic time measurement, originally proposed by Bunning (1936, 1960) and later expressed more explicitly by Pittendrigh and Minis (1964). This model states that

photoperiodic reactions occur when light (external cue) coincides with a particular phase of an internal, light-entrained, circadian oscillation. In 'long-day' responsive animals, for example, the photosensitive or photo-inducible phase is often presumed to occur during a period 12-24 hours after dawn. Annual cycles in reproductive or other functions arise as a consequence of the seasonally-changing photoperiod, which periodically exposes the photosensitive phase to light. This model can also accomodate the persistence of annual cycles under constant photoperiodic conditions if the phase relationship between the circadian rhythm and its entraining light-dark cycle is subject to circannual variations (Figure 3.42A). Circannual changes in the phase relationship between the circadian rhythm and the light-dark cycle would permit the alternate exposure of the circadian photosensitive phase to light and darkness, resulting in circannual rhythms of overt functions such as reproduction. Skeleton photoperiod regimes (e.g 6 hours main photoperiod plus a 2 hour minor photoperiod positioned at various intervals during the subsequent 18 hours of darkness) have provided evidence for the involvement of circadian mechanisms in photoperiodic time measurement in several teleost fish including the stickleback (Baggerman, 1972, 1980, 1985), catfish (Sundararaj and Vasal, 1976), medaka, Oryzias latipes (Chan, 1976) and mummichog, Fundulus heteroclitus (Day and Taylor, 1984); photosensitivity maxima generally occurred within a period 12-16 hours following the subjective dawn. Moreover, Baggerman (1980, 1981, 1985) reported a seasonal change in the circadian photosensitivity rhythm with sticklebacks transferred from ambient to 'short' daylengths between September and April becoming more reproductively responsive to this photoperiod the later they were moved. She therefore suggested that the stickleback exhibits a circannual rhythm in the phase relationship between a circadian rhythm in photosensitivity and its entraining light-dark cycle (Baggerman, 1985).

Skeleton photoperiods have also been employed to test for circadian rhythms of photosensitivity in two salmonids. Thus, Thorarensen and Clarke (1989) found that a photoperiod of 9L:6D:1L:8D stimulated the physiological parameters associated with smoltification of coho salmon almost as effectively as 16L:8D, and was much more

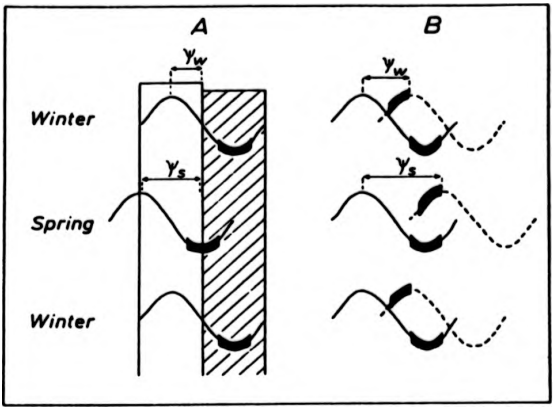


Figure 3.42: Schematic representation of two models that derive circannual rhythms from circadian rhythms. The overt circannual rhythm results from A) changes in the phase-angle difference (Ψ) between a circadian rhythm and its entraining light-dark cycle; in this example circannual variations in the phase-relationship between the circadian rhythm and the light-dark cycle expose the circadian photosensitive phase (indicated by the black block) to light during the spring but not during the winter B) changes in the phase-angle difference between two circadian rhythms; in this example circannual variations in the phase-relationship between two circadian rhythms cause specific circadian phase-points (indicated by the black blocks) to coincide, and initiate the photoperiodic response, in spring but not in winter (from Gwinner, 1986; after Gwinner, 1973).

effective than 10L:14D. They therefore proposed that an endogenous circadian rhythm of photosensitivity measures daylength in this species. In a more comprehensive study Duston and Bromage (1986a) exposed rainbow trout to skeleton photoperiods comprising of a main light period of 6 hours with additional 2 hour light pulses positioned 10-12, 12-14 or 14-16 hours after the subjective dawn. They reported a slight, but significant, advance in the timing of first maturation (at the end of the second year of the experiment) in fish subjected to the light pulse 12-14 hours after the subjective dawn, when compared to fish maintained under 6L:18D and the other skeleton photoperiod regimes. They therefore concluded that the rainbow trout possesses a circadian photosensitivity maxima at about 12-14 hours after the subjective dawn, consistent with the finding of the present study that 'long' daylengths of between 12 and 22 hours are equally effective for the advancement of maturation (experiments 3 and 4). Although suggestive of endogenous circadian involvement in photoperiodic time measurement, however, their results are not entirely convincing. Firstly, differences in spawning time between groups were relatively small, and spawning occurred in the 12-14 hour group at approximately the same time as fish maintained under natural conditions. Secondly, 24-hour skeleton photoperiods are considered inadequate for the conclusive demonstration of circadian involvement in photoperiodic time measurement (Follett, 1981). More powerful experimental protocols such as T-cycles (cycles with a period within the range of entrainment but differing from 24 hours; Pittendrigh and Minis, 1964) and resonance photoperiods (short photoperiods combined with extended periods of darkness; Nanda and Hamner, 1958) are thought to provide definitive evidence of circadian organisation in daylength measurement (for details see discussions in Elliott, 1981; Pittendrigh, 1981b; Follett et al., 1981). Only resonance procedures have been employed in fish, and only in one species, the rainbow trout (Duston and Bromage, 1986a). This study produced equivocal results, in that spawning was advanced to a similar extent in fish exposed to photoperiods of 6L:42D, 6L:48D and 6L:54D when compared to fish maintained under 6L:18D. The light period would only be expected to illuminate the photosensitive phase of a free-running (in DD) circadian rhythm, and hence advance maturation in the same manner as 'long' days, in fish

maintained under 6L:48D and 6L:54D; the photosensitive phase of the fish subjected to 6L:42D should not have been illuminated and hence they would have been expected to spawn at the same time as fish maintained under 6L:18D. Before considering the hypothesis that overt circannual rhythms are the result of circannual changes in a circadian rhythm of photosensitivity it should therefore be noted that, at present, the existence of the latter remains unproven in the rainbow trout.

Gwinner (1981, 1986) suggests that the above hypothesis is not very plausible for species that show circannual rhythms under a wide variety of photoperiods. The rainbow trout clearly falls into this category, exhibiting free-running cycles under 6L:18D, 18L:6D and LL (Duston and Bromage, 1986a). The approximately 6 month periodicity of the reproductive cycle observed under long photoperiods could be explained by the photosensitive phase remaining permanently illuminated irrespective of seasonal changes in its position. If this were the case the periodicity of the rhythm would be determined by the minimum period required for oocyte development. Although the model can accomodate the persistence of circannual rhythms in LL it cannot account for their occurrence in DD, in which any circadian photosensitive phase would never be exposed to light. Circannual rhythms in DD have not yet been demonstrated in the rainbow trout but there is evidence for their occurrence in another salmonid, the brook trout (Poston and Livingstone, 1971), and in the catfish (Sundararaj et al., 1982), a species thought to possess a circadian rhythm in photosensitivity (Sundararaj and Vasal, 1976). It is also difficult to reconcile this model with the existence of strains of rainbow trout which spawn bi-annually under natural photoperiod conditions (Aida et al., 1984; Lou et al., 1984). There is therefore little evidence that the 'external coincidence' model is applicable to the rainbow trout.

The third hypothesis is based on the 'internal coincidence' model for photoperiodic time measurement, initially suggested by Pittendrigh (1960, 1972). This model states that the seasonal changes in daylength result in alterations in the phase relationships of two or more circadian oscillators within an organism. Photoperiodic reactions occur when these internal rhythms assume a particular phase relationship to each other, i.e. when certain

phase-points of two or more oscillators coincide. This model can also accomodate the persistence of annual cycles under constant photoperiodic conditions if it is assumed that the phase relationship between circadian oscillators varies with a circannual periodicity, such that coincidence between certain circadian phase-points occurs only at a particular phase of the circannual cycle (Figure 3.42B). Circannual rhythms of overt functions such as reproduction would therefore result from circannual changes in the phase relationship between circadian oscillators. Thus, Meier (1976) proposed that changes in the phase-relationship between the circadian rhythms in production and secretion of prolactin and corticosterone may underly circannual rhythmicity in functions such as testicular activity in the white-throated sparrow, Zonotrichia albicollis. Neither the present or previous work on the rainbow trout provide evidence to support this model, although one advantage is that it can accomodate the persistence of circannual rhythms under DD (light only entrains the circadian oscillations, photoperiodic reactions are a function of their internal phase relationship), reported in at least one salmonid (Poston and Livingstone, 1971). Moreover, the existence of two oscillators has been postulated to explain the splitting of the daily activity pattern of brown trout into 2 components (dawn and dusk) which occurs during the transition from a mainly diurnal pattern in winter to a mainly nocturnal pattern in summer (Eriksson, 1973; Pittendrigh and Daan, 1976). Thus, internal coincidence may underly the annual activity cycle of this salmonid.

Although it is an attractive proposition there is no reason per se that circadian rhythms should be involved in the generation or expression of circannual rhythms, and evidence to support any of the models discussed above is, at best, tenuous. As Gwinner (1981) suggests, circannual rhythms may originate independently of circadian rhythms and the latter may be of significance only in connection with the process of synchronization, which may be a separate physiological phenomenon. In this respect the next chapter considers whether the entrainment of the circannual clock by the seasonally-changing photoperiod is mediated by seasonal changes in a circadian rhythm of the pineal gland hormone, melatonin.

CHAPTER FOUR

PATTERNS OF MELATONIN SECRETION AND THEIR ROLE IN THE TRANSMISSION OF PHOTOPERIODIC INFORMATION TO THE REPRODUCTIVE AXIS

4.1 Introduction

Although it is clear that photoperiod times reproduction in salmonids the mechanisms responsible for the transmission of photic information to the neuroendocrine system controlling reproduction remain to be elucidated. In many vertebrates the pineal gland (hereafter referred to as the pineal) converts photic information into a circadian rhythm of melatonin secretion, and, in certain seasonally-breeding mammals, the duration of the night-time increase in this hormone determines the reproductive response (reviewed by Goldman and Darrow, 1983; Karsch, 1984; Bittman, 1985; Tamarkin et al., 1985; Bartness and Goldman, 1989; Ebling and Foster, 1989). To determine whether a similar mechanism operates in salmonid fish this chapter primarily investigates the role of melatonin in the processing of photoperiodic information in the rainbow trout, Oncorhynchus mykiss.

Although other tissues are capable of synthesising melatonin (Ralph, 1981a; Pang and Allen, 1986), the principal source of this hormone in most vertebrates is the pineal (Pang, 1985). The pineal arises as an evagination of the roof of the diencephalon (Hamasaki and Eder, 1977; Ralph, 1983). For an excellent general review of the functional morphology of the pineal complex in fish see McNulty (1984a).

In salmonid fish the pineal consists of an end-vesicle located dorsomedially to the telencephalon and attached to the roof of the diencephalon (between the habenular and posterior commissures) by a hollow stalk, which is in open communication with the third ventricle (Hamasaki and Eder, 1977; Yasutake and Wales, 1983). The rainbow trout pineal lacks a blood-brain barrier (Omura et al., 1985) and the capillaries of the end-vesicle appear widened in comparison to capillaries in other portions of the brain (Ali et al., 1987), features consistent with an endocrine function. No specialised system of portal vessels exists between the pineal and other brain regions (Ali et al., 1987). The pineal complex of some fish contains a second component, the parapineal organ, homologous to the parietal eye of certain reptiles (Ralph, 1983). Although reasonably prominent in cyclostomes this organ is highly regressed in teleosts, and its function is unknown (McNulty, 1984a). Thus, although a small parapineal organ is present in the rainbow

trout (Rudeberg, 1969), the parapineal organ will not be considered further.

During the course of evolution the pineal has evolved from a directly photoreceptive organ capable of transmitting information to the brain via a neural pathway, but also endowed with a secretory capacity, to an entirely secretory organ, dependent on sympathetic neural input (Hamasaki and Eder, 1977; Oksche, 1983). The concept that mammalian pinealocytes have developed from the pineal photoreceptor cells of lower vertebrates was initially proposed independently by Collin (1971) and Oksche (1971). They subsequently established relationships between pineal photoreceptors, rudimentary (modified) photoreceptors and secretory pinealocytes and suggested that these cells were representatives of a single cell line (Collin and Oksche, 1981). Photoreceptor cells are common in the pineal of fish and amphibians, rudimentary photoreceptors are predominant in the pineal of chelonians, lacertilians and birds, and secretory pinealocytes are characteristic of mammals (Figure 4.1: for a review see Korf and Oksche, 1986). During these transformations several characteristics of the pineal, such as the production of melatonin, have been conserved in all vertebrates (Korf and Oksche, 1986; Vivien-Roels and Pevet, 1986). Thus, despite the considerable morphological and physiological diversity of the vertebrate pineal (Figure 4.1) there may be a number of similarities between pineal function in different vertebrate groups, and hence much is to be gained from a comparative approach. The following review therefore seeks to establish a background to the present work by surveying pineal function in both lower and higher vertebrates, with emphasis on rhythmic melatonin production and the role of melatonin in reproduction.

The fish pineal may convert photic information into either a neural or a humoral signal. Although this chapter is principally concerned with the endocrine functions of the pineal, specifically with regard to melatonin secretion, a brief review of current knowledge of pineal neural pathways will provide a background for the discussion of possible mechanisms regulating melatonin synthesis and release, and serve as a reminder that a hormonal signal is not the only mechanism by which the pineal could relay photic information. The fish pineal is composed of 3 types of cells: photoreceptor (sensory)

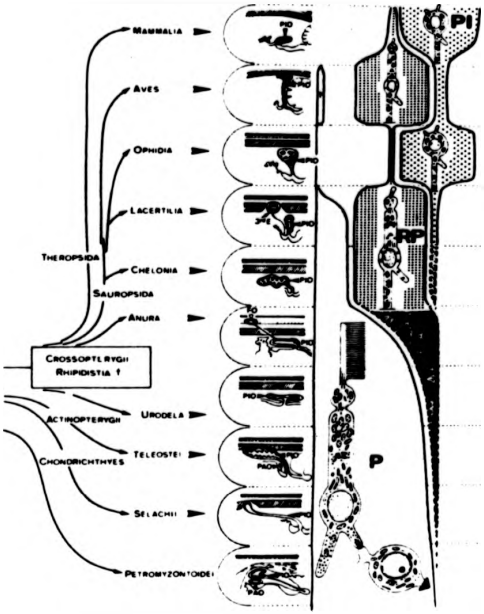


Figure 4.1: Diagrammatic representation of the diversity of the vertebrate pineal. Panels show (centre) sagittal sections through the diencephalic roof of vertebrates and (right) the gradual transformation of the photoreceptor cell during phylogeny (FO, frontal organ; P, photoreceptor; PAO, parapineal organ; PI, pinealocyte; PIO, pineal gland; RP, rudimentary photoreceptor; 3rd E, third (parietal) eye: from Gern and Karn, 1983).

cells, ganglion cells and supporting (interstitial) cells (Tamura and Hanyu, 1976; McNulty, 1984a). In the rainbow trout the photoreceptors synapse directly with the ganglion cells, the axons of which descend the pineal tract to the brain, except for a small population of photoreceptors possessing long axons which project directly to the brain; a small population of interneurons may provide an integrating network (Ekstrom and Korf, 1985; Ekstrom, 1987; Ekstrom and Meissl, 1988; Meissl and Ekstrom, 1988). The supporting cells are thought to participate in the exchange of nutrients and metabolites between the vascular supply and the photoreceptor and ganglion cells, and may play a role in the turnover of photopigments (McNulty, 1984a).

The rainbow trout pineal lies beneath a pigment-free area of the skull apparently specialised for light penetration (Dodt, 1966, 1973). Extracellular recordings of nervous activity from the exposed pineal end-vesicle and pineal tract of anaesthetised/immobolised and bilaterally-enucleated rainbow trout have demonstrated that during darkness a spontaneous nervous discharge emanates from both single ganglion cells and the axons comprising the pineal tract, and that this activity is inhibited by exposure to light in an intensity-dependent manner (Dodt, 1963, 1973; Morita, 1966; Hanyu and Niwa, 1970). Inhibition of the spontaneous discharge was produced by any strong light within the visible range with the exception of the red end (Hanyu and Niwa, 1970). The teleost pineal is therefore directly photosensitive and functions principally as a luminance detector (achromatic response), although a few chromatic cells (inhibited by short wavelengths, excited by longer wavelengths) may be present (Morita, 1966; Dodt, 1973).

The central projections and terminations of the pineal nerve tract of the rainbow trout have been traced by Hafeez and Zerihun (1974) using cobalt chloride iontophoresis. They concluded that the tract consists only of pinealofugal (afferent) fibres which project over an extensive sensorimotor area in the brain; terminations were observed in the lateral habenular nucleus, pretectal area, di- and mesencephalic periventricular grey, dorsomedial and dorsolateral thalamic nuclei, nucleus of Darkschewitsch, dorsal tegmentum and possibly the preoptic nucleus. Although peptidergic axons of central origin

have recently been demonstrated in the pineal of the stickleback, Gasterosteus aculeatus, coho salmon, Oncorhynchus kisutch and Atlantic salmon, Salmo salar (Ekstrom et al., 1988), and a central noradrenergic innervation of the pineal appears to exist in the stickleback (Ekstrom et al., 1986), there is currently no convincing evidence for pinealopetal (efferent) innervation of the rainbow trout pineal. In addition to the conventional axo-dendritic synapse between the photoreceptor and ganglion cell, however, axo-axonic synapses between nerve cell axons of unknown origin and photoreceptors have been described in rainbow trout and brook trout (Salvelinus fontinalis) pineals (Omura, 1979; Omura and Ali, 1980). Since the former are predominant in dark-adapted pineals and the latter in light-adapted pineals, it has been suggested that the presynaptic side of the axo-axonic synapse serves to inhibit signal transmission from photoreceptors to ganglion cells, and also that these inhibitory nerve endings may originate in the brain (Omura and Ali, 1980).

The role of the eyes in the regulation of pineal function in salmonids is controversial. The eyes are also derived embryonically from the diencephalon (Tamura and Hanyu, 1978; Dodt, 1987) and visual and pineal nerve fibres overlap in certain areas of the brain of some teleosts, although it is not known if they make contact (Ekstrom and Meissl, 1989). Smith and Weber (1976a) claimed that bilateral enucleation of rainbow trout abolished a diurnal variation in the activity of hydroxyindole-o-methyltransferase (HIOMT; the enzyme resonsible for the final conversion step in the synthesis of melatonin), whereas surgical capping of the pineal area with black polythene had no effect. In a similar blinding/pineal masking study on the rainbow trout Hafeez et al. (1978), using nuclear and nucleolar diameter as an indirect indication of the level of cellular functional activity, reported that pineal photoreceptor cells were affected solely by incident light in the pineal region, but that pineal supporting cells were only affected by photic input from the eyes. They therefore hypothesised that the pineal of the rainbow trout may function as a photocomparator in which photic inputs from the eyes and directly to the pineal affect the supporting and photoreceptor cells respectively; in this respect it should be mentioned that pineal supporting cells from rainbow trout exhibit stable resting

potentials which are unaffected by light changes (Meissl, 1986). Gern et al. (1978b) found that severing the optic tract of rainbow trout did not abolish the plasma melatonin rhythm, nor did it cause a reduction in amplitude. This suggests that pineal melatonin production depends on direct photoreception rather than indirect regulation via the eyes, in concurrence with the directly photosensitive nature of the pineal indicated by electrophysiological studies. There was, though, an increase in the amplitude of the melatonin rhythm after optic-tract section, which was attributed to stress (Gern et al., 1978b). At present, therefore, the possibility that indirect photic input from the eyes contributes to pineal function in the rainbow trout cannot be excluded.

Since the isolation of melatonin (N-acetyl-5-methoxytryptamine) from bovine pineals by Lerner et al. (1958) the principal components of the biochemical pathway leading to its synthesis have been resolved (Figure 4.2; Klein, 1979, 1985), primarily from work with rats, Rattus norvegicus. There is evidence for a similarly active pineal indole metabolism in a number of fish species including lampreys, Lampetra planeri (Meiniel, 1979; Meiniel and Hartwig, 1980; Meiniel and Vivien-Roels, 1980), eels, Anguilla anguilla (van Veen et al, 1982), sticklebacks (van Veen et al., 1980, 1984; Ekstrom and van Veen, 1984) goldfish, Carassius auratus (McNulty, 1984b, 1986) and pike, Esox lucius (Falcon, 1984; Falcon and Collin, 1985; Falcon et al., 1985, 1988, 1987, 1989). Furthermore, the pike pineal is capable of synthesising all indoles that are known to occur in the pineal of higher vertebrates (Falcon et al., 1985).

Early in vivo studies demonstrated selective uptake of 14C-labelled 5-hydroxytryptophan (5-HTP; Oguri et al., 1968), and the synthesis of serotonin (5-hydroxytryptamine) from exogenous 5-HTP (Hafeez and Quay, 1969), by the pineal of rainbow trout. HIOMT activity was first demonstrated in the pineal and retina of rainbow trout by Quay (1965), and Fenwick (1970a) was the first to identify melatonin in the pineal of a salmonid (chinook salmon, Oncorhynchus tshawytscha). More recent studies have focused on the influence of daily light-dark cycles on the synthesis and secretion of indoles. The rhythms in indole metabolism characteristic of many higher vertebrates are illustrated in Figure 4.2. Pineal serotonin profiles are variable among species; for

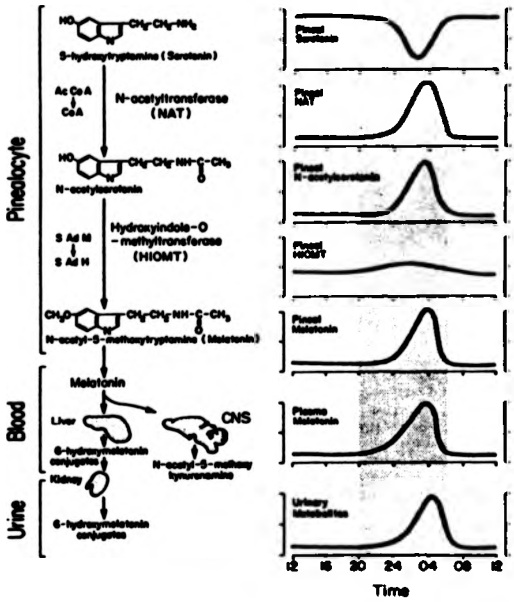


Figure 4.2: Biosynthesis and metabolism of melatonin (left) and daily rhythms in indole metabolism (right), as known to exist in mammals (S Ad M, S-adenosyl-methionine; S Ad H, S-adenosyl-homocysteine: from Reiter, 1990).

example, serotonin levels are high during the photophase (light-period) and low during the scotophase (dark-period) in the rat (Klein, 1979), but show two peaks, close to the light-dark transitions, in the chicken, Gallus domesticus (Binkley, 1988). There is currently little information on diurnal serotonin profiles in fish. Meissl (1978) reported that the pineal serotonin content of rainbow trout was slightly greater in the photophase than the scotophase, but the difference was not significant. In contrast, van Veen et al. (1982) found a marked diurnal variation in pineal serotonin content of the eel, with lowest levels at mid-photophase and at the light-dark transition, and highest levels at mid-scotophase and at the dark-light transition.

In higher vertebrates N-acetyltransferase (NAT) activity exhibits pronounced diurnal variations with a many-fold increase in darkness which closely parallels the daily rhythms in N-acetylserotonin and melatonin production (Figure 4.2). Since diurnal changes in HIOMT activity are small or absent NAT has been considered to be the rate-limiting enzyme for melatonin synthesis (Klein and Weller, 1970; Klein, 1979). Recent studies have indicated that this may also be true in fish. Marked diurnal fluctuations in pineal NAT, with peak levels during the scotophase, have been observed in both the pike (Falcon et al., 1986, 1987) and the rainbow trout (Figure 4.3a; Morton and Forbes, 1988). In the pike, the diurnal rhythms in pineal N-acetylserotonin and melatonin production paralleled that of NAT activity (Falcon et al., 1986). Some controversy, however, has surrounded the presence of diurnal fluctuations in HIOMT activity. Hafeez and Quay (1970a) reported that HIOMT activity was independent of light and dark in pineals of non-anadromous rainbow trout. In contrast, Smith and Weber (1974,1976a), working with an anadromous strain of rainbow trout, found HIOMT specific activity was minimal 1 hour before the onset of darkness but underwent a 2-fold increase during the scotophase of a variety of photoperiods. In the lamprey, Geotria australis, a 4-5 fold increase in pineal HIOMT was observed during the first 4 hours of darkness (Joss, 1977). A diel cycle in HIOMT specific activity was also observed in chinook salmon by Birks and Ewing (1981), but they concluded that this reflected changes in pineal protein content rather than changes in enzyme activity per se. More recently, no significant diurnal

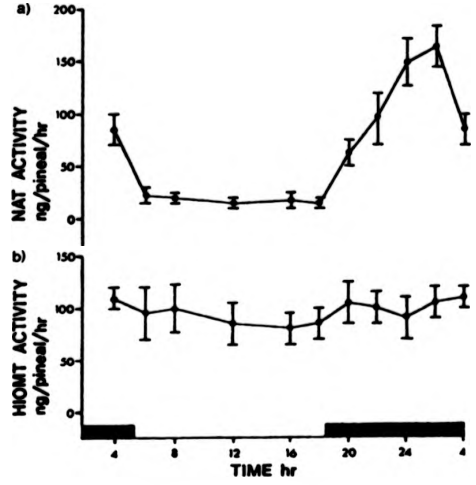


Figure 4.3: Diurnal fluctuations (mean ± 1SEM) in a) N-acetyltransferase (NAT) and b) hydroxyindole-o-methyltransferase (HIOMT) activity in rainbow trout pineal glands during mid-summer. The solid bars on the x-axis denote the scotophase (from Morton and Forbes, 1988).

variations in HIOMT activity were detected in pike pineals (Falcon et al., 1986, 1987) or in rainbow trout retinae (Gern et al., 1984a) or pineals (Figure 4.3b; Morton and Forbes, 1988). Thus, in the majority of fish studied, diurnal changes in HIOMT activity appear to be of very low amplitude or absent, a situation similar to that in higher vertebrates (Figure 4.2: Klein, 1979; Reiter, 1990).

Diurnal rhythms in melatonin production have been detected in the vast majority of animals so far investigated. These include man (Kennaway et al., 1977; Bireau et al., 1981), common laboratory animals such as the rat (Lynch, 1971; Kennaway et al., 1977; Johnson et al., 1982), various hamster species (Goldman et al., 1982), rhesus monkey, Macaca mulatta (Reppert et al., 1979) and marmoset, Callithrix jacchus jacchus (Guerin and Matthews, 1990), domesticated animals such as the horse, Equus caballus (Kilmer et al., 1982), various types of cattle (Hedlund et al., 1977; Kennaway et al.,1977; Doi et al., 1987) and sheep, Ovis aries (Rollag and Niswender, 1976; Kennaway et al., 1977; Yellon and Longo, 1987), goat, Capra hircus (Mori et al., 1987) and chicken (Pelham and Ralph, 1972; Pelham, 1975; Kennaway et al., 1977), and some more exotic species such as the camel, Camelus dromedarius (Kennaway et al., 1977), lemmings (Dicrostonyx species; Reiter et al., 1990), tammar, Macropus eugenii (McConnell, 1986a,b), Japanese quail, Coturnix coturnix japonica (Underwood et al., 1984), green sea turtle, Chelonia mydas (Owens et al., 1980), scincid and iguanid lizards (Kennaway et al., 1977; Firth et al., 1979; Underwood, 1985b) and the neotenic tiger salamander, Ambystoma tigrinum (Gern and Norris, 1979; Gern et al., 1983). Even an animal lacking a distinct pineal, the nine-banded armadillo, Dasypus novemcinctus, exhibits a diurnal rhythm in circulating melatonin (Harlow et al., 1981). There are, however, a few exceptions currently known which do not always express diurnal rhythms in melatonin production: a genetically deficient inbred laboratory mouse, Mus domesticus (Menaker, 1985), the domestic pig, Sus scrofa, at certain times of the year (Reiter et al., 1987), and certain animals undergoing hibernation (Vivien-Roels and Arendt, 1983; Heldmaier and Lynch, 1986; Jansky, 1986).

The most striking feature of the melatonin rhythm is that melatonin synthesis and

release is always higher at night than during the day (Figure 4.2). This is true irrespective of whether animals are nocturnally or diurnally active. Moreover, the duration of increased melatonin production is proportional to the duration of the scotophase. That pineal or circulating melatonin concentrations remain elevated for longer in animals maintained in 'short' photoperiods than in those subjected to 'long' photoperiods has been demonstrated in a number of species including the Syrian hamster, Mesocricetus auratus (Roberts et al., 1985; Skene et al., 1987), Turkish hamster, Mesocricetus brandti (Darrow et al., 1986), Djungarian hamster, Phodopus sungorus (Goldman et al., 1982, 1984), white-footed mouse, Peromyscus leucopus (Petterborg et al., 1981), ferret, Mustela furo (Baum et al., 1986), mink, Mustela vison (Ravault et al., 1986), cat, Felis catus (Leyva et al., 1984), cattle (Stanislewski et al., 1988), sheep (Rollag and Niswender, 1976; Arendt et al., 1979; Lincoln and Almeida, 1981; Lincoln et al., 1982; Bittman et al., 1983; Almeida and Lincoln, 1984a,b; Bittman and Karsch, 1984; Yellon et al., 1985; Karsch et al., 1986; English et al., 1988) tammar, (McConnell et al., 1986a,b), Japanese quail (Cockrem and Follett, 1985; Underwood and Siopes, 1985), and the box turtle, Terrapene carolina triunguis (Vivien-Roels et al., 1988).

Clearly, the cyclic production of melatonin is a ubiquitous phenomenon in higher vertebrates. All fish investigated to date also exhibit diurnal rhythms of melatonin synthesis and secretion. This was first demonstrated by Gern et al. (1978a) who reported that plasma melatonin levels in mid-scotophase were approximately twice those in mid-photophase in rainbow trout maintained in a 12L:12D photoperiod. Subsequent studies on the rainbow trout confirmed this observation (Gern et al., 1978b; Owens et al., 1978). More recently, Duston and Bromage (1986b) reported that serum melatonin levels were elevated for a period corresponding to the duration of darkness in rainbow trout maintained under 8L:16D or 16L:8D. Burton and Gern (1983) were also able to demonstrate a diurnal rhythm in pineal melatonin content in this species, with peak melatonin levels occurring during the scotophase. The pineal of the rainbow trout is a major source of circulating melatonin since pinealectomy causes a significant reduction in plasma melatonin levels at mid-scotophase, and a slight, but significant reduction in

melatonin levels at mid-photophase (Gern et al., 1978b). However, a diurnal rhythm in melatonin secretion is still detectable in pinealectomised rainbow trout, suggesting that extra-pineal tissues also release melatonin into the circulation (Gern et al., 1978b). The eyes are likely alternative sources since the retinae of rainbow trout are able to synthesise melatonin (Gern et al., 1978c; Gern and Ralph, 1979; Gern and Karn, 1983), although release of retinal melatonin into the blood has not yet been demonstrated. In pike, maintained under a 10.5L:13.5D photoperiod, melatonin levels in the plasma paralleled those in the pineal, and were elevated for the approximate duration of the scotophase (Falcon et al. 1987,1989). Alda (1989) stated that plasma melatonin levels in goldfish were elevated during the scotophase and that the pattern of secretion varied according to whether the fish were acclimated to a 'long' or 'short' photoperiod, but gave no further details. In two other cyprinids, the dace, Leuciscus leuciscus (Brook, 1989) and the common carp, Cyprinus carpio (Kezuka et al., 1988), the duration of increased melatonin was proportional to the duration of the scotophase under both long (18L:6D or 16L:8D) and short (6L:18D or 8L:16D) daylengths. Thus, in the few species of fish so far examined melatonin production is elevated at night and reflects the duration of the scotophase.

The pattern of nocturnal melatonin production in mammals varies among species. Reiter (1983, 1986, 1987, 1988) has identified 3 different patterns of melatonin production in mammals (Figure 4.4) which he arbitrarily designated as types A (or I), B (or II) and C (or III). In animals possessing a type A pattern melatonin production remains low until several hours after the onset of darkness, after which it rises rapidly to reach a short-term peak during the latter half of the scotophase, and then declines to basal levels before or at the time of light onset. This pattern is typical of several rodent species including the Syrian hamster (Panke et al., 1979; Tamarkin et al., 1979; Hastings et al., 1987) and the Mongolian gerbil, Meriones unguiculatus (Reiter et al., 1980a). In type B, melatonin production begins to increase at or shortly after (or even slightly before) the onset of darkness and rises gradually towards a peak at mid-scotophase, following which melatonin levels gradually decline, reaching basal levels close to the time of light onset. This pattern appears to be the most common (Reiter, 1987) and is found, for example, in

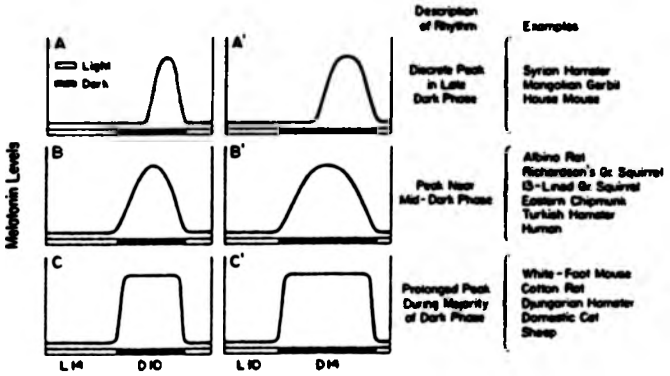


Figure 4.4: Different patterns (arbitrarily designated A, B and C) of pineal melatonin production in mammals with examples of species in which these patterns are known to occur. In each case an extension of the scotophase is associated with an increase in the duration of elevated melatonin production (A', B' and C': from Reiter, 1988).

the rat, (Johnson et al., 1982) and in humans (Kennaway et al., 1977; Birau et al., 1981). The third pattern, type C, is characterised by a rapid increase in melatonin production almost immediately after the onset of darkness, maintenance of high levels for the duration of the scotophase, and a rapid decline to basal levels either just before or at the time of light onset. Animals possessing this pattern of melatonin production include the Djungarian hamster (Goldman et al., 1982; Illnerova et al., 1984) and the sheep, (Rollag and Niswender, 1976; Lincoln et al., 1985). The physiological significance, if any, of these differences is unknown, but they might be expected to have some bearing on the mechanism by which the melatonin signal is interpreted. It is also not yet known whether such a classification is appropriate to other vertebrate groups since an insufficient number of species have been examined.

From the preceding discussion it is clear that patterns of melatonin secretion in many vertebrates do not merely follow light-dark changes. Depending on the species and the photoperiod, increased melatonin production may begin before lights-off, may be delayed until several hours after darkness onset, and may decline before lights-on. This suggests that, rather than having a direct driving influence on melatonin production (inhibition by light/stimulation by dark), the light-dark cycle entrains an endogenously generated rhythm of melatonin synthesis. The involvement of endogenous timing mechanisms in the regulation of pineal melatonin synthesis and release has been confirmed in many species by measuring pineal and circulating, cerebrospinal fluid or urinary melatonin (or N-acetyltransferase) in animals transferred from an acclimatory photoperiod to constant darkness (DD). Thus, the persistence in DD of melatonin rhythms previously synchronized to a light-dark cycle has been demonstrated in vivo (Figure 4.5) in the rhesus monkey (Perlow et al., 1981; Reppert et al., 1981), horse (Klimer et al., 1982), sheep (Rollag and Niswender, 1976; Lincoln and Almeida, 1981; Almeida and Lincoln, 1984; Lincoln et al., 1985; Earl et al., 1990) rat (Ralph et al., 1971; Rivest et al., 1981), Syrian hamster (Tamarkin et al., 1980), Djungarian hamster (Yellon et al., 1982), chicken (Lynch and Ralph, 1970; Ralph et al., 1974, 1975; Cassone and Menaker, 1983), quail (Cockrem and Follett, 1985), pigeon, Columba livia (Foa and

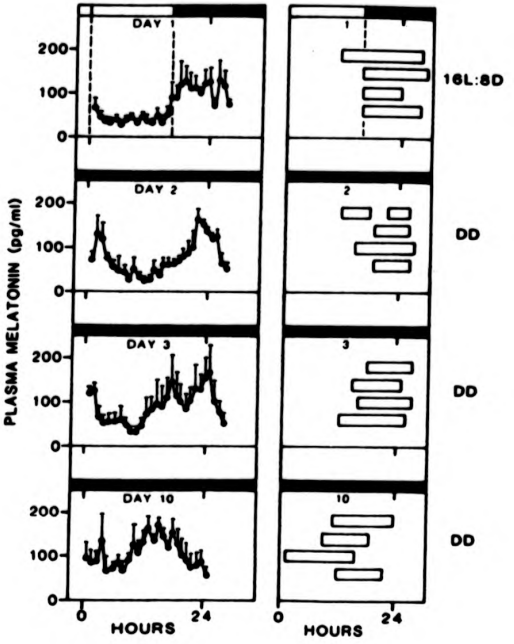


Figure 4.5: Persistence of melatonin rhythms in male Soay sheep transferred from 16L:8D to constant darkness (DD). Graphs show mean (± 1SEM) plasma melatonin concentrations for the group of 4 animals (left) and the timing of peak melatonin production for individual rams (open bars, right) for samples collected at hourly intervals over 24 hour periods on days 1 (last day under 16L:8D), 2, 3 and 10. Solid bars denote DD (from Lincoln et al., 1985).

Menaker, 1968), desert iguana, Dipsosaurus dorsalis (Janik and Menaker, 1990) and goldfish (Aida et al., 1989).

In mammals persistence of the pineal melatonin rhythm in DD requires neural regulation (Underwood and Goldman, 1987), but the pineals of several lower vertebrates also exhibit circadian rhythms of melatonin secretion in vitro (Figure 4.6). This area of research was pioneered by Binkley and co-workers in the late 1970's who studied N-acetyltransferase rhythms in pineal cultures, prior to the widespread availability of radioimmunoassays for melatonin. These studies (reviewed by Binkley, 1979), augmented by those of other groups (Deguchi, 1979a,b; Kasal et al., 1979), showed that pineal N-acetyltransferase rhythms in chickens are synchronized to the light-dark cycle, and persist in DD, in vitro as well as in vivo. Subsequent investigations on chickens, quail and starlings (Sturnus vulgaris) have demonstrated that pineal melatonin rhythms can also be entrained to light-dark cycles and will persist in DD (or dim red light) in vitro (Takahashi et al, 1980; Takahashi, 1981: cited in Menaker and Wisner, 1983; Cockrem and Follett, 1984). In chickens this occurs even when only small fragments of the pineal (Takahashi and Menaker, 1984) or dispersed pineal cell cultures (Robertson and Takahashi, 1988a,b; Zatz et al., 1988) are used. Thus, the chick pineal possesses an endogenous circadian oscillator, and is probably multi-oscillatory in nature. However, the melatonin rhythm in chickens in DD becomes heavily damped within several days both in vitro (Takahashi et al., 1980; Robertson and Takahashi, 1988a,b) and, after superior cervical ganglionectomy, in vivo (Cassone and Menaker, 1983), whereas the rhythm under a light-dark cycle, or in intact hens in DD, continues unabated. In the absence of photic input, therefore, the chick pineal appears to require sympathetic neural input via the superior cervical ganglia as in mammals.

Pineal melatonin rhythms have also been investigated in vitro in several reptilian species, all iguanid lizards. Menaker and Wisner (1983) demonstrated temperature-compensated circadian rhythms of melatonin secretion in isolated pineals of the green anole lizard, Anolis carolinensis, maintained in DD. Since these rhythms persisted for up to 10 cycles, considerably longer than in the chicken, Underwood and Goldman (1987)

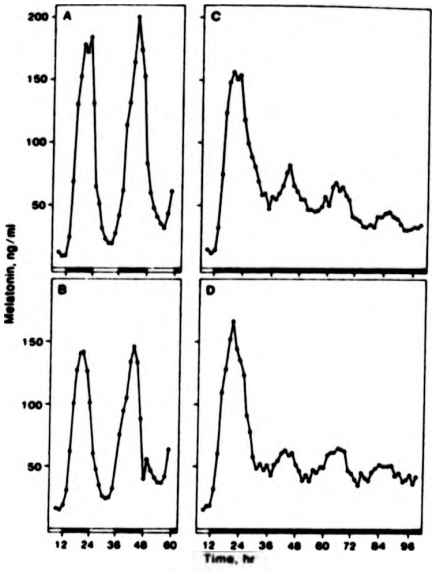


Figure 4.6: Persistence of melatonin rhythms in individual chicken pineals transferred from a 12 hours light:12 hours dim red light photoperiod regime (A and B) to constant dim red light (C and D). Each point represents a single determination of the melatonin concentration in the culture medium plotted at the start of each 90 minute collection interval from the flow-through culture apparatus. The light-dark cycle is indicated at the bottom of each graph (from Takahashi et al., 1980).

have suggested that the pineal of Anolis may be more autonomous than the chick pineal. Persistence of pineal melatonin rhythms in vitro has also been shown for Sceloporus occidentalis subjected to DD (Menaker, 1985). However, a third lizard, the desert iguana, does not exhibit self-sustaining rhythms of pineal melatonin secretion in vitro when maintained in DD; rather, it produces continuously high concentrations of melatonin (Menaker, 1985; Janik and Menaker, 1990). The latter species does, though, possess an extra-pineal oscillator since circulating melatonin rhythms persist in vivo: melatonin synthesis may therefore be driven via sympathetic innervation in this lizard (Janik and Menaker, 1990). Unfortunately, the role of potential neural inputs to the pineals of lower vertebrates has not yet been studied (Underwood, 1989).

Clearly, the majority of avian and reptilian vertebrates so far investigated appear to possess an intra-pineal oscillator(s) or clock. However, only a very limited number of species have been examined and, even within a single taxonomic family, the iguanid lizards, differences in the mechanisms regulating the production of melatonin are already apparent. It may be no coincidence that in the lizard which does not appear to possess an intra-pineal clock regulating melatonin production (the desert iguana) pinealectomy has no effect on locomotor rhythms, whereas in a lizard which does possess a pineal oscillator (the green anole lizard) behavioural rhythmicity is abolished by pinealectomy (Janik and Menaker, 1990). There has been little related work on fish although recent reports suggest that pineal melatonin rhythms are self-sustaining in vitro in the pike (Falcon et al., 1989) and the goldfish (Aida et al., 1989; Kezuka et al., 1989; Iigo et al., 1991), but not in the rainbow trout (Gern and Greenhouse, 1988). These studies will be referred to in more detail in the discussion to this chapter.

The name 'melatonin' reflects the melanosome-aggregating properties of the hormone (Lerner et al., 1958). Lower vertebrates undergo two kinds of rapid colour change: diurnal changes (blanching at night) and background adaptation (for a comprehensive account of pigmentation physiology see Bagnara and Hadley, 1973). The first indication of pineal involvement in colour change was provided by von Frisch (1911, cited in Oksche and Hartwig, 1979) who observed that illumination of the pineal area in blinded minnows,

Phoxinus laevis, caused darkening of the body (melanosome dispersion), whereas shading the head of blind fish resulted in the aggregation of melanosomes; moreover, the melanophore response was temporarily abolished by pinealectomy. Soon after, McCord and Allen (1917) discovered that bovine pineal extracts caused blanching in tadpoles, Rana pipiens. Subsequently, Young (1935) reported that pinealectomy abolished diurnal colour changes in intact larval and blind adult lampreys, Lampetra planeri, leaving them permanently dark. More recent studies are indicative of pineal and/or melatonin involvement in colour change in some fish, but not others, often dependent on developmental stage (Bagnara and Hadley, 1973; Gern et al., 1981; Dodd and Munro, 1983). In the rainbow trout bilateral enucleation reduced night-time pallor in juvenile fish maintained on a dark background and pinealectomy of blinded fish abolished the rhythmic colour change; pinealectomy alone, however, had no effect (Hafeez and Quay, 1970b). Administration of pharmacological doses of melatonin to the same fish caused dose-dependent body blanching and antagonised background adaptation following transfer from a white to a black background (Hafeez, 1970). In young anadromous fish of the same species Smith and Weber (1976b) claimed that changes in environmental background colour cause dramatic alterations in the pattern of pineal HIOMT activity. In contrast, Owens et al. (1978) found no relationship between plasma melatonin levels and background adaptation in adult rainbow trout, and found no significant diurnal colour change in fish maintained under ambient daylength. Gern et al. (1981) therefore suggest that, as in several other species, melatonin may be important in colour change in young rainbow trout, but not in older fish. However, neither melatonin nor pineal extracts caused melanosome aggregation in an in vitro bioassay utilising scales from juvenile rainbow trout; it seems likely that a hypothalamic peptide stored in the pituitary, melanin-concentrating hormone, is the most important melanosome-aggregating hormone in the rainbow trout (Rance and Baker, 1979; Kishida et al., 1989).

Although the pineal and its endocrine secretions may be involved in the regulation of a wide variety of daily and seasonal physiological and behavioural events it is their function in the control of reproduction which has attracted the greatest attention. Not surprisingly,

the role of the pineal and melatonin in the control of seasonal reproduction has been most thoroughly studied in mammals. As intimated previously, melatonin secretion in mammals is regulated by photic information perceived by the eyes and relayed to the pineal via a complex sympathetic neural pathway incorporating the suprachiasmatic nuclei (responsible for the circadian nature of the melatonin rhythm) and the superior cervical ganglia (Moore and Klein, 1974; Moore, 1978). Early studies indicated that pinealectomy or melatonin administration were able to influence reproductive activity in a number of photoperiodic mammalian species; however, the results were regarded as contradictory in that both anti- and progonadal effects were reported, or even no effect at all (reviewed by Turek and Campbell, 1979; Reiter, 1980). A significant advance towards understanding the mode of action of melatonin was provided by Tamarkin et al. (1976). They found that daily injections of melatonin administered in the morning had no effect on the gonadal activity of Syrian hamsters maintained on a stimulatory 'long' photoperiod, whereas the same dose administered in the late afternoon caused testicular regression in males and anoestrus in females, an effect similar to that observed on exposure to 'short' photoperiods. These results indicated that the reproductive system possessed a diurnal rhythm in sensitivity to melatonin, and, since the injection of melatonin in the afternoon had no effect on gonadal function in pinealectomised animals, the authors suggested that melatonin may affect reproduction indirectly, by acting within the pineal itself to regulate the release of another factor. However, a subsequent study (Tamarkin et al., 1977) demonstrated that three injections of melatonin administered daily over a 6-hour interval did cause testicular regression and anoestrus in pinealectomised Syrian hamsters, thus supporting an alternative hypothesis that single daily injections were effective when administered in the late afternoon because they summated with endogenously produced melatonin to increase the duration of exposure to the hormone. This line of thought eventually culminated in an elegant series of experiments in the Djungarian hamster and the sheep. In juvenile Djungarian hamsters pinealectomy prevented both 'short' photoperiod-induced inhibition and 'long' photoperiod-induced stimulation of gonadal development (Carter and Goldman, 1983a,b). However, subcutaneous infusion of

melatonin for 8, 9, 10 or 12 hours per day caused testicular regression in pinealectomised Djungarian hamsters previously maintained under a stimulatory long photoperiod (16L:8D), whereas melatonin infusion for 4 or 6 hours daily did not inhibit gonadal development (Carter and Goldman, 1983a; Goldman et al., 1984). Conversely, infusion of melatonin for 4 or 6 hours per day stimulated testicular development in pinealectomised hamsters raised under an inhibitory short daylength (10L:14D), whereas 8 or 12 hour infusions did not stimulate gonadal growth (Carter and Goldman, 1983b). The time of day at which infusion commenced was not important (Carter and Goldman, 1983a,b). Moreover, a 9 or 12 hour infusion pattern, which included a 2 or 3 hour melatonin-free period, failed to induce testicular regression in pinealectomised hamsters raised under a 'long' photoperiod, indicating that the amount of melatonin received per day was also unimportant (Goldman et al., 1984). Clearly, infusion of melatonin for 8-12 hours mimicked the effects of 'short' days and infusion for 4-6 hours mimicked the effects of 'long' days on reproductive development. Similar results have recently been reported in adult Djungarian hamsters (Bartness and Goldman, 1988a,b), indicating that the duration of the nocturnal increase in melatonin secretion is the feature of the melatonin rhythm responsible for the reproductive response to photoperiod in this species.

Bittman and colleagues have reported similar findings in the Suffolk ewe. They monitored the reproductive state by measuring serum luteinizing hormone (LH) levels in ovariectomised animals treated with constant-release oestradiol-17β implants. Exposure to long photoperiods (16L:8D) potentiates the negative feedback effects of oestradiol on LH release (reduced LH; anoestrous), whereas exposure to short daylengths (8L:16D) decreases the negative feedback potency of oestradiol on LH secretion (increased LH; breeding condition: for a review see Karsch et al., 1984). The effects of artificial changes between 'long' and 'short' photoperiods on reproductive activity were abolished by pinealectomy (Bittman et al., 1983a,b). However, infusion of melatonin for 16 hours per day to pinealectomised sheep produced a similar rise in LH secretion to that observed in pineal-intact animals transferred from 'long' to 'short' days (Bittman et al., 1983b, 1985). Conversely, melatonin infusion of pinealectomised sheep for 8 hours daily invoked

a decline in LH levels similar to that seen in controls transferred from 'short' to 'long' days (Bittman and Karsch, 1984; Bittman et al., 1985). In these experiments the duration of the infusion always matched the duration of the scotophase of the artificial photoperiod. To confirm the importance of melatonin in determining the reproductive response it was necessary to mismatch the melatonin rhythm and the photoperiod. When pinealectomised sheep maintained under a stimulatory 'short' daylength were infused with a long-day pattern of melatonin (8 hours per day) LH levels were suppressed (Bittman and Karsch, 1984), whereas pinealectomised sheep subjected to an inhibitory 'long' photoperiod responded to a short-day infusion pattern (16 hours per day) with an increase in serum LH (Yellon et al., 1985). Measurements of circulating melatonin in these studies confirmed that the levels attained by infusion were within the normal physiological range for this species. Moreover, as in the hamster, the time of day during which the infusion was administered to the sheep was not important (Wayne et al., 1988).

Consistent with these findings Dowell and Lynch (1987) reported that a 10-hour pulse of melatonin ('short-day') administered via a cannula to the hypothalamus of the white-footed mouse caused testicular regression in animals maintained under a stimulatory long (16L:8D) photoperiod, irrespective of the time of day of administration. A single 5-hour pulse ('long-day') or two 5-hour pulses separated by a 3-hour melatonin-free interval were without effect. More recently, Maywood et al. (1990) have reported a similar ('short-day') response to 10 hour infusions of melatonin in male Syrian hamsters maintained under a long (16L:8D) photoperiod. Constant-release melatonin implants have also been shown to mimic the effect of a 'short-day' on the reproductive response of a number of animals including the weasel, Mustela erminea (Rust and Meyer, 1969), red deer, Cervus elaphus (Lincoln et al., 1984), sheep (Lincoln and Ebling, 1985; Nowak and Rodway, 1985; English et al., 1986), goat (Deveson et al., 1989) and silver fox, Vulpes vulpes (Forsberg et al., 1990). In conjunction these studies provide compelling evidence that the reproductive response to changes in daylength in certain mammals is determined by the (uninterrupted) duration of the nocturnal increase in melatonin secretion.

In contrast, evidence for the involvement of the pineal and melatonin in avian reproduction is unconvincing. The eyes and pineal are not essential for photoperiodically-induced gonadal development in many birds in which the primary photoreceptors effecting the reproductive response to photoperiodic change appear to be located in the brain, probably in the hypothalamus (reviews: Menaker, 1971; Yokoyama et al., 1978; Oliver and Bayle, 1982; Follett et al., 1985). For example, pinealectomy had no effect on the gonadal response to photoperiod in the house finch, Carpodacus mexicanus (Hamner and Barfield, 1970), Harris's sparrow, Zonotrichia querula (Donham and Wilson, 1970), the house sparrow, Passer domesticus (Menaker et al., 1970) the Japanese quail (Homma et al., 1972; Siopes and Wilson, 1974), the female chicken (Harrison, 1972; Johnson and van Tienhoven, 1984), and the male turkey, Meleagris gallopavo (Siopes and El Halawani, 1989). However, delayed sexual maturity has been reported after pinealectomy in both male (Cogburn and Harrison, 1977) and female (Sharp et al., 1981) chickens, and female turkeys (Siopes and Underwood, 1987). Pinealectomy also inhibited reproductive activity in the duck, Anas platyrhinchos, but only in the breeding season immediately following surgery (Cardinali et al., 1971). In the Indian weaver bird, Ploceus philippinus. Balasubramanian and Saxena (1973) reported that pinealectomy in winter caused precocious testicular recrudescence, which was further accelerated under a long (18L:6D) photoperiod, and also enabled the birds to respond to a non-stimulatory photoperiod (9L:15D) as though it were stimulatory. A further study found that pinealectomy during the breeding season prevented gonadal regression in this species (Saxena et al., 1979). In contrast, Haidar and Ghosh (1990) recently reported that pinealectomy of the Indian jungle bush quail, Perdicula asiatica, had no effect in the non-breeding phase of the annual reproductive cycle, but caused testicular regression during the periods of gonadal recrudescence and breeding associated with increased photoperiod and temperature.

A lack of effect of pinealectomy on avian reproduction in many experiments could be attributed to the melatonin secreted into the circulation by the eyes. Thus, the retinae make a significant contribution to the melatonin rhythm in the blood of birds such as the

Japanese quail (Underwood et al., 1984) and the pigeon (Foa and Menaker, 1968). However, the reproductive response to photoperiod was unaffected in Japanese quail which were both blinded and pinealectomised (Siopes and Wilson, 1974), and melatonin injections had no effect on gonadal function in this species (Homma et al., 1967; Follett et al., 1985). Similarly, Turek and Wolfson (1978) reported that constant-release melatonin implants had no effect on photoperiod-induced gonadal growth in the white-throated sparrow, Zonotrichia albicollis, the fox sparrow, Passerella iliaca, and the slate-coloured junco, Junco hyemalis. Furthermore, melatonin administration failed to alter the photorefractory condition of the white-throated sparrow (Turek and Wolfson, 1978) and the canary, Serinus canarius (Storey and Nicholls, 1978). Melatonin has been reported to affect avian reproduction in some studies, however. Administration of melatonin 3 times weekly stimulated testicular development and comb growth in juvenile chickens, but inhibited these reproductive parameters in maturing and adult birds (Balemans, 1972). In a subsequent investigation melatonin had no effect in young cockerels but caused testicular regression in more mature birds (Balemans et al., 1977). It should be noted, however, that the effects of 5-methoxytryptophol (a derivative of 5-hydroxytryptophan) were equal to or greater than those of melatonin and poor experimental design rendered these studies difficult to interpret (see discussion in Ralph, 1981a). Thus, despite the highly photoperiodic nature of the reproductive response in birds, the results of studies attempting to elucidate the role of the pineal and melatonin in avian reproduction are inconsistent (for more detailed reviews see Ralph, 1978, 1981a) and hence it is not yet possible to attribute either with a role in this process.

Although reptiles have received little attention compared to birds and mammals there is some evidence that the pineal and melatonin may be involved in reproductive function in certain members of this class. In green anole lizards maintained on a 'short' photoperiod-warm temperature regime, pinealectomy stimulated ovarian and testicular development during the period of reproductive quiescence in winter, but was without effect during, and just subsequent to, the spring/summer breeding season (Levey, 1973; Underwood, 1981, 1985a). When the operation was performed in autumn gonadal development was

stimulated in males maintained under warm temperature and either 'long' (normally stimulatory) or 'short' (normally inhibitory) daylengths (Underwood, 1981, 1985a). The progonadal effects of pinealectomy were blocked by administration of melatonin either by injection (Levey, 1973) or by constant-release implants (Underwood, 1981, 1985a), although the latter author was unable to block gonadal growth by morning or afternoon injections (Underwood, 1985a). Pinealectomy in winter also stimulated testicular development in the Indian garden lizard, Calotes versicolor under both ambient and 'long' photoperiod, and gonadal regression was inhibited in animals exposed to a 'short' photoperiod and pinealectomised in early summer (Haldar and Thapliyal, 1977; Thapliyal and Haldar, 1979). Daily injections of melatonin during the early breeding season caused gonadal regression in this species irrespective of the time of day of administration, although injections given in the early morning or late afternoon were most effective (Misra and Thapliyal, 1979). Testicular regression was also produced during the breeding season by melatonin injections in another lizard, Callisaurus draconoides (Packard and Packard, 1977) and by constant-release melatonin implants in the tortoise, Testudo hermanni (Vivien-Roels, 1985). A recent report by Haldar and Pandey (1989) also indicates a role for the pineal in testicular function of the Indian chequered water snake, Natrix piscator: pinealectomy inhibited gonadal development during the later stages of gonadadal recrudescence and in the breeding season, inhibited gonadal collapse subsequently, and prevented the maintenance of full regression during the sexually quiescent phase. It should be noted that temperature may be a more important environmental cue than photoperiod for the control of seasonal reproduction in reptiles (Licht, 1984). In this respect, several studies have reported an effect of temperature on the pattern of melatonin secretion in reptiles (Owens and Gern, 1981; Vivien-Roels and Arendt, 1981, 1983; Underwood, 1985b; Vivien-Roels et al., 1988; Firth and Kennaway, 1989), and pinealectomy (Firth et al., 1988) or melatonin administration (Cothran and Hutchison, 1979; Erskine and Hutchison, 1981) can alter thermoregulatory behaviour. Thus, the pineal and/or melatonin may mediate the effects of both photoperiod and temperature on seasonal reproduction (and other daily and seasonal events) in reptiles.

Temperature also appears to play a more important role than photoperiod in the regulation of reproductive activity in amphibians (Paniagua et al., 1990). However, a small number of studies on frogs have indicated a role for melatonin in amphibian reproduction. O'Connor (1969) found that melatonin inhibited the in vitro ovulation of mature oocytes of Rana pipiens, and daily injections of melatonin 6 hours prior to the onset of darkness inhibited gonadal development in Hyla cinerea maintained under a 'long' photoperiod-warm temperature regime (de Vlaming et al., 1974). Interestingly, gonadal growth was also inhibited in Rana ridibunda maintained under a similar photoperiod-temperature regime even though the daily injections were given at the beginning of the scotophase (Delgado et al., 1983). Moreover, pinealectomy of Rana esculenta, inhibited spermatogenesis under favourable photoperiod and temperature conditions (Rastogi et al., 1976). Delgado and Vivien-Roels (1989) suggest that, as in some birds, melatonin synthesised in the eyes makes a considerable contribution to the melatonin rhythm in the blood in Rana perezi, but the significance of this observation is unknown. As temperature is able to modify melatonin rhythms in Rana perezi (Delgado and Vivien-Roels, 1989) and the neotenic tiger salamander (Gern et al., 1983) melatonin may be able to integrate information on both photoperiod and temperature in amphibians, as suggested for reptiles.

There is evidence that the pineal and melatonin are involved in reproduction in a number of fish species. The goldfish undergoes gonadal recrudescence in spring, a process inhibited by 'short' photoperiods and accelerated by 'long' photoperiods (Fenwick, 1970b). When goldfish maintained under 8L:16D were pinealectomised in late winter/early spring gonadal development was stimulated, thus reversing the inhibitory effect of the 'short' photoperiod (Fenwick, 1970b; de Vlaming and Vodicnik, 1978; Vodicnik et al., 1978). In contrast, when goldfish maintained under 16L:8D or 15.5L:8.5D were pinealectomised at about the same time the stimulatory effect of the 'long' photoperiod was blocked, resulting in gonadal regression (de Vlaming and Vodicnik, 1978; Vodicnik et al., 1978). Pinealectomy of goldfish during the period from late summer to early winter had no effect on gonadal development (Fenwick, 1970b; de Vlaming and Vodicnik, 1978; Vodicnik et al., 1978). Several workers have presented

evidence that the pineal influences reproductive activity in the goldfish by modulating GTH secretion; from late summer to early winter pinealectomy had no effect on circulating GTH levels, whereas in late winter/early spring pinealectomy depressed or abolished the diurnal rhythm in circulating GTH of fish maintained under 'long' photoperiods and promoted a diurnal rhythm in serum GTH in fish maintained under a 'short' daylength (Vodicnik et al., 1978; Hontela and Peter, 1980). Hontela and Peter (1980) therefore concluded that, in the spring, the pineal stimulates gonadal development under a 'long' photoperiod by promoting a diurnal rhythm in circulating GTH, and suppresses gonadal development under a 'short' daylength by inhibiting the rhythm in serum GTH.

de Vlaming (1975) conducted a detailed study of another cyprinid, the golden shiner, Notemigonus crysoleucas, which spawns in late spring/early summer under conditions of 'long' days and warm temperature; final gonadal maturation is inhibited by 'short' photoperiods or low temperatures. When shiners maintained under 9L:15D and warm temperatures (25°C) were pinealectomised in early spring gonadal development was stimulated as in the goldfish, but pinealectomy in late winter had no effect; when fish maintained under 15.5L:8.5D and warm temperatures were pinealectomised in late winter/early spring or late spring gonadal regression or a delay in final maturation occurred respectively (de Vlaming, 1975). Moreover, pinealectomy caused significant changes in gonadal activity only in fish maintained at warm temperatures. An effect of pinealectomy on GTH levels was also claimed for this species (de Vlaming and Vodicnik, 1977).

The Japanese killifish or medaka, Oryzias latipes, a cyprinodontid which spawns in late spring/summer, has been the subject of a series of similar studies by Urasaki and colleagues (Urasaki,1972, 1973, 1976, Urasaki et al., 1982). They reported that pinealectomy of fish maintained under 8L:16D in early winter prevented gonadal regression, whereas pinealectomy in winter or spring inhibited the gonadal recrudescence observed under long (≥13L:11D) photoperiods.

Another group of fish which have attracted attention are the catfish. The Asian catfish, Heteropneustes fossilis, spawns in July-August; 'long' photoperiods and warm

temperatures stimulate ovarian development and 'short' photoperiods during the post-spawning period suppress ovarian activity (Garg, 1988a). Under LL or DD (in which spawning always occurs eventually; section 3.1), 14L:10D, 12L:12D, and 8L:16D (25°C), pinealectomy inhibited ovarian development and vitellogenin synthesis during the post-spawning period (September-January), but had no effect at other times (Garg, 1988a,b). In contrast, pinealectomy accelerated ovarian development and vitellogenin synthesis during the post-spawning period in fish maintained under 9L:15D (25°C), but also had no effect at other times (Garg, 1988a).

Unlike the majority of species studied, the grey mullet, Liza ramada, spawns in mid-winter; 'short' photoperiods stimulate ovarian recrudescence and exposure to 'long' photoperiods, LL, or DD appears to arrest ovarian growth (Abraham and Sagi, 1984). However, when ovarian growth was arrested in mullet by exposure to a 'long' photoperiod during the winter breeding season, pinealectomy accelerated gonadal development in fish transferred to 8L:16D (as in cyprinids, the medaka and the Asian catfish), but had no effect on fish maintained on 16L:8D (Sagi et al., 1983).

The effects of melatonin on reproduction have been investigated in a number of fish species, usually by the administration of daily injections. Exceptions are two early studies by Krockert (1936; cited in de Vlaming et al., 1974) who observed that the appearance of secondary sexual characteristics was delayed in guppies, Poecilia reticulata, fed bovine pineals. Subsequently, intraperitoneal injections of melatonin have been shown to inhibit the stimulatory effects of a 'long' photoperiod on gonadal recrudescence in the goldfish (Fenwick, 1970a), medaka (Urasaki, 1972), golden shiner (de Vlaming, 1975), stickleback (Borg and Ekstrom, 1981) and killifish, Fundulus similis (de Vlaming et al., 1974). Interestingly, the timing of the melatonin injection (2 or 8 hours after lights-on) in the killifish was not important, but, whereas melatonin administration to fish maintained under 'short' days had no effect in mid-winter, the same treatment in late spring retarded gonadal development (de Vlaming et al., 1974). Gonadal growth was also inhibited in grey mullet administered melatonin under a naturally decreasing photoperiod in the autumn, shortly before the mid-winter breeding season. In Asian catfish maintained

under 12L:12D and 25°C, conditions which maintain ovarian activity, melatonin treatment inhibited vitellogenesis and induced follicular atresia when given 2 months prior to the summer spawning, and caused ovarian regression when administered during the spawning period; the higher dose of melatonin used also caused a significant reduction in the number of pituitary gonadotrophs on both occasions (Sundararaj and Keshavanath, 1976). In the latter study withdrawal of the treatment abolished the inhibitory effects of melatonin. Melatonin treatment in spring similarly arrested ovarian recrudescence in another catfish, Mystus tengara, maintained under 12L:12D (Saxena and Anand, 1977), and caused a reduction in circulating levels of oestradiol-17β, oestrone and 17αOH-P in catfish, Clarias batrachus exposed to naturally-increasing daylength and temperature (Nayak and Singh, 1987). In contrast to most investigations, Borg and Ekstrom (1981) also observed a pronounced progonadal effect in female sticklebacks maintained under 8L:16D and administered the lower of two doses of melatonin at the end of the breeding season.

In conjunction these st. ies indicate that the pineal and/or melatonin can exert either pro- or anti-gonadal effects depending on the time of year and the photoperiod-temperature regime to which the fish are exposed; in general, the pineal appeared to inhibit or stimulate reproductive development under 'short' and 'long' days respectively, and melatonin usually inhibited reproduction. However, the mode of action remains unclear. It is possibly important to note that most of the studies examining the role of melatonin in fish reproduction were short-term experiments utilising small species of fish readily maintained under laboratory conditions. Moreover, in the majority of these species photoperiod may not be the primary environmental cue for reproduction; temperature may be of equal or greater importance. This complicates the interpretation of experiments designed to assess the role of the pineal and melatonin in reproduction. In contrast, photoperiod is by far the most important zeitgeber for the control of reproduction in salmonids (section 3.1). There appear to be no reports of the effects of pinealectomy or melatonin on salmonid reproduction, however, almost certainly because of the long-time scale required for such experiments and the facilities necessary for

maintenance of salmonid broodstock.

The preceding review illustrated that the majority of vertebrates investigated exhibit an endogenous circadian rhythm in melatonin production entrained to the light-dark cycle, the precise pattern of which varies among species. Moreover, in some animals (e.g. certain mammals), but not others (e.g. many birds), this hormonal profile of seasonally-changing daylength synchronizes the reproductive cycle with the external environment. Although melatonin rhythms have been demonstrated in the rainbow trout, no previous study has utilised sufficiently frequent sampling to accurately define the patterns of melatonin secretion under different photoperiod regimes, and there has been no reported investigation of the role of melatonin in salmonid reproduction. Furthermore, although the enormous number and diversity of teleost species provides an ideal source for comparisons of melatonin rhythms in animals occupying a variety of different ecological niches, very few species have so far been examined. The experiments described in this chapter are divided into three sections. Section A aims to define the patterns of melatonin secretion under various photoperiod regimes and to investigate the nature of the mechanisms governing the generation of melatonin rhythms in the rainbow trout. In section B patterns of melatonin secretion are examined in two other commercially important fish, one of which, the Atlantic salmon, is closely related to the rainbow trout, whereas the other, the Nile tilapia, Oreochromis niloticus, is a sub-tropical species. Finally, section C investigates the ability of intra-peritoneal melatonin implants to mimic the effects of changes in daylength on the timing of reproduction in the female rainbow trout.

4.2 Section A: Patterns of Melatonin Secretion in the Rainbow Trout

Studies in higher vertebrates have indicated that 1) melatonin is rapidly secreted from the pineal after synthesis and hence blood melatonin levels accurately reflect pineal melatonin production, 2) patterns of melatonin release vary in a species-specific manner, 3) the duration of elevated melatonin changes in accordance with changes in the duration of the scotophase, and 4) melatonin secretion is under endogenous circadian control (Underwood and Goldman, 1987; Reiter, 1988). The experiments described in this section utilised frequent measurements of circulating melatonin under various constant photoperiod regimes in order to accurately define the 24-hour patterns of melatonin secretion in the rainbow trout and to investigate the importance of endogenous mechanisms in the generation of melatonin rhythms in this species.

Experiment 1 compared melatonin profiles in juvenile (1+) rainbow trout maintained under either long (16L:8D) or short (8L:16D) daylengths. Experiment 2 similarly investigated whether a rhythm in melatonin secretion could be detected under long (16L:8D) days in 4.5 month old fry. The effect of a 12-hour advance in the onset of the scotophase was examined in experiment 3 by measuring blood melatonin levels during the transfer of broodstock from a long (16L:8D) to a short (6L:18D) daylength. Experiment 4 investigated whether a rhythm in melatonin secretion could be detected in rainbow trout maintained in constant darkness after previous synchronization to either long (16L:8D) or short (6L:18D) days.

4.2.1 General methods

The rainbow trout used in experiments 1-4 came from a variety of domesticated stocks details of which are provided separately for each experiment. In experiments 1, 3 and 4 fish were maintained in circular photoperiod tanks (section 2.1.1; Figure 2.1). The tanks were supplied with constant temperature (7.5-8°C) spring water (experiments 3 and 4a) or chlorine filtered mains water of seasonally-varying temperature (experiments 1 and 4b; see experimental protocols for the temperature on a particular sampling date). Tungsten filament light bulbs provided a light intensity of 25-30 lux at the water surface (experiments 3 and 4a) or approximately 200 lux 0.5m. below the light source (experiments 1 and 4b; see section 2.1.1 for details). Prior to the experimental period fish were fed ad libitum with a commercial trout diet. Blood sampling (section 2.1.4) began 5-10 minutes before each time point and was generally completed within 20 minutes (maximum 30 minutes). A dim red light (Safelight; Kodak Ltd., Hemel Hempstead, Herts., U.K.) facilitated sampling during the dark and a damp cloth was used to cover the eyes and pineal area of the larger (broodstock) fish. A small long-handled net allowed the individual capture of juvenile fish from heavily-stocked tanks during darkness and individual fish were only sampled once during the course of each experiment. Serum (experiments 3 and 4a) or plasma (experiments 1, 2 and 4b) melatonin content was measured by radioimmunoassay (section 2.6). To minimise the effects of inter-assay variation on the melatonin profiles obtained in experiments 1 and 4b, each assay contained only one sample per treatment from each time point and equal numbers of parallel samples from each photoperiod regime (experiment 1) or day number (experiment 4b).

Stringent precautions prevented any light exposure during the experimental dark periods. All sources of external light (doors, windows, air vents) were shielded with industrial grade black polythene sheeting. Throughout experiment 4b the tanks were also covered with an extra layer of black sheeting which extended down to ground level; two vertical slits allowed access during darkness. The dim red light was turned away from the tanks when fish were removed and anaesthesia and blood sampling were performed over 2 metres away. During anaesthesia fish were protected from the dim red light by black

sheeting.

Initial analysis of the hormone data in each experiment revealed that the sample variances were heterogenous (section 2.7.3). The Kruskal-Wallis test (section 2.7.5) was therefore used for the preliminary analysis of hormone profiles. Due to the unavoidably small number of observations in each sample, however, the non-parametric multiple comparisons procedure (section 2.7.5) was not suitable for the detection of differences in melatonin concentrations between time points. Consequently, after log transformation of the data to reduce the heterogeneity of the sample variances, the results were analysed by one-way analysis of variance followed by a parametric multiple comparisons procedure (section 2.7.5). In each experiment the conclusion reached using one-way analysis of variance and the Kruskal-Wallis test was identical.

4.2.2 Experiment 1: Patterns of melatonin secretion in rainbow trout maintained under long (16L:8D) and short (8L:16D) daylengths

4.2.2.1 Protocol

Approximately 300 one-year old rainbow trout of mixed sex were transferred on May 11 from ambient daylength (16L:8D; latitude 56°09'N) to constant photoperiods of 16L:8D (lights on from 0400 to 2000 hours) or 8L:16D (lights on from 0800 to 1600 hours). The time switches controlling each photoperiod tank (section 2.1.1) were accurate to within 1 second of each other. After 2 months exposure to 8L:16D blood samples (section 2.1.4) were taken at hourly intervals over a 24-hour period (July 15-16). At this time the mean weight of the fish was 210g. Fish were similarly blood sampled after 3 months exposure to 16L:8D (August 12-13). Under both photoperiods the water temperature was 15°C and 5-7 fish were sampled at each time point.

4.2.2.2 Results

Under both 'long' and 'short' daylengths plasma melatonin concentrations were significantly higher ($p \leq 0.001$) during the scotophase than the photophase (Figures 4.7 and 4.8). Melatonin was, however, detectable in the majority of samples taken during the photophase with mean values ranging from 39 to 79pg/ml under 16L:8D and 33 to 92pg/ml under 8L:16D. Under both photoperiods melatonin levels increased rapidly after the onset of darkness, reaching approximately 300pg/ml 30 minutes after lights-off, remained elevated at about 500-600pg/ml for the remainder of the scotophase and had returned to daytime levels 30 minutes after lights-on. Scotophase levels were slightly higher under 'long' (mean amplitude 619pg/ml) than 'short' (mean amplitude 536pg/ml) days but the difference was not statistically significant, and there was no statistically significant increase in melatonin before lights-off, or decrease prior to lights-on, under either photoperiod.

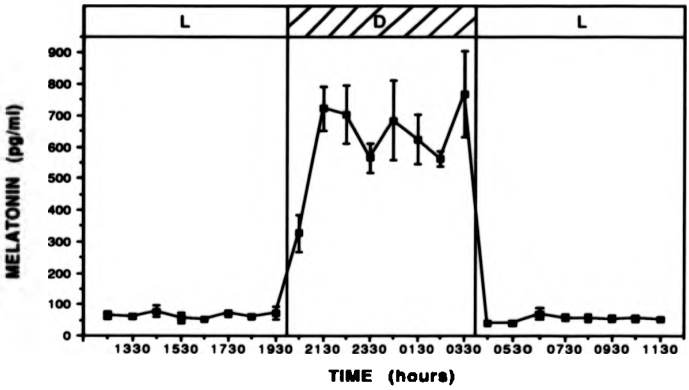


Figure 4.7: Diurnal changes in plasma melatonin concentrations in 1-year old rainbow trout maintained under a 16L:8D photoperiod regime. Each point represents the mean (± 1SEM) of 5-6 fish. The open bars (L) indicate the photophase, the hatched bar (D) the scotophase.

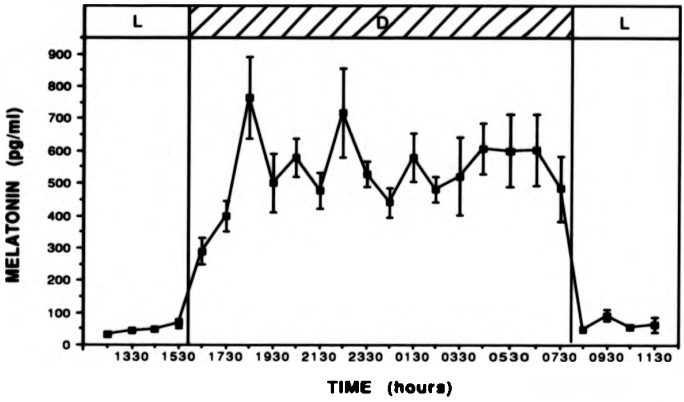


Figure 4.8: Diurnal changes in plasma melatonin concentrations in 1-year old rainbow trout maintained under an 8L:16D photoperiod regime. Each point represents the mean (± 1SEM) of 4-6 fish. The open bars (L) indicate the photophase, the hatched bar (D) the scotophase.

4.2.3 Experiment 2: Melatonin secretion in 4.5 month old rainbow trout fry maintained under a long (16L:8D) daylength.

4.2.3.1 Protocol

Rainbow trout eggs obtained from photoperiodically advanced fish (section 3.2.3) were fertilised with all-female (XX) milt (milt obtained from parents possessing the XX genotype which were masculinised by treatment with methyltestosterone) and laid down in a hatchery tray on October 8. The eggs hatched on October 31-November 1 and swim-up and first feeding commenced on November 16. Throughout this period the eggs and alevins were maintained under a dim 16L:8D photoperiod (15W pearl tungsten filament light bulb; lights on from 0600 to 2200 hours). On November 18 the resulting fry were transferred to a small (0.3 x 0.4m.) grey plastic tank with an initial water depth of 0.1m. The water depth was gradually increased until it was necessary to transfer the fish to a similar larger tank (0.65 x 0.45m.) on February 24. Both tanks were supplied with mains water passed through an activated charcoal/gravel filter at a flow rate of 3 litres/minute (Figure 4.9). Additional aeration was provided by a small aquarium pump. The fry were also exposed to a constant 16L:8D photoperiod (60W pearl tungsten filament light bulb providing a light intensity of 220 lux at the water surface).

When the fish were approximately 4.5 months (142 days) old blood samples were taken over a 24-hour period (March 22-23). Individual fish were anaesthetised (section 2.1.2) and rinsed in deionised water prior to removal of excess water with absorbent tissue paper. Blood was drawn by capillary action from the severed caudal dorsal aorta into heparinised microcapillary tubes (L.I.P. Ltd., Shipley, W. Yorkshire, U.K.). Rubber tubing attached to the microcapillary tubes allowed the blood to be gently blown out into polystyrene tubes (LP3; Luckham's Ltd.). The blood from 10 fish was pooled at each sampling time to obtain duplicate 150-250µl aliquots of plasma for the melatonin assay (section 2.6). Water temperature during the sampling period was 11°C.

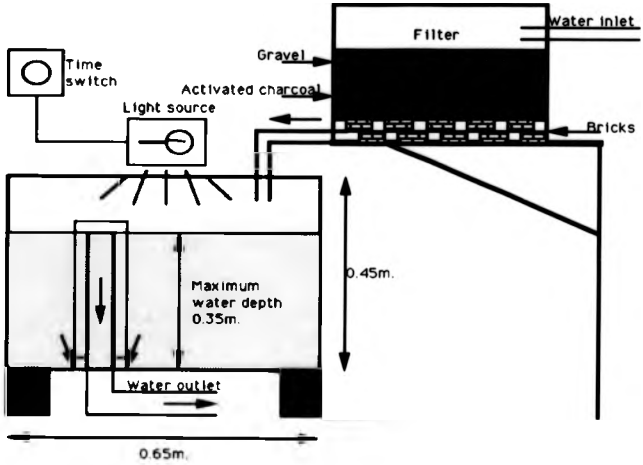


Figure 4.9: Diagram illustrating the main features of a rectangular fish holding tank, with a charcoal/gravel filtered mains water supply, designed for the maintenance of rainbow trout fry (not to scale).

4.2.3.2 Results

Although statistical analysis is not possible because only a single pooled sample was obtained for each time point it is clear that plasma melatonin concentrations in fry were markedly elevated during the scotophase relative to the photophase (Figure 4.10). The pattern of melatonin secretion was similar to that obtained under a 'long' daylength in experiment 1 (section 4.2.2; Figure 4.7). Melatonin levels, which ranged from 77 to 112pg/ml in the photophase, increased rapidly after the onset of darkness, reaching approximately 350pg/ml 30 minutes after lights-off, remained elevated at about 500-600pg/ml for the remainder of the scotophase, and had returned to daytime levels 30 minutes after lights-on.

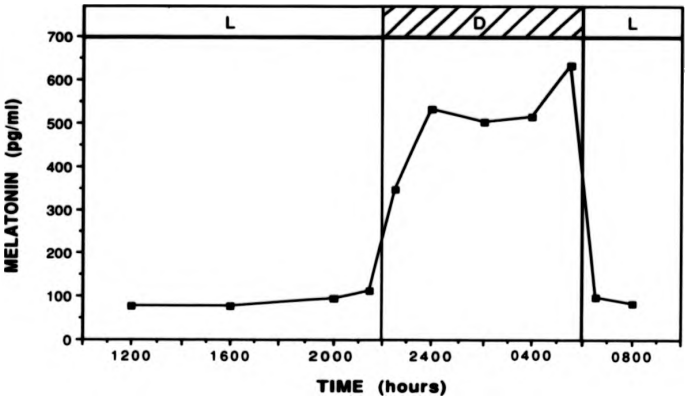


Figure 4.10: Diurnal changes in plasma melatonin concentrations in 4.5-month old rainbow trout fry maintained under a 16L:8D photoperiod regime. Each point represents the melatonin concentration in plasma pooled from 10 fish. The open bars (L) indicate the photophase, the hatched bar (D) the scotophase.

4.2.4 Experiment 3: Patterns of melatonin secretion in rainbow trout during transfer from a long (18L:6D) to a short (6L:18D) daylength.

4.2.4.1 Protocol

Two-year old virgin female rainbow trout broodstock were maintained under 18L:6D (lights on from 0800 to 0200 hours) for 4 months prior to an abrupt reduction in photoperiod to 6L:18D (lights on from 0800 to 1400 hours) on May 15 (section 3.2.3). Blood samples (section 2.1.4) were taken from 3-4 fish at each of the following time points (L=light; D=dark):

Date	14/5	14/5	15/5	15/5	15/5	15/5	15/5	15/5	15/5
Time	0830	1430	0130	0230	0500	0730	0830	1100	1330
Photoperiod	L	L	L	D	D	D	L	L	L
Date	15/5	16/5	16/5	16/5	16/5	16/5	17/5	17/5	
Time	1430	0130	0730	0830	1330	1430	0130	0730	
Photoperiod	D	D	D	L	L	D	D	D	

Water temperature was constant at 7.5°C.

4.2.4.2 Results

The patterns of melatonin secretion during transfer from a 'long' to a 'short' daylength are shown in Figure 4.11 (the 0830 samples taken on 14/5 were haemolysed and were therefore not assayed). Under 18L:6D there was a significant increase ($P \leq 0.05$) in plasma melatonin levels from approximately 110pg/ml in the photophase to about 180pg/ml 30 minutes after lights-off. Subsequently, there was a significant decrease ($P \leq 0.01$) in melatonin from about 190pg/ml 30 minutes prior to lights-on to about 100pg/ml 30 minutes after light onset. Advancing the onset of the scotophase by 12 hours by transferring the fish to 6L:18D also produced a significant increase ($P \leq 0.01$) in melatonin 30 minutes after lights-off, a rise of similar amplitude to that observed under

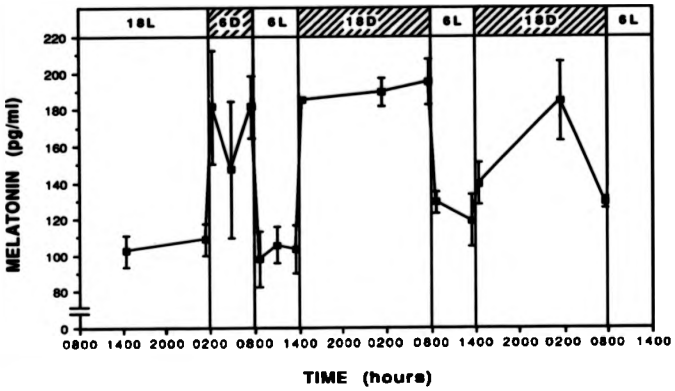


Figure 4.11: Diurnal changes in serum melatonin concentrations in 2-year old rainbow trout during transfer from an 18L:6D to a 6L:18D photoperiod regime. Each point represents the mean (± 1SEM) of 3-4 fish. The open bars (L) indicate the photophase, the hatched bars (D) the scotophase.

18L:6D. Melatonin levels remained elevated 30 minutes before lights-on but had significantly decreased ($P \leq 0.05$) to about 130pg/ml 30 minutes after light onset. Surprisingly, of the 3 samples obtained during the scotophase of the second day of exposure to 6L:18D only the sample obtained at 0130 hours contained significantly elevated melatonin ($P \leq 0.05$) in comparison with the previous photophase levels.

4.2.5 Experiment 4: Patterns of melatonin secretion in rainbow trout maintained in constant darkness (DD) after exposure to a) short (6L:18D) or b) long (16L:8D) daylengths.

4.2.5.1 Protocol

a) 'Short' days to DD

Two and three-year old female rainbow trout broodstock were maintained under 6L:18D (lights on from 0800 to 1400 hours) from May 15 (section 3.2.3). In early December the time clocks were re-adjusted to switch the lights on from 0730 to 1330 hours. At 1330 hours on January 8 the fish were transferred to DD. At this time the population consisted of approximately equal numbers of 3 and 4-year olds. Blood samples (section 2.1.4) were taken from 2-5 fish at approximately 2-hour intervals over 24-hour periods on January 8-9 (Day 1), January 10-11 (Day 3) and January 17-18 (Day 9). The water temperature was 7.5°C on each of the 3 sampling days.

b) 'Long' days to DD

Approximately 300 one-year old rainbow trout of mixed sex, previously sampled in experiment 1 (section 4.2.2), were maintained under 16L:8D (lights on from 0400 to 2000 hours) from July 16. On August 13 the fish were re-distributed between the 2 photoperiod tanks so that each contained equal numbers of fish previously exposed to either 'long' or 'short' days in experiment 1. At 2000 hours on September 19 the fish were transferred to DD. At this time their mean weight was 310g. Blood samples (section 2.1.4) were taken from 6-8 fish at 2-hour intervals over a 72-hour period from September 19-22 (Days 1-3), and from 5 fish at 2-hour intervals over a 24-hour period from September 24-25 (Day 6). The water temperature was 12°C on each of the 4 sampling days.

4.2.5.2 Results

a) 'Short' days to DD

Plasma melatonin concentrations increased rapidly after transfer to DD (1300 vs 1400 hours; $P \leq 0.05$) and remained elevated throughout the experimental period (Figure 4.12). Mean photophase levels ranged from approximately 83 to 100pg/ml and mean scotophase levels from approximately 109 to 252pg/ml (mean amplitude 170pg/ml). There was a significant variation ($P \leq 0.05$) in melatonin levels over time after transfer to DD and visual inspection of Figure 4.12 suggested that this variation may be indicative of an endogenous circadian rhythm in melatonin secretion. However, no significant rhythmicity was detected when the data were subjected to autocorrelation and integrated periodogram analysis (Statgraphics statistical package, STSC Inc., Rockville, U.S.A.).

b) 'Long' days to DD

Plasma melatonin concentrations increased rapidly after transfer to DD (1900 vs 2100 hours; $P \leq 0.01$) and remained elevated throughout the experimental period (Figure 4.13). Mean photophase levels ranged from approximately 40 to 52pg/ml and mean scotophase levels from about 300 to 700pg/ml (mean amplitude 473pg/ml). However, there was no evidence of endogenous rhythmicity; melatonin levels did not significantly vary over time after transfer to DD ($P \geq 0.4$). This conclusion was supported by autocorrelation and integrated periodogram analysis (Statgraphics statistical package), which also failed to detect a rhythm in melatonin secretion in DD.

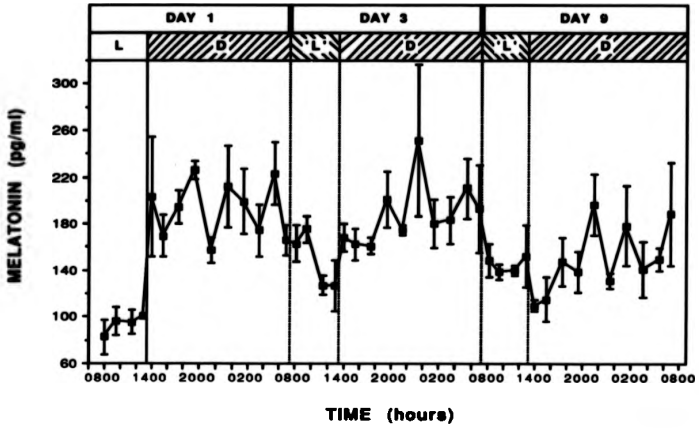


Figure 4.12: Serum melatonin concentrations in 3 and 4-year old rainbow trout maintained under constant darkness (DD) after previous exposure to a 6L:18D photoperiod regime. Each point represents the mean (± 1SEM) of 2-5 fish. The open bar (L) indicates the photophase prior to transfer to DD. The hatched bars indicate DD; periods corresponding to the scotophase and photophase of the preceding photoperiod are signified by D and 'L' respectively.

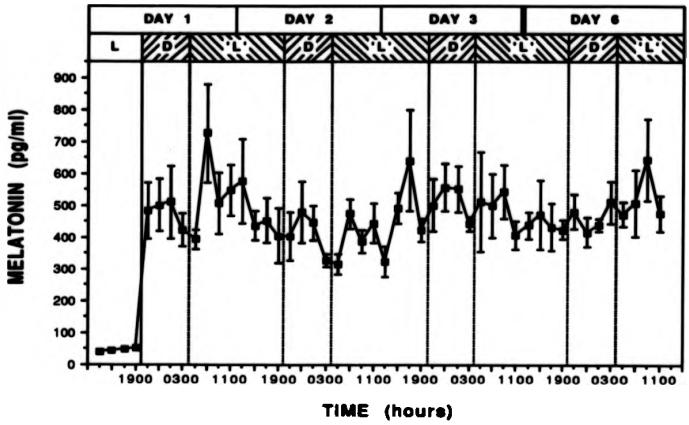


Figure 4.13: Plasma melatonin concentrations in 1-year old rainbow trout maintained under constant darkness (DD) after previous exposure to a 16L:8D photoperiod regime. Each point represents the mean (± 1SEM) of 5-7 (days 1-3) or 4-5 (day 6) fish. The open bar (L) indicates the photophase prior to transfer to DD. The hatched bars indicate DD; periods corresponding to the scotophase and photophase of the preceding photoperiod are signified by D and 'L' respectively.

4.2.6 Summary of Results: Section A

1. In all experiments plasma melatonin concentrations in rainbow trout were significantly higher during the scotophase than during the photophase.
2. The nocturnal increase in circulating melatonin accurately reflected the duration of the scotophase in both juvenile and adult rainbow trout maintained under long (16L:8D or 18L:6D) or short (6L:18D or 8L:16D) artificial daylengths.
3. All significant increases and decreases in melatonin levels coincided with the light-dark transitions under 16L:8D and 8L:16D; there was no evidence for 'anticipatory' increases or decreases prior to lights-off and lights-on or for a delay in secretion after the onset of darkness.
4. The melatonin rhythm immediately re-adjusted to a transfer from a long (16L:6D) to a short (6L:18D) photoperiod accomplished by advancing the onset of the scotophase by 12 hours.
5. Circulating melatonin levels remained continuously elevated after transfer to DD; an endogenous circadian rhythm of melatonin secretion could not be detected.
6. Circulating melatonin was detected during the photophase in all experiments.
7. There were no significant differences in the amplitude of the melatonin rhythms under 'long' or 'short' photoperiods in fish of similar age.
8. The amplitude of the melatonin rhythm was greater in adult fish than in juvenile fish.

4.3 Section B: Patterns of Melatonin Secretion in Two Other Teleost Fish: the Atlantic Salmon and the Nile Tilapia.

Although closely related to the rainbow trout, the Atlantic salmon undergoes a much more complex life cycle (Figure 4.14) in which photoperiod appears to entrain not only maturation (Eriksson and Lundquist, 1980; Lundquist, 1980; Johnston et al., 1990; Taranger et al., 1991) but also the major physiological, morphological and behavioural adaptations associated with seaward migration (collectively known as smoltification), which are necessary for life in the sea (Thrush and Bromage, 1988; Duston and Saunders, 1990). The seasonal dependence of both of these major developmental conversions places even greater constraints on the development of the multi-million pound salmon farming industry than those experienced by trout farmers. An appreciation of the mechanism(s) by which salmon transduce photoperiodic information is therefore of interest from both a comparative and a commercial viewpoint. In this respect, Lindahl and Wetterberg (1986) have reported rhythms in circulating melatonin, with evidence for an endogenous circadian component, in Baltic salmon (also Salmo salar), and Smith and Weber (1980) suggested that the pineal and melatonin may be involved in the migratory behaviour of anadromous salmonids. In a preliminary study, therefore, experiment 5 utilised frequent measurements of circulating melatonin to define the 24-hour pattern of melatonin secretion in Atlantic salmon parr maintained under ambient photoperiod conditions.

The tilapias are cichlid fish (family; Cichlidae) which occur naturally in the tropical and sub-tropical areas of Africa and the Levant, and have been distributed throughout the warmer countries of the world for fish farming. The natural distribution of the Nile tilapia encompasses the coastal rivers and lakes of Israel (33°N) and Egypt through Sudan, Chad, and Nigeria to the equatorial lakes of Uganda and Ethiopia (for further details see Trewavas, 1983; Lowe-McConnell, 1988). Well-suited to the low technology farming systems of developing nations the Nile tilapia is one of the most important tilapia species for aquaculture (Lowe-McConnell, 1988). At the higher latitudes breeding has a strong seasonal component, which may be timed with reference to cycles of temperature and

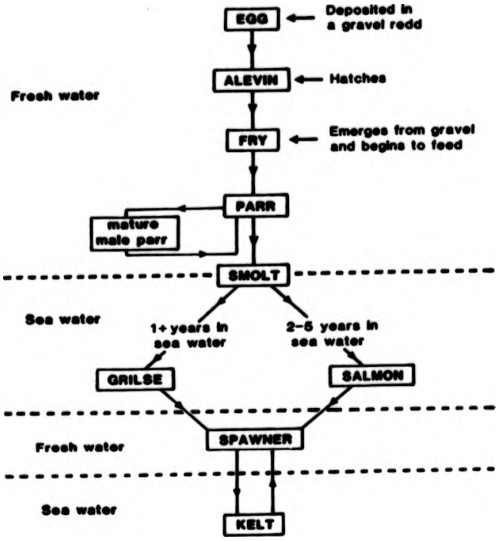


Figure 4.14: Life cycle of the Atlantic salmon (from Laird and Needham, 1988).

rainfall, but farther south the breeding season becomes more extended and close to the equator there is no evidence for breeding seasonality (Trewavas, 1983; Munro, 1990). In marked contrast to the rainbow trout and Atlantic salmon, a role for photoperiod in the control of seasonal rhythms has not been demonstrated in the Nile tilapia and hence it was of interest to investigate whether patterns of melatonin secretion could be detected in this species. Experiment 6 therefore attempted to measure circulating melatonin in Nile tilapia maintained under a constant 12L:12D photoperiod.

4.3.1 Experiment 5: Melatonin secretion in Atlantic salmon parr under a natural 12.5L:11.5D photoperiod

4.3.1.1 Protocol

Approximately 300 potential S2 Atlantic salmon parr (parr destined to smolt after 2 years in freshwater) were maintained under ambient photoperiod (51°20°N, 70% shade netting) and constant water temperature (10°C) from hatching. Blood samples (section 2.1.4) were taken from 8-10 fish (20-40g.) at hourly intervals over a 24-hour period from September 15-16 (approximately 12.5 hours daylength). Light intensity was measured immediately before and after each sampling time. Serum samples were subsequently assayed for melatonin (section 2.5); those taken between 1800 (15/9) and 0800 (16/9) hours were assayed individually, whereas those obtained between 0900 (15/9) and 1700 (15/9) hours were divided into pools each containing equal volumes of serum from two fish. Results were analysed as described in section 4.2.1.

4.3.1.2 Results

Serum melatonin concentrations varied inversely with light intensity, with levels significantly higher ($P \leq 0.01$) during the scotophase than the photophase (Figure 4.15). Mean values ranged from 87 to 130pg/ml during the majority of the photophase and from 219 to 306pg/ml during the majority of the scotophase (light-dark transitions excluded). An unexpectedly high mean value of 179pg/ml was recorded at 1000 hours, but this was not statistically significantly different from the majority of other photophase levels. Between 1700 and 1800 hours, about one hour before the onset of darkness, mean serum melatonin levels increased in the absence of a measured change in light intensity (which was approximately 50 lux; Figure 4.15), although the increase was only significant ($P<0.05$) relative to the 1400 hours sample, and there was no significant difference between the 1800 and 1900 hours samples. Interestingly, however, the first significant ($P \leq 0.01$) decrease in melatonin levels the following morning occurred at a much lower light intensity (approximately 5 lux).

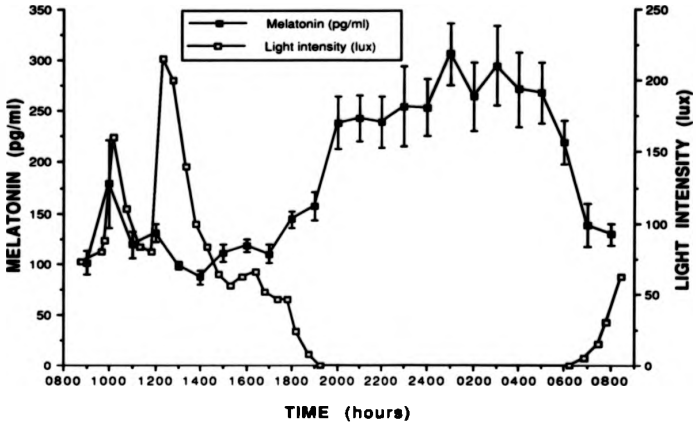


Figure 4.15: Diurnal changes in serum melatonin concentrations in Atlantic salmon parr maintained under a natural daylength of approximately 12.5L:11.5D. Between 0900 and 1700 hours each point represents the mean (± 1SEM) of 4-5 pooled samples containing equal volumes of serum from 2 fish. Between 1800 and 0800 hours each point represents the mean (± 1SEM) of 8-10 individual fish.

4.3.2 Experiment 6: Melatonin secretion in Nile tilapia maintained under a 12L:12D photoperiod.

4.3.2.1 Protocol

Approximately 40 (2:1; female:male) three-year old Nile tilapia broodstock (mean weight 740g.) were maintained in a 4000 litre fibreglass tank supplied with recirculated mains water maintained at a constant 27-28°C. These fish were descended from stock originally obtained from a wild population resident in Lake Manzala, Egypt (31°20'N) in 1979 (McAndrew and Majumdar, 1983) and had been maintained under a 12L:12D photoperiod from hatching. Blood samples (section 2.1.4) were initially taken from 4 fish at either midday or midnight and then from 2-5 fish at each of 7 time points over a 24-hour period (June 24-25). Serum melatonin was measured in duplicate 500µl aliquots using an un-validated adaptation of the radioimmunoassay described in section 2.6. The standard curve was prepared with charcoal-stripped pooled serum collected from tilapia during the photophase. Results were analysed as described in section 4.2.1.

4.3.2.2 Results

A preliminary assay of 2 pools of tilapia serum collected at midday and midnight indicated that melatonin was undetectable in the photophase but was present at a concentration of approximately 80pg/ml in the scotophase. The subsequent assay of samples taken over a 24-hour period, however, detected mean scotophase melatonin levels of less than 25pg/ml. The decreased slope of the standard curve in this assay indicated a deterioration of the radiolabel and, since the assay has also not been adequately validated for the measurement of melatonin in tilapia serum, it must be emphasised that the results obtained in this study should be regarded as qualitative rather than quantitative. Notwithstanding these reservations, it is clear that there was a diurnal rhythm of melatonin secretion in the Nile tilapia under a 12L:12D photoperiod; there was a significant variation ($P \leq 0.01$) in serum melatonin concentrations over time (Figure 4.16). Melatonin was undetectable (≤8pg/ml) in the majority of samples taken during the

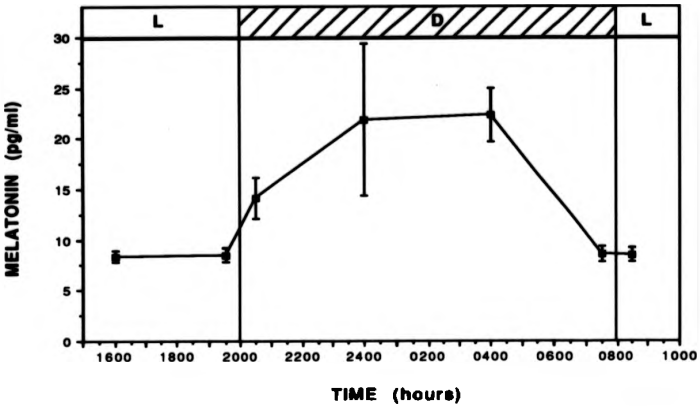


Figure 4.16: Diurnal changes in serum melatonin concentrations in 3-year old Nile tilapia maintained under a 12L:12D photoperiod regime. Each point represents the mean (± 1SEM) of 2-5 fish. The open bars (L) indicate the photophase, the hatched bar (D) the scotophase.

photophase. Although melatonin levels were higher in all 4 fish sampled 30 minutes after lights-off (2030 hours) than in any of the 6 fish sampled in the preceding photophase differences between means were not statistically significant (probably due to small sample size). Samples taken at 2400 and 0400 hours, however, both had significantly higher (vs 1930 hours, $P \leq 0.05$; vs 1600 and 0830 hours, $P \leq 0.01$) melatonin contents than those taken during the photophase. It is notable that a significant decrease ($P \leq 0.01$) in mean serum melatonin content occurred between 0400 and 0730 hours, 30 minutes before lights-on.

4.3.3 Summary of Results: Section B

1. In both Atlantic salmon parr and adult Nile tilapia circulating melatonin concentrations were significantly higher during the scotophase than during the photophase.
2. The nocturnal increase in circulating melatonin accurately reflected the duration of the scotophase in Atlantic salmon parr.
3. An increase in serum melatonin levels occurred prior to the onset of darkness in Atlantic salmon parr.
4. A significant decrease in serum melatonin levels occurred prior to light onset in adult Nile tilapia.

4.4 Section C: The Role of Melatonin in the Transduction of Photoperiodic Information in the Reproductive Axis of the Female Rainbow Trout.

In certain seasonally-breeding mammals the pineal gland converts photic information into a circadian rhythm of melatonin secretion, the pattern of which determines the reproductive response (section 4.1: reviewed by Goldman and Darrow, 1983; Karsch, 1984; Bittman, 1985; Tamarkin et al., 1985; Bartness and Goldman, 1989; Ebling and Foster, 1989). The role of the pineal gland and melatonin in lower vertebrates is less clear, but there is some evidence, principally derived from cyprinids at present, that both are involved in some way in the timing of reproduction in teleost fish (section 4.1: reviewed by de Vlaming and Olcese, 1981). This section describes experiments designed to test the hypothesis that melatonin mediates the effects of photoperiod on reproduction in the female rainbow trout.

The principal techniques available to investigate the role of the pineal and melatonin in reproductive physiology are 1) pinealectomy and 2) provision of exogenous melatonin, or a combination of both. The second technique was chosen in this study in order to assess the ability of melatonin to mimic the effects of photoperiodic change. Exogenous melatonin can be administered by several routes including feeding, injection, timed infusion, and implantation of mini-osmotic pumps or Silastic packets/tubes. Silastic implants are a convenient vehicle for the long-term delivery of melatonin and hence were the method of choice in this study. They have been successfully used to investigate the effects of melatonin on reproduction in a variety of animals including the green anole lizard (Underwood, 1985a), the hedgehog, Erinaceus europaeus (Fowler and Racey, 1990), several hamster species (Goldman et al., 1982), sheep (Lincoln and Ebling, 1985; Nowak and Rodway, 1985; English et al., 1986) and red deer (Lincoln et al., 1984). Although not previously used for the administration of melatonin to fish, Silastic tubes have proven effective for the long-term administration of steroids to a number of fish including grass carp, Ctenopharyngodon idella (Jensen et al., 1973), milkfish, Chanos chanos (Lee et al., 1986a,b,c) and brook trout (Lessman and Habibi, 1987). Experiment 7 was a

preliminary experiment conducted to assess the effectiveness of a range of implants for the controlled release of melatonin. The design of experiments 8 and 9 was based on the observation that November-December spawning rainbow trout subjected to a reduction from a 'long' to a 'short' photoperiod in May respond with a considerable advance in spawning time (section 3.2). If melatonin does mediate the effects of photoperiod on reproduction an equal advance in spawning time would be predicted in fish which received melatonin implants (= 'short day') in May. Conversely, administration of melatonin from January or February might be expected to block the effects of an artificial 'long' photoperiod, resulting in the delay of spawning characteristically observed on exposure to a constant 'short' photoperiod (section 3.2.4). Thus, experiments 8 and 9 examined the ability of melatonin implants to mimic a 'short day' photoperiod.

4.4.1 General Methods

The fish used in experiments 7 and 8 came from an established domesticated stock with a natural spawning period of November-December (the same strain was used in experiments 1-4 in chapter 3), and were maintained in 2 rectangular fibreglass tanks supplied with constant temperature (12°C) recirculating mains water (section 2.1.1; Figure 2.3). Cool white fluorescent tubes provided a light intensity of approximately 60 lux at the water surface. The fish used in experiment 9 came from an established domesticated stock with a natural spawning period of January-February, and were maintained in 3 circular photoperiod tanks supplied with chlorine-filtered mains water of seasonally-varying temperature (section 2.1.1; Figure 2.1). Tungsten filament light bulbs provided a light intensity of approximately 200 lux 0.5m. below the light source (see section 2.1.1 for details).

A variety of implants were prepared from Silastic medical-grade sheeting (0.005in., non-reinforced) and tubing (Dow Corning Ltd., Reading, Berks., U.K.). Silastic packets were made in 1, 2 and 2.5 cm^2 sizes (Plate 4.1). The sheeting was cut to size with a scalpel blade, washed in a hot water/mild soap solution to remove surface contaminants, rinsed in deionised water and allowed to dry. A thin layer of Silastic medical adhesive silicone type A (Dow Corning Ltd.), dispersed 1:1 in hexane (Analar; BDH Ltd.), was used to bond 2 identical squares of sheeting on 3 sides, prior to sterilization in an autoclave (121°C/30 minutes). After addition of crystalline melatonin (Sigma Chemical Company Ltd.) the open ends of the packets were sealed with sterilized (160°C/6 hours) adhesive and they were ready for use. Silastic tubing implants were made in 2 cm (1.02 x 2.16 mm internal/external diameter) and 2.5 cm (3.35 x 4.65 mm) lengths. One end was sealed with adhesive prior to decontamination and sterilization in the same way as sheeting. After addition of crystalline melatonin, or melatonin dissolved in absolute ethanol and castor oil (1:9; Sigma Chemical Company Ltd.), the open ends of the tubes were sealed with sterilized adhesive and they were ready for use. Packets and tubes intended for control fish were constructed in an identical fashion but contained no melatonin.

Fish were starved for 3 days prior to implantation of Silastic packets or tubes into the

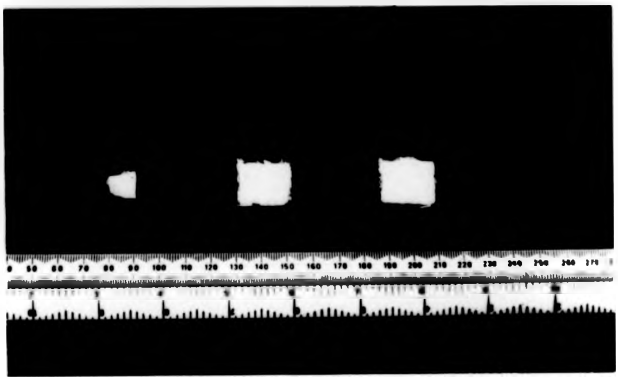


Plate 4.1: Examples of Silastic packets used to investigate the effects of long-term administration of melatonin on the timing of reproduction in female rainbow trout (from left, 1, 2 and 2.5 cm^2 packets containing 10, 100 and 100mg melatonin respectively).

peritoneal cavity. The site of implantation is shown in plate 4.2. A 1cm incision was made slightly above and behind the pelvic fins of an anaesthetised fish and the muscle layers eased apart. The implant was carefully introduced into the peritoneal cavity and the wound sealed with dental tissue glue (Orahesive; kindly donated by the Ministry of Agriculture, Fisheries and Food, Lowestoft, U.K.) containing one part to three Cicatrin antibiotic powder (The Wellcome Foundation Ltd., London, U.K.). Fish were tagged and panjetted (section 2.1.3) to aid identification and replaced in their tanks. Additional aeration was provided to aid recovery which generally occurred within 5 minutes. When a large number of fish were to be implanted only a small number were implanted initially and their satisfactory recovery ensured over 3 days prior to implantation of the remaining fish. Control fish and implanted fish were maintained in separate tanks.

GSI and hormone data were analysed by Student's t-test for homogenous variances (section 2.7.4) or one-way analysis of variance followed by a parametric multiple comparisons procedure (section 2.7.5).

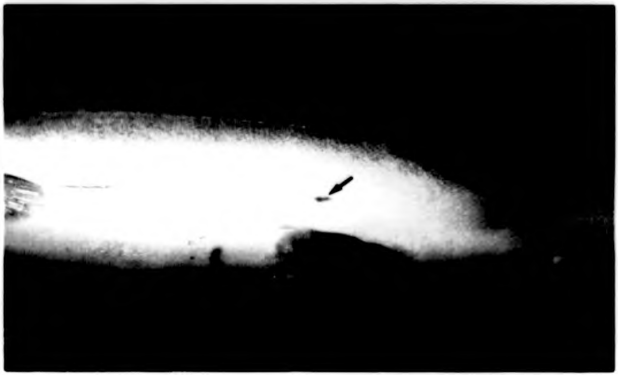


Plate 4.2: Site of implantation of Silastic packets and tubes containing melatonin (the healed wound is indicated by the arrow).

4.4.2 Experiment 7: Assessment of a range of implants for the administration of melatonin to rainbow trout.

4.4.2.1 Protocol

Two-year old female rainbow trout maintained under continuous light were implanted with 1 cm^2 Silastic packets containing either 0, 5, 10 or 20mg melatonin or with 2 cm Silastic tubes containing either 5 or 10mg melatonin (2-3 fish per implant type). A single fish was injected with microcapsules containing 10mg melatonin and made from a polymer under development as a controlled release device at Aston University, U.K. (kindly prepared by P. Kelly). Blood samples (section 2.1.4) were taken over a 4-month period and duplicate 500µl aliquots of serum assayed for melatonin (section 2.6) against a buffer standard curve.

4.4.2.2 Results

The implants were well tolerated by the fish with no deaths occurring during the monitoring period. The melatonin release profiles are illustrated in Figure 4.17. Only the 10 and 20mg Silastic packets showed any promise producing mean serum melatonin concentrations approximately 2.5 times those of the controls after 28 days. After 55 days, however, no elevation of melatonin levels was apparent in any of the implanted fish.

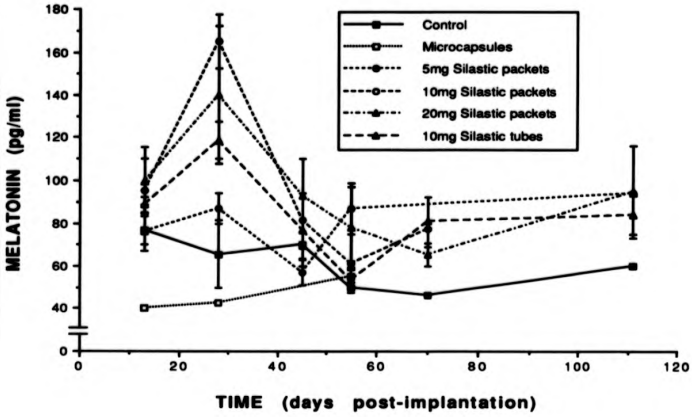


Figure 4.17: Serum melatonin concentrations (mean ± 1SEM) in 2-year old rainbow trout following the administration of a variety of implants containing melatonin (n=1-3).

4.4.3 Experiment 8: Ability of melatonin implants to mimic a 'short day' photoperiod in female rainbow trout maintained under long (18L:6D) days I.

4.4.3.1 Protocol

Approximately 60 two-year old virgin female rainbow trout were transferred from natural photoperiod (latitude 52°30'N) and constant temperature (7.5-8°C) conditions to a simulated natural photoperiod and constant temperature (12°C) on February 2. The fish gradually became acclimatised to their new environment and most had begun to feed again after about 2 weeks. Groups of fish were implanted according to the following protocol:

Group 1a - Controls; each fish received one empty Silastic packet (2 cm^2) on either February 16 (2 fish) or February 19 (6 fish).
Group 1b - Controls; fish were subjected to a sham-operation on either May 5 (3 fish) or May 8 (16 fish).
Group 2 - 'Short-day' from February; each fish received one Silastic packet (2 cm^2) containing 100mg melatonin on either February 16 (4 fish) or February 19 (8 fish).
Group 3 - 'Short-day' from May; each fish received 3 Silastic packets (2.5 cm^2) each containing 100mg melatonin on either May 5 (4 fish) or May 8 (12 fish).

On February 25 the fish were transferred from simulated natural photoperiod (10.5L:13.5D) to 18L:6D. Blood samples (section 2.1.4) were taken at mid-photophase from 2-8 of the fish which received implants in February, at 2-3 week intervals over a 2 month period. Duplicate 500µl aliquots of serum were assayed for melatonin (section 2.6) against a buffer standard curve. At monthly intervals from September to November blood samples were collected from the surviving fish in each group for analysis of serum calcium (section 2.4).

4.4.3.2 Results

Melatonin

The melatonin release profile of the February implanted fish is illustrated in Figure 4.18. After 6 days mean plasma melatonin concentrations reached 367 (±179; n=2)pg/ml; no controls were sampled at this time. At approximately 3, 6 and 8 weeks post-implantation plasma melatonin concentrations were relatively constant with mean levels of 135 (±20; n=4), 121 (±11; n=8) and 132 (±14; n=5)pg/ml respectively, compared to mean levels of 64 (±6; n=4), 62 (±7; n=4) and 63 (±4; n=3)pg/ml respectively in the controls. On each sampling occasion plasma melatonin concentrations were significantly higher ($P \leq 0.01$) in the melatonin implanted fish than in the controls.

Spawning

Approximately two-thirds of the fish died between April and July (principally June). The majority of these experienced difficulty in maintaining their position in the water and were found to be infected with the swim-bladder parasite, Ancyclostoma cystidicola. There was no detectable difference between members of groups which died during this period, all of which had immature ovaries (reflected by a GSI of ≤1). The remaining fish appeared to feed and behave normally from July onwards until the control fish (groups 1a and 1b) died when the pump maintaining the water recirculation system in their tank failed on November 17. To enable a comparison between the GSI of the melatonin implanted fish and the controls, groups 2 and 3 were also killed on November 22. The GSI's are tabulated below:

Group	Treatment	Mean GSI (±1SEM)	n
1a	Control-February	6.57 (±1.38)	4
1b	Control-May	10.27 (±1.28)	8
1a + 1b	Control-combined	9.04 (±1.07)	12
2	Melatonin-February	10.88 (±2.10)	4
3	Melatonin-May	7.38 (±1.99)	5

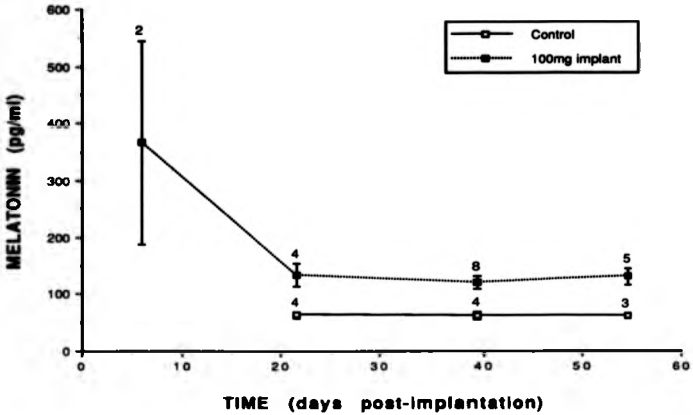


Figure 4.18: Serum melatonin concentrations (mean ± 1SEM) in 2-year old rainbow trout following the administration of Silastic packets containing 100mg melatonin. The number of fish examined on each sampling date is indicated above the error bars.

The figures for Gp 1a include one fish which had just ovulated. There were no statistically significant differences in GSI between any of the groups.

Calcium

Changes in serum calcium levels during the period from early September to early November are illustrated in Figure 4.19. Calcium levels were basal in September and October and appeared to be increasing in all groups by early November, although the difference between October and November levels was only significant ($P \leq 0.05$) in Gp. 2 (melatonin-February).

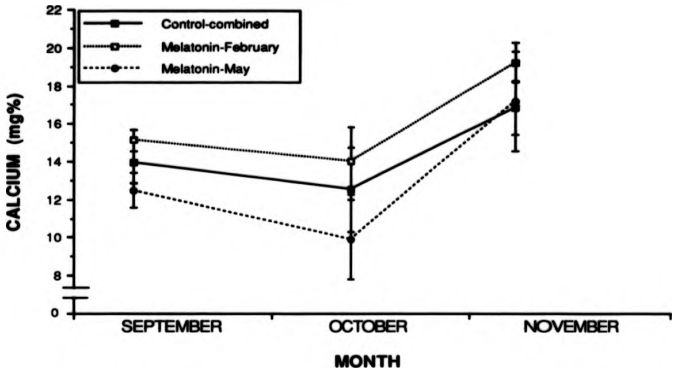


Figure 4.19: Changes in total serum calcium levels (mean ± 1SEM) during the latter part of the reproductive cycle of female rainbow trout administered Silastic packets containing melatonin in either February or May (Control; n=7-10: Melatonin-February; n=3-4: Melatonin-May; n=4).

4.4.4 Experiment 9: Ability of melatonin implants to mimic a 'short day' photoperiod in female rainbow trout maintained under long (18L:6D) days II.

4.4.4.1 Protocol

Approximately 100 two-year old virgin female rainbow trout were transferred from natural photoperiod (latitude 56°N) and seasonally-varying water temperature to 18L:6D (also seasonally-varying water temperature) on either January 13 (7.5L:16.5D) or February 2 (8.5L:15.5D). Unfortunately, the majority of these fish died within a short time, apparently because of a deterioration in water quality shortly after their arrival. The 14 surviving fish had regained weight and were feeding normally by May and were divided into 2 equal groups as follows:

Group 1 - Controls; each fish received one 2.5cm Silastic tube, containing 200µl ethanol:castor oil (1:9) mixture only, on May 6.
Group 2 - 'Short-day' from May; each fish received one 2.5cm Silastic tube, containing 5mg melatonin dissolved in 200µl ethanol:castor oil (1:9) mixture, on May 6.

Blood samples (section 2.1.4) were taken at mid-photophase on July 7 (n=3) and October 5 (n=14), and duplicate 500µl aliquots of serum were assayed for melatonin (section 2.6) against a buffer standard curve. Between October 26 and November 3 all of the fish stopped feeding and 10 died, apparently in response to a further deterioration in water quality. The remaining 4 fish did not regain condition and were killed on November 10. Gonad and body weights were recorded for calculation of the GSI (section 2.3.3).

4.4.4.2 Results

Melatonin

Approximately 2 months post-implantation (July 7) serum melatonin levels of 76pg/ml (n=1) and 93 (± 27; n=2)pg/ml were recorded for the control and melatonin implanted fish respectively. At 5 months post-implantation (October 5) mean melatonin

levels were 40.69 (±5.10; n=7)pg/ml in the control fish and 57.17 (±6.15; n=7)pg/ml in melatonin implanted fish. These differences were not statistically significant.

Spawning

The GSI's are tabulated below:

Group	Treatment	Mean GSI (±1SEM)	n
1	Control	3.58 (±0.61)	6
2	Melatonin-May	4.77 (±0.86)	6

Gp. 1 also contained an ill-conditioned submissive fish which had lost weight since implantation (GSI=0.27) and Gp. 2 a male (GSI=0.03), both of which were excluded from the group data above. There was no statistically significant difference in GSI between the 2 groups.

4.4.5 Summary of Results: Section C

1. Implantation of Silastic packets containing 100mg of melatonin into female rainbow trout elevated plasma melatonin concentrations, measured at mid-photophase, to twice those observed in the controls, for a period of at least 2 months.

2. Neither experiment examining the effects of melatonin on reproduction reached completion (spawning); however, Silastic implants containing melatonin implanted in either February or May had had no significant effect on the GSI when the experiments were terminated the following November.

4.5 Discussion

4.5.1 Patterns of melatonin secretion in the rainbow trout

The present study demonstrates a clear diurnal rhythm in circulating melatonin in rainbow trout maintained under both 'long' and 'short' daylengths. Serum and plasma melatonin levels in experiments 1-4 were within the range reported for a variety of fish and higher vertebrates (Table 4.1). Specifically, melatonin levels in juvenile rainbow trout measured during the scotophase, at approximately 500-600pg/ml, were similar to those observed at mid-scotophase by other workers studying rainbow trout of approximately the same age (Gern et al., 1978b; Owens et al., 1978); it should be noted, however, that a cross-reacting contaminant detected in chloroform-extracted trout plasma may have caused an over-estimate of melatonin concentrations in the assay used by Gern and colleagues (Gern et al., 1978b). Similarly, nocturnal melatonin levels in broodstock fish, at approximately 200pg/ml, were comparable to those previously reported in adult rainbow trout (Gern et al., 1978a, b; Duston and Bromage, 1986b), although lower than the levels of approximately 400pg/ml observed in 2-year old fish by Owens et al. (1978).

It should be noted that circulating melatonin was also detected during the photophase in experiments 1-4, although the concentrations were always much lower than those measured in the scotophase. Low blood melatonin levels in the photophase have been reported in a number of different vertebrate species (Table 4.1), and photophase concentrations within the range detected in this study have been reported in several previous studies on the rainbow trout (Gern et al., 1978a, b; Owens et al., 1978; Duston and Bromage, 1986), and also in the pike (Falcon et al., 1989) and common carp (Kezuka et al., 1988). A permanently high baseline does not appear to be a characteristic of the melatonin assay used in the present work, since melatonin levels in individual fish were occasionally below the limit of detection. It is possible that a high concentration of one or more cross-reacting molecules was present in the serum or plasma samples, but, in view of the high specificity of the antibody used in this study (section 2.6), and the agreement

Table 4.1: Day-night changes in melatonin concentrations (pg/ml serum or plasma) in a variety of vertebrate species.

Class	Species	Photophase	Scotophase	Ref. No.
Mammals	Calf	19	200	1
	Camel	29	221	2
	Donkey	24	108	3
	Human	23	97	4
	Pig	22	76	5
	Rat	6	75	6
	Rhesus monkey	32	88	7
	Sheep	10	240	8
Birds	Chick	50	200	9
	Pigeon	20	858	10
	Quail	10	400	11
	Sparrow	94	840	12
Reptiles	Scincid lizard	35	240	13
	Sea turtle	60	143	14
	Tortoise	20	180	15
Amphibians	Tiger salamander	174	249	16
Fish	Baltic salmon	<2	53-204	17
	Coho salmon	25-40	-	18
	Common carp	23-104	220-540	19
	Dace	<5	600-1200*	20
	Pike	70-120	170-210	21
	Rainbow trout	81	153	22
		124-264	254-596	23
		85-164	248-637	24
		35-100	150-316*	25

Adapted from Binkley, 1988. References: Binkley, 1988 (1-16); Lindahl and Wetterberg, 1986 (17); Gern et al., 1984b (18); Kazuka et al., 1988 (19); Brook, 1989 (20); Falcon et al., 1989 (21); Gern et al., 1978a (22); Gern et al., 1978b (23); Owens et al., 1978 (24); Duston and Bromage, 1986b (25). *=unvalidated assays.

with previous studies utilising different antibodies, it appears more probable that circulating melatonin is present during the photophase. Confirmation, however, awaits further validation of the assay using concentrated extracts of serum or plasma obtained during the photophase and/or gas-chromatography-mass-spectrometry (GC-MS), the most specific technique currently available.

The source of melatonin present in the photophase is unknown but it is probably primarily of extra-pineal origin since pinealectomy of rainbow trout has been reported to cause a considerable reduction in circulating melatonin levels in the scotophase but only a slight (although still significant) decrease in the photophase (Gern et al., 1978b, c). Several extra-pineal sites of putative melatonin synthesis have been identified in higher vertebrates including the eyes, the Harderian gland and the gut (Ralph, 1981b; Pang and Allen, 1986). The rainbow trout retina contains HIOMT (Quay, 1965; Quay et al., 1969; Gern et al., 1984) and is capable of melatonin synthesis (Gern et al., 1978c; Gern and Ralph, 1979; Gern and Karn, 1983). Moreover, in marked contrast to the majority of species investigated (Pang and Allen, 1986), the melatonin content of rainbow trout retinae was significantly higher during the photophase than the scotophase (Gern et al., 1978c). Thus, the melatonin detected during the photophase in the present study may have originated from the eyes. More recent studies by Karn (1983; cited by Gern and Karn, 1983), however, have reported a significant nocturnal elevation in retinal melatonin in the rainbow trout, thus contradicting earlier findings. Fish do not possess a Harderian gland and to my knowledge the fish gut has not been examined for melatonin. However, Gern (cited in Ralph, 1981b) was able to detect circulating melatonin in rainbow trout which had been both pinealectomized and enucleated, suggesting that extra-pineal sites other than the eyes may also be capable of producing melatonin.

There was considerable variation in the amplitude of the nocturnal increase in circulating melatonin between experiments with mean scotophase levels of approximately 500-600pg/ml in the juvenile fish sampled in experiments 1, 2 and 4b, but only about 200pg/ml in the 2-4 year old broodstock used in experiments 3 and 4a. This cannot be explained by strain differences since the fish used in experiments 2 , 3 and 4a were of the

same strain. Moreover, the juveniles used in experiment 2 were of the same sex (all female) as the adults used in experiments 3 and 4a. These results are in accord with those of Gern et al. (1978b) who reported mid-scotophase plasma melatonin levels of 596pg/ml in 1-year old and 254pg/ml in 2-year old rainbow trout, and also with those from an earlier study in which mid-scotophase levels of 153pg/ml were observed in 4-year old rainbow trout (Gern et al., 1978a). A similar relationship between age and the amplitude of night-time increases in pineal or blood melatonin levels has been observed in one other lower vertebrate, the scincid lizard, Trachydosaurus rugosus (Firth et al., 1979), and a number of higher vertebrates including the Syrian hamster (Reiter et al., 1980a, 1982; Pang and Tang, 1983), rat (Reiter et al., 1981; Pang et al., 1984), Mongolian gerbil (Reiter et al, 1980b) and rhesus monkey (Wilson and Gordon, 1989). In humans a decline in circulating melatonin levels with age has been reported in some studies but not others (see discussions in Wurtman et al., 1983; Lang, 1986;), although the current consensus is that a decline does occur between early childhood and fully grown adolescence (Silman, 1991). Young et al. (1988), recently reported that human pineal output is not age-dependent and concluded that the decrease in circulating melatonin levels observed during human growth and sexual maturation is due to the increase in body mass. This cannot adequately account for the differences in circulating melatonin in this study, however, since fish weighing less than 10g, (experiment 2) had similar scotophase levels to those weighing 300g (experiment 1), which, in turn, were only three times higher than those in fish weighing up to 4500g (experiment 3). In rats the reduction in pineal melatonin synthesis associated with old age appears to be due to a decline in HIOMT activity, rather than an alteration in pineal responsiveness to neural stimulation (Dax and Sugden, 1988). A similar mechanism would be in accordance with the directly photosensitive, exclusively pinealofugal, nature of the rainbow trout pineal.

Clearly, the relationship between age and nocturnal melatonin concentrations in this study is complicated not only by the possible effects of size, but also by the sexual maturity of the animals. In the female rhesus monkey the rate of the decrease in nocturnal melatonin secretion was related not only to age but also to the timing of sexual maturation,

with lower serum melatonin levels occurring in monkeys ovulating for the first time than in immature animals of similar age (Wilson and Gordon, 1989). In the domestic cat, Leyva et al. (1984) have found that significantly lower concentrations of melatonin are present during periods of follicle development than during periods of ovarian inactivity within a cycle. Since there is a negative correlation between plasma LH and melatonin levels in humans, Wurtman (1986) speculated that the high nocturnal melatonin levels observed in young children compared to adolescents act as a brake on puberty and that the subsequent decline in these levels allows the mechanisms governing adult development to be expressed. In this respect Silman (1991) suggests that puberty occurs in humans when a combination of constant melatonin output from the pineal and increasing body mass (see Young et al., 1988) results in circulating melatonin falling below a minimum critical concentration, at which point it is no longer able to inhibit the pulatile release of GnRH. This hypothesis is supported by the observation that the amplitude of the nocturnal increase in circulating melatonin in certain infertile women, in whom pulsatile GnRH secretion does not occur, is approximately double that measured in fertile subjects (Berga et al., 1988; Brzezinski et al., 1988). Conversely, Reiter et al. (1981) suggested that the depression of pineal melatonin synthesis in old female rats may be related to the cessation of reproductive activity in these animals. However, a causal relationship between the amplitude of the melatonin rhythm (rather than the rhythm per se) and reproductive competence in higher vertebrates has yet to be demonstrated.

There is even less evidence for such a relationship among lower vertebrates although unusually low levels of circulating melatonin have been reported in female green sea turtles during mating and nesting (Owens et al., 1980). Interestingly, Fenwick (1970) observed that the pineal melatonin content of immature chinook salmon was six times that of mature fish, although the age of these fish, which may spend 1-5 years at sea before returning to freshwater to spawn, was not reported, and the mature salmon were sampled under a shorter daylength than the immature fish.

Clearly, comparisons between the processes of sexual maturation in fish and mammals are of limited value, although, as detailed in chapter 1, there are many similarities

between the neuroendocrine mechanisms controlling reproduction in fish and higher vertebrates. Puberty can be defined as the time of first spawning in rainbow trout but the neuroendocrine changes associated with reproductive development in virgin fish begin at least 1 year before ovulation (Elliott et al., 1984; Sumpter, 1984; Sumpter et al., 1984). However, it is not known whether these changes had started in the 1-year old fish sampled in the current work since they were not retained until spawning and may subsequently have matured at either 2 or 3 years of age.

At least two further factors may explain the differences in amplitude of nocturnal melatonin secretion observed in this study. Firstly, light intensity can have a pronounced effect on melatonin synthesis. Certainly, when dealing with a light sensitive hormone, an increase in light intensity during the photophase might be expected to produce a more clearly defined secretion pattern (Lincoln et al., 1985). In the present study the juvenile fish were indeed exposed to a higher light intensity (>200 lux at the water surface) than the broodstock (25-30 lux at the water surface). However, in Gern et al.'s (1978b) investigation the 2-year old fish were subjected to a much higher light intensity (9,900 lux) than the 1-year old fish (700 lux). It therefore seems unlikely that the differences in amplitude of nocturnal melatonin secretion between juvenile and adult fish were due to differences in light intensity during the photophase. An alternative explanation is that the amplitude of melatonin secretion is temperature dependent. In cold-blooded vertebrates especially it has been suggested that melatonin may serve to integrate information on both photoperiod and temperature (Vivien-Roels, 1981, 1985). Certainly, fluctuations in temperature would be expected to be accompanied by substantial changes in metabolic/enzymatic activity in poikilotherms. Most research on this topic has been conducted in reptiles, which may experience large variations in environmental temperature and in which temperature appears to be an important proximate cue for the control of seasonal reproductive activity (Licht, 1984). A temperature-induced increase in the amplitude of pineal and/or circulating melatonin levels during the scotophase has been reported in the green sea turtle, the loggerhead turtle, Caretta caretta (Owens and Gern, 1981), the tortoise, Testudo hermanni (Vivien-Roels and Arendt, 1981, 1983),

and the box turtle (Vivien-Roels et al., 1988). In the latter two species there were clear day-night differences in melatonin levels at 25-30°C but these changes were abolished at 5°C, irrespective of time of year and photoperiod (winter/'short' day or summer/'long' day) or state of arousal (hibernating or aroused); in the box turtle the amplitude of the melatonin rhythm showed a marked increase at each of 3 increasing temperatures (15, 20 and 27°C). Melatonin rhythms were similarly abolished in the frog, Rana perezi, at 6°C, but a clear rhythm was present at 25°C, again regardless of time of year and photoperiod. In contrast, a clear plasma melatonin rhythm was observed at 10°C (12L:12D) in another amphibian, the neotenic tiger salamander, but the rhythm was abolished in animals maintained continuously at 20°C (Gern et al., 1983). Furthermore, the pineal melatonin rhythm of the green anole lizard can be entrained to a 24-hour temperature cycle with increased melatonin levels occurring during the cool phase of a 32°C/20°C (12:12 hours) temperature cycle even when the cool phase occurs during the day (Underwood, 1985b). Moreover, in the sleepy lizard, Tiliqua rugosa, 6 hour thermoperiods administered at different phases of a 12L:12D photoperiod caused phase-shifts of the melatonin rhythm, although melatonin levels were not observed to peak in the photophase in this species (Firth and Kennaway, 1989). Thus, temperature can modify melatonin secretion in a number of poikilotherms, although the effect produced by a particular change in temperature (whether it is an increase or decrease in amplitude) can vary with species.

In the present study the sampling temperatures were 11, 15 and 8°C for the 4.5 month-old fry, 1-year old juveniles and broodstock respectively. Thus, the much lower amplitude of the melatonin rhythm in the broodstock may be attributable to the lower temperatures experienced by these fish relative to the juveniles. The lower temperature experienced by the fry relative to the 1-year old fish may also explain why no age-related difference in melatonin levels was apparent between these groups (age-related and temperature effects need not be mutually exclusive). Temperature has been shown to affect the accumulation of cyclic-AMP, which is thought to be a second messenger in melatonin synthesis, and also NAT activity, in rainbow trout pineals in vitro, with synthesis declining rapidly either side of a maximum at about 15°C (Falcon and Collin,

1989). Morton and Forbes (1989) found that the capacity of pineal HIOMT to produce melatonin was diminished with decreasing temperature in the rainbow trout. However, they also reported that the affinity of HIOMT for its substrate, N-acetylserotonin, increases with decreasing temperature. Thus, the decrease in reaction velocity with decreasing temperature appears to be counteracted by the increase in affinity of the enzyme for the substrate, resulting in a constant rate of melatonin production. The authors suggest that this mechanism enables pineal HIOMT to accurately regulate melatonin production over a wide range of temperatures in the rainbow trout (Morton and Forbes, 1989). Moreover, an increased melatonin secretion at mid-scotophase in 2-year old relative to 1-year old rainbow trout was observed by Gern et al. (1978b) even though the two groups of fish were maintained at the same temperature. Additionally, Lindahl and Wetterberg (1986) reported a much higher nocturnal elevation in plasma melatonin levels in Baltic salmon (Salmo salar) in March than in December, although the temperature was similar on each sampling occasion. Thus, although salmonids may be exposed to considerable seasonal variations in temperature, and temperature is able to modulate seasonal reproductive events (section 1.2), a dependence of melatonin secretion on temperature remains unproven.

Experiments 1-3 demonstrate that the nocturnal increase in circulating melatonin accurately reflects the duration of the scotophase in both juvenile and adult rainbow trout. The pattern of melatonin secretion in rainbow trout maintained under both 'long' and 'short' photoperiods was similar to that arbitrarily classified as type C (or type III) in higher vertebrates (Figure 4.4: Reiter, 1983, 1986, 1987, 1988). Information on patterns of melatonin secretion in other fish is limited to a few species. Notwithstanding the reservation that only four samples were taken during the scotophase, the plasma melatonin profile of pike maintained under a natural 10.5L:13.5D photoperiod (Falcon et al., 1989) appears similar to that observed in the rainbow trout in the present study. In Lindahl and Wetterberg's (1986) 'four-seasons' study of the Baltic salmon the duration of the nocturnal increase in melatonin secretion (assessed at 2-5 hour intervals) was, in general, directly proportional to the duration of the scotophase, but no pattern type

clearly predominated. In common carp maintained under long (16L:8D) or short (8L:16D) daylengths plasma melatonin levels (assessed at 2 hour intervals) increased rapidly after the onset of darkness and remained elevated for the remainder of the scotophase, as in the rainbow trout; under 'short' days, however, two peaks in circulating melatonin were observed (Kezuka et al., 1988). Brook (1989) reported that the melatonin rhythm in the dace most closely resembled a type B (or type II) pattern under a long photoperiod (18L:6D) and a type C pattern under short days (6L:18D); interestingly, she also observed two peaks in circulating melatonin under 'short' days. Thus, in the two cyprinid fish for which data is available the melatonin rhythm exhibits two peaks under 'short' daylengths. Similar patterns have been observed in some studies on humans and sheep maintained under 'short' photoperiods (Arendt, 1979; Arendt et al., 1985). There was, however, no evidence for a similar biphasic pattern of melatonin secretion in rainbow trout maintained under 'short' days in the present study. No previous studies on fish have employed such frequent sampling intervals as those used in the present investigation; it is possible that the apparent existence of more than one peak in circulating melatonin observed in some studies is an artifact caused by the pulsatile nature of melatonin release (Bittman et al., 1983; English et al., 1987; Gern and Greenwood, 1988; Stanislewski et al., 1988). The only previous investigation on the effect of photoperiod on the pattern of melatonin secretion in the rainbow trout (Duston and Bromage, 1986b) also indicated that the duration of the nocturnal melatonin rise was proportional to the duration of the scotophase but suggested a type B pattern; this discrepancy between the two studies is no doubt due to the fact that in the earlier work only 2 and 3 samples were taken during the scotophase under 'long' and 'short' daylengths respectively.

The results of experiment 2 demonstrate that rainbow trout possess a distinct melatonin rhythm at only 4.5 months of age; the pattern of secretion in these young fish was almost identical to that observed in 1-year old fish maintained under the same photoperiod (16L:8D) in experiment 1. To my knowledge no previous studies have examined circulating (or pineal) melatonin levels in very young fish. However, the

ontogenetic development of the pineal, with reference to substances thought to be involved in photoreception and phototransduction, has been investigated in two species. Opsin and serotonin immunoreactive photoreceptors were present in the pineals of embryonic sticklebacks 72-80 hours after fertilisation, but immunoreactive retinal photoreceptors could not be demonstrated until just after hatching at 144 hours post-fertilisation (Ekstrom et al., 1983; van Veen et al., 1984). Similarly, S-antigen, α-transducin, opsin and serotonin immunoreactive photoreceptors were present in the pineals of Atlantic salmon 30 days prior to hatching whereas no immunoreactivity to these substances was observed in retinal photoreceptors until after hatching, which occurred at 110 days post-fertilisation (Ostholm et al., 1987). Thus, in two fish with very different rates of embryonic development, the developing pineal is probably capable of perceiving light information much earlier than the eyes. Moreover, the presence of serotonin in embryonic pineal photoreceptors may be indicative of an active indoleamine metabolism. This early differentiation of pineal photoreceptors suggests that a functional pineal is important for the successful development of young fish. In this respect, Ostholm et al. (1987) suggested that early differentiation of pineal photoreceptors may enable salmon fry to change skin colour and show negative phototactic behaviour in order to locate dark areas in the gravel and hence avoid predators. Moreover, a mechanism that allowed early photoperiodic entrainment of diurnal and/or seasonal rhythms might be expected to optimise the survival chances of alevins and fry. The results of experiment 2 show that by 4.5 months of age circulating melatonin provides a hormonal template of photoperiodic information in rainbow trout fry. Thus, the photoperiodic entrainment of diurnal and seasonal rhythms in salmonid (and perhaps other) fish may be mediated by diel and seasonal changes in patterns of melatonin secretion from a very early age.

4.5.2 Generation of melatonin rhythms in the rainbow trout.

An important finding of the present work is that the distinct diurnal rhythms in circulating melatonin demonstrated in rainbow trout maintained under 'long' and 'short' daylengths (experiments 1-3) did not persist in DD (experiment 4). This is in complete

contrast to the situation prevalent in those higher vertebrates so far investigated, which show persistence in DD of in vivo melatonin rhythms previously synchronized to a light-dark cycle (see section 4.1; Figure 4.5). Only one other study has examined in vivo patterns of melatonin secretion in fish maintained in DD. Aida et al. (1989) transferred goldfish from 12L:12D to DD and took blood samples at 4-hour intervals on days 1-3, 7-8 and 14-15. They reported that plasma melatonin levels on days 1-3 were higher during the period corresponding to the scotophase of the acclimatory photoperiod than during the period corresponding to the photophase, but that clear daily cycles had disappeared after 7-8 days in DD, and melatonin concentrations remained permanently elevated by days 14-15. These findings are indicative of free-running endogenous rhythms of melatonin secretion in individual fish, which became desynchronized after a period in DD. Workers in the same laboratory found that cultured goldfish pineals from fish maintained under a 12L:12D photoperiod continued to express a circadian rhythm of melatonin secretion in DD for up to 4 days, with significantly higher melatonin levels associated with the period corresponding to the scotophase of the acclimatory photoperiod (Aida, 1989; Kezuka et al., 1989; Iigo et al., 1991). Similarly, Falcon et al. (1989) have demonstrated that rhythms in pineal N-acetyltransferase and melatonin persist in vitro for at least 3 days in the pike; significantly elevated levels were again principally associated with the subjective scotophase of the previous (10L:14D) photoperiod although the melatonin rhythm exhibited a slight phase-shift with maximal secretion occurring slightly later on successive days. Moreover, even fractions of pike pineals are capable of releasing melatonin for 3 days in DD (Falcon and Collin, 1989). Thus, both the goldfish and the pike appear to possess an intra-pineal oscillator (or oscillators) which participates in the regulation of melatonin secretion.

In contrast, Gern and Greenhouse (1988) were unable to demonstrate persistence of the melatonin rhythm in isolated rainbow trout pineals maintained in DD; although clear rhythms in melatonin secretion were present in cultures maintained under 12L:12D or 14L:10D, melatonin levels remained continuously elevated after transfer to DD (although the amplitude of the increased melatonin production gradually decreased over time).

Interestingly, Meissl et al. (1990) were also unable to demonstrate a circadian rhythm in neural output during long-term recordings from pineal ganglion cells of isolated rainbow trout pineals. The results obtained in vivo in the present study are in agreement with those obtained in vitro by Gern and Greenhouse (1988) and therefore support their contention that the rainbow trout pineal does not contain a circadian oscillator capable of regulating melatonin release. Additionally, however, they provide evidence that melatonin release in vivo is not controlled by an extra-pineal circadian clock. This concurs with the findings of Hafeez and Zerihun (1974) who reported that the pineal tract innervation of the rainbow trout was exclusively pinealofugal, although pinealopetal innervation via another route, perhaps including the eyes (Smith and Weber, 1976; Hafeez et al., 1978), cannot be discounted (see section 4.1). If a central peptidergic innervation of the pineal occurs in the rainbow trout, as has been demonstrated for the Atlantic salmon and the coho salmon (Ekstrom et al., 1988), the present results also suggest that it is does not function to relay information from a central oscillator to the mechanism regulating melatonin secretion. To my knowledge the desert iguana is the only other non-mammalian vertebrate so far shown not to possess an intra-pineal oscillator, but melatonin rhythms did persist in DD in vivo indicating that an extra-pineal circadian clock regulates melatonin secretion in this species (Janik and Menaker, 1990). Firth et al. (1979) were unable to demonstrate a free-running rhythm of melatonin secretion in vivo in sleepy lizards, but they probably employed insufficient sampling points (6-hour intervals); moreover, they detected an 'anticipatory' increase in plasma melatonin prior to darkness onset, suggestive of an endogenous rhythm of melatonin secretion in this species.

It must be borne in mind that the pulsatile nature of melatonin release from rainbow trout pineals (Gern and Greenhouse, 1988) or rapid desynchronization of the rhythms of individual fish may have masked endogenous rhythmicity. As the day-night changes in circulating melatonin were so distinct in fish exposed to light-dark cycles, however, and since persistence of melatonin rhythms in vivo has been clearly demonstrated in DD in the sheep (Rollag and Niswender, 1976; Almeida and Lincoln, 1984; Lincoln et al., 1985), a species also known to exhibit episodic secretion of melatonin (Bittman et al., 1983;

English et al., 1987), it seems unlikely that endogenous rhythmicity was concealed by pulsatile secretion in the present study. Moreover, previous in vivo studies in a number of animals including the goldfish (Aida et al., 1989), desert iguana (Janik and Menaker, 1990), quail (Cockrem and Follett, 1985), sheep (Rollag and Niswender, 1976; Almeida and Lincoln, 1984; Linoln et al., 1985) and rhesus monkey (Perlow et al., 1981; Reppert et al., 1981) have shown that the melatonin rhythms of individual animals previously entrained to a light-dark cycle drift out of phase only slowly in DD (Figure 4.5) and hence retain sufficient synchrony in the days following transfer to DD for the persistence of the rhythm to be clearly demonstrated using groups of animals. There remains the possibility that one hundred years of domestication has lead to the disappearance of circadian control of melatonin secretion in farmed rainbow trout, and that such a mechanism may be retained in wild fish. However, this argument is unconvincing since circadian control has been retained in other domesticated species investigated (see above and section 4.1). Melatonin production in the rainbow trout appears, therefore, to be a direct response to darkness.

Additional support for this conclusion is provided by the patterns of melatonin secretion observed in experiment 1 in which all significant increases and decreases in circulating melatonin were coincident with the light to dark or dark to light transitions; there was no evidence for 'anticipatory' increases or decreases prior to lights-off and lights-on or for a delay in secretion after the onset of darkness. In contrast, patterns of melatonin secretion in many higher vertebrates are entrained to the light-dark cycle and do not merely follow light-dark changes (Figure 4.4: Underwood and Goldman, 1987; Reiter, 1988). There is limited evidence that this may also be the case in some fish. Thus, a significant increase in plasma melatonin before lights-off has been reported in common carp maintained under 16L:8D (Kezuka et al., 1988), and decreases in melatonin content prior to light onset have been observed in pike pineals sampled under a natural 10.5L:13.5D photoperiod (Falcon et al., 1987) and in the plasma of Baltic salmon at several daylengths between October and March (Lindahl and Wetterberg, 1986). In addition, the results of experiments 5 and 6 (to be discussed in more detail later) indicate

that an increase in plasma melatonin may have occurred before a detectable decrease in light intensity in the Atlantic salmon and that plasma melatonin had returned to daytime levels prior to light onset in the tilapia.

The results of experiment 3, in which rainbow trout were transferred from 18L:6D to 6L:18D, accomplished by advancing the onset of the scotophase by 12 hours, also provide evidence that melatonin production in this species is a direct response to darkness. On the day of transfer to 6L:18D there was an immediate increase in circulating melatonin levels which, at 30 minutes after lights-off, had reached a similar amplitude to that observed at the same time point under 18L:6D (Figure 4.11). Serum melatonin concentrations under 6L:18D remained elevated until 30 minutes prior to lights-on, but, as under 18L:6D, had returned to daytime levels 30 minutes after light onset. Thus, the pattern of melatonin secretion adjusted immediately to the change in photoperiod (although it is not clear why the melatonin rhythm was less distinct on the second day after transfer). This result is consistent with those of Gern and Greenhouse (1988) who reported that pulses of darkness applied to superfused rainbow trout pineals caused a rapid increase in melatonin secretion whether they were administered during the early or late stages of the photophase. In contrast, exposure of cultured pike pineals to darkness at midday did not induce an immediate rise in melatonin release (Falcon et al., 1989), a result in accordance with the proposed circadian nature of melatonin production in this species.

Illnerova and colleagues have conducted a series of studies similar to that employed in the present work to examine the re-entrainment behaviour of melatonin rhythms in rats and Djungarian hamsters. When rats were transferred from 16L:8D (lights off 2000 to 0400 hours) to 8L:16D by a symmetrical extension (lights off 1600 to 0800 hours) or afternoon/evening prolongation (lights off 1200 to 0400 hours) of the scotophase the pineal N-acetyltransferase rhythm required 6 days to adjust to the new photoperiod, although re-entrainment was quicker when the scotophase was extended into the morning hours (lights off 2000 to 1200 hours: Illnerova et al., 1986). Similarly, when rats were subjected to an 8-hour advance phase-shift of a 12L:12D photoperiod (lights off 1800 to 0600 hours⇒lights off 1000 to 2200 hours) adjustment of the pineal N-

acetyltransferase rhythm took 5 days, whereas re-entrainment occurred almost immediately following an 8-hour delay phase-shift (=lights off 0200 to 1400 hours: Illnerova et al., 1987). Re-entrainment of pineal melatonin and N-acetyltransferase rhythms occurred more gradually in Djungarian hamsters taking 4-6 weeks to adjust to a change from 16L:8D to 8L:16D accomplished by a symmetrical extension of the scotophase (Illnerova et al., 1984). Hastings et al. (1987) observed a similarly gradual re-entrainment of pineal melatonin rhythms in Syrian hamsters with adjustment to a change from 16L:8D to 8L:16D occurring over a period of 8 weeks, regardless of whether the change in photoperiod was achieved by an 8-hour advance or 8-hour delay phase-shift. Moreover, Lynch et al. (1978) found that human plasma and urinary melatonin rhythms took 5-7 days to re-entrain to a 12-hour phase-shift (accomplished by extending the photophase), and Reppert et al. (1981) reported that adjustment of the melatonin rhythm in the cerebrospinal fluid of rhesus monkeys subjected to a 12-hour phase-shift (accomplished by extending the scotophase) required 3 days. Thus, in at least 4 higher vertebrates, and possibly the pike, re-entrainment of the melatonin rhythm to a phase-shift of the light-dark cycle occurs not immediately, as in the rainbow trout, but gradually, although there are marked differences in the rate of re-entrainment which may occur over several days or weeks, depending on the species.

It should also be noted from the preceding discussion that, in rats at least, the rate of re-entrainment to a change from a 'long' to a 'short' photoperiod may also depend on whether the increase in duration of the scotophase is accomplished by advancing its onset, delaying its offset, or a combination of the two. It is therefore relevant to compare the results of the present work with studies in which a similar protocol was adopted to achieve the change in daylength. In experiment 3 the melatonin rhythm of the rainbow trout adjusted immediately to a change from a 'long' to a 'short' photoperiod achieved by a 12-hour advance in the onset of the scotophase. In rats, Djungarian and Syrian hamsters, however, when a transfer from long (16L:8D) to short (8L:16D) days was accomplished by an advance in the onset of the scotophase (this includes symmetrical extensions of the scotophase) re-entrainment of the melatonin rhythm to the new photoperiod always

occurred gradually over several days or even weeks (Ilinerova et al., 1984, 1986; Hastings et al., 1987). Gradual re-entrainment through a series of transients is a characteristic response of a circadian rhythm to a phase-shift of the zeitgeber (although the speed of re-entrainment to asymmetrical phase-shifts appears to depend on the free-running period of the entrained rhythm; Aschoff, 1981). Thus, there would appear to be fundamental differences between the mechanisms regulating melatonin secretion in the rainbow trout and certain higher vertebrates, especially rodents.

4.5.3 Melatonin secretion in the Atlantic salmon.

The pattern of melatonin secretion observed in Atlantic salmon parr under a natural 12.5L:11.5D photoperiod was similar to that observed in rainbow trout maintained on artificial 'long' and 'short' daylengths. Thus, serum melatonin concentrations increased rapidly as darkness fell, remained elevated for the remainder of the night and returned to daytime levels as light intensity increased the following morning. Melatonin levels during the scotophase, at 200-300 pg/ml, were within the range reported for other vertebrates, including the rainbow trout (Table 4.1), but were slightly higher than those observed in Baltic salmon parr (also Salmo salar: i.e. same species, different geographical location) by Lindahl and Wetterberg (1986). In contrast to the present work, however, the latter authors were unable to detect melatonin during the photophase, and hence the day-night change in melatonin concentration in the two studies was similar.

Interestingly, between two of the sampling times just prior to the onset of darkness (1700 and 1800 hours), there was a small rise in circulating melatonin in the absence of a detectable change in light intensity (~50 lux). This may represent an 'anticipatory' increase in melatonin levels, suggesting the involvement of endogenous mechanisms in the regulation of melatonin secretion in Atlantic salmon parr. This result, however, should be treated with caution; firstly, because the increase was statistically significant ($P \leq 0.05$) relative to only one of the preceding photophase samples, secondly, because the light intensity measured shortly after the 1800 hours sampling point was reduced considerably (to 24 lux), and thirdly, because there was no significant difference between melatonin

levels at 1800 and 1900 hours, during which time the light intensity decreased to close to the limit of detection of the lightmeter (from 24 to 8 lux). Nevertheless, the first significant decrease in melatonin levels the following morning (between 0600 and 0700 hours) occurred at a light intensity of approximately 5 lux, suggesting that melatonin production in the Atlantic salmon can be suppressed at a much lower light intensity than that experienced by the fish which showed an increase in melatonin levels prior to the onset of darkness. As previously mentioned, Lindahl and Wetterberg (1986) reported decreases in plasma melatonin before dawn in Baltic salmon parr, also suggestive of endogenous circadian control of melatonin secretion, although they did not observe this phenomenom on all sampling occasions, nor did they detect any increases in circulating melatonin prior to the onset of darkness, possibly because their daytime samples were not taken very close to dusk. Experiments utilising square-wave artificial photoperiods, similar to those used to study the rainbow trout in experiments 1-3, and maintenance in DD, are required to clarify the role of endogenous mechanisms in the regulation of melatonin rhythms in the Atlantic salmon. Interestingly, Weber and Smith (1980) speculated that the pineal and melatonin may play a role in the migratory behaviour of anadromous salmonids. In view of this, and of the close phylogenetic links between rainbow trout and Atlantic salmon (see Sanford, 1990), possible differences in the mechanisms regulating melatonin secretion in these species merit further attention.

4.5.4 Melatonin secretion in the Nile tilapia.

Notwithstanding the reservations about the quality of the radioimmunoassay (section 4.3.2.2), experiment 6 demonstrated a distinct diurnal rhythm of circulating melatonin in the Nile tilapia. To my knowledge this is the first time that a rhythm in melatonin secretion has been reported in cichlid fish. Moreover, there was a significant decrease ($P \leq 0.01$) in serum melatonin before lights-on suggesting that, in contrast to the rainbow trout, endogenous circadian mechanisms are involved in the generation of the melatonin rhythm in this species. The fish used in this study had been maintained in the laboratory under a constant 12L:12D photoperiod and temperature of 27-28°C since birth. Tilapia

are multiple spawners (Rana, 1988), and under these laboratory conditions the strain of tilapia used in the current study exhibit spawning cycles throughout the year (McEwen, personal communication). However, the fish used were descendents of a stock recently (1979) acquired from Lake Manzala, Egypt. At this latitude (31°20'N) they would be exposed to considerable seasonal variations in temperature and daylength, experiencing a photoperiod of approximately 14L:10D at the summer solstice and about 10L:14D at the winter solstice. Nile tilapia originating from the lakes of the Nile delta breed between April and August with peak spawning in May and June (Trewavas, 1983; Munro, 1990). At the limit of its northern range in Israel (33°N) breeding is even more restricted, occurring in April and May (Trewavas, 1983; Munro, 1990). In contrast, the breeding season becomes more extended farther south and close to the equator there is no evidence for breeding seasonality (Trewavas, 1983; Munro, 1990). It is therefore conceivable that, at higher latitudes, photoperiod provides an important environmental cue for the timing of reproduction in the Nile tilapia, which may be encoded in the diurnal rhythm of melatonin secretion demonstrated in experiment 6.

Information on the effects of photoperiod on reproductive activity of tilapia is scarce. Billard claimed that no gonadal development occurred in Oreochromis esculentus maintained under 6-8 hour photoperiods (although this species occurs naturally in equatorial regions) and Bruton suspected that the breeding of Oreochromis mossambicus in South Africa was correlated with daylength (personal communications cited in Balarin and Hatton, 1979). It has also been suggested that courtship behaviour of tilapia maintained in aquaria may be triggered by a 'long' photoperiod (Cridland, 1962; Goldstein, 1970: both cited in Balarin and Hatton, 1979). Clearly, however, the role of photoperiod and melatonin in the control of reproduction in tilapia requires futher investigation. A comparison of Nile tilapia indigenous to Egypt or Israel with those native to equatorial regions may provide useful information in this respect.

The importance of temperature for reproduction in tilapia is well documented; tilapia generally spawn between 20-23°C and maintenance of fish at temperatures below 20°C can be used to prevent reproduction (Balarin and Hatton, 1979). Thus, in the Nile delta

the Nile tilapia starts spawning when the air temperature rises to 19°C in April, spawning peaks as the temperature increases to 22°C and 24°C in May and June respectively, but decreases as the temperature rises even higher in July and August, with no spawning occurring from September onwards (Trewavas, 1983). Interestingly, Geraldes (1980) reported that the temperature required for reproduction in Nile tilapia was 2°C greater under a 10L:14D photoperiod (25°C) than under 13L:11D (23°C), suggesting an interaction of photoperiod and temperature in the control of breeding in this species. The possibility exists, therefore, that in the Nile tilapia, melatonin is able to integrate information on seasonal changes in temperature as well as photoperiod, as discussed earlier for other species.

Whether or not melatonin is involved in mediating the effects of photoperiod and/or temperature on seasonal breeding in the Nile tilapia, the pattern of melatonin secretion may be important in the entrainment of diurnal rhythms in this species. For example, the fish used in the present study usually spawn in the evening (10-12 hours into the photophase of the 12L:12D photoperiod; McEwen, personal communication). In contrast, Tilapia zilli maintained in the same laboratory usually spawn in the morning (McEwen, personal communication). That spawning is usually restricted to a particular time of day has also been shown in another tilapia, Oreochromis mossambicus, in which spawning has only been observed in the afternoon coincident with a peak in the number of territorial males (Munro and Singh, 1987). These observations suggest that the Nile tilapia possesses a circadian rhythm in spawning activity which may be entrained by the daily light-dark cycle; this entrainment may be mediated by the diurnal rhythm in melatonin secretion. The Nile tilapia also appears to possess circadian rhythms in otolith growth (Tanaka et al., 1981) and respiratory rate (Ross and McKinney, 1988), which are entrained by the daily light-dark cycle. Thus, the pattern of melatonin secretion demonstrated in this study may mediate the entrainment of a variety of circadian rhythms by photoperiod in the Nile tilapia. The apparent presence of an endogenous circadian rhythm of melatonin secretion in the Nile tilapia, in which at least some physiological and behavioral rhythms probably possess an endogenous circadian component, may be

indicative of a more complex circadian organisation in this species than in the more primitive rainbow trout, in which there is no convincing evidence for the involvement of endogenous circadian mechanisms in the control of diurnal rhythms (discussed in more detail later).

4.5.5 The role of melatonin in the transmission of photoperiodic information to the reproductive axis.

Experiments 1-3 clearly demonstrated that circulating melatonin is elevated for the duration of the scotophase in the rainbow trout and therefore provides this fish with accurate information on both daily and calendar time. In view of the highly photoperiodic nature of the reproductive response in the rainbow trout (chapter 3) the hypothesis that melatonin mediates the photoperiodic entrainment of seasonal reproduction in this species is extremely attractive. Unfortunately, neither experiments 8 or 9, which were designed to test this hypothesis, reached completion, due to mortalities before spawning. However, measurements of the GSI obtained at the enforced termination of each experiment did not indicate a difference in maturation rate between controls and fish implanted with melatonin in either January/February or May. Moreover, there was no significant difference in serum calcium levels between groups during the latter stages of experiment 8. In contrast, the ability of constant-release implants containing melatonin to modify the timing of reproduction has been demonstrated in several higher vertebrates. Silastic packets containing melatonin administered subcutaneously to rams during exposure to 'long' days induced all the reproductive changes normally observed upon exposure to 'short' days, and blocked the subsequent response to photoperiodic change (Lincoln and Ebling, 1985). Intravaginal melatonin implants prepared from Silastic tubing advanced the onset of oestrous in prepubertal and adult ewes maintained under natural daylength if they were administered in July, but not in May (Nowak and Rodway, 1985). Similarly, English et al. (1986) reported that Silastic packets implanted subcutaneously in June, but not in April or May, advanced the onset of ovarian cyclicity by 5-10 weeks in maiden adult ewes maintained under natural photoperiod. A comparable effect was observed in

ewes receiving a slow-releasing intraruminal soluble glass bolus containing melatonin in July (Poulton et al., 1987). Goats receiving melatonin implants from April after previous exposure to 2 months of 'long' days gave birth 2-3 months in advance of the natural spring breeding season (Deveson et al., 1989). Subcutaneous administration of Silastic packets containing melatonin to male red deer at the nadir of the sexual cycle in May advanced autumnal sexual development by more than 1 month (Lincoln et al., 1984). The autumnal onset of reproductive activity was also advanced by several months in male silver foxes administered constant-release melatonin implants from June, an effect similar to that observed in animals exposed to artificial 'short' days at this time (Forsberg et al., 1990). Thus, there is ample evidence that constant-release melatonin implants can mimic the effects of a 'short' photoperiod in a number of seasonally-breeding mammals. Unfortunately, similar long-term experiments utilising constant-release melatonin implants have not been reported in lower vertebrates.

Although the results of the present study provide no evidence for the hypothesis that changes in patterns of melatonin secretion mediate the effects of changes in photoperiod on reproductive timing in the rainbow trout it should be emphasised that, aside from the fact that neither experiment reached completion, the failure to detect an effect of melatonin may have been due to an inadequate release of the hormone from the Silastic implants. Measurements of circulating melatonin in experiment 8 clearly demonstrated that Silastic packets are capable of providing long-term elevation of circulating melatonin levels in the rainbow trout; over a 2-month period melatonin levels in implanted fish were approximately double those in control fish (Figure 4.18). However, apart from the burst in levels measured 6 days post-implantation, serum melatonin concentrations always remained below those measured during the scotophase in experiments 1-4 (although measurements were taken only from the February implant group, which received only one 100mg implant per fish, and not from the May implant group, which received three 100mg implants per fish). It is therefore possible that the elevation in circulating melatonin levels produced by the implants was insufficient to 'over-ride' the natural rhythm in melatonin production which occurred in response to the long (18L:6D)

photoperiod, and which may have been of sufficient amplitude to mask the effects of the implants. Certainly, Silastic tubing appears inappropriate for the long-term administration of melatonin to rainbow trout since no increase in circulating melatonin concentrations could be detected 2 or 5 months after the implantation of Silastic tubes containing melatonin in experiment 9. The inability to achieve long-term elevation of melatonin levels within the normal night-time physiological range for this poikilothermic species is probably principally related to the effects of temperature (≤12°C) on release rate. The proximity of intra-peritoneal implants to the hepatic portal circulation may also be a contributing factor since melatonin is rapidly metabolised by the liver. Clearly, a more thorough examination of the releasing properties of a range of implant types in cold-water fish is necessary before the experiments described in the present work are repeated.

4.5.6 Interpretation of the melatonin signal

The rainbow trout is unique among the small number of vertebrates studied to date in that neither intra- or extra-pineal endogenous circadian clocks appear to be involved in the regulation of melatonin secretion in this species. However, the absence of endogenous circadian regulation of melatonin secretion in the rainbow trout does not preclude the hypothesis that the photoperiodic entrainment of diurnal and seasonal rhythms in this species is mediated by diel and seasonal changes in melatonin profiles. In the context of the present work the mechanism by which melatonin may mediate the effects of photoperiod on seasonal events such as reproduction is of interest. Two main hypotheses have been proposed to explain how animals interpret seasonal changes in the melatonin signal (Karsch, 1986; Reiter, 1987, 1988). The first contends that the duration of the nocturnal increase in melatonin secretion codes for daylength, the second that a photoperiodic response is dependent on the phase relationship between the melatonin rhythm and a circadian rhythm of sensitivity to melatonin which is entrained by the light-dark cycle (a form of internal coincidence; see section 3.4.12). Although these hypotheses are not mutually exclusive, experiments in which 'long' or 'short' day patterns

of melatonin have been administered at different times of the day do not support the phase hypothesis (see section 4.1) and the duration hypothesis currently appears to be the most generally applicable and most widely accepted of the two. Even in the Syrian hamster, in which evidence for the phase hypothesis was strongest (reviewed by Stetson and Watson-Whitmyre, 1986), convincing evidence against a role for phase has been provided by recent work which demonstrated that 10-hour infusions of melatonin, chosen to mimic a 'short-day' pattern of melatonin release, elicit a 'short-day' reproductive response (gonadal regression/atrophy) in pinealectomised animals when delivered both at non-24 hour intervals and alternately during the day and night, results consistent instead with the duration hypothesis (Maywood et al., 1990).

Should melatonin prove to be important in the timing of seasonal events in the rainbow trout, both hypotheses may be considered plausible. Although the melatonin rhythm itself is not generated endogenously in the rainbow trout the phase hypothesis remains feasible for this species since the exogenously generated seasonal change in patterns of melatonin secretion means that elevated melatonin would coincide with different circadian phases at different times of the year. If the circadian phase of the melatonin-sensitive period also varied seasonally such a mechanism would be even more flexible. However, the patterns of melatonin secretion described in the present study, with melatonin levels remaining elevated throughout the scotophase, are more clearly consistent with the duration hypothesis, although it should be remembered that the duration of melatonin release per se probably provides no information on direction of change of photoperiod. In this respect several mammalian studies have demonstrated that the duration of elevated melatonin secretion reflects the prevailing daylength irrespective of photoperiodic history (Hastings et al., 1986; Hoffmann et al., 1986; Robinson and Karsch, 1987). Information on direction of change of photoperiod must therefore be processed downstream of the pineal via, an as yet unknown, neural mechanism which operates as an 'interval timer' and is able to compare the current melatonin pattern with the preceding pattern of melatonin secretion held in memory. Recent evidence suggests that this mechanism may also require information on the duration of the melatonin-free interval (Maywood et al., 1990;

Hastings et al., 1991), and in poikilothermic animals such as the rainbow trout. It might be postulated that the neural machinery responsible for interpreting the melatonin signal is also capable of extracting information on seasonal changes in temperature which may be encoded in the melatonin rhythm, possibly by alterations in amplitude.

The apparent importance of the duration of melatonin secretion suggests that the physiological basis of the circadian rhythm in photosensitivity thought to underly photoperiodic time measurement (section 3.4.12) is inherent in the circadian rhythm of nocturnal melatonin production; the effects of skeleton photoperiods, resonance photoperiods and T-cycles (section 3.4.12) can be attributed to the truncation of the melatonin signal by light applied during the subjective night (Hastings et al., 1985, 1989). A similar mechanism may explain the effects of skeleton photoperiods in teleost fish (section 3.4.12), including the rainbow trout, in which melatonin production is strictly under exogenous control, as the direct suppressive effect of light would still serve to shorten the duration of uninterrupted melatonin secretion. The finding that melatonin production does not oscillate in DD in the rainbow trout may also provide an explanation for the equivocal results obtained with resonance photoperiods (section 3.4.12: Duston and Bromage, 1986a) as light pulses would simply provide occasional interruptions to a constantly elevated melatonin signal rather than falling in the subjective night of a circadian oscillation in melatonin secretion.

A pertinent question is 'Why should melatonin production be under endogenous circadian control in other vertebrates investigated, but not in the rainbow trout?'. Hastings et al. (1989) suggest there is adaptive value in using a self-sustaining oscillatory mechanism for the generation of melatonin rhythms; such a mechanism is able to interpolate between samples of the light-dark cycle to define subjective day and night and will be resistant to intermittent exposures to darkness. For example, the suppression of melatonin levels which occurs in nocturnal animals at dawn will not be reversed by subsequent exposure to darkness (such as on return to a burrow; Hastings et al., 1989), neither will melatonin levels increase in diurnal species should they seek shelter in darkness during the day. In contrast, exposure of rainbow trout to darkness during the day

would result in an immediate increase in melatonin production (as in experiment 3) and hence continuous sampling of the light-dark cycle is required if melatonin rhythms are to provide an accurate representation of daylength. Perhaps the absence of circadian control over melatonin secretion in the rainbow trout is compensated for by the comparatively long exposure to a change in daylength (compared to some other vertebrates) required to elicit a reproductive response (section 3.4.8). For an animal that requires a year or more to complete reproductive development this may not be an unfavourable mechanism. Aside from the possibility that the rainbow trout may represent the primitive state, the ecology of the species may also be significant. Rainbow trout generally inhabit shallow open water and hence are likely to be exposed to the complete light-dark cycle. Moreover, the rainbow trout pineal is able to detect light at low intensities (mean threshold of 7.8×10^{-3} lux; Morita, 1966) and hence may still function efficiently under conditions such as flooding or ice cover during which light transmission is inhibited. If melatonin rhythms were abolished for short periods under such conditions, however, this would be expected to have little effect on long-term rhythms such as the reproductive cycle since the circannual clock would simply free-run during this period. The absence of endogenous circadian control of melatonin secretion may not, therefore, be a disadvantage for the rainbow trout.

4.5.7 Non-reproductive functions of melatonin.

If melatonin does not mediate the effects of photoperiod on reproduction in the rainbow trout the question that arises is: what does it do?. Although large quantities of melatonin can cause melanosome-aggregation in rainbow trout (Hafeez, 1970) it is unlikely that melatonin is of major importance in colour change in this fish under natural conditions (section 4.1). In their theory of the evolution of melatonin's functions and effects Gern (1981), and Gern and Karn (1983), state that the original role of melatonin was probably to maximise photoreceptor cell function within the pineal and retinae themselves, and that the resulting rhythmic secretion of melatonin into the circulation for elimination by the liver facilitated the evolution of melatonin's role in the timing of certain daily and seasonal rhythms. Thus, melatonin has been implicated in several aspects

of rhythmic photoreceptor metabolism (Besharse and Dunis, 1982; Gern and Karn, 1983; Pang and Allen, 1986) such as renewal of the photoreceptor outer segments (disc shedding) and aggregation of melanosomes within the pigment epithelium (uncovering the outer segments and allowing increased surface area for photoreception at night). In this respect intraocular or systemic injection of melatonin during the photophase has been shown to cause melanosome aggregation within the retinal pigment epithelium of the rainbow trout (Cheze and Ali, 1976). Moreover, Meissl et al. (1990) recently reported an intra-pineal action of melatonin in the rainbow trout, whereby the hormone reversibly inhibited neural output from pineal ganglion cells, suggesting that melatonin may be involved in the regulation of neural output from the pineal, especially at night when melatonin concentrations are high. This evidence for intra-retinal and intra-pineal functions of melatonin in the rainbow trout, which is a relatively primitive teleost (Batten and Ingleton, 1987), is consistent with the hypothesis that the original role of melatonin was within the synthesising organs themselves.

It is possible that the rainbow trout represents the primitive state, in which the role of melatonin is limited to intra-pineal and intra-retinal functions such as those discussed above. However, the widespread distribution of melatonin receptors in the rainbow trout brain (Aggelopoulos and Demaine, 1990) argues against such a restricted role. Another possibility is that melatonin is involved in the synchronization of daily rhythms in the rainbow trout, as previously suggested for the tilapia. There is considerable evidence for the involvement of the pineal and melatonin in circadian organisation, especially in lower vertebrates (reviews by Underwood and Groos, 1982; Underwood, 1989). The importance of the pineal in the regulation of circadian rhythms was first demonstrated in the house sparrow in which it was discovered that the free-running rhythm of locomotor activity in DD was abolished by pinealectomy (Gaston and Menaker, 1968). Subsequent studies showed that the rhythm of locomotor activity could be restored by transplanting the pineal of another sparrow into the eye of the pinealectomised bird, and that the rhythm of the recipient assumes that of the donor (Zimmerman and Menaker, 1975, 1979). Moreover, constant-release Silastic implants can alter the free-running period of activity rhythms

or produce arrhythmicity in house sparrows (Turek et al., 1976), and daily melatonin injections can entrain activity rhythms in pinealectomised starlings (Gwinner and Benzinger, 1978). In combination these data suggest that, in certain birds, the pineal participates in the regulation of circadian activity rhythms via the rhythmic secretion of melatonin. The influence of the pineal and melatonin varies between species, however. Thus, the pineal appears to play a major role in the regulation of circadian rhythms in passerine birds, such as the house sparrow, but only a minor role in gallinaceous birds, such as the Japanese quail, in which pinealectomy and melatonin administration had little or no effect on locomotor activity (Simpson and Follett, 1981). Pinealectomy also has major effects on circadian activity rhythms in a number of lizard species, such as the green anole lizard (reviewed by Underwood, 1988). Moreover, constant-release Silastic implants can alter the free-running period of activity rhythms or produce arrhythmicity, and daily melatonin injections can entrain activity rhythms in at least one species (Underwood, 1988). As in some birds, these data are consistent with the hypothesis that the rhythmic secretion of melatonin from the pineal of certain lizards serves to entrain other components of the circadian system. It should be noted, however, that pinealectomy has no effect on circadian locomotor rhythms in the desert iguana (Janik and Menaker, 1990), a further demonstration that the influence of the pineal on circadian organisation in lower vertebrates is not universal. Nevertheless, even in mammals, in which pinealectomy has little or no effect on the circadian system, and the SCN are generally considered to be the major (if not the sole) generators of circadian rhythms (including the rhythm in pineal melatonin secretion; Underwood and Goldman, 1987), recent work in rodents has indicated that daily melatonin injections can entrain circadian activity rhythms, suggestive of an important role for melatonin in the synchronization of daily rhythms in some higher vertebrates (reviewed by Armstrong, 1989).

There is also evidence that the pineal and melatonin may be involved in circadian organisation in a number of fish, although it should be noted that rhythmicity in some species may be under strictly exogenous control (Rusak, 1981). Pinealectomy has been found to cause changes in the free-running periods of several fish maintained in DD

including the lake chub, Couesius plumbeus, the burbot, Lota lota, and the white sucker, Catostomus commersoni (Kavaliers, 1979a, 1980a, 1981a), and in the latter species also resulted in the splitting of circadian activity into a number of free-running components (Kavaliers, 1979b). Garg and Sundararaj (1986) reported that free-running rhythms in locomotor activity observed in Asian catfish in DD became arrhythmic after pinealectomy. Pinealectomy also abolished the free-running rhythm in locomotor activity observed in river lampreys, Lampetra japonica, maintained in DD, and the rhythm was restored when the pineal from another fish was transplanted into the pinealectomised fish (Samejima et al., 1987). This suggests that, in the lamprey at least, the pineal may participate in the regulation of circadian activity rhythms via the rhythmic secretion of melatonin, as appears to be the case in some birds and lizards. In this respect, a marked decrease in swimming activity and schooling behaviour (suspected to be under endogenous circadian control) has been reported in the damselfish, Chromis viridis, in the days following injection of melatonin (Sparwasser, 1987).

In salmonids, endogenous circadian rhythms of swimming activity have been claimed for the pink salmon, sockeye salmon, the brown trout and the brook trout (Godin, 1981, and references therein). In the sockeye salmon, pinealectomy caused an increase in swimming activity (Byrne, unpublished: cited in Hafeez, 1970), and administration of melatonin by injection inhibited activity, but only during the photophase of a 12L:12D photoperiod (Byrne, 1970). Administration of melatonin by injection has also been shown to inhibit swimming activity in the rainbow trout, but this may simply have been a reflection of the toxic effects of high doses of melatonin on locomotor ability (impairment of normal smooth body movements/lateral tilting) since the dose levels used were considered to be pharmacological in nature (Hafeez, 1970). This view is supported by the observation that pinealectomy had no effect on swimming activity in fish maintained under identical conditions in the same study (Hafeez, 1970). Moreover, in a study started since the completion of the work described in this thesis, rainbow trout administered constant-release melatonin implants which achieved circulating melatonin levels approximately double those detected in juvenile fish at night, exhibited no obvious inhibition of

locomotory activity or the feeding response during the photophase of a 16L:8D photoperiod (personal observation). In addition, recent attempts to correlate activity and feeding rhythms with pineal rhythms in the rainbow trout have been unsuccessful; both activity and feeding were found to be extremely labile with fish alternating between diurnal, crepuscular and nocturnal activity, with no evidence for a circadian component (E. Morgan, personal communication). This lability suggests rainbow trout may be opportunists able to take advantage of a large variety of food sources as and when they become available. In this respect Matty and Majid (1980) concluded that feeding in the rainbow trout is mainly governed by the degree of gut distension, rather than by a biological clock, with feeding activity during the photophase peaking every 8-10 hours if food is made constantly available. To date, therefore, there is little evidence to suggest that the pineal and/or melatonin are involved in the synchronization of rhythms in activity and feeding in the rainbow trout. As previously mentioned, the absence of an endogenous component coupled to the regulation of melatonin synthesis and secretion, and the lack of convincing evidence for the involvement of endogenous circadian mechanisms in the control of behavioural rhythms or in photoperiodic time measurement (see section 3.4.12), suggests that circadian organisation in the rainbow trout, and possibly other salmonids, may be less complex than in some other species of fish and other vertebrate groups.

4.5.8 Alternative mechanisms for the transmission of photoperiodic information to the reproductive axis.

It should be noted that the putative pineal-mediated effects on reproduction in some fish (section 4.1) could be attributed to neural signals or to pineal products other than melatonin. Electrophysiological studies have clearly demonstrated that neural pathways are transmitting photic information (section 4.1), but, unfortunately, the role of neural outputs from the pineal of lower vertebrates has not been studied (Underwood, 1989). McNulty (1984) believes that the wide distribution in the brain of the rainbow trout of nerve terminals emanating from the pineal tract (Hafeez and Zerihun, 1974) is an

indication of the relative importance of neural pathways in pineal-mediated photosensory responses. As previously mentioned, however, melatonin receptors are also widely distributed in the brain of the rainbow trout and other lower vertebrates (Aggelopoulos and Demaine, 1990; Martinoli et al., 1991), in contrast to the more localised distribution found in the mammalian brain (Morgan and Williams, 1989). This suggests that both neural and humoral pathways may be important in a wide range of pineal-mediated responses in the rainbow trout and other lower vertebrates, although their precise roles may be different. With regard to humoral signals, a large number of pineal substances other than melatonin have been identified in many vertebrates, although they may not all be synthesised in situ (Pevet, 1982a). These include other 5-methoxyindoles (Pevet, 1982b), proteins and peptides (Pevet, 1982a; Ebadi et al., 1989), all of which have been implicated in the control of reproduction.

The importance of extra-pineal photoreceptors in the photoperiodic control of reproduction in salmonids is also unknown. In the goldfish Delahunty et al. (1979) reported that optic tract section caused ovarian regression in spring and reduced serum oestradiol-17β levels in both spring and autumn. They therefore proposed that the stimulatory effects of increasing daylength on ovarian growth in spring are primarily mediated by retinal pathways, a conclusion in contrast to previous studies asserting the importance of the pineal in this process (de Vlaming and Vodicnik, 1978; Vodicnik et al., 1978). Blinding similarly prevented the photoperiodic stimulation of ovarian development in the catfish, Mystus tengara (Saxena, 1980). In contrast, Shiraishi (1965) reported no difference in the gonadal response of intact and blinded ayu-fish, Plecoglossus altivelis (sometimes classified as a salmonid species: Tamura and Hanyu, 1978) to 'long' and 'short' photoperiods, and bilateral enucleation of sticklebacks did not prevent the development of secondary sexual characteristics which occurred in response to a 'long', but not a 'short', photoperiod (Borg, 1982). The reproductive response of the Japanese killifish to photoperiodic change was also conserved after blinding, although to a lesser extent than in intact fish (Urasaki, 1973, 1976). Garg and Jain (1985) reported that the photoperiodic stimulation of gonadal development can occur in the absence of the

eyes in the Indian murrel, Channa punctatus, and blinding also had little effect on ovarian development in Asian catfish maintained under DD or LL (Garg, 1988b). Thus, the eyes appear to be involved in mediating the effects of photoperiod on reproduction in some fish but not others.

In addition to the eyes another class of extra-pineal photoreceptors may be involved in the transmission of photoperiodic information to the reproductive axis in fish. Investigations by von Frisch (1911), Scharrer (1928) and Hartwig (1975) (all cited in Oksche and Hartwig, 1979) have indicated that the brain of the European minnow, Phoxinus phoxinus, contains light sensitive regions in the diencephalon (in the vicinity of the third ventricle) and in the antero-dorsal hypothalamus. The existence of encephalic photoreceptors has also been suggested by studies in eels (van Veen et al., 1976) and lake chub (Kavaliers, 1980b,1981b), in which locomotory responses to light were conserved in fish subjected to both blinding and pinealectomy; in contrast, masking the brain area of blind eels from light disrupted locomotor activity patterns. A notable study in this context is that of Day and Taylor (1983) who found that mummichogs, Fundulus heteroclitus, which had been both bilaterally enucleated and pinealectomised, were still able to perceive daylength; as in intact fish ovarian development occurred in response to a 'long', but not a 'short', photoperiod. This indicates that neither the eyes or the pineal have an essential photoreceptive or endocrine role in reproduction in this species. Thus, as in some birds (section 4.1), the photoperiodic control of reproduction in some fish may be mediated by encephalic rather than retinal or pineal photoreceptors. The relative importance of pineal, retinal and encephalic photoreceptors in the mediation of the effects of light (and dark) on a particular physiological or behavioural function appears, therefore, to vary among species.

4.5.9 Summary

The present work demonstrates that the nocturnal increase in circulating melatonin accurately reflects the duration of the scotophase in both juvenile and adult rainbow trout maintained under either 'long' or 'short' artificial photoperiods. Melatonin production in

the rainbow trout is a direct response to darkness; there is no evidence for endogenous circadian regulation of melatonin secretion as in some other vertebrates. Patterns of circulating melatonin in the Atlantic salmon and Nile tilapia also reflect the prevailing photoperiod, but melatonin secretion may be under endogenous circadian control in these species. Although the seasonally-changing pattern of melatonin secretion clearly provides the rainbow trout with accurate information on both daily and calendar time the results of experiments designed to test the hypothesis that melatonin mediates the photoperiodic entrainment of seasonal reproduction were inconclusive.

CHAPTER FIVE

GENERAL CONCLUSIONS
AND
SUGGESTIONS FOR FUTURE WORK

The principal aim of the experiments described in this thesis was to clarify the mechanisms by which photoperiod entrains the endogenous circannual rhythm or clock which, it is proposed, controls maturation in the female rainbow trout. Chapter 3 examined the effects of a range of daylengths on reproductive timing in order to determine which features of the photoperiodic signal are important for the entrainment of the clock, and chapter 4 investigated the potential of changes in patterns of melatonin secretion to convey photoperiodic information to the reproductive axis. This chapter reviews the main conclusions and questions arising from the present study and makes some suggestions for future work.

Depending on the timing of exposure in relation to the phase of the annual reproductive cycle, abrupt changes in photoperiod can either advance or delay spawning and the changes in serum calcium, oestradiol-17ß and testosterone which accompany maturation in the female rainbow trout. These effects can be interpreted as corrective phase advances or phase delays of the endogenous circannual clock which controls reproduction. The results of the present study provide convincing evidence that the direction of change of daylength is the feature of the photoperiodic signal responsible for the entrainment of the circannual clock. Thus, the same photoperiod may be perceived by the fish as 'long' or 'short' providing it is longer or shorter than that to which they have been previously exposed. Moreover, maturation can be advanced even in fish which do not experience an increase in daylength in spring (i.e. remain on a winter photoperiod) provided they receive a decrease to an even shorter photoperiod prior to the summer solstice. Daylength per se (absolute daylength), and the magnitude of change in daylength, were shown to be of little importance in the entrainment process. Clearly, the rainbow trout reads daylengths comparatively, with reference to the preceding photoperiod, rather than absolutely. The traditional concept of a rigid 'critical' daylength for reproductive function is therefore not applicable to the rainbow trout. Consequently, extreme care should be taken in both the design and interpretation of future photoperiod experiments to account for the influence of photoperiodic history.

Further evidence for the preceding conclusions may be obtained by an experiment in

which the natural decrease in daylength is artificially accelerated during the last quarter of the year so that December spawning rainbow trout are exposed to, for example, a constant 2L:22D photoperiod from the time of the winter solstice. If the conclusions of the present work are correct, increasing the photoperiod to 8L:16D in January should cause a similar advance in spawning time to, for example, increasing the daylength from 8L:16D to 14L:10D at this time. Similarly, a reduction in daylength from 8L:16D to 2L:22D the following May should cause another advance in spawning time comparable to that which would be obtained following a reduction from 14L:10D to 8L:16D. In contrast, spawning would be expected to be delayed and desynchronized in constant daylength controls (2L:22D, 8L:16D), a comparison of which would also allow any effect of the initial photoperiod manipulation to be distinguished.

The present study provides strong supportive evidence for the proposition that maturation in the rainbow trout is ultimately under endogenous circannual control. Firstly, the desynchronization of spawning times observed in fish maintained under constant 'short' days indicates that the circannual rhythms of individual fish were free-running with variable periodicity, a characteristic feature of endogenous clocks under constant conditions. Secondly, the timing of each change in photoperiod, relative to the phase of the reproductive cycle, was shown to be an important determinant of spawning time. An essential property of endogenous clocks is that they possess a differential sensitivity to the phase-shifting effects of the zeitgeber such that a particular time cue causes phase-shifts of different magnitude and sign depending on the phase at which the rhythm is perturbed. This property was clearly demonstrated in the current work in which it was possible to construct a partial phase-response curve to describe the effects of 2 month periods of LL applied at different phases of the reproductive cycle. In this respect the entrainment behaviour of circannual clocks can be considered analogous to that of circadian oscillators. It should be noted that the experiments conducted concentrated on photoperiod regimes designed to advance spawning and hence the phase-response curve obtained is biased towards perturbations causing advance phase-shifts. It would therefore be desirable to perform additional experiments with LL periods applied at times likely

either to cause phase delays, or to have no effect (i.e. occur in a 'dead zone'), in order to obtain a more complete phase-response curve.

The proportion of fish responding to short periods of LL is dependent on both the duration of the light period, and, most importantly, its position in relation to the phase of the reproductive cycle. The minimum period of exposure to LL capable of advancing maturation in a majority of rainbow trout was 1 month. This suggests that the rainbow trout is unable to recognise a 'new' daylength as the current photoperiod immediately, and hence cannot perceive a change in daylength, by comparison with the preceding photoperiod(s) held in memory, until a minimum period of time has elapsed. Under natural conditions, where the photoperiod is changing only gradually, such a mechanism may prevent misinterpretation of the prevailing photoperiod due to the influence of other environmental factors such as moonlight, coloured water or extensive cloud cover. One month may, however, be an overestimate of the time required for an individual to register a new daylength since a minority of fish responded to only 2 weeks LL with an advance in spawning. The provision of less heterogenous populations by grading fish into, for example, early, mid- and late spawners, may enable the situation to be clarified in future studies.

Differences in the proportion of rainbow trout responding with an advance in maturation to 1 and 2 month periods of LL applied at different times close to the preceding ovulation may be explained by a 'gating' mechanism. However, this hypothesis remains unproven. It would be interesting to know if the circannual clock of the non-responding fish was initially advanced by exposure to LL, but, the fish being physiologically incompetent to mature at this stage, was subsequently re-entrained to ambient daylength, or whether the LL periods were completely ignored. Maintenance of fish under constant conditions after exposure to LL to ascertain whether the fish subsequently spawn close to the normal time or express an advance in spawning the following year should differentiate between these two possibilities. The possibility that the circannual clock re-sets at ovulation might also be considered, as this would explain why only a few fish, perhaps the earliest spawners, responded to LL periods applied prior to the natural winter spawning

period. The importance of elevated GTH levels to the recruitment of a fresh batch of oocytes and the possibility that ovulation was prevented by high temperatures, and/or atresia occurred in some groups, should also be examined. A detailed histological study of ovarian samples taken at frequent intervals from fish exposed to a variety of LL treatments would provide an efficient method of investigating most of these possibilities.

Exposure of rainbow trout to short periods of LL provides a simple, cheap and predictable method for the production of out-of-season eggs without the need for blackout facilities. In 3 consecutive experiments over 90% of females exposed to LL for 2 months from January to March spawned again in a 6-week period in July and August, approximately 5 months in advance of their natural spawning time. Similarly treated males produced milt throughout the spawning period of the females. Moreover, spawning was delayed by 2-3 months in a high proportion of fish subjected to LL from July to September. Short periods of LL have considerable potential for use not only on trout farms but also in salmon farming where the size and location of broodstock tanks often precludes the construction of blackout facilities, and where it is sometimes desirable to apply photoperiod treatments to fish maintained in sea cages. When applying the method on a farm for the first time it will be necessary to conduct a preliminary examination of the effects of LL applied at different times of the year since the optimum time for application of LL is likely to vary according to the natural spawning time and strain of the species under investigation. Although the technique is readily applicable on farms with seasonally-fluctuating water temperatures it may be necessary to check fish for maturity at more frequent intervals when spawning coincides with high summer temperatures in order to retain egg viability. Where a borehole water supply is available it may prove advantageous to transfer the fish to constant temperature water prior to spawning, preferably when the temperatures of the seasonally-changing and borehole supplies are about equal.

Patterns of melatonin secretion in the rainbow trout accurately reflect the prevailing photoperiod, with levels elevated for the duration of darkness. This pattern of secretion is similar to that arbitrarily classified as type C in higher vertebrates. In contrast to many

higher vertebrates, however, melatonin production in the rainbow trout is not under endogenous circadian control; all significant changes in melatonin levels coincide with the light to dark or dark to light transitions, and the melatonin rhythm does not persist in DD. Moreover, the melatonin rhythm immediately re-adjusts to the new photoperiod when rainbow trout are transferred from 'long' to 'short' days. Melatonin production in the rainbow trout appears, therefore, to be a direct response to darkness. Clearly, serial sampling of individual animals maintained in DD is required in order to exclude the possibility that endogenous rhythmicity was masked in the present study by pulsatile release of melatonin or rapid desynchronization of the melatonin rhythms of individual fish. This technique was not appropriate to the small fish used in the present work, but in future studies it may be possible to cannulate some of the larger broodstock for serial sampling in order to confirm the findings presented in this thesis.

There were no significant differences in the amplitude of melatonin rhythms in rainbow trout of comparable age maintained under either 'long' or 'short' daylengths (but otherwise identical conditions) suggesting that the amplitude of the melatonin rhythm does not provide the fish with information on daylength. However, there were considerable differences in amplitude between juvenile and adult fish which may be related to age, degree of sexual maturation, temperature or light intensity. The experiments described in this thesis were not designed to detect age-related differences in melatonin secretion and hence it was not possible to ascertain the cause(s) of the difference in amplitude of nocturnal melatonin secretion between juvenile and adult fish. Clearly, a longitudinal study of melatonin secretion under conditions of constant temperature and light intensity (during the photophase) is indicated. Comparison of sexually mature fish with sterile fish (for example, triploids) of the same age/size, or of precociously mature males with immature fish of the same age/size, may clarify the relationship between sexual maturation and circulating melatonin levels. Examination of the effects of temperature and light intensity on the amplitude of melatonin rhythms would be relatively simple to perform in groups of sibling fish maintained under otherwise constant conditions. It should, of course, be recognised that none of the possible causes of the observed variation

in amplitude of the melatonin rhythm are mutually exclusive.

The pattern of circulating melatonin in 4.5 month old rainbow trout fry was similar to that observed in juvenile and adult fish, indicating that the pineal is capable of transducing photic information into a humoral signal even in very young fish. In combination with observations that photoreceptor differentiation occurs earlier in the pineal than the eyes in the two teleosts so far studied (section 4.5) this result suggests that a functional pineal is important for the successful development of young fish. In view of this, and of the putative role of melatonin in the photoperiodic entrainment of diurnal and seasonal rhythms, it would be interesting to measure melatonin in eggs and alevins to ascertain at which stage of development rhythmic melatonin production commences.

The pattern of circulating melatonin in Atlantic salmon parr under a natural daylength was similar to that observed in rainbow trout under artificial photoperiods, with the exception that a small 'anticipatory' rise in melatonin secretion was detected prior to the onset of darkness, possibly suggesting the involvement of endogenous mechanisms in the generation of melatonin rhythms in salmon. In view of the difference in complexity between the life cycles of the Atlantic salmon and the non-anadromous strain of rainbow trout utilised in the present work, possible differences in the mechanisms regulating melatonin secretion in these species merit further attention. In this respect, examination of melatonin profiles in fish maintained under square-wave artificial photoperiods and in DD should enable the role of endogenous mechanisms in the generation of melatonin rhythms in Atlantic salmon to be elucidated.

A distinct diurnal rhythm in circulating melatonin was also detected in a sub-tropical strain of Nile tilapia. A role for photoperiod in the control of seasonal rhythms has not been demonstrated in this species and hence it would be of interest to investigate whether patterns of melatonin secretion reflect the seasonally-changing photoperiod, whether they can be influenced by other environmental variables such as temperature, and if melatonin can affect the timing of reproduction in tilapia. A comparison of the effects of photoperiod and melatonin on the timing of reproduction in tilapia indigenous to sub-tropical regions, which experience considerable seasonal variations in daylength and temperature, with

those native to equatorial regions, may provide useful information in this respect.

The pattern of circulating melatonin observed in the Nile tilapia differed from those seen in the rainbow trout and Atlantic salmon in that a decrease in melatonin levels occurred prior to lights-on. This suggests that, in contrast to the rainbow trout, endogenous circadian mechanisms are involved in the generation of melatonin rhythms in the Nile tilapia. This may be indicative of a more complex circadian organisation in the latter species, in which the possibility that melatonin mediates photoperiodic entrainment of circadian rhythms should be investigated. In this respect it would be beneficial to aquaculturists to alter the spawning behaviour of some tilapia species so that the spawning times of individuals were synchronized and spawning occurred at the most convenient time of the day for egg collection; if melatonin is able to entrain daily rhythms in spawning behaviour such alterations may be achieved by appropriately timed melatonin administration.

As with other vertebrate groups it is becoming apparent that patterns of melatonin secretion, and the importance of melatonin to circadian organisation and the timing of seasonal events such as reproduction, may show considerable variation in teleosts. There are over 20,000 teleost species occupying an immense variety of ecological niches, and it would be of interest to investigate whether differences in patterns of melatonin secretion, and the role of melatonin in daily and seasonal physiological and behavioural events, correlate with the individual life-history tactics and the environmental pressures to which a particular species is exposed. In this context future studies might consider the effects of light intensity and wavelength on melatonin production, factors which may be of greater importance to animals living in aquatic environments than to terrestrial animals. For example, fish could be exposed to equal photon densities of light of different wavelengths to ascertain their ability to suppress melatonin secretion. To discriminate the direct suppressive effects of light per se from those of a change in light intensity melatonin production could be studied in fish exposed to 24-hour cycles composed of light of different intensities (e.g. 'high':'medium', 'medium':'low', 'low':zero). Much of the preliminary work on melatonin rhythms in different species could be conveniently

conducted using pineal cultures.

Although the duration of the nocturnal increase in melatonin secretion clearly provides the rainbow trout with an accurate representation of daylength the results of the implant studies provide no evidence that melatonin is involved in the transmission of photoperiodic information to the reproductive axis. However, since neither experiment reached completion (spawning), and the implants did not increase plasma melatonin to physiological levels, these results cannot be regarded as conclusive. Given the highly photoperiodic nature of the reproductive response in salmonids, and the apparent similarities in the mechanisms underlying this response in salmonids and certain seasonally-breeding mammals in which melatonin has been shown to be important, the hypothesis that melatonin mediates the photoperiodic entrainment of seasonal reproduction remains extremely attractive. It is therefore suggested that, providing high-release rate melatonin implants are available, a priority of future work should be to repeat the experiments on reproduction described in this thesis. It would also be interesting to assess the ability of constant-release melatonin implants to influence the timing of smoltification, the other major developmental conversion under photoperiodic control in salmonids. Additionally, the possibility that timed infusions of melatonin could be used to mimic daylength in future studies should be investigated, although this technique may prove technically difficult in fish.

It should be recognised that there is no a priori reason why melatonin should mediate the effects of photoperiod on reproduction or other seasonal events in salmonids, and hence the possible contribution of other factors should not be ignored. In this context the role of extra-pineal photoreceptors in the transmission of photoperiodic information to the reproductive axis is unknown as is the function of neural outputs from the pineal and pineal products other than melatonin. Although pinealectomy experiments may help to distinguish the relative importance of these factors (for example, can pinealectomised animals respond to changes in daylength?) there have been no reports of the long-term effects of pinealectomy on reproduction in salmonid fish. Unfortunately, pinealectomy necessarily removes both the humoral and neural outputs of the pineal and hence one can

never be sure to which an effect should be attributed. The replacement of melatonin rhythms in pinealectomised fish by timed infusions or the development of specific melatonin receptor antagonists to nullify the effects of melatonin in intact fish may overcome these problems and allow the functions of neural pathways to be differentiated from those of melatonin and/or other pineal hormones. In view of the widespread distribution of melatonin receptors in the brain of the rainbow trout (section 1.5) the characterisation of receptors at different target sites, which may be associated with different physiological and behavioural functions, also deserves urgent attention.

In conclusion, the work presented in this thesis demonstrates that the direction of change of daylength is the feature of the photoperiodic signal responsible for the entrainment of the endogenous circannual rhythm which controls reproduction in the female rainbow trout. Seasonal changes in daylength are reflected in the seasonally-changing pattern of melatonin secretion which provides accurate information on both daily and calendar time. Information on the direction of change of daylength may therefore be conveyed to the reproductive axis via changes in patterns of melatonin secretion. Elucidation of the role of melatonin (if any) in the transmission of photoperiodic information to the reproductive axis should be the first priority of future work.

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