The Role Of Melatonin And The Pineal Gland In The Photoperiodic Control Of Reproduction And Smoltification In Salmonid Fish.

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

by

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August 1996

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:

Signature of Supervisor: _____

Date: 6th June 1997

ABSTRACT

The timing of seasonal events in salmonids is thought to be controlled by endogenous circannual rhythm(s) which are entrained by the seasonally-changing daylength. This thesis investigates the role of the pineal gland in the perception of the photoperiodic zeitgeber and the subsequent transmission of this information to the brain through neural or hormonal pathways.

Melatonin biosynthesis by isolated rainbow trout pineal glands was shown to exhibit a differential response to graded photic or thermal stimuli. *In vitro* experiments were carried out at 10±0.5°C as this provided optimum melatonin levels for radio-immuno assay analysis together with a pineal longevity of up to 14 days. By incorporating a variety of light intensities into the light/dark cycle, the salmonid pineal gland was shown to synthesise significantly different levels of melatonin even when light levels varied by only 0.5 lux. Early work on the salmonid pineal suggested it was unresponsive to red light, having a spectral sensitivity which peaks between 500 and 550 nm, this study has revealed that the pineal is also capable of responding to wavelengths between 660 to 800 nm, at which pineal reception was previously thought to be severely limited.

No endogenous rhythm of melatonin secretion was observed within the isolated rainbow trout pineal gland. Both Atlantic salmon and Atlantic halibut pineals exhibited elevated levels of melatonin in response to the dark phase, however, they also appeared capable of maintaining this rhythm in the absence of external stimuli. This provides the first evidence that the daily rhythm of melatonin production in these species is controlled by

an endogenous circadian oscillator located within the pineal gland.

The pinealectomy technique developed during the course of this thesis successfully abolished the diel rhythm of melatonin secretion and, together with an enucleation procedure, enabled the pineal to be identified as the predominant source of the dark phase melatonin in Atlantic salmon and rainbow trout. However, the lateral eyes did contribute significantly to plasma melatonin levels in both species.

Long term experiments, involving pinealectomy and/or implantation of melatonin, were used to investigate the role of the pineal gland in the timing of rainbow trout maturation and smoltification in Atlantic salmon. Pineal removal at the summer or winter solstices did not significantly alter the timing of smoltification. However, significantly higher blood serum osmolarities following seawater challenge tests were observed in smolts implanted with melatonin. This, together with a significant growth increase shown by salmon parr within 1 month of implantation, indicates that melatonin may directly affect the development of salmonids through either a physiological response or by influencing the entrainment of endogenous rhythms. The increased growth observed in the implanted parr is also thought to be responsible for the unimodal population distribution and high percentage of S1 smolts within this group.

Investigations into the role of the pineal gland in the timing of spawning in rainbow trout found that pineal removal at the summer solstice caused a 6 week delay in spawning time compared to intact fish. However, no clear effects on spawning time were observed when pineal removal, with or without

melatonin implantation, was performed to coincide with the change from long to short daylengths which is known to advance spawning times. Although no significant effect in spawning times was observed between groups, the 4 month spawning period of the pinealectomised group compared to 1 month in the sham-pinealectomised fish also suggested that pineal removal may have caused a desynchronisation in spawning time. Pinealectomy and/or implantation did not alter egg size or fecundity, but plasma calcium levels were shown to be significantly lower in the pinealectomised trout over the spawning period.

To summarise, the pineal gland and melatonin play a significant role in salmonid development. It is suggested that melatonin can influence biological systems through a direct physiological action while the pineal gland may synchronise circannual events through the photoneuroendocrine transduction of seasonal environmental information

ACKNOWLEDGEMENTS

I would firstly like to express my sincere thanks to my supervisors, Professor Niall Bromage and Dr Clive Randall who have provided advice and co-operation throughout this period of study. I would also like to thank all past and present staff and students at the Institute of Aquaculture for their advice and assistance, especially, Dr Mark Thrush, Dr Briony Campbell, David Nickell, Neil Duncan, Mike Bruce, Ann Gilmore, Kevin Coward and Dr Sirawut Klinbunga all of whom have endured my endless practical jokes over the last four years.

I would like to acknowledge that enucleations in sections 3.2.11 and 3.2.12 were carried out by Professor Niall Bromage.

The research for this thesis was conducted at the University of Stirling and Buckieburn Research Facility and I am grateful to Niall Auchinachie and Paul Finnigan for maintenance of experimental fish at Buckieburn.

Thanks to the Biotechnology and Biological Sciences Research Council for the provision of a research studentship.

And finally, thank you to Vikki for all you have done over the last 4 years.

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GLOSSARY OF COMMON AND SCIENTIFIC NAMES USED IN THIS THESIS

Atlantic salmon	*Salmo salar*
brown trout	*Samlo trutta*
rainbow trout	*Oncorhynchus mykiss*
humpback salmon	*Oncorhynchus gorbuscha*
chinook	*Oncorhynchus tshawytcha*
chum salmon	*Oncorhynchus keta*
coho	*Oncorhynchus kisutch*
amago salmon	*Oncorhynchus rhodurus*
sockeye salmon	*Oncorhynchus nerka*
steelhead trout	*Oncorhynchus mykiss*
masu salmon	*Oncorhynchus masu*
brook trout	*Salvelinus fontinalis*
Arctic charr	*Salvelinus alpinus*
goldfish	*Carassius auratus*
golden shiner	*Notemigonus crysoleucas*
American eel	*Anguilla rostrata*
European eel	*Anguilla anguilla*
channel catfish	*Ictalurus punctatus*
freshwater catfish	*Clarius batrachus*
catfish	*Mystus tengara*
Indian catfish	*Heteropneustes fossilis*
murrel	*Channa punctatus*
common carp	*Cyprinus carpio*
silver carp	*Hypophthalmichthys molitrix*
bighead carp	*Aristicthys nobilis*
flounder	*Platichthys flesus*
dab	*Limanda limanda*
turbot	*Scophthalmus maximus*
medaka	*Oryzias latipes*
Atlantic halibut	*Hippoglossus hippoglossus*
river lamprey	*Lampetra japonica*
lamprey	*Petromyzon marinus*
grey mullet	*Liza ramada*
white sucker	*Catostomus commersoni*

European Minnow	*Phoxinus phoxinus*
mosquito fish	*Gambuscia affinis*
lake chub	*Couesius plumbeus*
lamprey	*Petromyzon marinus*
hammerhead shark	*Sphyrna lewini*
guilthead sea bream	*Sparus aurata*
zebrafish	*Chichlasoma nigrofasciatum*
anolis Lizard	*Anolis carolinensis*
Richardson's ground squirrel	*Spermophilius rich ardsinii*
house sparrow	*passer domesticus*
domestic pig	*Siudae scofa*
red deer	*Cervus elaphus*
Djungarian hamster	*Phodopus sungorus*
Syrian hamster	*Mesocricetus auratus*
sheep	*Ovis aries*
White-footed mouse	*Peromyscus leucopus*

I would like to dedicate this thesis to my family who have offered support and encouragement throughout my academic career.

Chapter 1

General Introduction

1.1 Introduction

Salmonids (Order *Salmoniformes*, Class *Osteichthyes*), originally native to the northern temperate zone, are now found in almost all waters of the world's continents except for Antarctica. This study concentrates on the rainbow trout (*Oncorhynchus mykiss* Walabum, formerly *Salmo gairdneri* Richardson) which is now a recognised member of the Pacific *Salmonidae* (Kendall, 1988) and the Atlantic salmon. Rainbow trout are indigenous to the west coast of North America and were initially introduced to other parts of North America with the aim of enriching the fauna, but later with a view to its economic value. Its reputation as an excellent food and sport fish has since ensured its world-wide distribution and over the last 120 years large naturalised populations have emerged in South America, Europe, Scandinavia, temperate regions of Africa and Australasia (MacCrimmon, 1972).

A common feature of all salmonid species is their dependence on fresh water to reproduce. However, they do not always spend their entire life within the freshwater environment and many, such as the Atlantic salmon, may migrate between the sea and freshwater lochs and rivers several times during their lives. In contrast, several species, such as the rainbow trout, have strains that remain in fresh water throughout their life cycle and also have anadromous strains (steelheads).

Taylor and Taylor (1977) defined migration as "a fundamental biological response to adversity", and in the case of salmon the energetic cost of migration and osmotic adaptation (smoltification) is far outweighed by exploiting the richer food

resources available within the marine environment. As a consequence the reproductive strategies of anadromous and landlocked species differ significantly.

In the rainbow trout, females never mature in their first year of life. However some, depending on their size and growth rate, will mature in their second year, and the majority of the remaining females will have spawned by the end of their third year. In contrast, a minority of males (commonly known as precocious males) may mature as 1 year old fish but most will mature in their second or third years. When maintained under temperate conditions spawning occurs every year, usually between the months of October and February in the northern hemisphere, and may be repeated for up to 6 years (Laird and Needham, 1988). Within the confines of a culture system rainbow trout mature and ovulate but will not spawn and hence require the eggs to be artificially removed from the body cavity (stripped).

The majority of wild Atlantic salmon spawn between October and December, although this may extend to February in some strains. As with most salmonids the eggs are deposited in a 'redd' excavated by the female to a depth of approximately 40cm. On hatching the yolk-sack fry (alevins) remain within the spawning gravels until first feeding whereupon the positively phototactic fry 'swim-up' to the light and enter the water column. As the fry develop into parr their body colouration develops a distinctive vertical banding (parr marks); this stage of their life cycle may last from 1 to 8 years depending upon food availability and body size (see Thorpe, 1989, for a detailed review). During this time males may become sexually mature (precocious males) and attempt to fertilise the eggs of returning females. The

majority of parr will, however, undergo major physiological and behavioural changes at some point in their first 8 years in preparation for their downstream migration as smolts (Figure 1.1) during late spring/early summer. The length of time spent at sea again varies with the individual; some fish mature and return to fresh water after only one winter (grilse), while others may remain on the feeding grounds for up to 5 years before returning to spawn. Once spawning has occurred the majority of spent fish (kelts) die, but a small percentage (usually females) will return to sea and may return to spawn again in the future (for detailed life history patterns see Laird and Needham, 1988).

Both species are of significant economic value in Britain as the majority of the market is supplied by cultured fish. Improved husbandry techniques and greater efficiency within the industry have resulted in a steady increase in the annual production of both species over the last 20 years. In 1995 the Scottish salmon harvest increased by 9% to 70,060 tonnes compared to 64,060 tonnes the previous year, and the 1996 total is expected to increase by a further 19% to a projected 83,300 tonnes (Scottish office Agriculture and Fisheries Department annual production survey, 1995). At 15000 tonnes/annum the rainbow trout is now the second most important species of cultured fish in Britain after the Atlantic salmon. Of the total production 3000 tonnes goes for restocking fisheries and the remainder is sold directly for consumption (N.Bromage pers. comm.).

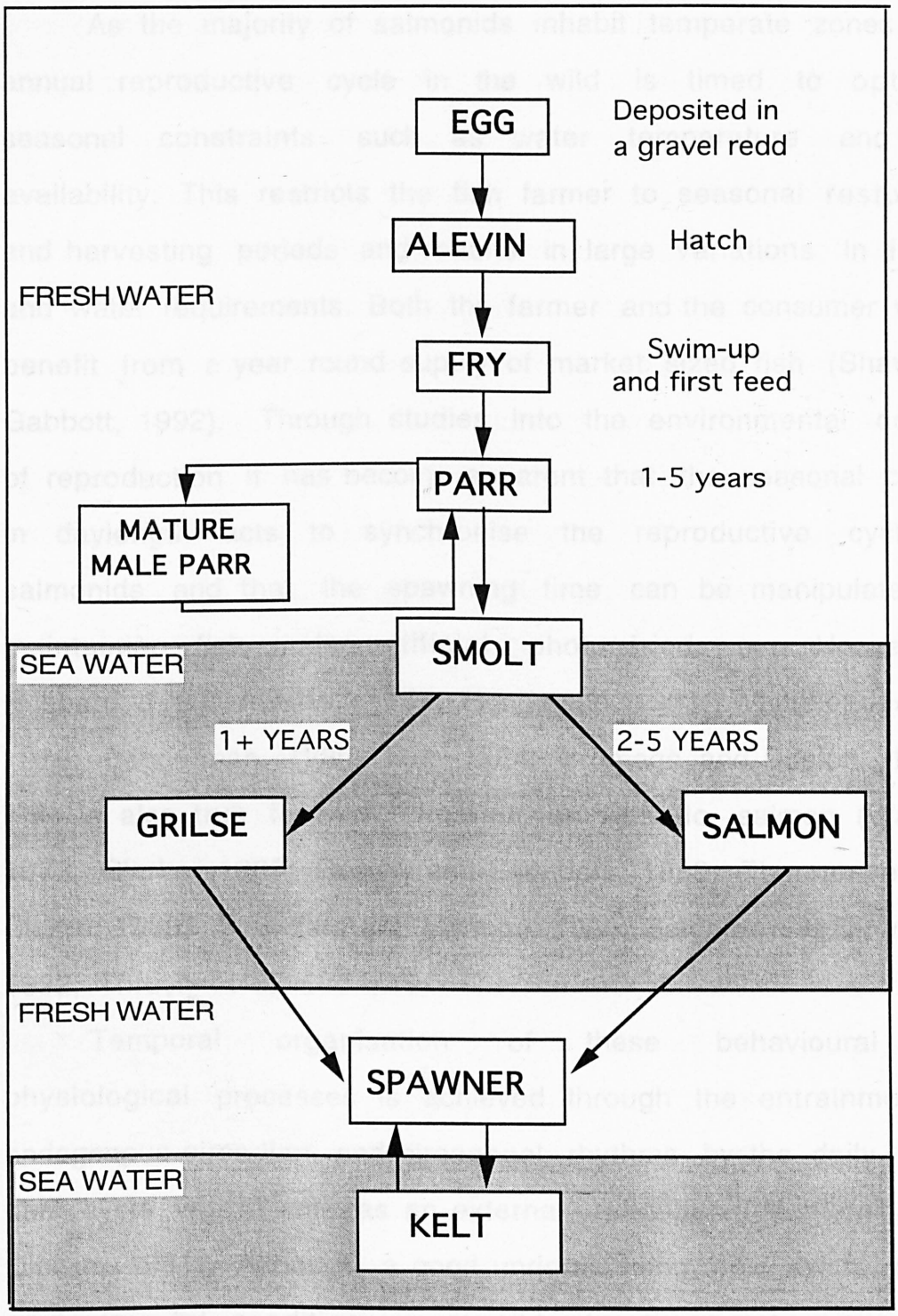


Figure 1.2

Life history of the Atlantic salmon (adapted from Laird and Needham, 1988).

As the majority of salmonids inhabit temperate zones their annual reproductive cycle in the wild is timed to optimise seasonal constraints such as water temperature and food availability. This restricts the fish farmer to seasonal restocking and harvesting periods and results in large variations in labour and water requirements. Both the farmer and the consumer would benefit from a year round supply of market sized fish (Shaw and Gabbott, 1992). Through studies into the environmental control of reproduction it has become apparent that the seasonal change in daylength acts to synchronise the reproductive cycle of salmonids and that the spawning time can be manipulated by maintaining fish under artificial photoperiods (e.g. Hoover and Hubbard, 1937; Allison, 1951, Henderson, 1963; Whitehead *et al.*, 1978; Baggerman, 1980; Bye, 1984; Bromage and Duston, 1986). This is also true for smoltification in Atlantic salmon (Wagner, 1973; Clarke, 1989; Duston and Saunders, 1989; Thorarensen and Clarke, 1989; Saunders and Harmon, 1990; Gaignon and Quemener, 1992; Thrush et al. 1994).

Temporal organisation of these behavioural and physiological processes is achieved through the entrainment of endogenous circadian and circannual rhythms by the daily light-dark cycle which acts as an external zeitgeber (de Vlaming and Olcese, 1981). Although a good understanding now exists of the environmental conditions responsible for the synchronisation of seasonal events (discussed in detail in chapters 4 and 5) little is known of the pathway(s) or mechanisms of this entrainment .

In 1911, Karl von Frisch produced a report in which he made a series of remarkable observations on the colour change mechanisms of the minnow. From his experiments von Frisch

concluded that the melanophore reaction to ambient illumination was determined by an area of the brain beneath the parietal spot. He suggested that the pineal gland may function as a light-sensitive receptor and by removing the pineal was able to abolish the melanophore response for a limited period. From this work von Frisch suggested the pineal to be the principal but not sole site of extraocular photoreception. This pioneering study concurs with more recent studies on the teleost pineal, a gland described by Osche and Hartwig (1975) as the second level of the photoneuroendocrine system in vertebrates. We now know that the pineal contains photoreceptor cells which have the ability to detect ambient light conditions and relay the photic information to various areas of the brain via neural or hormonal pathways (Falcon, 1979; Meissl and Ekstrom, 1988; Iigo, *et al.*, 1991; Kusmic *et al.*, 1992; Max and Menaker, 1992).

The pineal gland in teleosts, developed through an evagination of the dorsal thalamus, is positioned beneath the parietal bones (within the *fossa*) on the dorsal surface of the brain. This optimises protection of the gland while ensuring it receives maximum illumination (Dodt and Nauheim, 1973). The saccular, well vascularised, end vesicle is connected to the posterio-dorsal diencephalon via the epiphyseal stalk. The pineal gland is composed of 3 main types of cell: photosensory cells, interstitial glial cells and sensory ganglia. The outer segments of the sensory cells protrude into the pineal lumen while their inner basal processes synapse with sensory neurones. As well as this obvious neural link the photoreceptors have also been shown to be capable of synthesising a number of indolic compounds (reviewed by Zachmann *et al.*, 1992). Of these, melatonin (5-methoxy-N-

acetyltryptamine) displays marked daily fluctuations in relation to the light/dark cycle, with increased production at night relative to levels during the day (reviewed by Gern et al., 1992). This nycthemeral rhythm in melatonin production has led several authors to suggest that it has a role as an internal zeitgeber in vertebrates (Armstrong, 1989; Falcon and Collin, 1989; Underwood, 1989).

Strong evidence indicates that melatonin biosynthesis is followed by immediate release from the pineal (Falcon *et al.*, 1992; Zachmann *et al.*, 1992) and that in many of the teleost species so far investigated (pike, goldfish, whitesucker) the rhythmic secretion of melatonin is governed by intrapineal circadian oscillators (Falcon *et al.*, 1989, 1994; Kezuka *et al.*, 1989; Iigo *et al.*, 1991; Zachmann *et al.*, 1992). In contrast, in the rainbow trout, hammerhead shark and possibly the lamprey the melatonin rhythm appears to be directly controlled by ambient illumination (Gern and Greenhouse, 1988; Randall *et al.*, 1991, 1995; Boilliet *et al.*, 1993; Okimoto and Stetson, 1995).

Unfortunately, study of the pineal gland *in vivo* is more problematic, as no neural or endocrine blockers have yet been developed, the abolition of pineal innervation or the melatonin signal requires surgical removal of the whole gland. Techniques have been developed to allow this to be conducted on a number of species: medaka, Urasaki (1973); goldfish, Delahunty *et al.* (1978), Vodicnik *et al.* (1978); catfish, Davis *et al.* (1982), Nayak and Singh (1987), Khan and Joy (1990); carp, Popek *et al.* (1994); rainbow trout, Gern *et al.* (1978), Popek *et al.* (1992).

When the importance of photoperiod to the synchronisation of spawning times became apparent many authors used pineal

removal experiments to assess the possible role of the pineal in the transduction of the photoperiodic signal. The results obtained from these studies (discussed in detail in section 5.5) varied enormously depending upon species, stage of gonadal development, and the ambient photoperiod at the time of pineal removal (de Vlaming, 1975; de Vlaming and Vodicnik, 1978; Vodicnik *et al.*, 1978; Abraham and Sagi, 1984; Garg, 1988). Only Popek *et al.* (1992) have used this technique to study the involvement of the pineal in salmonid maturation. This study, in conjunction with their work on carp (Popek *et al.*, 1991), led the authors to suggest that the pineal acts in a stimulatory capacity during vitellogenesis but has no influence over ovarian maturation immediately preceding ovulation. Similarly, changes in photoperiod able to influence spawning time if applied during vitellogenesis have no effect if applied less than 1 month before spawning takes place (Randall, 1992). As the trout pineal continues to receive photoperiodic information, and produces a corresponding melatonin profile, regardless of the stage of gonadal development, this suggests that the endogenous rhythm of reproduction enters a period of unresponsivness to pineal entrainment in the month preceding ovulation (Randall, 1992). Consequently, despite the pineal acting as a neuroendocrine photometer, its signal appears to be selectively acted upon by the areas of the brain which are responsive to entrainment.

Although the effects of photoperiod on salmonid reproduction and smoltification have been extensively studied very little is known about the reception and transmission of photoperiodic information or whether the pineal and melatonin are responsible for the entrainment of the endogenous rhythms

associated with seasonal events in the salmonid life cycle. The work described in this thesis was designed to elucidate the function of the pineal in reproduction and smoltification in salmonids using both *in vivo* and *in vitro* tecniques.

1.2 Aims of thesis

The major aims of the thesis are three fold. Each aim is discussed in detail below.

1. To investigate the environmental control of melatonin biosynthesis by the salmonid pineal gland and its production of circulating plasma melatonin levels.

The rainbow trout pineal gland synthesises melatonin in direct response to the ambient illumination. This makes it an ideal subject for illumination and temperature studies as there is a complete absence of an endogenous melatonin rhythm and therefore any response observed must be a direct result of the stimulus. Initial work in this chapter concentrated on developing optimum conditions for pineal survival within the culture system. This entailed experiments with various media and culture temperatures in order to obtain the optimum amplitude of melatonin, while retaining the maximum longevity.

The photic sensitivity of the rainbow trout pineal was also studied under a range of intensities and wavelengths with the aim of discovering the minimum variation between the light and dark phase light levels required to produce a significant variation in melatonin levels. As mentioned, the rainbow trout pineal synthesises melatonin in direct response to ambient illumination, but it is still not known whether the Atlantic salmon shares this

characteristic or whether it exhibits an endogenous rhythm of melatonin secretion in common with most other species so far studied. To address this question Atlantic salmon pineals were maintained under constant conditions over several light/dark cycles to obtain melatonin profiles in the absence of an external stimulus. A similar but more preliminary study was also conducted on Atlantic halibut pineal glands.

Sections 3.2.10 onwards focused on the source of circulating plasma melatonin in rainbow trout and Atlantic salmon. Initial work used *in vitro* techniques to establish whether the pineal tract was capable of secreting melatonin in response to the light/dark cycle, primarily as an aid to developing techniques to allow the pineal to be removed from a range of salmonid age groups. Together with enucleation experiments these studies enabled the identification of the contribution of pineal melatonin to the circulating plasma melatonin levels in juvenile Atlantic salmon and rainbow trout. The pinealectomy technique developed during the course of this chapter was subsequently used in chapters 4 and 5.

2. To investigate the role of melatonin and the pineal gland on the development of *S.salar* parr and the parr-smolt transformation. In addition to determining the sensitivity of the pineal to environmental influences this thesis also investigates the role of the pineal in the entrainment of seasonal events. Chapter 4 was designed to investigated the role of the pineal and melatonin in the growth and development of Atlantic salmon parr and their transformation into seawater-tolerant smolts.

Photoperiod would appear to be the primary exogenous cue for the entrainment of smoltification in Atlantic salmon (Duncan *et al.*, 1994; Stefansson *et al.*, 1994; Thrush *et al.*, 1994). However, the mechanisms underlying the transmission of photic information to the target sites involved in the behavioural and physiological changes associated with smoltification remain unclear despite studies by Grau *et al.*, 1982 and Rouke, 1994 on *Oncorhynchus* spp. The experiment in section 4.2 attempted to address this question through the use of pinealectomy performed alone or in conjunction with the administration of constant-release melatonin implants. This experiment began at the winter solstice to assess whether pinealectomised parr would still be able to perceive the increasing daylength. In a similar experiment, initiated at the summer solstice (section 4.3), salmon parr were either pinealectomised, sham-pinealectomised and melatonin implanted, or left intact. Melatonin implants were used to establish the importance of the natural daily rhythm of melatonin and to determine whether a constantly elevated melatonin signal could mimic the effects of a short day signal, as reported in sheep (Lincoln and Almeida, 1981), or whether it would act as a 'blindfold' (masking the natural rhythm in melatonin release) and hence cause the endogenous clock to free-run.

Following preliminary observations made in sections 5.2 and 5.3, the work carried out in section 5.4 investigated the possible effects of melatonin implants on the growth rate of Atlantic salmon parr and in particular the bimodal distribution of the juvenile population. This involved the melatonin implantation of parr ranging from 3-11g in June (5 months post hatch) which

were then weighed at 2 week intervals up until the time of smoltification the following June when the ratio of S1 to S2 fish was assessed.

3. In addition to the pineal's influence on smoltification this thesis also explored its role in the transduction of photic information with regards to the entrainment of the endogenous circannual rhythm of reproduction in the rainbow trout.

As artificial changes from long to short day photoperiod are known to produce advances in the spawning times of several months in salmonids (Bromage *et al.*, 1984, 1992; Takashima and Yamada, 1984) the point of change from long to short daylengths was deemed the most appropriate time to remove the pineal gland in order to assess its importance in the perception of changes in daylength. Sections 5.2 and 5.3 used compressed square wave photoperiod regimes designed to advance maturation by up to 6 months. During these experiments pinealectomy and/or administration of constant-release melatonin implants were performed parallel to the change from long to short day photoperiods.

In section 5.4, pinealectomy at the summer solstice, ensured the pineal was absent over both the decreasing daylengths of autumn and the increasing daylengths of spring during gonadal development.

To summarise, the experiments in this thesis were designed to assess the importance of the pineal gland and its main secretory product melatonin on the perception and transduction of photoperiodic information. This is of particular interest in

salmonids which rely on the seasonally-changing photoperiod to synchronise annual rhythms in growth, reproduction and smoltification.

Chapter 2

General Materials and Methods

2.1 Experimental animals

Two salmonid species were used for the majority of the experiments presented here: rainbow trout and Atlantic salmon. All salmon used were of an Otterferry/Mowi cross supplied by Howietoun Fisheries (Sauchieburn, Stirling, UK.). The trout were of South African origin unless otherwise stated.

2.1.1 Maintenance

Fish were maintained within a range of freshwater systems designed to meet the specific requirements of each experiment. The salmon parr in experiments 1-3 in chapter 5 were maintained in circular 400 L fibreglass tanks (diameter 0.8m, depth 0.6m) at the University of Stirling's freshwater research facility at Buckieburn. The tanks were in a flow-through system supplied from a reservoir 1km from the site at a rate of approximately 5 L per minute and at ambient seasonal temperatures (1-17^{o}C, Figure 2.1). Tanks were made light-proof with industrial grade black plastic surrounding a plastic pressure-pipe frame secured to the tank with access provided by an overlap in the cover which could be sealed using a velcro strip. Light was supplied by 60 watt pearl tungsten filament light bulbs housed within waterproof bulkhead lamps (model EB.10; J. and G. Coughtrie Ltd., Glasgow, UK.) positioned 1.5m above the water surface (Figure 2.2) providing an intensity of 20-30 lux at the water surface (lightmaster photometer; Evans Electroselenium Ltd., Halstead, UK.) with 95% of the wavelengths between 560-800 nm (Macam SR 3010 Spectroradiometer). Simulated natural photoperiods were controlled by a light sensor (designed in house by D.Milroy using a D-Eem B92

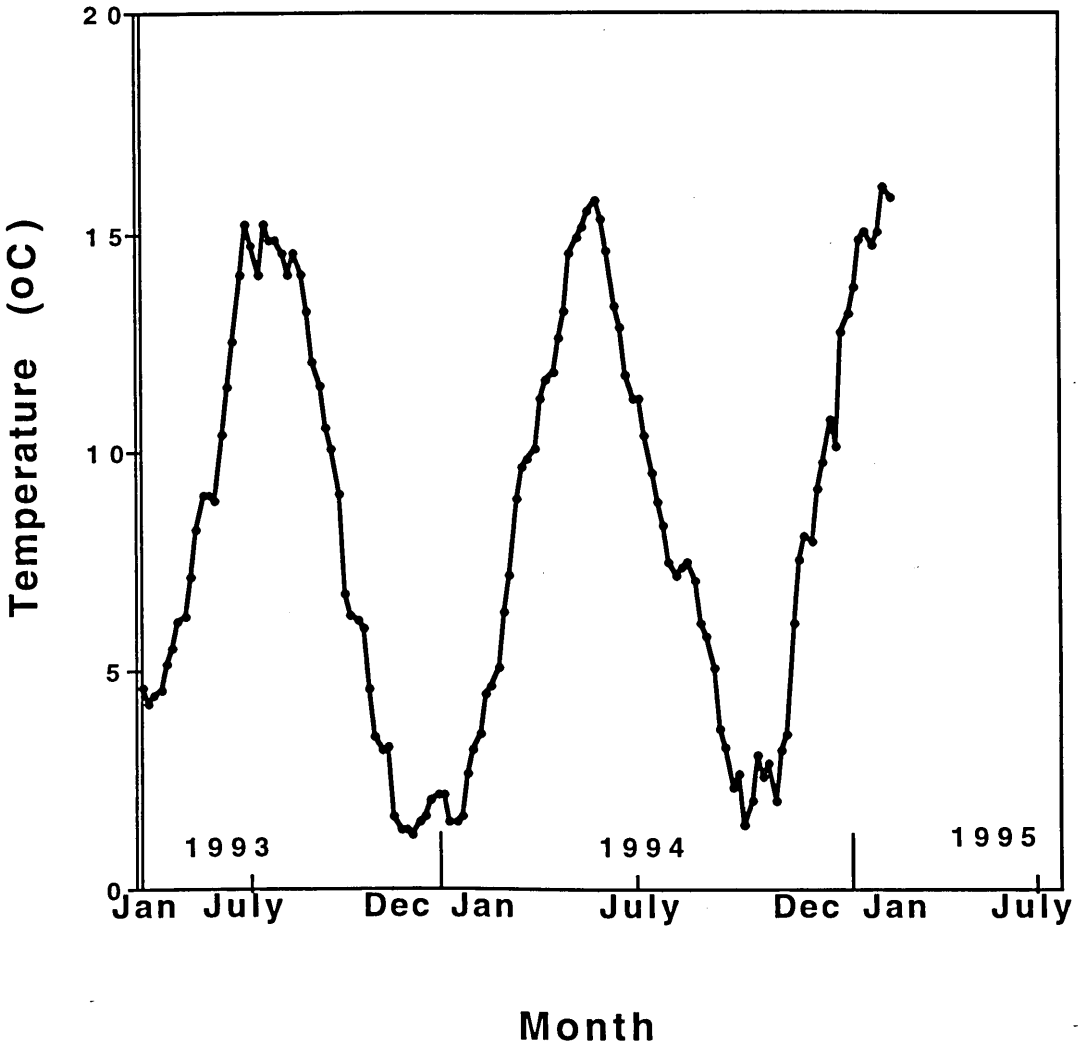


Figure 2.1

Seasonal temperature fluctuations recorded at Buckieburn Research facility from January 1993 to July 1995.

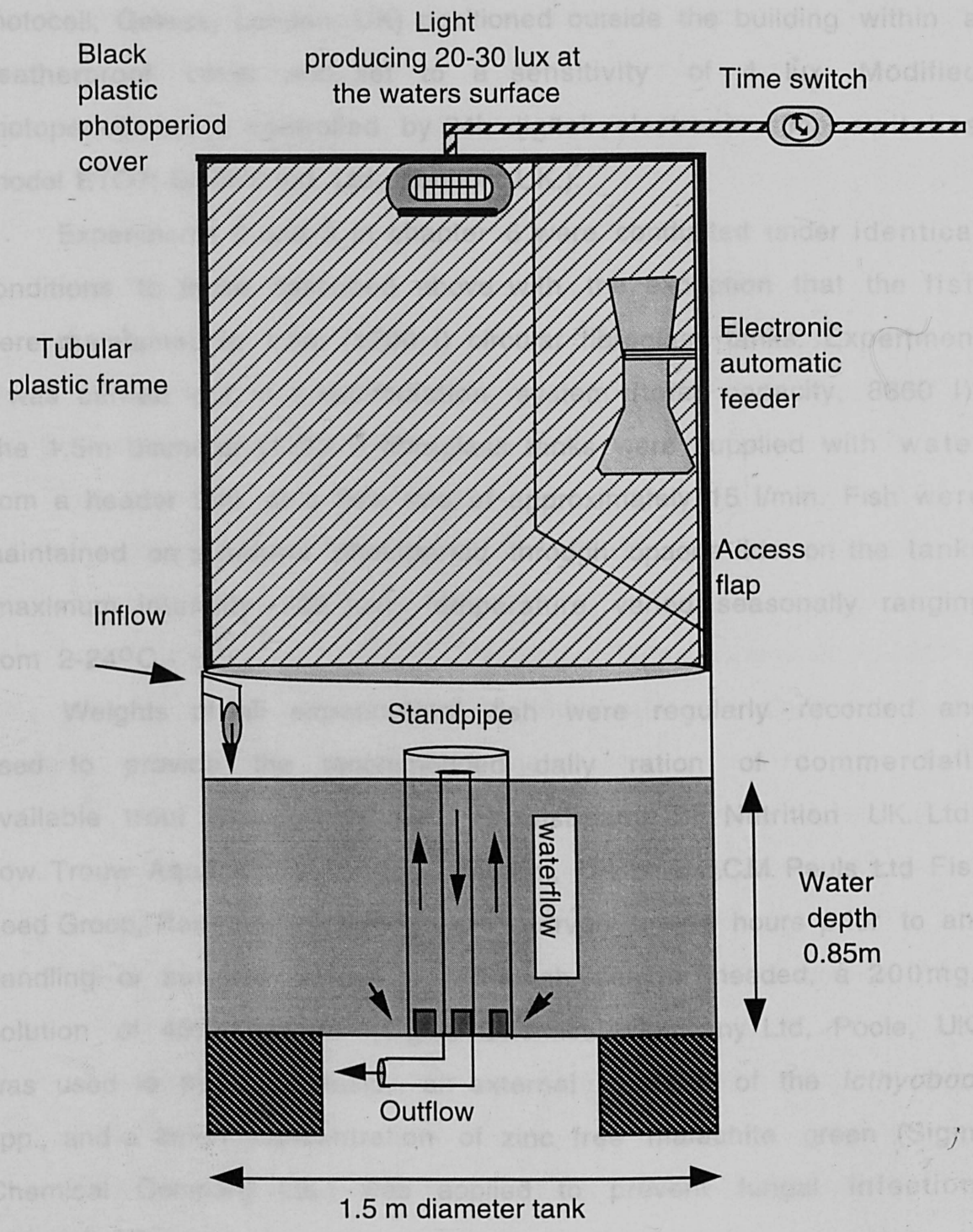


Figure 2.2

Diagram of a typical tank and photoperiod cover (not to scale).

photocell; Gewiss, London, UK) positioned outside the building within a weatherproof cover and set to a sensitivity of 4 lux. Modified photoperiods were controlled by 24h digital electronic time switches (model ETO7; Smith's Ind. Ltd., London, UK.).

Experiments 2 and 3 in chapter 6 were conducted under identical conditions to those described above with the exception that the fish were maintained in 1.5m (1200 l) circular fibreglass tanks. Experiment 1 was carried out in a recirculation system (total capacity, 8360 l). The 1.5m diameter (1200 l) fibreglass tanks were supplied with water from a header tank at a flow rate of approximately 15 l/min. Fish were maintained on a natural photoperiod through opaque lids on the tanks (maximum intensity 700 lux); temperature varied seasonally ranging from 2-24°C.

Weights of all experimental fish were regularly recorded and used to provide the recommended daily ration of commercially available trout and salmon diets (Mainstream; BP Nutrition UK. Ltd., now Trouw Aquaculture UK. Ltd., Witham, UK. or B.O.C.M. Pauls Ltd Fish Feed Group, Renfrew, UK.). Fish were starved for 24 hours prior to any handling or surgical procedure. Although seldom needed, a 200mg/l solution of 40% formalin (Sigma Chemical Company Ltd, Poole, UK.) was used to treat costiasis, an external parasite of the *Icthyobodo* spp., and a 2mg/l concentration of zinc free malachite green (Sigma Chemical Company Ltd.) was applied to prevent fungal infections (*Saprolgenia* spp.).

2.1.2. Anaesthesia.

Anaesthesia was used whenever fish were handled to reduce stress and damage to the fish. A 1:20,000 concentration of 2-phenoxyethanol (Sigma Chemical Company Ltd.) was used which

allowed 4-5 minute operations to be carried out without gill perfusion. Fish were placed in aerated fresh water to recover and full recovery was usually seen within 5 minutes; no mortalities were recorded using this procedure.

2.1.3. Blood Sampling.

Blood samples were always taken via the caudal dorsal aorta of anaesthetised fish. Depending on the amount of blood required 1, 2 or 5ml syringes were used (Terumo Europe N.V., Leuven, Belgium). For fish over 250g a 21G sterile hypodermic needle was used (Gillette UK. Ltd., Middlesex, UK.), below this weight 23 or 25G needles were used as appropriate (Gillette UK. Ltd.). For collection of plasma, syringes were rinsed with ammonium heparin (4mg/ml, Sigma Chemical Company Ltd.), whereas serum was obtained by withdrawing the blood into clean syringes and allowing it to clot. The plasma or serum from both methods was then transferred to new polystyrene tubes (LP3; Luckhams Ltd., Burgess Hill, Sussex, UK.) on ice prior to centrifugation (CT422; Jouan Ltd. Herts, UK.) at 2500 rpm for 15 minutes at 4°C. The supernatant was transferred to polystyrene tubes (LP3; Luckhams Ltd.) if over 1 ml in volume and polypropylene microcentrifuge tubes (Life Sciences International UK. Ltd.) if 1 ml or less, and stored at -70°C until analysis.

Many of the blood samples were taken during the dark period. This was achieved by removing the fish from the tank in total darkness, placing it in an anaesthetic bath and securing the bath lid. After 1 minute the fish was removed from the anaesthetic, a damp cloth placed over the head region, and the blood sample taken under a dim red light (λ=670-800 nm; 0.2 lux at 0.5m).

2.2 Surgical procedures 2.2

2.2.1 Pinealectomy

The following method provided a quick and effective technique for pineal removal (pinealectomy; pinx) without the need for gill perfusion. The fish were anaesthetised and a 0.5cm horizontal incision was made posterior to the pineal window, which is situated within the cranial bone and is clearly visible in young salmonids through the skin. A flap of tissue was then lifted anterior to reveal the pineal. In the pinx group, the pineal stalk was cut at the point of attachment to the diencephalon; the pineal removed with forceps; and the area cleaned by suction using a pipette. A similar procedure was used for sham-pinealectomised (sham-pinx) fish except that the pineal was left intact. The overlying tissue was then replaced and a 3:1 mixture of orahesive powder (Squibb and Sons Ltd., Hounslow, Middlesex) and cicatrin antibiotic (The Wellcome Foundation Ltd., London) applied over the incision. On larger fish sutures were used to hold the flap of skin in place before application of the orahesive powder.

A 7% mortality rate was experienced with salmon parr and juvenile trout and a 14% mortality rate for trout over 250g. After an interval of three months external differentiation of pinealectomised and control fish was impossible. Twelve weeks after the operation, a 0.5ml blood sample was taken from each fish 3 hours after lights out. Two weeks later this procedure was repeated 3 hours into the light phase. Samples were stored at -70°C prior to assay for melatonin (section 2.4). The absence of the pineal was confirmed by dissection at autopsy 15 months after the operation.

2.2.2 Melatonin Implantation.

Melatonin implants ('Regulin'; Schering Agrochemicals, Alexandria, Australia) were used in a number of the experiments to permanently elevate plasma melatonin in excess of night-time physiological levels with the aim of masking the natural rhythm in circulating melatonin levels. The implants contained 18mg of melatonin and utilised a polymer coating to allow a slow and constant release. An implanter (Schering) was used to administer the implants intramuscularaly 1cm below the dorsal fin. The degree of increased plasma melatonin was found to be dependant on the body weight of the fish. Although day and night-time melatonin levels in implanted fish were slightly different this was not significant (Kruskal-Wallis test, $P>0.05$). Plasma levels of melatonin in the implanted fish did not decrease significantly over a 12 month period (Dunn's Test $P>0.05$) in any of the experiments in which implants were used.

2.2.3 Identification

To distinguish individual fish (section 5.3), intra-muscular electronic tags (Avid Tags ; Norco, Ca., U.S.A.) were inserted 10 mm below the dorsal fin. This was achieved by making a 5mm incision in the skin 1 cm below the dorsal fin and injecting the tag anteriorly, positioning it approximately 35mm in front of the initial incision and 5mm below the skin. The tag reader was then used to scan the tag to ensure it was functioning before a 3:1 mixture of orahesive powder (Squibb and Sons Ltd.) and cicatrin antibiotic (The Wellcome Foundation Ltd.) was applied to the area of incision. No mortalities were experienced as a result of this method and the failure rate of the tags was less than 1%.

When quick accurate identification of post-spawned fish was required each fish was marked on its ventral surface between the pelvic fins with alcian blue dye (1% w/v in water; Sigma Chemical Company Ltd.) immediately after the eggs were stripped. The dye was applied using a Panjet (F.H. Wright, Dental MFG Company Ltd., West Dundee, UK.) which fires the dye under high pressure into the dermal layer. This method has the advantages of: being performed in seconds; leaves little possibility of infection as no incision is required; and lasted for up-to six months before the mark had to be renewed.

2.3 Spawning Assessment.

Maturation experiments in chapter 6 required accurate assessment of the spawning times of mature rainbow trout. Fish were examined every month and blood samples taken to evaluate blood calcium as an index of vitellogenin or yolk protein (section 2.6) and 17β-oestradiol levels (section 2.5). From these parameters the time of ovulation could be estimated and the frequency of examinations shortened to two week intervals accordingly. The maturation of the females was characterised by: a darkening of the ventral surface; a distention of the abdomen as the eggs swell inside the body cavity; and, just prior to ovulation, the swollen papilla extended from the vent. As rainbow trout do not release their eggs in captivity they must be manually 'stripped'. This is achieved by holding the fish at approximately 45 degrees with the head upwards and applying gentle pressure to the abdomen starting at the pectoral fins and moving towards the vent. Spawning time was defined as the point at which eggs could be stripped from the body cavity.

2.3.1 Fecundity.

To allow accurate measurements of the stripped eggs they first had to be water-hardened. The eggs were stripped into a clean dry beaker then rinsed three times in fresh water and allowed to stand for 45 minutes while the eggs absorbed water. During this time the eggs expand, become hard to the touch and are then safe to be measured.

Once water-hardening was complete the eggs were sieved to remove the water, poured into a measuring cylinder and the volume recorded to the nearest 10 ml. Using this volume the fecundity of the donor female was calculated from the following equations based on those from von Bayer (1950):

Total Fecundity = (antilog Y)(Z/1000)

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

$Y = -0.283X + 5.41$

X = egg diameter (mm)

Z = egg volume (ml)

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

Springate (1985) used actual egg counts to validate these equations. He found a significant correlation ($P < 0.001$) between his own measurements and those made using von Bayer's methods.

2.3.2 Egg Diameter.

To calculate the mean egg diameter for each individual spawning the number of water hardened eggs which could be positioned along a 120mm V-shaped groove was recorded (to the nearest half egg). The mean egg diameter could then be calculated using :

Mean egg diameter (mm) = 120/Number of eggs within 120mm

Springate (1985) validated this equation using caliper measurements.

2.3.3 Gonadosomatic Index.

Gonadal development of fish prior to ovulation was assessed by sacrificing the fish and recording the gonad weight which was then expressed as a percentage of body weight, the gonadosomatic index (GSI) :

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

2.4 Melatonin Radioimmunoassay.

Melatonin present in culture media and blood plasma was measured using a direct radioimmunoassay adapted from Randall, *et.al.* (1995) as follows:

2.4.1 Assay Buffer.

Tricine buffer was freshly prepared the day before each assay and stored at 4°C overnight. All chemicals used were of Analar grade as supplied by BDH Chemicals Ltd.. The following buffer chemicals

were dissolved in 150ml of nanopure water in a polystyrene specimen container (Sterlin Ltd., Hounslow, UK.) at approximately 50°C for 30 minutes.

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

2.4.2 Radiolabel.

Tritiated melatonin ([O-methyl-^{3}H]melatonin) was supplied by Amersham International Ltd. in 250μCi quantities with a specific activity of 70-85 Ci/mmol. This stock label was used to prepare an intermediate solution by diluting 20μl in 2ml of absolute ethanol Fisons Ltd.). The intermediate solution was stored in 20ml glass vials (Canberra Packard, Berks., UK.) at -20°C. The working solution was freshly prepared for each assay by diluting the intermediate solution with assay buffer to give an activity of approximately 4000 dpm/100μl (~ 20μl of the intermediate solution in 10ml of buffer).

2.4.3 Antibody.

Freeze-dried sheep anti-melatonin antiserum (Stockgrand Ltd., Surrey, UK.) was reconstituted with 2ml of nanopure water to provide an intermediate solution. This was divided into 100μl aliquots and stored at -20°C in polystyrene tubes (LP3; Luckhams Ltd.). The working solution was prepared by diluting one 100μl aliquot to 20ml with assay buffer.

2.4.4 Melatonin-free Plasma.

Melatonin-free plasma was used to allow a direct comparison to be drawn between the standard curve and the plasma samples.

Melatonin was removed from the plasma (collected during the photophase) by charcoal stripping according to the following protocol:

1. Prepare a 10%w/v suspension of charcoal (activated, untreated; Sigma Chemical Company Ltd.) in plasma in 20ml polystyrene 'universal' containers (Sterlin Ltd., Hounslow, Middx., UK.).
2. Shake suspension for 60 minutes on ice in a shaking water bath.
3. Centrifuge at 1500 rpm (4°C) for 30 minutes.
4. Decant supernatant and re-suspend 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 (4°C) for 30 minutes (L8-55M ultracentrifuge; Beckman Instruments Inc., High Wycombe, Bucks, UK.).
8. Filter supernatant through Millex-GV 0.22µm filters (Millipore S.A., Molsheim, France).
9. Divide the resulting pooled plasma into 6ml aliquots and store in polystyrene 'Bijou' bottles (Sterilin Ltd.) at -20°C.

Each new pool was assayed against the previous pool to ensure no melatonin was present.

2.4.5 Standards.

A stock solution of 1mg/ml melatonin (N-acetyl-5-methoxytryptamine; Sigma Chemical Company Ltd.) was diluted in 10ml of absolute ethanol (Analar; Fisons Ltd.). This intermediate solution was stored at -20°C and used to prepare fresh standards for each assay. Serial dilutions of 250µl aliquots were prepared from a working

solution of 1ng/ml to provide standards from 3.9-250 pg/tube. A further working solution (2ng/ml) was used to provide a 500 pg/tube standard (Figure 2.3).

2.4.6 Assay Protocol.

All samples and standards were assayed in duplicate according to the following protocol:

1. Prepare a series of melatonin standards in polystyrene tubes (LP3; Luckhams Ltd.) to give a range of dilutions from 0-500pg/250µl.
2. Add 250µl of assay buffer to the sample tubes plus 450µl of buffer to a further two tubes which are used to calculate the non-specific binding (NSB).
3. Add 250µl of melatonin-free plasma or melatonin-free L15 culture medium to the NSB and standard tubes (from 0pg up to 500pg to avoid contamination).
4. Add 250µl of samples to sample tubes.
5. Add 200µl of antibody to each tube except the NSBs and incubate at room temperature for 30 minutes.
6. Add 100µl of tritiated melatonin to each tube, vortex and incubate at 4°C for 18 hours.
7. Add 500µl of charcoal [dissolve 1 'Separex' dextran-coated charcoal tablet (Steranti Research Ltd.) in 50ml of assay buffer and stir on ice for 30 minutes] to each tube, vortex and incubate at 4°C for 15 minutes.
8. Centrifuge at 3000 rpm (4°C) for 15 minutes.
9. Transfer 1ml of supernatant to 6ml polyethylene scintillation vials (Canberra Packard Ltd.) and add 4ml of scintillation fluid (Ultima Gold; Canberra Packard Ltd.). Transfer 4ml of scintillation fluid to

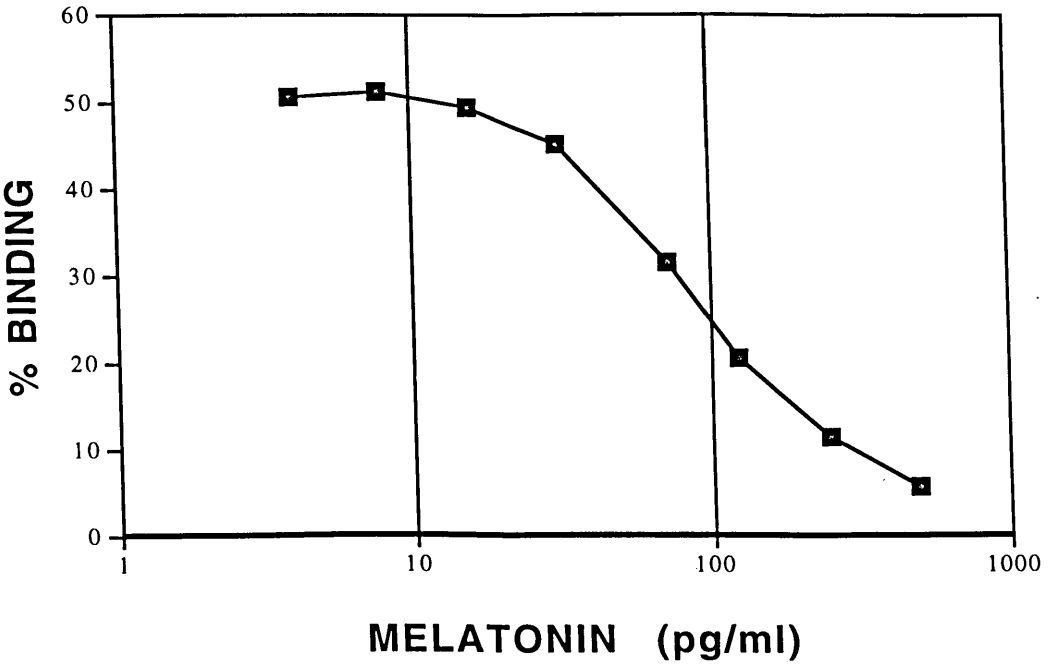


Figure 2.3

A typical standard curve obtained from a radioimmunoassay of melatonin. The hormone concentration within a sample is obtained from the intersect of the percentage binding in the sample.

3 empty vials. Add 100µl of tritiated melatonin to two of the vials for calculation of the total radioactivity. Use the remaining vial of scintillation fluid to calculate the background radioactivity.

10. Vortex the vials and count the radioactivity for 10 minutes in a scintillation counter (1900TR LSA; Canberra Packard Ltd.).

Assay disintegration per minute (dpm) values were converted to pg/tube using the 'Assayzap' computer program (Elsevier Biosoft) for the Apple Macintosh.

2.4.7 Quality Control & Validation.

The sensitivity of the assay (i.e. the minimum amount of melatonin able to be statistically distinguishable from zero) was 3.9pg/tube. Pooled plasma with a melatonin content of approximately 350pg/tube was used to check the reproducibility of measurements between assays, i.e. for quality control. The intra-assay coefficient of variation was 0.85% and the inter-assay coefficient of variation was 2.47%.

Serial dilutions of pooled rainbow trout and Atlantic salmon plasma were used to obtain inhibition curves (Figures. 2.4 and 2.5). When plotted against the standard curve it was observed that both curves were parallel to the standard curve and no statistically significant differences in the slopes were found (ANOVA), indicating that the melatonin in the standards was immunologically similar to that in the samples.

Assay validation was performed by Dr C. Randall (see Randall et al. 1995).

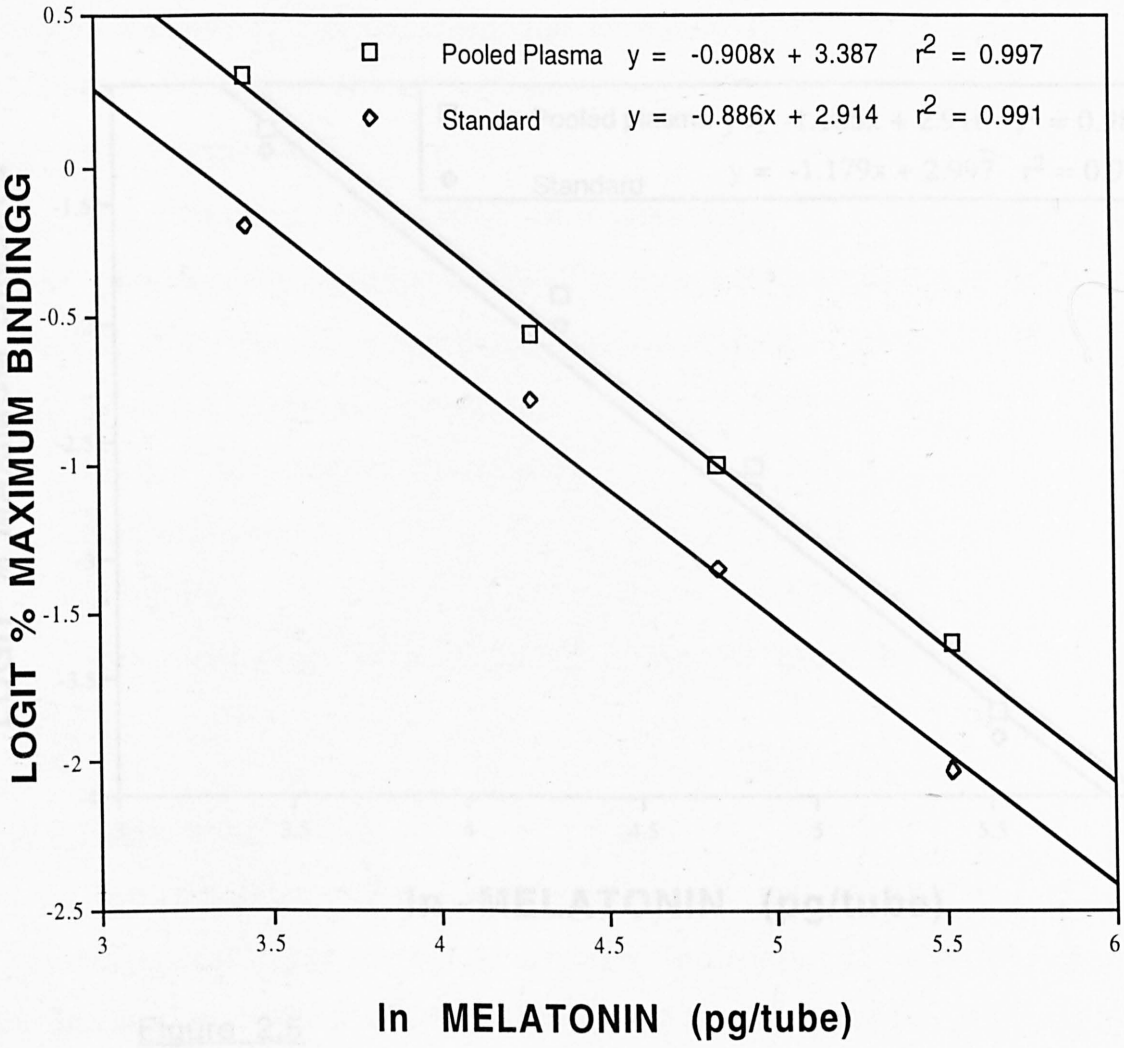


Figure 2.4

Parallelism of an inhibition curve obtained from a serial dilution (1:2) of 250 µl aliquots of pooled rainbow trout plasma (collected during the dark phase) with the melatonin assay standard curve. Each point represents the mean of duplicate measurements, the x-axis denotes the natural log of the melatonin content in the standards.

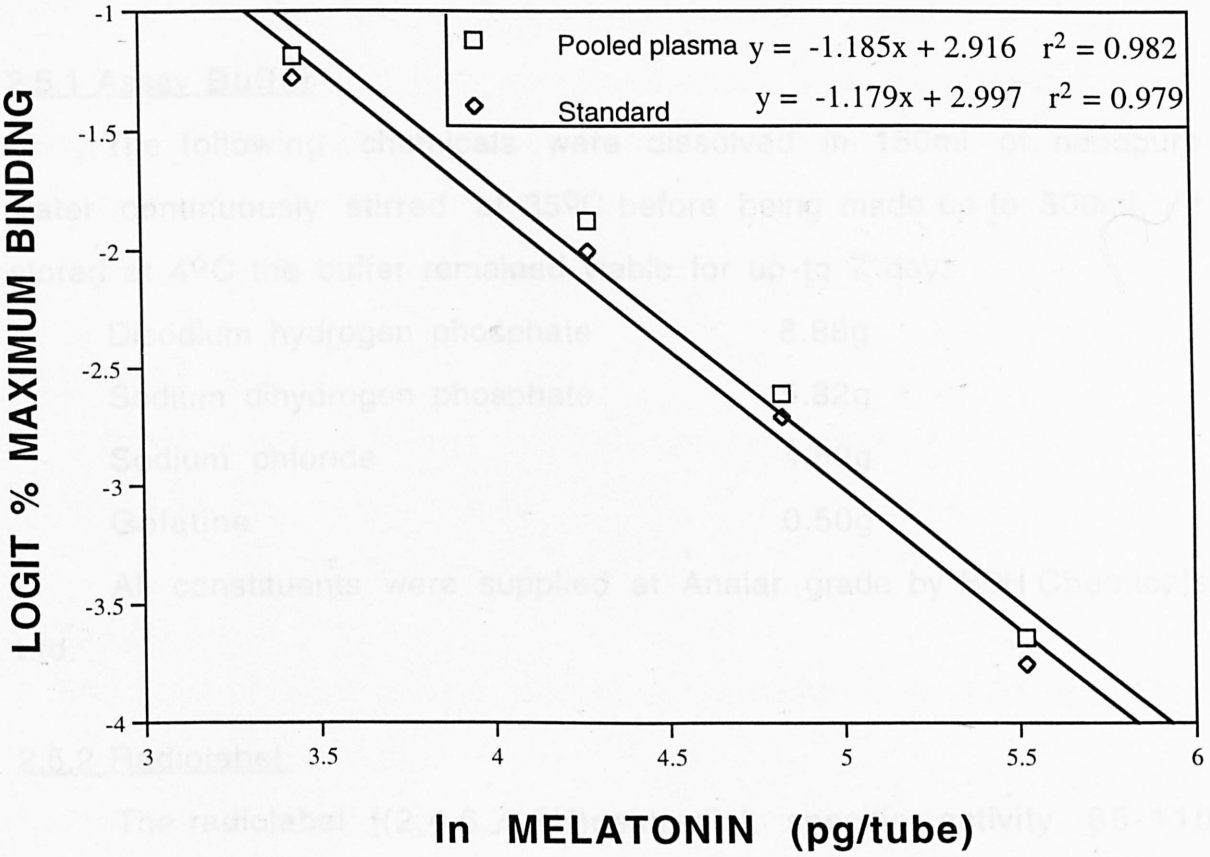


Figure 2.5

Parallelism of an inhibition curve obtained from a serial dilution (1:2) of 250 µl aliquots of pooled Atlantic salmon plasma (collected during the scotophase) with the melatonin assay standard curve. Each point represents the mean of duplicate measurements, the x-axis denotes the natural log of the melatonin content in the standards.

2.5 Oestradiol Radioimmunoassay.

Plasma samples were analysed for oestradiol-17β using a protocol developed from Duston and Bromage (1987) as follows:

2.5.1 Assay Buffer

The following chemicals were dissolved in 150ml of nanopure water continuously stirred at 35°C before being made up to 500ml. If stored at 4°C the buffer remained viable for up to 7 days.

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

All constituents were supplied at Analar grade by BDH Chemicals Ltd.

2.5.2 Radiolabel.

The radiolabel [(2,4,6,7,-^{3}H)oestradiol, specific activity 85-110 Ci/mmol] was supplied by Amersham International Ltd. (Amersham, Bucks, UK.) in 250μCi batches. An intermediate solution was prepared by diluting 20μl of the stock label in 2ml of absolute ethanol. This was then used to prepare a working solution of approximately 20,000 dpm/100ml (approximately 45μl of the intermediate solution in 10ml of assay buffer).

2.5.3 Antibody.

Freeze-dried rabbit anti-17β-oestradiol antiserum (Steranti Research Ltd., St. Albans, Herts., UK.) was reconstituted with 1ml of assay buffer. This was then stored in 100μl aliquots at 4°C in polystyrene tubes (LP3; Luckhams Ltd.).

2.5.4 Standards.

A standard of 100ng/ml was prepared by dissolving 1μg of dry oestradiol-17β (Steranti Research Ltd.) in 10ml of absolute ethanol and stored at -20°C. A new working solution was prepared for each assay by diluting 100μl of stock standard to 1ml with absolute ethanol. The standard curve used 100μl aliquots of the working solution to provided serial dilution ranging from 7.8-1000 pg/tube.

2.5.5 Assay Protocol.

Samples and standards were always assayed in duplicate within 3 months of the extraction date.

Extraction.

1. Add 50μl of each plasma sample to separate polypropylene tubes (LP3; Luckhams Ltd.).
2. Add 1ml of ethyl acetate (BDH Chemicals Ltd.) to each tube and stopper.
3. Spin tubes on a rotary mixer for 1hr.
4. Centrifuge tubes at 1500 rpm (4°C) for 10 min.
5. Store at 4°C if not to be assayed immediately.

Assay.

1. Transfer 100μl of extract to separate polypropylene tubes (LP3; Luckhams Ltd.).
2. Prepare serial dilutions of the standard (0-1000pg/100μl) with ethanol in polypropylene tubes (LP3; Luckhams Ltd.).
3. Dry down extracts in a vacuum oven at less than 35°C then cool tubes to 4°C.

4. Add 100µl of anti-oestradiol-17β to each tube.
5. Add 100µl of tritiated oestradiol-17β to each tube
6. Vortex each tube and incubate at 4°C for 18 hours.
7. Add 500µl of dextran-coated charcoal to each tube (dissolve one 'Separex' dextran-coated charcoal tablet in 50ml of assay buffer and stir on ice for 30 minutes prior to use). Vortex and incubate at 4°C for 15 minutes.
8. Centrifuge at 2000 rpm (4°C) for 10 minutes.
9. Transfer 400µl of supernatant to 6ml polyethylene scintillation vials (Canberra Packard Ltd.) and add 4ml of scintillation fluid (Ultima Gold; Canberra Packard Ltd.). Transfer 4ml of scintillation fluid to 3 empty vials and add 100 µl of tritiated oestradiol to two of the vials for calculation of the total radioactivity. Use the remaining vial of scintillation fluid to calculate the background radioactivity.
10. Vortex for 10 seconds and count the radioactivity in a scintillation counter (1900TR LSA; Canberra Packard).

Assay dpm values were converted to pg/tube using the 'Assayzap' computer program (Elsevier Biosoft) for the Apple Macintosh.

2.5.6 Quality Control & Validation.

The sensitivity of the assay (i.e. the minimum amount of oestradiol statistically distinguishable from zero) was 7.8 pg/tube. Pooled plasma with an oestradiol content of approximately 13 ng/tube was used for quality control. The intra-assay coefficient of variation was 4.55% and the inter-assay coefficient of variation was 6.59%.

A pool of rainbow trout plasma was used to obtain an inhibition curve (Figure. 2.6). When plotted against the standard curve it was

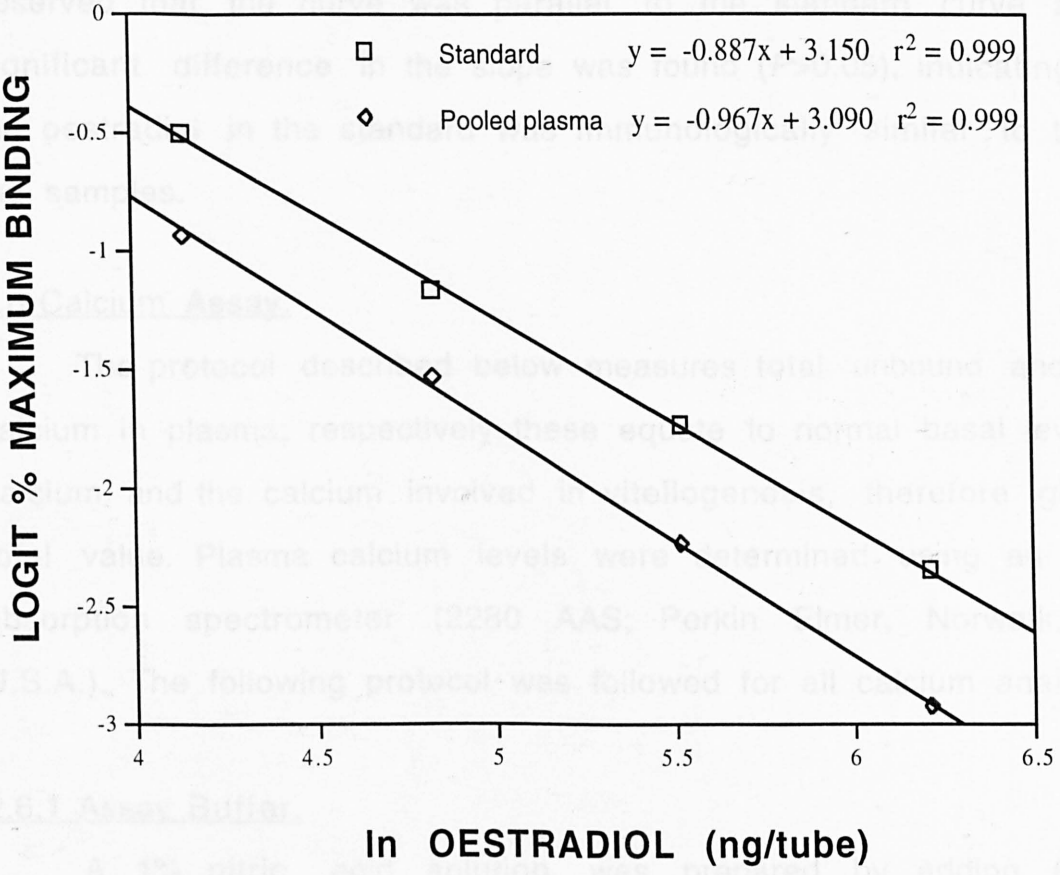


Figure 2.6

Parallelism of an inhibition curve obtained from a serial dilution (1:2) of 250 µl aliquots of pooled rainbow trout plasma with the oestradiol assay standard curve. Each point represents the mean of duplicate measurements. The x-axis denotes the natural log of the oestradiol content in the standards.

observed that the curve was parallel to the standard curve and no significant difference in the slope was found ($P>0.05$), indicating that the oestradiol in the standard was immunologically similar to that in the samples.

2.6 Calcium Assay.

The protocol described below measures total unbound and bound calcium in plasma; respectively these equate to normal basal levels of calcium and the calcium involved in vitellogenesis, therefore giving a total value. Plasma calcium levels were determined using an atomic absorption spectrometer (2280 AAS; Perkin Elmer, Norwalk, Con., U.S.A.). The following protocol was followed for all calcium analyses.

2.6.1 Assay Buffer.

A 1% nitric acid solution was prepared by adding 6ml of concentrated nitric acid (Sigma Chemical Company Ltd.) to 6 litres of deionised water. 60g of lanthium chloride was added to the 6 l of 1% nitric acid to produce a solution of 1% lanthium chloride in 1% nitric acid. The lanthium chloride is added to prevent interference from non calcium ions while aspiration is taking place on the atomic absorption spectrometer.

2.6.2 Calcium Standards.

Standards were prepared by diluting a commercially available calcium stock standard of calcium chloride (Sigma Chemical Company Ltd., UK.) with buffer. Fresh standards were prepared for each assay. Two standards (2mg/l and 4mg/l) were prepared as follows:

A) 2 mg/l = 400 µl stock standard made up to 200ml with buffer

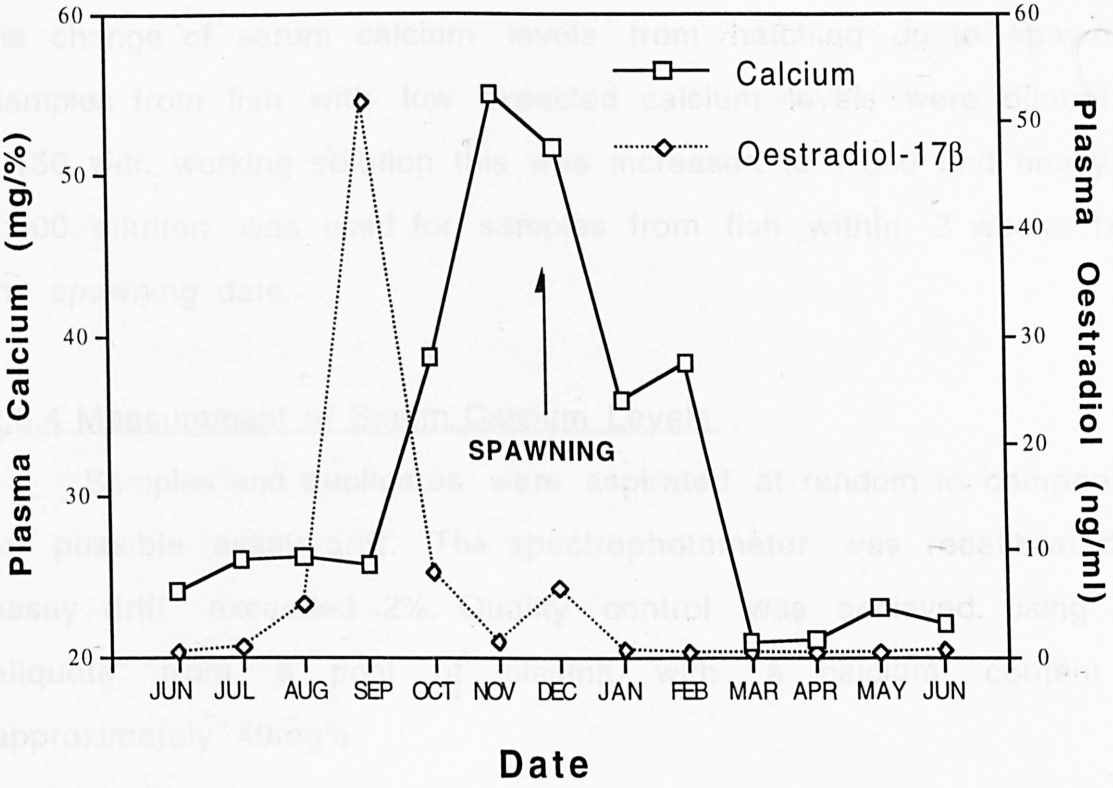


Figure 2.7

Seasonal changes in plasma calcium and oestradiol levels in female rainbow trout.

B) 4 mg/l = 800 µl stock standard made up to 200 ml with buffer

2.6.3 Sample Dilutions.

The sample dilution's used in the assay depended on the amount of calcium expected in the sample which is directly related to the stage of vitellogenesis. Figure 2.7 (adapted from section 5.4) shows the change of serum calcium levels from hatching up to spawning. Samples from fish with low expected calcium levels were diluted at 1:150 with working solution this was increased to 1:250 and finally a 1:500 dilution was used for samples from fish within 3 weeks from the spawning date.

2.6.4 Measurement of Serum Calcium Levels.

Samples and duplicates were aspirated at random to compensate for possible assay drift. The spectrophotometer was recalibrated if assay drift exceeded 2%. Quality control was achieved using 2ml aliquots from a pool of plasma with a calcium content of approximately 40mg%.

2.7 Assessment of Parr Smolt Transformation.

The transformation of juvenile Atlantic salmon from the freshwater parr stage of their life history to the anadromous smolt phase was assessed using three techniques. The first used the change in condition factor between parr and smolt. The other methods assessed the ability of smolts to osmoregulate. In experiment 5.3, a Sea Water Challenge Test was used to minimise the number of mortalities as the experimental number (n) was low. A Sea Water Tolerance Test was applied to experiments 4.2 and 4.4 as this tested more fully the abilities of the fish to osmoregulate and hence provided

a more accurate reflection of the parr/smolt ratio within the experiment.

2.7.1 Condition Factor.

During smoltification experiments approximately 50 fish were sampled at 14 day intervals to assess length, weight and condition factor. Anaesthetised fish were weighed to 0.1g (Sartorius L12000s digital balance; Sartorius Instruments Ltd., Epsom, Surrey, UK.) and the lengths recorded to 0.1cm. The condition factor was calculated using the following:

$$\text{Condition Factor (K)} = [\text{weight (g)} \times 100] / \text{length (cm)}^3$$

The condition factor provides an estimate of the timing and degree of transformation from parr to smolt for each individual. A typical value for a parr would be 1.20 whereas the condition factor of a smolt is approximately 0.90.

2.7.2 Sea Water Challenge Test.

Sea water challenge tests were adapted from those described by Blackburn and Clarke (1987) and conducted at 21 day intervals. A salinity of 35‰ was chosen as inconclusive results had previously been obtained using 30‰ (N. Duncan, pers. comm.). The following protocol was adopted:

1. 50 l plastic bags of artificial sea water (35‰) were prepared by dissolving 1.75 kg of Instant Ocean (Animal House, Bartlet, UK.) in 50 l of fresh water.

2. The bags of sea water were contained within a tank (2 x 2m) of circulating water held at 10°C by a cooler and heater system (Grant Instruments Ltd., Cambridge, UK.).
3. The sea water was aerated (Rena 101 air pump; Animal House, Bartlet, W.Yorks., UK.) and left for 24h to ensure all the salt had dissolved. The salinity was also checked with a salinity refractometer (S/Mill; Atago Oc. Ltd., Japan.).
4. Fifteen fish from each group were put into a separate bag of sea water for 24h after which the fish were removed and 0.2ml blood taken.
5. The blood was placed in 1.5ml microcentrifuge tubes (Life Sciences UK., Ltd.) and allowed to clot before centrifugation at 13,000 rpm for 5 minutes.
6. The supernatant was then drawn off and the osmolarity measured in triplicate using a 3MO Plus Advanced Micro-Osmometer (Advanced Inst. Inc., Needham Heights, Massachusetts, U.S.A.).

2.7.4 Sea Water Tolerance Test.

This test provides an accurate assessment of the sea water hypoosmoregulatory ability of parr and smolts. The test is based on the assumption that the parr cannot osmoregulate at 37.5‰ so will not survive the 96h. The high salinity also places stress on the smolts and hence the number of individuals in the experiment is considerably reduced after each test. The following protocol was adopted:

1. 50 l plastic tanks (0.9mØ) of artificial sea water (37.5‰) were made by dissolving 1.875kg of Instant Ocean (Animal House, Bartlet. W.Yorks., UK.) in 50 l of fresh water.

2. Each tank of sea water was contained within a tank (2 x 1m) being fed with fresh water at ambient temperature.
3. The sea water was aerated (Rena 101 air pump; Animal House, Bartlet. W.Yorks., UK.) and left for 24h to ensure all the salt had dissolved. The salinity was also checked with a salinity refractometer (S/Mill; Atago Oc. Ltd., Japan.).
4. Each tank was used to test 20 fish from the designated group. Mortalities were recorded every 12h from the time of introduction for 96h.
5. The LT50 for each group was then calculated as follows:

$$CPM = 100 \times (2M-1 / 2P)$$

where CPM = cumulative percentage mortalities
M = cumulative mortalities
P = 'n' number at the beginning of the test

The CPM was then plotted against the time (hours) on Log_2cycles X probability graph paper. The LT50 can then be extrapolated from the intersect of the slope at 50% CPM.

2.8 Organ Culture Systems.

The cultures in chapter 3 and section 4.2 were carried out under static conditions. All other cultures used the flow-through system described below. L15 culture medium with L-glutamine and 10% heat activated foetal bovine serum (Sigma Chemical Co. Ltd.) was used in section 3.2.2. The pH of the medium was adjusted to 6.8 and the osmolarity maintained as near as possible to salmonid serum i.e. ~330 mOsm. Lighting was supplied by 150w tungsten-halogen bulbs (Ealing Electro-Optics, Watford, U.K.) and measured using a photometer (Lightmaster photometer; Evans Electroselenium Ltd., Halstead, UK.) at

the beginning and end of each experiment. Unless stated the pineals were illuminated by a white light source. When specific wavelengths were required these were produced using interference bandpass filters (Ealing Electro-Optics, Watford, U.K.).

2.8.1 Static Cultures.

Pineals were placed in culture media on ice immediately after dissection. The pineals were then transferred to 24 well culture plates (Sigma Chemical Co. Ltd.) containing 1ml of fresh media and acclimated at the desired temperature in an air atmosphere prior to the commencement of the trial. At the comencement of the experiment the pineals were again transferred to fresh media and maintained under the chosen photoperiod within a light box. Pineals were transferred to fresh media every 12h, constant darkness (D:D) groups being changed under a dim red light (λ>730 nm). An empty chamber of culture media was held under light and dark conditions for 12h to serve as a blank control. Each sample of media was stored at -70°C immediately after collection until assayed for melatonin

2.8.2 Flow-through Culture System.

After dissection pineals were again maintained in L15 medium with L-glutamine plus 10% heat activated foetal bovine serum (Sigma Chemical Co. Ltd.) prior to being transferred to the culture system (Figure 2.8). The flow-through system used a P3 peristaltic pump (Pharmacia Fine Chemicals Ltd., Uxbridge, Middx., UK.) with 0.38mm IØ tubing (116-05323-04; Gradko Int. Ltd., Winchester, Hants., UK.) to provide a continuous supply of medium through Silastic tubing (Medical Grade Silastic; Dow Corning Co., Mi., U.S.A.). A 2ml syringe (Terumo Europe N.V., Belgium) was used as a 0.5ml culture chamber with a 50µm

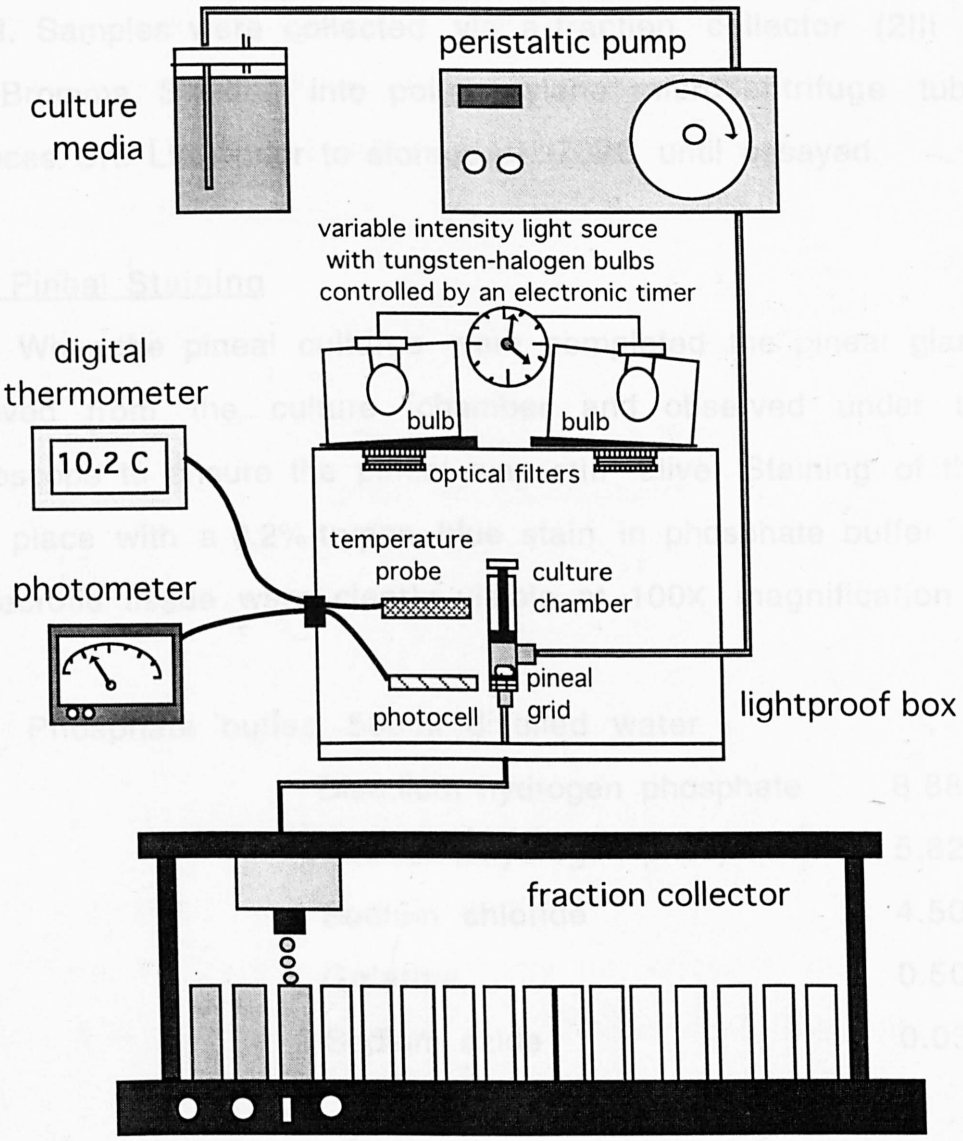


Figure 2.8

Diagram of flow-through culture system as used in section 3.2

grid (Plastok, Wirral, Merseyside, UK.) acting as a platform for the pineal. Samples were collected via a fraction collector (2III Multirac LKB; Bromma, Sweden) into polypropylene microcentrifuge tubes (Life Sciences UK. Ltd.) prior to storage at -70°C until assayed.

2.8.3 Pineal Staining

When the pineal cultures were completed the pineal glands were removed from the culture chamber and observed under the light microscope to ensure the pineal was still alive. Staining of the glands took place with a 0.2% trypan blue stain in phosphate buffer and signs of necrotic tissue were clearly visible at 100X magnification

Phosphate buffer: 500ml distilled water

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. at Analar grade)

2.9 Statistical Analysis.

The statistical techniques used in this thesis are described in Sokal and Rohlf (1981) and Bailey (1995). All calculations were performed using the Minitab Statistical Package (Release, 8) on an Apple Macintosh (LC III) computer. Where appropriate, additional 'in house' software for non-parametric analysis (courtesy of Dr. Mark Thrush) was executed within Minitab.

2.9.1 Estimation of the Population Mean.

The arithmetic mean ($\overline{X}$) was used to provide an estimation of the population mean (μ). In all cases $\overline{X}$ was used in conjunction with the standard error of the mean (SEM) to indicate the sample distribution. This is denoted in the text by ($\overline{X}$±SEM).

Arithmetic mean $(\overline{X}) = \frac{\sum X}{n}$

where: $\sum X$ = sum of observed samples

n = number of observations

Standard error of the mean (SEM) = $\frac{s}{\sqrt{n}}$

where: s = sample standard deviation = $\sqrt{\frac{\sum X^2 - \frac{(\sum X)^2}{n}}{n-1}}$

2.9.2 Parametric Tests and Assumptions.

Parametric techniques assume the observations were made at random and that test variances were independent. They also require the data to be normally distributed and sample variances (s^2, the square of the sample standard variation) to be homogeneous. Failure to comply with any of these criteria will at the very least reduce the test's power and may invalidate the test altogether. Therefore, all data were analysed for normality and homogeneity before further tests were applied.

2.9.3 Normality of Distribution and Sample Variances

To assess the normality of sample distributions the normal scores (N-scores) of the samples were calculated and correlated with

their sample values. The resulting correlation coefficient (*r*) was then compared to tabulated values. Sets with an *r* value greater than that in the tables (at *P*=0.05) were defined as significantly departed from normality (Shapiro-Wilk test).

Comparisons of sample variances were assessed using the F-test for the comparison of two samples or Bartlett's test if there were three or more samples.

$F_s = \frac{s_1^2}{s_2^2}$ where s_1^2 and s_2^2 are the greater and lesser variances.

Degrees of freedom $v_1, v_2 = n_1-1, n_2-1$

If the F_s value was less than the tabulated value at *P*=0.05 the sample variances were treated as homogeneous; if F_s was greater than or equal to the tabulated value the variances were concluded to be heterogeneous.

Bartlett's Test was used for multiple comparisons of variances, i.e. to test the homogeneity of more than two samples. It is based on an estimation of Chi square (χ^2) which can then be compared to a tabulated value.

$\chi^2 = \frac{M}{C}$ with (*a*-1)degrees of freedom

$$M = (2.3026)\left[(\sum f_i)\log s^{-2} - \sum f_i \log s_i^{-2}\right]$$

$$C = 1 + \frac{1}{3(a-1)}\left[\sum \frac{1}{f_i} - \frac{1}{\sum f_i}\right]$$

where: $\bar{s}^{-2} = \frac{\sum f_i s_i^2}{\sum f_i}$

If the tabulated value at P=0.05 was greater than the calculated χ^2 value it was concluded that the variances were homogeneous; if the value in the tables proved to be lower than the calculated χ^2 value the samples were treated as heterogeneous.

2.9.4 Comparison of Two Samples.

If the sample variances were found to be normally distributed and homogeneous, their means were compared using the Student's t-test with a pooled estimation of the variance. If normally distributed but with heterogeneous variances the means were compared using the Student's t-test but incorporating a separate estimate for each variance. This reduces the degrees of freedom of the critical t, thus reducing the probability of a type-1 error.

2.9.5 Multiple Comparisons.

A one-way analysis of variance (ANOVA) was used for the preliminary analysis of three or more sample means with a normal distribution and homogeneous variances. Significant differences between means ($P \leq 0.05$) were compared with a multiple range test.

$$t_s = \frac{\overline{X_1} - \overline{X_2}}{\sqrt{s^2\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$ with $(n_1=n_2)$ - 2 degrees of freedom

where $\overline{X_1}$ and $\overline{X_2}$= sample means

n_1 and n_2= number of observations in each sample

s^2= error mean square, as calculated by the ANOVA

Means were considered significantly different if their calculated t_s was greater than the tabulated value for t at p=0.05. If sample variances were heterogeneous or if one or more sample variations did not exhibit a normal distribution, the Kruskal-Wallis test (a non-parametric equivalent of ANOVA) was used to compare three or more sample means. Differences between pairs were compared using Dunn's multiple comparison test as follows:

$$Q_{0.5,k} = \frac{\overline{R_2} - \overline{R_1}}{SE}$$

where: $\overline{R_2}$ and $\overline{R_1}$= mean ranks of the two samples (e.g. $\overline{R_1} = \frac{R_1}{n_1}$)

k = number of groups

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

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

t=number of ties for a given value

n_1 and n_2= number of observations in each sample

2.9.6 Outlier Detection

For detection of outlying variants (r)with a sample size of up to n=25 Dixon's Test was used:

$$r = \frac{y_3 - y_1}{y_{n-2} - y_1}$$

n= total number of observations in the group

Y_1= the first variant and suspected outlier

Y_3= the third variant in ascending or decending order from Y_1

For sample sizes with n>25 Grubbs Test was employed:

$$r = \text{outlier probability} = (Y_1 - Ym) / s$$

Y_1= suspected outlier

Ym=sample mean

s= sample standard deviation

Chapter 3

The pineal gland and its control of circulating melatonin levels in salmonids

3.1 Introduction

3.1.1 Teleost pineal photoreception

The perception of light is perhaps the most important sensory ability found in animals. This has led to the development of a vast array of photoreceptive organs capable of analysing the spatio-temporal environment in which the animal lives. Visualisation of these signals requires image-forming apparatus combined with an oculomotor system and a complex neural network to receive and interpret the information. Not surprisingly, in view of their predominant importance and accessibility as discrete organs, the lateral eyes have been the subject of intensive study over the centuries. Although extra-ocular photoreceptors (EOPs) are known to exist (reviewed in Groos, 1982) little is known of their location or function. More recently, several photoreceptive tissues have been identified within the teleost brain and, in particular, the pineal gland. Unlike the lateral eyes, it is suspected their main role is to monitor changes in irradiance (Foster *et al.*, 1994). As the principal fluctuations in light intensity within the natural environment are a direct result of the day/night photoperiod, it has been suggested that extra-retinal photoreception is principally involved in monitoring the seasonally-changing photoperiod (Aschoff, 1981; Groos, 1982). As the inhibition of melatonin synthesis is now known to be a direct response to the illumination of pineal photoreceptors (Falcon *et al.*, 1986), it seems reasonable to assume that melatonin may act as a hormonal messenger of photoperiodic information.

Despite the importance of the seasonal changes in daylength to many of the salmonid life stages (Bromage *et al.*, 1982; Duston and Saunders, 1990) little is known about either the perception of these changes or how photoperiodic information is translated into signals which co-ordinate seasonal developmental events such as reproduction and smoltification. In an attempt to address these questions, the experiments in this chapter were designed to investigate pineal melatonin synthesis in response to environmental manipulation in the Atlantic salmon and rainbow trout. Initial *in vivo* work concentrated on developing a technique for simple and efficient pineal removal in an attempt to establish the primary source of nocturnal plasma melatonin and to provide a method of abolishing the daily melatonin rhythm in salmonids. Once the pineal had been established as the primary site of nocturnal melatonin synthesis, work began on assessing environmental sensitivity of the pineal gland. For this *in vivo* and *in vitro* techniques were used to assess the pineal's response to temperature and photic manipulation. Surgical techniques developed in the course of this work were then utilised predominantly in the experiments described in chapters 4 and 5.

The existence and function of EOPs has only become apparent during the last hundred years. VonFrisch in 1911 (cited in Groos 1982) reported the presence of EOPs in the European minnow and Kavaliers (1981) reported circadian rhythms of photosensitivity by non-pineal extra-retinal photoreceptors in the lake chub which, it has been suggested, may be located within the diencephalon, paraphysis and third ventricle (Oche and Hartwig, 1975; Hartwig and vanVeen, 1979), although dermal photosensitivity cannot be ruled out (VonFrisch in 1911).

Subsequently, both the pineal and deep encephalic photoreceptors of the diencephalon have been identified as EOPs in lower vertebrates and birds (Yokoyama et. al., 1978). By contrast, all mammals so far investigated seem to have lost the ability to receive light extraretinally.

As to the function of EOPs in non-mammalian vertebrates, we must first consider why both EOPs and ocular photoreception co-exist. As mentioned earlier, the eye provides a spatial image of the environment through the focused representation of photons sampled at a particular moment in time. In doing this the eye measures brightness at a precise point in space (radiance), but not over the complete field of view (irradiance). To allow irradiance to be measured, photons from all directions must fall uniformly on a matrix of photoreceptors. This is exactly the result achieved by the pigmented epithelium and overlying tissue which scatters light before it is received by encephalic photoreceptors (Foster *et al.*, 1994). Mammals have overcome this by evolving two distinct groups of ocular projections which serve visual or circadian nuclei. Retinal projections innervate the visual centres of the brain while retino-hypothalamic projections lack spatial order and project randomly to the SCN so producing a form of irradiance detection (Cooper et. al., 1993; cited in Foster *et al.*, 1994). Groos (1982) suggests the most appropriate generalisation that can be drawn about the irradiance detection of EOPs is their role in the entrainment of circadian rhythms to the seasonally changing photoperiodic cycle (see also van Veen *et al.*, 1976; Kavaliers, 1980; Aschoff, 1981).

Localisation studies of deep brain photoreceptors have relied upon the identification of the photoreceptor proteins opsin

and chromophore (11-*cis* retinol) within the suspected tissues using antibodies raised against these photoreceptor proteins. Silver and co-workers (1988) successfully labelled cerebrospinal fluid (CSF) contacting neurones within the septal and tuberal areas of the avian brain with anti-opsin antibody but failed to clearly identify opsin-like proteins using Western blots. Intense immunostaining of CSF-contacting neurones in the lateral ventricle ependyma of the lizard *Anolis carolinensis* was also reported by Foster *et al.* (1993), who, using Western blot analysis, were able to identify a single 40kD protein sharing similar characteristics to vertebrate cone opsins. Foster *et al.* (1989) also identified retinoids associated with phototransduction in the fore-brain areas of *Anolis* labelled by anti-opsin antibodies. Using HPLC analysis they were able to show the presence of the vitamin A_2-derived photopigments, chromophores 11-*cis* and all-*trans*-3,4-didehydroretinalaldehyde.

Of the limited work conducted on fish, Garcia-Fernandez and Foster (1994) reported an anti-rod opsin antisera labelled population of CSF-contacting neurones within the postoptic commissural nucleus and ventral hypothalamic nucleus of the larval lamprey (amnocoetes). The same neurones were also labelled for the retinal photoreceptor G-protein (alpha-transducin). Further fish species have produced opsin-like labelling within the neurosecretory cells of the nucleus magnocellularis preopticus (NMPO). Goldfish NMPO cells were immunopositive to anti-rod opsin antibody and negative to anti-cone antibodies. In contrast, the mosquito fish and Atlantic salmon proved immunonegative to anti-rod but positive to anti-cone antibodies (Foster *et al.*, 1994). This recent work strongly

suggests the presence of multiple encephalic photopigments and at least two types of EOP cells outwith the pineal gland but as yet no conclusive proof of their existence has been found.

The photoreceptive ability of the pineal gland in teleosts was first reported by the German scientist Karl von Frisch (1911). In a series of pioneering experiments he was able to localise the pineal gland as the principal, but not exclusive, site of extra-ocular photoreception responsible for a light-sensitive melanophore response in the European minnow. The early 1960s saw a resurgence of interest in the pineal resulting in a number of neurophysiological and ultrastructural investigations (reviewed by Dodt, 1963; Dodt and Nauheim, 1973; Hamasaki and Eder, 1977; Oksche and Hartwig, 1979; Groos, 1982).

Studies into the ontogenetic development of photoreceptive tissues in fish have consistently shown that pineal photoreceptor differentiation occurs significantly earlier than retinal development (Ekstrom *et al.*, 1983; Ostholm *et al.*, 1987; Omura and Oguri, 1993). Pineal photoreceptors endowed with photoreceptive outer segments and signal transmitting synapses appeared only 15 days post-fertilisation in the rainbow trout (Omura and Oguri, 1993). By 21 days after fertilisation these photoreceptors were well developed compared to the retinal photoreceptors which only appeared after 27 days. In Atlantic salmon, the pineal is morphologically and functionally mature within 440 degree-days of fertilisation which is at least 30 degree-days prior to the retina becoming functional (Ostholm *et al.*, 1987). Comparative time differences have also been reported in the three-spined stickleback (Ekstrom *et al.*, 1983). In all cases, the authors proposed the early development of pineal

photoreceptors to be instrumental in the entrainment of a circadian rhythm and possibly to allow a phototactic response in predator avoidance.

3.1.2 Teleost pineal morphology

Structurally, the pineal complex develops from a circumscribed area of the diencephalic roof, between the habenular and posterior commissures, forming a rostrocaudal groove. In teleosts, the pineal gland proper (*epiphysis cerebri*) is often accompanied by a much reduced parapineal organ which is located to the left of the pineal stalk and consists of a single capillary lumen surrounded by a small number of photoreceptor cells and neurones supported by interstitial cells (Ekstrom *et al.*, 1983; Korf, 1994). The parapineal, originally presumed to be present only in juvenile fish, has now been identified in the adult rainbow trout as well as in a number of other species (Rudeberg, 1969 and van Veen *et al.*, 1980; cited in Ekstrom *et al.*, 1983). However, its function is unknown.

When fully developed, the pineal gland proper appears as a large saccular structure located on the dorsal surface of the telencephalon beneath the specialised parietal foramen of the skull (Figure 3.1). This overlying area within the heavily pigmented ectomenix is often referred to as the pineal window in recognition of its transparent specialisation. Morita (1966) discovered that this region of reduced meningeal melanophores allows approximately 10% of the incident light to penetrate through the overlying tissue into the pineal fossa of the juvenile rainbow trout. This was substantially reduced to between 1-4% in adults. In comparison, Nordtog et. al. (1994) reported that 4% of

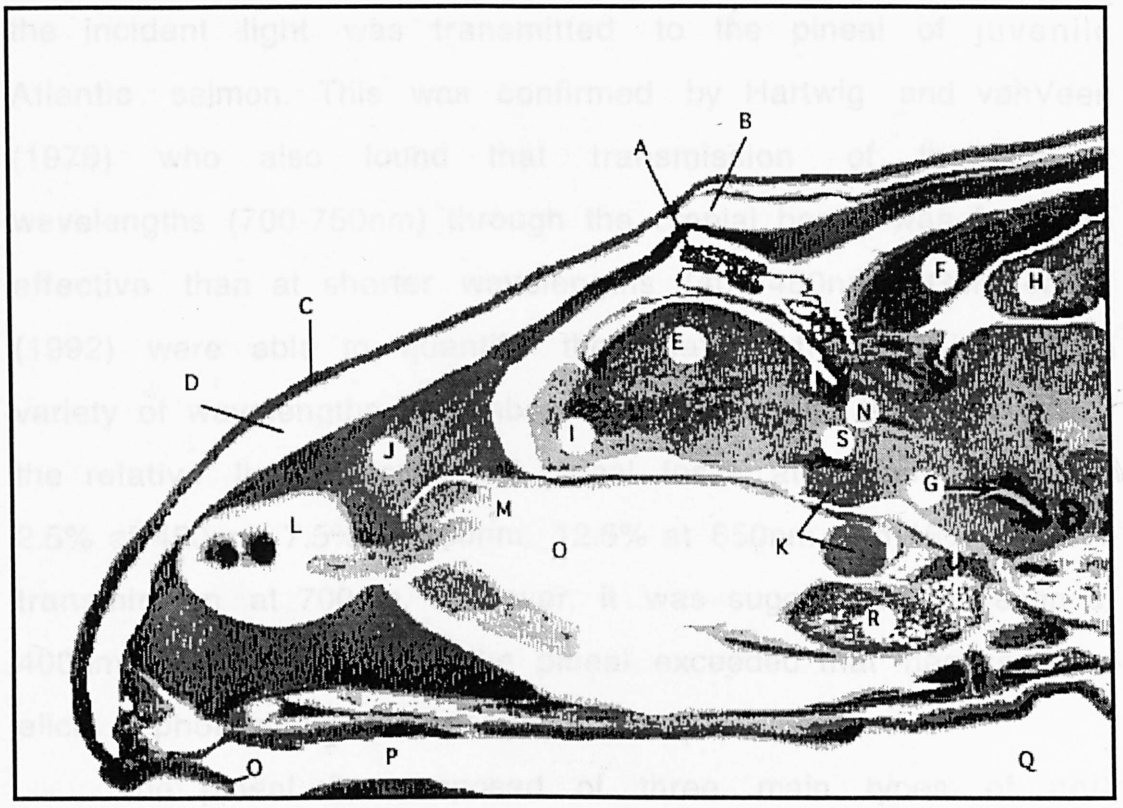


Figure 3.1

Sagittal section of the brain area of a 1 month old rainbow trout, x45.

A	pineal gland	B	pineal fossa
C	epidermis	D	mesenchymal tissue
E	telencephalon	F	optic tectum
G	ventral hypothalamus	H	mesencephalon
I	olfactory bulb	J	cartilage
K	optic nerves	L	olfactory lumen
M	olfactory nerve	N	diencephalon
O	oral valve	P	oral region
Q	pharangeal region	R	pituitary
S	hypothalamus		

Adapted from Yasutake and Wales (1983).

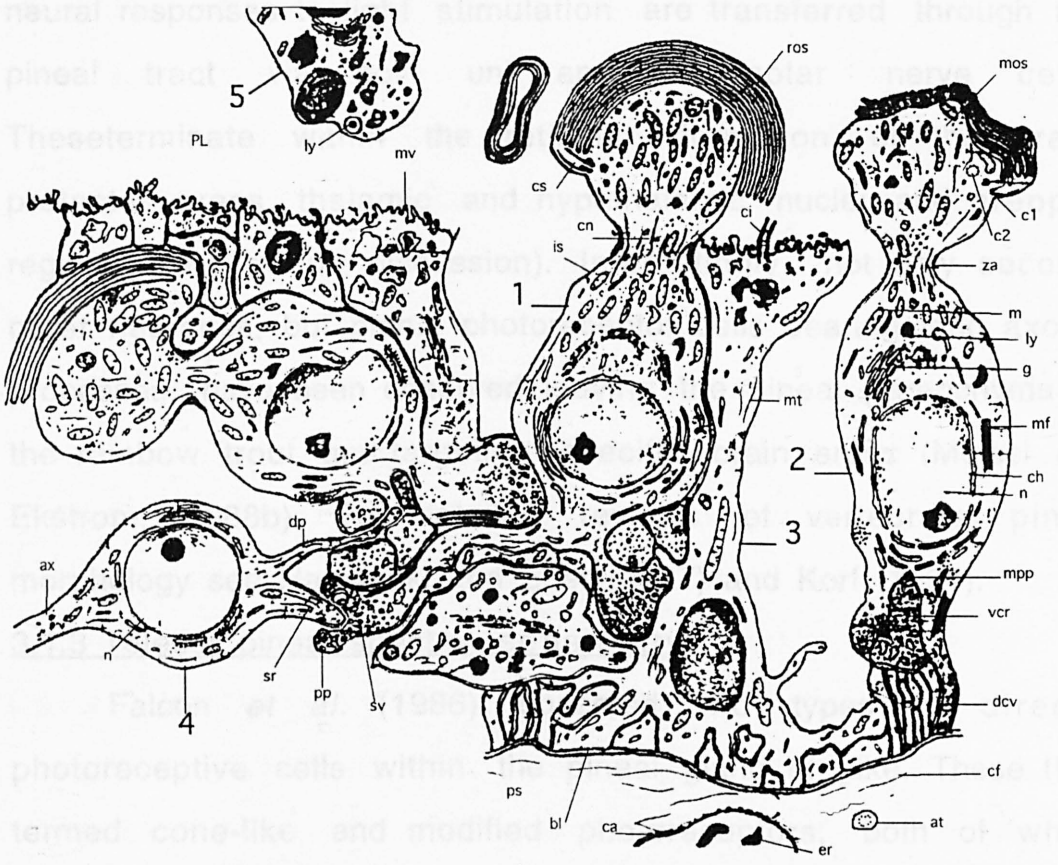
the incident light was transmitted to the pineal of juvenile Atlantic salmon. This was confirmed by Hartwig and vanVeen (1979) who also found that transmission of the longer wavelengths (700-750nm) through the cranial bones was far more effective than at shorter wavelengths (400-450nm). Gern *et al.* (1992) were able to quantify the relative transmission at a variety of wavelengths in rainbow trout and found that only 1% of the relative light entered the pineal fossa at 400nm, rising to 2.5% at 450nm, 7.5% at 550nm, 12.5% at 650nm and 16% relative transmission at 700nm. However, it was suggested that even at 400nm the light reaching the pineal exceeded that necessary to elicit a photoreceptor response.

The pineal is composed of three main types of cell: pinealocytes (photoreceptive); supporting (glial) cells; and neurones, which together form the pineal parenchyma. This is separated from the adjacent connective tissue layer (capsule) and the capillaries by a basal lamina (Korf, 1994). The supporting cells possess laminar cytoplasmic projections forming sheaths which surround the receptor cells; the presence of rough-ER, well developed Golgi apparatus and free ribosomes are indicative of their biosynthetic activity (Hamasaki and Eder, 1977). The pineal tract (*tractus pinealis*) services the receptor and supporting cells of the pineal parenchyma and as such consists of the microvasculature of the pineal (*epiphyseales*-artery and *vena cerebri anterior*-vein) and pineal ganglion (Syed *et. al.*, 1987). These run dorsally from the pineal end-vesicle to the subcommisural organ, diffusing at the posterior commisure.

As the pinealocytes within the pineal end-vesicle show significant variation they are usually sub-divided in accordance

with their structure and neuronal apparatus into true pineal photoreceptors and modified pineal photoreceptors; the latter are thought to be the precursors of pinealocytes *sensu stricto* found in mammalian pineals (Falcon, 1984; Falcon *et al.*, 1989; Korf, 1994). Of these pinealocytes, poikilothermic vertebrates are unique in possessing true pineal photoreceptor cells (Figure 3.2). These cells are morphologically similar to those of the lateral eyes and both are derived from ependymal cells. Photoreceptor cells consist of inner and outer segments connected via ciliary processes with a 9+2+0 microtubule structure. The outer segment of pineal photoreceptors protrudes into the pineal lumen and is formed through successive basoapical invagination of the plasma membrane resulting in a stacked disc arrangement as found in retinal cones. Unlike the retina, these outer segments are shorter than would be expected in cones, being restricted to a maximum of 450 discs but usually less depending upon species. The organelles of the inner segment closely resemble those found in the photoreceptors of the lateral eye, containing: the cell nucleus; high concentrations of mitochondria; and a basal synaptic region. Teleost basal processes originate from the perikaryon. Like the retinal cones their enlarged terminals contain numerous electron lucent synaptic vesicles surrounding synaptic ribbons. Unlike retinal inner segments the presence of dense-cored vesicles is observed in the cytoplasm as well as the photoreceptor terminals.

Information from pineal photoreceptors is received by dendrites of intrapineal second-order neurones which form synapses with the basal processes. In the pineal, the dendrites do not interdigitate deeply into the photoreceptor cells as seen in the retina but remain within distinct regions. Light stimuli from



Fiure 3.2 Pineal parenchyma structure in teleosts. 1) Typical photoreceptor cell. 2) modified photoreceptor cell. 3) Glial (interstistial) cell. 4) sensory neurone. 5) phagocytotic cell (macrophage).

at	axon terminal	ax	axon
bl	basal lamina	c1	axial centriole
c2	distal centriole	ca	capillary
cf	collagen fibre	ch	chromatin
ci1	cilium (9+1)	cn	cell neck
cs	connecting segment (9+0)	dcv	dense cored vesicle
dp	dendritic process	mos	modified outer segment
er	erythrocyte	g	golgi complex
is	inner segment	ly	lysosome
m	mitochondrion	mf	microfilaments
mt	microtubules	mv	microvilli
n	nucleus	PL	pineal lumen
pp	photoreceptor cell process	mpp	photoreceptor cell process
ps	perivascular space	ros	regular outer segment
sr	synaptic ribbon	sv	synaptic vesicle
vcr	vesicle crowned ribbon	za	zonula adherens

Modified from Falcon, 1979.

neural responses to light stimulation are transferred through the pineal tract via both uni and multi-polar nerve cells. Theseterminate within the reticular formation of the brain, pretectal areas, thalamic and hypothalamic nuclei and preoptic region (see previous discussion). Interestingly, not only second-order neurones, but pineal photoreceptor cells bearing long axonal processes, have been observed leaving the pineal parenchyma of the rainbow trout and targeting specific brain areas (Meissl and Ekstrom, 1988b). For detailed reviews of vertebrate pineal morphology see Hamasaki and Eder (1977) and Korf (1994).

3.1.3 Teleost pineal spectral sensitivity

Falcon *et al.* (1986) observed two types of directly photoreceptive cells within the pineal gland of pike. These they termed cone-like and modified photoreceptors, both of which were found to produce indoles in response to the light/dark cycle. The presence of at least two different types of true photoreceptors has also been suggested for the rainbow trout (Meissl and Ekstrom, 1988a; Max and Menaker, 1992; Kusmic *et al.*, 1993). Their evidence for this is based on spectrophotometric measurements obtained from pineal photoreceptors which have shown the presence of multiple action spectra in the trout pineal. Initially, Meissl and Ekstrom (1987) reported that the spectral sensitivity of all intracellular recorded photoreceptors peaked between 520-530 nm. In their 1992 paper, Max and Menaker found a sensitivity peak of around 500 nm which agreed with the work of Dodt (1963) who also reported a λmax of 507 nm. However, in acknowledging Meissl and Ekstrom's work, Max and Menaker suggested that there may be two photoreceptor types each with

its unique photopigment, which would explain the conflicting results.

At present, the known visual pigments all contain a common protein (opsin) which is conjugated with a prosthetic group, an aldehyde of vitamin A (retinine). The diversity of visual pigments observed in fish is based on a series of species-specific opsins and the presence of two retinines. Of these, the aldehyde of vitamin A1, with a single double bond in its ring structure, is named rhodopsin. The second pigment derived from vitamin A2 (porphyropsin) has two double bonds (see Munz 1971 for a detailed account). At rest, the photopigment is in the 11-cis isomeric configuration but on absorption of a photon the complex straightens to the all-trans form. This is thought to mobilise calcium ions and the protein α-transducin which change the permeability of the plasma membrane to the passage of sodium ions so altering the polarity of the photoreceptor and generating a nerve impulse (Lythgoe, 1988). Another protein, S-antigen, acts to desensitise the gated action channels once the nerve impulse has passed and the calcium-binding protein recoverin initiates recovery of the photoreceptor cell from the light adapted state (Korf, 1994). As rhodopsins and porphyropsins have dissimilar aldehyde groups, the spectral wavelength responsible for initiating a response has also been shown to differ. Rhodopsins have a spectral sensitivity peaking at the lower end of the wavelength range (i.e. 500 nm) whereas porphyropsins generally peak at a higher wavelength (i.e. >550nm) (Munz, 1971; Lythgoe, 1980; Levine and McNichol, 1982; McFarland, 1986).

It would appear then that within the pineal photoreceptors of teleosts there exists two visual pigments or, as in the ocular

photoreceptors, paired pigments favouring vitamin A1 or A2 (Bowmaker, in Douglas and Djamgoz, 1990). This was verified in rainbow trout by Kusmic *et al.* (1993) who obtained two distinct action spectra: the first with an absorption curve peaking at 463 nm, which best fitted the rhodopsin based template and the second peaking at 561 nm, similar to the porphyropsin template. They also suggested that the results of Max and Menaker (1992) may represent the spectral sensitivity of the pineal as a whole due to the convergence of information from both receptor types. This idea was also presented by Ekstrom and Meissl (1988) who reported coupling between pineal photoreceptors in the trout. They suggested that this may reflect an electrical coupling via gap-junctions which may function as part of a signal averaging system.

Light and dark adaptation by the trout pineal is primarily a luminance response. This is characterised by their reduced ganglion time-to-peak response to illumination (Hanyu and Niwa, 1970; Meissl and Ekstrom, 1988b). In some cases, this was shown to be 10 times slower than equivalent retinal rod or cone responses in lower vertebrates (Kusmic *et al.*, 1992). However, the inability to discriminate rapidly-changing light intensities through neural responses would seem to be consistent with the suggested role of these cells in responding to the slowly varying daily light intensity.

As mentioned earlier, Falcon *et al.* (1986) observed that photoreceptor cells within the pineal gland of pike produce a neuroendocrine response to the light/dark cycle. By 1992, Falcon *et al.* provided convincing evidence that synthesis of methoxyindoles took place within photoreceptor cells. In this

study they were able to identify and localise a compound similar to HIOMT in pike and trout photoreceptors. Melatonin (N-acetyl-5-methoxytryptamine), now recognised as the principal indole synthesised by the pineal, was first isolated from bovine pineals by Lerner (1958). Since then its daily rhythmic secretion has been identified in a large number of fish species (reviewed in Zachmann *et al.*, 1992). Falcon *et al.* (1985) have shown the pineal gland in pike to be equally capable of synthesising all indoles identified in the pineals of higher vertebrates.

3.1.4 Melatonin biosynthesis within the pineal gland

The first step in the biosynthetic pathway of melatonin is the conversion of tryptophan to 5-hydroxytryptophan (5HTp) through the enzymatic action of tryptophan hydroxylase (Figure 3.3). 5-hydroxytryptophan is then decarboxylated by the aromatic amino-acid decarboxylase to produce serotonin which is converted to N-acetylserotonin by serotonin N-acetyltransferase (NAT). A second enzyme, hydroxyindole-O-methyltransferase (HIOMT), methylates N-acetylserotonin to produce melatonin. Two other indoles, 5-hydroxyindole acetic acid and 5-hydroxytryptophol, are also formed through the catalysis of serotonin by monoamine oxydase (MAO). Methylation of these compounds by HIOMT leads to the formation of 5-methoxyindole acetic acid and 5-methoxytryptophol, respectively. Direct methylation of serotonin has also been shown to yield 5-methoxytryptamine (Falcon *et al.*, 1992). Selective uptake and metabolism of melatonin by pineal photoreceptor cells has been observed in the goldfish (McNulty, 1986) and pike (Falcon *et al.*, 1985) and the lipophilic nature of the melatonin molecule is thought to result in its immediate and

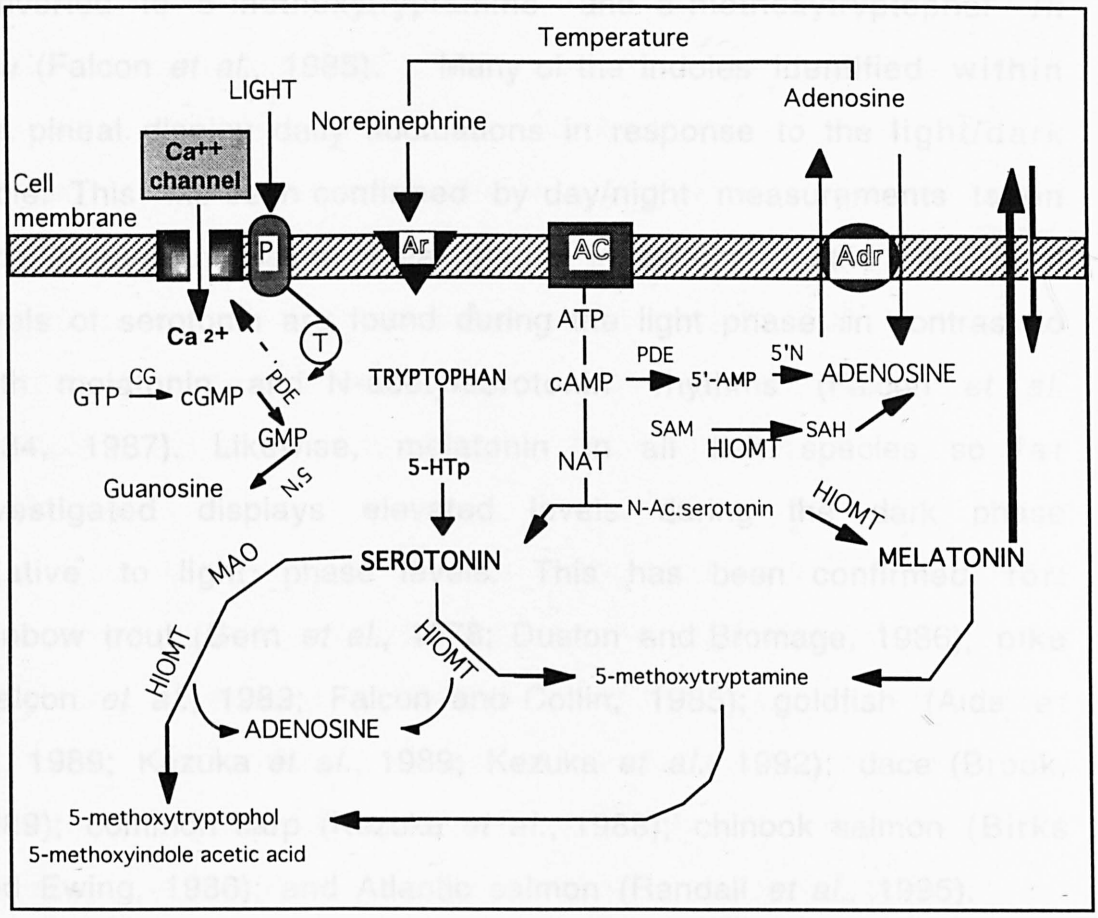


Figure 3.3

Melatonin biosynthetic pathway in teleost pineal photoreceptor cells. Ar: Adrenoreceptor; Adr: Adenosine receptor; AC: Adenyl cyclase; P: Photons, T: Transducin; PDE: Phosphodiesterase; cGMP: cyclic GMP; N-Ac serotonin: N-acetylserotonin; CG: Guanyl cyclase; 5´´N: 5´-nucleotidase; MAO: Monoamine oxydase; HIOMT: Hydroxyindole-O-methyltransferase; T: Transducin; cAMP: cyclic AMP; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; NAT: serotonin N-acetyltransferase; 5-HTp: 5-hydroxytryptophan. (adapted from Falcon *et al.*, 1992)

passive release across the plasma membrane following biosynthesis. However, melatonin not directly released may be converted to 5-methoxytryptamine and 5-methoxytryptophol *in situ* (Falcon *et al.*, 1985). Many of the indoles identified within the pineal display daily fluctuations in response to the light/dark cycle. This has been confirmed by day/night measurements taken both *in vivo* and using pineal glands in culture. In the pike, high levels of serotonin are found during the light phase, in contrast to both melatonin and N-acetylserotonin rhythms (Falcon *et al.* 1984, 1987). Likewise, melatonin in all fish species so far investigated displays elevated levels during the dark phase relative to light phase levels. This has been confirmed for: rainbow trout (Gern *et al.*, 1978; Duston and Bromage, 1986); pike (Falcon *et al.*, 1989; Falcon and Collin, 1985); goldfish (Aida *et al.*, 1989; Kezuka *et al.*, 1989; Kezuka *et al.*, 1992); dace (Brook, 1989); common carp (Kezuka *et al.*, 1988); chinook salmon (Birks and Ewing, 1986); and Atlantic salmon (Randall *et al.*, 1995).

Not surprisingly then, NAT also shows increased concentrations within trout and pike pineals during the dark phase (Morton and Forbes, 1988; Falcon *et al.*, 1987, 1989) and inhibited activity under illumination both *in vivo* and *in vitro* (Falcon *et al.*, 1989). In contrast, HIOMT activity in pike, trout and salmon pineals was found to remain constant throughout the 24 h light/dark cycle (Hafeez and Quay, 1970; Birks and Ewing, 1981; Falcon *et al.*, 1987; Morton and Forbes, 1988). However, Smith and Weber (1976) reported distinct daily variations in the HIOMT concentrations in steelhead trout. As with melatonin, they found HIOMT levels were elevated during the dark phase, which they

suggested was due to photic stimulation from the eyes as enucleation appeared to abolish the HIOMT response.

Fluctuations in NAT levels may reflect variations in cyclic-adenosine monophosphate (cAMP) levels over the light cycle. cAMP acts as a secondary messenger, participating in the control of NAT activity and therefore melatonin production (Thiabult *et al.*, 1993; see also Figure 3.3). Thibault and co-workers (1993) also suggested that cAMP production was governed by the presence of intrapineal circadian oscillators which are entrained through photoperiodic and temperature cycles, but this has not been confirmed.

Also of interest is the importance of extracellular adenosine and Ca^{2+} concentrations to the production of cAMP and melatonin. Begay *et al.* (1994) observed that Ca^{2+} was necessary for the dark-induced rise in melatonin production by photoreceptor cells. Entry into the photoreceptor cell is thought to occur via L-type Ca2+ channels, possibly during cell depolarisation in the dark. Cell polarisation in response to the light/dark cycle has been most thoroughly studied in retinal photoreceptors and is now known to occur in response to changes in cyclic GMP (cGMP) concentration (Pugh and Lamb, 1990). Light excitation of the photopigment (rhodopsin) activates transducin (a GMP-binding protein) which in turn activates cGMP phosphodiesterase (PDE). The subsequent decrease in cGMP closes the cationic channels and results in cell hyperpolarization (reviewed in Falcon *et. al.*, 1992). This mechanism is consistent with the findings of Falcon *et al.* (1990) who reported a 40% decrease in cGMP levels within dissociated trout pineal cells. With the onset of darkness transducin activity ceases and guanyl

cyclase activation once again accelerates the cGMP metabolic cycle, thereby causing the closure of the cationic channels and cell depolarization. Furthermore, it has been suggested that a Ca^{2+}/calciprotein complex acting at two different sites within the cell was able to regulate cAMP metabolism at one position and directly affect melatonin synthesis at the other. Falcon and Collin (1989) were able to show that adenosine produced by the pineal in trout and pike was able to modulate cAMP levels and NAT activity. By removing extracellular adenosine or using a blocker to the plasmalemma adenosine carrier system, a decrease in adenosine concentration was produced which resulted in elevated NAT activity. They suggested that this may act as a feed-back mechanism to regulate melatonin production via adenosine receptors in the cell membrane coupled to adenyl cyclase.

The idea of a self-regulating system controlling melatonin synthesis was also discussed by Begay *et al.* (1992) who found that dissociated trout pineal photoreceptor cells proportionately increased melatonin production in relation to a decrease in the concentration of external melatonin in the culture media. Yanez and Meissl (1995) observed similar results with the addition of radiolabelled 2-iodomelatonin to the perfusate of a complete pineal gland of a rainbow trout in culture. As the labelled melatonin could easily be recognised from native melatonin using liquid chromatography, a reduction in native melatonin production was evident after addition of the labelled indole. This would indicate the presence of an autocrine or paracrine action of the pineal. As no melatonin binding sites were located in the pineal gland of either the goldfish or Atlantic salmon (Martinoli *et al.*,

1991; Ekstrom and Vanecek, 1992; Iigo *et al.*, 1994a, 1994b), the mechanism by which melatonin concentration is measured still remained unanswered.

Meissl *et al.* (1990) further proposed that there is a close interaction between the endocrine and neural outputs of the trout pineal. They found that melatonin exerted a dose related inhibition of the post-synaptic discharge from second-order neurones in the pineal. So, from the evidence presented, it would appear that pineal photoreceptor cells may have the ability to modulate both melatonin synthesis and the neural output from ganglion cells by means of a negative feedback mechanism.

3.1.5 The rhythmic release of melatonin by the pineal gland

Of all the teleost species shown to exhibit cyclic changes in melatonin secretion (extensively reviewed by Zachmann *et al.*, 1992), the rainbow trout, hammerhead shark and possibly the lamprey are unique in displaying a direct response to illumination (Gern and Greenhouse,1988; Boilliet *et. al.*, 1993; Okimoto and Stetson, 1995; Randall *et al.*, 1991, 1995). This has been confirmed using periods of constant darkness and varied combinations of light and dark both *in vivo* (Randall *et al.*, 1991; Max and Menaker, 1992; Alvarino *et al.*, 1993) and *in vitro* (Gern and Greenhouse, 1988; Gern *et al.*, 1992; Bolliet *et. al.*, 1993). The remainder of species so far studied are all capable of maintaining a rhythmic secretion of melatonin in the absence of any external entrainment. Goldfish, transferred to constant darkness from a 12L:12D cycle maintained a rhythmic secretion of melatonin corresponding to the previous 12L:12D regime for up to 7 days, after which time the rhythm disappeared and melatonin levels remained elevated (Aida *et al.*, 1989). The same results were

observed when the pineals were maintained in culture under constant darkness, with the melatonin rhythm lasting for up to 4 light/dark cycles (Aida *et. al.*, 1989; Kezuka *et al.*, 1989; Iigo *et al.*, 1991). Similarly, Falcon et. al. (1989) demonstrated an endogenous rhythmicity in melatonin and NAT levels from cultured pike pineals maintained under constant darkness. Both secretions were observed to fluctuate with a periodicity of greater than 24 h. Furthermore, short periods of darkness applied during the light phase did not induce an immediate rise in either NAT or melatonin. A rapid response was only observed during the subjective dark phase, indicating the presence of a refractory period during the subjective light phase. These results support the idea of an endogenous circadian oscillator located within the pineal of all species so far investigated with the exception of the but rainbow trout, hammerhead shark and lamprey which appear to release melatonin in direct response to the ambient photoperiod.

In mammals, circadian organisation is dominated by the suprachiasmatic nucleus (SCN) which is thought to act as a 'master pacemaker' controlling a wide array of behavioural and physiological rhythms (see review by Arendt, 1995). Cassone *et al.* (1993) suggested that mammalian pineal melatonin serves to synchronise the circadian oscillators within the SCN; photic information is thought to be transferred to the pineal through neural connections between the ocular photoreceptors and pinealocytes. As no area homologus to the mammalian SCN has been positively identified in fish (Holmqvist *et al.*, 1992), it is thought that their endogenous circadian rhythm is independent of the lateral eyes and may be generated from within the pineal

photoreceptor cells. This has been confirmed using dissociated pike pineal cells which, when separated into typical and modified photoreceptor cell fractions, behaved in the same way as the whole gland (Bolliet *et al.*, 1994). Under constant conditions, both fractions displayed an endogenous circadian rhythm of melatonin secretion which Bolliet suggested was the result of individual cellular circadian oscillators. According to Bolliet *et al.* (1994) the loss of the endogenous rhythm from complete pineals, both *in vitro* and *in vivo* (see, Aida et. al., 1989; Kezuka *et al.*, 1989; Iigo *et al.*, 1991), was probably due to a desynchronization (uncoupling) between the cellular 'clocks' or the gradual dampening of individual oscillators deprived of entrainment.

Although the presence of photoreceptors in the teleost pineal gland is strongly indicative of its photoreceptive function, it is also considered to act as a transducer for thermal information in ectotherms (Zachmann *et al.*, 1992; Tabata and Meissl, 1993; Falcon *et al.*, 1992, 1994). Early mammalian studies showed that melatonin administration and pinealectomy resulted in variations in the entrainment of daily core temperature rhythms. This was also reported for the rhythm of deep body temperature in the house sparrow (reviewed by Badia *et al.*, 1993). Seasonal variations in thermoregulation such as daily torpor cycles and hibernation are also known to be photoperiod dependant in some species, with melatonin implants altering non-shivering thermogenesis as regards brown adipose tissue in selected rodents (Badia *et al.*, 1993).

Temperature studies on ectotherm pineals have mainly concentrated on *in vivo* experiments involving amphibians and reptiles (Menaker and Wisner, 1983; Menaker, 1985; Janik and

Menaker, 1990; Paniagua *et al.*, 1990). However, some direct effects of temperature on pineal melatonin have been reported in fish. In both pike and whitesucker, low temperatures have been shown to dampen the circadian oscillators responsible for driving the endogenous melatonin rhythm (Zachmann *et al.*, 1992; Bolliet *et al.*, 1994). Falcon *et al.* (1994) found that in the absence of the light/dark cycle, the thermoperiod could be used to synchronise the circadian oscillators within the pineal of pike. These authors were able to show a direct correlation between the temperature and amplitude of the melatonin rhythm. Further evidence that temperature can affect the melatonin biosynthetic pathway was supplied by Falcon and Collin (1989) and Thibault *et al.* (1993) using cultured pike and trout pineals, respectively. Their work provided the first evidence that forskolin induced cAMP formation and activity, which in turn controls NAT production, is temperature dependent. These results have been supported by *in vivo* measurements of plasma melatonin at varying ambient temperatures which showed that, melatonin production in Atlantic salmon, pike and rainbow trout was greater at high temperatures (Falcon and Collin, 1989; Max and Menaker, 1992; Randall *et al.*, 1995). By contrast, Zachmann *et al.* (1992) found that peak plasma melatonin measurements were associated with lower temperatures.

Tabata and Meissl (1993) went as far as to propose temperature to be the primary entraining cue for the rainbow trout pineal melatonin rhythm. They based this on observations concerning ganglion cell activity at varying temperatures, in which the optimum operating temperature for pineal neural activity was measured at between 10 and 20°C. Above this range

ganglion spike discharges were significantly diminished, whilst below this, the neurones became insensitive to light. They also reported that at temperatures below 7°C the hormonal response to light disappeared altogether. However, this does not agree with the findings of this chapter (discussed later).

Temperature may play an important role in the regulation of melatonin production in the teleost pineal. However, photoperiod is still considered to the primary zeitgeber in the entrainment of endogenous rhythms, at least for species inhabiting temperate latitudes. For fish living near (or, 'close to') the equator this may not be the case as the annual photocycle displays very little seasonal change. Under these conditions, the seasonal rains or temperature would appear to be the most likely synchronising cue for reproductive development (Munro *et al.*, 1990).

The irradiance sensitivity of fish pineal glands has had little attention when compared to the spectral properties of the photoreceptors (discussed earlier). Max and Menaker (1992) reported that light pulses of increased intensity applied during the dark phase proportionately inhibited melatonin production. They found the minimum intensity required to elicit an inhibition response from cultured rainbow trout pineals was 8.6 log photons/cm^2/s. The sensitivity and responsiveness of the pineals were also found to vary with temperature and the intensity required to suppress melatonin production decreased as the culture temperature increased with the maximum response occurring at 21°C.

3.1.6 Aims of chapter 3

The work carried out in section 3.2.3 addressed 3 main objectives.

Firstly to assess the photic sensitivity of the salmonid pineal. This followed on from the work of Max and Menaker (1992) who revealed that the rainbow trout pineal displays a differential response to varied intensities of light phase. The work presented here again uses a range of light intensities but, unlike Max and Menaker's work, the dark phase is substituted by a low level of illumination by white light. As major concentrations of salmonids are to be found north of the Arctic circle where periods of constant light are experienced during much of the year it seems reasonable to assume that the synchronisation of spawning is entrained by the daily variation in light intensity rather than a light/dark cycle as occurs at more temperate latitudes. Therefore, in the present work, a series of experiments were designed to measure the response of rainbow trout pineals to varying light intensities and wavelengths when the dark phase was substituted by a period of reduced illumination. This was to assess whether, as expected, the variation in the photocycle intensity mediated the level of melatonin production rather than acting as a strict on/off stimulus resulting in a complete inhibition response induced by the light phase.

Secondly, the absence of an endogenous circadian rhythm of melatonin synthesis in rainbow trout is well documented (discussed earlier) but little is known for other salmonids. Therefore, investigations into the possibility of an endogenous component in the pineal of Atlantic salmon were conducted, despite there being little evidence of this *in vivo* (Randall *et. al.*, 1995). The unexpected opportunity to study the pineal of the Atlantic halibut also led to investigations into its ability to synthesise melatonin and the presence of endogenous rhythms. To

my knowledge neither of these species have previously been studied *in vitro*.

The third objective of this was to identify possible sources of plasma melatonin within Atlantic salmon and rainbow trout. This involved *in vivo* studies which required the development of a simple and effective method of pineal removal for both juvenile and adult salmonids. Once this had been achieved pinealectomy was employed alone, and in conjunction with enucleations, to assess the contribution of the pineal and retina to dark phase melatonin synthesis. This pinealectomy technique was subsequently used in the reproduction and smoltification experiments described in chapters 4 and 5.

3.2.1. The effects of temperature on the rate of melatonin secretion and the longevity of rainbow trout pineal glands cultured under a 12L:12D photoperiod at a constant 5, 10 or 15 °C.

3.2.1.1 Objectives

This experiment was designed to assess the optimum temperature for salmonid pineal culture with respect to the amplitude of the melatonin profile produced by a 12L:12D photoperiod and for the survival of the pineal glands.

3.2.1.2 Material and Methods

To obtain the pineals for culture, female rainbow trout were first killed by decapitation. If only the pineal was required a longitudinal section was made at a level dorsal to the orbits. This traversed through the brain which could then be lifted with forceps to expose the pineal gland. After the efferent arteries had been incised, the pineal tract could then be cut at the base of the diencephalon and the pineal removed.

The pineals were removed from female trout (weight approximately 120g) and cultured within a flow-through system (section 2.8.2) under a 12L:12D photoperiod at 5, 10 or 15°C (N=4 in each case). Although melatonin was measured for only 2 complete light cycles, the pineals were maintained in culture until signs of necrosis were observed after which the pineals were removed and studied under a light microscope (section 2.8.3) to assess the viability of the pineal glands. Samples were stored at -70°C and assayed for melatonin within 4 months (section 2.4).

3.2.1.3 Results

The amplitude of the melatonin rhythm from pineals cultured at 5°C was by far the lowest (40±2 pg/ml of culture media in the light phase, 920±137 pg/ml in the dark phase) of the three temperatures. Light and dark phase levels of melatonin were significantly different ($P<0.05$) at each temperature (Figure 3.4). Dark phase levels of melatonin at 5°C were also significantly lower ($P<0.05$) than those at 10°C (400±65 pg/ml light phase, 6565±383 pg/ml dark phase), which in turn were significantly lower ($P<0.05$) than those at 15°C (295±64 pg/ml light phase, 9800±482 pg/ml dark phase). Only light phase levels at 5°C and 10°C showed significant variation ($P<0.05$).

In terms of prolonging the life of the pineals, 5°C was significantly more successful than either 10°C or 15°C ($P<0.05$), and pineals survived significantly longer at 10°C than 15°C ($P<0.05$) The average period of apparent viability in culture of a pineal cultured at 5°C was 12.5 ±0.29 days compared to 7.75±0.47 days at 10°C and 3 days at 15°C.

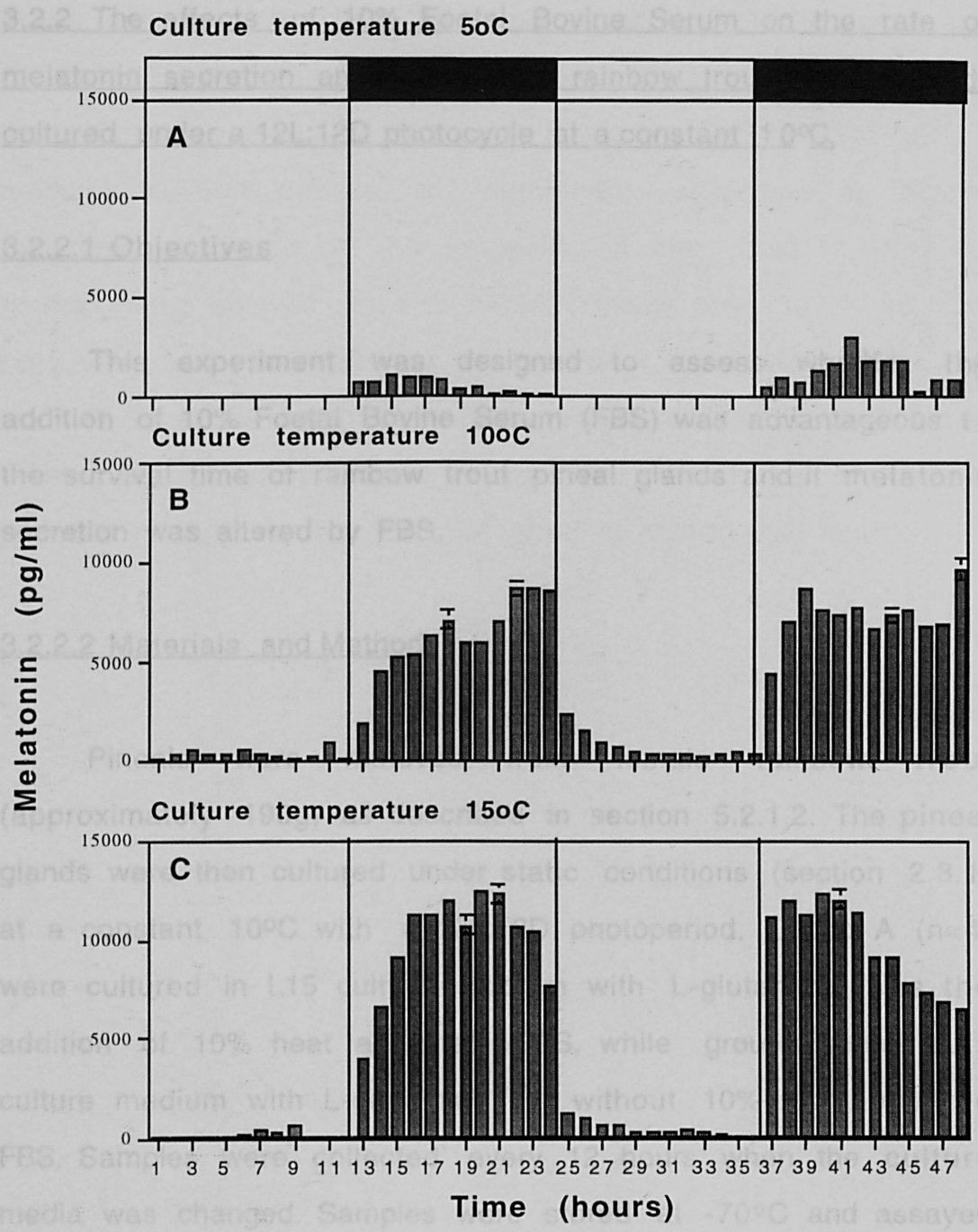


Figure 3.4

Changes in melatonin release (mean ±1SEM, n=4) from rainbow trout pineal glands cultured under: A 5oC; B 10oC; and C 15oC. All pineals were maintained on a 12L:12D photoperiod (indicated above graph A). Unless shown the SEM values were too small to be depicted.

3.2.2 The effects of 10% Foetal Bovine Serum on the rate of melatonin secretion and longevity of rainbow trout pineal glands cultured under a 12L:12D photocycle at a constant 10°C.

3.2.2.1 Objectives

This experiment was designed to assess whether the addition of 10% Foetal Bovine Serum (FBS) was advantageous to the survival time of rainbow trout pineal glands and if melatonin secretion was altered by FBS.

3.2.2.2 Materials and Methods

Pineals were removed from female rainbow trout (approximately 190g) as described in section 5.2.1.2. The pineal glands were then cultured under static conditions (section 2.8.1) at a constant 10°C with a 12L:12D photoperiod. Group A (n=4) were cultured in L15 culture medium with L-glutamine plus the addition of 10% heat activated FBS, while group B used L15 culture medium with L-glutamine but without 10% heat activated FBS. Samples were collected every 12 hours when the culture media was changed. Samples were stored at -70°C and assayed for melatonin (section 2.4) within 4 months of collection.

3.2.2.3 Results

Significant variation ($P<0.05$) was observed between light and dark phase melatonin levels within groups. Group A produced a light phase level of 535±54 pg/ml of culture media and a dark

phase level of 4975±1542 pg/ml compared to 171±26 pg/ml and 6702±1189 pg/ml respectively from group B (Figure 3.5). However, the addition of 10% heat activated FBS to L15 culture medium did not provide any significant advantage to either melatonin secretion or the longevity of the pineal glands as neither group showed signs of necrotic tissue after 132 hours and both were still responding to the light/dark cycle at this time.

It must be noted that both groups showed significantly higher dark phase melatonin levels after 132 hours in culture, possible reasons for this are discussed in section 3.3.

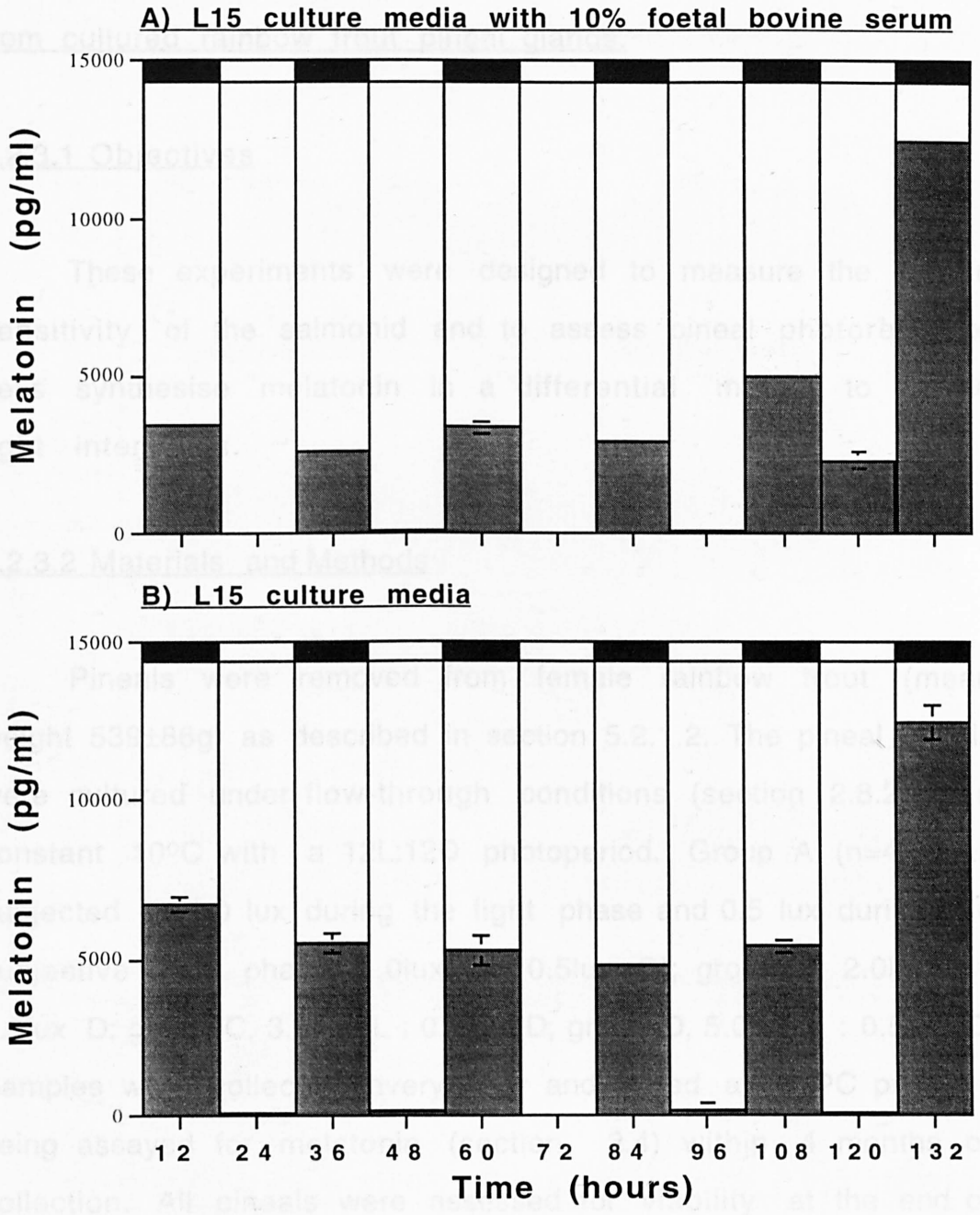


Figure 3.5

Changes in melatonin secretion from dissociated rainbow trout pineal organs maintained within static conditions under a 12L:12D photoperiod in L15 with L-glutamine culture media either with (A) or without (B) 10% foetal bovine serum. Unless shown the SEM values were too small to be depicted.

3.2.3 The effects of light intensity on melatonin secretion rates from cultured rainbow trout pineal glands.

3.2.3.1 Objectives

These experiments were designed to measure the photic sensitivity of the salmonid and to assess pineal photoreceptor cells synthesise melatonin in a differential manner to varied light intensities.

3.2.3.2 Materials and Methods

Pineals were removed from female rainbow trout (mean weight 539±86g) as described in section 5.2.1.2. The pineal glands were cultured under flow-through conditions (section 2.8.2) at a constant 10°C with a 12L:12D photoperiod. Group A (n=4) was subjected to 1.0 lux during the light phase and 0.5 lux during the subjective dark phase (1.0lux L : 0.5lux D); group B, 2.0lux L : 0.5lux D; group C, 3.0lux L : 0.5lux D; group D, 5.0lux L : 0.5lux D. Samples were collected every hour and stored at -70°C prior to being assayed for melatonin (section 2.4) within 4 months of collection. All pineals were assessed for viability at the end of the experiments as described in section 2.8.3.

3.2.3.3 Results

All groups except group B showed significant variation ($P<0.05$) between light and dark phase melatonin samples (Figure 3.6). A Dunn's Test carried out on the light and dark samples from

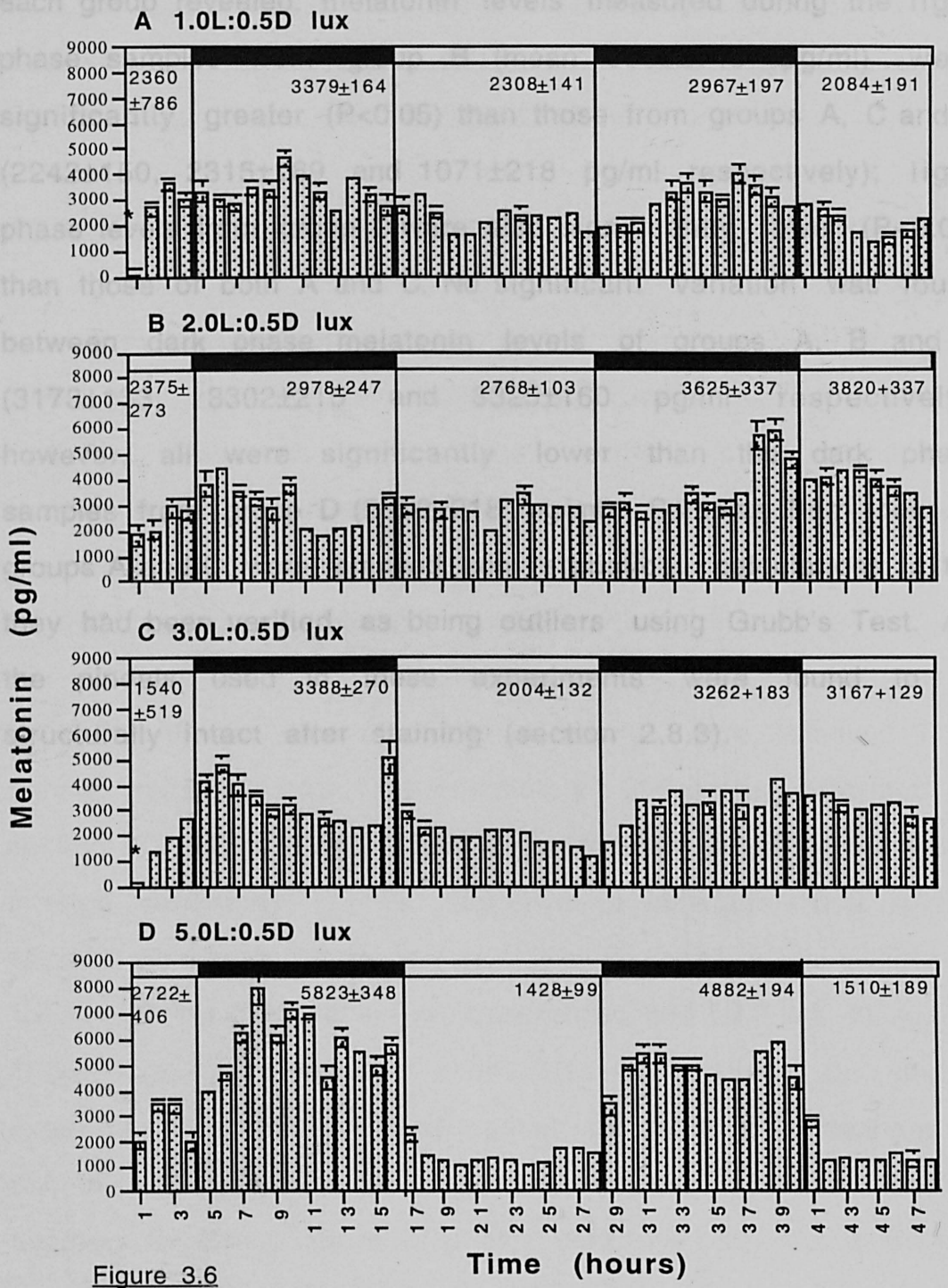


Figure 3.6

Changes in melatonin secretion (mean±1SEM, n=4) from rainbow trout pineals maintained under a 12L:12D photoperiod under a range of intensities at a constant 10∞C. Outlier values were selected using Grubb's Test and are indicated by *. Mean melatonin levels for each phase are displayed within each cycle. Unless shown the SEM values were too small to be depicted.

each group revealed: melatonin levels measured during the light phase samples from group B (mean 3053±146 pg/ml) were significantly greater ($P<0.05$) than those from groups A, C and D (2242±150, 2315±169 and 1071±218 pg/ml respectively); light phase levels from group D were also significantly lower ($P<0.05$) than those of both A and C. No significant variation was found between dark phase melatonin levels of groups A, B and C (3173±133, 3302±215 and 3325±160 pg/ml respectively), however, all were significantly lower than the dark phase samples from group D (5352±218 pg/ml). Samples from hour 1, groups A and C, were removed from statistical calculations after they had been verified as being outliers using Grubb's Test. All the pineals used in these experiments were found to be structurally intact after staining (section 2.8.3).

3.2.4 An *in vivo* and *in vitro* study to assess whether illumination at wavelengths above 640nm can inhibit melatonin secretion from rainbow trout pineal gland photoreceptors.

3.2.4.1 Objectives

Previous workers have reported that the rainbow trout pineal gland is unable to respond, through melatonin inhibition, to 'red' light. This study examined the sensitivity of the pineal both *in vivo* and under culture conditions to wavelengths above 640nm.

3.2.4.2 Materials and Methods

For the *in vitro* study pineal glands were removed from female rainbow trout (approximately 260g) as described in section 3.2.1.2. The pineal glands were maintained under flow-through conditions (section 2.8.2) at a constant 10°C with a 12L:12D photoperiod. Group A (n=4) was exposed to white light of 1.0 lux during the subjective dark phase and 50.0 lux at a λ of 650-800nm during the light phase. This was achieved by using an experimental grade longpass optical filter with a stopband of 650nm and passband of 800nm (Edmund Scientific Optics, supplied by Ealing Electro-Optics, Watford, U.K.). Group B (n=4) was exposed to white light of 1.0 lux during the subjective dark phase and white light of 50.0 lux during the light phase. Samples were collected every hour and stored at -70°C prior to being assayed for melatonin (section 2.4) within 4 months of collection. All pineals were assessed for viability at the end of the experiments as described in section 2.8.3.

The *in vivo* study also used fish of approximately 260g which were maintained as described in section 2.1.1. At the time the experiment took place the ambient water temperature was 10°C. Group A (n=28) was maintained under a 12L:12D photoperiod with the light phase supplied by a tungsten halogen bulb fitted with a Kodak safelight filter producing 120 lux at the water surface (λ,668-800nm). Group B (n=28) was held under similar conditions except that the light phase was supplied by a tungsten halogen bulb supplying 120 lux at the water surface without the red filter. Both groups were acclimated to the tank conditions for 2 weeks prior to blood sampling which took place mid-light and mid-dark phase (section 2.1.3). Samples were stored at -70°C before being assayed for melatonin as described in section 2.4.

3.2.4.3 Results

Both groups of pineal glands in culture (Figure 3.7) showed significant differences ($P<0.05$) between light and dark phase melatonin levels but no significant difference was observed between groups A and B during the dark phase (10328±459 pg/ml and 12200±193 pg/ml, respectively). When the lights were on the melatonin level in group A, with the red light, (3583±150 pg/ml) was significantly greater ($P<0.05$) than in group B (1891±99 pg/ml). All the pineals appeared to be in good condition when observed under the microscope at the end of the experiments.

In vivo studies using red and white light sources revealed a significant variation in plasma melatonin levels within the groups when the light and dark phases were compared ($P<0.05$).

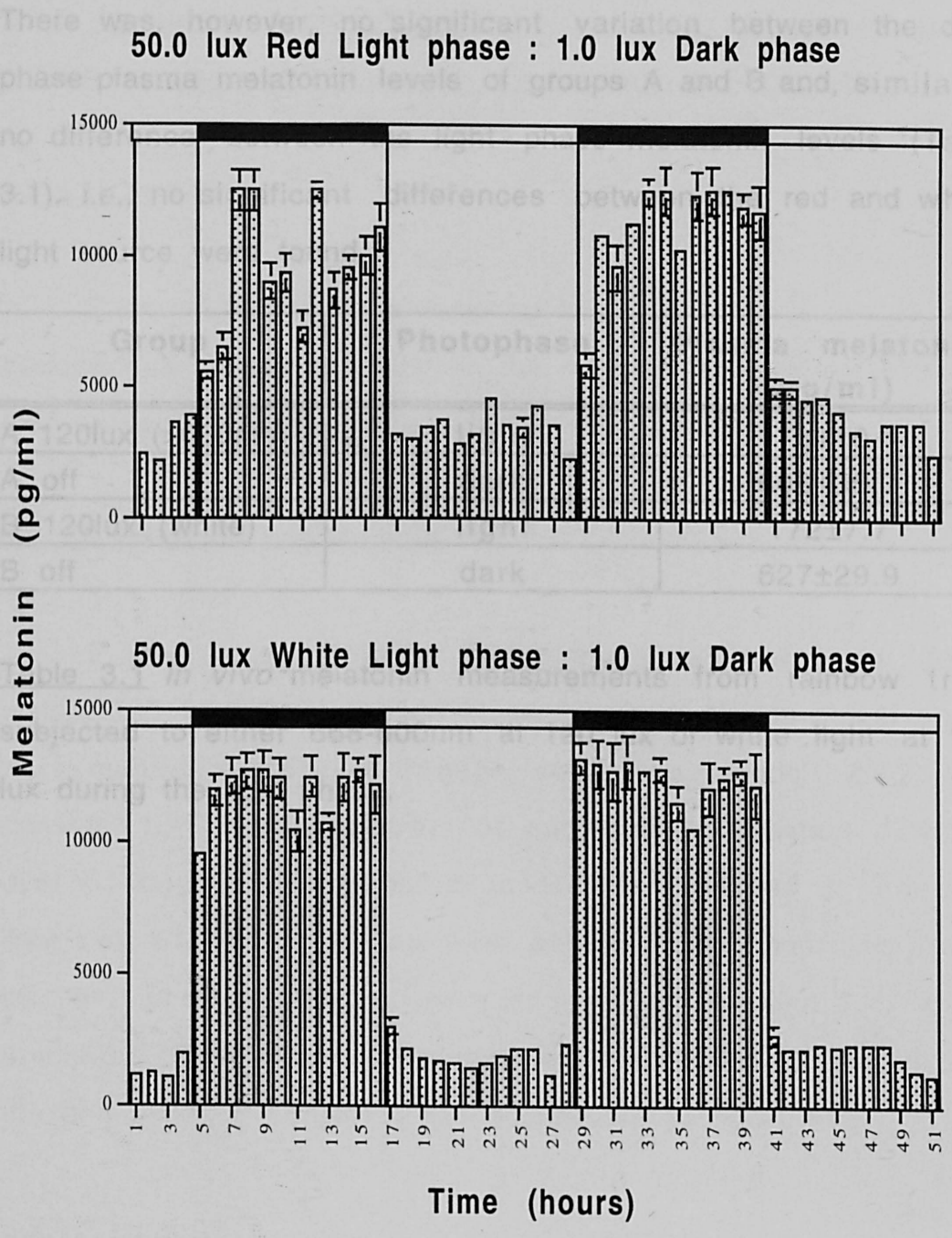


Figure 3.7

Changes in melatonin secretion (mean±1SEM, n=4) from rainbow trout pineal glands maintained on a 12L:12D photoperiod at a constant 10oC. Light phases were supplied at either 50 lux white light or 50 lux at wavelengths between 660 and 800nm; the dark phase was supplied at 1.0 lux white light in both cases. Unless shown the SEM values were too small to be depicted.

There was, however, no significant variation between the dark phase plasma melatonin levels of groups A and B and, similarly, no difference between the light phase melatonin levels (Table 3.1), i.e., no significant differences between the red and white light source were found.

Group	Photophase	Plasma melatonin (pg/ml)
A 120lux (>660nm)	light	178±10.1
A off	dark	606±35.7
B 120lux (white)	light	172±7.7
B off	dark	627±29.9

Table 3.1 *In vivo* melatonin measurements from rainbow trout subjected to either 668-800nm at 120 lux or white light at 120 lux during the light phase.

3.2.5 Melatonin secretion from rainbow trout pineal glands cultured under continuous light and constant dark conditions.

3.2.5.1 Objectives

These cultures were designed to confirm the absence of an endogenous melatonin rhythm in the rainbow trout pineal when no external entrainment was provided.

3.2.5.2 Materials and Methods

Pineals were removed from female rainbow trout (mean weight 603±21g) as described in section 3.2.1.2. The pineal glands were cultured under flow-through conditions (section 2.8.2) at a constant 10°C. Group A (n=4) was subjected to constant darkness over 61 hours, while group B (n=4) was subjected to constant light over 61 hours. Samples were collected every hour and stored at -70°C prior to being assayed for melatonin (section 2.4) within 4 months of collection. All pineals were assessed for viability at the end of the experiments as described in section 2.8.3.

3.2.5.3 Results

Figure 3.8 shows the large variation between the melatonin produced by rainbow trout pineals maintained under constant darkness (6623±195) and constant light (139±13). Apart from the significant variation between light and dark measurements ($P<0.05$) melatonin secretion from

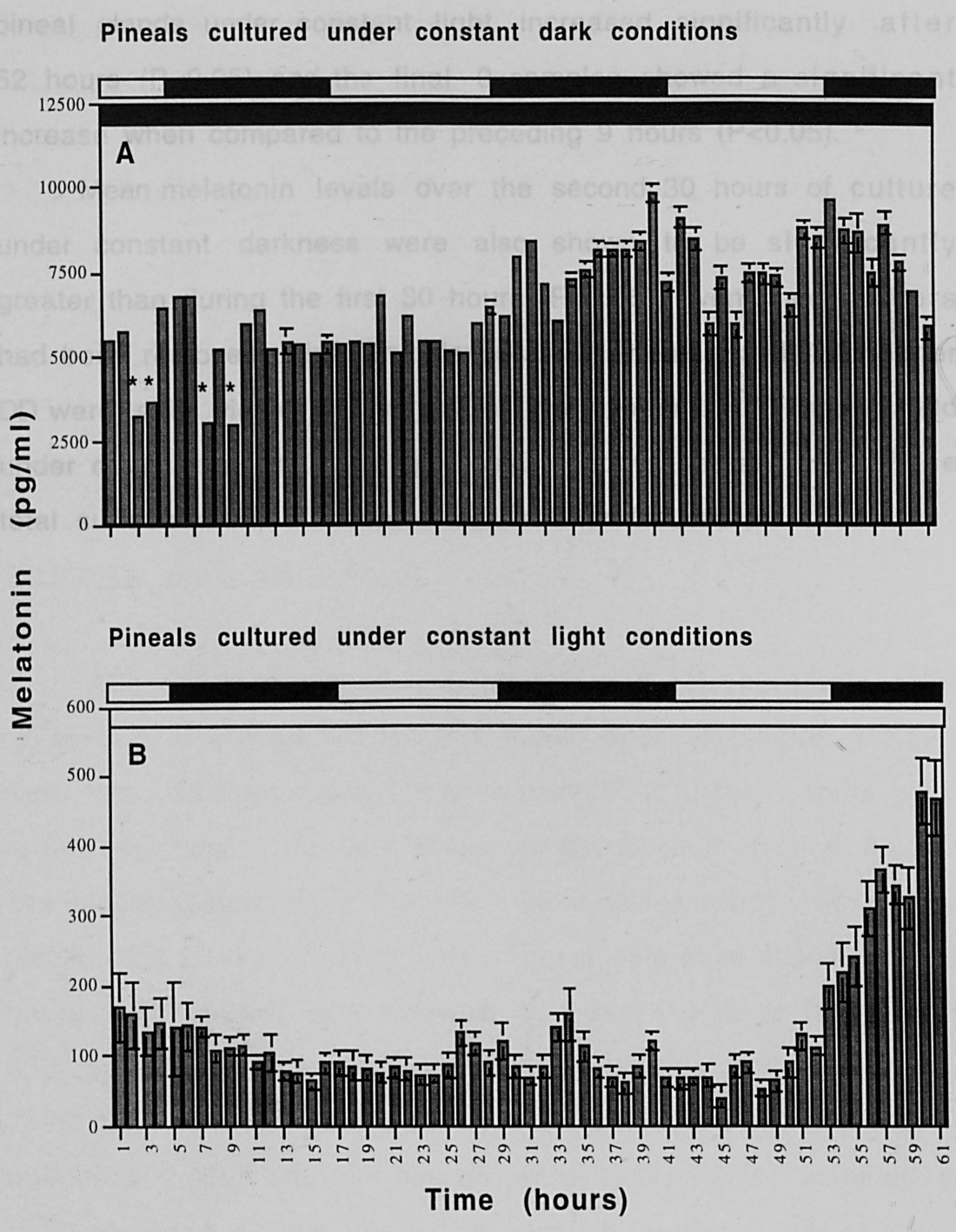


Figure 3.8

Changes in melatonin secretion (mean ± 1SEM) from rainbow trout pineal glands cultured under constant dark (A) and constant light (B) conditions at a constant 10∞C. The photoperiod under which the fish were maintained prior to decapitation is depicted above each graph and outlier values selected by Grubb's Test are shown by * . Unless shown the SEM values were too small to be depicted.

pineal glands under constant light increased significantly after 52 hours ($P<0.05$) and the final 9 samples showed a significant increase when compared to the preceding 9 hours ($P<0.05$).

Mean melatonin levels over the second 30 hours of culture under constant darkness were also shown to be significantly greater than during the first 30 hours ($P<0.05$), even once outliers had been removed using Grubb's Test. All pineals maintained under DD were still viable at the end of the experiment. Pineals held under continuous light developed areas (approximately 10% of the total surface area) of necrotic tissue after 61 hours.

3.2.6 Do endogenous rhythms exist within cultured Atlantic salmon pineal glands maintained under constant conditions ?

3.2.6.1 Objectives

Although the pineal gland of the rainbow trout has been reported to lack an endogenous rhythm of melatonin secretion, no *in vitro* work has yet been conducted on the Atlantic salmon pineal gland.

3.2.6.2 Materials and Methods

Atlantic salmon were maintained on a 12L:12D photoperiod (described in section 2.1.1) for 1 month prior to culture. Pineals were removed from 4 fish (mean weight 97.9±10.3g) 3 hours prior to the beginning of the dark phase, as described in section 3.2.1.2. The pineal glands were cultured under flow-through conditions (section 2.8.2) at a constant 10°C. The pineals were exposed to 16 hours of continuous dark followed by 9 hours of light (see Figure 3.9). Samples were collected every 30 minutes and stored at -70°C prior to being assayed for melatonin (section 2.4) within 4 months of collection. All pineals were assessed for viability at the end of the experiments as described in section 2.8.3.

3.2.6.3 Results

Mean light and dark phase levels (509±256 pg/ml and 6627±113pg/ml, respectively) showed significant variation

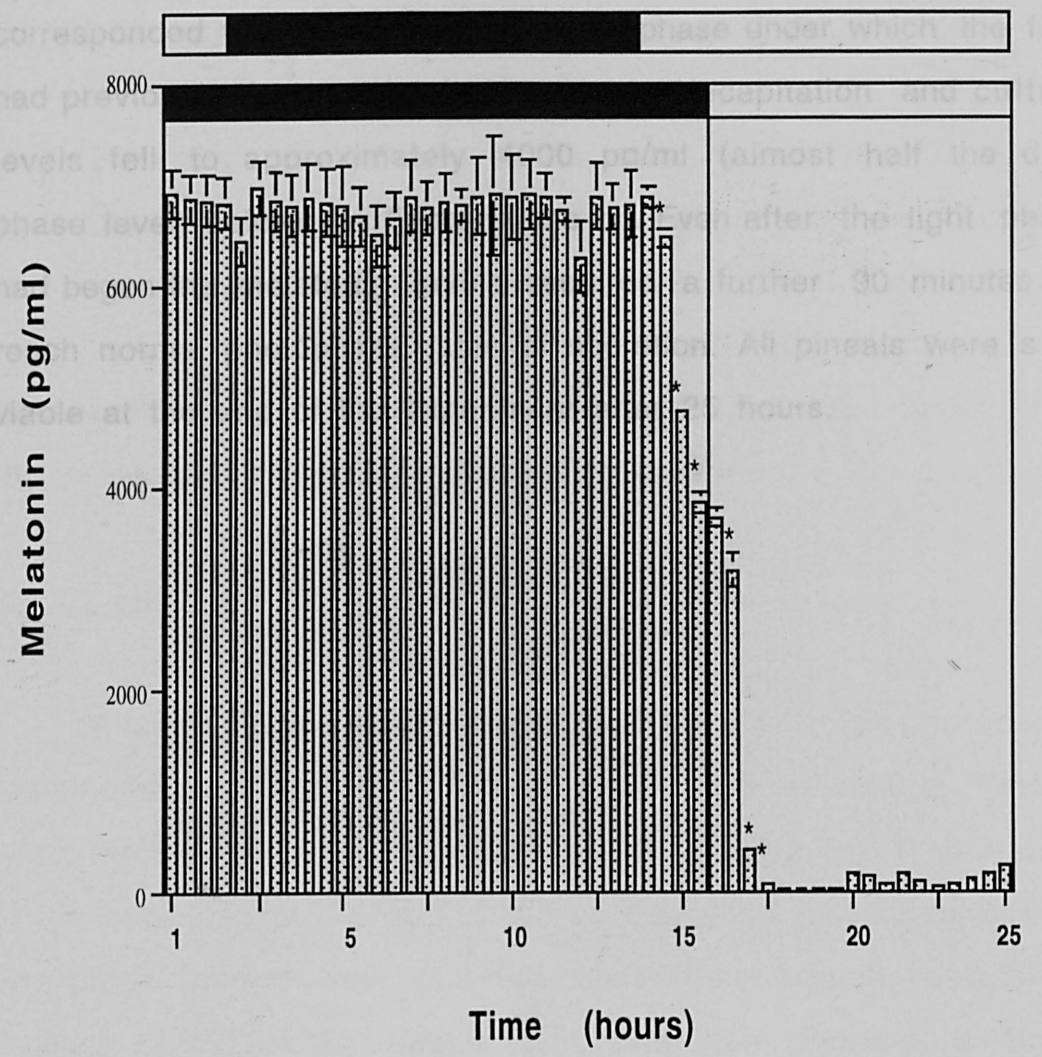


Figure 3.9

Changes in melatonin secretion (mean ±1SEM) by salmon pineal glands maintained under a 16D:9L photoperiod at a constant 10oC. Previous photoperiod prior to culture is depicted above and melatonin measurements significantly different ($P<0.05$) from the preceeding value are depicted by *. Unless shown the SEM values were too small to be depicted.

($P<0.05$). Interestingly, melatonin levels began to decrease 2 hours before the light phase was initiated (significant decreases are indicated by an asterisk (*) in Figure 3.9). This decrease corresponded with the end of the dark phase under which the fish had previously been maintained prior to decapitation and culture levels fell to approximately 4000 pg/ml (almost half the dark phase level) before the lights came on. Even after the light phase had begun the melatonin levels required a further 90 minutes to reach normal photophase rates of secretion. All pineals were still viable at the end of the experiment after 25 hours.

3.2.7 The effects of constant darkness on the melatonin rhythm produced by cultured Atlantic salmon pineal glands.

3.2.7.1 Objectives

Following the initial results from experiment 3.2.6 this experiment was designed to further investigate the possible presence of an endogenous oscillator within the Atlantic salmon pineal gland by maintaining the pineals over a longer time period so encompassing an increased number of light/dark cycles from the previously entraining (12L:12D) photoperiod.

3.2.7.2 Materials and Methods

Atlantic salmon were maintained on a 12L:12D photoperiod (described in section 2.1.1) for 1 month prior to culture. Pineals were removed from 4 fish (mean weight 100±12.6g) 1 hour prior to the beginning of the light phase as described in section 3.2.1.2. The pineal glands were cultured under flow-through conditions (section 2.8.2) at a constant 10°C. The new photoperiod under which the pineals were maintained consisted of 5 hours of light at 50 lux followed by 51 hours of constant darkness, then a further 9 hours of light, to ensure that the pineals were still able to respond to illumination (see Figure 3.10). Samples were collected every hour and stored at -70°C prior to being assayed for melatonin (section 2.4) within 4 months of collection. All pineals were assessed for viability at the end of the experiments as described in section 2.8.3.

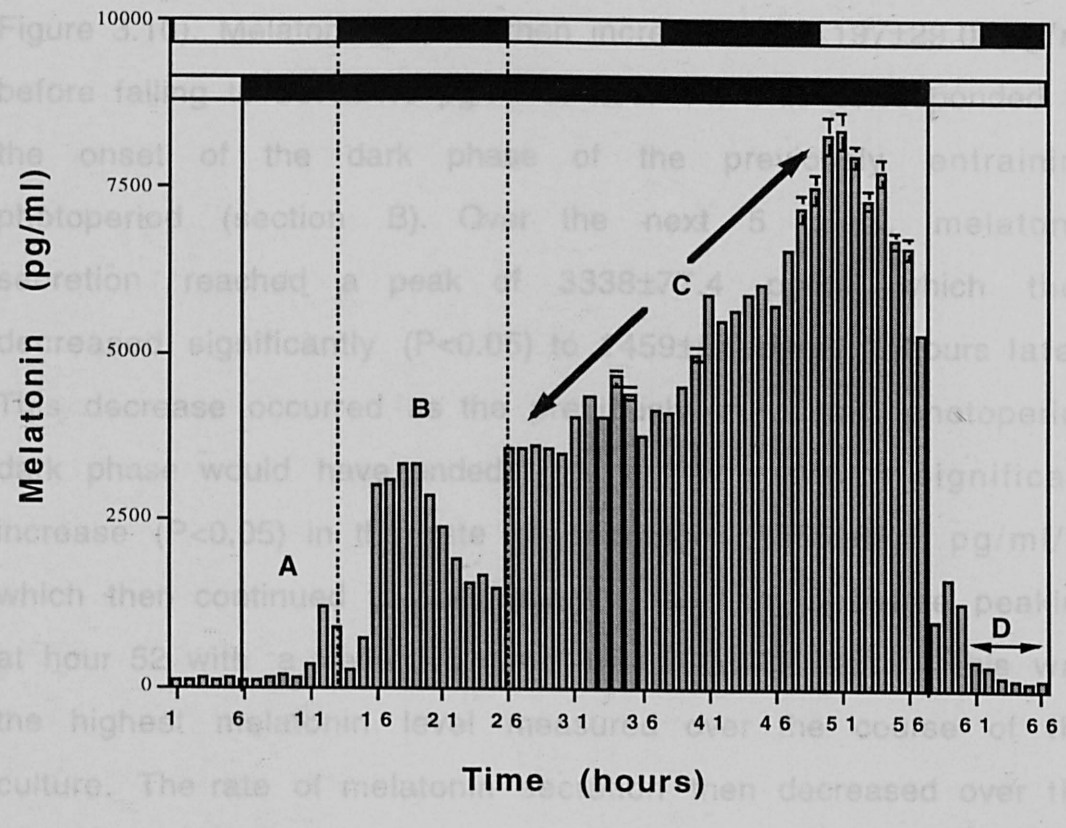


Figure 3.10

Changes in melatonin secretion (mean ± 1SEM) from Atlantic salmon pineals maintained at a constant 10oC under a L:D:L (5:51:9) photoperiod. The photoperiod under which the fish were maintained previous to decapitation is depicted above. Sections A, B, C and D are discussed in the text. Unless shown the SEM values were too small to be depicted.

3.2.7.3 Results

During the initial 5 hours of light, pineal melatonin levels remained low (118±13.1 pg/ml). Surprisingly, melatonin production did not increase significantly immediately after the illumination was switched off (Figure 3.10) and the following 5 hours of darkness had a mean level of 138±18.1 pg/ml (section A, Figure 3.10). Melatonin levels then increased to 1197±29.0 pg/ml before falling to 257±21.0 pg/ml at hour 14. This corresponded to the onset of the dark phase of the previously entraining photoperiod (section B). Over the next 5 hours, melatonin secretion reached a peak of 3338±77.4 pg/ml which then decreased significantly ($P<0.05$) to 1459±54 pg/ml 6 hours later. This decrease occurred as the previously entraining photoperiod dark phase would have ended. The next hour saw a significant increase ($P<0.05$) in the rate of secretion (3555±89.8 pg/ml/h) which then continued to rise steadily (section C) before peaking at hour 52 with a melatonin level of 8360±268 pg/ml. This was the highest melatonin level measured over the course of the culture. The rate of melatonin secretion then decreased over the next 6 hours before reaching a significantly lower rate ($P<0.05$) of 5271±96 pg/ml/h. This decrease coincided with the onset of a light phase from the previous photoperiod under which the fish had been maintained. When the light was again switched on at hour 58, there was an immediate and significant decrease ($P<0.05$) to 1020±53 pg/ml followed by a further decrease to light phase levels (mean 180±40.5 pg/ml, section D) not significantly different from the initial 5 hours, thus

demonstrating that the final 9 hours of light were successful in inhibiting melatonin secretion.

The melatonin inhibition over the final 9 hours together with cell staining techniques confirmed the viability of the pineals after 66 hours of culture.

3.2.8 The presence of circadian rhythms of melatonin secretion in the pineal gland of the Atlantic halibut.

3.2.8.1 Objectives

As no previous work had been carried out on melatonin secretion from the pineal gland of Atlantic halibut, when the opportunity arose to work on a limited number of halibut pineals, they were investigated for a light/dark rhythm of melatonin secretion and the presence of an endogenous circadian rhythm.

3.2.8.2 Materials and Methods

The pineal glands of 6 halibut broodstock from the Sea Fish Industry Authority's Marine Research Unit at Ardtoe (latitude, 56.75°N) were collected on the 10th September, six months prior to normal spawning time. The fish, with a mean weight of 15.5kg, had previously been maintained under a natural photoperiod (approximately 12L:12D in September) and ambient temperature in 4m diameter tanks. The halibut were decapitated and a coronal section made, starting anterior to the eyes, in order to expose the brain. The brain tissue was then lifted from the skull cap to expose the pineal, the stalk cut at the point of attachment to the diencephalon and the pineal placed in culture media on ice (L15 medium with L-glutamine plus 10% heat activated FBS). Pineals were transferred to 24-well culture plates, each well containing 1ml of fresh medium and acclimated at 10°C in an atmosphere of 100% air for 2h prior to the commencement of the trial. At 2000hrs, the pineals were again transferred to fresh medium and

maintained under either a 12L:12D photoperiod (n=3; lights on 0800-2000hrs, intensity 160 lux supplied by a tungsten-halogen lamp) or DD (n=3) within a light-proof area of the culture cabinet. Pineals were subsequently transferred to fresh media every 12h. Additional wells containing 1ml of culture media were exposed to either light or dark conditions for 12h to serve as blank controls for the radioimmunoassay. Samples of medium were stored at -70°C immediately after collection until assayed for melatonin (section 2.4).

3.2.8.3 Results

Pineal glands maintained under L12:D12 (Figure 3.11a) exhibited a melatonin profile with peak values during the dark phase (range 1230-2600 pg/ml) and reduced levels associated with the light phase (range 489-1002 pg/ml). Under DD, the rhythm in pineal melatonin secretion persisted indicating a free-running rhythm (Figure 3.11b). Over the culture period, differences in the levels of melatonin between night (or subjective night) and day (or subjective day) were significant ($P<0.05$) in both groups. Although the LD group exhibited slightly higher levels of melatonin, individual time points showed no significant differences from their counterparts in the DD group ($P>0.05$).

Both groups also showed a significant reduction ($P<0.05$) in the levels of dark (or subjective dark) phase melatonin over the 72 hours

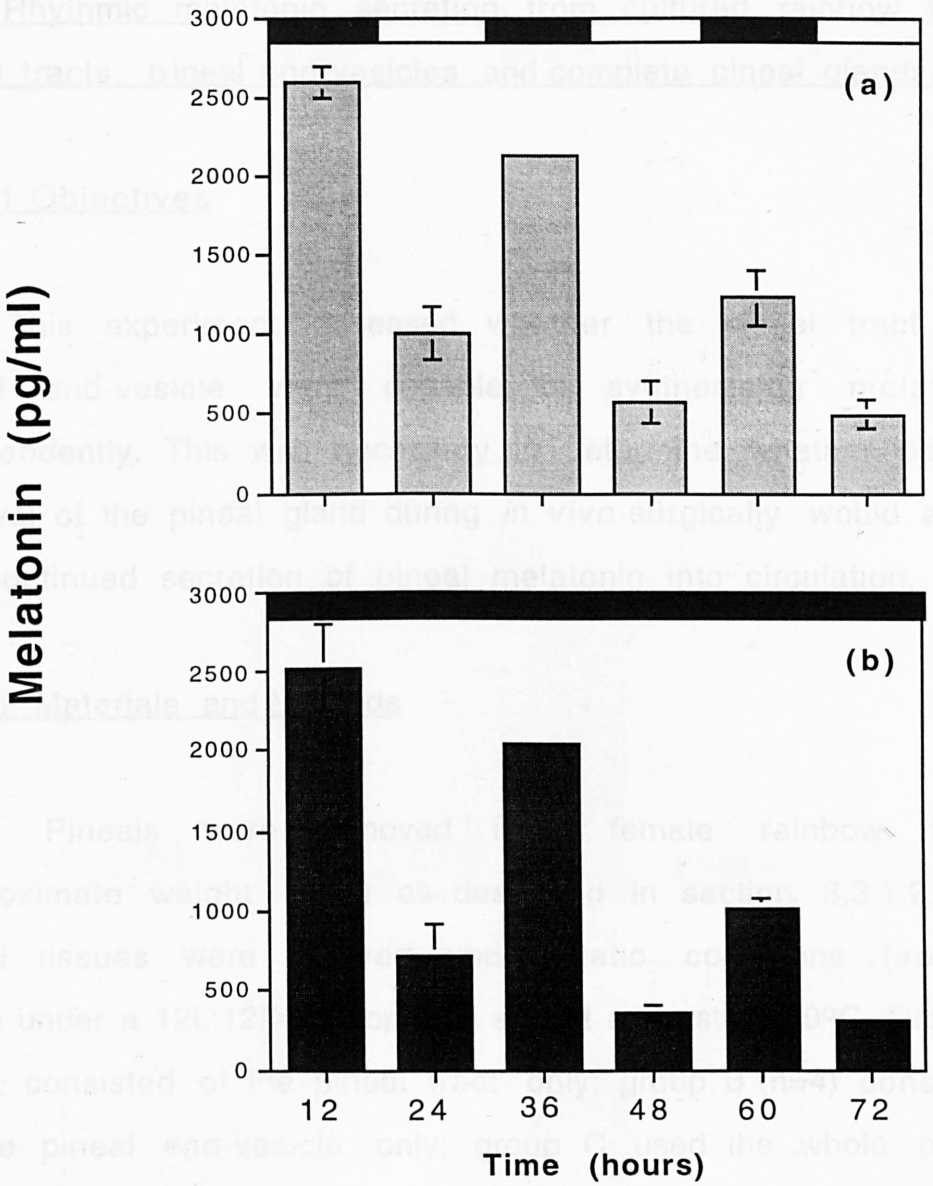


Figure 3.11

Changes in melatonin secretion (mean ±1SEM, n=3) from Atlantic halibut pineal glands maintained for 72 hours under a) 12L:12D and b) constant darkness at a constant 10oC. Photoperiods are shown above graphs. Unless shown the SEM values were too small to be depicted.

3.2.9 Rhythmic melatonin secretion from cultured rainbow trout pineal tracts, pineal end-vesicles and complete pineal glands.

3.2.9.1 Objectives

This experiment assessed whether the pineal tract and pineal end-vesicle were capable of synthesising melatonin independently. This was necessary to determine whether partial removal of the pineal gland during *in vivo* surgically would allow the continued secretion of pineal melatonin into circulation.

3.2.9.2 Materials and Methods

Pineals were removed from female rainbow trout (approximate weight 250g) as described in section 3.2.1.2. The pineal tissues were cultured under static conditions (section 2.8.1) under a 12L:12D photoperiod and at a constant 10°C. Group A (n=4), consisted of the pineal tract only; group B (n=4) consisted of the pineal end-vesicle only; group C, used the whole pineal (end-vesicle plus pineal tract) in the culture. Samples were collected every hour and stored at -70°C prior to being assayed for melatonin (section 2.4) within 4 months of collection.

3.2.9.3 Results

All groups showed significant variations between mean light and dark phase melatonin levels during the first 48 hours of culture ($P<0.05$), Figure 3.12. However, if individual sample times were compared no significant differences were observed in any of

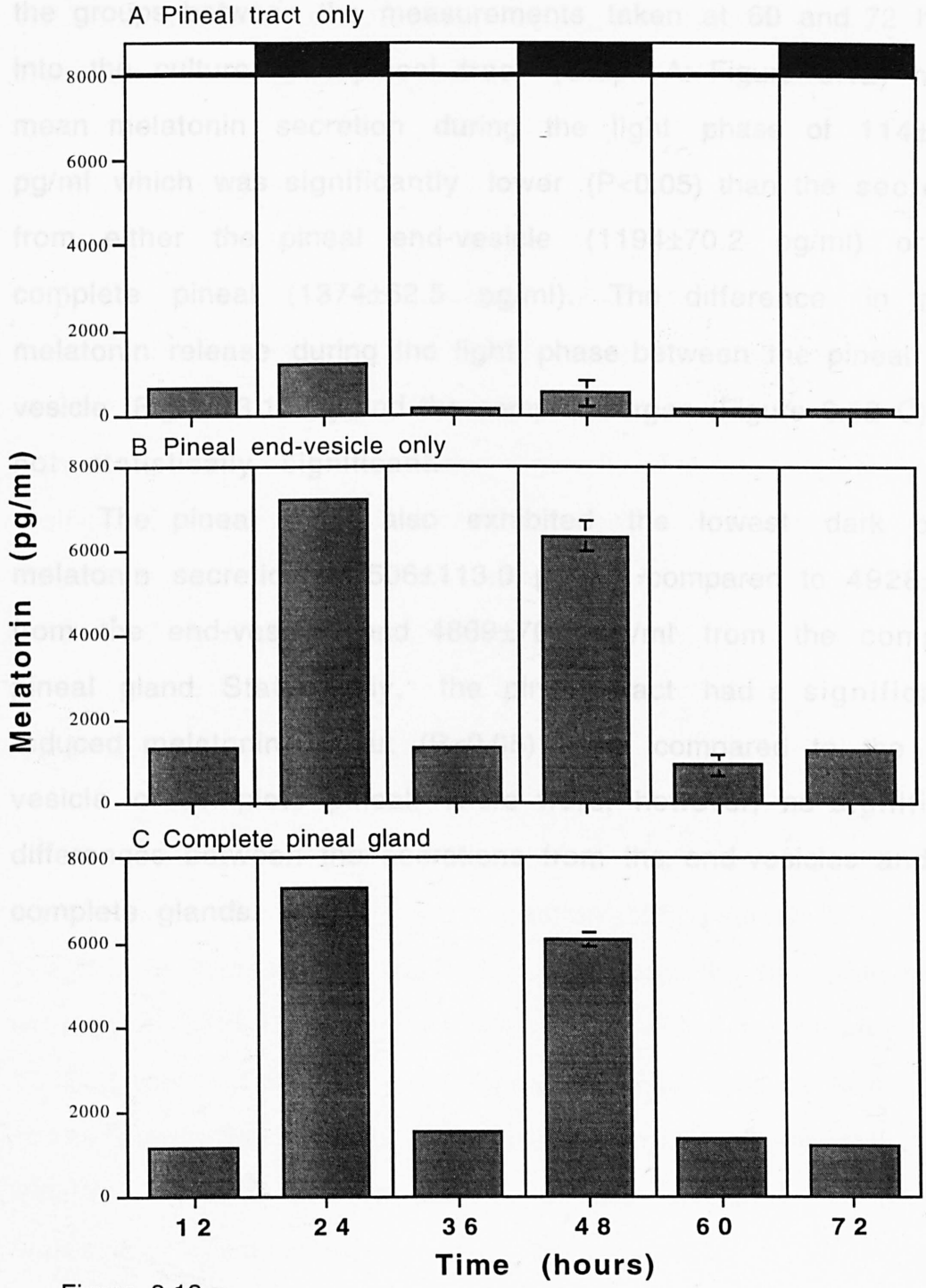


Figure 3.12

Changes in melatonin secretion (mean ±1SEM) from: (A) cultured rainbow trout pineal tract; (B) pineal end vesicle; and (C) complete pineal gland maintained at a constant 10°C under a 12L:12D photoperiod. Unless shown the SEM values were too small to be depicted.

the groups between the measurements taken at 60 and 72 hours into the culture. The pineal tract (Graph A: Figure 3.12) had a mean melatonin secretion during the light phase of 114±15.0 pg/ml which was significantly lower ($P<0.05$) than the secretion from either the pineal end-vesicle (1194±70.2 pg/ml) or the complete pineal (1374±62.5 pg/ml). The difference in mean melatonin release during the light phase between the pineal end-vesicle (Figure 3.12 B) and the complete organ (Figure 3.12 C) was not statistically significant.

The pineal tract also exhibited the lowest dark phase melatonin secretion of 506±113.0 pg/ml, compared to 4928±801 from the end-vesicle and 4869±706 pg/ml from the complete pineal gland. Statistically, the pineal tract had a significantly reduced melatonin output ($P<0.05$) when compared to the end-vesicle or complete pineal. There were, however, no significant differences between the secretions from the end-vesicles and the complete glands.

3.2.10 The effect of pineal removal on circulating melatonin levels in Atlantic salmon parr

3.2.10.1 Objectives

The Atlantic salmon pineal synthesises melatonin in a distinct diel rhythm with elevated levels in response to the hours of darkness. As both smoltification and maturation in Atlantic salmon are known to be under the influence of photoperiod, a method of pineal removal was developed to allow future *in vivo* long-term experiments to be conducted into the pineal's influence on the timing of these seasonal events.

3.2.10.2 Materials and Methods

Thirty one-year old salmon parr (Otterferry/Mowi cross) were maintained (described in section 2.1.1) under a constant 12L:12D photoperiod. At the time of surgery, the fish weighed an average of 55.3 ± 8.3 g and the ambient water temperature was 13°C. Groups of ten fish were pinealectomised (see section 2.2.1), sham-pinealectomised or left intact. After an interval of 3 months, external differentiation of the operated groups was impossible. Twelve weeks after the operation a 0.5 ml blood sample was taken from each fish 3 hours into the dark phase (section 2.1.3). To allow complete recovery and a return to ambient stress levels from the first blood sample, the parr were left for 14 days before this procedure was repeated 3 hours into the light phase. Samples were stored at -70°C prior to assay for

melatonin (section 2.4). The absence of the pineal was confirmed by dissection at autopsy 15 months after the operation.

3.2.10.3 Results

Control and sham-pinealectomy groups exhibited diel fluctuations in melatonin levels (Figure 3.13). However, after removal of the pineal gland, dark phase levels of circulating melatonin were significantly reduced ($P< 0.001$) from means of 598±19.3 pg/ml and 612±29.7 pg/ml in control and sham-pinealectomised groups to 96±6.5pg/ml in pinealectomised fish. Light phase levels of control, sham-pinealectomised and pinealectomised groups did not differ significantly, having means of 63.3±4.5 pg/ml, 70.3±3.2 pg/ml and 64.2±1.3 pg/ml, respectively. Interestingly, a significant variation ($P<0.01$) between day and night-time levels of melatonin remained following pinealectomy. Autopsies revealed no pineal remnants in any of the pinealectomised fish.

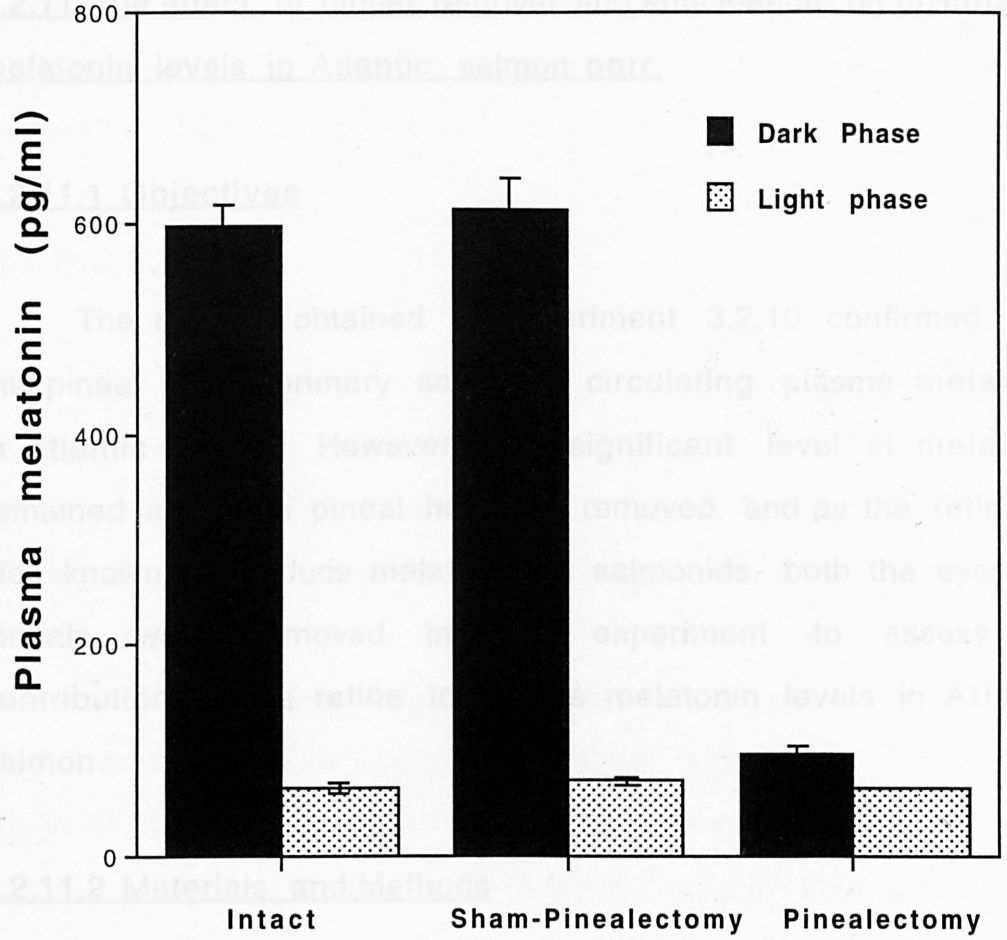


Figure 3.13

Circulating plasma melatonin levels (mean ± 1SEM) recorded during the mid-light and mid-dark phase from pinealectomised, sham-pinealectomised and intact Atlantic salmon parr. Unless shown the SEM values were too small to be depicted.

3.2.11 The effect of pineal removal and enucleation on circulating melatonin levels in Atlantic salmon parr.

3.2.11.1 Objectives

The results obtained in experiment 3.2.10 confirmed that the pineal is the primary source of circulating plasma melatonin in Atlantic salmon. However, as a significant level of melatonin remained after the pineal had been removed, and as the retina is also known to produce melatonin in salmonids, both the eyes and pineals were removed in this experiment to assess the contribution of the retina to plasma melatonin levels in Atlantic salmon.

3.2.11.2 Materials and Methods

Thirty one-year old salmon parr were maintained (as described in section 2.1.1) under a constant 12L:12D photoperiod. At the time of surgery, the fish weighed an average of 64 ± 11 g and the ambient water temperature was 8°C. Groups of 10 fish were pinealectomised (see section 2.2.1), pinealectomised plus enucleated or left intact. Enucleation was achieved by incising the cornea and severing the optic nerve to allow removal of the eye. The orbit was then cleaned and tissue glue applied to its surface. Twelve weeks after the operation a 0.5 ml blood sample was taken from each fish 3hr into the dark phase (section 2.1.3). To allow complete recovery and a return to ambient stress levels from the first blood sample, the parr were left for 14 days before this procedure was repeated 3hr into the light phase. Samples

were stored at -70°C prior to assay for melatonin (section 2.4). The absence of the pineal was confirmed by dissection at autopsy 4 months after the operation.

3.2.11.3 Results

Day and night-time levels of melatonin in intact fish showed significant variation ($P<0.05$) with levels of 36.0±5.5 pg/ml during the light phase and 289.1±31.5 pg/ml during the dark phase (Figure 3.14). Neither the pinealectomised group (light phase 38.6±3.5 and dark phase 36.71±6.1 pg/ml) nor the enucleated plus pinealectomised group (light phase 16.9±1.0 and dark phase 19.5±1.9 pg/ml) revealed significant differences between the light and dark phase melatonin levels. Dark phase levels in intact fish were significantly greater than those in the pinealectomised or enucleated plus pinealectomised groups. The pinealectomised fish also had significantly greater dark phase melatonin levels than the enucleated plus pinealectomised fish ($P<0.05$). Light phase melatonin secretion did not differ significantly between the intact and pinealectomised fish, but in both it was significantly greater than in the enucleated plus pinealectomised fish. No trace of pineal glands was found during autopsy after 4 months.

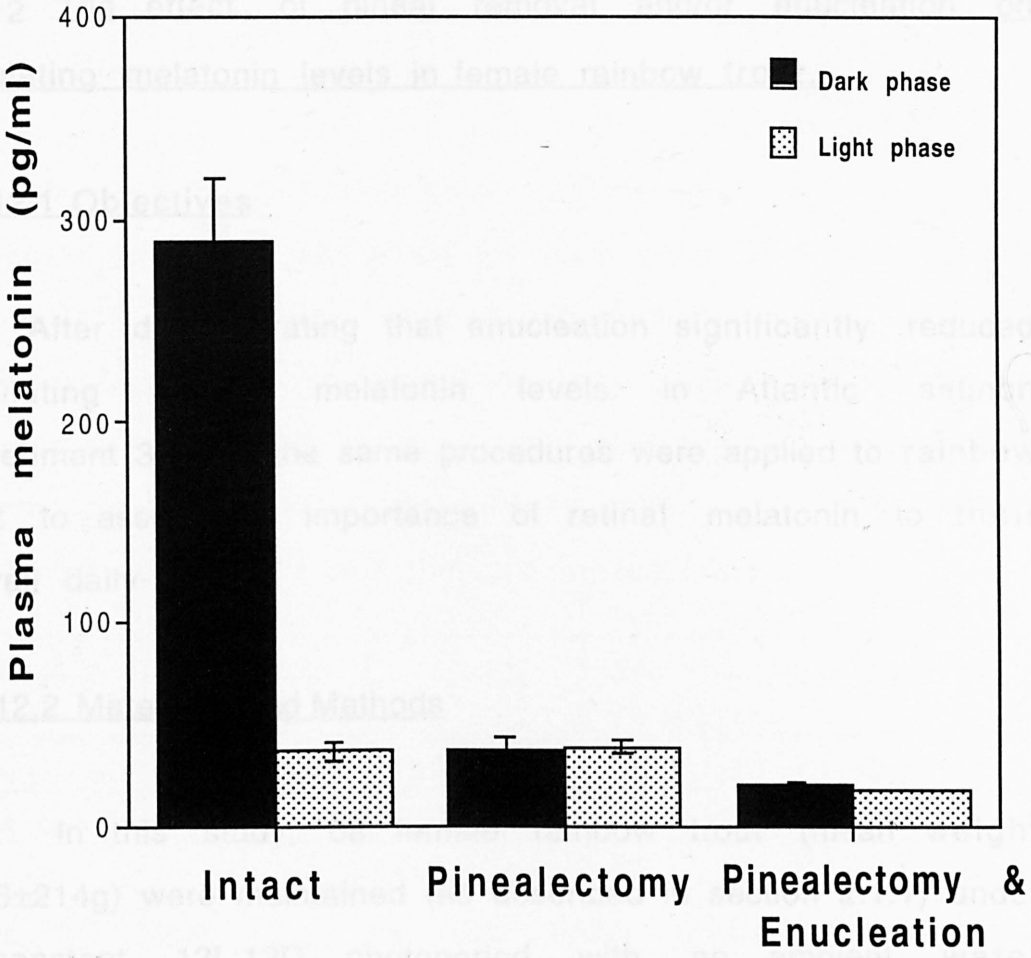


Figure 3.14

Circulating plasma melatonin levels (mean ± 1SEM) recorded during mid-light and mid-dark phase from intact, pinealectomised and enucleated & pinealectomised Atlantic salmon parr. Unless shown the SEM values were too small to be depicted.

3.2.12 The effect of pineal removal and/or enucleation on circulating melatonin levels in female rainbow trout.

3.2.12.1 Objectives

After demonstrating that enucleation significantly reduced circulating plasma melatonin levels in Atlantic salmon (experiment 3.2.11), the same procedures were applied to rainbow trout to assess the importance of retinal melatonin to their overall daily rhythm.

3.2.12.2 Materials and Methods

In this study, 68 female rainbow trout (mean weight 1236±214g) were maintained (as described in section 2.1.1) under a constant 12L:12D photoperiod with an ambient water temperature of 2°C at the time of surgery. Groups of 17 fish were: pinealectomised (section 2.2.1); pinealectomised plus enucleated; enucleated only; or left intact. Enucleation was achieved as described in section 3.2.11.2. Three weeks after the operation, a 2.0 ml blood sample was taken from each fish at the mid-dark phase (section 2.1.3). A further blood sample was obtained from each fish 2 weeks later at the mid-light phase. Samples were stored at -70°C prior to assay for melatonin (section 2.4). The absence of the pineal was confirmed by dissection at autopsy at the end of the experiment.

3.2.12.3 Results

Mean plasma melatonin levels (pg/ml±1SEM) taken mid-dark and mid-light phase are given for each group in Table 3.2 and Figure 3.15. All groups showed significantly lower ($P<0.05$) plasma melatonin levels during the light phase than during the dark phase. Light phase levels revealed no significant differences between the intact and pinealectomised groups and the enucleated or between enucleated plus pinealectomised groups.

Treatment	Enucleation	Intact	Enucleation/ Pinealectomy	Pinealectomy
light phase	30.0±5.7	51.0±6.0	17.0±3.3	45.0±5.8
dark phase	79.5±4.5	119.0±5.1	52.8±6.1	64.3±4.2

Table 3.2 Mean light and dark phase levels of melatonin (pg/ml±1SEM) from: enucleated; intact; enucleated plus pinealectomised; and pinealectomised female rainbow trout.

The pinealectomised plus enucleated group was observed to have significantly lower ($P<0.05$) levels during the light phase than either the intact or pinealectomised fish.

Dark phase melatonin levels in the intact fish were significantly greater ($P<0.05$) than in all other groups. The only other significant variation in the dark phase was found between the enucleated fish and the pinealectomised plus enucleated group ($P<0.05$). Autopsies revealed no pineal remnants in any of the pinealectomised fish

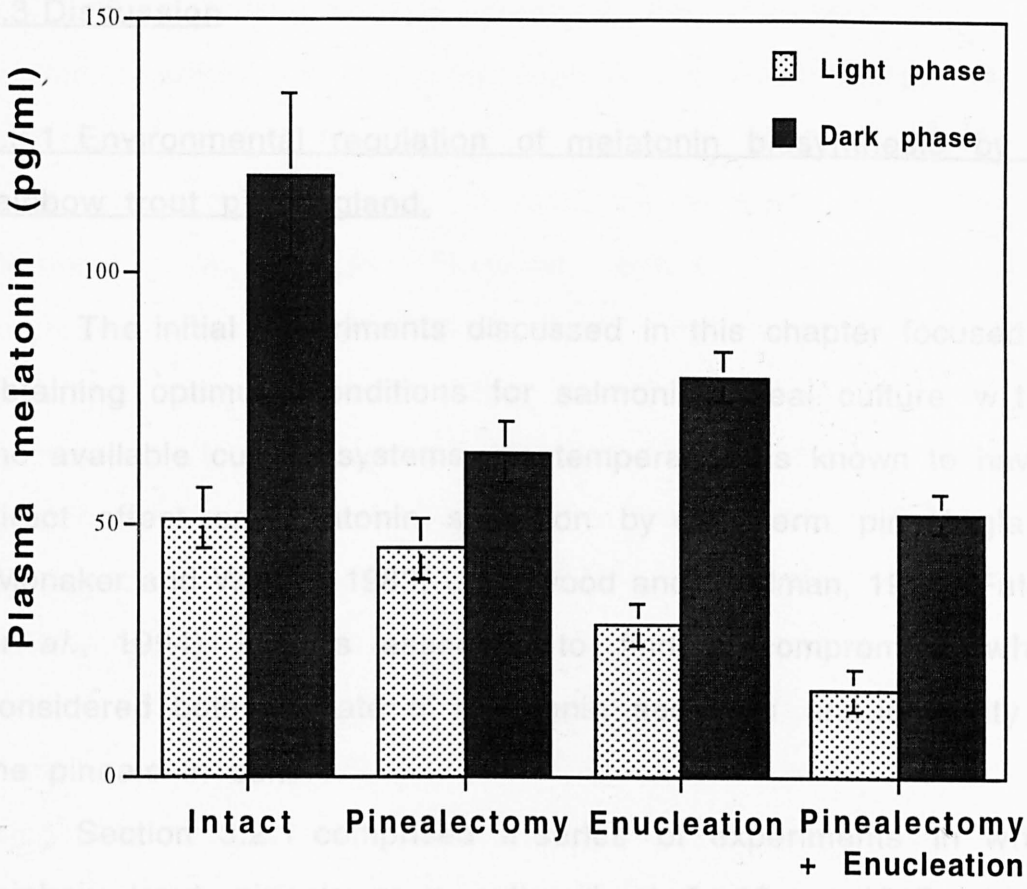


Figure 3.15

Circulating plasma melatonin levels (mean ±1SEM) recorded mid-light and mid-dark phase from rainbow trout which had undergone pinealectomy, enucleation, pinealectomy plus enucleation or were left intact.

3.3 Discussion

3.3.1 Environmental regulation of melatonin biosynthesis by the rainbow trout pineal gland.

The initial experiments discussed in this chapter focused on obtaining optimum conditions for salmonid pineal culture within the available culture systems. As temperature is known to have a direct effect on melatonin secretion by ectotherm pineal glands (Menaker and Wisner, 1983; Underwood and Goldman, 1987; Falcon *et al.*, 1994) it was essential to find a compromise which considered both the rate of melatonin secretion and longevity of the pineals in culture.

Section 3.2.1 comprised a series of experiments in which rainbow trout pineals were cultured at 5, 10, or 15°C under a 12L:12D photoperiod. From this work it is clear that, although at 5°C the pineal glands could be maintained for up to 12.5 days, the amplitude of melatonin secretion was significantly reduced. This has also been reported in pike (Bolliet *et al.*, 1994). The mechanism behind temperature dependant melatonin production is now thought to occur at an earlier stage in the biosynthetic pathway. Evidence that forskolin induced cAMP formation is temperature dependant has been provided by Falcon and Collin (1989) and Thibault *et al.* (1993), suggesting that cAMP formation regulates NAT production which in turn controls the synthesis of melatonin. *In vivo* studies into melatonin synthesis have also reported a greater amplitude of secretion at increased temperatures (Falcon and Collin, 1989; Max and Menaker, 1992; Randall *et al.*, 1995).

Although significantly reduced, the light/dark rhythm of melatonin production remained, even at 5°C. Interestingly, Tabata and Meissl (1993) reported a varied level of spike discharges from pineal ganglion cells in response to light stimulation at different temperatures. However, below 7°C trout pineal neurones became insensitive to light. This suggests that below 7°C trout rely on a hormonal signal to relay photoperiodic information from the pineal. Max and Menaker (1992) suggested that increased melatonin secretion in conjunction with increased temperatures may enhance the fish's ability to discriminate between seasons. This is possible during the spring and autumn months when equal daylengths can arise but one would expect the warmer ambient conditions in the autumn to result in a greater amplitude of melatonin during the dark phase. Additionally this hypothesis cannot explain why trout spawning out of season due to photoperiod manipulation are apparently oblivious to the temperature cycle.

Unfortunately, reduced survival times were concomitant with higher temperatures and increased melatonin production. This was emphasised at 15°C, where, although the melatonin output was significantly greater than at 10°C, the pineals only remained viable for 3 days. Previous studies on the rainbow trout and pike have found maximum melatonin production to occur at 15-20°C, however, no reference has been given as to the longevity of the cultures (Falcon and Collin, 1989; Max and Menaker, 1992; Thibault *et al.*, 1993; Falcon *et al.*, 1994). With these considerations in mind, 10°C was chosen as the most appropriate temperature for salmonid pineal culture within this culture system. This allowed cultures to be maintained for up to 8 days

while the pineals were able to maintain melatonin production at a level suitable for measurement by our radioimmunoassay technique.

Once an optimum temperature had been decided, tests on the culture media were undertaken (section 3.2.2). The media suggested for salmonid tissue culture was L15 Leibovitz with L-glutamine and 10% heat activated foetal bovine serum (S.Millar, pers. comm.). After several trials it was found that the foetal bovine serum did not significantly enhance either the melatonin production or the longevity of the pineals but merely introduced another possible source of infection. It was therefore decided to withhold the foetal bovine serum from the media which was used thereafter with L-glutamine and adjusted for pH and osmolarity.

Results from *in vivo* photoreceptive studies on teleosts can be difficult to interpret if specific photoreceptors are to be isolated due to several possible sites of photosensitivity. Of these, the lateral eyes, the pineal gland and deep encephalic photoreceptors provide the three main areas of possible photoreception, although dermal sensitivity cannot be overlooked (Oche and Hartwig, 1975; Hartwig and vanVeen, 1979). Tissue and organ culture, therefore, provides an ideal opportunity to study specific photoreceptive organs in isolation without interference from other sites.

The series of experiments performed using organ culture in section 3.2.3 was designed to establish whether the rainbow trout pineal gland merely receives photic information, and consequently inhibits melatonin production as a result of anything but absolute darkness, or whether a specific threshold illumination must be reached before the pineal is able to respond

to the light source. During these experiments, the 'dark' phase was supplied by a white light source at a constant 0.5lux. The reasons for this were two-fold. Firstly, a period of complete darkness is seldom achieved in the natural environment. Consequently, the pineal gland must have the ability to distinguish between the high illumination of the daytime and the low level of illumination experienced at night. Secondly, as this experiment was designed principally to observe whether the pineal responded to varying levels of illumination with equal levels of melatonin synthesis, there needed to be a measurable difference between the illumination in light and dark phase. This could not be achieved using complete darkness, as even the lowest form of illumination during the light phase could be considered as infinitely brighter than the dark phase.

From this work it has become apparent that pineal photoreceptors are able to respond to relatively small changes of illumination through the production of melatonin. Even with light phase levels as low as 1.0 lux (a light/dark variation of only 0.5 lux), a significant difference between light and dark phase melatonin levels was still observed although the variation in amplitude between the light and dark phases was not as large as at 5.0 lux. Interestingly, when the light level reached 5.0 lux, not only was melatonin suppression during the light phase significantly greater, but the amplitude of the dark phase measurements increased despite the dark phase illumination remaining constant, i.e. the rhythm had clearer definition. This suggests that the light phase irradiance not only prevents melatonin production during this period but the magnitude of the difference between light and dark phase illumination governs the

rate of melatonin secretion during the dark period. The results described here agree with similar work by Max and Menaker (1992) who found a differential response to varying intensities of illumination despite returning to complete darkness during the dark phase. Gern *et al.* (1992) and Yanez and Meissl (1996) also reported a quantitative increase in melatonin production following a stepwise reduction of the irradiance using neutral density filters and, although similar, his results were obtained from successive reductions in the light intensity so allowing the pineal photoreceptors to adapt to each step before the next reduction.

Neural studies of the trout pineal have also found a graded response to light stimulation in which hyperpolarisation responses to photic stimulation were inversely related to the intensity of the adapting illumination (Hanyu and Niwa, 1970; Meissl and Ekstrom, 1988). In their 1992 review, Gern *et al.* posed the question, "Does light inhibit or does darkness permit melatonin production". They proposed that darkness permitted melatonin synthesis and that light, depending on the irradiance power, activates a series of events that decrease melatonin production. After consideration of the results obtained in the present study I suggest we can also add that darkness permits melatonin synthesis, the amplitude of which depends upon the irradiance power of the light phase.

Section 3.2.4 investigated the pineal's ability to respond to wavelengths in the range of 660-800nm. This was initially carried out *in vivo* under a light phase of either white light or red light between 660 and 800 nm supplied at 120 lux. The results of this work clearly showed that melatonin synthesis was

significantly reduced by both forms of illumination. However, as the lateral eyes were intact during this study no definite conclusions can be made as to the spectral sensitivity of the pineal gland. Work on the *in vivo* spectral sensitivity of rainbow trout has been carried out by Molina Borja *et al.* (1990) who showed that the daily activity patterns of juvenile rainbow trout were entrained by red light with a main spectral wavelength of 650nm. Again, no assumptions could be made as to which photoreceptor population was responsible for receiving the stimulus.

A far more accurate method of addressing pineal sensitivity is to isolate the organ from other areas of photoreception. In this way, any hormonal response recorded must be derived solely from the pineal photoreceptors. Many authors have already examined the action spectra of the trout pineal *in vitro* but, to my knowledge, they have all relied upon the electrophysiological response from hyperpolarised photoreceptor ganglia. From this work, it is now known that maximal spectral sensitivity occurs between 463 and 561nm in the rainbow trout (Dodt, 1963; Hanyu and Niwa, 1970; Meissl and Ekstrom, 1988; Max and Menaker, 1992; Kusmic *et al.*, 1993). However, this overlooks the pineal's hormonal response to illumination or assumes that the hormonal and neural responses occur in unison.

From personal observations made in section 3.2.4 (Figure 3.7), it was apparent that melatonin secretion was significantly reduced at 660-800nm supplied at 50 lux. However, despite melatonin synthesis equalling that in the control group during the dark phase, light phase levels were significantly greater than under a white light source of equal intensity. These results

conflict with those of Gern *et al.* (1992) who found no melatonin inhibition above 675nm. The reason for this disparity is unknown and the fact that melatonin synthesis was not inhibited to the same degree by wavelengths between 660 and 800nm compared to the broad spectral band covered by white light stimulation suggests that not all photoreceptors were inhibited by the long wavelengths and so melatonin continued to be synthesised. This work lends support to the to the suggestion that there are two or more photoreceptor types with varying concentrations of photopigments derived from vitamin A1 and A2 (Max and Menaker, 1992; Kusmic *et al.*, 1993). Through the development of this paired pigment system (Bowmaker, cited in Douglas and Djamgoz, 1990) or photoreceptor coupling (Ekstrom and Meissl, 1988), the trout pineal has the ability to respond to a wide range of spectral information; the response observed in section 3.2.4 is possibly the result of the photoreceptors sensitive to the red waveband inhibiting melatonin synthesis, while other photoreceptors unable to respond to the red wavelengths continue to allow the production of melatonin. Zatz *et. al.* (1988) found that cultured chick pineals were able to perceive red light. However, its effect on melatonin production was dependant on whether the red light alternated with white light or darkness. Under a red/dark light cycle melatonin was inhibited by the red light, but when a red/white light photoperiod was used the, melatonin production increased under the red light. Unfortunately, no reference was given as to the intensity of the light sources and therefore the red light may simply be being interpreted as a period of low intensity lighting.

The pattern of melatonin secretion from superperfused trout pineal glands maintained under constant darkness or continuous light (section 3.2.4) suggests the absence of any endogenous component to the daily melatonin rhythm. This conforms with the results of *in vivo* studies by Randall *et al.* (1991) and of several *in vitro* investigations (Gern and Greenhouse, 1988; Begay *et al.*, 1992; Max and Menaker, 1992). Unlike rainbow trout, hammerhead shark (Okimoto and Stetson, 1995) and possibly the lamprey (Bolliet *et al.*, 1993), all other species of fish so far studied possess an endogenous melatonin rhythm in the absence of an external zeitgeber, i.e. goldfish (Kezuka *et al.*, 1988; Iigo *et al.*, 1991, 1994); pike (Falcon *et al.*, 1989); whitesucker (Zachmann *et al.*, 1991); zebrafish (Cahill, 1994); and Atlantic halibut (section 3.2.8). The increase in melatonin levels observed during the final 9 hours of the trout pineal cultures under constant light is thought to be a consequence of pineal necrosis. This may allow the release of cell metabolites from ruptured cells resulting in an increased release of melatonin during the final 9h of culture. When compared to melatonin levels observed under DD conditions this 250 pg/ml rise was not considered to be an indication of an endogenously driven increase in response to darkness.

From the work presented here and that of other groups, it is apparent that, unlike the other teleost species studied so far, the rainbow trout pineal gland does not behave as an endogenous pacemaker but is more analogous to an endocrine photometer. This is emphasised by the square wave profile of melatonin secretion from the pineal gland in culture together with the differential synthesis of melatonin in response to a graded stimulus, as

opposed to an all or nothing reaction. Whether each photoreceptor is capable of a varied production rate of melatonin or increased recruitment of photoreceptors occurs according to the level of stimulation remains unknown. Secondly, the pineal is able to respond to a wide spectrum of wavelengths, suggesting its adaptation to the daily variation in the visible spectrum. In this sense, red light detection would be especially valuable to trout which by nature are crepuscular feeders, when longer wavelengths are dominant.

3.3.2 Endogenously driven melatonin synthesis in the pineal gland of the Atlantic salmon and Atlantic halibut.

As mentioned in section 3.3.1 the pineal gland of all teleosts so far studied except the rainbow trout, hammerhead shark and possibly the lamprey possess an endogenously driven melatonin rhythm (Gern and Greenhouse, 1988; Kezuka *et al.*, 1988; Falcon *et al.*, 1989; Iigo *et al.*, 1991, 1994; Randall *et al.*, 1991; Zachmann *et al.*, 1991; Max and Menaker, 1992; Bolliet *et al.*, 1993; Okimoto and Stetson, 1995). Strangely, this list does not extend to the Atlantic salmon which as the U.K.'s main cultured species has several of its life cycle stages artificially manipulated by photoperiod (see section 5.1 for more detail). Although salmon are known to have a daily rhythm of melatonin, with an increased amplitude during the dark phase (Lindhal, 1986; Randall *et al.*, 1995), and the possibility of an endogenous rhythm has been suggested (Randall *et al.*, 1989), it is still unknown whether an endogenous component exists within the Atlantic salmon pineal.

In an attempt to discover whether the Atlantic salmon can continue to produce rhythmic secretions of melatonin, pineal cultures were undertaken in the absence of an external stimulus. In the first of these (Figure 3.9), salmon were maintained on an artificial 12L:12D photoperiod for 1 month prior to decapitation. Under culture, the pineals were maintained on 16 hours of constant darkness which overlapped the previous dark phase by 2 hours on each side. The results of this experiment revealed a premature decrease in melatonin secretion with the first significant decrease occurring 1 hour 30 minutes before the light phase began. Of greater importance is the fact that the initial decrease was observed 30 minutes after the previously entraining photoperiod would have entered its light phase. Interestingly, both Gern *et al.* (1992) and Max and Menaker (1992) both found it took 30 minutes to fully inhibit melatonin synthesis from the point illumination was supplied to isolated pineals. The fall in melatonin secretion seemed to plateau at approximately half its dark adapted level before a further decrease was observed when the light phase was initiated. Within 1 hour, light phase levels of melatonin secretion had been reached (mean 509±256 pg/ml).

From these results, it would appear that some photoreceptors ceased melatonin production in anticipation of the expected illumination in response to the previously entrained photoperiod under which the fish had been maintained for 1 month prior to decapitation. It seems unlikely that we are seeing a differential response as reported in the rainbow trout (section 3.3.1) as there was no exposure to a graded stimulus. As mentioned in section 3.1, pike are known to exhibit both typical and modified photoreceptor cells within the pineal gland (Falcon

et al., 1989). It would also appear that the functionality of these receptor types may differ as only typical photoreceptors are synaptically connected to second-order neurones and are found within the distal regions of the pineal containing afferent connections. By contrast, the 'avian like' modified photoreceptors, although located within the distal region, are also found in the medial area of the gland which is devoid of neurones. Bolliet *et al.* (1994) were able to show that both types of photoreceptors exhibit circadian properties of melatonin production under constant conditions suggesting neural responses to photic stimulation are confined to the typical photoreceptors whereas both types are involved in the hormonal response. It is possible, then, that the Atlantic salmon pineal contains populations of photoreceptors with endogenous circadian rhythmicity, as well as photoreceptors lacking any endogenous component (as in the rainbow trout). Therefore, in experiment 3.6, the photoreceptors with an endogenous rhythm may have been responding to the previous photoperiod while the directly photosensitive photoreceptors were only inhibited from synthesising melatonin by the ambient illumination.

The case for an endogenous component in the pineal of Atlantic salmon is further strengthened by the results of section 3.7. Again, a period of continuous darkness was applied to the pineals in culture but, following the results of experiment 3.6, the dark period now covered 2 of the previous light/dark cycles. In this case, the melatonin level in the culture media did not increase significantly until 5 hours after the dark period began. Even then, a large increase in the rate of melatonin synthesis was not observed until the approximate start of the dark phase under

which the salmon had previously been entrained. This level of production was then shown to decrease up to the point when the second light phase would have occurred (see section B, Figure 3.10). Melatonin production within section B (Figure 3.10) bears a close resemblance to a type B (or II) pattern of melatonin secretion as described by Reiter (1988). This describes the peak in melatonin production occurring mid-dark phase followed by a gradual decrease to the expected time of illumination. Although salmonid pineal cultures have previously been shown to exhibit a type C (or III) form of secretion, displaying a square wave profile, this has always been observed when the fish were entrained to a specific photoperiod (Gern and Greenhouse, 1988; Kezuka *et al.*, 1988; Falcon *et al.*, 1989; Iigo *et al.*, 1991, 1994; Randall *et al.*, 1991; Zachmann *et al.*, 1991; Max and Menaker, 1992, Bromage *et al.*, 1995). It may, therefore, be the case that, in the absence of photoperiodic entrainment, a limited population of photoreceptors still have the ability to continue the rhythmic secretion of melatonin and in doing so revert to a type B pattern of secretion.

Following the second phase of secretion (section B, Figure 3.10), melatonin production increased steadily up to hour 51 after which a decrease was observed prior to the lights going off, as observed in experiment 3.6. These results strongly indicate the presence of an endogenous rhythm of melatonin secretion in the Atlantic salmon pineal in the absence of photic entrainment. Interestingly, the patterns of melatonin secretion produced by the cultured salmonid pineals closely resemble those of pike pineals which have been maintained under constant darkness (J. Falcon pers. comm.). It is clear, however, that if circadian oscillators are present within the salmon pineal the population as a whole

exhibits only a weak overt rhythm, possibly due to the rapid uncoupling of photoreceptors leading to asynchrony between sub-populations or individual cells. It is also notable that such endogenous rhythms are only displayed by isolated pineals and are not revealed *in vivo*. Obviously, more work is required before the presence of an endogenous rhythm within the pineal of the Atlantic salmon can be confirmed.

Section 3.2.8 was aimed at assessing the pattern of melatonin secretion from cultured Atlantic halibut pineal glands obtained from adult fish. These are difficult to obtain due to the high value of the broodstock and as such this experiment provides the first evidence of a rhythmic release of melatonin from the halibut pineal gland. Under a period of continuous darkness, it was shown that the rhythm in pineal melatonin secretion persisted, indicating a free-running rhythm (Figure 3.11). This suggests that the halibut pineal contains some form of endogenous circadian oscillator which regulates the rhythmic production of melatonin similar to that found in: the goldfish (Kezuka *et al.*, 1989; Iigo *et al.*, 1994); pike (Falcon *et al.*, 1989); whitesucker (Zachmann *et al.*, 1992); and zebrafish (Cahill, 1994).

This study provides the first evidence that melatonin release from the Atlantic halibut pineal is able to encode information on daylength which may be used to entrain rhythms in reproduction and other seasonal events. The endogenous control of melatonin secretion in halibut may be an important adaptation to its environment. Since some of its time is spent at depths of up to 1000m, and as little or no light penetrates below 200m (Kampa, 1970), there may be no consistent entrainment of the endogenous clock by the light/dark cycle. The presence of an

endogenous rhythm would therefore help to maintain internal synchronisation in the absence of exogenous photoperiodic cues.

3.3.3 The role of the pineal gland and other possible sources in the day/night change in circulating plasma melatonin levels in salmonids.

This final section concentrates on the importance of the pineal gland to the daily rhythm of circulating melatonin in salmonids. As the emphasis of this thesis was to investigate the importance of pineal involvement in the timing of reproduction and smoltification in salmonids, a method of pineal removal was required. This led to the development of a technique which allowed the pineal gland to be removed quickly and effectively at various stages of the life cycle without detrimental effects to the survival of the fish (Porter *et al.*, 1996).

Before pineal removal was attempted, organ cultures were performed on trout pineal end-vesicles (*epiphysis cerebri*), the pineal stalk (*tractus pinealis*), and complete gland. This was to assess whether previous methods of pineal removal using suction (Gern *et al.*, 1978) could leave parts of the pineal stalk intact, and if so, whether they could continue to produce melatonin.

From Figure 3.12A, it is clear that the pineal stalk is capable of producing melatonin in a rhythmic manner in response to the light/dark cycle although at a significantly reduced rate compared to the complete pineal. As for the pineal end-vesicle, melatonin levels were elevated during the dark phase and reduced during the light phase. No significant addition was made to the melatonin secretion of the pineal end-vesicle when left attached

to the stalk (Figure 3.12B) compared to the end-vesicle only (Figure 3.12C). Histological studies carried out by Omura (1979) on the trout pineal reported that the pineal tract running from the pineal end-vesicle to the posterior commissure consisted mainly of nerve fibres, although, Falcon (1979; cited in Bolliet *et al.*, 1994) found that the pineal stalk of the pike contained numerous afferent neurones and typical photoreceptor cells. From the results of the cultures presented here, it appears that the pineal stalk of the rainbow trout does indeed contain photoreceptive cells and is capable of synthesising melatonin. Consequently, it was decided to perform pineal removals by incising the stalk at the point of attachment to the diencephalon to ensure complete removal.

The results of the surgical procedures carried out in sections 3.2.10 to 3.2.12 clearly show the pineal gland to be the principal source of plasma melatonin during the dark phase in both rainbow trout and Atlantic salmon. The daily plasma melatonin levels from intact rainbow trout and Atlantic salmon were shown to fluctuate with the light/dark cycle, displaying an increased amplitude during the dark phase. This is in agreement with the findings of Gern *et al.* (1978), Owens *et al.* (1978), Burton and Gern (1983), Duston and Bromage (1986), and Randall *et al.* (1991,1995). For the first time, it has been demonstrated that removal of the pineal gland in Atlantic salmon significantly reduces dark phase plasma melatonin levels but does not alter light phase levels (Porter *et al.*, 1996). Similar results were found in rainbow trout (section 3.2.12; Gern *et al.*, 1978) and have also been reported in goldfish (Kezuka *et al.*, 1992). In agreement with the findings of Gern *et al.* (1978), the daily rhythm of

plasma melatonin persisted following pinealectomy. This was also observed in salmon in experiment 3.2.10 but not in experiment 3.2.11. The lack of variation in the latter experiment may be due to the low ambient temperature at the time of sampling causing an overall reduction of melatonin production. Temperature effects on *in vitro* melatonin synthesis were observed in section 3.2.1 and have been reported in pike (Falcon *et al.*, 1987) and *in vivo* for rainbow trout (Randall *et al.*, 1995).

Once the pineal had been confirmed as the main source of melatonin production, attention focused on the origin of the remaining light and dark phase levels. As yet the retinal photoreceptors are the only other melatonin synthesising tissues known in fish (Gern *et al.*, 1978; Gern and Ralph, 1979; Falcon and Collin, 1991; Zachmann *et al.*, 1991; Meissl and Brandstatter, 1992). With this in mind, the effects of bilateral enucleation either alone, or in conjunction with pinealectomy, were examined.

Enucleation alone significantly reduced dark phase plasma melatonin concentrations in rainbow trout and, in conjunction with pinealectomy, produced significantly lower melatonin levels during both light and dark phases in Atlantic salmon and lower light phase levels in rainbow trout. The low melatonin levels observed in experiment 3.2.12 can be attributed to the low water temperatures at the time of the experiment (2°C). Although the results indicate that both the eyes and pineal provide a significant contribution to circulating plasma melatonin levels in Atlantic salmon and rainbow trout, further trials at higher water temperatures will need to be performed before any conclusions can be made. Interestingly, Kezuka *et al.* (1992) found that pinealectomy plus enucleation had no effect on light phase

melatonin levels in the goldfish. However, dark phase levels were significantly lower than with pinealectomy alone. In comparison, Gern *et al.* (1978) reported that by severing the optic tract light phase plasma melatonin levels were actually increased compared to pinealectomised and intact fish. They proposed this increase may be stress induced due to sensory deprivation.

The origin of the remaining circulating plasma melatonin is still unknown and will require further investigation. However, it would appear, in rainbow trout at least, that peak production is still associated with the dark phase. As to the site of secretion, the presence of deep encephalic photoreceptors in fish (Garcia-Fernandez and Foster, 1994; Foster *et al.*, 1994) would suggest that other areas of the brain may be able to synthesise melatonin as do pineal and retinal photoreceptors. Reiter *et al.* (1981) identified the mammalian Harderian gland as a source of melatonin in the Richardson's ground squirrel, however, as fish do not possess a Harderian gland, this can be ruled out. The gastrointestinal tract has also been identified as a possible site of melatonin synthesis in higher vertebrates (Heuther, 1993) and may also prove to be a source in fish, although as yet no work has been conducted in this area.

3.3.4 Summary of Chapter 3

To summarise, chapter 3 concentrated on the factors influencing melatonin biosynthesis from the pineal gland of Atlantic salmon and rainbow trout. Initial work revealed that optimum conditions for salmonid pineal culture within the systems used required pineals to be maintained at a constant

10°C in L15 culture media with L-glutamine; this provided reliable melatonin synthesis at an increased longevity. The trout pineal exhibited a differential response to a graded photic stimulus, rather than responding in an all or nothing manner. This agrees with the observations of Max and Menaker (1992). Contrary to the findings of Gern *et al.* (1992) melatonin inhibition was found to occur at wavelengths between 660 and 800nm, but at a reduced level. It is suggested that this may indicate a limited population of trout pineal photoreceptor cells possess the ability to detect wavelengths at the red end of the visible spectrum.

The absence of an endogenous rhythm of melatonin within the isolated rainbow trout pineal confirms previous work (Gern and Greenhouse, 1988; Begay *et al.*, 1992; Max and Menaker, 1992). Similar to the rainbow trout the Atlantic salmon produces a light/dark rhythm of melatonin in response to the ambient photoperiod. Interestingly it appears that, unlike the trout, the isolated pineal may contain photoreceptor cells capable of maintaining an endogenous rhythm of melatonin secretion in the absence of an external stimulus. However, if an endogenous component does exist within the salmon pineal its influence over melatonin production appears weak and may indicate that only selected photoreceptors contain endogenous oscillators. Another possibility is that there is rapid desynchronization (or uncoupling) between cells once entrainment by an external stimulus is removed. No such ambiguity exists as to the presence of an endogenous circadian oscillator within the Atlantic halibut pineal. Section 3.2.8 provided for the first time convincing evidence of an endogenous circadian oscillator within the pineal

of Atlantic halibut, which maintained a rhythmic secretion of melatonin on the absence of an external stimulus.

Pineal removals were carried out on Atlantic salmon and rainbow trout to determine the principal source of plasma melatonin in salmonids. From this work the pineal was found to be the predominant source of dark phase melatonin synthesis in Atlantic salmon and through the course of enucleation studies the lateral eyes were also shown to make a significant contribution to melatonin levels. This is in agreement with previous studies on rainbow trout (Gern *et al.*, 1978) and goldfish (Kezuka *et al.*, 1992).

The pinealectomy tecnique developed here (Porter *et. al.*, 1996) was subsequently used to study the long tern effects of the pineal on smoltification and reproductio in salmonids.

Chapter 4

The Effect of Melatonin and the Pineal Gland on the Development of S.salar parr and the Parr-Smolt Transformation

4.1 Introduction

The exploitation of the freshwater environment by salmonids varies enormously with genus (Thorpe, 1987). Many remain in fresh water for the whole of their life cycle, while others migrate to sea and then return to fresh water to spawn (anadromy), following which they may die. However, even within stocks of the same species there is a disparity in the period of time different individuals remain at sea. The degree of dependence on the freshwater environment is well illustrated by the Pacific salmon (*Oncorhynchus*) species: humpback salmon occupy fresh water for embryonic development and reproduction only; chinook and chum salmon can spend several months in fresh water before migrating to sea; coho remain within well defended territories for up to three years before moving downstream; immature sockeye salmon may spend days or up to four years in their spawning lakes before migrating, or alternatively may remain landlocked (in the case of sockeye these are termed 'kokanee'). This flexibility in life history strategy observed with the Pacific salmon is also typical of the *Salmo* and *Salvelinus* genera, which possess many species with both anadromous and non-migrant strains (Hoar, 1988).

At present both the synchronising effect of the seasonal photoperiod on the behavioural and physiological processes underlying sea water adaptation (smoltification) and the physiology of the teleost osmoregulatory system are well documented (see later). However, the mechanisms behind the transduction of photic information into these developmental changes are little understood. This chapter investigates the

neuroendocrine transfer of information by the pineal gland in juvenile Atlantic salmon over the course of the parr-smolt transformation. Pinealectomy, and the administration of constant release melatonin implants, were used to assess the pineal's role in the entrainment of endogenous rhythms and the direct effects of melatonin on both growth and osmoregulation.

Why some individuals within a species should 'decide' to go to sea in their first, second or third spring was addressed by Taylor and Taylor (1977) who defined migration as 'a fundamental biological response to adversity'. However, the energy required to perform the adaptation and migration to sea is related to the growth rate and energy reserve of the individual (Thorpe, 1986,1989), favouring the larger fish in the year class. Growth rates do not occur uniformly throughout a sibling population of salmon but result in a bimodal length frequency distribution (Thorpe *et al.,* 1980; Kristinsson, 1985; Nicieza *et al.,* 1994). The disparity in growth rates begins in late summer and is most pronounced prior to salt-water migration (Thorpe *et al.,* 1980). This split in the population means the lower modal group (LMG) may forgo smoltification until the following spring; these fish are known as S2's. By contrast the upper modal group (UMG) undergo sea water adaptation in their first year (S1's) (Metcalfe *et al.,* 1986). By entering the marine environment, these individuals are able to utilise a greater food resource and so obtain a growth advantage over the remaining freshwater population (Thorpe, 1987). The advantage of migration to sea is confirmed by the smaller size and lower breeding weights of landlocked freshwater stocks.

Calow and Townsend (1981) described growth as 'an organism's means of reaching its reproductive state', which is the primary function of spending time at sea, to gain a growth advantage and hence reproductive success. However, Policansky (1983) concluded that maturation occurs as soon as it is developmentally possible, which seems to be the strategy taken by a percentage of immature males in every year class. As growth rates and maturation are positively correlated (Alme, 1959; Thorpe, 1986), it has been suggested that these fish, sometimes known as precocious males, are in some way aware of their specific growth rate and on reaching a critical value become sensitive to photoperiodic stimulation of their gonadotropic hormone system (Scott and Sumpter, 1983; Scott *et al.*, 1984; Thorpe, 1986; Duston and Saunders, 1992). This may also be the case with salmonid maturation at sea (Saunders, 1986). As 'precocious males' is now an established term used throughout aquaculture and fisheries literature to describe male salmonids which mature while still at the parr stage of development it will be used during the course of this thesis. There is also evidence that early maturation, in part, is a heritable characteristic (Thorpe *et al.*, 1983). Thorpe (1987) suggested that maturation and smolting are mutually exclusive ensuring initiation and development of only one system at any one time (Figure 4.1).

Hoar (1976) observed that the normally aggressive and territorial parr begin to shoal during migration. Another behavioural change reported by Kalleberg (1958) was the move from the river bed to take up station mid-way in the water column. This, together with a reduced swimming ability, produces

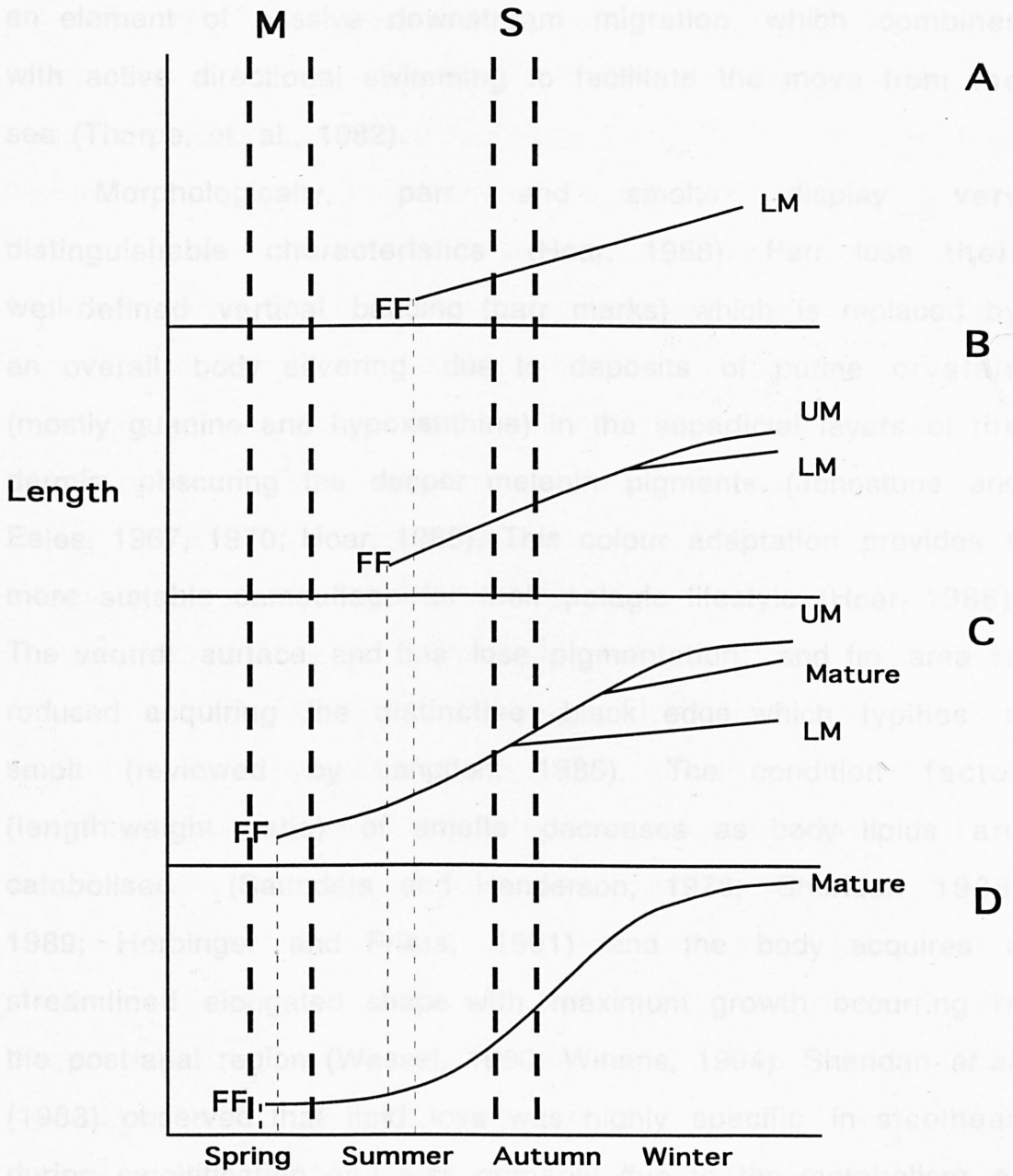


Figure 4.1

Thorpe's proposed growth curves for Atlantic salmon parr development. M: sensitive period for maturation; S: sensitive period for growth; FF: first feeding; UM: upper modal group; LM: lower modal group.

A) Poor developmental conditions
B) Moderate developmental conditions
C) Good developmental conditions
D) Excellent developmental conditions
(for further information see Thorpe, 1987).

an element of passive downstream migration which combines with active directional swimming to facilitate the move from the sea (Thorpe, et. al., 1982).

Morphologically, parr and smolts display very distinguishable characteristics (Hoar, 1988). Parr lose their well-defined vertical banding (parr marks) which is replaced by an overall body silvering due to deposits of purine crystals (mostly guanine and hypoxanthine) in the superficial layers of the dermis, obscuring the deeper melanin pigments (Johnstone and Eales, 1967, 1970; Hoar, 1988). This colour adaptation provides a more suitable camouflage for their pelagic lifestyle (Hoar, 1988). The ventral surface and fins lose pigmentation, and fin area is reduced acquiring the distinctive black edge which typifies a smolt (reviewed by Langdon, 1985). The condition factor (length:weight ratio) of smolts decreases as body lipids are catabolised (Saunders and Henderson, 1978; Sheridan 1986, 1989; Herbinger and Friars, 1991) and the body acquires a streamlined elongated shape with maximum growth occurring in the post-anal region (Wessel, 1990; Winans, 1994). Sheridan *et al.* (1983) observed that lipid loss was highly specific in steelhead during smoltification and was primarily due to the metabolism of triglyceride stores in the liver, intestinal mesentery and red muscle. Carbohydrate metabolism in Atlantic salmon has also been indicated by a reduction in liver glycogen and elevated blood glucose levels (Wendt and Saunders, 1973).

Of greater importance to smolt survival is the development of an efficient osmoregulatory system which will enable the transfer from fresh water to the hyperosmotic marine environment. However, the ability to tolerate high salinities is

not solely related to smoltification but is also highly correlated to the size of the fish (Clarke, 1982), probably as a consequence of a decreased surface area to volume ratio, and in turn reduced ion and water fluxes. This does not represent true adaptation to the marine environment which requires an active system of ion and water regulation over osmotic and ionic gradients.

Teleost rehydration in sea water is achieved by constant ingestion of sea water and increased absorption across the intestine together with a reduction in urine volume. However, this results in an excess salt load within the body which must be excreted, principally via the gills. To achieve this, the gills and operculum contain large mitochondrion-rich chloride cells which excrete monovalent ions through the activity of the sodium/potassium-stimulated enzyme adenosine triphosphate (Na^+K^+-ATPase). During the process of smoltification, the chloride cells increase in size and number and the external surface of the cells develop pits with increased invagination occurring in the cell walls bordering afferent blood vessels (Conte, 1980; Langdon and Thorpe, 1984). These changes are associated with the well documented rise in Na^+K^+-ATPase activity which occurs during saltwater adaptation (McCormick *et al.*, 1987; Boeuf *et al.*, 1989; Prunet *et al.*, 1989; Saunders *et al.*, 1989; Stefansson *et. al.*, 1994).

Participation of the endocrine system including the pituitary, inter-renal and thyroid systems during sea water adaptation of Atlantic salmon was first recognised by Hoar (1939) who observed a seasonal hypertrophy of thyroid follicles and questioned the role of the two main thyroid hormones thyroxine (T_4) and triiodothyronine (T_3). T_4 levels have since been

shown to increase approximately two months in advance of downstream migration followed by a subsequent rise in T_3 levels (Dickhoff *et al.*, 1978; Specker *et al.*, 1984; McCormick *et al.*, 1987; Boeuf *et al.*, 1989; Prunet *et al.*, 1989; Yamada *et al.*, 1993). The exact function of the thyroid hormones is as yet unclear, although administration of T_3 and T_4 to salmon parr has been shown to have varied effects. Boeuf *et al.* (1994) found no change in Na^+K^+-ATPase activity or sea water adaptation following T3 administration while Higgs *et al.* (1982) and Dickhoff *et al.* (1985) reported an increased salinity tolerance. Miwa and Inui (1985) and Omeljaniuk and Eales (1986) found this adaptation was short-lived as Na^+K^+-ATPase activity had not been initiated despite the occurrence of body silvering (Ikuta *et al.*, 1985) and elongation (McBride *et al.*, 1982; Sullivan *et al.*, 1987).

Further work on the endocrine system has investigated the importance of the pituitary gland and especially its adenohypophyseal hormones, growth hormone (GH) and prolactin (PL) on the parr/smolt transformation. The initiation and seasonal increase in GH occurs in response to an increasing photoperiod, (Bjornsson *et al.*, 1989, 1994). These authors reported an out-of-season rise in GH following exposure of salmon parr to an increasing daylength. Several studies have reported improved hypo-osmoregulatory ability of salmon parr following the administration of GH (Clarke *et al.*, 1977; Collie, 1989; Madsen, 1990; Almendras *et al.*, 1993). Smolt morphology was also altered but to a lesser degree. Constant release GH implants were used by Boeuf *et al.* (1994) to elevate Na^+K^+-ATPase activity in parr during their first winter in fresh water

and on transfer to sea water; this also led to an improvement in growth rates and hypo-osmoregulatory ability.

Unlike GH levels there is a reduction in prolactin in Atlantic salmon and rainbow trout when transferred to higher salinities. Prolactin, described as a freshwater adapting hormone by Prunet and Boeuf (1989), appears to be of greater importance during the upstream migration by the adults as it regulates osmotic homeostasis by decreasing gill permeability and retaining Na^+ ions (reviewed by Hirano, 1986).

The interrenal gland was implicated in smoltification by Thorpe *et al.* (1987) who found increased blood cortisol levels during the period of transfer in Atlantic salmon. However, administration of cortisol to parr failed to produce any smolt characteristics (Langdon *et al.*, 1984), although Forest *et al.* (1973) induced increased Na^+K^+-ATPase activity together with body silvering in the eel. Injections of adrenocorticotropin (ACTH), however, did stimulate Na^+K^+-ATPase activity (Langdon *et al.*, 1984) and fin darkening but, as no particular pattern of secretion in relation to smolting has been demonstrated, clearly more work is required before its role can be properly assessed.

With ambient temperature known to be a primary factor in the somatic growth of poikilotherms, it is not surprising to find that temperature influences a number of the processes involved in salmonid smoltification. Although previous studies suggested that temperature plays only a minor role in the onset of smoltification (Hoar, 1988; Johnstone and Saunders, 1981), combinations of temperature and photoperiod manipulation have been successfully used to obtain under-yearling sockeye (Clarke *et al.*, 1978), chinook and coho (Clarke *et al.*, 1989) smolts.

Temperature has also been shown to be a significant factor in the development of physiological smolt characteristics in Atlantic salmon (Zaugg and McLain, 1976; Duston and Saunders, 1994; McCormick, 1994). Elevated temperature advanced smoltification through increased growth and elevated Na^+K^+-ATPase activity (Stefansson *et al.*, 1994). However, higher temperatures were shown to shorten the length of the smoltification window through the advanced loss of sea water tolerance characteristics (Wagner, 1973; Duston, 1991; McCormick, 1994).

The annual photoperiod cycle appears to be the primary environmental cue that entrains the endogenously driven regulation of osmotic and ionic balance (Baggerman, 1959, Hoar, 1976; Folmar and Dickhoff, 1980; Clarke, 1989; Duston and Saunders, 1990). The use of seasonally-compressed photocycles or phase-advanced photoperiods is now an established means of advancing the parr-smolt transformation in Atlantic salmon (Komourdjian *et al.*, 1976; Clarke *et al.*, 1985; Bromage and Duston, 1986; Bjornsson *et al.*, 1989; Duston and Saunders, 1990; Stefansson *et al.*, 1992; Duncan *et al.*, 1994; Stefansson *et al.* ,1994; Thorarensen and Clarke, 1994; Thrush *et al.*, 1994). However, a number of salmonids which lack a 'typical' smoltification stage do not respond to photoperiod during juvenile development, nor do they initiate the parr-smolt transformation in the same manner as the majority of species (Table 4.1; reviewed by Clarke, 1989).

The mechanism by which photoperiod initiates smolting is still unclear although hormonal induction through GH and T_4 seems likely. Grau *et al.* (1981, 1982) reported T_4 surges, precisely timed with the 'new moon' phase of the lunar cycle. This

has also been observed prior to smoltification in Atlantic (Boeuf and Prunet, 1985, Prunet et. al., 1989), masu and amago salmon (Yamauchi *et al.*, 1984).

Photoperiod Controlled (typical smolts)	**Photoperiod Controlled** (non-typical)
Atlantic salmon *S.salar*	Chum salmon *O.keta*
Steelhead trout *O.mykiss*	Humpback salmon *O.gorbuscha*
Chinook (stream-type) *O.tshawytcha*	Chinook (ocean-type) *O.tshawytcha*
Coho salmon *O.kisutch*	Brook trout *S.fontainalis*
Masu salmon *O.masu*	Arctic charr *S. alpinus*
Sockeye salmon *O.nerka*	

Table 4.1. Classification of salmonids according to photoperiod control of juvenile development and initiation of smolting (Clarke, 1989).

Considering the widely recognised effects of photoperiod on smoltification and the well established importance of the pineal gland as a phototransducer (Gern and Greenhouse, 1988; Morton and Forbes, 1988), it is surprising that very few studies have investigated the role of either the pineal gland or its main endocrine product, melatonin, in this process. As the production of melatonin in salmonids is directly related to the hours of darkness (Randall, 1989, 1991), it would provide an ideal mechanism by which parr could accurately determine the yearly seasons and hence the timing of sea water migration.

Consequently, this chapter investigates the importance of the pineal gland and the contribution of melatonin to the timing

and development of sea water adaptation in Atlantic salmon. In order to determine whether the rhythmic secretion of melatonin by the pineal is essential to the parr-smolt transformation, experimental alterations were made to the daily melatonin signal. This was achieved by either removing the signal at source (i.e. pinealectomy) or by abolishing the melatonin rhythm by 'masking' information derived from the endogenous melatonin rhythm through the introduction of a constant elevated level of melatonin using melatonin implants. Pinealectomy was performed in order to determine whether the pineal is essential to the transmission of photoperiodic information.

Melatonin implantation has previously been performed on higher vertebrates where, in some species, it has been shown to mimic a short-day (long-night) photoperiod (O'Callaghan *et al.*, 1991). At present very little work has been conducted on the effects of melatonin implants on smoltification. Rourke (1994), treated steelhead parr with constant release melatonin implants in mid-November and achieved a significant increase in gill Na^+K^+-ATPase by December 4th, although sea water adaptation times were not affected. Randall and Bromage (pers. comm.) found no significant variation in the timing or acquisition of smolt characteristics of potential S2 smolts implanted with melatonin at the autumn equinox or winter solstices. To date, no other studies have investigated the effects of melatonin implants on sea water adaptation.

Pineal removal in teleosts has been shown to significantly reduce the amplitude of the night-time increase in melatonin secretion in rainbow trout (Gern et al., 1978), goldfish (Kezuka *et al.*, 1992) carp (Popek et al., 1994) and Atlantic salmon (Porter et

al., 1996) although the rhythm was not completely abolished. Pinealectomy has been reported to stimulate, inhibit or have no effect on gametogenesis in: the goldfish (Fenwick, 1970; Kezuka *et al.*, 1992); the medaka (Urasaki, 1972); the golden shiner (deVlaming, 1975; deVlaming and Olcese, 1981); the grey mullet (Sagi *et al.*, 1983); the channel catfish (Davis *et al.*, 1986) and the rainbow trout (Popek *et al.*, 1992), depending on the temperature and light regimes utilised (discussed in detail in section 5.1). The mechanisms by which the pineal affects gonadal development remain unclear, although it is presumed to act via either a neural or hormonal messenger acting on gonadotropin secretion. Hontela and Peter (1980) and Vodicnik *et al.* (1978) found the results of pineal removal in goldfish to be dependant on photoperiod, temperature and the stage of sexual development. The pineal stimulated gonadal development and promoted a daily cycle of GTH under a long-day photoperiod but suppressed development of the gonads under short-days. Popek *et al.* (1992, 1994) detected no obvious effect of pinealectomy on reproduction in the common carp but they did report a 2 week delay in the spawning time of rainbow trout.

The primary aim of the experiments described in this chapter was to assess the effect of intra-muscular melatonin implants and/or pinealectomy on the timing of saltwater adaptation, growth rate and maturation in Atlantic salmon parr

As the seasonal change in photoperiod and specifically the change in daylength is recognised to induce smoltification (Eriksson and Lundqvist, 1982; Duston and Saunders, 1990), experiment 1 was designed to assess if the pineal was involved in detecting this change and if so, whether the information was

relayed to the sympathetic nervous system via neural or endocrine (melatonin) transduction. This was achieved by removing the pineal, and therefore the main source of melatonin, carried out in conjunction with or without melatonin implantation, so masking any remaining natural melatonin rhythm with a constant high level of the hormone. Section 4.2 utilised potential S1 smolts and began on the winter solstice as the photoperiod changed to an increasing daylength. Section 4.3 used similar treatments but this time started on the summer solstice and used S2 smolts. This allowed precocious male maturation to be observed within the groups.

Section 4.4 was undertaken to provide more information on a possible growth increase as a result of the melatonin implants. This increase was observed at points during experiment 1 but was not evident in the final length/weight measurements. This had also been noted by Randall and Bromage (pers. comm.). The fish in this experiment were given melatonin implants as soon as they had reached 3.5g and then were observed throughout their subsequent development.

4.2 The effects of pineal removal and constant release melatonin implants applied at the winter solstice on smoltification of potential S1 smolts.

4.2.1 Objectives

This experiment was designed to establish whether pinealectomy, performed on the winter solstice, would prevent potential S1 smolts from interpreting the spring increase in daylength, and hence affect the timing of smoltification. The winter solstice hold photoperiod (8L 16D) was used to mimic the possible free running effect of pinealectomy from 22 December.

4.2.2 Materials and Methods

A mixed sex population of Atlantic salmon parr was reared from hatch and maintained at ambient temperature (Figure 2.1) under natural photoperiod (Lat. 56°03' N) at the Buckieburn Research facility. In November, the stock was graded and the top grade of fish (potential S1s) ranging from 15.2-25.6g (mean Wt. 21.4 ± 3.2g) was transferred to 0.8m diameter tanks with 100 fish per tank (section 2.1.1). All tanks were illuminated under simulated natural photoperiod (SNP) and kept at ambient temperatures. Groups of 200 fish to be maintained on SNP (Figure 4.2) were either:

* left intact (controls)
* pinealectomised (section 2.2.1)
* pinealectomised and implanted with constant-release melatonin implants (section 2.2.2)
* sham-pinealectomised and sham-implanted.

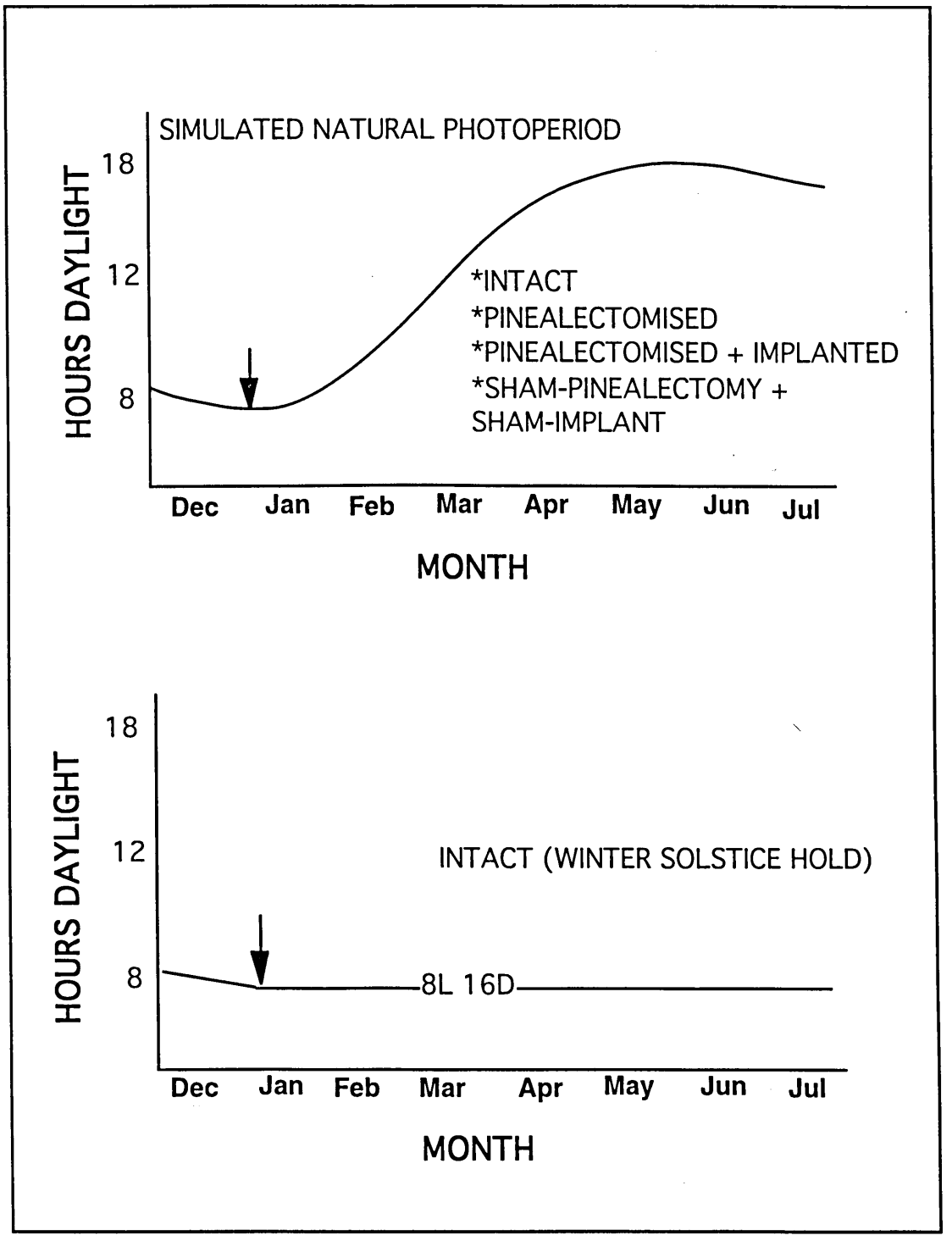


Figure 4.2

Diagram of the protocol for experiment 4.2 in which groups of 200 fish maintained on a simulated natural photoperiod were; left intact, pinealectomised, pinealectomised and implanted with constant release implants or sham-pinealectomised and sham-implanted. Fish held under a winter solstice hold photoperiod (8L 16D) were left intact.

All the operations were performed between 20-22 December. A further group of 200 individuals was left intact but placed on a winter solstice-hold photoperiod (LD 8:16) from the 22 December (Figure 4.2).

At 3 weekly intervals, morphometric measurements from 100 fish per group were taken (section 2.7.1) and a further 20 individuals from each group were used for sea water tolerance tests (section 2.7.3) which began on 17 January. Day and night-time blood samples were taken (section 2.1.3) in March and again at the end of the experiment in August and assayed for melatonin (section 2.4) to ensure that the pinealectomy operations had been successful and that the melatonin implants were continuing to release high levels of melatonin. On 6 July the water supply to the pinealectomy groups was unfortunately turned off accidentally so terminating study of these groups. The remaining groups were maintained for a further two months until smolt characteristics had regressed.

4.2.3 Results

Growth

There were no significant differences in the weights among the groups from the start of the experiment (mean weight 31.26 ± 1.8g) in December to the beginning of May (Figure 4.3). Fish maintained on a short day-hold photoperiod and those which had been both pinealectomised and implanted showed a decreased rate of growth at the end of May, while the pinealectomy group revealed increased growth over this month. Only the pinealectomy and short day groups were significantly different ($P<0.01$).

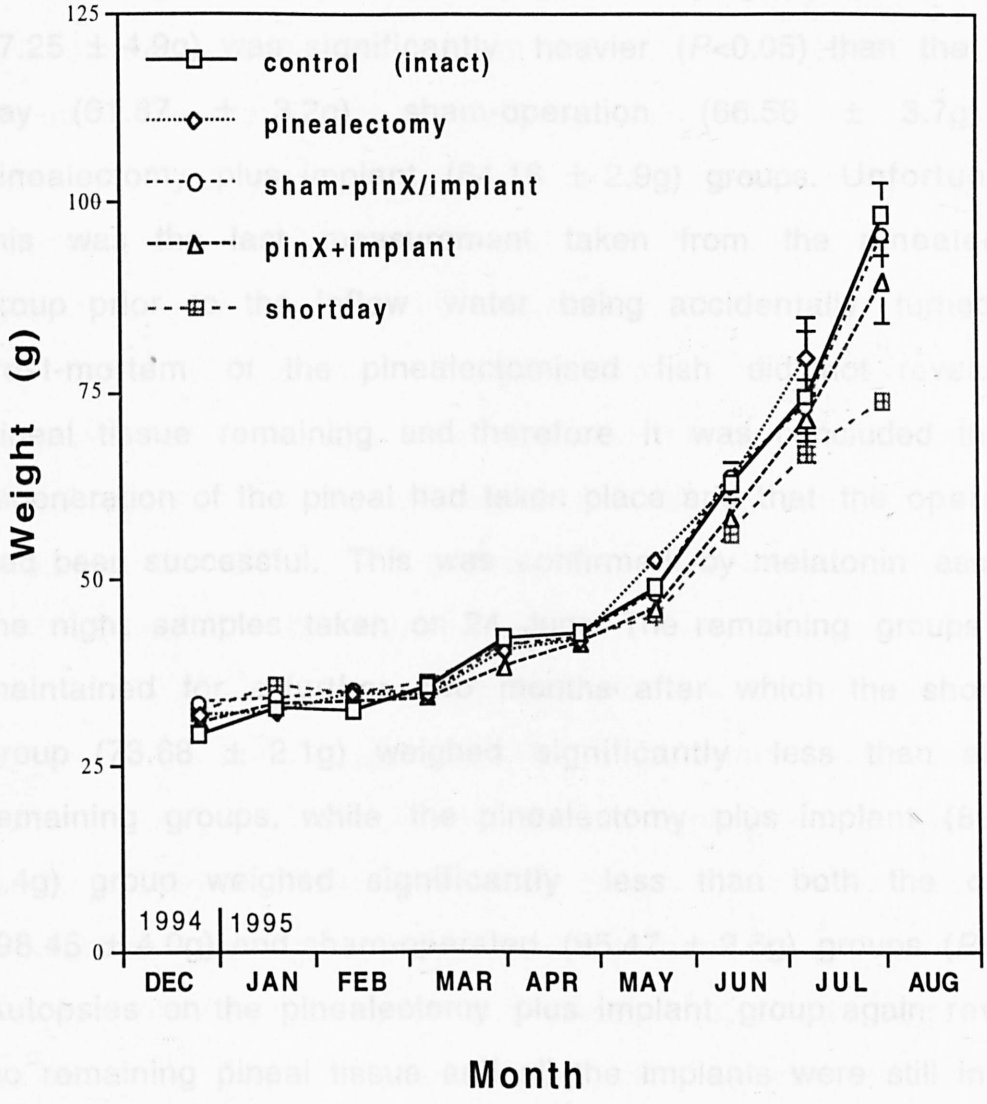


Figure 5.3

Changes in weight (mean ± S.E.M.) of potential S1 smolts under a range of photoperiod and melatonin manipulations initiated on December 22nd. Unless shown the S.E.M. values were too small to be depicted.

By the end of June, the pinealectomy group (mean weight 87.25 ± 4.9g) was significantly heavier ($P<0.05$) than the short day (61.87 ± 3.2g), sham-operation (66.56 ± 3.7g) and pinealectomy plus implant (64.16 ± 2.9g) groups. Unfortunately, this was the last measurement taken from the pinealectomy group prior to the inflow water being accidentally turned off. Post-mortem of the pinealectomised fish did not reveal any pineal tissue remaining and therefore it was concluded that no regeneration of the pineal had taken place and that the operations had been successful. This was confirmed by melatonin assay on the night samples taken on 24 June. The remaining groups were maintained for a further two months after which the short day group (73.68 ± 2.1g) weighed significantly less than all the remaining groups, while the pinealectomy plus implant (89.32 ± 5.4g) group weighed significantly less than both the control (98.45 ± 4.0g) and sham-operated (95.47 ± 2.6g) groups ($P<0.05$). Autopsies on the pinealectomy plus implant group again revealed no remaining pineal tissue and all the implants were still in place

Condition Factor

Condition factor and the results of the sea water tolerance tests were both closely correlated as a decreasing condition factor is accepted as a means of estimating the stage reached in the parr-smolt transformation (Farmer *et al.*, 1978). The condition factor begins to decrease in preparation for smoltification. This continues until a minimum level is reached whereupon the majority of salmon will have acquired sea water adaptability and the subsequent increase in condition factor signifies the closure of the smolt window.

The groups began to show sea water adaptability between the 12 March and the 20 April at which time the condition factor began to decrease (Figure 4.4), by which time all groups had achieved 100% survival rates in the sea water tolerance tests. Initially, the mean December condition factor was 1.12 (±0.07) and, although a general drop in condition factor was observed, no significant differences occurred between the groups until the beginning of June, when the condition factor of the short day group was found to be lower than that of the pinealectomy plus implanted fish ($P<0.01$). This was followed at the end of June by a rapid increase in the condition factor of the pinealectomy group from 1.02 to 1.07, in contrast to all the other groups which continued to fall. Apart from the pinealectomy plus implanted fish (Figure 4.4) the pinealectomised group was significantly greater ($P<0.05$) than the other groups. This rise appears to indicate the closing of the smolt window for the pinealectomised fish as sea water tolerance test survival rates also began to fall at this time. The final measurement of condition factor revealed the pinealectomy plus implant group to be significantly greater than the others at 1.03 ($P<0.01$) with the control, short day and sham-operation fish finishing on 0.93, 0.92 and 0.94 respectively.

Sea water Tolerance

The sea water tolerance test provides a direct indication of the percentage of the population which have achieved the ability to osmoregulate in sea water. As the test involves the direct exposure to salinities (37.5‰) in excess of the ambient marine environment (35‰) for up to 96 hours, fish without fully developed osmoregulatory systems fail to survive the test

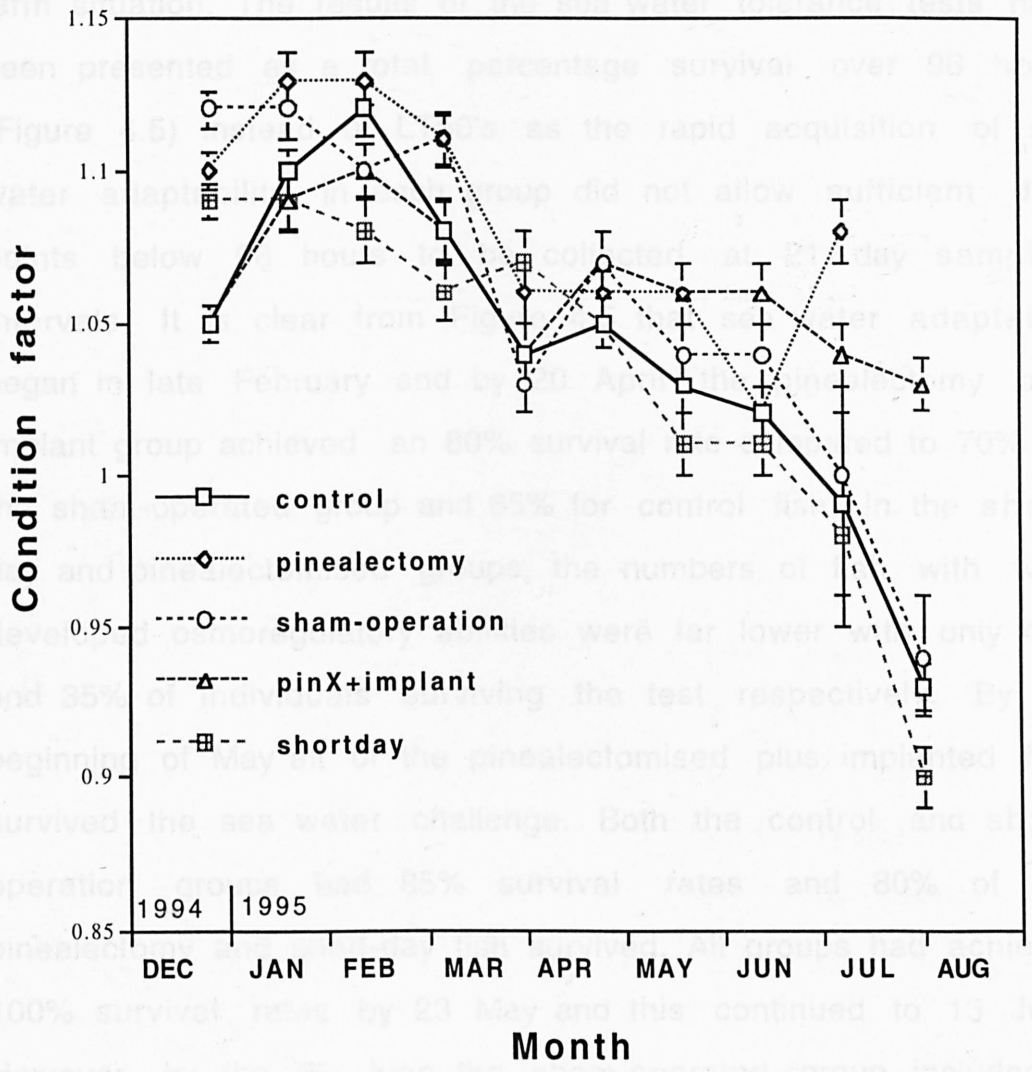


Figure 4.4

Changes in condition factor (mean ± S.E.M.) of potential S1 smolts under a range of photoperiod and melatonin manipulations initiated on December 22nd. Unless shown the S.E.M. values were too small to be depicted.

leaving only fully adapted smolts. The simplicity and conclusive results of the sea water tolerance test lends itself well to the farm situation. The results of the sea water tolerance tests have been presented as a total percentage survival over 96 hours (Figure 4.5) instead of LT50's as the rapid acquisition of sea water adaptability in each group did not allow sufficient data points below 96 hours to be collected at 21 day sampling intervals. It is clear from Figure 4.5 that sea water adaptation began in late February and by 20 April the pinealectomy plus implant group achieved an 80% survival rate compared to 70% for the sham-operated group and 65% for control fish. In the short-day and pinealectomised groups, the numbers of fish with well developed osmoregulatory abilities were far lower with only 40% and 35% of individuals surviving the test respectively. By the beginning of May all of the pinealectomised plus implanted fish survived the sea water challenge. Both the control and sham-operation groups had 85% survival rates and 80% of the pinealectomy and short-day fish survived. All groups had achieved 100% survival rates by 23 May and this continued to 13 June. However, by the 30 June the sham-operated group included 2 mortalities giving a 90% survival rate and the pinealectomised fish attained only 75% survival, while the remaining groups maintained a 100% survival rate. Again, this would seem to suggest a narrower smolt-window for the pinealectomised fish.

Observations regarding external smolt characteristics were made over the course of the experiment. Although these provide a good indication of smolt status it is open to individual discretion and therefore was only used as an estimate of smoltification within a group. All groups began to show individuals with a

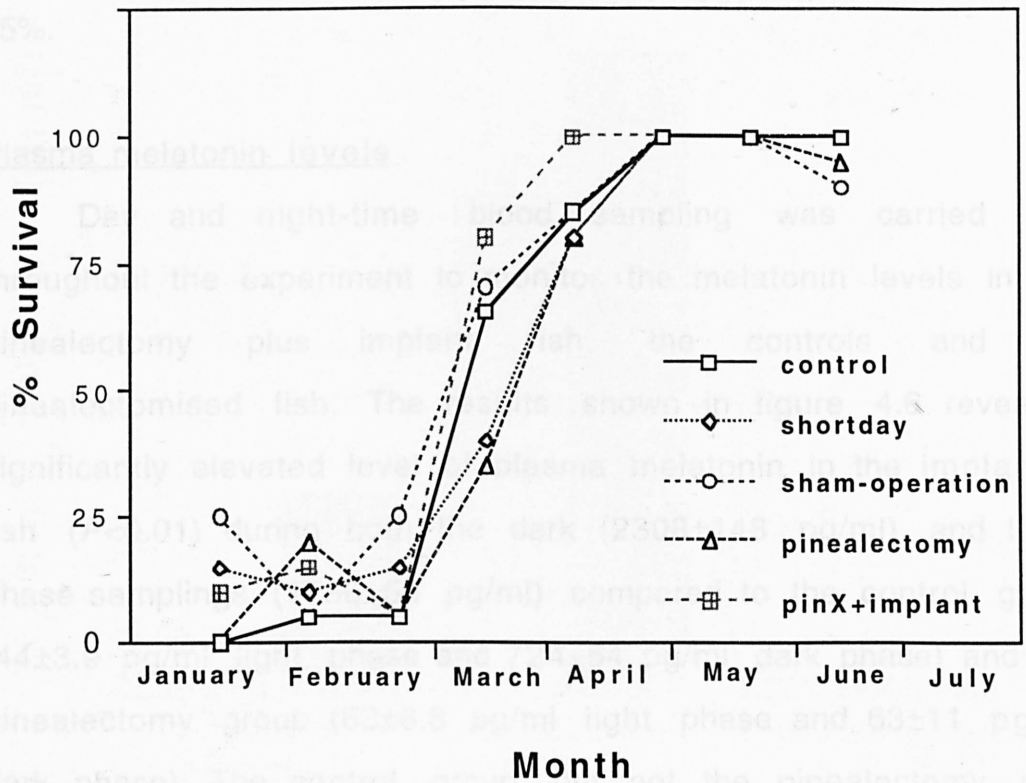


Figure 4.5

Changes in % survival from seawater tolerance tests of potential S1 smolts under a range of photoperiod and melatonin manipulations initiated on December 22nd.

silvery appearance by the beginning of February. By March approximately 75% of the pinealectomy plus implanted group and control fish displayed silver flanks and dark edges to their fins, compared to 60% of the sham-operation group and approximately 50% of the short-day and pinealectomised fish. In July, the number of parr remaining in each group was about 15%, except for the control and short-day fish in which this number was nearer 25%.

Plasma melatonin levels

Day and night-time blood sampling was carried out throughout the experiment to monitor the melatonin levels in the pinealectomy plus implant fish, the controls and the pinealectomised fish. The results shown in figure 4.6 reveal a significantly elevated level of plasma melatonin in the implanted fish ($P<0.01$) during both the dark (2308±148 pg/ml) and light phase samplings (1790±56 pg/ml) compared to the control group (44±3.9 pg/ml light phase and 724±54 pg/ml dark phase) and the pinealectomy group (63±6.8 pg/ml light phase and 63±11 pg/ml dark phase). The control group, but not the pinealectomy plus implanted fish revealed significant variation between day and night-time levels of melatonin ($P<0.05$). The melatonin levels of the pinealectomised fish did not differ from those of the control group during the photophase but were significantly lower during the scotophase ($P<0.01$). Blood samples taken at four monthly intervals throughout the experiment revealed no significant intra-group variations.

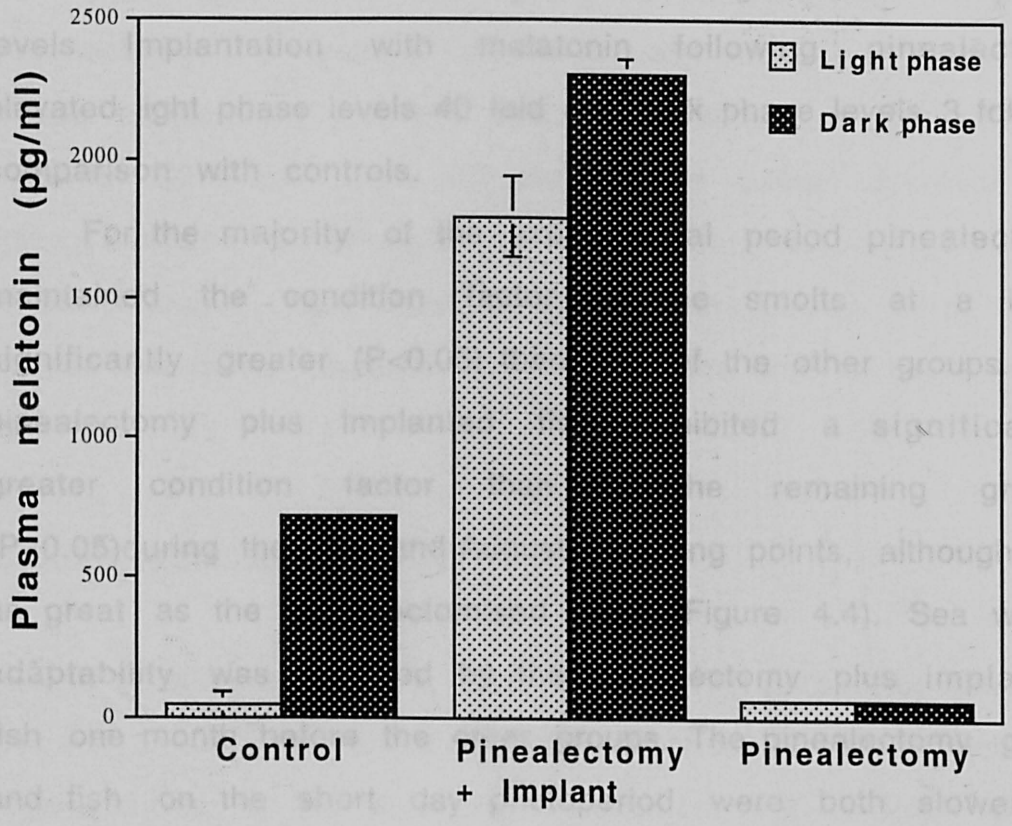


Figure 4.6

Plasma melatonin levels (mean ± S.E.M.) collected during mid-light and mid-dark phase in pinealectomised, pinealectomised plus melatonin implanted or control (intact) potential S1 Atlantic salmon parr. Unless shown the S.E.M. values were too small to be depicted.

4.2.4 Summary of results

In this experiment both pinealectomy and pinealectomy plus implantation were successful in manipulating physiological levels of melatonin in Atlantic salmon parr (Figure 4.6). Pinealectomy reduced nocturnal melatonin to the extent that no significant difference remained between light and dark phase levels. Implantation with melatonin following pinealectomy elevated light phase levels 40 fold and dark phase levels 3 fold in comparison with controls.

For the majority of the experimental period pinealectomy maintained the condition factor of the smolts at a level significantly greater ($P<0.05$) than that of the other groups. The pinealectomy plus implanted fish exhibited a significantly greater condition factor than in the remaining groups ($P<0.05$)during the July and August sampling points, although not as great as the pinealectomised fish (Figure 4.4). Sea water adaptability was achieved by the pinealectomy plus implanted fish one month before the other groups. The pinealectomy group and fish on the short day photoperiod were both slower in developing sea water tolerance; however, 100% survival rates were achieved at the same time as the control and sham-pinealectomy fish (Figure 4.5). Condition factor and sea water tolerance results suggest that pinealectomy of salmon parr at the winter solstice reduces the time period during which the fish are physiologically adapted for successful sea water transfer.

4.3 The effects of pineal removal and administration of constant release melatonin implants at the summer solstice on smoltification of potential S2 smolts.

4.3.1 Objectives

This experiment was designed to establish whether pinealectomy (i.e. removal of the primary melatonin signal and pineal innervation) on the summer solstice, would prevent potential S2 smolts from interpreting the autumn decrease and spring increase in daylength, and subsequently affect the timing of smoltification. Also investigated were the effects of constant release melatonin implants applied from the time of the summer solstice.

4.3.2 Materials and Methods.

The animals used in this experiment were the smallest grade of a population obtained from potential S2 stock which had been retained at the Buckieburn Research Unit in fresh water instead of being transferred to sea in their first year. They formed the lowest size mode from a population of mixed sex salmon parr ranging from 7.3-35.1g, with a mean weight of 21.3±5.2g. The fish were transferred to 0.8m diameter tanks 8 weeks prior to the beginning of the experiment on 22 June (summer solstice). All groups were maintained on a simulated natural photoperiod (lat. 56°03' N) and ambient temperature.

On the 21 and 22 of June 3 groups of 110 individuals were either:

* pinealectomised
* sham-pinealectomised and implanted with a constant release melatonin implant
* left intact to act as controls

Groups of 15 fish from each group were sea water challenged (section 2.7.2) at 3 weekly intervals beginning in August and continuing through to the following July. Length and weight data were also collected at these times. Blood samples were taken from 8 individuals per group during the mid-light phase and mid-dark phase in July, December and May for melatonin radioimmunoassay to confirm the effects of the pinealectomy and melatonin implantation. Male pre-smolts were judged to have matured when milt production began (i.e., when the fish were 'running').

4.3.3 Results

Growth

There were no significant differences in weight among the groups between June and November (Figure 4.7); all groups showed a rapid increase in growth with mean weights ranging from 21g to 50g over this period. Pinealectomised fish exhibited an enhanced growth during November and December and were significantly heavier ($P<0.05$) than the controls; by contrast, the melatonin implanted group was significantly smaller ($P<0.05$) than both the control and pinealectomised groups. All groups showed a reduced growth rate over the colder winter months

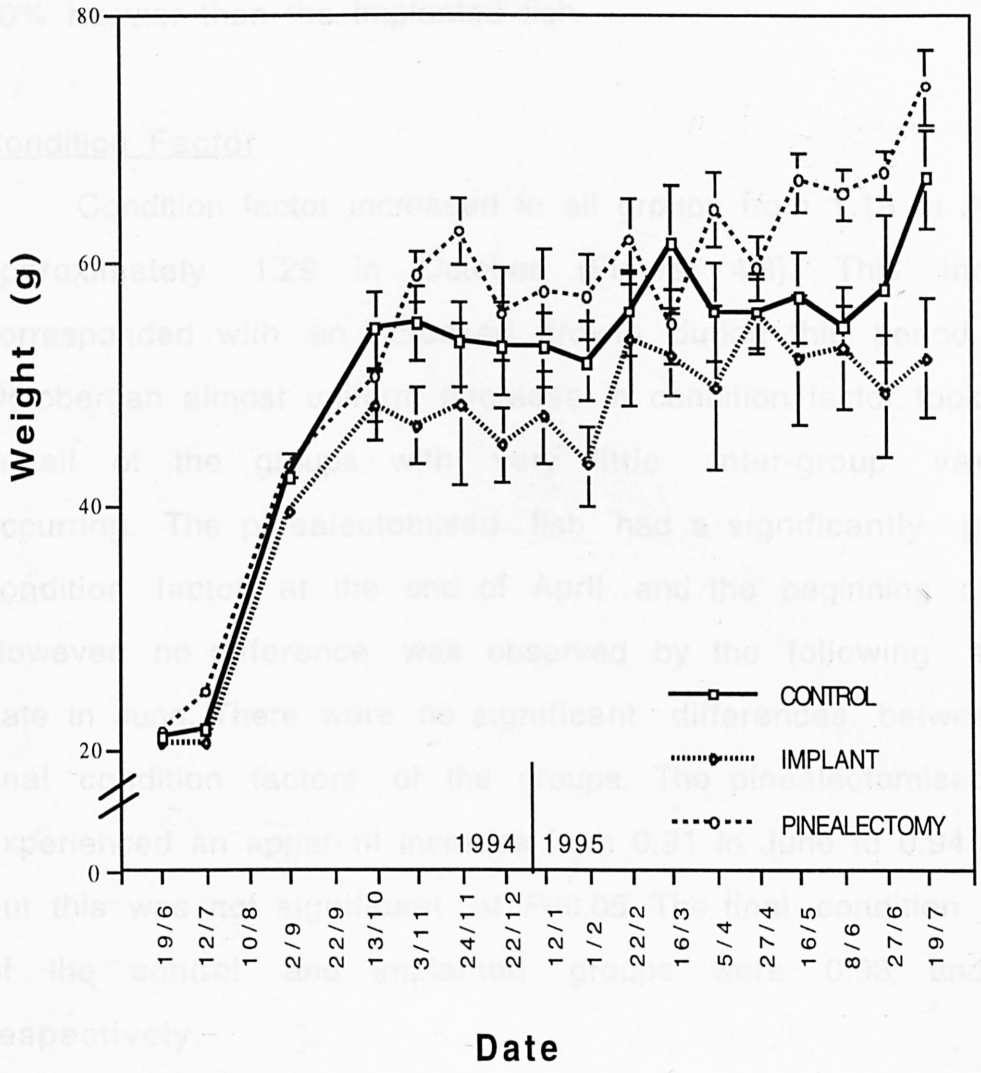


Figure 4.7

Changes in weight (mean ± S.E.M.) of potential S2 smolts after pinealectomy or melatonin implantation on June 22nd. Unless shown the S.E.M. values were too small to be depicted.

before growth resumed in May, by which time the pinealectomised fish had a significant ($P<0.05$) growth advantage over the other treatments. By the final sampling date in July, the pinealectomised fish were 10% larger than the control group and 30% heavier than the implanted fish.

Condition Factor

Condition factor increased in all groups from 1.15 in June to approximately 1.29 in October (Figure 4.8). This increase corresponded with an increased growth during this period. From October an almost uniform decrease in condition factor took place in all of the groups with very little inter-group variation occurring. The pinealectomised fish had a significantly greater condition factor at the end of April and the beginning of May. However, no difference was observed by the following sample date in June. There were no significant differences between the final condition factors of the groups. The pinealectomised fish experienced an apparent increase from 0.91 in June to 0.94 in July but this was not significant at $P<0.05$. The final condition factor of the control and implanted groups were 0.93 and 0.89 respectively.

Sea water Challenge Test

The sea water challenge test assesses the ability of parr and pre-smolts living in freshwater to osmoregulate when placed directly into a hyper-osmotic environment for 24 hours. This

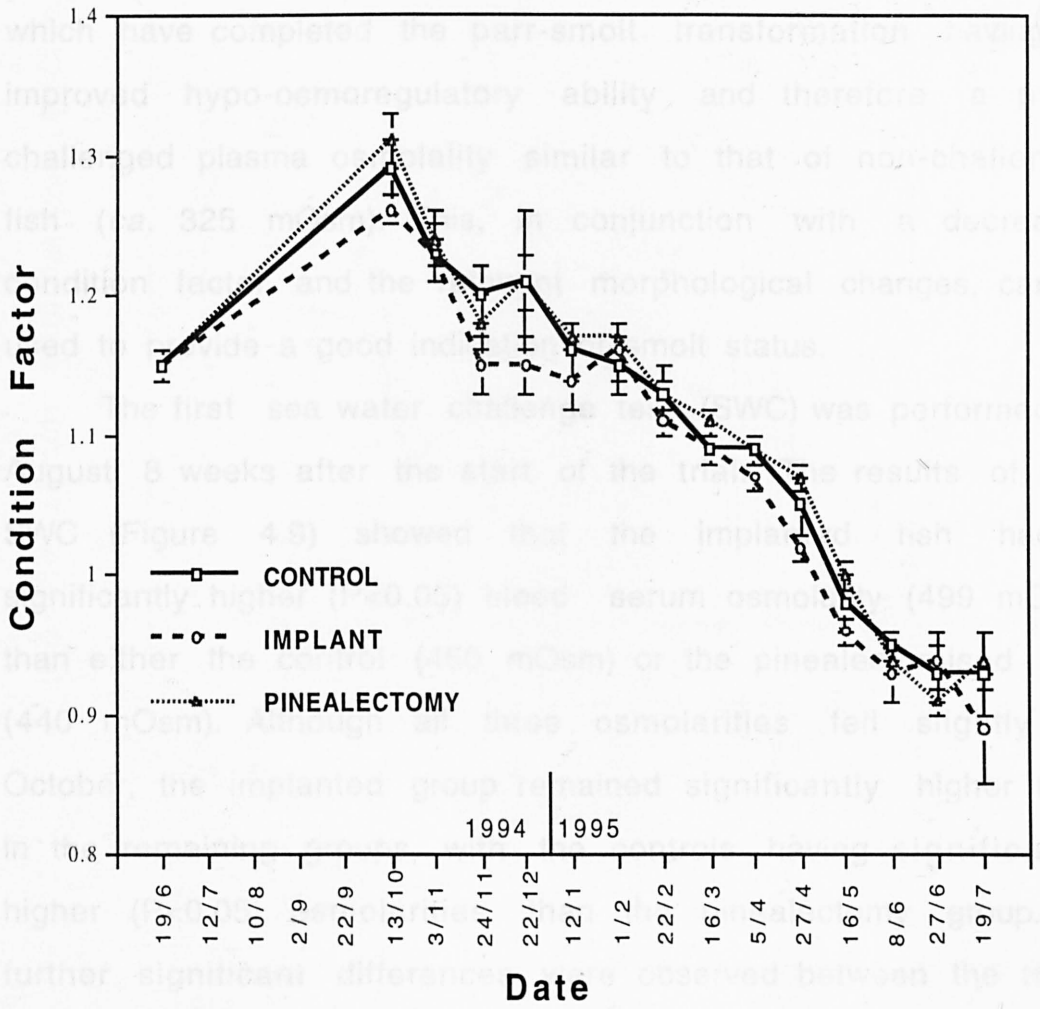


Figure 4.8

Changes in condition factor (mean ± S.E.M.) of potential S2 smolts after pinealectomy or melatonin implantation on June 22nd. Unless shown the S.E.M. values were too small to be depicted.

typically results in an immediate rise in plasma ionic concentration (Bath and Eddy, 1979) which can be measured using total serum osmolality (Duston and Saunders, 1990) or plasma/serum sodium (Blackburn and Clarke, 1978). The amplitude of the increased plasma ion concentration provides a convenient physiological index of smolt status, with individuals which have completed the parr-smolt transformation having an improved hypo-osmoregulatory ability and therefore a post-challenged plasma osmolality similar to that of non-challenged fish (*ca.* 325 mOsm). This, in conjunction with a decreased condition factor and the relevant morphological changes, can be used to provide a good indication of smolt status.

The first sea water challenge test (SWC) was performed in August, 8 weeks after the start of the trial. The results of this SWC (Figure 4.9) showed that the implanted fish had a significantly higher ($P<0.05$) blood serum osmolality (499 mOsm) than either the control (460 mOsm) or the pinealectomised fish (440 mOsm). Although all three osmolarities fell slightly by October, the implanted group remained significantly higher than in the remaining groups, with the controls having significantly higher ($P<0.05$) osmolarities than the pinealectomy group. No further significant differences were observed between the three groups until May when the control and pinealectomised fish reached their minimum serum osmolarities of 329 and 321 mOsm respectively. This indicates completion of their sea water adaptability when compared to the osmolarity of unchallenged fish of 325 mOsm. At this time the implanted fish showed a significantly higher ($P<0.05$) osmolarity (366 mOsm) having reached their minimum in April (337 mOsm). In May the levels in

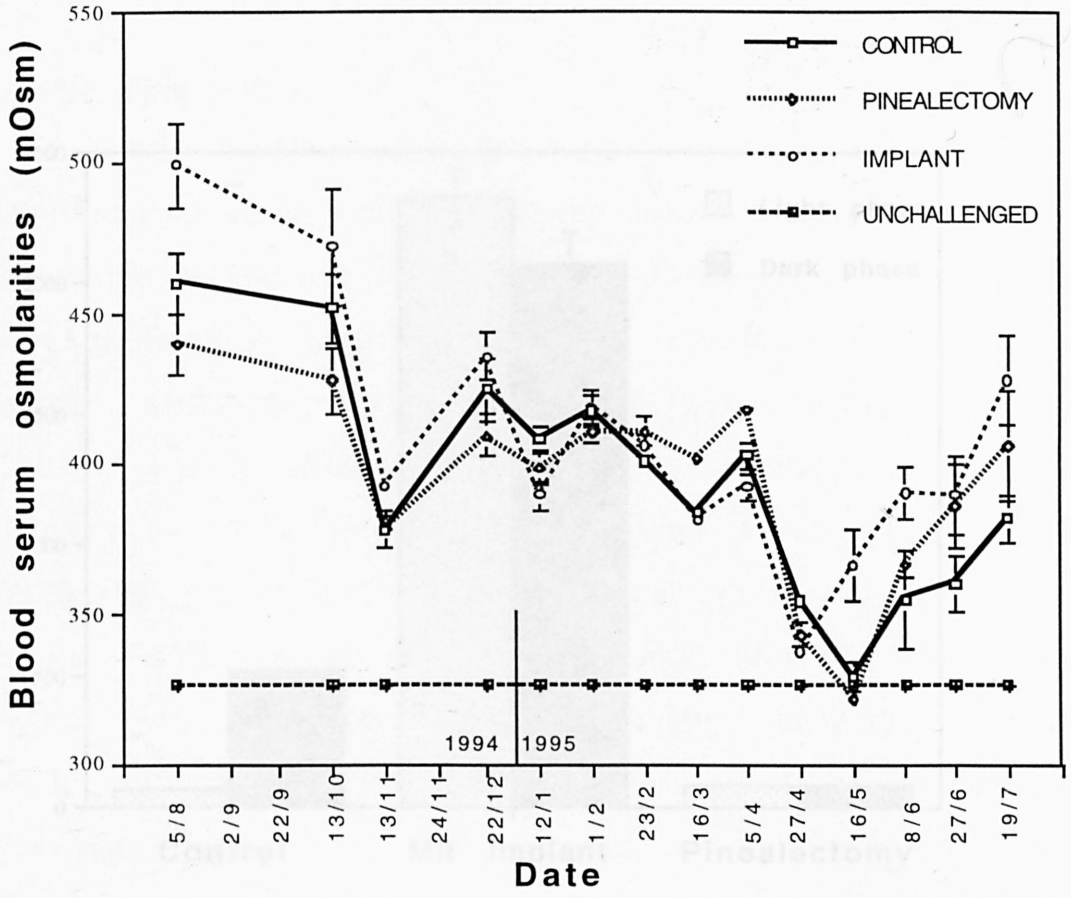


Figure 4.9

Changes in blood serum osmolarities in potential S2 Atlantic salmon smolts after seawater challenge, either pinealectomised, melatonin implanted or left intact (control) from June 22nd. Unless shown the S.E.M. values were too small to be depicted.

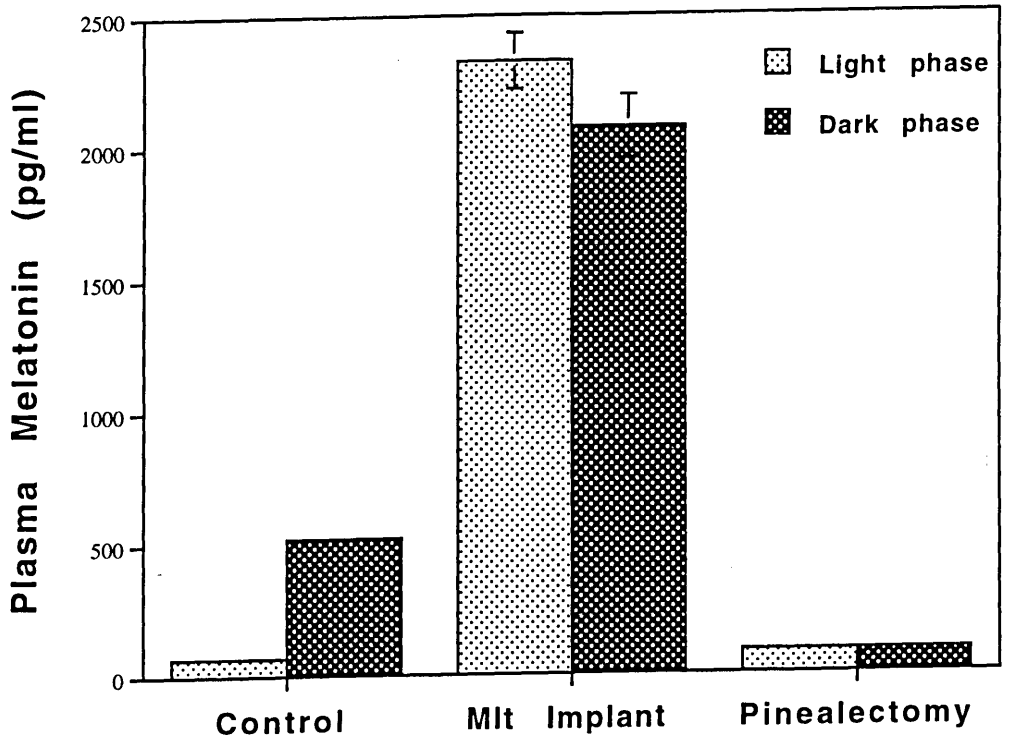


Figure 4.10

Plasma melatonin levels (mean ± S.E.M.) collected during mid-light and mid-dark phase in pinealectomised, implanted or intact potential S2 Atlantic salmon parr. Unless shown the S.E.M. values were too small to be depicted.

the implanted fish began to increase (as the smolting window closed). A further three SWC tests confirmed a continued rise in blood serum osmolarities in all the groups, with the final test in July revealing that the osmolarities of the implanted fish (428 mOsm) were significantly greater than the controls (381 mOsm).

Plasma melatonin levels

Blood samples revealed no significant intra-group differences in blood plasma melatonin between those taken at the start, middle and end of the experiment. Only the control group had significantly different levels of melatonin (Figure 4.10) between samples collected in the light (67±9 pg/ml) and dark (520±12 pg/ml) ($P<0.01$). The implants produced a 33 fold increase in normal physiological day-time levels of melatonin (2326±107 pg/ml) and a 4 fold increase in normal night-time values (2075±119 pg/ml). Pinealectomy abolished the daily rhythm of circulating melatonin (light, 85±5pg/ml; dark, 84±4pg/ml) reducing dark phase melatonin levels to the same as light phase levels in the control group, and to significantly less than the normal night-time levels ($P<0.05$).

Male maturation

As a mixed sex population of potential S2 smolts were used in this experiment, maturation of a number of the males was expected. Maturation began in October and ended in May in all three groups, with neither pinealectomy nor implantation having any affect on the timing of male maturation.

There were clear differences in numbers of mature males in the different groups (Figure 4.11). In January the control group

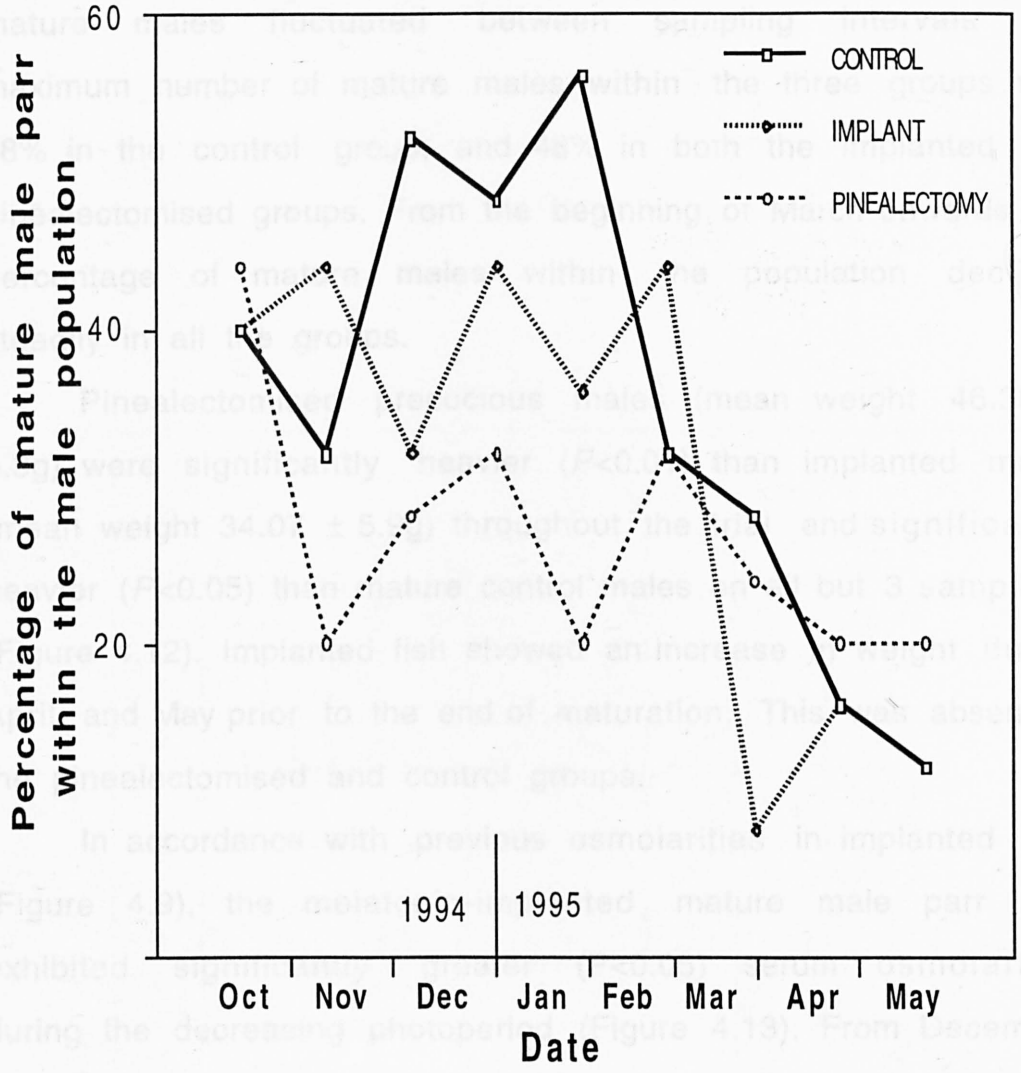


Figure 4.11

Changes in percentage numbers of mature Atlantic salmon pre-smolts which had been pinealectomised, implanted with melatonin or left intact from 22 June 1994.

had 58% of mature males within the population compared to 30% within the pinealectomised group. Although the numbers of mature males fluctuated between sampling intervals the maximum number of mature males within the three groups was 58% in the control group, and 48% in both the implanted and pinealectomised groups. From the beginning of March onwards the percentage of mature males within the population declined steadily in all the groups.

Pinealectomised precocious males (mean weight 46.38 ± 5.3g) were significantly heavier ($P<0.01$) than implanted males (mean weight 34.07 ± 5.9g) throughout the trial and significantly heavier ($P<0.05$) than mature control males on all but 3 samplings (Figure 4.12). Implanted fish showed an increase in weight during April and May prior to the end of maturation. This was absent in the pinealectomised and control groups.

In accordance with previous osmolarities in implanted fish (Figure 4.9), the melatonin-implanted mature male parr also exhibited significantly greater ($P<0.05$) serum osmolarities during the decreasing photoperiod (Figure 4.13). From December, however, there was no significant difference observed between the groups. Curiously, all mature males acquired a degree of sea water adaptability by May, shown by a decrease in blood serum osmolarity, despite the absence of any external signs of smoltification.

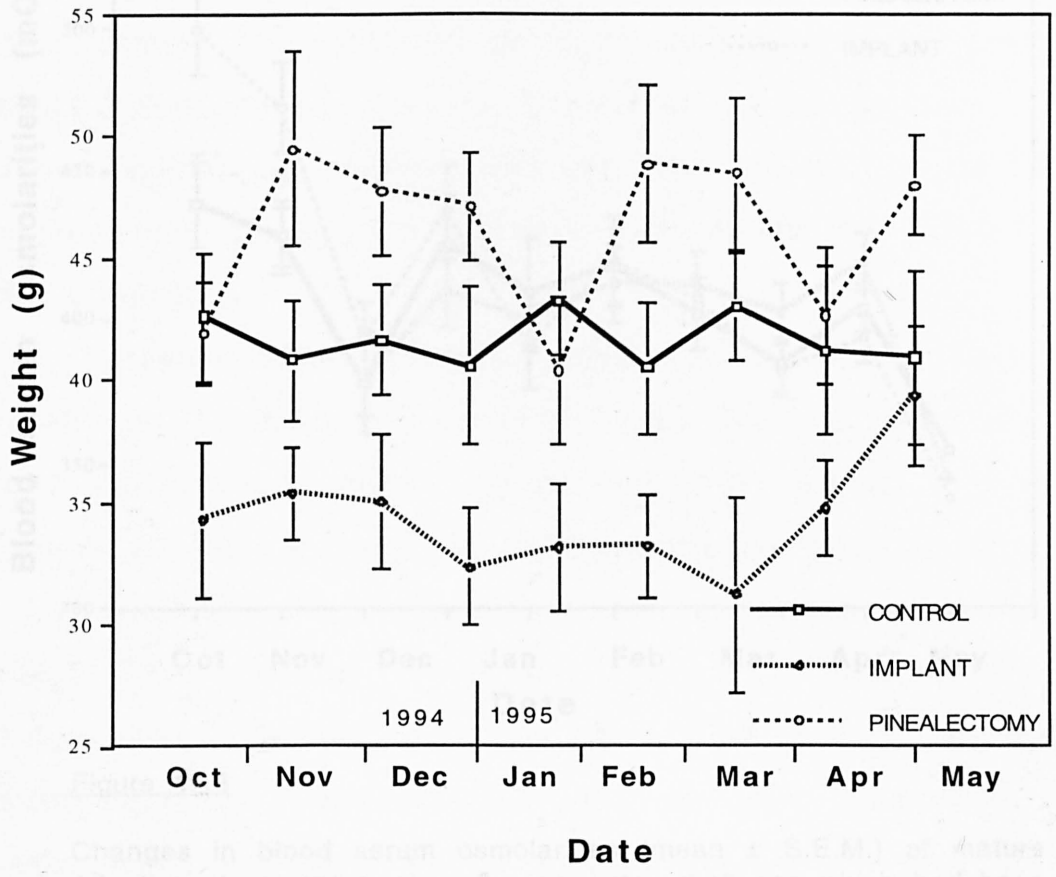


Figure 4.12

Changes in weights (mean ± S.E.M.) of mature Atlantic salmon pre-smolts which had been pinealectomised, implanted with melatonin or left intact from 22 June 1994.

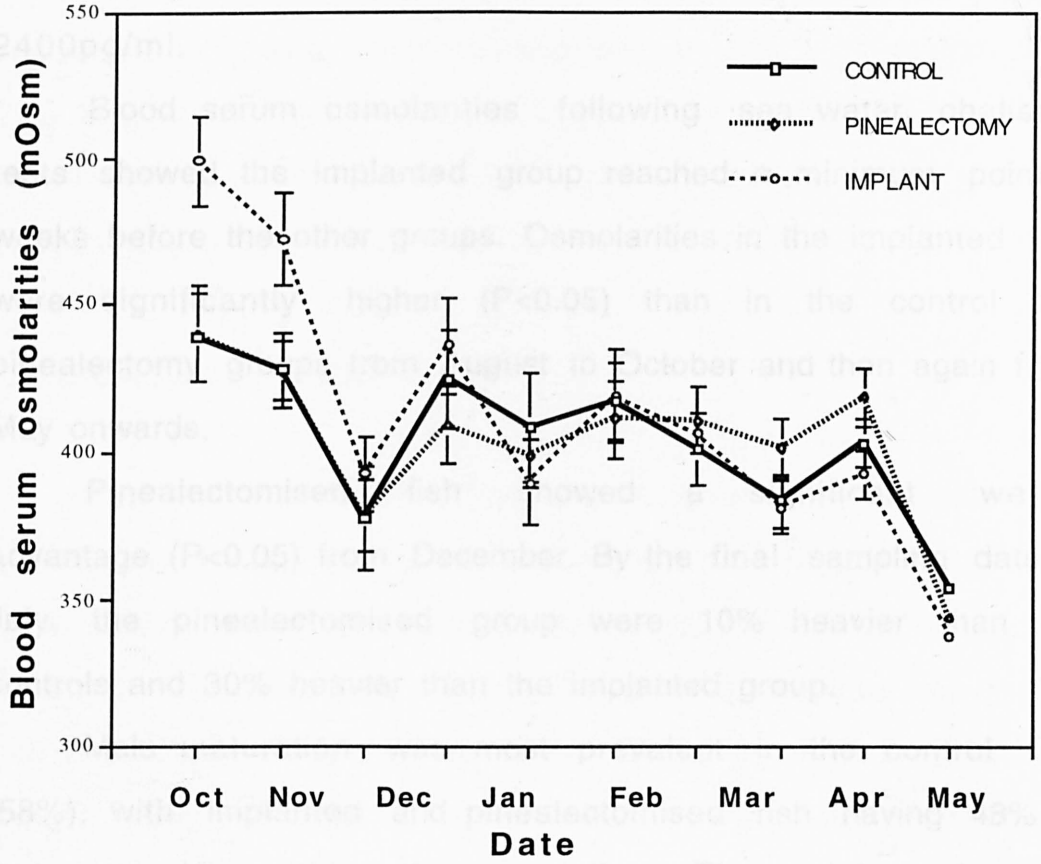


Figure 4.13

Changes in blood serum osmolarities (mean ± S.E.M.) of mature Atlantic salmon pre-smolts after seawater challenge which had been pinealectomised, implanted with melatonin or left intact from 22 June 1994. Unless shown the S.E.M. values were too small to be depicted.

4.3.4 Summary of Results

Dark phase blood samples revealed that melatonin levels in pinealectomised fish had been reduced to those of light phase levels while in the melatonin implanted fish the difference between light and dark phase levels and ranged between 2000-2400pg/ml.

Blood serum osmolarities following sea water challenge tests showed the implanted group reached a minimum point 3 weeks before the other groups. Osmolarities in the implanted fish were significantly higher ($P<0.05$) than in the control and pinealectomy groups from August to October and then again from May onwards.

Pinealectomised fish showed a significant weight advantage ($P<0.05$) from December. By the final sampling date in July, the pinealectomised group were 10% heavier than the controls and 30% heavier than the implanted group.

Male maturation was most prevalent in the control fish (58%), with implanted and pinealectomised fish having 48% of mature males within the population. The weight advantage observed in the pinealectomised parr was also present in the males which were significantly heavier than the controls, which in turn were heavier than the implanted fish. Notably, all mature fish were able to osmoregulate when challenged and no difference between these groups was observed after December.

4.4 The effects of constant release melatonin implants administered at the summer solstice on the length-frequency distribution of a population of Atlantic salmon parr

4.4.1 Objectives

This experiment was designed to investigate the effect of constant release melatonin implants on the growth, population structure and timing of smoltification in Atlantic salmon parr. This involved either; melatonin implantation, sham-implantation or maintaintenence on a short day photoperiod to give an increased duration of elevated dark phase melatonin.

4.4.2 Materials and Methods

The fish in this experiment were taken from a mixed-sex population of Atlantic salmon which had been reared from hatch (in February) under natural photoperiod (56°03' N) and at ambient temperature (Figure 2.1). In June the entire population was graded into three fractions and those in the middle grade (3.3-10.6g with a mean weight of 5.6g at 5-months post hatch) were selected. On the 23 June, 3 groups of 100 fish were either: sham-implanted and transferred to a short day (6L:18D); sham-implanted and held on a simulated natural photoperiod; or implanted with a constant-release melatonin implant and held on a simulated natural photoperiod. Length and weight data were collected from the groups every 14 days and blood samples collected from 10 individuals per group in December and again at the end of the trial. A sea water tolerance test was conducted on 50 fish from

each group when condition factor indicated that their sea water hypo-osmoregulatory ability was maximal.

4.4.2 Results

Growth

Within 14 days of implantation with melatonin, fish in this group exhibited a weight advantage over control and short day fish. After only 1 month these differences were statistically significant ($P<0.05$). Subsequently, the implanted group remained significantly heavier ($P<0.05$) than the fish held on a short day for the duration of the trial (Figure 4.14). From August until December, the control group also weighed significantly less than the implanted group ($P<0.05$). During this period, fish in the implanted group were, on average, 24% heavier than those in the control group and 34% heavier than the short day fish. Although the implanted fish remained heavier than the control group, the difference was not statistically significant again until June ($P<0.05$). After the SWT test, as expected, the mean weights of the control and implant groups were similar (52.3g and 53.4g respectively) as the lower modal class did not survive the SWT test and therefore all the remaining individuals belonged to the same modal class (S1's).

Length-frequency distribution

Figure 4.15 (a and b) shows length-frequency distributions for the experimental groups at 2 monthly intervals throughout the trial. The results of n-score tests for normality (Shapiro and Wilk, 1965) within populations revealed that, although the short

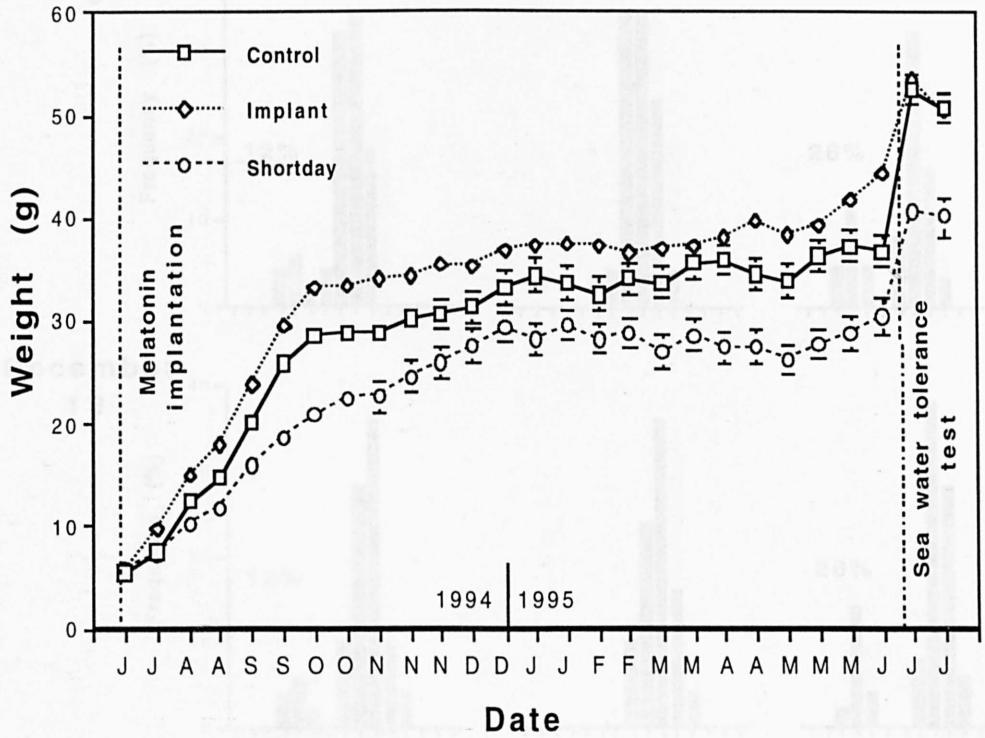


Figure 4.14

Changes in weight (mean ± S.E.M.) of potential S1 smolts maintained on simulated natural photoperiod [implanted with melatonin or intact (controls)] or held under constant short day from the summer solstice. The melatonin implantation and seawater tolerance test dates are depicted by vertical dashed lines. Unless shown the S.E.M. was too small to be depicted.

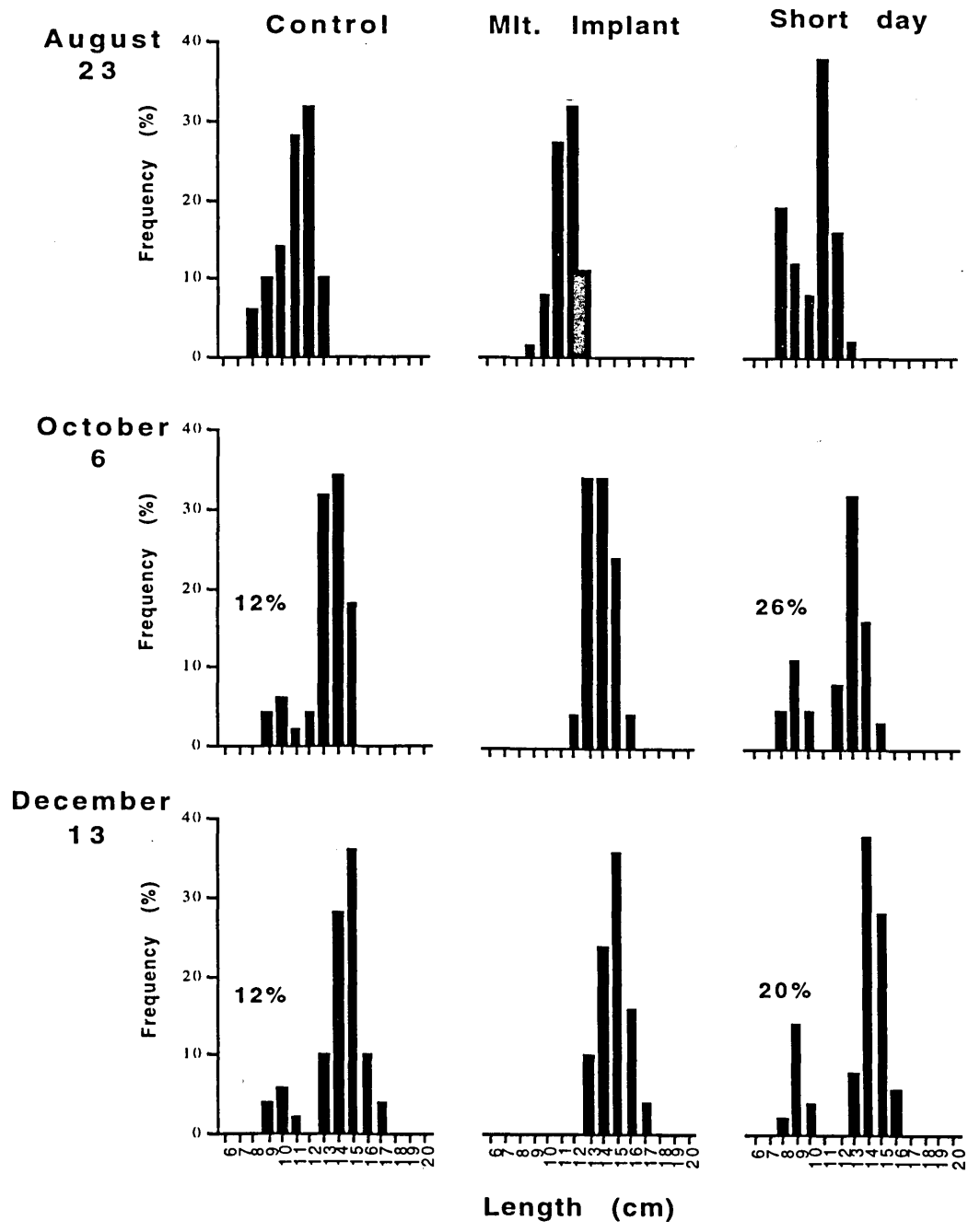


Figure 4.15a

Length-frequency distributions of 3 populations of potential S1 Atlantic salmon smolts during 1994 which had been maintained under a simulated natural photoperiod and either implanted with constant release melatonin implants or left intact or held under a constant short day from the summer solstice. The percentages of fish within the lower modal group are included for each date.

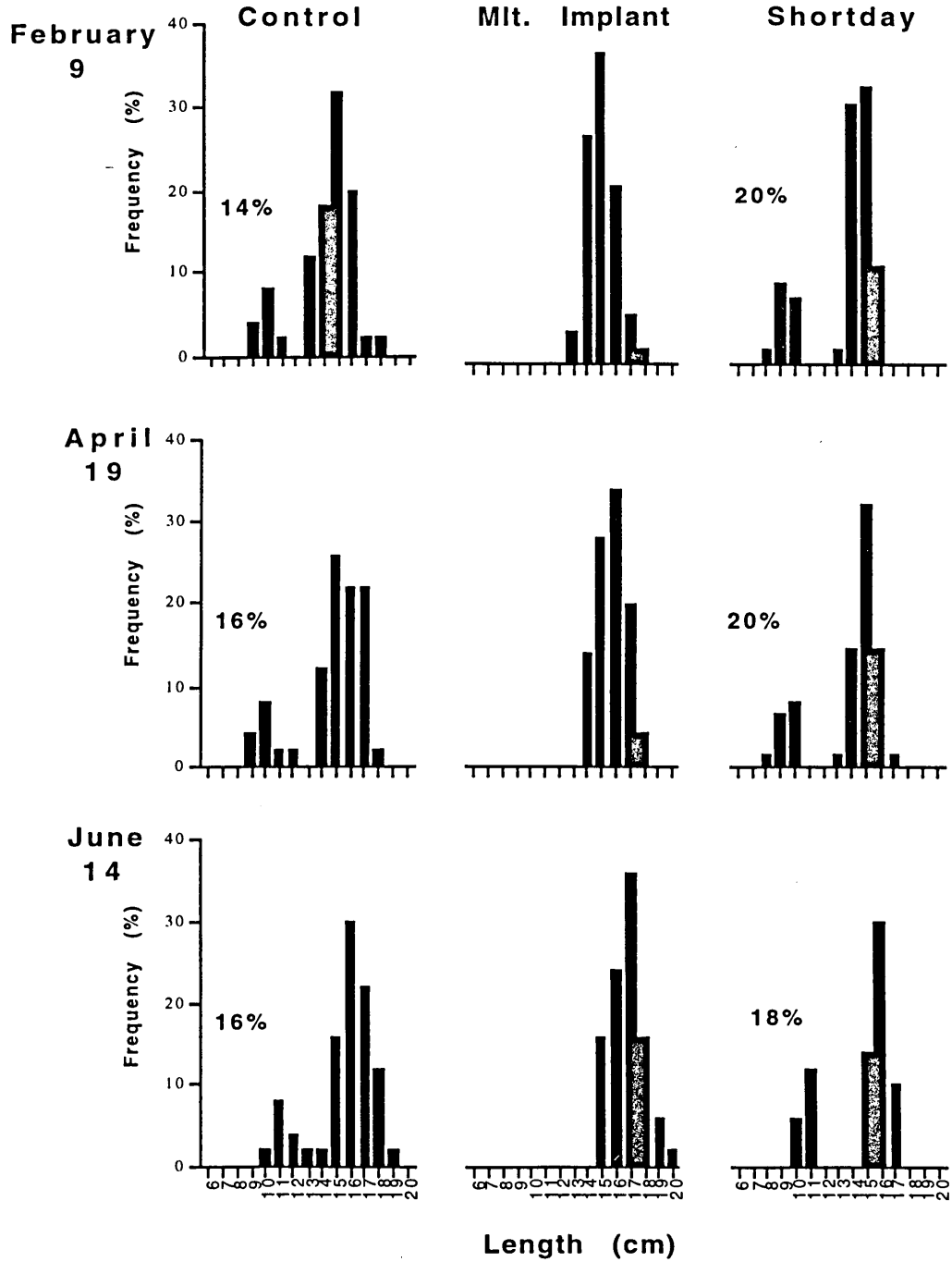


Figure 4.15b

Length-frequency distributions of 3 populations of potential S1 Atlantic salmon smolts during 1995 which had been maintained under a simulated natural photoperiod and either implanted with constant release melatonin implants or left intact or held under a constant short day from the summer solstice. The percentages of fish within the lower modal group are included for each date.

day group did not appear normally distributed, all groups had a statistically normal distribution in August, although by October both short day and control groups had a bimodal population structure. This indicated a split in life strategies with the lower-mode containing potential S2 fish (parr) and the upper-mode including potential S1 smolts. By the following June all of the implanted fish were within the upper-mode, while 16% of the control group consisted of lower-modal fish as did 18% of the short day fish. Fungal infection, especially within the short day tank, caused mortalities, predominantly from the smaller fish in the lower-modal group (LMG). This occurred in the spring and autumn as the water temperature ranged between 4 and 8°C and so reduced the size of the LMG. It is estimated that this accounted for an 18% decrease in the number of fish in LMG of the short day fish and a 5% decrease in the LMG of the controls.

Condition factor

To obtain a fair representation of the condition factor of the three groups a distinction was made between the upper and lower modal class within each group. From the length-frequency distribution of each population the divergence in modal classes was found to occur at 13cm. Therefore, the condition factor of both control and short day groups are given for the upper and lower modal classes (Figure 4.16).

The short day fish showed significant variation ($P<0.05$) in the condition factor of the upper and lower modal groups during January and February and again in May and June as the condition factor of the smolting fish decreased rapidly. The upper modal class of the control group also displayed a significantly lower

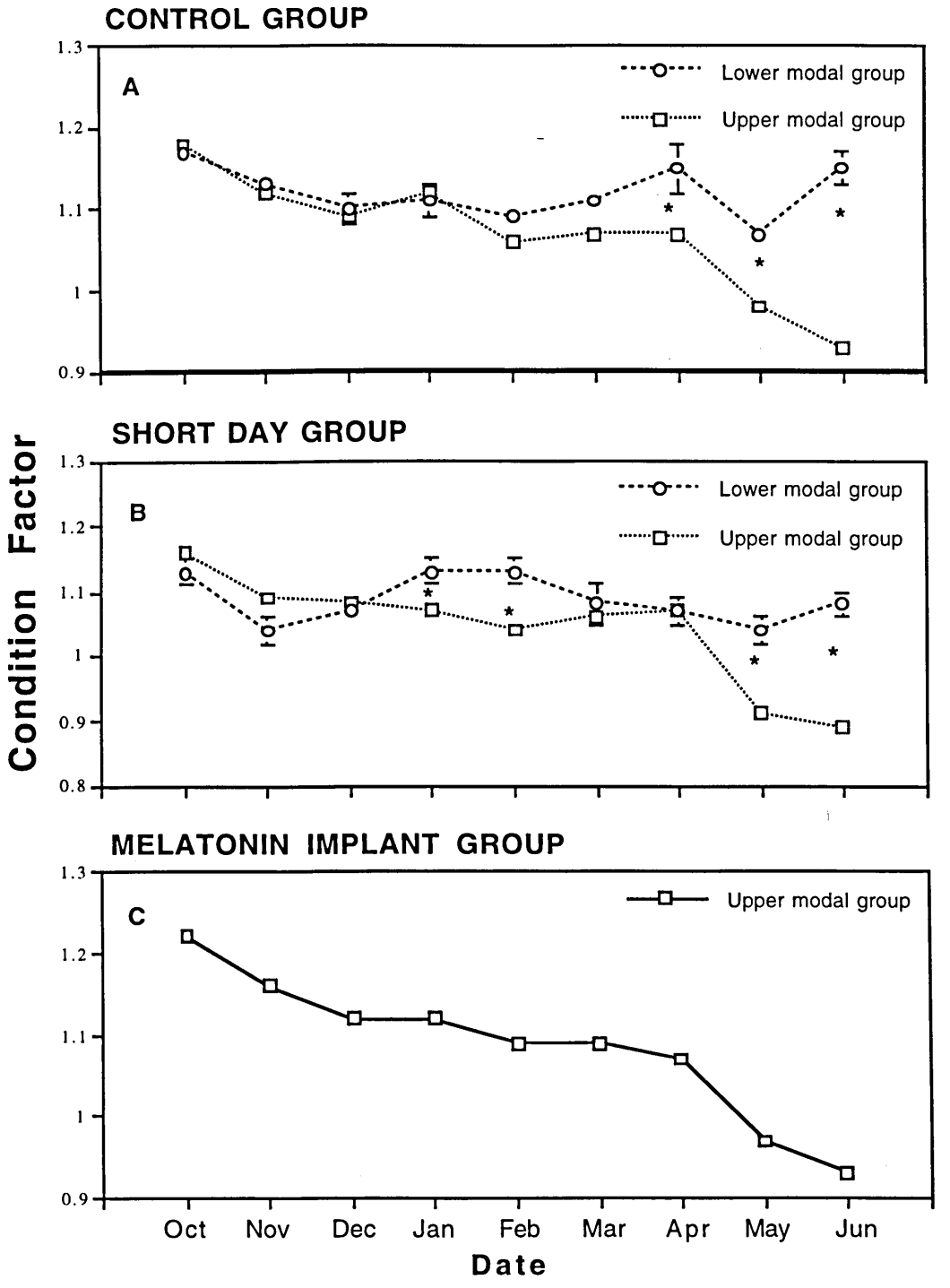


Figure 4.10

Changes in condition factor (mean ± S.E.M.) of potential S1 and S2 smolts maintained under: A) simulated natural photoperiod and implanted with melatonin B) simulated natural photoperiod and sham implanted or C) held under a constant short day and sham-operated. Significant variations are shown by * , unless shown the S.E.M.s were too small to be depicted were too small to be depicted.

condition factor ($P<0.05$) compared to the potential S2 fish from April onwards.

A comparison of the upper modal fish within the three groups showed that the implanted fish had a statistically higher ($P<0.05$) condition factor than the short day fish at all but the May sampling point. This was also found to be the case between the control and short day groups from January onwards.

Sea water tolerance test

A sea water tolerance test performed on 50 individuals from each group revealed a 92% survival rate in the implanted fish; 76% survival in the short day group; and 60% in the controls. This would suggest that melatonin implantation reduced the occurrence of S2 parr to 8% compared to 24% and 40% in the short day and control fish respectively.

External morphology, body silvering and loss of parr marks was noted in all groups in late February. The number of fish displaying smolt characteristics increased in all groups between February and June but was more marked in the melatonin implanted group. Interestingly, although all the fish in the implanted group were in the upper mode and the majority were sea water tolerant, a few fish (approximately 5%) retained parr marks.

Plasma melatonin levels

Blood samples taken in December and again in June revealed no significant differences in melatonin levels within the groups. The implanted fish had plasma melatonin levels 165 times greater than the control and short day fish (Figure 4.17; $P<0.001$).

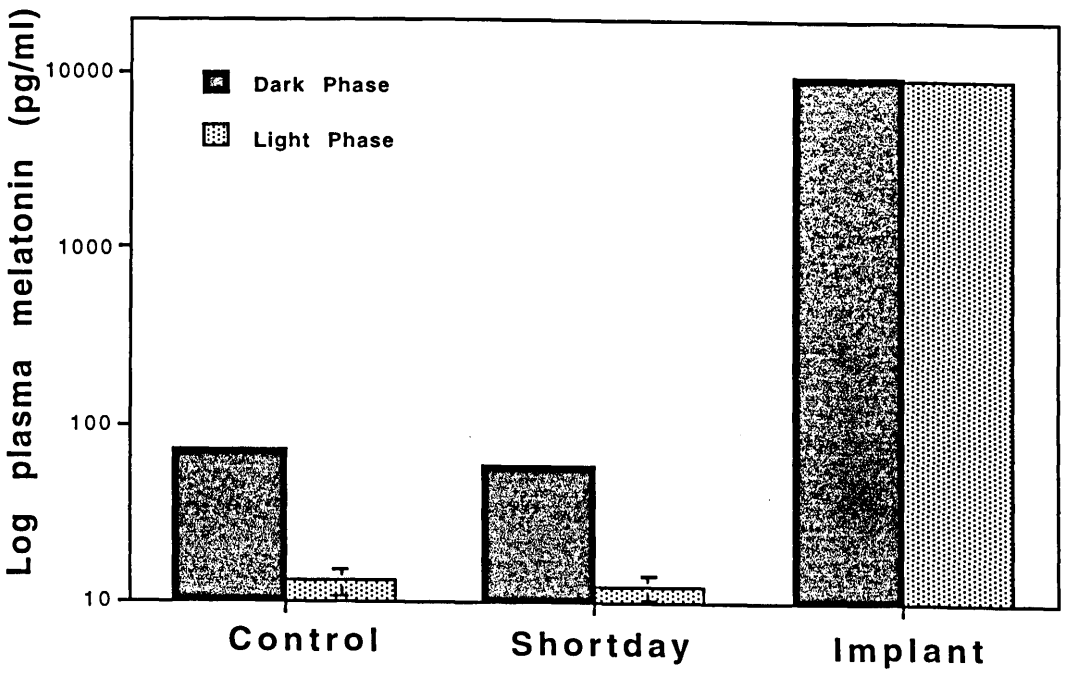


Figure 4.17

Plasma melatonin levels collected in June during mid-light and mid-dark phases in potential S1 smolts maintained under simulated natural photoperiod (pinealectomised or intact control), or held under constant short day photoperiod, from the summer solstice. Unless shown the S.E.M. values were too small to be depicted.

4.4.4 Summary of results

The results in this chapter clearly show a pattern of increased growth following the administration of constant release melatonin implants. The salmon parr implanted on the summer solstice were significantly heavier ($P<0.05$) than either the control or short day fish after only 1 month. This growth advantage was then maintained over the course of the experiment. In addition the bimodal split in the length-frequency distributions observed in the control and short day groups was absent from the implanted fish, all of which entered the upper-modal class. This was reflected in the 92% of the group which attained sea water tolerance within their first year (S1) compared to 76% of the short day group and 60% of the controls. Blood samples collected during the light phase confirmed the implants had increased melatonin levels by up to 165 times.

4.5 Discussion

The pinealectomy technique performed here for the first time on Atlantic salmon was utilised in an attempt to understand more fully the mechanisms and physiological processes by which sea water adaptation in salmonids is initiated. Pineal removal produced a positive influence on somatic growth in potential S1 and S2 smolts (experiments 1 and 2). A considerable growth advantage was also gained by implanting Atlantic salmon fry with constant release melatonin implants prior to their segregation into S1's and S2's. This resulted in an increased recruitment into the upper modal group of potential S1 smolts and hence a greater number of sea water tolerant fish in their first year.

4.5.1 The effects of pinealectomy and melatonin implantation on plasma melatonin levels

The results of pinealectomy and implantation on circulating melatonin levels in Atlantic salmon parr are illustrated in Figures, 4.6, 4.10 and 4.17 for experiments 1-3 respectively. As the quantity of melatonin within the implants and release rates could not be altered the disparity in circulating melatonin levels between groups appeared to depend on the weight of the implanted individuals. A similar age/size related variation has been reported previously in other salmonids (Gern *et al.*, 1978; Randall, 1992). This did not prove significant ($P > 0.05$) within each group, but, a four-fold increase was found between the fish in experiment 3 and experiments 1 and 2. The far higher levels associated with the melatonin implants in experiment 3 may

offer one explanation as to the apparent lack of growth stimulation in the melatonin implanted fish in experiments 1 and 2.

The levels of day and night-time melatonin measured in control groups from experiments 1 and 2 compare closely with those reported by Randall *et al.* (1995) and Thrush (1994). All control groups exhibited a type III or C pattern of melatonin secretion as described by Reiter (1988) which agrees with previous studies on Atlantic salmon (Thrush, 1994), rainbow trout (Randall *et al.*, 1991) and pike (Falcon *et al.*, 1989). It should also be noted that although pinealectomy removed the majority of the night time melatonin signal, the retina has also been suggested as a source of melatonin in teleosts (Falcon and Collin, 1991; Gern and Karn, 1983; Heuther, 1993; Zachmann *et al.*, 1992); thus the eyes may maintain a low amplitude rhythm of melatonin secretion in the absence of the pineal. Heuther (1993) reported 2 further melatonin synthesising tissues in higher vertebrates, the Harderian gland and the gastrointestinal tract. The first of these can be ruled out as teleosts do not posses a Harderian gland and to my knowledge no study has ever been made of melatonin synthesis by the gastrointestinal tract in fish, and clearly requires future investigation. Previous experiments have shown significant, but much reduced, differences between day and night-time levels of melatonin in pinealectomised Atlantic salmon parr (chapter 3; Porter *et al.*, 1996), Although no difference was observed in experiments 1 or 2, the presence of a low amplitude diel rhythm cannot be ruled out.

4.5.2 The effects of pinealectomy and melatonin implantation on growth rates of juvenile salmonids

The increased growth rate experienced by pinealectomised fish in experiments 1 and 2 occurred in parallel to the natural seasonal rhythm in growth observed in the control groups. It is as yet unclear how pineal removal is interpreted by the salmon parr. It may be perceived as a constant long day due to a reduction of nocturnal melatonin. This has been reported to promote growth in Atlantic salmon (Saunders and Harmon, 1990) and goldfish (Marchant et al., 1986). If the absence of the melatonin rhythm from the pineal is perceived as a period of continuous light, an inhibition of GH may have occurred as reported in Atlantic salmon by Bjornsson *et al.* (1995) who suggested that GH levels are strongly influenced by the seasonal change in daylength. However, it is still unknown as to whether the pineal is instrumental in the seasonal change in salmonid GH levels or whether the lateral eyes and/or deep encephalic photoreceptors may receive and transduce photic information. Clearly, future experiments involving pinealectomy during smoltification would benefit from measuring GH levels over the course of the parr-smolt transformation.

In experiment 1 the weight of the short day group was also significantly lower than the sham-operated, control and pinealectomised groups. This agrees with the findings of Lundqvist (1980) who reported significantly lower growth in immature Baltic salmon parr subjected to a constant short day regime (LD 6:18) and enhanced growth in constant long day fish (LD 20:4).

Although few studies have assessed the impact of melatonin implants in teleosts, Randall and Bromage (pers.

comm.) observed a reduction in growth parameters in Atlantic salmon post-smolts implanted in March, June and September and a slight increase in growth in December implanted fish. Work on various higher vertebrates has revealed that implants are able to mimic a short day photoperiodic signal although this was most noticeable when the implanted animals had previously been maintained on a long day photoperiod (Yates and Herbert, 1976; Thorpe and Herbert, 1977; Lincoln *et al.*, 1984; Lincoln and Ebling, 1985; English *et al.,* 1986; Poulton *et al.*, 1987; O'Callagan, 1991). It is possible then, that the suppressed growth in the short day and implanted groups may be the result of an increased daily duration of melatonin acting on growth rates. If this is the case it may also explain the increased growth of the pinealectomised groups, which received no exposure to elevated melatonin, as would be expected in fish held under constant light (LL). Weber and Smith (1980) suggested that the decreased duration of nocturnal melatonin experienced during the increasing daylength of spring was directly responsible for the decrease in plasma prolactin levels at this time, which impairs the parr's ability to osmoregulate in freshwater, therefore stimulating the initiation of downstream migration (Weber and Smith, 1980). The work of Vodicnik (1976) lends credence to this theory, as pineal removal, and hence the nocturnal rise in melatonin, decreased pituitary prolactin levels in goldfish. However, prolactin levels in parr have been shown to increase from February to July, unlike smolting fish which showed decreasing levels of plasma prolactin over this period (Prunet and Boeuf, 1989; Prunet *et al.*, 1989). Clearly, therefore, prolactin levels do not simply decrease in response to a decreased duration of nocturnal melatonin. A more

likely explanation is that photic information from the pineal may entrain an endogenous circannual rhythm of prolactin release which varies with the stage of salmonid development.

4.5.3 Measurement and entrainment of endogenous rhythms

Whether or not pinealectomy or melatonin implantation affect growth and smolt characteristics depends largely upon how the two treatments are perceived and interpreted by the brain. It is possible that the constant high levels of melatonin experienced with implantation may result in a down-regulation of melatonin receptors in the brain. However, even if specific melatonin binding sites are no longer responsive to the melatonin rhythm, neural signals from the pineal should still be transmitted. Indeed, through the work of Jiminez *et al.* (1995) and Ekstrom and Vanecek (1992) it is known that both melatonin binding sites and pineal efferent projections show a high degree of common localisation. Alternatively, following the removal of the melatonin rhythm (by either pinealectomy or implantation), parr may rely instead on an endogenous clock for the control of seasonal events.

The presence of endogenous oscillators controlling the development of rhythmic processes is well established. Gwinner (1981, 1986) has extensively covered its role in animals inhabiting environments subject to seasonal changes in photoperiod and temperature. He stated that a true endogenous rhythm should maintain its natural frequency (free-run) in the absence of exogenous cues, and that this should occur with a periodicity approximating to but significantly different from one

year. Such yearly or 'circannual' rhythms have been identified in more than 40 species to date (reviewed in Gwinner, 1986).

Endogenous control of seasonal occurrences in salmonids was suggested by Brown (1946) who demonstrated a free-running rhythm of growth in brown trout. Duston and Bromage (1986, 1991) showed that rainbow trout, maintained for 5 years and 3 spawning cycles in the absence of changing environmental cues, continued to mature at approximately yearly intervals, i.e. circannually. In Atlantic salmon Eriksson and Lundqvist (1982) reported a 10 month cycle in growth rate, condition factor and skin pigmentation, while Thrush *et. al.* (1994) proposed the presence of an endogenous component in the timing of sea water adaptation. Unfortunately the study of Eriksson and Lundqvist (1982) was only conducted over a 14 month period and therefore does not provide conclusive proof of an endogenous circannual rhythm of smoltification. However, giving the likelyhood of seasonal events in Atlantic salmon ultimately being under endogenous circannual control, the slight variations observed between groups in experiments 1 and 2 may partly be the result of endogenous control taking over in the absence of pineal entrainment.

Photoperiod undoubtedly has a strong influence on smoltification, although little is known about the mechanisms involved in this process. The daily oscillations of melatonin (Porter *et. al.*, 1996) are known to synchronise certain daily rhythms in lower vertebrates (Korf, 1994). It seems likely, therefore, that the pineal, either through neural or hormonal signals, is able to influence daily and seasonal cycles in teleosts. It has been shown that melatonin provokes the chromatic

response to darkness observed in fish and amphibians, i.e. paling of the skin in the dark and increased pigmentation during the light period (Rollag, 1988). Pineal removal has been shown to abolish the free-running rhythm in locomotor activity observed in the Asian catfish and river lamprey maintained in DD (Garg and Sundararaj, 1986; Samejina *et al.*, 1987). The melatonin rhythm in the lamprey was restored after transplantation of a pineal from another lamprey into the pinealectomised fish (Samejina *et al.*, 1987). This study would suggest that rhythmic secretion of melatonin plays a fundamental role in the organisation of circadian events, in the lamprey at least. It should be noted, however, that no differences in rhythmic locomotor activity were observed between groups in the present experiments.

Melatonin is thought to act on endocrine systems either directly (as with arginine vasotocin, see later) or through the transfer of external environmental information via the pineal to entrain the endogenous oscillators. Of these, the most likely role of the melatonin rhythm is in the entrainment of a range of hormonal and other processes associated with smoltification. It has been suggested that the entrainment of circannual events, e.g. reproduction in salmonids, is mediated via circadian processes. At present there are several alternative hypotheses as to how circadian cycles could be translated into yearly events.

The frequency demultiplication theory assumes that each circadian cycle is 'counted' with approximately 365 cycles accounting for 1 circannual cycle. Although only weak evidence for this mechanism has been obtained, by correlating locomotor activity and moult frequency in birds (Gwinner, 1986), there is experimental evidence against this theory in the timing of

reproductive function in salmonids. Duston and Bromage (1986) used resonance experiments, exposing rainbow trout to longer but fewer light-dark cycles than the ambient photoperiod, and achieved an advance in the timing of maturation. If the number of light-dark cycles were responsible for the timing of maturation then clearly a delay would have been expected. It is also apparent from their study that the total number of daylight hours was not used to synchronise events. This agrees with the present work (experiments 1 and 3) as smolts on continuous short days achieved sea water tolerance at approximately the same time as control fish. Furthermore, if the melatonin rhythm was used as an indication of the daily light/dark cycle then pinealectomy would be expected to prevent any such entrainment. Therefore, it seems unlikely that frequency demultiplication is the mechanism by which the circannual clock is set.

Bunning (1936, 1960) was responsible for the introduction of the 'external coincidence' model. This has since been developed and forms the basis of the second theory of time measurement. It relies on the light phase of the photoperiod (external influence) coinciding with an internal circadian rhythm of photosensitivity. Thus, in 'long-day breeding animals it is suggested that a photosensitive phase occurs between 12 and 24 hours after dawn (lights-on) which requires the long days of summer to illuminate the light-sensitive phase (Figure 4.18a).

Night-interruption experiments have been used to investigate the role of circadian cycles in time measurement in several fish species including: the stickleback (Baggerman, 1980, 1985); catfish (Sundararaj and Vasal, 1976); medaka (Chan, 1976); and mummichog (Day and Taylor, 1983). Night-interruption

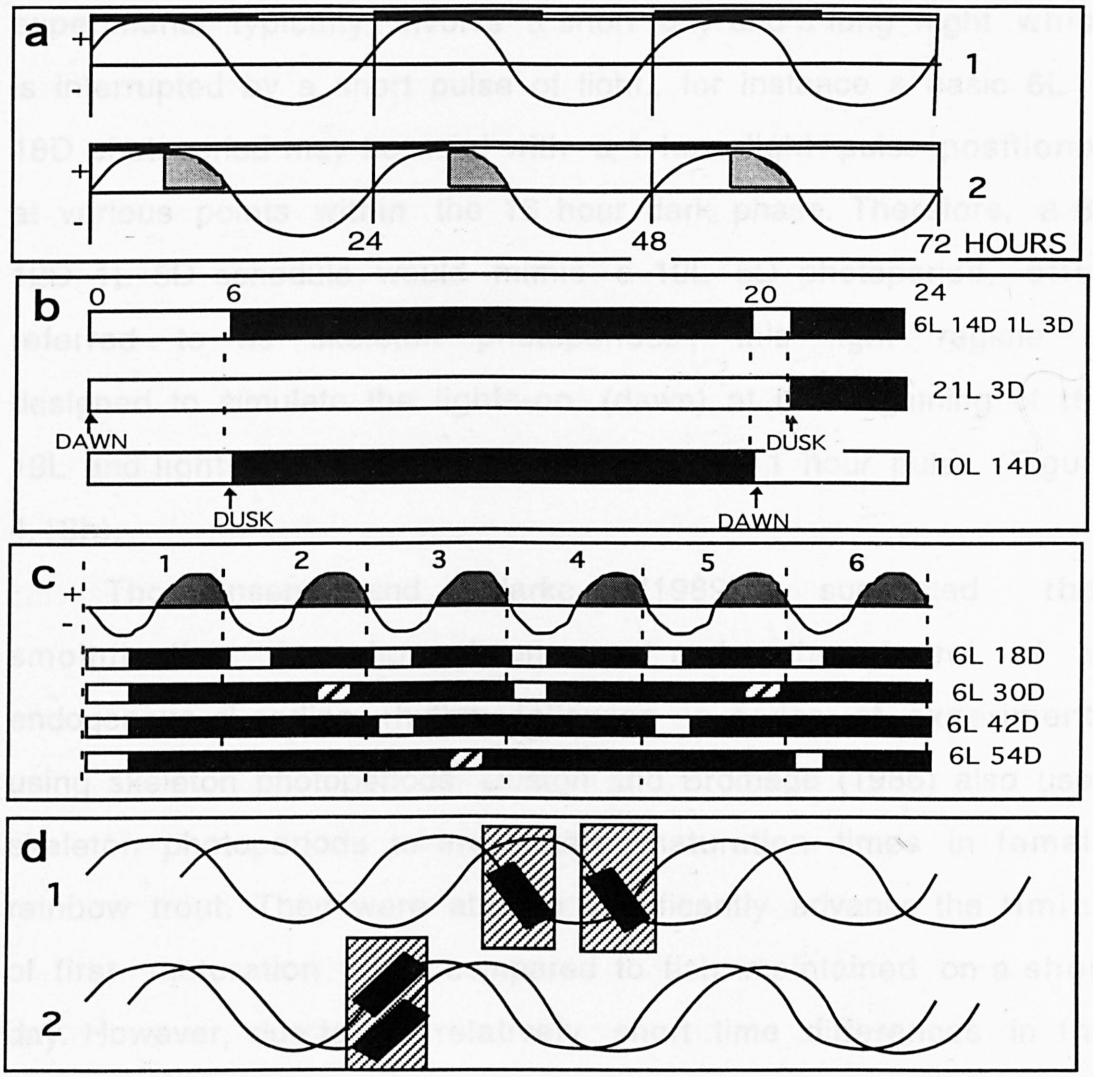


Figure 4.18

a) Simplified version of the Bunnings 'external coincidence' hypothesis. The sinusoidal wave represents the endogenous rhythm and the external photoperiod is displayed above. In figure a1, the light phase fails to fall on an area of +ve photosensitivity, while in 1b, the short night long days illuminates a +ve phase (shown by shadad area).

b) Possible interpretation of skeleton photoperiods, The first photoperiod shows the actual light cycle used, while the next 2 diagrams illustrate how the light cycles may be interpreted as either a long or short day photoperiod depending upon when the animal perceives the dawn.

c) A typical resonance experiment showing the positions of the light cycles in relation to the +ve photosensitive phase, stimulatory light phases are shown by hatched areas.

d) Schematic representation of the 'internal coincidence' model, d1, shows the phase relationship between 2 circadian rhythms where the +ve phase points (black block) do not coincide. d2 shows a case of internal coincidence.

experiments typically involve a short day and a long night which is interrupted by a short pulse of light., for instance a basic 6L 18D photoperiod may be used with a 1 hour light pulse positioned at various points within the 18 hour dark phase. Therefore, a 6L 12D 1L 5D schedule would mimic a 19L 5D photoperiod, often referred to as 'skeleton photoperiods', this light regime is designed to simulate the lights-on (dawn) at the beginning of the 19L and lights-off (dusk) at the end of the 1 hour pulse (Figure 4.18b).

Thorarensen and Clarke (1989), suggested that smoltification in coho salmon was under the control of an endogenous circadian rhythm following a series of experiments using skeleton photoperiods. Duston and Bromage (1986) also used skeleton photoperiods to investigate maturation times in female rainbow trout. They were able to significantly advance the timing of first maturation when compared to fish maintained on a short day. However, due to the relatively short time differences in the spawning times and ambiguous interpretation of night interruption photocycles they repeated this work using resonance experiments. In agreement with Duston and Bromage (1986), Earnest and Turek (1983) also provided evidence that skeleton photoperiods could be interpreted as either long or short day photoperiods in golden hamsters (Figure 4.18b).

Resonance photoperiods expose the animal to one of a series of light/dark cycles in which a fixed light phase of short duration is coupled with varying durations of darkness (Figure 4.18c). These experiments avoid entrainment of internal rhythms as the light/dark cycles are outwith a 24 hour period (Sumpter, 1990). once again the results obtained by Duston and Bromage (1986)

failed to provide conclusive proof of circannual rhythms of entrainment within the rainbow trout.

A further flaw in the external coincidence model is the persistence of circannual rhythms in the absence of a light/dark cycle. The rainbow trout has been shown to mature in the absence of a light-dark cycle (Duston and Bromage, 1986) and Poston and Livingstone (1971) revealed circannual rhythms in the brook trout held under DD. Baggerman (1980, 1985) found similar results in sticklebacks but suggested that the position of the 'photoinducable phase' does itself have a circannual rhythm and therefore changes over the year. However, it seems likely that, although no conclusive evidence exists to discredit this model, the external coincidence model is not used by salmonids.

The final timing mechanism, which was initially proposed by Pittendrigh (1972), relies on the phase relationship of two or more endogenous circadian rhythms (Figure 4.18d). As stimulation in this model results from the coincidence of phases of internal rhythms it negates the necessity for external environmental cues, although the internal rhythms are still thought to be entrained by the seasonal photoperiod. The underlying presence of endogenous rhythms allows this model to accommodate annual events such as maturation occurring in the absence of photoperiodic stimulation i.e. under LL or DD. The lack of evidence for this mechanism emphasises the difficulty in designing experiments to investigate this theory however its role in salmonid development cannot be excluded.

4.5.4 Possible melatonin/growth hormone interactions

Maximum growth in pinealectomised smolts coincided with that of the control groups i.e. during the spring and early summer when increasing daylength was accompanied by rising water temperatures. Unfortunately growth hormone (GH) was not measured during the experiments. Hence, it is not known whether pinealectomy or implantation altered the natural increase in plasma GH levels. There is a concomitant increased in growth rate and GH levels in spring and early summer which is possibly initiated by the increasing daylength in January (Boeuf, *et al.*, 1989; Prunet *et al.,* 1989; Bjornsson *et al.*, 1989, 1994; Stefansson *et al.*, 1991; Tanguy *et. al.*, 1994). Bjornsson *et al.* (1994,1995) confirmed that photoperiod is the major zeitgeber for this rise and, although temperature is known to stimulate GH levels (Bjornsson *et al.*, 1989), it seems that in Atlantic salmon at least it is not responsible for the initial increase (Bjornsson *et al.*, 1995).

The rise in GH occurs as part of the preparatory mechanisms for downstream migration (Prunet *et. al.*, 1989; Yada *et al.*, 1991,1992; Bjornsson *et al.*, 1989, Boeuf *et al.*, 1994). GH has recently been shown to directly affect the osmoregulatory ability of the gills by increasing the number of α-type chloride cells while also decreasing the number of β-type chloride cells (Prunet *et. al.*, 1993) which, together with an increased Na^+, K^+-ATPase activity, facilitates branchial excretion of Na^+. Exposure of coho, chum and Atlantic salmon, and rainbow trout, to sea water, after maintenance in fresh water, has been shown to elevate the amplitude of the transient increase in circulating GH levels

(Sweeting *et al.*, 1985; Hasegawa *et al.*, 1987; Ogasawara *et al.*, 1988; Bjornsson *et al.*, 1989; Boeuf et. al., 1989; Collie *et al.*, 1989; Young *et al.*, 1989; Sakamoto *et al.*, 1990 and Sakamoto and Hirano, 1991). The importance of GH in smoltification is evident when the *in vivo* effects of GH administration on salmonids are considered: these include a decrease in plasma Na^+ and Cl^-, a decrease in plasma Ca^{2+}/Mg^{2+}, increases in size and number of α-type gill chloride cells and gill Na^+, K^+-ATPase activity and an increase in intestinal proline absorption (see Prunet *et al.*, 1993; review by Sakamoto *et al.*, 1993; Boeuf *et al.,* 1994).

Considering the effects of photoperiod on GH levels the presence of a light-pituitary axis seems likely (Komourjian *et al.*, 1976, Bjornsson *et al.*, 1994, 1995). Ekstrom (1984) showed that the pineal possesses neural projections into the central brain areas of teleosts; this was further elaborated by Ekstrom and Korf (1985) and Jiminez *et al.* (1995) who identified pineal efferent projections within the optic tectum, dorsal and ventral thalamus, anterior hypothalamus and habenula. Significantly, these areas are also known to receive retinal innervation, suggesting an overlapping of photic stimulation in these areas.

The involvement of the pineal in osmoregulation was first suggested by de Vlaming *et. al.* (1979) who reported a change in serum osmolarity following pinealectomy in goldfish. Ostholm *et al.* (1992) also observed an increase in the number of acetylcholinesterase-positive cells, indicating increased neural activity within the pineal during the period of parr/smolt transformation. However, the ability of melatonin to exert a light dependant influence on the endocrine activity of teleosts has also been proposed (Martinoli *et al.*, 1991; Ekstrom and Vanacek,

1992). It is tempting to suggest a pineal/GH link on the evidence above, but, at the present time there is no conclusive evidence to link the pineal gland to GH regulation in salmonids although this area clearly requires further research.

4.5.5 The role of melatonin in salmonid osmoregulation

Recent work (Ekstrom and Vanacek, 1992; Holmqvist *et al.*, 1994; Pang *et al.*, 1994) emphasised the link between melatonin and pituitary function. Both studies found extensive melatonin binding (sites) within the optic tectum and hypothalamic optic nucleus (HON) in Atlantic salmon. Similar results were reported by Martinoli *et al.* (1991) in the goldfish. The appearance of pinealofugal termination sites (Holmqvist *et al.*, 1994) and increased retinal inputs (Ebbesson *et al.*, 1988) into the HON during the parr/smolt transformation strongly suggests the importance of photic information to these central brain areas during sea water adaptation.

Work by Holmqvist *et al.* (1994) has also shown that hypophysiotrophic arginine vasotocin (AVT), isotocin (IST) and dopamine (DA) neurones within the HON have the potential to be affected by external photic information. The actions of these neurohormones on the osmoregulatory and physiological state of salmonids is well documented. Dopamine is known to act on pituitary function, inhibiting control of prolactin (James and Wigham, 1984), thyrotropin (Olivereau *et al.*, 1988) and gonadotropin (Peter *et al.*, 1986) and stimulating GH release in goldfish (Wong *et al.*, 1993). Kahn and Joy (1988) revealed that *in vivo* melatonin administration to the catfish can act directly to influence either DA or norepinepherine (NA).

In teleosts AVT is a posterior-pituitary neurohormone, synthesised and stored in hypothalamic neurosecretory neurones before release from the neurohypophysis (Van den Dungen *et al.*, 1982). Arginine vasotocin is thought to play an important role in changing the osmoregulatory capability of salmonids during smoltification by influencing cells in the pituitary, kidney and gills (Peter, 1977; Peter and Fryer, 1983; Hyodo and Urano, 1991), however, no clear efffects have yet been demonstrated Furthermore, the distribution of AVT-immunoreactive fibres and binding sites within the pre-optico-hypophysial neurosecretory system has prompted suggestions as to its role as a neurotransmitter and/or a neuromodulator in the central nervous system (Ekstrom and Vanacek, 1992; Goosens *et al.*, 1977; Holmqvist *et al.*, 1994; Kulczykowska, 1995; Van den Dungen *et al.*, 1982). Notably, there is evidence that vasopressin and vasotocin (closely related to AVT and IST) are synthesised in the mammalian pineal organ (Olcese *et al.*, 1993; Reiter, 1975) and AVT has been detected in the fish pineal (Binkley, 1988). Furthermore, the greatest concentration of AVT-immunoreactive fibres coincides with areas of the brain which posses the highest concentrations of melatonin binding sites providing further evidence of an AVT/melatonin interaction (Ekstrom and Vanacek, 1992; Holmqvist *et al.*, 1994; Kulczykowska, 1995).

Kulczykowska's (1995) model for interactions between AVT and melatonin was principally derived from mammalian studies (see Ebels and Batemans, 1986). In this model he proposed that pineal melatonin synthesised during the scotophase, inhibits AVT production in the hypothalamic neurones (and possibly the pineal). However, it was also suggested that when photophase

melatonin levels fall below an inhibitory threshold level this would allow increased activity within the AVT-neurones. The presence of a negative feedback loop was also proposed whereby an increase in the level of AVT has an inhibitory affect on melatonin production (Figure 4.19). This has been demonstrated in rats by Binkley (1988) who was able to decrease plasma melatonin levels through the administration of AVT.

Maintaining an osmotic equilibrium within; rainbow trout, flounder and medaka transferred from sea water to fresh water was shown to require a high level of neurohypophysial and plasma AVT indicating an increase in both synthesis and release (Haruta *et al.*, 1991; Perrot *et al.*, 1991). The opposite was found to be true when the fish were transferred from a low (freshwater) to high (sea water) osmotic gradient. This is corroborated by the high levels of pro-vasotocin mRNA found in euryhaline fish in fresh water and by the return to basal levels following transfer to sea water (Hyodo and Urano, 1991).

These findings offer a possible explaination to the anomalies in the sea water adaptation of the implanted smolts in this chapter. In experiment 1 the melatonin implants appear to have had no effect on the osmoregulatory ability of the juvenile fish until the parr/smolt transformation began (Figure, 4.5), whereupon an advancement of 1 month in attainment of hypo-osmoregulatory ability was observed in the groups, especially in comparison with the pinealectomy and short day fish. Rourke (1994) also reported a significant increase in gill chloride cells and Na^+ K^+-ATPase activity in steelhead smolts treated with constant release melatonin implants and proposed that melatonin plays an important role in the smoltification process.

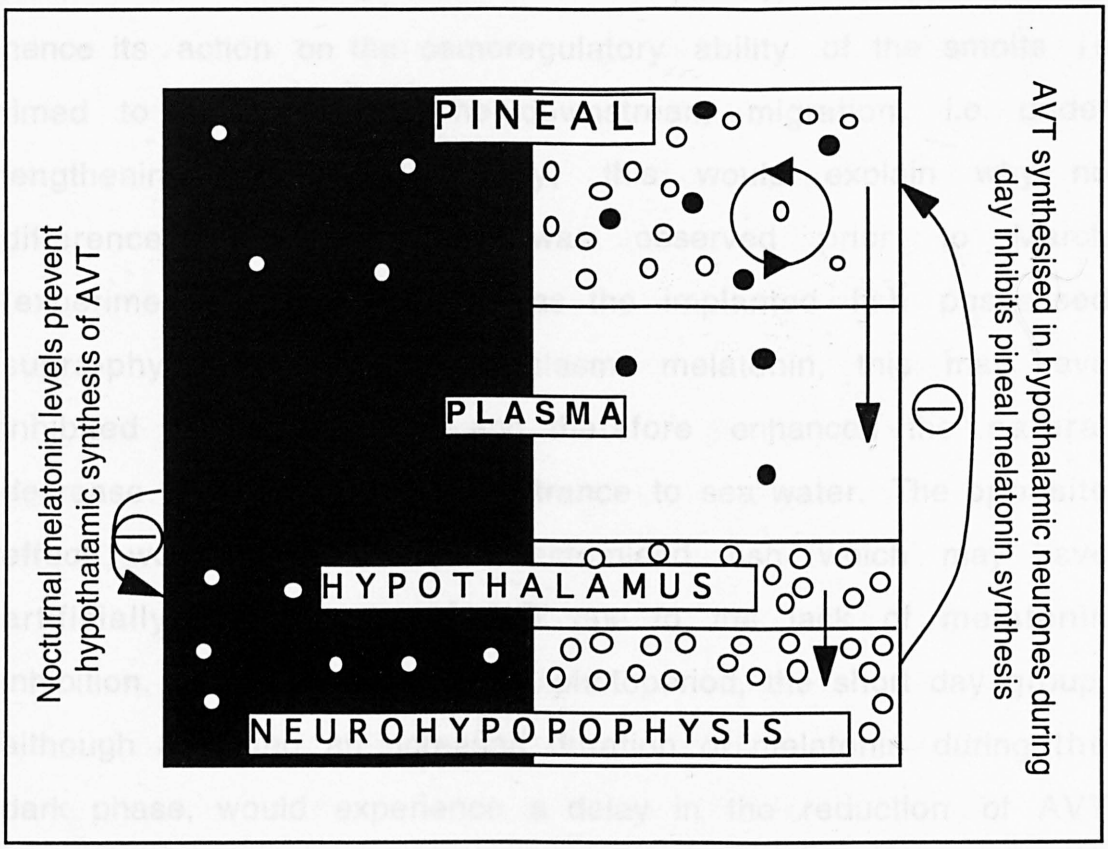


Figure 4.19

Kulczykowska´s proposed model for AVT-melatonin interactions in teleosts. Melatonin (0) synthesised in the pineal during darkness (shaded area) and released into the plasma inhibits argenine vasotocin synthesis in hypothalamic neurones and the pineal. Argenine vasotocin (0), synthesized according to osmotic stimuli in the hypothalamic neurones and released from the neurohypophysis, inturn inhibits melatonin synthesis in the pineal. From Kulczykowska (1995).

It is suggested that the action of AVT on sea water adaptation in Atlantic salmon is under photoperiodic control and hence its action on the osmoregulatory ability of the smolts is timed to coincide with the downstream migration, i.e. under lengthening photoperiod. Firstly, this would explain why no difference between groups was observed prior to March (experiment 1) and secondly, as the implanted fish possessed supra-physiological levels of plasma melatonin, this may have inhibited AVT production and therefore enhanced the natural decrease in AVT reported on entrance to sea water. The opposite effect would result in pinealectomised fish which may have artificially high levels of AVT due to the lack of melatonin inhibition. If AVT is mediated by photoperiod, the short day group, although receiving an increased duration of melatonin during the dark phase, would experience a delay in the reduction of AVT through the action of the short day photoperiod.

Experiment 2 showed that the lowest blood serum osmolarities in the implanted fish occurred 3 weeks prior to the minimum point of either the control or pinealectomised fish (Figure, 4.9). Although the serum osmolarity of the implanted fish was not significantly lower then the other groups at this time it signified the date of optimum sea water adaptation. This is in agreement with the results from experiment 1. However, in experiment 2 the osmolarities of the pinealectomised fish in this case did not differ significantly from those of the controls. Interestingly, Duston and Saunders (1990) reported that potential S1 smolts subjected to a constant short day photoperiod from December did not exhibit the large reduction in condition factor seen in the control group, although no variation was shown in the

timing of sea water tolerance. They suggested that the circannual clock controlling smoltification became desynchronised in the absence of external entrainment but, as no causal relationship exists between salinity tolerance and condition factor, sea water adaptation was maintained, whereas the decrease in condition factor required the increasing daylength of spring. These results are remarkably similar to those observed in the December pinealectomised plus implanted fish in the present study, suggesting a possible masking effect of the implants. However, similar results were not observed in either the summer solstice implanted group (experiment 2) nor the June implanted and short day hold groups in experiment 3. Future experiments may benefit from using artificial photoperiods to alter the timing of smoltification, thus eliminating the possibility of a free-running group spawning at the same time as the controls. It has also been suggested that, in order for the constant to be interpreted correctly, a daily melatonin-free interval is required. This was reported to occur in Djungarian and Syrian hamsters, which unlike the sheep, cannot respond to a constantly elevated melatonin signal but require a period of low amplitude melatonin within each 24 hour cycle (Lincoln and Ebling, 1985; Maywood *et al.*, 1990; Hastings *et. al.*, 1991). As this could only be supplied by timed infusions we can only acknowledge this may be the case in salmonids. Obviously, this area requires further study, preferably with greater numbers of fish and regular AVT and GH measurements.

The high osmolarities recorded in the implanted group between August and September may have resulted from the decreasing daylength 'switching off' the osmoregulatory system

and closing the smoltification-window. Alternatively, temperature may have become a major limiting factor. Wagner (1974) and McCormick (1994) showed that temperature has the ability to terminate the smolting process, possibly by controlling biochemical reaction rates. The implanted fish were the first group to experience a loss in sea water tolerance suggesting this was indeed the case.

4.5.6 Growth and the development of a bimodal length-frequency distribution within juvenile salmonid populations

Juvenile Atlantic salmon, whether reared in hatcheries or in natural stream ecosystems, often develop a bimodal length-frequency distribution within the population during the first year (Thorpe *et al.*, 1980). The upper modal group (UMG) of fish maintain feeding and continue growth, albeit at a reduced rate, throughout the winter, and complete smoltification to begin the sea water stage of their life histories the following spring. Fish within the lower modal group (LMG) become 'dormant' over the winter months, consuming only a maintenance ration, and possibly using stored lipids, resulting in a drop in condition factor (Herbinger and Friars, 1991; Higgins and Talbot, 1985). Normal growth resumes in the spring. However, these individuals forgo smolting until their second or even third year (Higgins, 1985; Thorpe, 1977; Thorpe *et al.*, 1980).

Experiment 3 revealed the growth advantage obtained by Atlantic salmon parr (3-10g) implanted with constant release melatonin implants in June (5-months post hatch). Of greater interest was the almost complete absence of a lower modal group (potential S2 smolts). Subsequently, 92% of the individuals from

this group achieved full sea water adaptability in the first year compared to 60% from the control group and 76% from the short day group (Figures, 4.15 a, b).

It is now accepted that the divergence in strategies within salmonids is strongly correlated to the growth and the size of the individual in relation to a threshold length (Bailey *et al.*, 1980; Berglund *et al.*, 1991; Nicieza, 1994; Skilbrei, 1991). However, the reason for the disparity in growth rates remains undetermined. Metcalf *et al.* (1986) demonstrated a photoperiod-induced change in feeding strategies of juvenile Atlantic salmon. From mid-summer onwards a proportion of the individuals studied exhibited reduced feeding appetite motivation regardless of competition, food abundance or temperature. Thorpe *et al.* (1989) also reported the development of distinct groups from late summer onwards, in agreement with experiment 3 in which all groups showed a normal distribution in August and the appearance of modal groups by October. Villarreal *et al.* (1988) also recognised this decline in feeding levels but found most fish continued to feed if given sufficient opportunity. It has been suggested the change in feeding pattern is influenced by photoperiod either directly (Clarke *et al.*, 1978, 1981; Higgins and Talbot, 1985; Metcalf *et al.*, 1986) or through the action of an endogenous rhythm initiating thyroxine (T_4) release (Villarreal *et al.*, 1983, 1988).

Recruitment into the UMG has been shown to have a strong genetic influence (Bailey *et al.*, 1980; Thorpe, 1977; Thorpe and Morgan, 1978, 1980) as does the incidence of precocious maturation in male parr. Thorpe *et al.* (1982) proposed that the LMG observed within the juvenile population was primarily a

consequence of the 'decision' to mature at an early stage and that this decision was made as the result of an individual exhibiting an increased growth rate and high food-conversion efficiency. It was suggested that fish with lower growth rates then comprised the UMG and, failing to mature, the parr then underwent sea water adaptation as S1's. An opposing view was put forward by Berglund *et al.* (1992), who suggested that precocious parr are a consequence of an individual failing to meet the threshold level required to smolt but achieving sufficient length to mature. Therefore, to achieve a high proportion of S1 smolts optimum growing conditions are needed to allow parr to reach the threshold length prior to the population split (Berglund *et al.*, 1992; Nicieza, 1994; Whitsel and Carmichael, 1994).

As mentioned earlier, mortalities were experienced especially in the short day LMG, through fungal infections. However, these mortalities cannot explain all of the changes in population structure observed within the short day fish and it is therefore suggested that a number of individuals originally found to be in the LMG were in fact recruited into the UMG between October and June. This contradicts the previous findings of Bailey *et al.* (1980) and Thorpe *et al.* (1980), who maintained that once a distinction between each modal class had been established there could be no movement between classes. However, maximum recruitment into the UMG has since been shown to be possible by extending the long day photoperiod into autumn or winter, so allowing some transfer of fish between the modal populations through an extension of the growing season (Clarke and Shelbourn, 1986; Saunders *et al.*, 1989; Skilbrei, 1991; Duston and Saunders, 1994). Duston and Saunders (1994) also found that by elevating

the water temperature to 10°C over the winter months a significant increase in the incidence of S1s could be achieved. Therefore, in the present study, melatonin may have masked the ambient photoperiod sufficiently to allow certain individuals to continue growing and therefore enter the UMG.

In experiment 3 the initial growth increase of implanted fish occurred within 3 months of implantation. Smythe and Lazarus (1974) reported an initial increase in GH after melatonin administration followed by a decrease in higher vertebrates. Although Bolton *et al.* (1987) reported a stunting effect of high GH levels in coho salmon, this was after sea water transfer and it may be that in the present trial an initial GH rise may have induced an increased growth rate which allowed the majority of implanted fish to attain a length above the threshold limit for the S1 strategy. Once on this course of development the behavioural and physiological changes associated with the UMG seem to have overridden any effects of growth inhibition by the melatonin and hence the initial growth advantage was maintained. It must be noted that during this trial the melatonin implants produced plasma melatonin levels of upto 100 times greater than normal physiological levels and 5 times greater than those measured in section 4.3 which resulted in a decrease in growth. When the groups were tested for saltwater tolerance the effects of the implants may have had a beneficial role as discussed earlier in this chapter.

4.5.7 Maturation within male salmon parr

As mentioned, precocious maturation is thought to be a consequence of low body weight in the autumn prior to

smoltification (Berglund *et al.* 1992) rather than the optimum strategy adopted by parr (Thorpe *et al.* 1982). Berglund *et al.* (1992) also reported that individual male parr may remain in fresh water and mature for two or more years in succession. If a size threshold does determine the decision to smolt or mature, an increased frequency of mature males within the smaller implanted fish in experiment 2 (Figure, 4.11) would have been expected. As this was not the case it may be that melatonin acts on growth, smoltification and the reproductive axis independently. Alternatively, if the pineal does entrain endogenous clock(s) then its/their disruption by either pinealectomy or implantation may be interpreted differently by different systems (i.e., smoltification and maturation). If the absence of the pineal does promote growth in potential S2 smolts then accordingly the majority of the population should attain the threshold size and forgo maturation to smolt. The reduced number of precocious parr recorded in the pinealectomised group support this suggestion.

It is suggested that the low number of mature males reported in the smaller implanted individuals may be a consequence of supra-physiological levels of melatonin on gonad development. Fenwick (1970) found melatonin administration (20μg/fish for 50 days) to have an inhibitory affect on gonad development in goldfish when maintained on long day photoperiods; he also observed melatonin concentrations to be six times higher in the pineal of juvenile chinook salmon than in mature fish. There is also growing support for the idea that large doses of melatonin, suitably administered to higher vertebrates at specific times of the seasonal cycle, can inhibit reproductive

activity possibly through modifying the feedback sensitivity to gonadal steroids at pituitary and hypothalamic levels (Arendt, 1992).

Thorpe *et al.* (1982) proposed that the larger fish in each year class during early summer were responsible for the incidence of mature parr the following spring. We would then have expected increased maturation within the larger pinealectomised parr in the present study. However, this was not the case. Therefore, it must be concluded that either migration is the preferred strategy adopted by larger individuals (Berglund *et al.*, 1992) or that pinealectomy reduced the number of mature male parr.

Pinealectomy is known to influence testicular and ovarian development as well as GTH secretion, although the outcome is dependant on species, photoperiod, temperature and stage of gonadal development. Popek *et al.* (1994) discovered that pinealectomy affected neither the amplitude nor rhythmicity of GTH release in carp. However, Hontela and Peter (1980) found it repressed the daily rhythms of GTH in goldfish and decreased gonadal development, depending on the stage of maturation and the ambient photoperiod. In Asian catfish pineal removal was ineffectual in altering female maturation during the pre-spawning and spawning periods, but during the preparatory phase (early light responsive period) it produced an accelerated growth in the ovary (Joy and Agha, 1989). In contrast, pinealectomy in the Japanese killifish reduced or completely eliminated ovulation, whereas enucleation had no effect on ovarian maturation and oviposition (Urasaki, 1973). However, Davis *et al.* (1986) found pineal removal or enucleation or both produced a

delay and reduction in spawning frequency, but none of these procedures were totally inhibitory. Most studies have concentrated on female maturation but de Vlaming (1975) found testicular development was retarded in the golden shiner if pinealectomised during final maturation and pinealectomy caused testicular regression if performed approaching the gonadal preparatory and pre-spawning season. Of the limited work on salmonids Popek and co-workers (1992) reported a 2 week delay in the spawning times of rainbow trout if pinealectomised 5 months prior to spawning but no effect was observed if performed 1 month before spawning. Clearly, the complex interactions between maturation and smoltification makes it difficult to establish the role of melatonin within each process.

From the information collected in experiment 2 it is proposed that melatonin and the pineal acts on growth in juvenile salmonids independently of maturation. However, the growth advantage gained by the pinealectomised parr did result in fewer precocious males being produced, likewise the smaller implanted fish had the lowest frequency of mature males. Pineal and melatonin manipulation of sea-going salmon may help distinguish between the effects on smoltification and maturation, although preliminary results of Randall *et al.* (pers. comm.) revealed no significant effects on the maturation of grilse. However, it is now apparent that the pineal and or melatonin does have a role to play in the development of Atlantic salmon parr and this area requires further study with larger numbers of fish and, if possible, regular measurements of plasma GH and AVT.

An important effect of melatonin implantation in parr was the increased serum osmolarities which were far higher than in

either the control or pinealectomised groups. Presumably, melatonin alters osmoregulation in the mature parr in the same manner as described earlier in the smolts. As before the high serum osmolarities were only recorded in the implanted fish during the period of decreasing photoperiod prior to the winter solstice. It is interesting to note that, although mature, the parr are still able to partially regulate their ion balance. As the implants have been shown to affect osmoregulatory ability it seems that melatonin, as suggested earlier, is acting on parr osmoregulation independently of the reproductive axis.

4.5.8 Summary of chapter 4

It appears from these results that the pineal and melatonin (although administered at a supra-physiological level) have the ability to significantly modify the development of salmonid smolts. It is proposed that melatonin acts in two ways. Firstly by a direct physiological response, as shown by the unusually high blood serum osmolarities observed in experiment 2 and the suggested link with AVT. Secondly, via the influence of melatonin and/or pineal innervation on endogenous rhythms, either by influencing the rhythms individually or possibly by the disruption of a central oscillator which acts to entrain other rhythms.

From the work in this chapter it is apparent that the administration of constant release melatonin implants and/or pineal removal significantly alters the growth rate of juvenile Atlantic salmon. This was shown conclusively in experiment 3 where implantation at the summer solstice resulted in a statistically significant growth advantage within 1 month. It is

believed that this increased growth allowed the threshold size for smoltification to be reached and consequently 92% of the population attained sea water tolerance within their first year compared to 60% of controls. No effects of pinealectomy were observed in relation to the timing of smoltification, however, melatonin implants appeared to advance the aquisition of a hypo-osmoregulator ability. Clearly, more work is needed in this area with special emphasis being placed on: the release rates of the implants used; the duration of implantation and possible removal of the implant, allowing a return to a natural melatonin rhythm; the possibility of, replacing the implants with timed infusions and thereby providing a melatonin-free interval; and the time of year at which implantation or pinealectomy is performed.

Chapter 5

The Role of Melatonin and the Pineal Gland in the Timing of Spawning in Rainbow Trout

5.1 Introduction

The rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri* Richardson) is now recognised as a member of the Pacific *Salmonidae* (Kendall, 1988). Introduced from western North America in the latter half of the 19th century, at 15000 tonnes/annum, it is now the second most important species of cultured fish in Britain after the Atlantic salmon.

For a species of such economic significance, large gaps still exist in our knowledge of the rainbow trout's photoreceptive and endocrine systems. Over the last 40 years, emphasis has been placed on understanding which environmental cues determine the spawning time of rainbow trout. To a large extent this has now been answered. However, we still have to address the questions relating to how these environmental changes influence the biological rhythms which control seasonal events in salmonids and what mechanisms underly the interpretion of these seasonal cues.

The pineal gland and specifically melatonin would appear to be of importance to the timing of daily and seasonal events in the life histories of salmonids. This, together with the photoreceptive abilities and efferent neural projections from the pineal gland suggest their importance to the entrainment of various endogenous rhythms, including reproduction.

This chapter aimed to investigate the contribution of the pineal gland and melatonin in the transmission of the seasonally changing photoperiodic information to the reproductive axis in rainbow trout. The importance of the pineal and melatonin to reproductive development were assessed by

removing the pineal gland at critical points in the seasonal photoperiod and/or through the administration of constant release melatonin implants to virgin female rainbow trout.

5.1.1 Melatonin and the pineal gland in relation to teleost reproduction

In mammals, photic information is relayed from the retina via the retinohypothalamic tract to the suprachiasmatic nucleus (SCN) and entrains an endogenous oscillator within the SCN (Hastings, 1985; Moore-Ede and Moline, 1985). This in turn entrains a pacemaker within the paraventricular nucleus of the hypothalamus (Klein, 1985). Finally the photic information is transfered to the pineal which releases a circadian rhythm of melatonin (Figure 5.1). All mammals so far studied exhibit increased melatonin levels during the dark phase and reduced levels during the light phase (Korf, 1994). The pig appears to be a possible exception to this and although Binkley (1988) reported normal patterns of daily melatonin secretion Reiter *et al.* (1987) found no expression of a rhythm at specific periods of the year and during hibernation.

Two points that should be considered when applying this mammalian model to fish are that, unlike teleosts, the mammalian pineal does not have the capacity to respond directly to light and secondly, the mammalian Harderian gland has been identified as a source of extra-pineal melatonin (Reiter *et al.*, 1981). As fish do not possess a Harderian gland and are able to synthesise melatonin in direct response to the

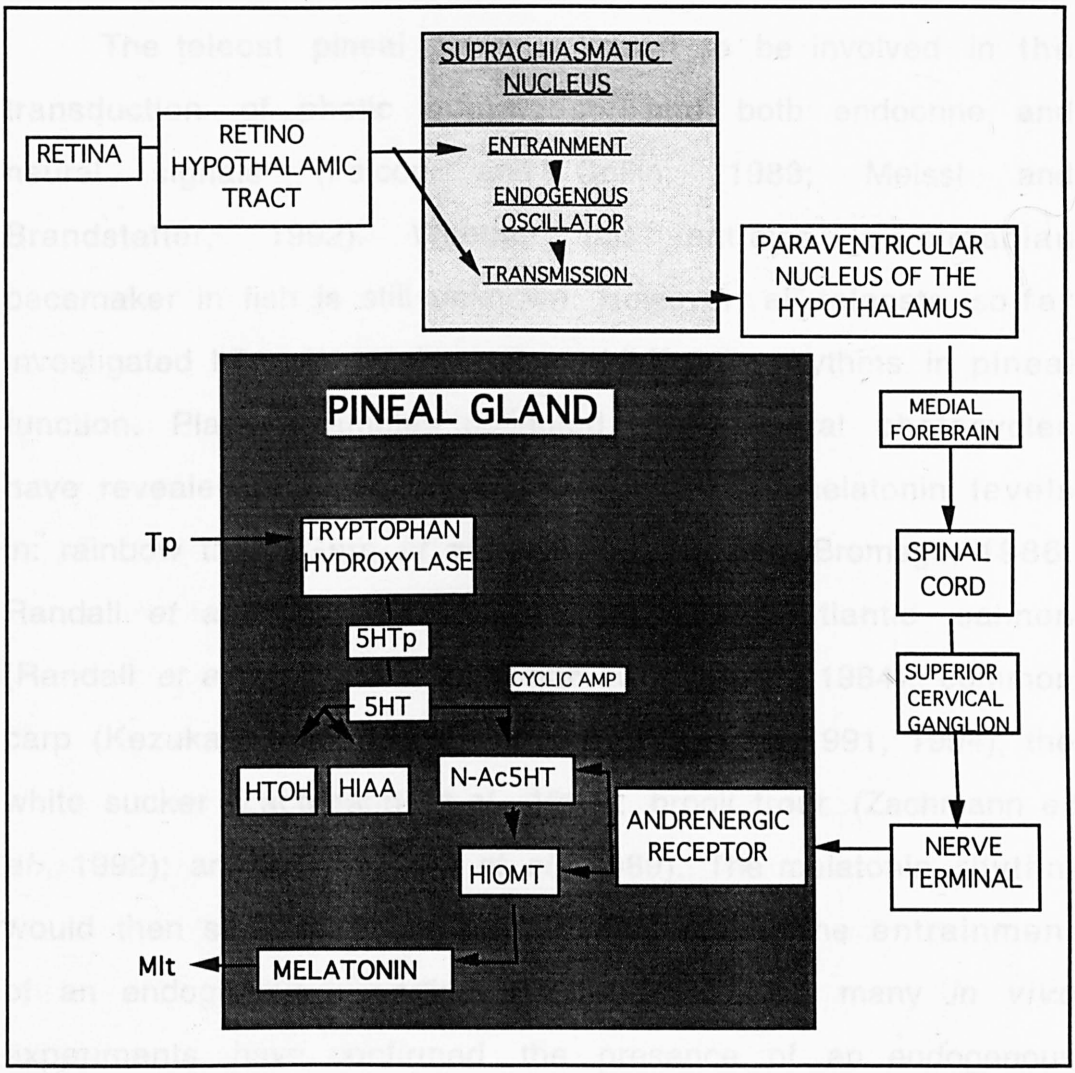


Figure 5.1

The mammalian melatonin rhythm generating system. 5HTp, 5 - hydroxytryptophan, 5HT, 5-hydroxytryptamine (serotonin); HTOH, 5-hydrotryptophol; HIAA, 5-hydroxyindole acetic acid; N-Ac5HT, N-acetylserotonin; HOIMT, hydroxyindole-O-methyltransferase. Modified from Klein (1985).

light/dark cycle through photosensitive pineal photoreceptors (Falcon *et. al.*, 1986) comparisons between the mammalian and teleost systems should be used with caution.

The teleost pineal gland is known to be involved in the transduction of photic information into both endocrine and neural signals (Falcon and Collin, 1989; Meissl and Brandstatter, 1992). Whether this entrains a circadian pacemaker in fish is still unknown. However, all teleosts so far investigated have been shown to exhibit daily rhythms in pineal function. Plasma samples collected over several photocycles have revealed day/night changes in circulating melatonin levels in: rainbow trout (Gern *et al.*, 1977; Duston and Bromage, 1986; Randall *et al.*, 1991; Alvarino *et al.*, 1993); Atlantic salmon (Randall *et al.*, 1995); coho salmon (Gern *et al.*, 1984); common carp (Kezuka *et al.*, 1988); goldfish (Iigo *et al.*, 1991, 1994); the white sucker (Zachmann *et al.*, 1991); brook trout (Zachmann *et al.*, 1992); and pike (Falcon *et al.*, 1989). The melatonin rhythm would then seem to be the ideal zeitgeber for the entrainment of an endogenous circadian oscillator. Indeed many *in vivo* experiments have confirmed the presence of an endogenous melatonin rhythm in fish (Zachmann *et al.*, 1991; Iigo *et al.*, 1991, 1994) although this is not the case for the rainbow trout (Randall *et al.*, 1991). *In vitro* cultures of isolated pineals from pike, goldfish, Atlantic halibut and possibly the Atlantic salmon (see chapter 3) have revealed that this endogenous rhythmicity persists in isolated pineals in the absence of the central nervous system (CNS) (Falcon *et al.*, 1987, 1989; Kezuka *et al.*, 1989; Iigo *et al.*, 1991, 1994; Bolliet *et al.*, 1994). However, the rainbow trout pineal has been shown to be

directly photoreceptive revealing no endogenous rhythm of melatonin secretion under constant conditions (Gern and Greenhouse 1988; Max and Menaker, 1992).

The development of 2-[^{125}I]iodomelatonin (a melatonin analogue) has allowed effective labelling of high-affinity melatonin binding sites (Vakkuri *et al.*, 1984) within piscine tissues (Martinoli *et al.*, 1991). Since this discovery the teleost brain has been under investigation in the hope that a knowledge of binding site distribution would lead to a greater understanding of the melatonin signal transduction mechanism and its role in the transmission of environmental information to the brain-pituitary-gonad axis. Iigo *et al.* (1994) reported a circadian rhythm in the concentration and distribution of melatonin binding sites within the goldfish brain as did Ekstrom and Vanecek (1992) in the Atlantic salmon. Both studies found that binding site density increased during the photophase and was inversely correlated with the concentration of circulating plasma melatonin. Interestingly, no significant variation was observed in the affinity of the 2-[^{125}I]iodomelatonin binding.

To date most melatonin binding site research has been carried out within the brain. However, binding sites have also been reported in peripheral tissues including: the intestine and gonads of the gilthead seabream (Molina-Borja *et al.*, 1994); the heart of Atlantic salmon, Arctic charr and rainbow trout (Pang *et al.*, 1994); and the retina of the goldfish (Iigo *et al.*, 1992). From work carried out by Kezuka *et al.* (1992) it was suggested that melatonin produced in the goldfish retina is not released into general circulation and so retinal binding sites may

mediate the action of locally produced melatonin for diel processes such as disc shedding and retinomotor movements (Iigo *et al.*, 1994).

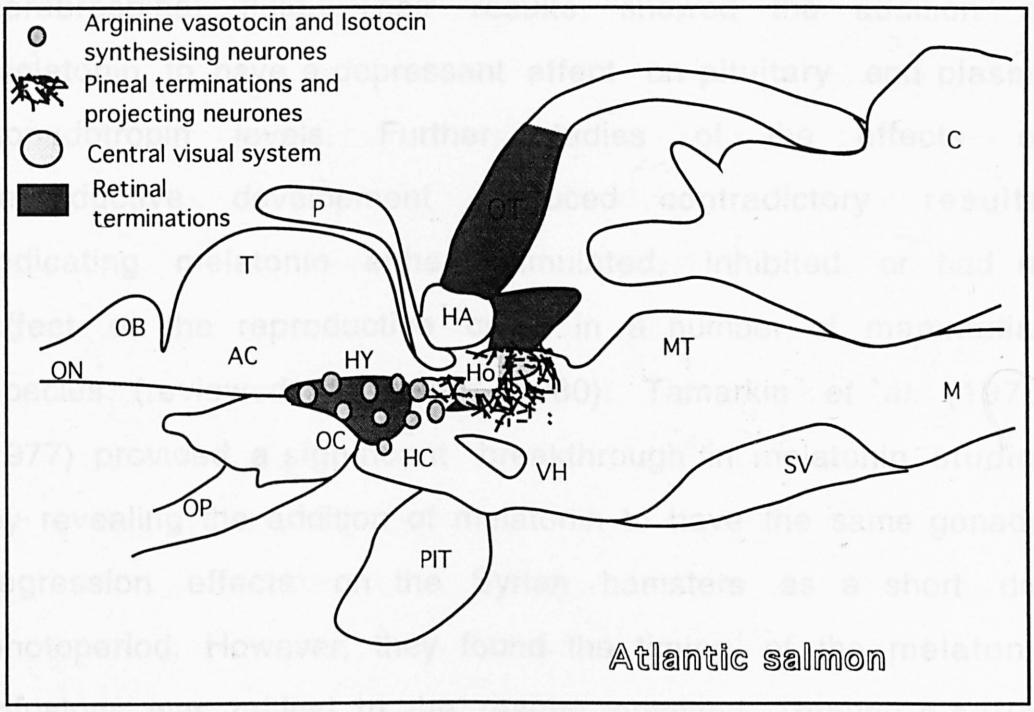
Melatonin binding site distribution is remarkably similar in both the goldfish, Atlantic salmon and rainbow trout (Martinoli *et al.*, 1991; Ekstrom and Vanecek, 1992; Davies *et al.*, 1994; Iigo *et al.*, 1994a, 1994b). All the studies so far carried out have found that the greatest concentration of high-affinity binding sites were associated with: the optic tectum; preoptic and pretectal areas; the thalamus; and the inferior lobes of the hypothalamus. Intermediate concentrations were located within the molecular layer of the cerebellum, medulla oblongata and telencephalon. No binding was observed in the pineal of these species. Iigo *et al.* (1994) reported low binding site concentrations in the olfactory bulb and pituitary, but, Martinoli *et al.* (1991), Ekstrom and Vanecek (1992) and Davies *et al.* (1994) all reported a complete lack of pituitary binding sites. These studies would suggest that melatonin binding sites are mainly concentrated in areas already known to be important to sensory and neuroendocrine functions.

The work of Holmqvist *et al.* (1994) provides further evidence of the possible link between photic information (from pineal and retinal innervation) and melatonin binding sites within the neurotransmitter/hormone systems in the brain of Atlantic salmon, indicating both neural and endocrine transfer of the light/dark information to similar areas of the brain. Using neural tract tracing in combination with immunocytochemical localisation of tyrosine hydroxylase, arginine vasotocin (AVT) and isotocin (IST), Holmqvist *et al.*

(1994) revealed retinal and pineal terminations (reticulate) plus pineal tectal projecting neurones distributed throughout the central visual system and hypophysiotrophic system. In particular, the central optic nuclei, i.e. the hypothalamic optic nucleus, the habenular optic nucleus, the dorsomedial optic nucleus, and periventricular pretectal optic nucleus, receive both pineal and retinal innervation and possess projections to the optic tectum. Pineal efferent projections have also been traced in goldfish by applying a concentrated solution of horseradish peroxidase (HRP) to the pineal organ, and then revealed using immunocytochemistry with an anti-HRP antiserum (Jiminez, 1995). This work compares favourably with that of Holmqvist (1994), revealing immunoreactive fibers located in the dorsolateral thalamic nucleus, habenular region, pretectalis area and optic tectum. Together, these results suggest the pineal may convey photoperiodic information through hormonal and neural signals to circadian and reproductive systems within teleosts (Figure 5.2).

5.1.2 The effects of melatonin administration in teleosts

Most early work on the reproductive and endocrine effects of melatonin administration concentrated on mammals (reviewed in Reiter, 1980; Bittman, 1985; Underwood and Goldman, 1987; Armstrong, 1989; Cassone, 1990). Initial experiments carried out by Fraschini and Martin (1970) and Kamberi *et al.* (1971) (cited in Reiter, 1975) used timed infusions of melatonin applied directly to neural structures adjacent to the anterior pituitary gland or infused into the



P pineal gland
OT optic tectum
C cerebellum
HA habenular
Ho habenular optic nucleus
MT tegmentum of the midbrain
T telencephalon
OB olfactory bulb
ON olfactory nerve
AC anterior commissure
HY hypothalamus
OC optic chiasma
OP optic nerve
HC horizontal commissure
PiT pituitary
VH ventral hypothalamus
SV saccus vasculosus
M medulla oblongata
SCO subcommissural organ
APB anterior pineal bundle
PPB posterior pineal bundle
AP area pretectalis
Ndl nucleus dorsolateralis thalmi

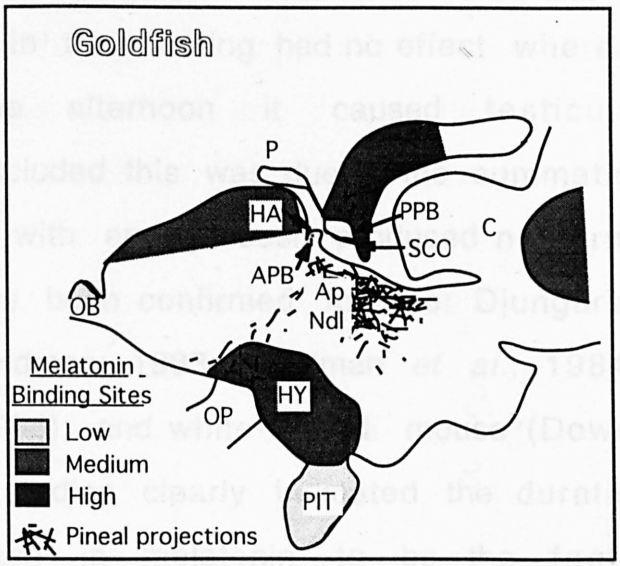


Figure 5.2

Schematic representations of midsagittal sections through the brains of Atlantic salmon and goldfish showing main areas of pineal and retinal innervation and melatonin binding sites. Atlantic salmon illustration is based on figure from Holmqvist *et al.*, 1994; and goldfish diagram is based on a figure from Jimenez *et al.*, 1995.

cerebrospinal fluid. Their results showed the addition of melatonin to have a depressant effect on pituitary and plasma gonadotropin levels. Further studies of the effects on reproductive development produced contradictory results, indicating melatonin either stimulated, inhibited or had no effect on the reproductive cycle in a number of mammalian species (reviewed in Reiter, 1980). Tamarkin *et al.* (1976, 1977) provided a significant breakthrough in melatonin studies by revealing the addition of melatonin to have the same gonadal regression effects on the Syrian hamsters as a short day photoperiod. However, they found the timing of the melatonin infusions was critical to the results obtained. Through a series of experiments Tamarkin *et al.* (1977) discovered that melatonin administration in the morning had no effect whereas if administered in the afternoon it caused testicular regression. Tamarkin concluded this was due to the summation of the afternoon infusion with endogenously produced nocturnal melatonin. This has since been confirmed for the: Djungarian hamster (Carter and Goldman, 1983; Goldman *et al.*, 1984); sheep (Bittman *et al.*, 1983); and white-footed mouse (Dowell and Lynch, 1987). These studies clearly indicated the duration of the nocturnal increase in melatonin to be the factor responsible for altering the reproductive axis in these species. More recently the use of constant release melatonin implants have also been shown to act as an artificial short day photoperiod in some mammals (Lincoln *et al.*, 1984; Nowak and Rodway, 1985; Deveson et al., 1989; Forsberg *et al.*, 1990).

Some early studies on melatonin administration to fish agreed with the mammalian work. Urasaki's work on Japanese

killifish (1972) showed melatonin treatment to have the same effect in reducing the gonadosomatic index as subjecting the fish to short photoperiods; a similar result was obtained with the catfish (Joy and Agha, 1991). However, subsequent studies have shown the outcome of melatonin administration is dependent on a number of physiological and environmental factors (Joy and Khan, 1991; Begay *et. al.*, 1994).

Saxena and Anand (1977) found that melatonin produced an anti-gonadotrophic effect in catfish which was more effective in arresting ovarian recrudescence than a short day photoperiod but only during the early preparatory phase of gonadal development. This was also found to be the case by Joy and Khan (1991), who reported that melatonin inhibited the normally stimulatory effect of long days on ovarian development in catfish and Sundaraj and Keshavanath (1976), who found that melatonin inhibited vitellogenesis and induced follicular atresia in catfish during the prespawning period while during the spawning period melatonin induced significant ovarian regression. Melatonin proved stimulatory to growth and weight gain in goldfish but only under a short day regime; no change was recorded under long days (deVlaming, 1980).

The importance of photoperiod, and the dose of melatonin was emphasised by the work of Borg and Ekstrom (1981) who observed that melatonin, when given at 4μg/day, produced an antigonadal effect in male and female sticklebacks kept under long days, but proved ineffectual if applied at 0.8μg/day under similar conditions. When the fish were maintained under short day photoperiods, a progonadal response was observed, but only when administered at 0.8μg/day and only with female

sticklebacks. Studies on the effects of melatonin administration on other hormones have also shown marked dose-related responses. Nayak and Singh (1987) produced excellent results on the dose-related consequences of melatonin administration on sex steroids and thyroid hormones in catfish during the prespawning period. Generally melatonin had an increasingly inhibitory effect on testosterone, oestradiol-17β, thyroxine (T_4) and triodothyroxine (T_3) when applied at concentrations of 25-200μg/day. Whereas, at 400μg/day, a stimulatory effect on T_3,T_4 and testosterone was observed. Interestingly, Begay *et al.* (1994) were able to show that oestradiol-17β stimulated melatonin release from cultured trout pineal photoreceptor cells in a dose-dependant manner suggesting sex steroids may also modulate melatonin release through a feedback mechanism.

Finally, although little evidence exists of melatonin binding sites within the pituitary (Iigo *et al.*, 1994), melatonin has nonetheless been shown to increase the size of pituitary gonadotropes in the goldfish (Fenwick, 1970) and reduce pituitary gonadotrope numbers in the catfish (Sundaraj and Keshavanath, 1976).

To summarise, melatonin administration by either timed infusion or implantation has been shown to alter the timing and development of the reproductive axis in selected species. As previously discussed the results of melatonin administration are dependant on species, sex, dose given, stage of gonadal development when administered, ambient photoperiod and temperature. As little is known about the effects of exogenous melatonin administration in salmonids, experiment 2 in this

chapter was designed to assess whether, as in some teleosts, constant-release melatonin implants can be used to mimic a short day photoperiod in the rainbow trout.

If this did indeed prove to be correct it could provide a cost effective and convenient commercial alternative to the problem of maintaining salmonids under artificial photoperiod regimes. This would be especially true in the case of salmonid culture employing the use of offshore cages where constructing light-proof covers to simulate a short daylength is impractical. As our understanding of the salmonid life cycle increases the more opportunities arise to manipulate the stages of greatest commercial significance to the aquaculturist. At present this is achieved using artificial photoperiods to advance spawning, enhance seawater tolerance and reduce the incidence of grilsing (Bromage and Duston, 1986; Johnstone et. al., 1987; Duston and Saunders, 1992). If, however, melatonin was found to be the internal cue entraining an endogenous circannual rhythm of reproduction, implants could be developed to mimic the effects of external photoperiods which could then be administered at the appropriate time in the seasonal cycle as already practiced in livestock farming eg. sheep.

5.1.3 The influence of the pineal gland on gonad development

The pineal gland is known to be responsible for the majority of the circadian melatonin rhythm in fish (Falcon *et al.*, 1987, 1989; Kezuka *et al.*, 1989; Iigo *et al.*, 1991; Zachmann *et al.*, 1991a and b; Max and Menaker, 1992) and pineal removal has been shown to significantly decrease, if not abolish, the

melatonin rhythm in all teleosts so far studied (Gern *et al.*, 1978; Kezuka *et al.*, 1991; Porter *et al.*, 1996). The consequences of pinealectomy can vary depending upon a number of factors. In the goldfish, pinealectomy is only effective from late winter to early summer. During this period, fish held under short day photoperiods were shown to experience gonadal stimulation after pinealectomy, whereas fish maintained under long day photoperiods underwent gonadal regression (deVlaming and Vodicnik, 1978; Vodicnik *et al.*, 1978). Similar results were reported in: the catfish (Garg, 1988) grey mullet (Abraham and Sagi, 1984) and golden shiner (deVlaming, 1975). To date the only study carried out concerning the effects of pinealectomy on maturation in salmonids was undertaken by Popek and co-workers (1992). Popek *et al.* (1992) removed the pineal from maturing female rainbow trout at 1 and 5 months prior to spawning (10-30 April). They found that pinealectomy performed directly preceding spawning had no significant effect. However, when carried out during vitellogenesis (5 months before spawning) ovulation was prevented in 20% of females and spawning was delayed by 2 weeks in the remainder. Although this study suggests that the pineal may be involved during the final stages of maturation, it fails to incorporate the change from short to long daylengths which are known to play an important part in the timing of maturation in salmonids.

The pineal's role in the reproductive process may not be limited to its photoreceptive abilities. Just as melatonin can alter plasma gonadotropin (GtH) levels, so pinealectomy caused a reduction in serum GtH levels in the goldfish (Vodicnik *et al.*, 1978; Hontela and Peter, 1980) although no such reduction was

observed in the carp (Popek *et al.*, 1994). Nevertheless, the effects of pinealectomy are not restricted to the reproductive axis. Delahunty and co-workers (1978) discovered that pineal removal caused a significant decrease in goldfish serum K^+, Ca^{2+} and PO_4^{3-} ions, but only during late February, while de Vlaming and Vodicnik (1979) discovered that liver and hepatic glycogen levels were reduced and plasma lipid and glucose levels could be increased depending on the time of year and ambient photoperiod. As with melatonin administration, the thyroid hormones (T3 and T4) would appear to be under the influence of the pineal at certain times of the reproductive cycle (Nayak and Singh, 1987). Goudie *et al.* (1983) have also suggested that the pineal may have an influence on locomotor activity as this showed a significant reduction in catfish during the dark period when compared to controls.

The results described above indicate that the pineal plays a significant role in the control of daily and seasonal physiological and behavioural events in teleosts. Considering the apparent importance of the pineal it is surprising that little work has been conducted on salmonids, which, being strongly influenced by the seasonal photoperiodic cycle, and of considerable commercial significance, would seem an ideal species for study. The experiments in this chapter aim to assess the effects of pineal removal and melatonin administration on the reproductive cycle of the rainbow trout under both natural and artificial photoperiod regimes.

In order to discern the role of melatonin and the pineal gland we must first understand the mechanisms involved in salmonid reproduction. For this reason, brief reviews are

included, outlining the ovarian cycle and neuroendocrine pathways involved in rainbow trout maturation.

5.1.4 The ovarian cycle of rainbow trout.

Female rainbow trout first mature at the end of their second or third year, by which time the ripe ovaries account for 12-20% of the body weight of the fish. The paired ovaries are positioned below the kidney and swim bladder, suspended by a pair of mesenteries (mesovaria). The ovaries are supplied via the dorsal aorta and ovarian vein (for a more detailed account see Bromage and Cumaranatunga, 1988).

For the first 4 months after hatching, the ovary is comprised solely of primary oogonia and, by the process of mitotic division, secondary oogonia. After this period, although other oocyte stages may exist in the developing ovary, it appears that oogonia are always available for recruitment. Oogenesis begins at about 4 months with the first meiotic prophase of secondary oogonia into stage 1 oocytes (Figure 5.3). This is often referred to as the chromatin nucleolar stage and within 1 month, stage 2 oocyte development is apparent. Cumaranatunga (1988) divided this into 3 sub-stages (2a, 2b and 2c) depending on the position of the migrating Balbiani bodies, which appear close to the nucleus at stage 2b but have moved to the cell periphery by stage 2c. Stage 2 development may last up to 3 months. Nine months after hatching, most ovaries contain stage 3 (late perinuclear stage) oocytes. By this time, the Balbiani bodies have disappeared and the cell cytoplasm has become acidophilic as opposed to basophilic.

The following stage of development was originally referred to as a period of endogenous vitellogenesis but is now known as the vesicle stage or cortical alveoli stage. Stage 4 (vesicle stage) oocytes are apparent within the first year of life and as the name suggests are characterised by the formation of cytoplasmic vesicles. Again, depending upon the number and position of the vesicles, this stage can be subdivided into 4a and 4b, 4a having fewer vesicles located close to the cell membrane and 4b showing concentrated vesicle accumulations around the nucleus. Although stage 4 oocytes proceed to fully developed eggs, vesicle stage oocytes are subsequently present throughout all stages of ovarian development.

Exogenous vitellogenesis begins at stage 5 (also known as the peripheral yolk granule stage). This sees oocyte sequestration of a lipophosphoprotein-calcium complex (vitellogenin) synthesised by the liver (Tyler *et al.*, 1987). Incorporation of vitellogenin takes place through receptor-mediated endocytosis and results in large increases in cell volume. This absorption of yolk continues throughout stage 6 which sees the migration of the nucleus towards the periphery of the cytoplasm. The oocyte reaches its maximum size in the mature ovary during stage 7. During this stage, the nuclear envelope ruptures and the micropyle becomes apparent within the zona radiata.

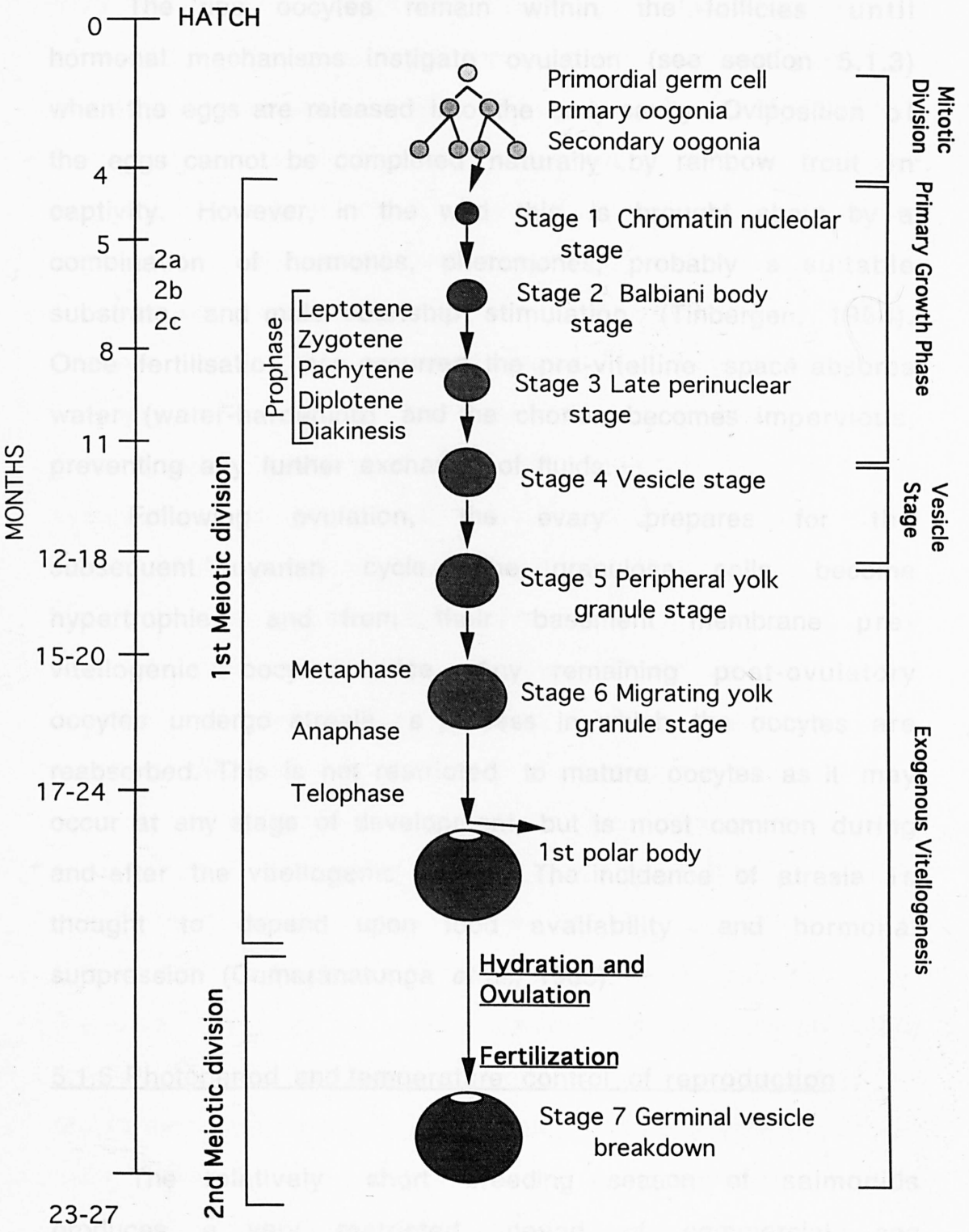


Figure 5.3

Model showing the cytological stages of oocyte development in the female rainbow trout (based on Bromage and Cumaranatunga, 1988).

The ripe oocytes remain within the follicles until hormonal mechanisms instigate ovulation (see section 5.1.3) when the eggs are released into the body cavity. Oviposition of the eggs cannot be completed naturally by rainbow trout in captivity. However, in the wild this is brought about by a combination of hormones, pheromones, probably a suitable substrate, and male courtship stimulation (Tinbergen, 1953). Once fertilisation has occurred the pre-vitelline space absorbs water (water-hardening) and the chorion becomes impervious, preventing any further exchange of fluids.

Following ovulation, the ovary prepares for the subsequent ovarian cycle. The granulosa cells become hypertrophied and from their basement membrane pre-vitellogenic oocytes arise. Any remaining post-ovulatory oocytes undergo atresia, a process in which the oocytes are reabsorbed. This is not restricted to mature oocytes as it may occur at any stage of development, but is most common during and after the vitellogenic phases. The incidence of atresia is thought to depend upon food availability and hormonal suppression (Cumaranatunga *et al.*, 1985).

5.1.5 Photoperiod and temperature control of reproduction

The relatively short breeding season of salmonids produces a very restricted period of commercial egg availability. This, in turn, results in each year class of fish reaching marketable size at approximately the same time so causing a depreciation in their market value. This seasonality of the harvest also means the biomass of the farm increases

from the time the eggs are bought in until the fish are harvested, putting a seasonal stress on the stocking densities of the ponds and hence water, oxygen and labour demands of the farm. Not surprisingly then, there is great commercial interest in producing a year round supply of eggs to allow a wider harvest period to provide fresh trout throughout the year.

As with most salmonids living at temperate latitudes, rainbow trout have an annual reproductive cycle which is primarily influenced by the seasonally-changing photoperiod. This ensures spawning within the breeding population is synchronised to within 6-8 weeks and the fry hatch when environmental conditions are optimum for their survival. Baker (1938) termed these optimal conditions for offspring and parental survival, 'ultimate factors'. These include: water quality and oxygenation; maximum food abundance, and therefore minimum competition; and minimal predation.

As exogenous vitellogenesis in the rainbow trout takes approximately 6 months to complete, oviposition to hatch usually requires 300 degree/days (30 days at 10°C), and a further 200 degree/days (20 days at 10°C) are required until the larvae 'swim-up' and are capable of feeding, the complete reproductive cycle must be initiated at least 8 months prior to the forthcoming 'ultimate' conditions. This is achieved through using reliable environmental changes, 'proximate' factors, which allow the synchronisation of endogenous rhythms and provide an indication of the approaching ultimate factors (Baker, 1938; Munro *et al.*, 1990). In salmonids, photoperiod is the primary proximate cue for the timing of reproduction

(deVlaming, 1972; Lam, 1983; Scott and Sumpter, 1983; Bye, 1984; Bromage and Duston, 1986).

The seasonal variation in photoperiod is now known to synchronise the annual reproductive cycle in the rainbow trout but, whether the increasing or decreasing daylengths provided the stimulus for reproductive development was a point of speculation. It is now generally agreed that both photoperiods provide entrainment at specific stages of gonadal development. The increasing photoperiod of late winter and spring is now believed to initiate gonadal development while the decreasing photoperiod of late summer and autumn entrains the later stages of oocyte development (Bromage *et al.*, 1982; Scott and Sumpter, 1983; Bye, 1984; Scott *et al.*, 1984).

The role of environmental factors in the control of reproduction in teleosts has been intensively studied (for reviews see de Vlaming, 1972; Scott, 1979; Baggerman, 1980; Bye, 1984). More recently this knowledge has been used to artificially manipulate the spawning time of salmonids using modified temperatures or photoperiod cycles. Although photoperiod is considered the overriding environmental cue, temperature is also important to the reproductive process. Significant delays can be induced in the hatching time of rainbow trout fry by cooling the incubation water. This maintains the number of degree days to hatch but due to the lower water temperature the eggs take longer to incubate; a delay of 2 months or more can be induced in this manner (Bromage *et al.*, 1982). Temperature can also be used to delay ovarian development and it has been consistently shown that exposure to temperatures below 4°C can delay spawning time in

rainbow trout (Morrison and Smith, 1986; Nakari *et al.*, 1988; Davies and Bromage, 1991). Moreover, Tyler *et al.* (1987) reported that vitellogenin uptake into cultured ovarian follicles is decreased at lower temperatures, indicating a direct physiological effect. However, the option of temperature manipulation is restricted to a few sites with the ability to interchange between river and borehole water supplies, and so to the majority of farmers this is not a consideration. Also, in practice the alterations that can be achieved are relatively small compared to those obtainable by manipulating the daylength.

Photoperiod provides a more convenient, effective and cheaper method of altering spawning time in salmonids. Hoover and Hubbard (1937) were the first to use an artificial photoperiod to manipulate the spawning time of salmonids. They used a compressed (accelerated) seasonal light cycle to advance spawning time in the eastern brook trout by 4 months. Hazzard and Eddy (1951) and Henderson (1963) employed the same principle with equal success. Compressed photocycles have since been used to advance spawning in: grey mullet (Kuo *et al.*, 1974); gilthead sea bream (Bye, 1987); turbot and sea bass (Girin and Devauchelle, 1978); brown trout (Buss, 1980); and rainbow trout (Nomura, 1962; Kunesh *et al.*, 1974; Whitehead *et al.*, 1978; Bromage *et al.*,1982; Pohl *et al.*, 1982; Elliot *et al.*, 1984; Bromage, 1987). These studies on the rainbow trout revealed that a 12 month photocycle, compressed to 9 or 6 months could advance spawning by 6 and 12 weeks respectively; however, a 3 month compression of the seasonal photocycle proved to be outwith the range of entrainment

(Whitehead *et al.*, 1978). Similarly, expansion of the photocycle produces a delay in the spawning time. Bromage *et al.* (1984) used an 18 month extended seasonal light cycle to effect a 3 month delay in the spawning time of rainbow trout; a similar result was also reported by MacQuarrie *et al.* (1979) for pink salmon.

From a commercial aspect, manipulation of a seasonal photoperiod on a weekly basis is complex and prone to error. Consequently, a simpler photoperiod regime is to be preferred. This prompted the introduction of constant and square-wave light regimes. Bromage et al. (1984) and Scott et. al. (1984) initially introduced constant daylengths into seasonally-changing light cycles in a study using rainbow trout which had already undergone a 3 month advance. After completing one seasonal cycle compressed into 6 months, the daylength was reduced to a constant 8L:16D at the longest day. Spawning was advanced to the same degree as if the compressed photocycle had been continued, suggesting that the short daylength was the important factor during the decreasing phase of the photocycle rather than the gradual reduction in photoperiod. In contrast, Erikson and Lundqvist (1980) found that an abrupt change had no influence on maturation of precocious male Atlantic salmon. However, as a gradual step-like decrease in photoperiod proved stimulatory, they therefore concluded that the changing daylength synchronises ripening in Atlantic salmon by its 'differential effect' as opposed to the proportional difference between the photoperiod regimes. This effect has not been reported in subsequent salmonid studies.

Initially, constant long-days were thought to have an inhibitory effect on salmonid reproduction as early experiments resulted in delayed spawning times in: brook trout (Hazard and Eddy, 1951; Shirashi and Fukuda, 1966); sockeye salmon (Combs *et al.*, 1959; Shirashi and Fukuda, 1966); Atlantic salmon (Errikson and Lundqvist, 1980); chinook salmon (Johnson, 1984) and masu salmon (Takashima and Yamada, 1984). Rainbow trout were also shown to respond to constant long daylengths (Skarphedson *et al.*, 1982) or LL with a delay in maturation (Bourlier and Billard, 1984). However, Whitehead and Bromage (1980) and Bromage *et al.* (1982) reported that a long-day photoperiod, if applied from the summer solstice, resulted in no shift in the spawning time. This was later found to be a unique characteristic of the late spawning strain of trout used in these early experiments. Indeed subsequent work by Duston and Bromage (1986, 1987, 1988), Scott *et al.* (1984) and Skarphedinsson *et al.* (1982, 1985), revealed that long-days could stimulate an advance in maturation if exposure was confined to the early phase of the reproductive cycle.

The effect of constant short daylengths on salmonid spawning times has also been shown to be dependent on the stage of the reproductive cycle at which it is applied. Short-days (e.g. 6L:18D) applied soon after spawning had taken place delayed spawning in the rainbow trout (Duston and Bromage,1986, 1987), whereas, if applied during the second half of the reproductive cycle, spawning was advanced in sockeye salmon (Combs *et al.*, 1959; Shirashi and Fukuda, 1966) and rainbow trout (Whitehead and Bromage (1980).

One consideration to note is that whether a period of illumination is interpreted as a long or short photoperiod cannot be measured by daylight hours alone but is determined by the length of the preceding daylength. Hence, it is the disparity between daylengths that induces a response in salmonids rather than the actual hours of light or darkness.

To summarise the findings of the aforementioned authors, long-day photoperiods can advance maturation in salmonids if the exposure is during the early stages of the reproductive cycle. However, if applied later in the cycle, a delay in spawning time results. Conversely, short-day exposure during the latter part of the reproductive cycle will advance maturation, but if applied earlier will produce a delay. Therefore, the most stimulatory regime for advancing salmonid maturation using a square wave photoperiod regime would consist of a period of constant long-days followed by a direct change to short-days (Bromage *et al.*, 1984, 1992; Takashima and Yamada, 1984; Bromage and Duston, 1986; Bromage and Cumaranatunga, 1988).

5.1.6 Neuroendocrine regulation of reproduction in the rainbow trout.

The diurnal profile of melatonin secretion from the pineal gland is thought to synchronise seasonal events through the entrainment of endogenous rhythms. Despite the absence of conclusive evidence of the pineal's role in gonadal development in teleosts, high concentrations of melatonin binding within the hypothalamus (see section 5.1.1 for detailed discussion)

indicate a link between melatonin and the endocrine system. The administration of intrahypothalamic micro-implants in higher vertebrates, has provided direct evidence of melatonin's influence on the hypothalamus (Hastings, 1988). As melatonin did not stimulate gonadotropin release from perfused goldfish pituitaries *in vitro* (Somoza and Peter, 1991), melatonin may act on the reproductive axis through gonadotropin releasing hormone (GnRH) regulation.

Sherwood *et al.* (1983, 1984, 1987, 1993) identified two types of GnRH in the brain of chum salmon. Salmon-I GnRH (s-GnRH-I = $Trp^{7}Leu^{8}$GnRH) closely resembles mammalian LHRH. Structurally it differs only in amino acids 7 and 8 in the peptide sequence (Sherwood *et al.*, 1983). The second, identified by chromatographic cross-reactivity studies (Sherwood *et al.*, 1987), is known to be identical to chicken-II GnRH (c-GnRH-II = $His^{5}Trp^{7}Tyr^{8}$GnRH) and has been shown to maintain a highly conserved peptide structure through the course of evolution (Sherwood *et al.*, 1993). Therefore, unlike mammals, fish express at least two forms of GnRH. The presence of s-GnRH-I and c-GnRH-II have both been confirmed in the rainbow trout (Sherwood *et al.*, 1987; Okuzawa *et al.*, 1990) as well as the goldfish (Yu *et.al.*, 1988), masu salmon (Amano *et al.*, 1991) and most other species studied (for details see Breton *et al.*, 1993; Powell *et. al.*, 1994). Other forms of GnRH have also been demonstrated in fish, such as the chicken-I form (cGnRH-I) in winter flounder and hake and the mammalian GnRH in sturgeon (Sherwood and Coe, 1991), others like the skate and platyfish, exhibit four or more variant forms of GnRH (Billard, 1993; Magliuli-Cepriano et. al., 1994). As yet the biological

requirement for multiple GnRHs remains unresolved. Regulation of GnRH production is thought to involve the nucleus lateralis tuberalis (NLT) (Peter and Crim, 1978; Peter *et al.*, 1980) in the hypothalamus.

The distribution of s-GnRH-I and c-GnRH-II is known to vary with season and sexual maturity. Using an antisera raised against synthetic LHRH, Schafer *et al.* (1989) observed lower immunoreactivity to GnRH in the brains of previtellogenic and vesicle stage 4 rainbow trout than in fish which had undergone exogenous vitellogenesis. Okuzawa *et al.* (1990) reported increased concentrations of s-GnRH-I in the hypothalamus of immature rainbow trout compared to mature individuals, but pituitary concentrations of s-GnRH-I were greater in mature than immature trout. Amano *et al.* (1991,1993) reported increases in pituitary s-GnRH-I content in precocious male masu salmon from spring to autumn while seasonal changes in s-GnRH-I concentrations were reported in the olfactory bulbs, telencephalon and hypothalamus with high levels in the autumn and winter and low levels in the summer. s-GnRH-I concentrations in the hypothalamus and olfactory bulbs also increased significantly in association with testicular maturation during year 3. No distinct changes in c-GnRH-II concentrations were detected within the brain in relation to season or maturation. In the goldfish, the GtH-II ovulatory surge is accompanied by a dramatic decrease in pituitary, telencephalon and olfactory bulb s-GnRH-I content, suggesting a role in the release of gonadatropin (Breton *et al.*, 1993). From this work, the authors suggested that s-GnRH-I principally acts

to control GtH release in the brain, whereas c-GnRH-II is thought to function only as a neuromodulator.

In addition to their GnRH studies in goldfish, Peter and Paulencu (1980) have provided evidence of a gonadotropin release-inhibiting factor (GRIF). Subsequent work suggests the anterior preoptic area to be the source of this catecholamine now thought to be dopamine (Peter *et al.*, 1986). Chang *et al.* (1983) used injections of pimozide (a dopamine antagonist) or 6-hydroxydopamine (a catecholaminergic neurotoxin) to elevate GtH levels in the female goldfish but found dopamine or apomorphine (a dopamine agonist) significantly reduced serum GtH levels (reviewed by Stacey, 1984). Since this demonstration, hormonal spawning-induction in common carp, Chinese loach, Chinese bream, African catfish, European eel, coho salmon, and tilapia, has employed the use of dopamine antagonists in conjunction with GnRHs to promote GtH release (reviewed by Zohar, 1989).

Variations in GtH secretion during reproduction have been reported in a number of salmonids: Atlantic salmon; sockeye salmon; brook trout; brown trout (Crim *et al.*, 1975); and rainbow trout (Zohar *et al.*, 1982; Lou *et al.*, 1984). All studies found low levels of GtH during the cortical alveoli stage and early exogenous vitellogenesis in females and early spermatogenesis in males. During exogenous vitellogenesis in females, and just prior to sexual maturity in males, GtH values began to increase steadily and continued to rise during the final stages of vitellogenesis and peaked when spermiation and ovulation occurred. From the work of Lou and co-workers (1984), it is evident that the stage of gonadal development, and

environmental influences are equally important to plasma GtH levels. The preovulatory peak in GtH (Figure 5.3), together with the fact that in many species ovulation can be induced through administration of exogenous GtH, strongly implicates this hormone in the control of ovulation and spermiation (reviewed by Donaldson and Hunter, 1983; Bromage and Cumaranatunga, 1988).

Initially, at least two GtHs were thought to exist in teleosts: a low carbohydrate content (ConA-1) GtH capable of stimulating both vitellogenin uptake into the oocyte and, in certain species, steroidogenesis; and a high carbohydrate content (ConA-2) GtH responsible for stimulating steroidogenesis, oocyte maturation, ovulation and spermiation (Idler and Ng, 1983; Ng and Idler, 1983). Subsequent investigations by Kawauchi *et al.* (1987) isolated and characterised two distinct GtHs, GtH-I and GtH-II, that are homologous to tetrapod luteinizing hormone (LH) and follicle stimulating hormone (FSH). Synthesis of GtH-I and GtH-II is now known to take place in two distinctly different gonadotropes within the proximal pars distalis of salmonid pituitaries (Kawuchi *et al.*. 1987; Swanson *et al.*, 1989; Nozaki *et al.*, 1990; Naito *et al.*, 1993). The function of GtH-I and GtH-II are still unclear since both are able to stimulate gonadal steroidogenesis at all stages of the reproductive cycle so far examined (Swanson *et al.*, 1989). However, synthesis of the two GtHs seems to occur at different stages of the reproductive cycle. Consequently, Dickhoff and Swanson (1990) proposed that reproductive development up to spawning is regulated by GtH-I following which a surge of GtH-II is required to induce

ovulation. So, despite intensive investigation into teleost gonadotropins, we are still unclear as to the precise number and function of these hormones.

The preovulatory increase in GtH is thought to induce ovarian secretion of oestrone and oestradiol-17β which peaks 3-4 months before spawning takes place (Whitehead and Bromage, 1978; Bromage *et al.*, 1982; Whitehead *et al.*, 1983; Duston and Bromage, 1987). This increase in oestradiol-17β then stimulates the liver to synthesise and release vitellogenin. Peak blood plasma levels of vitellogenin occur at ovulation (Whitehead *et al.*, 1983 and reviewed in Bromage and Cumaranatunga, 1988). As calcium comprises a significant component of vitellogenin and can be easily measured from blood plasma samples, it provides a useful index of vitellogenin secretion. Both oestradiol-17β and calcium analysis were performed during the experiments in this chapter to accurately assess the stage of the reproductive cycle without having to sacrifice the fish.

Finally, with the preovulatory rise in oestrogens there is a parallel increase in testosterone levels with a peak approximately 1 week prior to ovulation (Scott *el. al.*, 1983). Although the precise function of testosterone is still unclear it may simply act as a precursor to oestradiol-17β or may also be involved in atresia as suggested by (Cumaranatunga, 1985). Finet et. al. (1988) further suggested that testosterone may regulate cAMP accumulation by the oocytes prior to the GTH stimulated pre-ovulatory surge in progestagens. Of these 17α-hydroxy-20β-dihydroprogesterone (17α20β-P) is vital to the induction of final maturation (Nagahama et. al.,

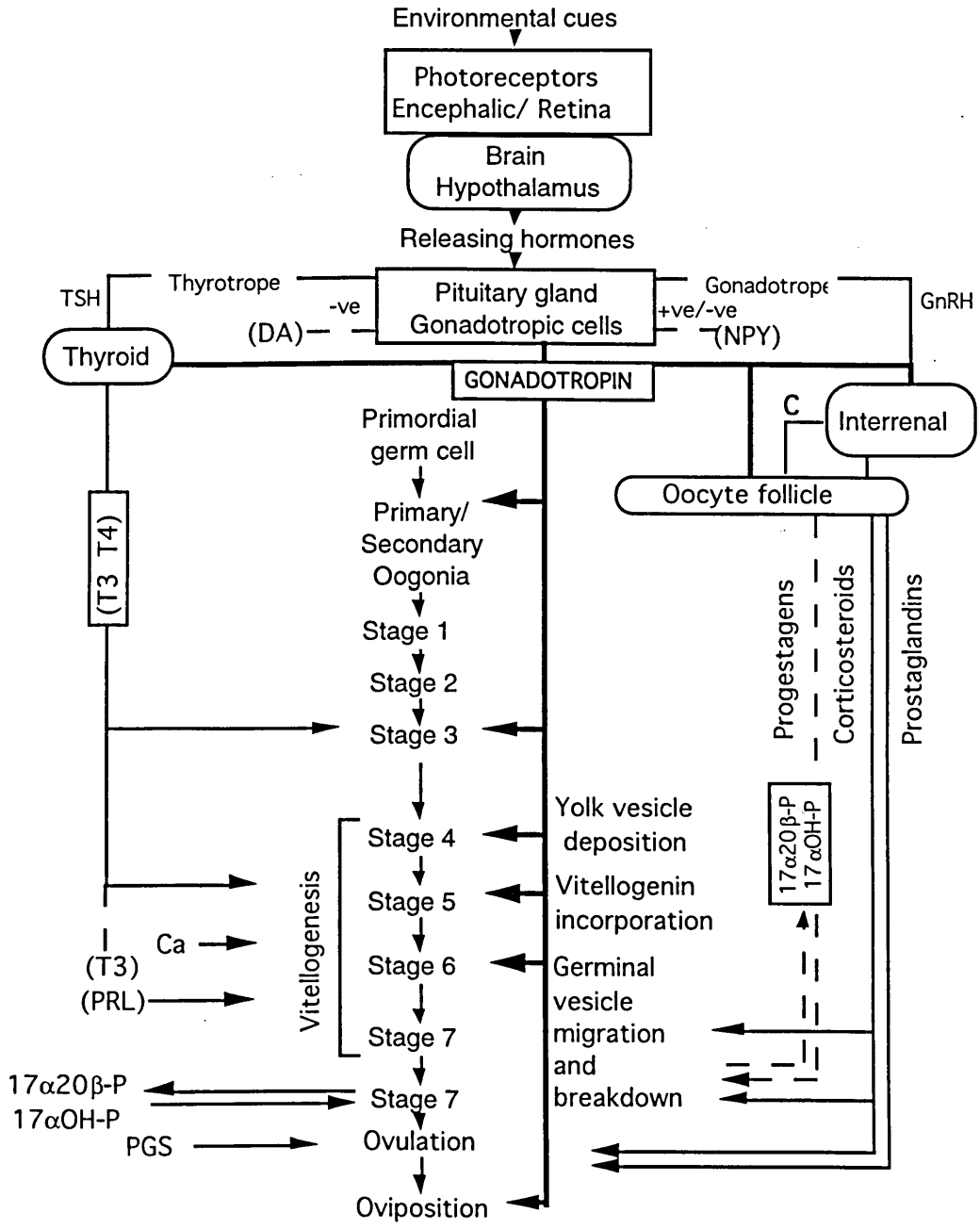


Figure 5.4

Diagram summarising the neuroendocrine system controlling the reproductive cycle of the female rainbow trout (TSH, thyroid stimulating hormone; DA, dopamine; NPY, neuropeptide Y; GnRH, gonadotropin releasing hormone; C, corticosteroids; T3 triiodothyronine; T4, thyroxine; Ca, calcitonin; PRL, prolactin; 17α20β-P,17α–hydroxy–20β-dihydroprogesterone;17αOH-P,17α–hydroprogesterone; PGR, prostaglandins).

1985) as its secretion by the ovaries occurs at stage 7 of oocyte development (see Figure 5.4). 17α20β-P synthesis takes place within the ovarian follicles where thecal cells produce 17α-hydroxyprogesterone (17α0H-P) which is then converted to 17α20β-P by the granulosa cell layer (Nagahama et. al., 1985).

5.1.7 Endocrine manipulation of spawning times

Endocrine manipulation in fish culture began in Brazil during the early 1930's and was aimed at inducing ovulation in cyprinids which would not spawn in captivity. Initially this involved the injection of pituitary homogenates (hypophysation). Since then the development of hormonal induction and synchronisation of ovulation and spermiation has developed to cover a wide range of reproductive hormones (Reviewed in Lam, 1982; Donaldson and Hunter, 1983; Billard, 1989; Zohar, 1989)

Hypophysation, utilising pituitary extract (PE), usually from salmon or carp, is still the most commonly used method of inducing spawning of broodstock (Billard, 1989; Lam, 1982), although alternatives are being sought to overcome the high cost and variable potency of PE. Partially purified teleost gonadotropins have been found to be effective when taken from: carp, chinook salmon (Donaldson *et al.*,1972); chum salmon (Idler *et al.*,1975); and rainbow trout (Breton *et al.*, 1976) and can be administered to many cultured species. Again, the cost of refining or partially purifying the hormone has proved prohibitive for many commercial applications although in future it may be economically viable to administer these

preparations to species with a high fecundity. Mammalian gonadotropins have also been investigated. One of these, Human-Chorionic Gonadotropin (HCG), has produced successful results but its effects varies with temperature and the stage of the reproductive cycle at which it is administered (Zanuy *et al.*, 1986). An immunity to HCG after several years of use has been reported in silver carp and bighead carp although this seems to be an isolated case (Anon, 1977).

Luteinising hormone-releasing hormone (LH-RH) and its analogues (LH-RHa) are both temperature independent and induce gonadotropin (GtH) secretion in teleosts (Crim et al., 1979, 1983, 1988). Superactive analogues are the more effective especially if used as implants (Crim and Glebe, 1984) although potency does seem to vary according to the stage of gametogenesis when applied, with pituitary responsiveness being far greater in the final stages. Zohar (1988) suggests that lack of final oocyte maturation, ovulation and spawning is the result of a failure to release GtH. This being the case, it would seem the use of GtH releasing hormones (GnRH) would provide the most effective therapy.

In many species mammalian GnRH analogue (GnRHa) is more active than piscine GnRH (Zohar, 1989) and is also cheaper to synthesise in a pure form. Other advantages of GnRH and their analogues are: a resistance to enzyme degradation by the pituitary; a low degree of biological species specificity allowing their use on a variety of fish; and the low doses required means costs are kept to a minimum. Trials have revealed that GnRHa is at its most effective when applied as a cocktail with domperidone, a dopamine antagonist (discussed

later), since dopamine acts as a gonadotropin release inhibitory factor (GRIF) Zohar (1989). As with LH-RHa, if GnRHa is administered in a single injection, the result in some species is a single surge of GtH and, as such, the use of GnRHa cellulose implants may prove more satisfactory as they produce an extended release period. This method of influencing spawning times seems preferable as the fish utilises endogenous GTH.

Although the use of hormones to manipulate the reproductive cycles of cultured species seems an ideal tool, in practice other factors must be considered. Primarily, whether the sale and possible consumption of offspring from hormonally modified fish is acceptable to the consumer or legislating bodies plus the cost involved in obtaining and purifying the hormone from a donor or synthesising an artificial compound. Secondly, as most salmonids are cultured within flow-through systems, the environmental implications of the release or 'leaking' of hormones into natural watercourses must be considered. Thirdly, the time of administration and its results are dependent on many factors: the stage of gonadal development; the time between injections and number given; the temperature of the water and half life of the hormone. In practice many hormones are only effective when applied close to the time of spawning otherwise egg quality deteriorates, and attempts to alter the reproductive cycle from an early stage have not yet been successful. Finally, the act of anaesthetising and injecting the fish is not only time consuming and introduces possible sites of infection, but is extremely stressful to the animal concerned. Considering the value of

many salmonid broodstock a safer alternative would be preferable.

Sections 5.1.6 and 5.1.7 provide a brief introduction to the hormonal mechanisms which co-ordinate gonadal maturation in salmonids. The reader should note that there are a number of endocrine components not discussed in the text (see Figure 5.4) which have been implicated in salmonid reproduction, these include neuropeptide-Y (NPY), calcitonin (Ca), thyroid hormones (T3, T4), prolactin (PRL) and prostaglandins (PGS) (for further information see Dickhoff *et. al.*, 1989; Breton *et al.*, 1990; Fouchereau-Peron *et. al.*, 1990).

In summary, the experiments described in this chapter attempt to address the role of melatonin and the pineal gland in conveying seasonal photoperiodic information to the reproductive axis of female rainbow trout. To achieve this pinealectomies and/or constant release melatonin implants were administered at specific times of the photocycle known from previous photoperiod experiments (Duston and Bromage, 1988; Randall and Bromage, 1992) to be critical to the timing of spawning in rainbow trout.

5.2 The effects of pineal removal on the timing of maturation in female rainbow trout subjected to a reduction from long to short day photoperiods.

5.2.1 Objectives

This experiment was designed to establish whether pinealectomy (pineal removal), which removes both the melatonin signal and the neural connections of the pineal to the brain would prevent recognition of the change from a long to short day photoperiod and therefore prevent an advance in spawning time of female rainbow trout.

5.2.2 Materials and Methods.

An all-female population of rainbow trout (South African stock) was reared from hatch and maintained at ambient temperature (Figure 2.1) under natural photoperiod (Lat. 56°03' N) at the Buckieburn Research facility. At the beginning of February 1994, 250 fish (mean weight 703±49g) were moved to 5 tanks (1.3m diameter) with a stocking rate of 50 fish per tank. All tanks were illuminated under simulated natural photoperiod (SNP) and maintained at ambient temperatures. On the 30 March the photoperiod in 4 of the tanks was changed to 18L:6D and fish in the fifth tank (mean weight 811±34g) remained on SNP (SNP control) to assess the natural spawning time of the stock. Three of the groups on the long day photoperiod were transferred to a short day (6L:18D) on 27 May. Of these: one group (mean weight 860±49g) was left intact

(short day control); one group (mean weight 688±56g) had their pineals removed (section 2.2.1) pinealectomy; and the last group (mean weight 901±47g) underwent a sham-pinealectomy. The fourth group (mean weight 835±63g) was maintained on a continuous long day of 18L:6D (long day control). These experimental protocols are illustrated in Figure 5.5.

At monthly intervals, body weight measurements from 25 fish per group were taken (section 2.7.1) and a further 10 individuals from each were blood sampled (section 2.1.3) to measure plasma calcium (section 2.6) and oestradiol (section 2.5). Unfortunately this was restricted at certain times over the summer months due to excessively high water temperatures and low dissolved oxygen levels, therefore to reduce stress it was decided to forgo some sampling dates. Day and night-time blood samples were taken from the sham-pinealectomy and pinealectomy groups in September and assayed for melatonin (section 2.4) to ensure that the pinealectomy operations had been successful. Once the fish had begun to show signs of maturing each group was assessed for spawning females every 2 weeks (section 2.3).

Due to the rapid growth of the fish, stocking densities required 20 individuals from each group to be removed on 28 July. All the fish from the short day tanks were sub-cutaneously tagged (section 2.2.3) and transferred to a 5m diameter tank with identical photoperiod, water conditions and feeding regimes to those they had been maintained under previously. The fish from the control and constant long day tanks were moved to separate 1.3m diameter tanks, again under identical conditions to the rest of their group. The sham-

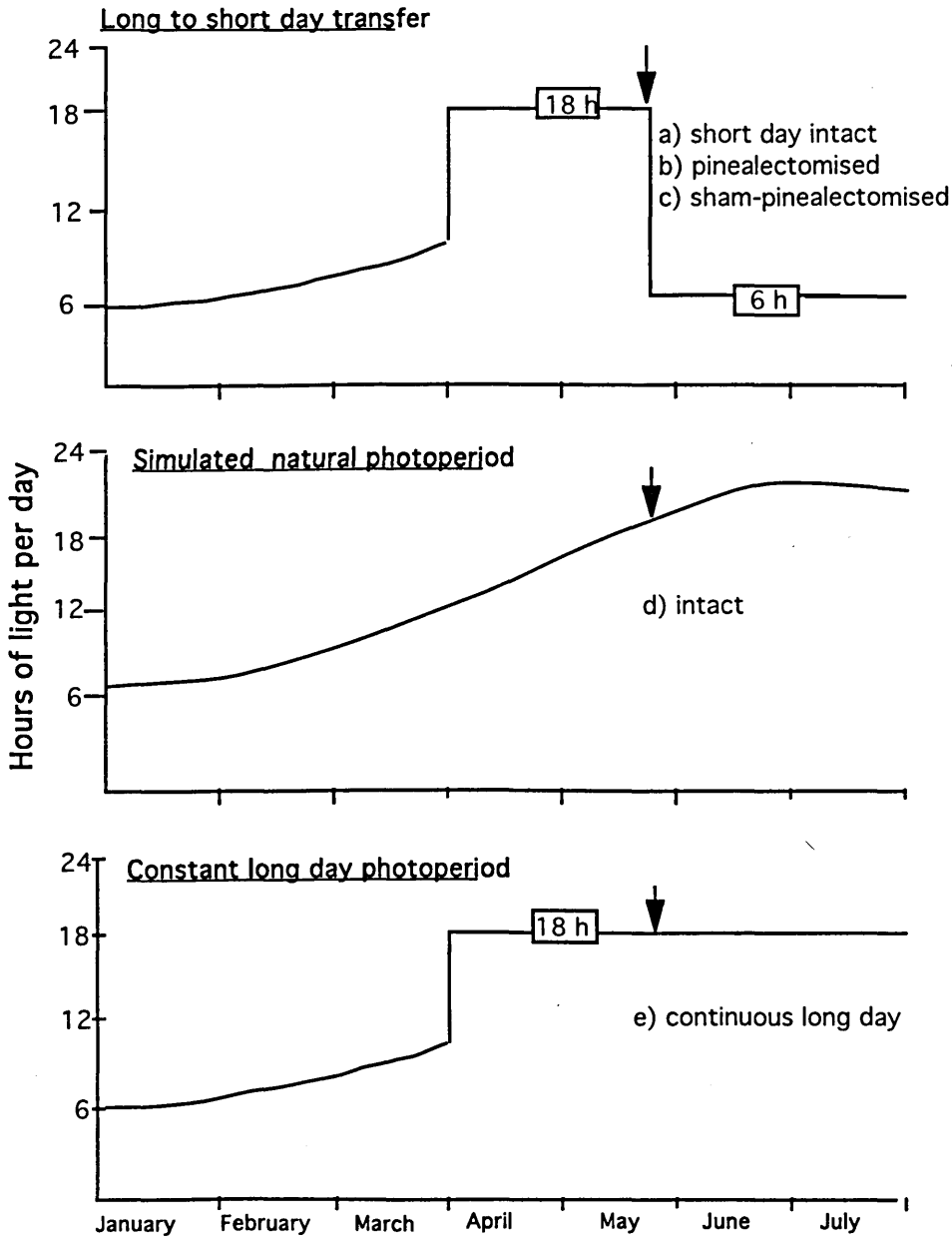


Figure 5.5

Model of the photoperiods and treatments used in the groups in experiment 1 chapter 5. The long day photoperiods were initiated on 30 March and the transfer to short days took place on 27 May. Pinealectomy and sham-operations were performed on the 27 May (arrowed).

pinealectomy and short day control groups were removed from the experiment on 10 January as all the maturing fish from the groups had spawned and the tanks were required for other experiments. By 3 May all groups had finished spawning and the experiment was terminated. Pinealectomised fish were autopsied as a further assessment of whether complete pineal removal had been achieved. This entailed dissection of the brain and a visual examination for presence of any pineal and pineal tract tissues.

5.2.3 Results

There were no significant differences between the weights of the groups at the end of the experiment (Figure 5.6) although the short day group did show a significantly lower ($P<0.05$) mean weight when compared to the remaining groups in December. The spawning profiles are shown in Figure 5.7. From these it can be seen that the square-wave shift in photoperiod was successful in advancing the spawning time of all the groups transferred to a short day. The pinealectomy, short day control and sham-pinealectomy groups spawned significantly earlier than the SNP control ($P<0.01$) and long day groups ($P<0.001$). No significant difference was found between the pinealectomy, short day control or sham-pinealectomy fish (after the removal of outliers using Grubb's Test), nor was the long day group significantly delayed in comparison to the SNP controls (5.7).

Interestingly, the plasma calcium levels for the pinealectomy group more closely reflect those of the SNP

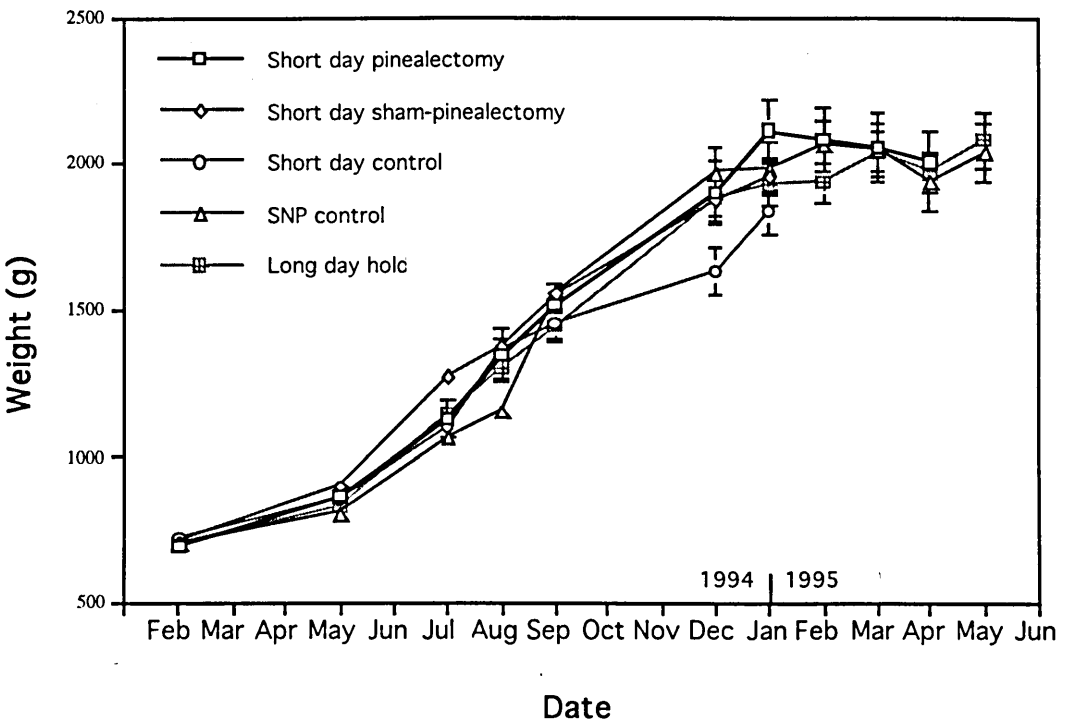


Figure 5.6

The effects of pinealectomy on the timing of changes in weight (mean ± 1SEM) in femals rainbow trout subjected to a range of photoperiod manipulations.

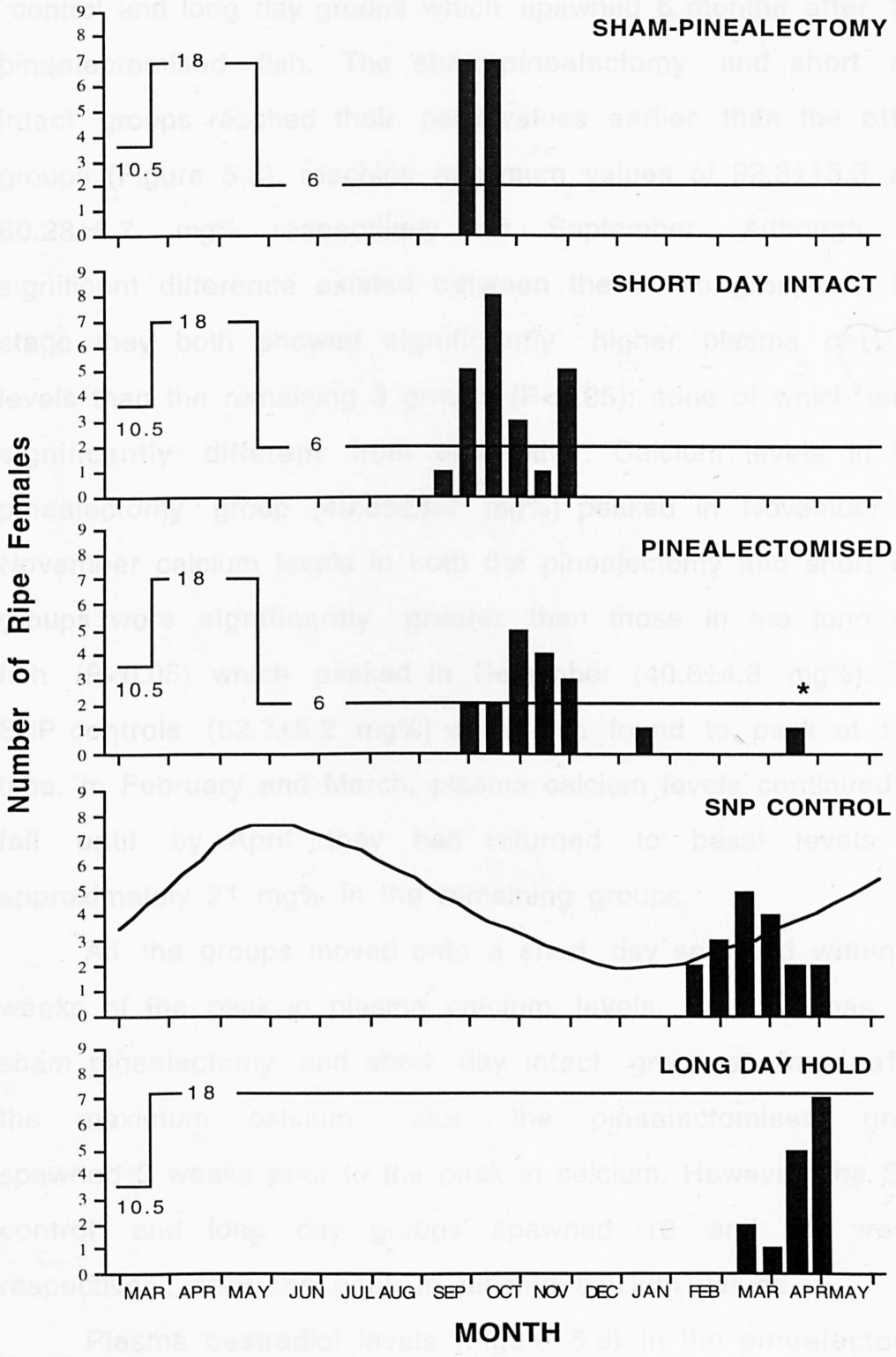


Figure 5.7

The effects of long days followed by short days on the spawning time of rainbow trout which had been pinealectomised, sham-pinealectomised or left intact. Two further groups were either maintained on a somulated natural photoperiod or constant long day hold. * denoted outliers detected using Grubbs Test.

control and long day groups which spawned 6 months after the pinealectomised fish. The sham-pinealectomy and short day intact groups reached their peak values earlier than the other groups (Figure 5.8), reaching maximum values of 92.3±13.3 and 60.28±6.7 mg% respectively in September. Although no significant difference existed between these two groups at this stage they both showed significantly higher plasma calcium levels than the remaining 3 groups ($P<0.05$); none of which were significantly different from each other. Calcium levels in the pinealectomy group (49.85±5.0 mg%) peaked in November. In November calcium levels in both the pinealectomy and short day groups were significantly greater than those in the long day fish ($P<0.05$) which peaked in December (40.6±4.3 mg%). The SNP controls (52.7±5.2 mg%) were also found to peak at this time. In February and March, plasma calcium levels continued to fall until by April they had returned to basal levels of approximately 21 mg% in the remaining groups.

All the groups moved onto a short day spawned within 3 weeks of the peak in plasma calcium levels, but, whereas the sham pinealectomy and short day intact group spawned after the maximum calcium value, the pinealectomised group spawned 3 weeks prior to the peak in calcium. However, the SNP control and long day groups spawned 10 and 14 weeks respectively, after the peak in plasma calcium values.

Plasma oestradiol levels (Figure 5.9) in the pinealectomy, sham-pinealectomy and long-short day intact groups appeared to have peaked before blood sampling began with levels in the pinealectomy group remaining at basal levels (mean level 2.35±1.3 ng/ml) throughout the sampling. However, oestradiol

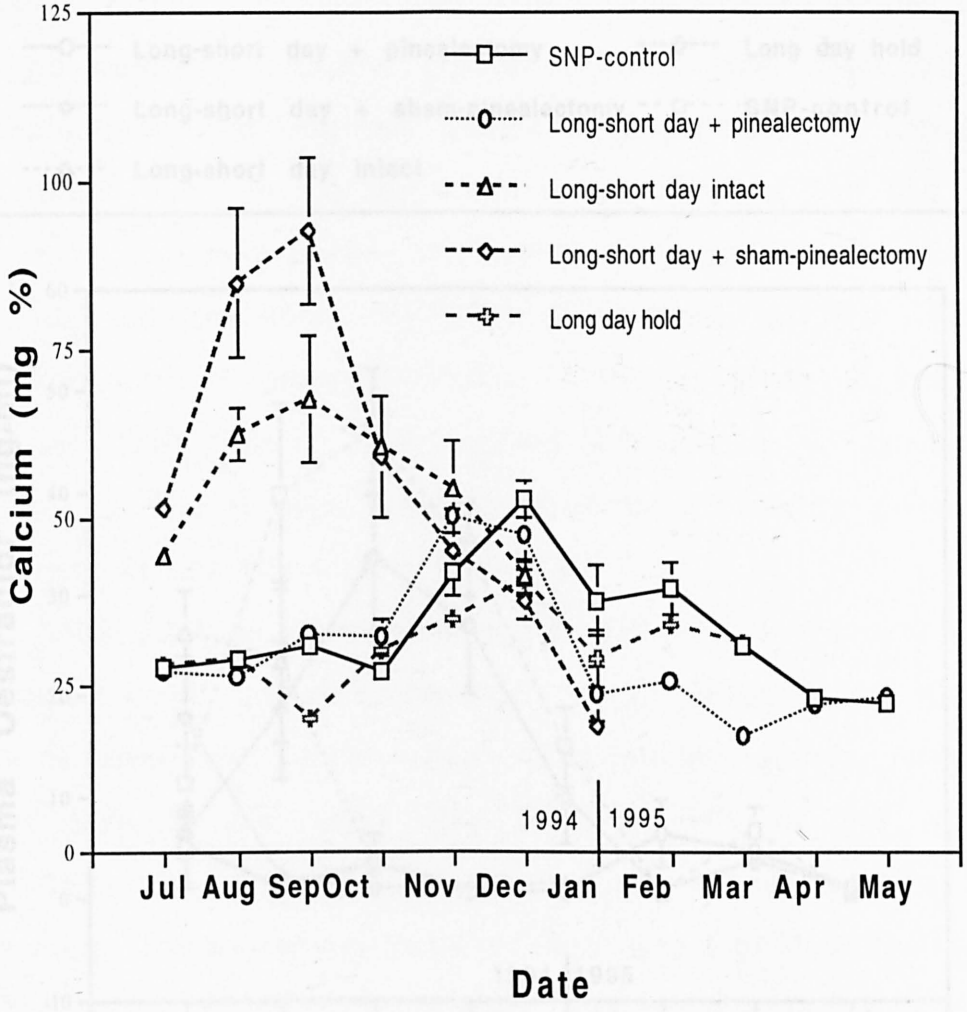


Figure 5.8

The effects of pinealectomy and sham-pinealectomy on the timing of changes in total plasma calcium levels (mean±1SEM) during maturation in female rainbow trout exposed to a constant long day followed by a constant short day photoperiod. Other groups included fish maintained on a simulated natural photoperiod (SNP- control) and a constant long day hold from 27 May.

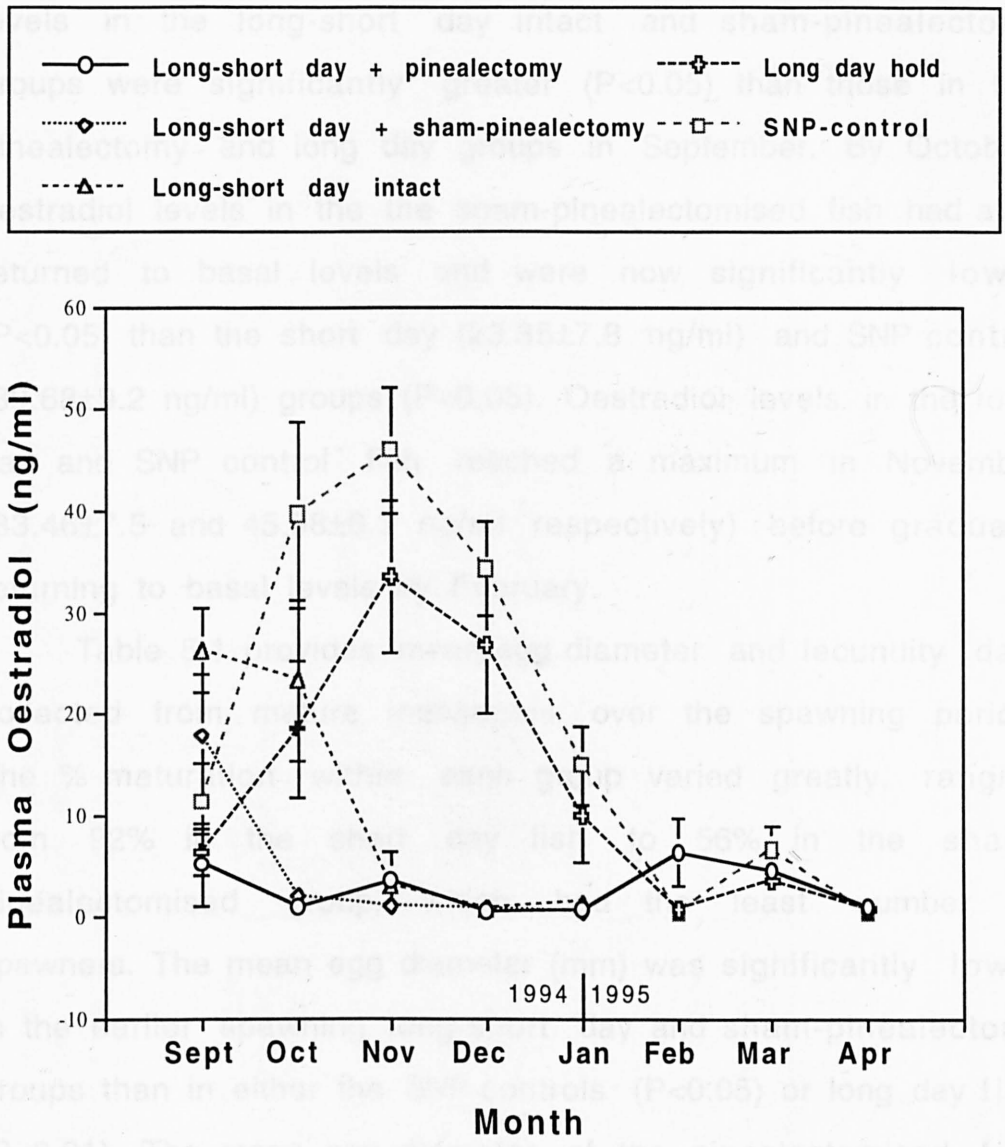


Figure 5.9

The effects of pinealectomy and sham-pinealectomy on the timing of changes in total plasma oestradiol levels (mean±1SEM) during maturation in female rainbow trout exposed to a constant long day followed by a constant short day photoperiod. Other groups included fish maintained on a simulated natural photoperiod (SNP- control) and a constant long day hold from 27 May.

levels in the long-short day intact and sham-pinealectomy groups were significantly greater ($P<0.05$) than those in the pinealectomy and long day groups in September. By October, oestradiol levels in the the sham-pinealectomised fish had also returned to basal levels and were now significantly lower ($P<0.05$) than the short day (23.35 ± 7.8 ng/ml) and SNP control (39.68 ± 9.2 ng/ml) groups ($P<0.05$). Oestradiol levels in the long day and SNP control fish reached a maximum in November (33.46 ± 7.5 and 45.98 ± 6.3 ng/ml respectively) before gradually returning to basal levels by February.

Table 5.1 provides mean egg diameter and fecundity data collected from mature individuals over the spawning period. The % maturation within each group varied greatly, ranging from 92% in the short day fish to 56% in the sham-pinealectomised group, which had the least number of spawners. The mean egg diameter (mm) was significantly lower in the earlier spawning long-short day and sham-pinealectomy groups than in either the SNP controls ($P<0.05$) or long day fish ($P<0.01$). The mean egg diameter of the pinealectomised fish was also significantly smaller ($P<0.05$) than that of the long day fish. Figure 5.10 confirms the variation ($P<0.01$) of mean egg diameter over time, with values ranging from 4.35 ± 0.09 mm in September to a peak in March (5.11 ± 0.04 mm) before decreasing again in April and May.

No significant difference was observed in the total fecundities of the groups. However, the relative fecundities of the short day groups were significantly higher than either the long day or SNP control groups ($P<0.05$); no significant differences were observed between the short day fish.

GROUP	A SNP Control intact	B Short day intact	C Sham- pinealectomy	D Pinealectomy	E Long day-hold
% Maturation	73	92	56	72	60
Egg Diameter (mm.)	4.87 ± 0.05	4.61 ± 0.06 a (P≤0.05) b (P≤0.001)	4.41 ± 0.08 a (P≤0.001) b (P≤0.001)	4.60 ± 0.08 b (P≤0.05)	4.92 ± 0.04
Total Fecundity	4010 ± 187	4105 ± 224	4348 ± 169	4090 ± 259	3872 ± 384
Relative Fecundity (N°. eggs/kg)	2114 ± 161	3554 ± 275 a (P≤0.01) b (P≤0.01)	3706 ± 249 a (P≤0.001) b (P≤0.001)	3321 ± 277 a (P≤0.05) b (P≤0.05)	2138 ± 167
Spawning Period	Feb. 7-April 19	Sept. 21-Nov. 30	Oct. 6-Oct. 21	Oct. 6-Feb. 7 (April 5 outlier removed)	March 7-April 19
Mid-spawning in each group from Sept. 21	173.67 ± 5.03	37.48 ± 4.48	23.0 ± 1.94	54 .0 ± 6.33	198.67 ± 3.93

Table 5.1

Egg size and fecundity data from groups in experiment 1 transferred from a long day photoperiod to a short day in May All measurements are given as a group mean (± 1SEM). The mid-spawning point for each group is given as the number of days from when the first fish in the population spawned. Each group contained 25 individuals. Groups significantly different from control (intact) are indicated by the superscript: a. A significant difference from the Long day group is indicated by the superscript, b.

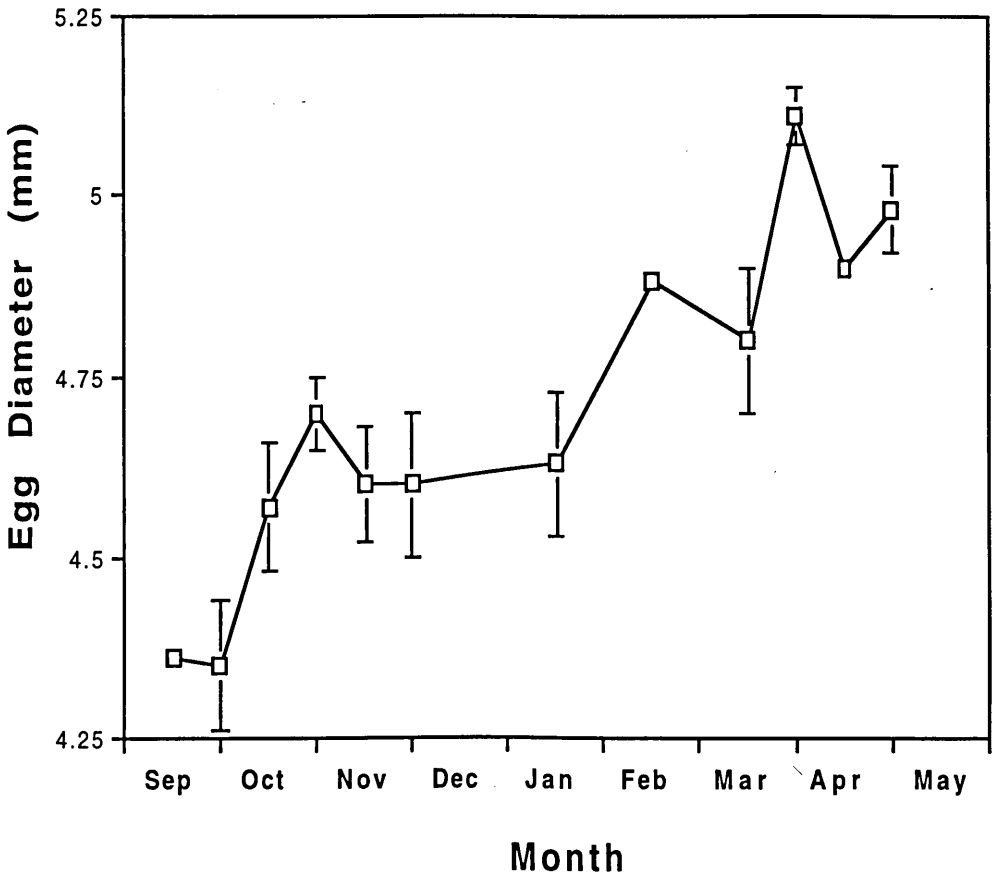


Figure 5.10

The relationship between egg size (mean ± 1SEM) and the timing of spawning in rainbow trout (pooled data from all groups in experiment 1, chapter 5).

Blood samples taken from pinealectomy and sham-pinealectomy groups (n=10) during mid-light and mid-dark phases of the light cycle (Figure 5.11) revealed that pineal removal had significantly reduced circulating plasma melatonin during the dark phase ($P<0.001$). Dark phase blood samples revealed that melatonin levels in pinealectomised fish (67.1±5.9 pg/ml) were not significantly greater than the light phase levels of pinealectomised and sham-pinealectomised fish (65.1±3.4 and 63,6±1.5 pg/ml), but were significantly lower than those of sham-pinealectomised dark phase samples ($P<0.01$).

Blood samples were also collected from each pinealectomised individual during the dark phase subsequent to the spawning date. From the results of these samples it was concluded that all the fish that reached maturity had indeed been successfully pinealectomised. Dark phase blood samples from pinealectomised fish ranged from 52.6 to 75.0 pg/ml, this was significantly lower ($P<0.05$) than the mean plasma melatonin level for sham-pinealectomised fish collected during the scotophase (230.4±22.8 pg/ml). This finding was reinforced by the results of the autopsies as no indications of pineal remnants were found in the pinealectomised fish.

5.2.4 Summary of Results of section 5.2

Melatonin levels collected from pinealectomised fish at the mid-dark phase (67.1±5.9 pg/ml) did not show significant variation when compared to those collected during the light phase (65.1±3.4 pg/ml). These were also not significantly different from light phase levels (63.6±1.5 pg/ml)in sham-

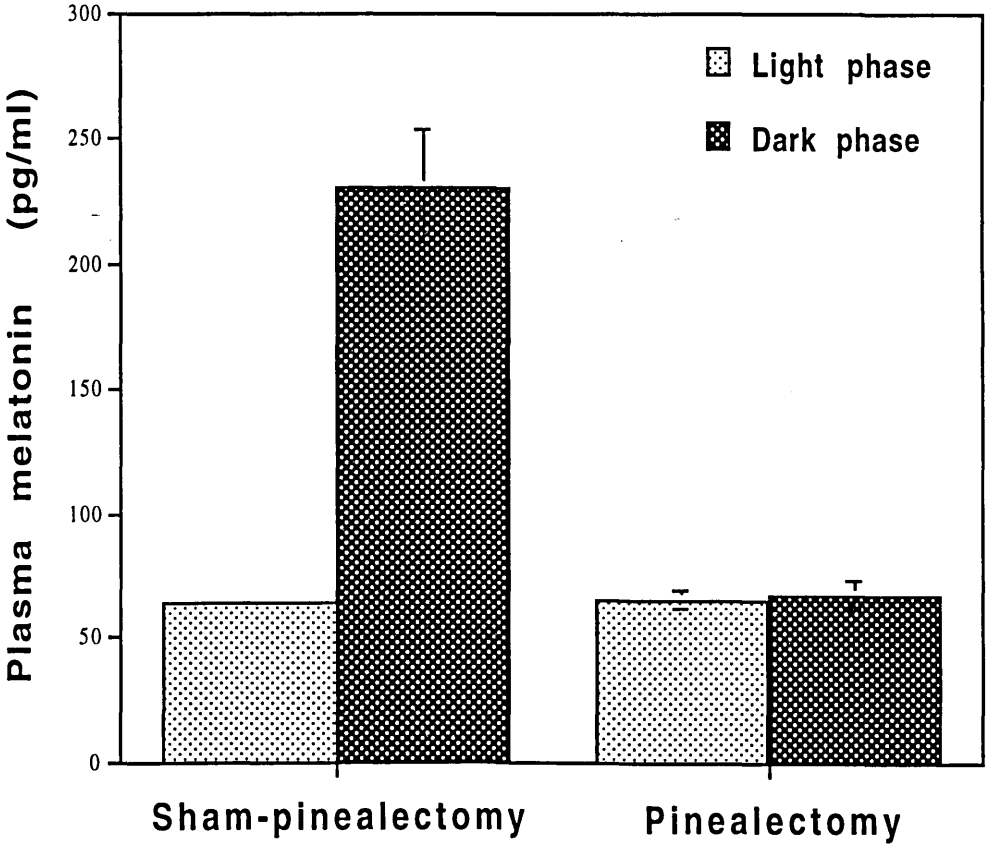


Figure 5.11

Plasma melatonin levels (±1SEM) collected during mid-light and mid-dark phase from pinealectomised and sham-pinealectomised female rainbow trout maintained under short day photoperiods.

pinealectomised fish. The transfer from long to short day photoperiods was successful in advancing the spawning times of the long-short day intact, sham-pinealectomy and pinealectomy groups, when compared to the SNP control and long day fish. Although no significant variation in spawning times was observed between the three groups once outliers had been removed, their presence together with the 4 month spawning period suggests a desynchronisation within the pinealectomised group, especially when compared to the sham-pinealectomised fish. This is reinforced by the mean plasma calcium profile for pinealectomised fish which closely followed that of the SNP control and long day groups showed a significantly lower amplitude ($P<0.05$) which reached a maximum value 14 weeks after either the long-short day intact or long-short day sham-pinealectomised fish. This change was not observed for plasma oestradiol levels. No variations in weights were observed between the groups over the course of the experiment.

Total fecundity showed no variations between groups, but, the relative fecundities of the advanced groups, although showing no inter-group variation, were significantly greater than those of the SNP control and long day fish ($P<0.05$). The difference between the pinealectomised group and SNP control was not statistically significant.

5.3 The effects of pineal removal and melatonin administration on the timing of maturation in female rainbow trout subjected to a transfer from long to short day photoperiods.

5.3.1 Objectives

After the inconclusive results of section 5.2 this experiment repeated the previous work using an earlier reduction from long to short days to accentuate the spawning advance. Two groups of fish did not receive the short day reduction in but instead one group was administered constant release melatonin implants to assess whether the implants were interpreted as a short day photoperiod. Similarly, pinealectomy alone, or in combination with implants, was performed to determine the importance of the pineal gland and melatonin during the maturation of female rainbow trout.

5.3.2 Materials and Methods.

An all-female population of rainbow trout (South African stock) were obtained from Trossachs Fish Farm, Callander, where they had been maintained under ambient temperature and natural photoperiod (Lat. 56°24' N). On January 20 1995, 7 tanks (1.3m diameter) each with 38 fish were transfered to a long day (18L:6D) photoperiod. A further 2 groups were exposed to a simulated natural photoperiod (SNP) and all the fish were maintained at ambient temperatures. From 1-4 May, five of the groups on long days were either: sham-implanted (mean weight 549±21g); pinealectomised (section 2.2.1) (mean weight 557±9g); sham-pinealectomised and administered a constant-release melatonin implant (section 2.2.2) (mean weight

572±14g); sham-pinealectomised (mean weight 573±20g); pinealectomised and administered a constant-release melatonin implant (mean weight 551±16g).

These groups were then transferred to a constant short day (SD) photoperiod (6L:18D) on 4 May. Of the groups on SNP, one group was sham-implanted (mean weight 588±16 g) and a second was left intact to assess the natural spawning time of the stock. The remaining 2 groups on the long day photoperiod (LD) remained on a constant long day but were either sham-implanted (mean weight 567±16 g) or administered constant-release melatonin implants (mean weight 576±17 g). These treatments are illustrated graphically in Figure 5.12.

At monthly intervals, body weight measurements were taken from 20 fish per group (section 2.7.1) and a further 10 individuals from each group were blood sampled (section 2.1.3) to measure plasma calcium (section 2.6). Day and night-time blood samples were taken from the sham-pinealectomy and pinealectomy groups in September and assayed for melatonin (section 2.4) to ensure that the pinealectomy operations had been successful. Once the fish began to show signs of maturing, each group was assessed for spawning females every 2 weeks (section 2.3).

Due to constant high temperatures and low rainfall during the summer months, water availability to the farm fell to critical levels. This required all flow rates to the tanks to be reduced drastically in order to save the experimental animals. Consequently dissolved oxygen levels fell to 5.7% despite the addition of air stones to each tank. As a result feeding rates were severely reduced and over a 1 month period feeding was

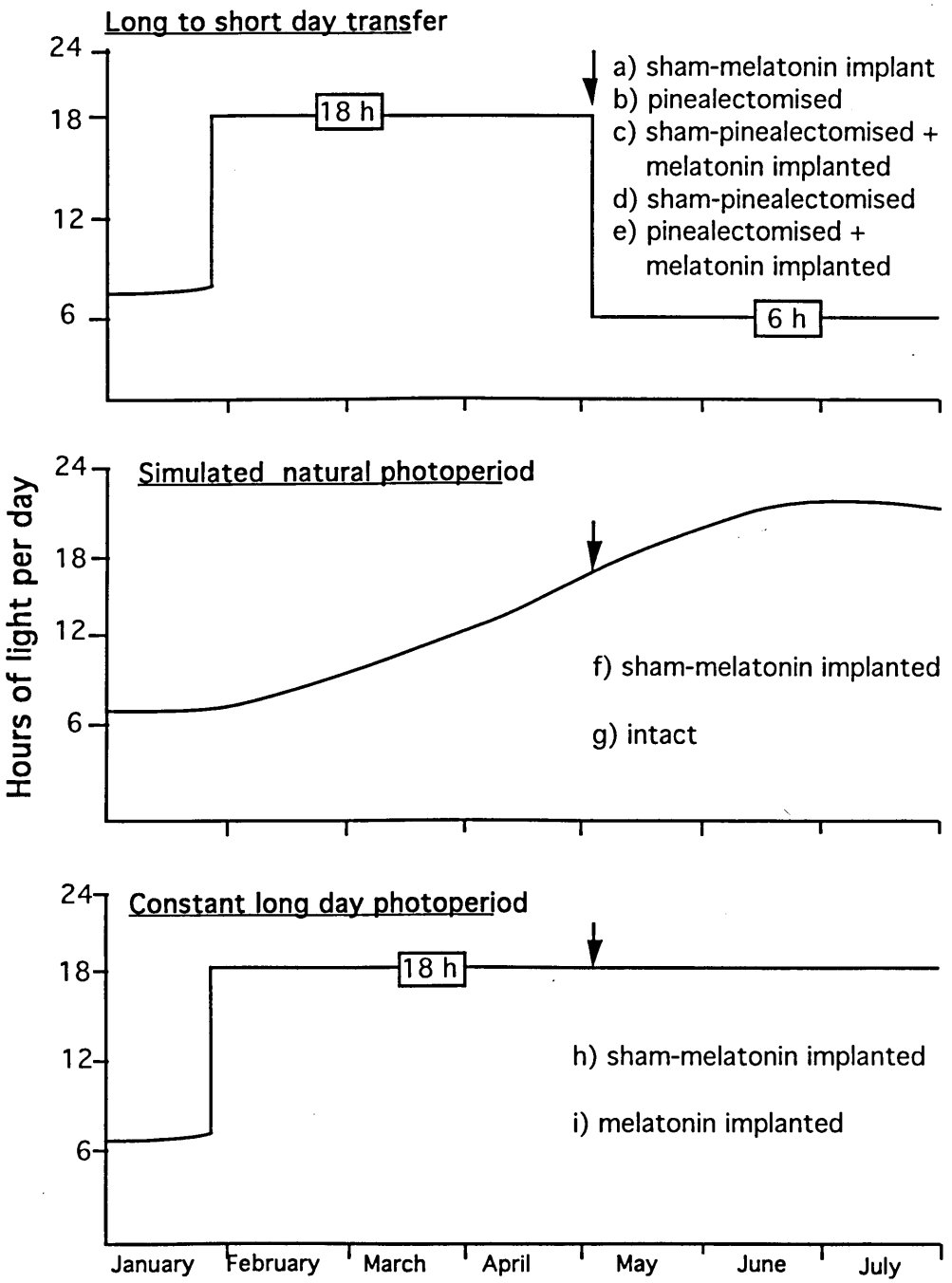


Figure 5.12

Experimental design of the photoperiods and melatonin treatments in groups a-i, experiment 2 chaper 6. The long day photoperiods initiated on 20 January and the transfer to short days took place May. Pinealectomy, sham-operations and melatonin implantation performed between 2 and 4 May (arrowed).

stopped altogether. During this time, no sampling was undertaken to minimise the stress to the fish. The consequences of these actions are discussed in section 5.5.

6.3.3 Results.

No significant variations between group weights were observed at the time of the operations and photoperiod changes in May (Figure 5.13). Despite the period of reduced feeding, by october the SNP sham-melatonin implanted group (889±26 g) was significantly heavier ($P<0.05$) than the short day groups which had been pinealectomised (662±41 g), sham-pinealectomised + melatonin implanted (697±24 g), and pinealectomised + melatonin implanted (731±40 g), and the long day groups which had been sham (679±28 g) and melatonin implanted (700±28 g). This weight advantage was maintained throughout November with SNP groups with sham-melatonin implants or left intact remaining heavier ($P<0.05$) than short day groups with sham-melatonin implants, pinealectomised, sham-pinealectomised + melatonin implants and pinealectomised + melatonin implants and the long day fish in the sham-melatonin implanted group being significantly heavier ($P<0.05$) than either the short day pinealectomised or short day sham-pinealectomised + melatonin implanted groups. Again in December the SNP sham-implant group was significantly ($P<0.05$) heavier than short day groups which had been

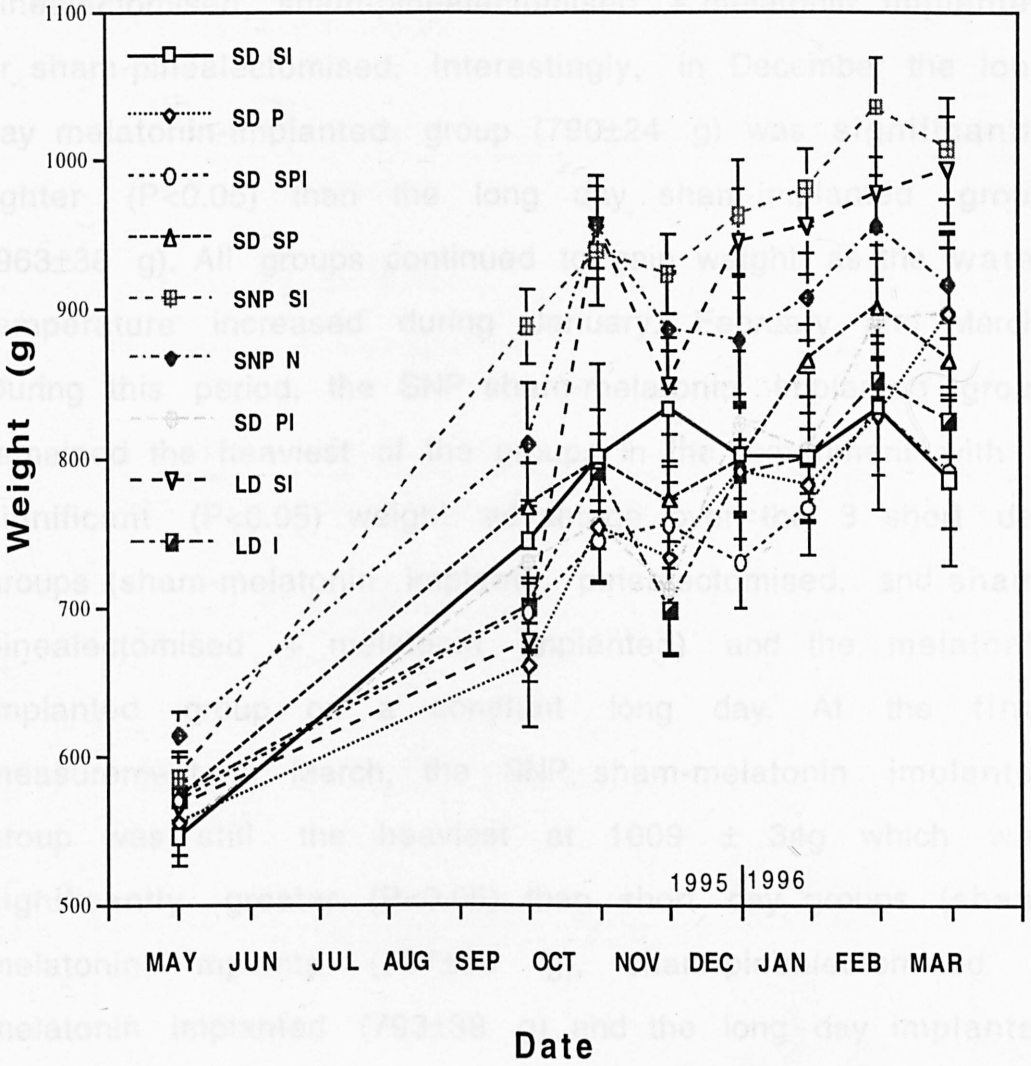


Figure 5.13

The effects of pinealectomy and melatonin implantation on the timing of changes in weights (mean ± 1SEM) of female rainbow trout subject to a range of photoperiod manipulations (SD SI, long-short day + sham-implant; SD P, long-short day + pinealectomised; SD SPI, long-short day + sham-pinealectomy plus melatonin implant; SD SP, long-short day + sham-pinealectomy; SNP SI, SNP sham-implant; SNP N, SNP intact; SD PI, long-short day + pinealectomy plus melatonin implant; LD SI, long day sham-implant; LD I, long day melatonin implant).

pinealectomised, sham-pinealectomised + melatonin implanted or sham-pinealectomised. Interestingly, in December the long day melatonin-implanted group (790±24 g) was significantly lighter ($P<0.05$) than the long day sham-implanted group (963±38 g). All groups continued to gain weight as the water temperature increased during January, February and March. During this period, the SNP sham-melatonin implanted group remained the heaviest of the groups in the experiment with a significant ($P<0.05$) weight advantage over the 3 short day groups (sham-melatonin implants, pinealectomised, and sham-pinealectomised + melatonin implanted) and the melatonin implanted group on a constant long day. At the final measurement in March, the SNP sham-melatonin implanted group was still the heaviest at 1009 ± 34g which was significantly greater ($P<0.05$) than short day groups (sham-melatonin implants (787±58 g), sham-pinealectomised + melatonin implanted (793±38 g) and the long day implanted group (826±24 g).

Spawning profiles, shown in Figure 6.14, reveal that all groups which received a change from long to short day photoperiods in May experienced an advance in their spawning times. Spawning in groups transferred from long to short days was advanced by approximately 4 and 5 months respectively, when compared to the SNP and constant long day groups. No significant variation ($P>0.05$) was observed between the different groups moved onto short daylengths. There was also no difference in the spawning times of the 2 groups on SNP and no difference between the 2 groups on constant long days. But,

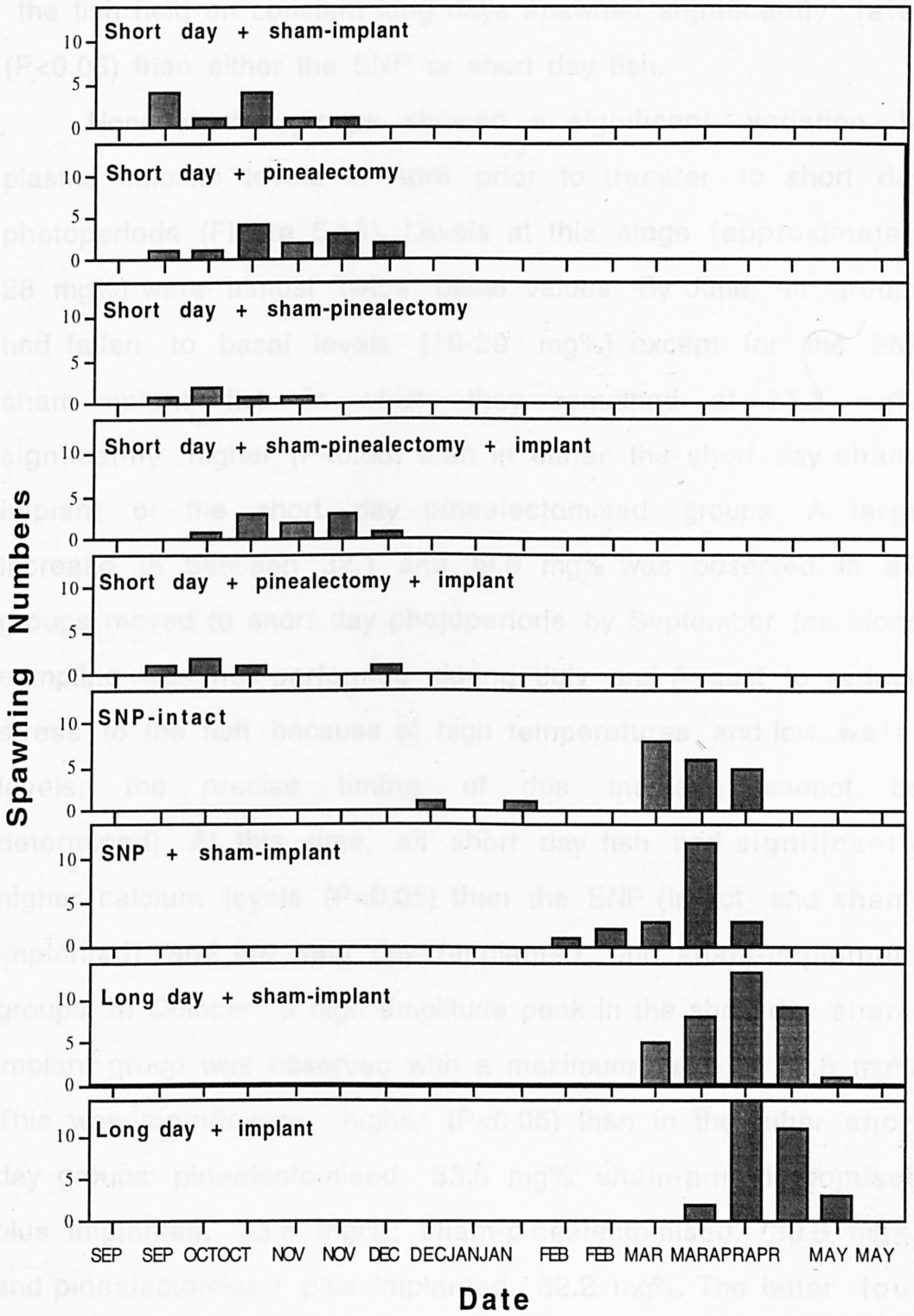


Figure 5.14

The effects of pinealectomy and melatonin implantation on the timing of changes in spawning times of female rainbow trout subject to a range of photoperiod manipulations short day sham-implant; short day pinealectomised; short day sham-pinealectomy plus melatonin implant; short day sham-pinealectomy; SNP sham-implant; SNP intact; short day pinealectomy plus melatonin implant; long day sham-implant; long day melatonin implant.

the fish held on constant long days spawned significantly later ($P<0.05$) than either the SNP or short day fish.

None of the groups showed a significant variation in plasma calcium levels in April prior to transfer to short day photoperiods (Figure 5.15). Levels at this stage (approximately 28 mg%) were almost twice basal values. By June, all groups had fallen to basal levels (18-20 mg%) except for the SNP sham-implant fish in which they remained at 27.3 mg%, signficantly higher ($P<0.05$) than in either the short day sham-implant or the short day pinealectomised groups. A large increase to between 32.1 and 39.8 mg% was observed in all groups moved to short day photoperiods by September (as blood sampling was not performed during July and August to reduce stress to the fish because of high temperatures and low water levels, the precise timing of this increase cannot be determined). At this time, all short day fish had significantly higher calcium levels ($P<0.05$) than the SNP (intact and sham-implanted) and the long day (implanted and sham-implanted) groups. In October a high amplitude peak in the short day sham-implant group was observed with a maximum value of 50.8 mg%. This was significantly higher ($P<0.05$) than in the other short day groups: pinealectomised, 33.6 mg%; sham-pinealectomised plus implanted, 33.8 mg/%; sham-pinealectomised, 30.5 mg%; and pinealectomised plus implanted, 32.2 mg%. The latter four groups also had significantly higher ($P<0.05$) plasma calcium levels than the long day or SNP groups in October. By December, the calcium levels in the SNP and long day groups began to increase while those in the short day groups remained at their previously elevated values. In the short day groups calcium

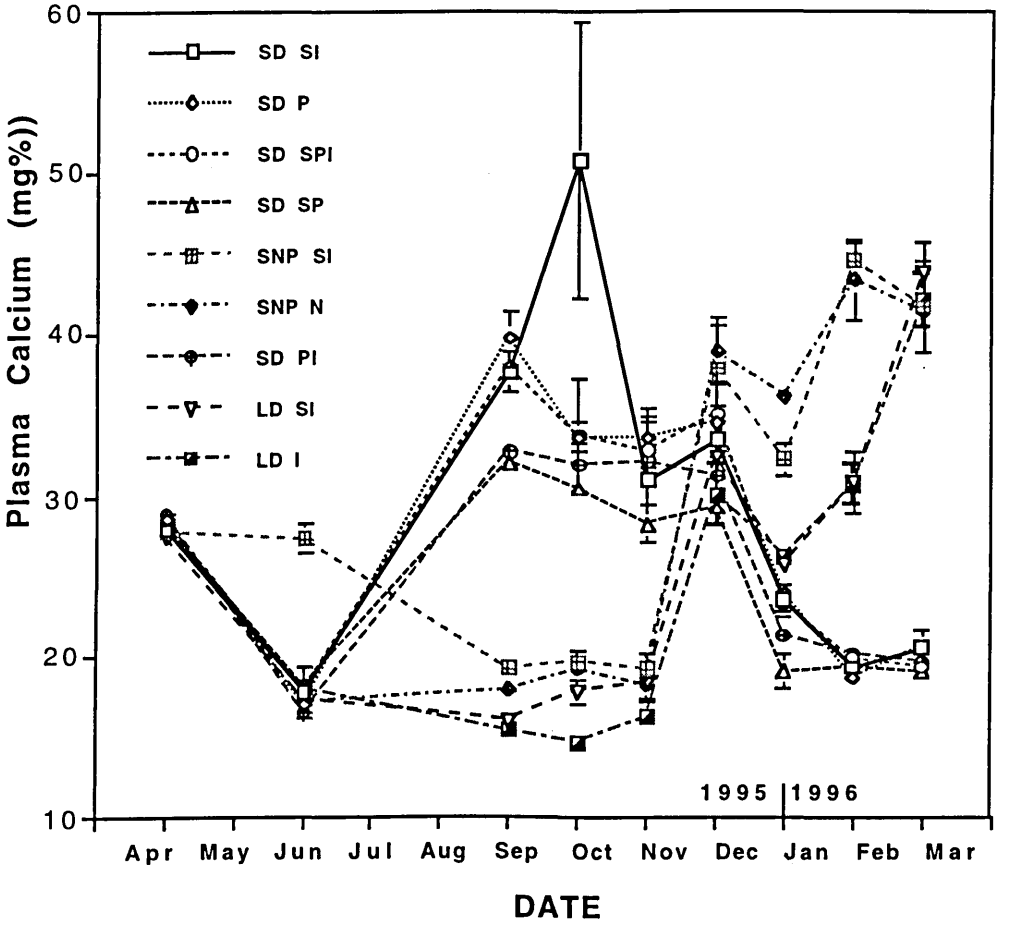


Figure 5.15

The effects of pinealectomy and melatonin implantation on the timing of changes in total plasma calcium levels (mean ± 1SEM) in female rainbow trout subjected to a range of photoperiod manipulations (SD SI, short day sham-implant; SD P, short day pinealectomised; SD SPI, short day sham-pinealectomy plus melatonin implant; SD SP, short day sham-pinealectomy; SNP SI, SNP sham-implant; SNP N, SNP intact; SD PI, short day pinealectomy plus melatonin implant; LD SI, long day sham-implant; LD I, long day melatonin implant).

levels started to fall in January with a small decrease also noted in the 2 long day and 2 SNP groups, although the overall trend in the latter 4 groups was upwards. By February, all short day groups had returned to basal levels, and the long day sham-implant and implanted groups calcium levels (43.8 and 42.1 mg% respectively) were significantly higher ($P<0.05$) than in the short day pinealectomised and sham-pinealectomised fish. Groups under a SNP had significantly higher ($P<0.05$) levels of plasma calcium than all of the groups on short days. The final measurement in March showed that the 2 long day groups had levels higher than those in the 2 SNP groups which may have peaked as a slight decrease in plasma calcium was noted. Again all 4 groups had mean calcium values which were significantly greater ($P<0.05$) than the fish under short day photoperiods.

Overall, it appears that the move from long to short daylengths advanced the peak in plasma calcium concentrations by approximately 5 months. This was shown by the short day groups peaking between September and October compared to the fish under SNP which produced a maximum value in February and the long day hold fish which had still not peaked by March. The variations in plasma calcium profiles are consistent with the differences in spawning times between the 9 groups. Peak calcium levels were observed approximately 2 weeks prior to the mean spawning time of all groups regardless of their photoperiod and treatment.

Table 5.2 provides mean egg diameter and fecundity data collected from mature individuals from groups transferred from a long day photoperiod to a short day in May. The % maturation within each group was predictably low due to the

GROUP	A Sham-Implant	B Pinealectomy	C Sham-pinealectomy	D Sham-pinealectomy + Implant	E Pinealectomy + Implant
% Maturation	44	52	20	40	20
Egg Diameter (mm)	3.56 ± 0.008	4.08 ± 0.07	3.97 ± 0.12	3.89 ± 0.14	3.86 ± 0.12
Total Fecundity	2530 ± 145	2114 ± 262	2526 ± 276	2716 ± 129	2568 ± 285
Relative Fecundity (N°. eggs/kg)	4082 ± 272	3899 ± 479	4430 ± 429	4182 ± 282	3971 ± 530
Spawning Period	Sept. 27-Nov. 24	Sept. 27-Dec. 6	Sept. 27-Nov. 9	Oct. 11-Dec. 6	Sept. 27-Dec. 6
Mid-spawning in each group from Sept. 27	22.0 ± 6.0	41.9 ± 6.0	21.0 ± 7.4	44.2 ± 5.7	26.6 ± 12.2

Table 5.2

Egg size and fecundity data from groups in experiment 2 transferred from a long day photoperiod to a short day in May. All measurements are given as a group mean (±1SEM). The mid-spawning point for each group is given as the number of days from when the first fish in the population spawned. Each group contained between 25-29 individuals. No significant differences were found between the groups.

period of high temperatures and reduced daily ration over July and August. Despite this, 52% of the pinealectomised group matured, compared to: 44% of the sham-implanted fish; 40% of the sham-pinealectomy plus implant group; and 20% of the fish in the pinealectomy plus implant and sham-pinealectomy groups. No significant difference ($P>0.05$) was observed between the short day photoperiod groups with respect to egg diameter (mm), total or relative fecundity or mid spawning time.

Plasma melatonin levels in this experiment were measured during the mid-dark and mid-light phase in December 1995 (Figure 5.16). Of the fish sampled, all groups implanted with constant release melatonin implants had significantly greater ($P<0.05$) levels of melatonin than fish without implants. However, the long day implanted group (i) (dark phase 981±94 pg/ml, light phase 469±6 pg/ml) and the short day pinealectomised plus implanted fish (e) (dark phase 982±100 pg/ml, light phase 655±39 pg/ml) showed levels of melatonin far in excess ($P<0.05$) of those measured in the short day sham-pinealectomy plus melatonin implant fish (c) (dark phase 290±80 pg/ml, light phase 125±6 pg/ml).

No significant variation in melatonin levels was found between long day sham-implant (dark phase 72±9 pg/ml, light phase 36±6 pg/ml); simulated natural photoperiod (dark phase 58±5, light phase 34±5 pg/ml); and short day sham-pinealectomised fish (dark phase 60±8, light phase 28±6 pg/ml). No difference between light phase melatonin levels in short day pinealectomised fish (30±2 pg/ml), short day sham pinealectomised, SNP intact and long day sham-melatonin were

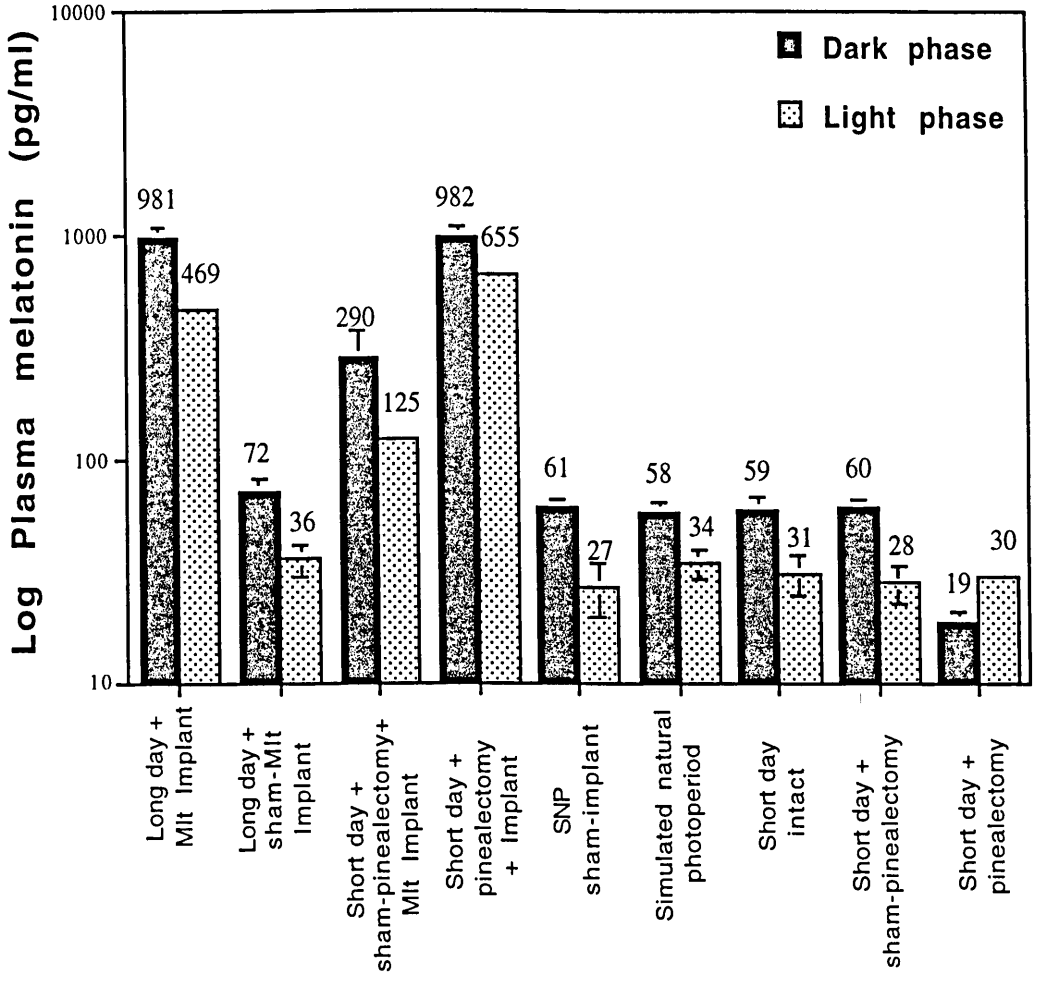


Figure 5.16

Plasma melatonin levels (±1SEM) collected during mid-light and mid-dark phase from female rainbow trout which had been: pinealectomised; sham-pinealectomised; implanted with melatonin; sham implanted; or left intact. The fish were maintained under constant long day, short day or simulated natural photoperiods. In some cases the SE bars were too small to be depicted.

found ($P>0.05$), however, dark phase melatonin levels were found to be significantly lower ($P<0.05$) in the short day pinealectomised fish (19±2 pg/ml) than the other 3 groups.

5.3.4 Summary of Results

Neither pinealectomy, melatonin implantation, nor a combination of the two had any significant effect on the spawning time of female rainbow trout exposed to a photoperiodic change, from constant long to constant short days, in May. However, this photoperiod manipulation was successful in advancing the spawning time of the groups concerned by 5 months compared to fish of the same stock maintained on a simulated natural photoperiod. No difference in fecundity or egg diameter was observed between the groups maintained on short days and, unlike experiments 1 and 3, no variation in plasma calcium levels was detected between these groups. Pinealectomy successfully reduced the dark phase melatonin levels of the control groups to between 58 and 70 pg/ml and those of the light phase levels to a mean 19 pg/ml. Melatonin implantation increased light and dark phase circulating melatonin by 13 times in long day implanted and short day pinealectomised plus implanted fish. Pineal removal did not appear to influence plasma melatonin levels in the implanted fish. However, short day sham-pinealectomised plus implanted individuals had levels only 4 times those of control fish. The reason behind this reduction is unknown.

5.4 The effects of pineal removal at the summer solstice on the timing of maturation in female rainbow trout maintained under a natural photocycle.

5.4.1 Objectives

The annual light cycle exerts a synchronising influence on the timing of maturation in salmonids. To assess the importance of the pineal gland in perceiving the change from the increasing daylengths of spring and summer and the decreasing photoperiod experienced during the latter half of the year, virgin female rainbow trout were pinealectomised on the summer solstice. The effect of pineal removal on the subsequent spawning time was then observed.

5.4.2 Materials and Methods.

An all-female population of rainbow trout (South African stock) were reared from hatch and maintained at ambient temperature (4.5-18°C) under natural photoperiod (Lat. 56°09' N). This was carried out at the University of Stirling within an outdoor recirculation system in 1.5m diameter tanks. In May 1994, 15 fish from the stock were sacrificed to obtain a mean gonadosomatic index (GSI) to ensure a large proportion of the fish would mature the following Spring. On 26 June, the pineals were removed from 45 fish (mean weight 107.4±7.3 g). These were maintained on a natural photoperiod at ambient temperature as were 40 intact control fish. The controls were left intact to minimise the number of mortalities due to the risk of infection which is far greater in a recirculation than a flow-through system. Indeed, 8 of the pinealectomised

individuals died of fungal infections within 3 weeks of the operation.

At monthly intervals, body weight measurements from 25 fish per group were taken (section 2.7.1) and a further 10 individuals from each group were blood sampled (section 2.1.3) for analysis of plasma calcium (section 2.6) and oestradiol (section 2.5). Day and night-time blood samples were taken from the sham-pinealectomy and pinealectomy groups in September and assayed for melatonin (section 2.4) to ensure that the pinealectomy operations had been successful. Blood samples were also collected from each pinealectomised individual during the dark phase subsequent to the spawning date. This was to verify that the dark phase melatonin level of each mature fish had been reduced to a level which indicated that the pineal had been completely removed. Once the fish began to show signs of maturing, each group was assessed for spawning fish every 2 weeks (section 2.3).

All pinealectomised fish were autopsied on completion of the experiment to confirm that no pineal remnants were still present and melatonin levels were rechecked.

5.4.3. Results.

No statistically significant differences in weight (Figure 5.17) were found except in January when the pinealectomised fish were heavier. Peak spawning time of the control group occurred on 5 January, 6 weeks prior to the peak spawning time of the pinealectomised group (Figure 5.18); this delay was

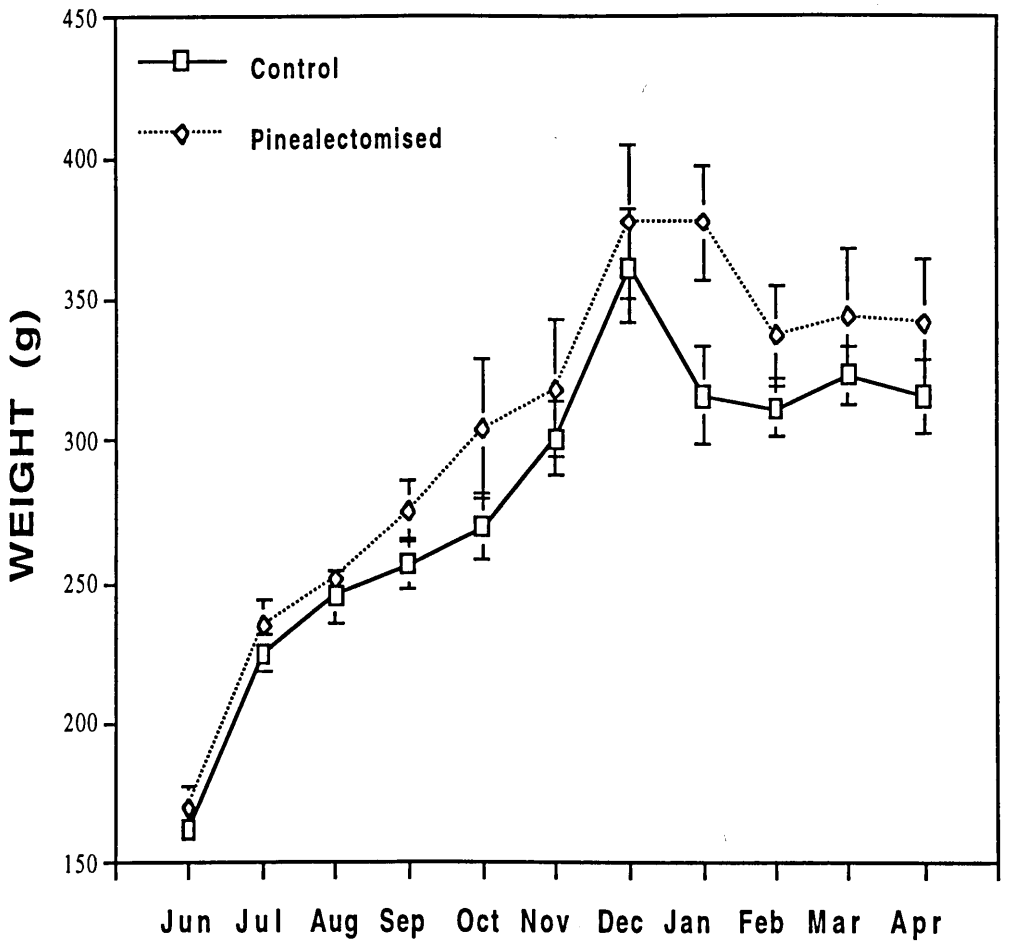


Figure 5.17

The effect of pinealectomy on the change in weight (mean ± 1SEM) of female rainbow trout pinealectomised on the summer solstice compared to intact controls.

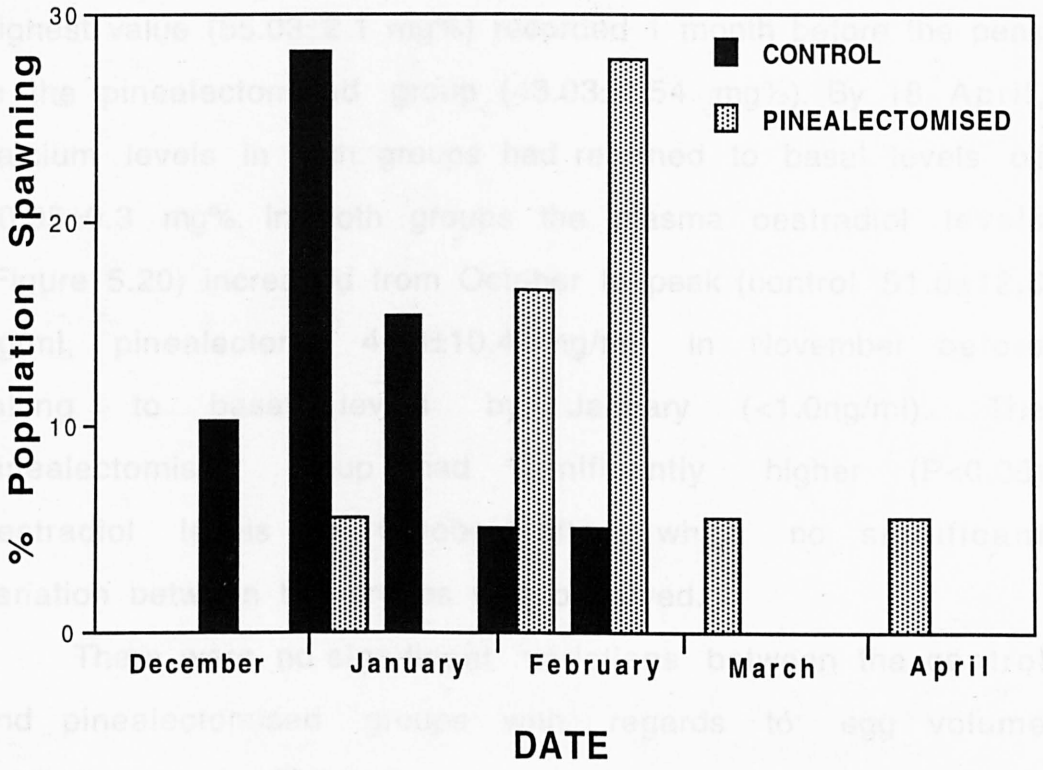


Figure 5.18

The effects of pinealectomy (on 22 June) on the timing of spawning in female rainbow trout.

statistically significant ($P<0.01$; Mann-Whitney test). The percentage of spawning individuals within the 2 groups were similar, with 73% maturing in the control group and 70% in the pinealectomised group. Plasma calcium levels of the control fish (Figure 5.19) were significantly greater than in the pinealectomised fish in December and January ($P<0.05$) with the highest value (55.03±2.1 mg%) recorded 1 month before the peak in the pinealectomised group (43.03±1.54 mg%). By 18 April, calcium levels in both groups had returned to basal levels of 20.95±0.3 mg%. In both groups the plasma oestradiol levels (Figure 5.20) increased from October to peak (control 51.8±12.6 ng/ml, pinealectomy 41.3±10.4 ng/ml) in November before falling to basal levels by January (<1.0ng/ml). The pinealectomised group had significantly higher ($P<0.05$) oestradiol levels in October after which no significant variation between the groups was observed.

There were no significant variations between the control and pinealectomised groups with regards to: egg volume (63.5±2.4 and 67.0±8.8 ml respectively); egg diameter (4.46±0.03 and 4.51±0.06 mm respectively); total fecundity (894±38 and 919±115 respectively); or relative fecundity (2487±108 and 2618±257 respectively) (Table 5.3).

Figure 5.21 shows light and dark phase levels of plasma melatonin collected from both control and pinealectomised fish in September. Only the dark phase sample from the control group (178.6±11.6 pg/ml) revealed a significant difference ($P<0.001$) compared to the control light phase sample (21.4±8.0) and both pinealectomy samples (dark; 30.66 ±6.6 and light; 27.4±9.3 pg/ml). Blood samples collected from each

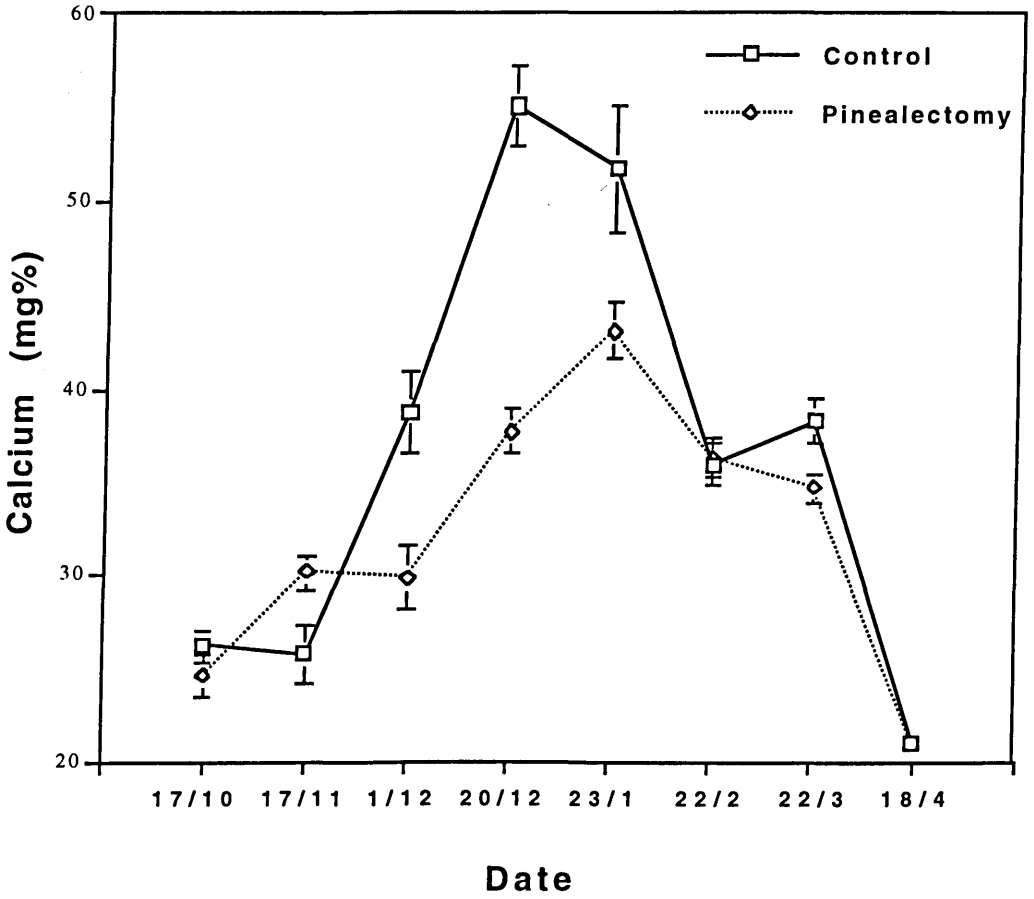


Figure 5.19

The effects of pinealectomy (22 June) on the seasonal changes of total plasma calcium (mean ± 1SEM) during maturation in female rainbow trout.

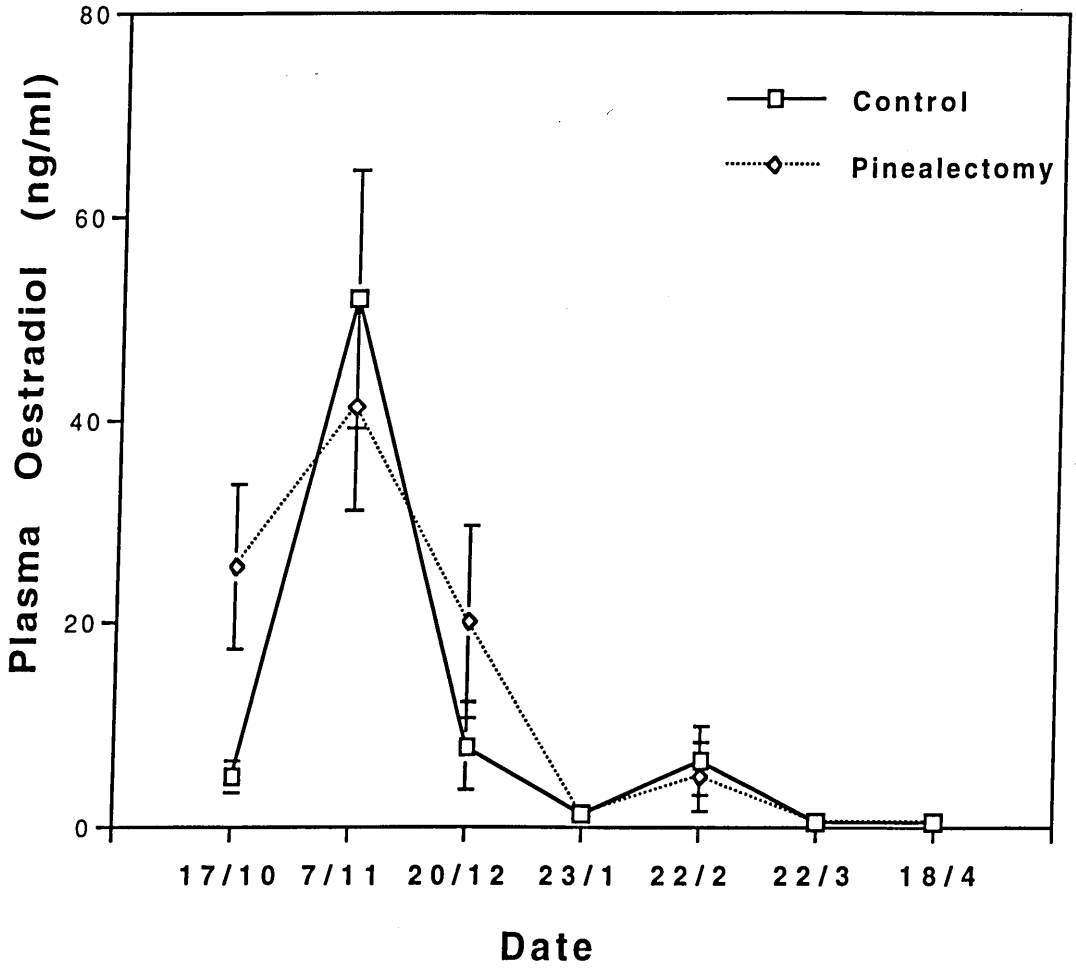


Figure 5.20

The effects of pinealectomy (22 June) on the seasonal changes of total plasma oestradiol (mean ± 1SEM) during maturation in female rainbow trout.

GROUP	% Maturation	Egg Diameter (mm)	Total Fecundity	Relative Fecundity	Spawning Period	Mid-spawning in each group from Dec. 20
Control	74	4.46±0.03	894±38	2487±108	Dec.20-Feb.22	44.3±3.7
Pinealectomy	70	4.51±0.06	919±115	2618±257	Jan.5-Apr.4	80.8±6.5

Table 5.3

Egg size and fecundity data from groups in experiment 3 maintained under a natural seasonal photoperiod and ambient temperature. All measurements are given as a group mean (±1SEM). The mid-spawning point for each group is given as the number of days from when the first fish in the population spawned. Each group contained between 25-30 individuals. No significant differences were found between the groups.

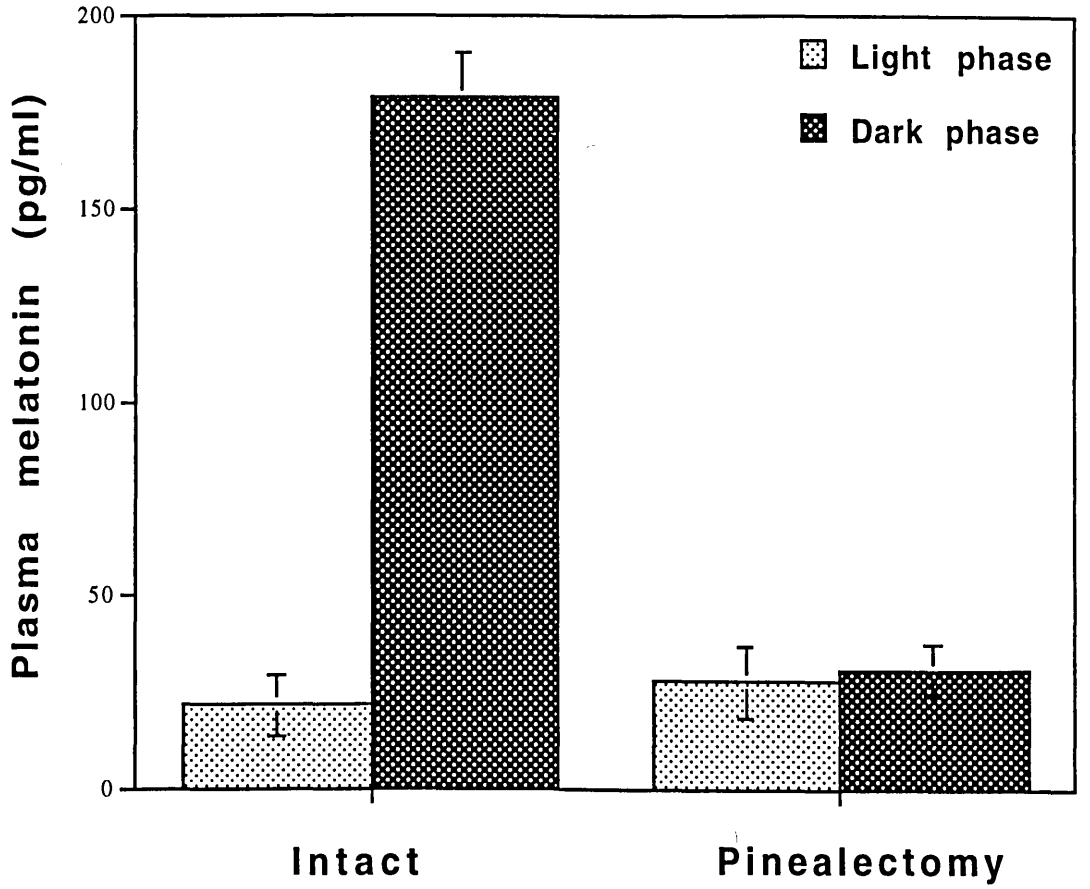


Figure 5.21

Plasma melatonin levels (±1SEM) collected during mid-light and mid-dark phase from pinealectomised and intact female rainbow trout maintained under a natural photoperiod.

pinealectomised individual during the dark phase subsequent to the spawning date indicated that all the fish that had reached maturity had indeed been successfully pinealectomised. The lowest value obtained from the analysis of the dark phase blood samples of pinealectomised fish was 15.6 pg/ml and the highest value was 85.8 pg/ml.

Autopsies on the fish at the end of the experiment revealed no remnants of pineal glands in any of the pinealectomised fish and no differences in melatonin levels within groups were found.

6.4.4 Summary of Results

Pinealectomy at the summer solstice produced a 6 week delay in the spawning time of female rainbow trout. Plasma calcium levels in the pinealectomised fish were also of a significantly lower amplitude and peaked 4 weeks after the control group. However, plasma oestradiol appeared unaffected by pineal removal. The: mean body weight; egg volume and diameter; and total and relative fecundities exhibited no significant differences between groups over the course of the experiment. Finally, pineal removal successfully reduced the dark phase circulating melatonin levels to almost six times that of the control dark phase values.

5.5 Discussion

5.5.1 The effects of pineal removal on spawning time in salmonid fish

The experiments in this chapter indicate that the pineal gland in salmonids has the ability to influence maturation in female rainbow trout. Whether this is through endocrine or neural pathways is as yet unclear. However, as melatonin implantation (although at a supra-physiological level) had no significant effect on the timing of spawning in rainbow trout, this may suggest that the pineal may act on the reproductive axis by means of a neural pathway.

Pinealectomy performed on the summer solstice was effective in producing a 6 week delay in ovarian maturation in rainbow trout. This provides compelling evidence that the pineal gland is involved with the timing of seasonal reproduction in salmonids. The mechanisms by which the pineal and/or melatonin control the spawning time in rainbow trout are still uncertain and are open to several lines of interpretation. These are discussed in detail below.

The results obtained from section 5.4 are consistent with the hypothesis that the lack of a pineal gland is interpreted as a period of continuous light (LL). Almost all previous photoperiod studies in which rainbow trout were exposed to a constant long day or LL photoperiod reported a 6 to 8 week delay in spawning (Allison, 1951; Shiraishi and Fukuda, 1966; Bromage *et. al.*, 1984, Bromage and Duston, 1986) which is

similar to the delay observed following pinealectomy at the summer solstice (see section 5.4).

The absence of a melatonin rhythm was evident from blood samples obtained from pinealectomised fish during the mid-light and mid-dark phases of the experiments. Pinealectomy may therefore be considered as a constant photoperiod. Since the pineal in rainbow trout is known to exhibit a pattern of melatonin secretion linked directly to phototransduction (Gern and Greenhouse, 1988; Randall *et al.*, 1991) and the pattern of melatonin secretion observed after pineal removal (Figures 5.11, 5.16 and 5.21) closely resembled that of rainbow trout exposed to periods of continuous light (Figure 5.22, Alvarino *et al.*, unpublished). It is therefore reasonable to assume that the lack of a nocturnal increase in circulating plasma melatonin levels in pinealectomised fish may be interpreted as a photoperiod of continuous light.

Pinealectomy effectively abolishes any neural signal from the pineal regarding changes in photoperiod. How the brain interpretes this lack of rhythmic innervation can only be speculated from neural studies on isolated photoreceptor cells. The work of Meissl and Ekstrom (1988) and Kusmic *et. al.* (1992) showed that when pineal photoreceptor cells were exposed to periods of constant illumination there was a sustained hyperpolarisation of the receptor membrane potential. This decreased membrane conductance is thought to produce a voltage dependant synthesis and secretion of indoleamines in relation to the environmental illumination (Kusmic *et. al.*, 1992). It is possible then that the lack of neural information from the pineal may also be interpreted as a period

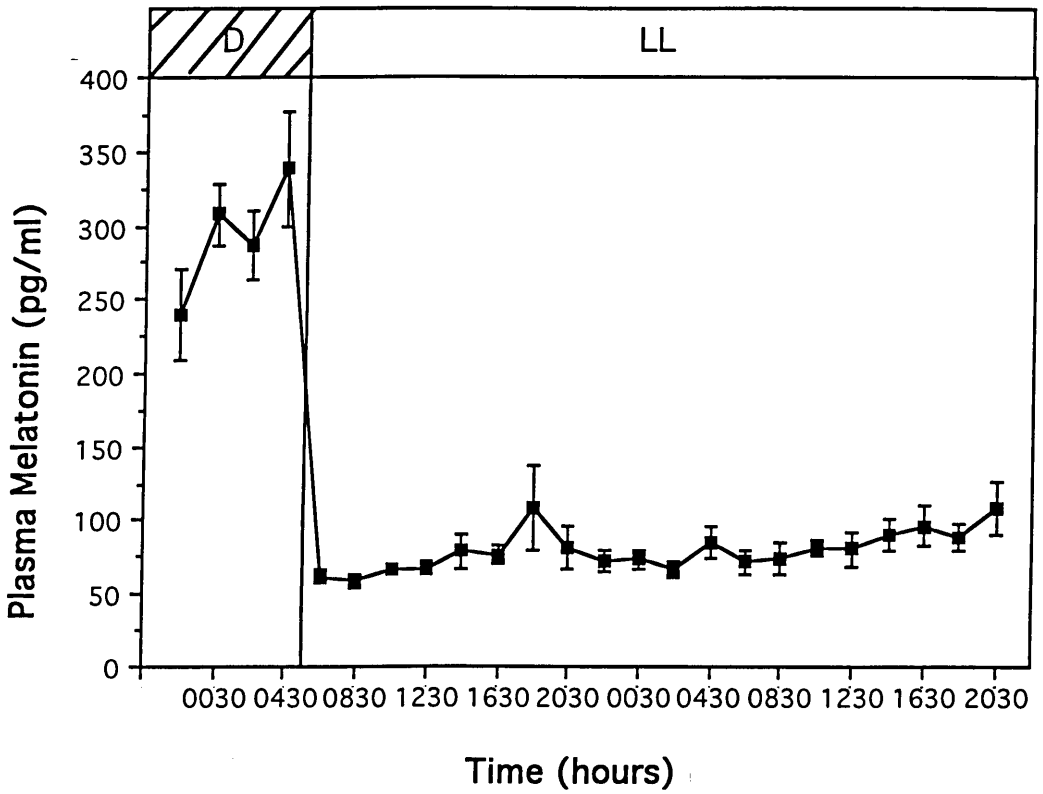


Figure 5.22

Plasma melatonin levels of rainbow trout maintained under constant darkness and constant light photoperiods. From Alvarino *et. al.*, unpublished.

of continuous light and therefore cause any biological rhythms entrained by this neural signal to free-run.

Early work by Allison (1951) reported that exposure to a constant long daylength from August 15 was responsible for a 6 week delay in the spawning time of rainbow trout. Subsequently, Shiraishi and Fukuda (1966) reported a 2-3 month delay in the maturation of sockeye salmon, brook trout, rainbow trout and amago salmon after exposure to either constant long days (16L:8D) or LL from June to February at a constant 10°C. Initially, Bromage *et al.* (1982) found no variation in spawning times of rainbow trout subjected to long day photoperiods (16:8D) from June 21. However, this work was carried out on a late spawning strain and subsequent studies have shown that long daylengths applied after the summer solstice delay gonadal development (see reviews in Bromage *et al.*, 1984, 1990; Bromage and Duston, 1986; Bromage and Cumaranatunga, 1988).

The histological study carried out by Bourlier and Billard (1984a) revealed distinct differences in gametogenesis induced by an LL photoperiod when compared to the control fish on a natural photoperiod. Male rainbow trout exhibited a marked difference in the proportion of germ cells within the testes and spermatids were present in the testicular lobules when spermiation began. The final spermiation yield was also greatly reduced in the LL males. Female trout were found to have a reduced follicular diameter and gonadosomatic index during vitellogenesis although relative fecundity and egg diameter did not vary significantly from the control group when spawning occurred 2 months after the controls. Significantly, plasma

calcium levels measured in trout pinealectomised at the summer solstice in the present work, reached a peak 1 month after the control fish. This is consistent with the delay in spawning time observed in the pinealectomised group and suggests that exogenous vitellogenesis took place 1 month later than control fish. As histology was not carried out on the maturing females, no comparisons can be made with regard to the GSI or follicular diameter during vitellogenesis.

Further studies have revealed that rainbow trout maintained under a continuous long day or LL photoperiod regime, from the summer solstice preceding the first reproductive cycle and continued until after the second cycle, showed a delay of between 1 and 2 months in the first spawning and an advance of up to 6 months for the second spawning cycle (Skarphedinsson *et al.*, 1982; Bourlier and Billard, 1984b; Scott *et al.*, 1984). Unfortunately, due to heavy mortalities after spawning, the groups in section 5.4 were not maintained for the second spawning cycle. This would have provided conclusive evidence that the absence of the pineal was being interpreted as an LL photoperiod and not merely free-running with a circannual periodicity

Drawing on this previous work, it is suggested that pineal removal at the summer solstice acts as a period of continuous light due to the abolition of either the daily melatonin rhythm or possibaly the removal of neural transmission to the brain. The alteration in spawning time would be the result of a corrective delay imposed on the reproductive cycle which is perceived to be running ahead of the endogenous circannual rhythm due to the pinealectomy being interpreted as a period of

LL (i.e. the fish perceives the lack of nocturnal melatonin or innervation from the pineal as an indication of an extended summer) and therefore delays the endogenous clock which is overtly expressed as a delay in spawning time (Bromage *et al.*, 1992; Randall and Bromage, 1992).

This explanation does not hold when considering the effects of pinealectomy applied on the change from long to short days in sections 5.2 and 5.3 which produced no significant variation in spawning time when compared to the intact fish exposed to the same long to short day change in photoperiod. It was clear that pineal removal was not interpreted as a long day in this instance, as the group maintained on a constant long day throughout the experiment matured 5-6 months after the short day groups. This apparent lack of response may be as Popek *et al.* (1992) suggested due to the pineals lack of influence over gonadal maturation during the latter stages of the cycle. In these experiments, unlike section 5.4 when gonadal development had still to be initiated, the trout had already been exposed to 3 months of constant long days to advance development of the reproductive cycle. It may be the case that the fish had reached a stage of gonadal development which was unresponsive to the influence of the pineal or that once the pinealectomies had been performed the endogenous circannual rhythm controlling the reproductive cycle was free-running.

Popek *et. al.* (1992) carried out the only previous investigation into the involvement of the pineal gland in the timing of reproduction in rainbow trout. They reported a small (2 week) delay in spawning times of trout pinealectomised 5 months prior to their natural spawning time, this is also

consistent with the pinealectomy being interpreted as continuous illumination. They observed that pineal removal 1 month before ovulation had no effect on the spawning time, this is to be expected as alterations in photoperiod at this time also have no effect. Unfortunately, no details were given as to whether pineal removal was confirmed to be successful, i.e. no dark phase melatonin measurements were taken, and no autopsies of the brain area were made on completion of the experiment. The incomplete removal of the pineal may explain why only a portion of the fish experienced delayed maturation. Of the fish that were delayed by 2 weeks, Popek et. al. (1992) suggested the pineal may act to stimulate gonadal maturation, especially during the vitellogenic stages but its influence over ovarian maturation is far less pronounced immediately preceding ovulation (during germinal vesicle migration). These suggestions were made following findings by Popek *et al.* (1991) when studying the role of the pineal in the development of carp ovaries. During this work they observed that removal of the pineal gland inhibited germinal vesicle migration resulting in a breakdown of their morphological structure. However, pinealectomy did not prevent ovulation when maturation was induced through the administration of a hypophysial homogenate.

Sections 5.2 and 5.3 in this chapter were designed to assess the importance of the salmonid pineal in transmitting information on changes in daylength. However, it appears from the results that the trout were either able to interpret the change in photoperiod despite the absence of the pineal gland, as spawning occurred over the same period as in the control

fish, or that the pineal is not essential to the completion of gonadal development at the time it was removed.

These equivocal results may also be explained by the presence of extrapineal photoreceptors; both ocular and deep encephalic photoreceptors have been identified in salmonids (see section 3.1). Holmqvist *et al.* (1994) were also able to identify specific areas of the central optic nuclei in the Atlantic salmon which received both retinal and pineal neural terminations. These sites have also been linked to areas of high density melatonin binding (Holmqvist, et. al., 1994). However, Kezuka *et al.* (1992) and Zachmann *et al.* (1992) observed that melatonin production by the retina in goldfish and whitesucker is discrete from the extraocular functions of the hormone, being instead instrumental in the synchronisation of circadian rhythms within the eye. Forester *et al.* (1994) proposed that the teleost pineal, unlike the retina, was capable of detecting spectral irradiance (see chapter 3, section 3.1 for more detailed discussion) which Groos (1981) suggested is the most appropriate spectral energy for the entrainment of endogenous rhythms to the seasonally-changing photoperiodic cycle (see also vanVeen et. al., 1976; Kavaliers, 1980; Aschoff, 1981). Consequently, it seems more likely that pineal, and not ocular, photoreception is responsible for the entrainment of the endogenous circannual clock in salmonids although, the lateral eyes cannot be ruled out as Davis et. al. (1982) reported earlier spawning times in sighted than in enucleated catfish.

It then suggests that either the lack of pineal causes a free-running of the endogenous rhythm or, as Popek (1992) suggested, that the trout in experiments 1 and 2 had reached a

stage of gonadal maturation (due to the 3 months of previous long days) where the influence of the pineal was less pronounced.

Previous findings from pinealectomy studies carried out on goldfish have all found that the effects of pineal removal are very much dependant on the time of year, the ambient photoperiod and stages of the reproductive cycle. Delahunty *et al.* (1978) reported that pineal removal significantly decreased goldfish serum K^+, Ca^{2+} and PO_4^{3-} ions but only during late February. While deVlaming and Vodicnik (1979) reported that liver and hepatic glycogen levels could be reduced and plasma lipid and glucose levels were increased depending on the time of year and ambient photoperiod under which pinealectomy was carried out. Thyroid hormones (T3 and T4) were also observed to be under pineal control during specific periods of the reproductive cycle (Nayak and Singh, 1987).

These factors are also critical to the outcome of pinealectomy on reproduction. The goldfish has been shown only to be responsive to pineal removal from late summer to early winter, during this period its effect can be either stimulatory or inhibitory depending upon whether the fish were under short or long day photoperiods, respectively (deVlaming and Vodicnik, 1978; Vodicnik *et al.*, 1978). Similar observations were made in catfish (Garg, 1988), grey mullet (Abraham and Sagi, 1984) and golden shiner (deVlaming, 1975). This agrees with the findings of the present study on rainbow trout which again, primaraly appear to be dependant on the stage of gonadal development when the pineal is removed.

In her review, Baggerman (1985) proposed the presence of a photo-reactivity threshold (PRT) in sticklebacks based on her 30 years of study with the species. The PRT is based on what Saunders (1982) referred to as a critical daylength below which the daily light phase has no effect on the reproductive cycle.

Using photoperiodic response curve experiments, Baggerman (1978) found that, like many birds, sticklebacks will not breed during the autumn months. This she attributed to the PRT being at such a level that even long day photoperiods could not overcome the threshold. Chan (1976) also suggested the presence of a PRT in the medaka which, as in Baggerman's theory, oscillates over the 12 month reproductive cycle producing periods when gonadal development may be stimulated or refractory to the same photoperiod. Finally, Baggerman (1980, 1982) found the PRT to be temperature dependant and so suggested in her 1985 review that seasonal breeding in the stickleback was based on an endogenous annual cycle of increasing and decreasing PRTs, the amplitude of which was determined by the ambient water temperature as her previous findings showed that a decrease from 20 to 15 ^{o}C significantly reduced the PRT.

Although Baggerman's PRT does not explain all the results reported in this chapter, it does introduce the idea of refractory periods within the seasonal response to the changing photoperiod. Duston and Bromage (1988) also proposed that rainbow trout may experience 'dead zone' periods when reproductive rhythms are unresponsive to entrainment by photoperiods. This, they termed gating mechanisms and

suggested its close association with the internal clock, which at specific times of the 12 month cycle is at the gate-open phase and allows fish having reached a specific stage in their reproductive development to proceed onto full maturation. Those fish failing to reach the threshold stage of development would then forgo spawning for that year. By altering the external zeitgeber (photoperiod), a phase shift in the internal clock is produced which in turn alters the timing of the gate in relation to the developmental stage.

The results of the experiments in this chapter provide contradictory evidence as to the pineal's role in controlling reproductive function in salmonids. It is possible, however, that a gating mechanism is involved allowing the pineal to exert an influence over gonadal development and possibly effecting a phase-shift in the internal clock, but only during specific stages of the reproductive cycle. It may be the case that the long days at the beginning of experiments 1 and 2 were sufficient to allow the fish to reach a stage of reproductive development whereby the pineal gland or brain was experiencing a period of unresponsiveness when the change from long days to short days occurred. Moreover, the lack of a pineal would be of little consequence to the spawning time of the groups providing the change in photoperiod from long to short days could be detected and acted upon by ocular or extrapineal photoreceptors. A further possibility is that the effects of pineal removal were interpreted as a period of constant illumination. Although this agrees with the findings of section 5.4 it does not explain why the pinealectomised groups in sections 5.2 and 5.3 did not exhibit a similar delay in the

spawning time as shown by the fish maintained on constant long days.

A more probable explanation is that pinealectomy caused the reproductive cycle to free run. In section 5.2, there apppeared to be a small delay in the spawning time of the pinealectomised fish when compared to the other fish exposed to long and short days. This delay did not prove statistically significant (after the removal of outliers) when compared to the sham-pinealectomised group. However, the distribution of the spawning profile for the group suggests a desynchronisation of the spawning times within the pinealectomised group relative to the sham-pinealectomised group. One explanation for this may be that the long days experienced at the start of the trial were sufficient to initiate and advance the early stages of gonadal development due to a phase shift of the internal clock in relation to the SNP groups. This has already been demonstrated by a number of authors (Bromage *et al.*, 1984, 1992; Takashima and Yamada, 1984; Bromage and Duston, 1986; Bromage and Cumaranatunga, 1988) who also suggested that short days during the latter stages of maturation may further advance and synchronise spawning times. In sections 5.2 and 5.3, once the change from short to long days had taken place, the intact fish were advanced as expected. However, it is suggested that the pinealectomised fish were by now exhibiting a free-running endogenous circannual rhythm due to the lack of pineal entrainment but were still advanced in comparison to the SNP groups due to the initial long day photoperiod. However, they will have lacked the synchrony of the intact fish. The long day hold fish, although initially advanced, were then delayed

during the latter stages of reproduction by the constant long day photoperiod, agreeing with the findings of Scott *et. al.* (unpublished, quoted in Bye, 1984) for rainbow trout with a natural spawning time in March.

Consideration must also be given to the fact that ambient temperatures were used during the course of all these experiments which, in the case of fish maintained at Buckieburn Research Unit, included temperatures of 1°C for significant periods of time during the winter months (see Figure 2.1). Similar winter temperatures were also observed within the recirculation system used in experiment 5.4, although, being a small enclosed body of water, the temperature fluctuations in this system were closely correlated to the ambient air temperatures which often resulted in summer water temperatures of up to 20°C. Low water temperatures, below 4°C, have been shown to inhibit ovulation in certain strains of rainbow trout (Morrison and Smith, 1986; Nakari *et al.*, 1988) and, although this is obviously not the case in sections 5.2 and 5.3, the effects of low water temperatures are known to delay gonadal development and spawning time by up to 1 month (Davies and Bromage, 1991). This reduction in the rate of development appears to be due to the direct effects of low temperatures on biological processes. The effects of temperature may therefore be exerting a bias on the spawning distribution between groups and although the results indicate that the South African strain of trout used is a late spawning strain (January-April), this may not have been the case under more constant temperature conditions. Consequently, the time interval between spawning in the advanced and delayed groups

cannot solely be attributed to the effects of photoperiod, as temperature will influence the maturation process.

To summarise, the results of pinealectomy experiments in this chapter indicate that the removal of the pineal gland in rainbow trout does influence the spawning time of this species. However, the mechanisms underlying the interaction of the pineal with the hypothalamic-gonadal axis are still unclear and will require further research. It is suggested that the pineal may act within a 'gating' mechanism resulting in periods when its influence may stimulate or unaffect the entrainment of the reproductive systems during the annual cycle. As yet, whether ambient photoperiod, time of year or stage of gonadal development determine the response to pineal removal in the rainbow trout is unknown. During unresponsive periods it appears the reproductive cycle free-runs if the pineal is removed with any previous phase shifts of the endogenous clock being maintained. If the pineal is removed during a stimulatory phase it would appear from experiment 3 that its absence is interpreted as a period of continuous light, possibly due to the daily melatonin rhythm being replaced with a constant low amplitude signal.

5.5.2 The results of melatonin implantation on the spawning time of salmonid fish

Considering a low amplitude melatonin rhythm may be interpreted as a period of continuous light, it could then be presumed that a high level of circulating melatonin may well have the effect of mimicking a period of constant darkness or a

constant short daylength. In fact, the effects of melatonin administration in teleosts have been shown to vary enormously depending upon the ambient photoperiod, sex and species of fish, stage of the reproductive cycle when administered and the concentration of melatonin used (see section 5.1). Table 5.4 presents a summary of other studies on the administration of melatonin in fish.

In contrast to the work of Urasaki (1972) and Joy and Agha (1991) in which daily intraperitoneal injections were used to study the effects of melatonin on gonadal development, the administration of constant release melatonin implants in section 5.3 was not interpreted as a period of constant short days. This became apparent as the long day implanted trout did not experience the advance shown in the fish transferred to a constant short day and instead exhibited the delayed spawning time observed in the long day sham-implant group. Furthermore, the melatonin implants did not appear to be interpreted as continuous darkness as this would have been expected to cause the reproductive cycle to free-run in a similar way to that which is thought to have occurred in the pinealectomised fish. However, it could also be argued that both the continuous long day photoreriod and melatonin implants caused a desynchronisation in the reproductive cycle and therefore both groups experienced a free-running rhythm after May 4th so masking the effects of the implants.

HYPOTHALAMUS	SPECIES	REFERENCE
Altered serotonergic and monoamine oxidase activity, depending on photothermal conditions and season	*Carassius auratus*	Olcese *et. al.*, 1981
Altered daily rhythm of monoamine oxidase	*Channa punctatus*	Khan and Joy, 1987
Changed 5-HT rhythm, depending on photoperiod and temperature	*Channa punctatus*	Khan and Joy, 1988
ENDOCRINE SYSTEM		
Increased size of pituitary gonadotropes	*Carassius auratus*	Fenwick, 1970
Reduction of pituitary prolactin activity	*Fundulus similis*	deVlaming *et al.*, 1974b
Inhibitory action on hypothalamo-hypophysial-ovarian system	*Heteropneustes fossilis*	Sundararaj and Keshavanath, 1976
Altered GTH levels in plasma, depending on time of injection and season	*Carassius auratus*	Hontela, 1984
Inhibitory effects on sex steroids and thyroid hormones during prespawning periods and depending upon dose level	*Clarius batrachus*	Nayak and Singh, 1987
GONADS		
Inhibitory effect upon gonad size	*Carassius auratus*	Fenwick, 1970
Inhibited gonad growth	*Oryzias latipes*	Urasaki, 1972,1976
Inhibited vitellogenesis and induced follicular atresia in the ovary during the prespawning period	*Heteropneustes fossilis*	Sundararaj and Keshavanath, 19,76
Inhibited gonad activity	*Fundulus similis*	De Vlaming *et .al.* 1974a
	Mystus tengara	Saxena and Anand, 1977
	Gasterosteus aculeatus	Borg and Ekstrom, 1981
No effects on ovarian activity or on vitellogenin levels	*Heteropneustes fossilis*	Garg, 1989
Inhibited ovarian vitellogenesis and induced atresia	*Heteropneustes fossilis*	Joy and Agha, 1991
INTRAPINEAL FUNCTION		
Modulation of neural activity	*Oncorhynchus mykiss*	Meissl *et .al.*, 1990

Table 5.4 Effects of melatonin in fish (Adapted from Zachmann *et al.* 1992)

It may also be the case that the implants merely raised the 'baseline' of the daily melatonin rhythm (Figure 5.16) which still allowed the constant long day photoperiod to be represented as a light/dark cycle in the melatonin profile. From the work presented in chapter 3 and the previous findings of Max and Menaker (1992) it has already been shown that it is the difference in amplitude between the light and dark melatonin profiles that distinguish the difference between night and day. Therefore, as there was a significant difference between light and dark phase levels of melatonin, it is possible that the fish could still 'read' the daylength. The reason for the significantly lower levels of melatonin shown in the short day sham-pinealectomy plus melatonin implant group is unknown but could be attributed to either: contamination introduced into the sampling procedure; radioimmunoassay contamination during analysis; or a reduced concentration of melatonin within the implants used in this group. Of these, the latter explanation seems the most likely as the standard error of the mean for the samples was low (±80 pg/ml dark phase and ±6 pg/ml light phase) and no abnormalities were found in any subsequent samples.

It would seem then that the melatonin implants did not produce any significant change in the spawning time in any of the implanted groups. This may indicate that the fish could still recognise the daylength, as light and dark phase melatonin levels were significantly different, or may possibly be due to: the timing of the implant in relation to the stage of gonadal development; the ambient photoperiod; or concentration of melatonin released from the implants. These results agree with

the findings of Nash *et. al.* (1995) who also found no alteration in the spawning times of rainbow trout. They also administered intramuscular melatonin implants, at various times of the year, which elevated plasma melatonin levels, but only for 8 weeks after implantation. They also repeatedly implanted at 2 month intervals to trout maintained under a natural photoperiod but in none of these experiments did they find any alteration in spawning time. Reiter (1988) proposed that the duration of the nocturnal increase in plasma melatonin was responsible for the perception of daylength, but in the case of constant release implants this interpretation of daylength is being denied to the fish. This lends support to recent work on mammals suggesting that a melatonin-free interval may also be required (Maywood, *et. al.*, 1990; Hastings *et. al.*, 1991). As with pineal removal there may be a gating mechanism or periods during the reproductive cycle when the endocrine system becomes insensitive to the daily rhythm of melatonin secretion from the pineal.

Further investigations are needed before any assumptions can be made as to the importance of melatonin to the timing of reproduction in rainbow trout. However, using alternative concentrations of melatonin applied at different times of the reproductive cycle and under varying photoperiods may result in the elucidation of the correct combination of factors required to obtain a response.

As there appeared to be no effect of externally administered melatonin in rainbow trout, it is possible that the pineal acts upon the reproductive system through neural signals. In this case melatonin implants would not be expected

to have a significant effect on salmonid reproduction as pineal photopreceptor cells would still be respondiong to the daily change in illumination and so would maintain their daily neural rhythm. The evidence for pineal innervation and its role as a photoneuroendocrine transducer is discussed in detail in section 3.1 and has been well documented by Ekstrom and Meissl (1990) and Korf (1994). However, the fact that the pineal of many teleost species is endowed with an endogenous rhythm of melatonin secretion and also that the daily rhythm of plasma melatonin in the rainbow trout precisely matches the light/dark cycle, suggests its importance as an internal zeitgeber. Considering the evidence regarding the role of melatonin within biological systems of fish (Table 5.4) and other vertebrate species (Urasaki et. al., 1972; Sundararaj and Keshavanath, 1976; Saxena and Anand, 1977; deVlaming, 1980; Borg and Ekstrom, 1981; Joy and Agha, 1991; Joy and Khan, 1991) it seems likely that melatonin plays an important physiological role in teleosts.

5.5.3 The effects of pinealectomy on the vitellogenic increase in plasma calcium levels in rainbow trout.

The variations in plasma calcium levels observed during the experiments in this chapter do not appear to have been reported elsewhere by other authors. It would appear, from the results of sections 5.2 and 5.3, that pinealectomy caused a delay in the peak calcium concentration within the blood plasma. In section 5.4, this occurred 4 weeks after the control groups reached a mean maximum value which is approximately

the same delay experienced between peak spawning times. However, the pinealectomised fish in experiment 1 reached a maximum value 8 weeks after the intact control group despite there being no significant variation in their spawning times, possibly indicating a desynchronisation of the spawning profile in the pinealectomised fish. It is unknown whether vitellogenin uptake by the oocytes was influenced by the absence of a pineal. Bourlier and Billard (1984) found a reduced follicle diameter and gonadosomatic index during vitellogenesis in trout exposed to LL photoperiods. Alternatively, the reduced amplitude of the calcium profiles in the present study may simply be a consequence of the later maturation times and therefore vitellogenesis occurring when ambient water temperatures are lower. This was observed by (Tyler *et al.*, 1987) who reported reduced Ca^{2+} uptake by cultured ovarian follicles during vitellogenesis at low temperatures and suggested temperature to have a direct physiological effect on reproductive development.

The calcium values in experiment 2 do not provide a reliable representation of the actions of pinealectomy and or melatonin implantation on reproductive development as the % maturation within each group was so low. This is probably related to the low food ration on which the fish were maintained during the summer months because of the drought conditions. It appears that many of the fish 'decided' to forgo spawning in their second year as the majority of individuals remained immature. Only the SNP and long day groups seemed unaffected by the reduced feeding, presumably due to their immaturity over the summer months, whereas the short day

groups would already have 'decided' whether to reproduce or forgo reproduction and continue somatic growth.

To summerise the results of chapter 5, it is proposed that the pineal gland in female rainbow trout acts as a photoneuroendocrine transducer relaying information from the external environment to the reproductive axis. It appears that, preceding and during gonadal development, the brain undergoes periods of sensitivity and unsensitivity towards pineal-mediated entrainment of the endogenous rhythm controlling the timing of spawning. This was confirmed in section 5.4 when pineal removal at the summer solstice resulted in a 6 week delay in the spawning time. It is suggested that the absence of the pineal gland was interpreted as a period of continuous light by the trout. Pineal removal immediately prior to a change in photoperiods produced an apparent desynchronization in the spawning profile of the group. In this case, it is suggested that in the absence of the pineal the brain may be refractory to external stimulation allowing the endogenous circannual rhythm of reproduction to free-run. However, we can only speculate as to how the brain interprets the complete absence of pineal photoperiodic information. It may be the case that in the absence of the pineal extrapineal photoreceptors are able to provide the nessessary entrainment. The administration of constant release melatonin implants produced no variation in spawning times, however, as in other species, this may be due to: the stage of gonadal development when applied; the concentration of melatonin used; the ambient photoperiod; or ambient temperature. Another possibility is that, as Hastings *et al.* (1991) suggested, a melatonin-free interval is required to

allow successive melatonin signals to be 'read' relative to a base line value.

This study clearly demonstrates that the pineal gland exerts an influence on the reproductive cycle of salmonids. However, more work is required before we can fully understand the mechanisms behind its actions.

Chapter 6

Conclusions and Suggestions for Future Work

The primary aim of this thesis was to investigate the role of the pineal gland and melatonin in the entrainment of endogenous circannual rhythms which co-ordinate the timing of seasonal events in salmonids. These events included gonadal maturation in the female rainbow trout and smoltification in the Atlantic salmon. Chapter 3 examined the effects of temperature, light intensity and an absence of exogenous stimulation on the melatonin rhythm in isolated rainbow trout and Atlantic salmon pineals *in vitro*. This chapter also included *in vivo* trials into the source of circulating plasma melatonin using pinealectomy and enucleation techniques. Chapter 4 detailed the effects of pinealectomy and constant release melatonin implants on the timing of smoltification and growth rates in immature Atlantic salmon parr. Pinealectomy and melatonin implantation were also performed during the experiments in chapter 5 which were designed to assess the importance of the pineal gland in the timing of maturation in the female rainbow trout. This chapter summarises the main findings of this work and suggests further studies within these fields of investigation.

The work undertaken in chapter 3 investigated the rates of melatonin secretion by the salmonid pineal glands in response to environmental stimulation from both temperature and light intensity. Both light and dark phase melatonin levels were significantly reduced at lower temperatures, probably as a result of temperature dependant cAMP formation at an earlier stage of the biosynthetic pathway (reviewed by Zachmann *et al.*, 1992). With this in mind the optimum temperature for salmonid pineal culture was found to be 10±1.5°C, as this combined a consistently

high level of melatonin secretion with acceptable longevity of the organ.

Illumination studies on isolated salmonid pineal glands revealed its acute sensitivity as a photometer, observed through the significant variation in levels of melatonin synthesis at light intensities varying by only 0.5 lux. From this it is now clear that the pineal exhibits differential melatonin synthesis in response to varying intensities of illumination. Furthermore, in response to Gern *et al.*'s (1992) question "Does light inhibit or darkness permit melatonin production" it is suggested that darkness permits melatonin synthesis, but that the amplitude of dark phase melatonin levels is dependant upon the irradiance power of the light phase.

Observations made in section 3.2.4 conflict with those of Gern *et al.* (1992) who reported no melatonin inhibition at wavelengths above 675nm. In contrast, *in vivo* and *in vitro* results presented in this thesis showed melatonin levels were significantly reduced by light at wavelengths between 660-800nm. Obviously as the lateral eyes and deep encephalic photoreceptors were intact during spectral studies *in vivo* their involvement in melatonin inhibition, although unlikely, cannot be ruled out. However, work on isolated pineal glands clearly discounts interference from other photoreceptor populations (retinal or deep encephalic photoreceptors). Interestingly, melatonin inhibition was significantly lower under red light than white light of the same intensity, suggesting that only specific photoreceptors containing photopigment concentrations responsive to longer wavelengths were inhibited. Clearly, more work in this area is required, covering an increased spectral

range and possibly using several age groups of fish, as it has been suggested that spectral variations may exist between mature and juvenile salmonid pineals (Bowmaker *et al.,* 1991; Thorpe and Douglas, 1993).

Rainbow trout pineal glands maintained in the absence of an external stimulus revealed no endogenous component to the daily melatonin rhythm, consistent with previous findings (Gern and Greenhouse, 1988; Begay *et al.*, 1992; Max and Menaker, 1992). This indicates a direct relationship between melatonin inhibition and light phase illumination. Similar studies on the Atlantic salmon pineal did not produce a pattern of secretion similar to that of the rainbow trout. Indeed, the melatonin profile observed closely resembled that of the pike (J.Falcon pers. comm.) suggesting for the first time that the salmon pineal *in vitro* does possess a population of endogenous oscillators within the pineal photoreceptors. Further work in this area should include the maintenance of the pineals under a range of temperatures as Boillet *et al.* (1994) discovered this to have significant consequences for the expression of the endogenous rhythm. It may also be possible to increase the clarity of the rhythm in culture through entrainment to alternative photoperiod regimes prior to decapitation. Furthermore, by increasing the time the pineal is maintained in culture the greater the opportunity for the pineal has to express an endogenous regulation of melatonin synthesis.

Finally, as the opportunity arose to obtain the pineal glands from Atlantic halibut they were also examined under culture conditions. This provided the first evidence of rhythmic secretion of melatonin from the Atlantic halibut pineal. Additionally the pineals maintained under constant darkness displayed a clear

endogenous rhythm of melatonin synthesis. It is suggested that this may be an adaptation to its environment as it spends much of its time below the photic zone. Consequently, an endogenous rhythm could be invaluable in maintaining an internal synchronisation in the absence of external cues.

The latter half of chapter 3 was primarily designed to identify the contribution of pineal and retinal melatonin to the daily variation in plasma melatonin levels in rainbow trout and Atlantic salmon. This was achieved through the development of a technique for removing the pineal from salmonids (Porter *et al.*, 1996) and comparing plasma melatonin levels both before and after pinealectomy. The results of *in vitro* studies revealed that the pineal tract (*tractus pinealus*) was also capable of producing a rhythmic secretion of melatonin, indicating that, complete removal of the pineal end vesicle plus the pineal tract was necessary to abolish the rhythmic secretion of melatonin.

The results obtained from these experiments clearly show the pineal to be the main source of nocturnal melatonin in salmonids, and are in greement with the previous findings of Gern *et al.* (1978) on rainbow trout and Kezuka *et al.* (1992) on goldfish. Bilateral-enucleation in conjunction with pinealectomy further reduced plasma melatonin levels indicating a pathway for retinal melatonin to enter the general circulation (Porter *et. al.*, 1995). However, even with the eyes and pineal removed there remained a significant level of melatonin within the blood, which in the case of the rainbow trout still exhibited a light/dark variation; this was also reported in rainbow trout by Gern *et. al.*(1978). The origin of this melatonin is unknown and clearly needs further investigation. Whether the deep encephalic

photoreceptors, as with those of the lateral eyes and pineal, are capable of synthesising melatonin is still unknown. Also, the Harderian gland and gastrointestinal tract have been identified as sites of secretion in higher vertebrates (Reiter *et al.*, 1981; Heuther, 1993), but as fish do not posses a Harderian gland the intestine should be considered in future *in vitro* work.

Chapter 4 investigated the effect of melatonin administration and removal of the pineal gland on the development of salmon parr and the parr-smolt transformation. Initial work concentrated on the participation of melatonin and the pineal gland in the timing and synchronisation of smoltification. However, preliminary results from these studies suggested that melatonin may influence growth rates in juvenile salmonids during particular times of the year and/or within specific age classes of fish. This prompted further trials which concentrated on the growth effects of melatonin and their consequences on the ratio of recruitment into the upper modal group of potential S1 smolts within a population.

This work provided conclusive evidence that constant release melatonin implants administered within the first 5 months post-hatch, significantly increased the growth rates of Atlantic salmon parr. It is also suggested that this growth advantage contributed to 92% of the implanted fish undergoing smoltification after their first winter compared to only 40% of the controls. It must, however, be noted that the melatonin implants administered in section 4.3 resulted in fish of below average weight, whereas the removal of the pineal gland produced an opposite effect. The disparity in results may be attributed to a number of factors. Firstly, the large variation in plasma

melatonin levels exhibited between the parr in the two experiments must be considered. This revealed plasma melatonin levels within the fish which displayed an increased growth to be 165 times greater than normal physiological levels and 5 times greater than the implanted fish in section 4.3 in which a decreased growth rate was reported. It is suggested that the increased levels exhibited by fish in section 4.4 were concomitant with their lower body weight. A similar relationship between body size and circulating melatonin levels was reported by Gern *et al.* (1978) and Randall (1992).

Age may also influence the effects of melatonin administration. The increased growth was observed in fish with a mean weight of approximately 5g, which may indicate a period of increased responsiveness to melatonin stimulation at this early stage of development. Interestingly, Fenwick (1969) observed that juvenile salmonids exhibit higher pineal melatonin levels than mature individuals, emphasising a possible developmental requirement in juveniles. Smythe and Lazarus (1974) reported a sharp increase in GH immediately following melatonin administration in mammals which suggests that melatonin has a functional significance to vertebrate development.

These preliminary experiments suggest a number of areas for future research. In particular, the influence of melatonin on salmonid growth rates should be investigated, including the administration of varying concentrations of melatonin (preferably at nearer physiological levels) to a range of age groups. Unfortunately, growth hormone levels were not measured over the course of the experiments in chapter 4. As this may help in our understanding of how melatonin affects salmonid growth it is

essential that regular measurements of GH, and possibly GnRH, are incorporated into future experiments. It is also suggested that the melatonin is administered at different times of the seasonal cycle and under a variety of photoperiod regimes.

Neither pinealectomy nor implantation of Atlantic salmon parr on the summer or winter solstices resulted in a significant variation in the timing of smoltification. As the ambient photoperiod is known to be the primary environmental cue in the synchronisation of smoltification this would suggest that either the pineal and/or melatonin are not involved in the interpretation of photoperiod or that in their absence the fish were able to receive photic information via alternative photoreceptors, i.e. the lateral eyes or deep encephalic photoreceptors. It must, however, be emphasised that previous authors have found pineal involvement in the entrainment of endogenous rhythms to occur at very specific stages during the annual cycle, and to be dependent on the photoperiod at the time of the operation (Vodicnik *et al.*, 1978; Abraham and Sagi, 1984; Khan and Joy, 1990). It has also been suggested that, in order for the constant melatonin signal supplied by the implants to have a significant effect, a daily melatonin-free interval is required. This was reported to occur in Djungarian and Syrian hamsters, which, unlike the sheep, require a period of low amplitude melatonin within each 24 hour cycle (Lincoln and Ebling, 1985; Maywood *et al.*, 1990; Hastings *et. al.*, 1991). Consequently, the development of tecniques to allow timed infusions of melatonin should be considered in future work.

As the precise timing of smoltification is difficult to measure precisely, future experiments may benefit from using a photoperiod which would advance the time of smoltification and

thereby provide an indication of whether pinealectomy or implantation produces a free-running circannual rhythm, which in the present work may have enabled the smolts to acquire sea water tolerance at a similar time to the controls. Greater numbers of individuals would also allow pinealectomies to be performed alone or in combination with melatonin implants, before the artificial photoperiod is applied, or at the point of change from long to short daylengths. This would also allow larger numbers of fish to be sea water tolerance tested and permit the effects of pinealectomy and melatonin implants to be studied once the fish had been transferred to sea water sites.

Finally, one recurrent feature of melatonin implanted parr and smolts was their varied osmoregulatory ability. It is suggested this may occur through the action of arginine vasotocin (AVT). This acts as a posterior-pituitary neurohormone which alters osmoregulation in salmonids by its action on pituitary, kidney and gill cells. Interestingly, AVT has been detected in the teleost pineal (Binkley, 1988) and its mammalian equivalent (vasotocin) is synthesised within the mammalian pineal (Olcese *et al.*, 1993). This has led both Binkley (1988) and Kulczykowska (1995) to suggest a negative feedback loop between melatonin and AVT in fish as a similar system has already been identified in rats (Binkley, 1988).

Chapter 5 studied the effects of melatonin administration and the pinealectomy on the spawning time and fecundity in rainbow trout. This work clearly showed that the pineal gland exerts a significant influence on the spawning time of female rainbow trout. Pinealectomy performed at the summer solstice resulted in a 6 week delay in ovarian maturation. This delay in

spawning time is consistent with the results of previous studies in which constant long days or LL were applied from the summer solstice (Bromage *et al.*, 1984; Bromage and Duston, 1986) and suggests that the lack of a pineal signal is perceived as a period of constant light (LL) by the fish. This theory is supported by the pattern of melatonin secretion observed in the pinealectomised fish which closely resembles that of rainbow trout maintained under an LL photoperiod (Alvarino *et al.* unpublished). Unfortunately, due to mortalities after spawning it was not possible to maintain the pinealectomised trout for a further spawning cycle. This may have confirmed whether the absence of the pineal was being interpreted as a period of LL as similar studies using LL, instead of pineal removal observed a 1-2 month delay in the first spawning cycle followed by a 6 month advance of the second spawning cycle (Skarphedinsson *et al.*, 1982; Bourlier and Billard, 1984; Scott *et al.*, 1984). In light of these findings it is important that, should this experiment be repeated, the fish are maintained at least until their second spawning cycle is completed in order to determine whether pinealectomy evokes the same response as an LL signal.

Although difficult, the maintenance of the fish on a constant temperature regime would be a great advantage to the interpretation of the results in almost all studies involved in circannual events. Despite the evidence promoting photoperiod as the primary environmental cue responsible for the synchronisation of reproduction and for that matter smoltification in salmonids it is still possible that temperature plays an important role in these events. It is suggested that the direct effects of temperature on reproductive development

(section 5.3) may have resulted in ovulation being prevented until temperatures rose above approximately 8°C. Figure 6.1 provides a simplified model illustrating the possible pathways for the transmission of environmental information to the reproductive axis of teleosts.

In the present work it would appear that the removal of the pineal at the summer solstice was perceived as a LL signal either through a lack of a rhythmic melatonin secretion or possibly because of an absence of neural transmission to the brain. Whichever of these is the case the fish seemed to interpret this as a continuation of the long day summer photoperiod, i.e. the endogenous clock was perceived to be running ahead of time. In response the fish then elicited a phase-delay in the endogenous clock which was observed overtly as a delay in gonadal development in order to ensure that the reproductive cycle if synchronised with the subjective time of the year.

In the remaining experiments in chapter 5 the absence of the pineal was clearly not perceived as a long day or LL photoperiod. Indeed no statistically significant variation was observed following pineal removal or implantation with melatonin although there was a tendency for spawning to be delayed and desynchronised in the pinealectomised fish relative to the sham-pinealectomised group. In these cases, because gonadal development had initially been advanced by a long day photoperiod, the brain may have entered a 'dead-zone' during

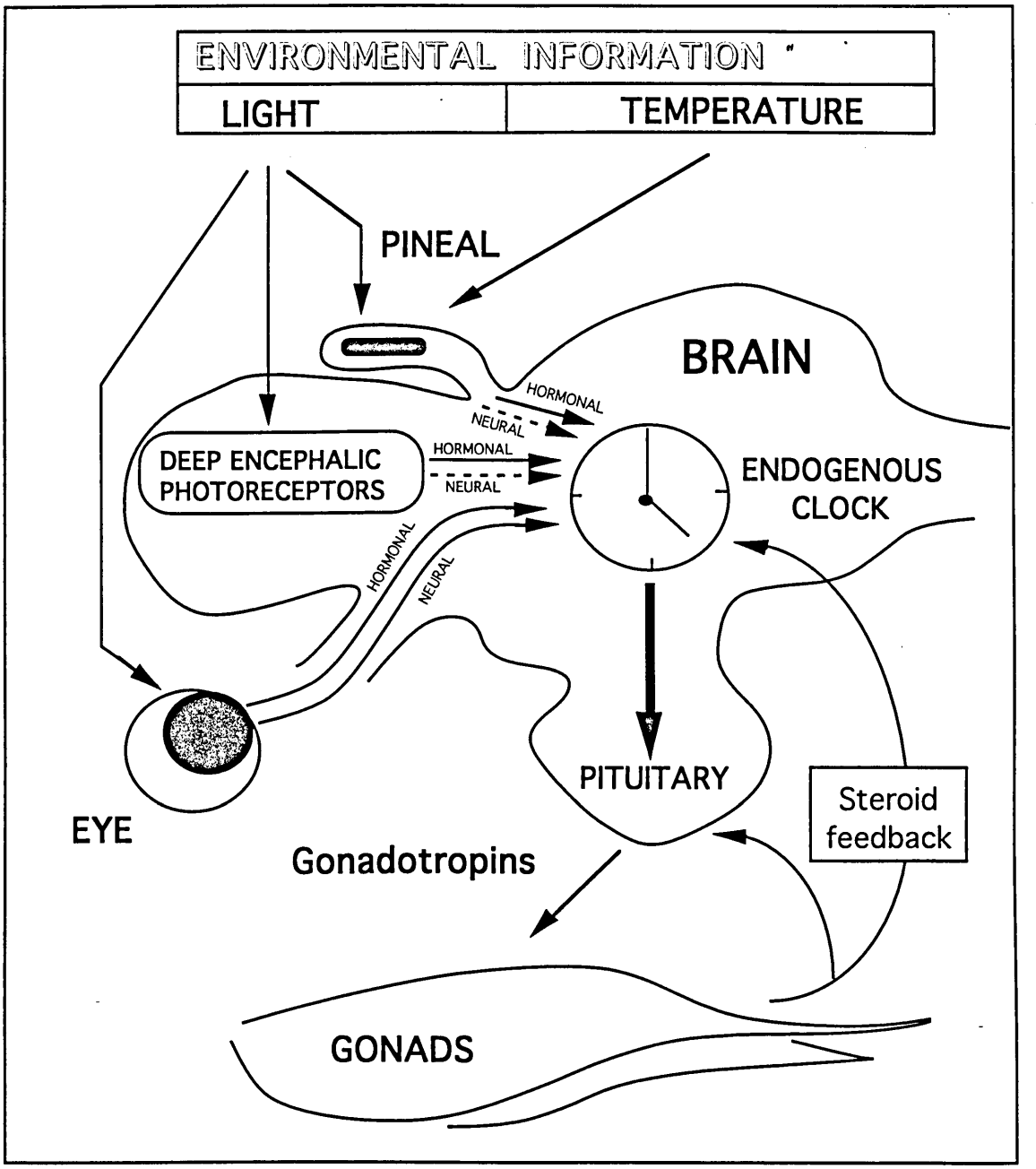


Figure 6.1

Possible endocrine pathways involved in the transfer of environmental information to the reproductive axis of teleost fish.

which phase of the annual cycle it was no longer responsive to pineal information (Popek *et al.*, 1992). It is also possible that, in the absence of the pineal, other populations of photoreceptors (lateral eyes or deep encephalic photoreceptors) are able to provide information on the prevailing photoperiod. Future experiments using 'capping' to restrict light from reaching encephalic photoreceptors via the cranial tissue may help to resolve the role of deep brain photoreceptors in fish. Previous authors have also reported varied outcomes to pineal removal in teleosts depending upon: the sex of the fish; species; stage of gonadal development when performed; and ambient photoperiod (Delahunty *et al.*, 1978; deVlaming and Vodicnik, 1978; Abraham and Sagi, 1984; Garg, 1988; Popek *et al.*, 1992, 1994). As the work presented in this study addresses only a small minority of these parameters there remains enormous potential within this area of research.

Finally, the administration of constant release melatonin implants appeared to have had no effect on the spawning time of female rainbow trout. The implants were clearly not interpreted as a period of short days as reported in several higher vertebrates and certain species of fish (Hastings *et al.*, 1991; Nash *et al.*, 1995). It is possible that the implants merely raised the basal level of melatonin, on to which, the daily melatonin rhythm was then super-imposed. As mentioned earlier the interpretation of melatonin implants by maturing rainbow trout may require the provision of a melatonin-free interval as reported in some higher vertebrates (Maywood *et al.*, 1990; Hastings *et al.*, 1991). One answer to these problems would be implants with alternating day/night release rates and/or periods

of suspended release, these are rapidly becoming a reality and would prevent the possibility of receptor down-regulation in response to constant high levels of melatonin. When implants capable of such release patterns become available it will allow a pattern of melatonin secretion to be produced which would mimic the natural rhythmic release of melatonin. This, in conjunction with pinealectomy, may finally answer some of our questions regarding the involvement of the pineal and melatonin in the reproductive cycle of fish.

The work presented here provides important advances in our understanding of the photoreceptive properties of the pineal gland with regards to melatonin production and the possible role(s) of melatonin and the pineal in the regulation of physiological and behavioural characteristics associated with salmonid development. Clearly, however, our knowledge of these systems is still far from complete.

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Appendix: Research Publications

Work presented in this thesis has been published in the following refereed articles:

Porter, M., Randall, C. and Bromage, N. (1995). The effect of pineal removal and enucleation on circulating melatonin levels in Atlantic salmon parr. In: 'Reproductive Physiology of Fish', Proceedings of the 5th International Symposium on the Reproductive Physiology of Fish., Goetz, F.W. and Thomas, P. (eds.), 2-8 July, Austin, Texas, USA., p 75.

Porter, M.J.R., Randall, C.F. and Bromage, N.R. (1996). The effect of pineal removal on circulating melatonin levels in Atlantic salmon parr. J. Fish. Biol. 48, 1011-1013.

The Effect of Pineal Removal and Enucleation on Circulating Melatonin Levels in Atlantic Salmon Parr

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Introduction

As in other vertebrates the pineal gland in salmonids is thought to use photoperiodic information to synchronize both daily and seasonal behavioral and physiological events. It is not yet clear whether this is achieved through neural or endocrine pathways. As melatonin is one of the main endocrine hormones responsible for phototransduction a simple and effective method of removing the two main sites of melatonin secretion was developed for future long-term experiments.

Materials and Methods

In study A, 30 one-year old salmon parr (Salmo salar L.) were maintained under a constant LD (12:12) photoperiod and ambient temperature. Groups of ten fish (mean weight 55.3g) were pinealectomised (pinx), sham-pinealectomised (sham-pinx) or left intact (control). The pinx and sham-pinx fish were anaesthetized and a 5mm horizontal incision was made posterior to the pineal window. A flap of tissue was then lifted anteriorly to reveal the pineal. In the pinx group the pineal stalk was cut at the point of attachment to the diencephalon and the pineal removed with forceps. The pineal was left intact in sham-pinx fish. The overlying tissue was then replaced and an orahesive powder with cicatrin antibiotic applied over the incision. Subsequent experiments on larger fish used sutures to hold the flap of skin in place. Pinealectomy plus enucleation was performed on ten individuals in study B. Enucleation was achieved by incising the cornea and severing the optic nerve to allow removal of the eye; the orbit was then cleaned and tissue glue applied to its surface.

Further work on large numbers of parr revealed a 7% mortality rate for pinealectomised fish and less than 1% mortalities in the control and sham-pinx groups. Twelve weeks after the operation a 0.5 ml blood sample was taken from each fish 3hr after lights out. Samples were taken under a dim red light (wavelength 650-800nm). Two weeks later this procedure was repeated 3hr into the light phase. Samples were stored at -70°C prior to assay for melatonin. The absence of the pineal was confirmed by dissection at autopsy 15 months after the operation.

Results

Diel fluctuations in melatonin levels were found in control and sham-pinx groups. However, after removal of the pineal gland, night-time levels of circulating melatonin were significantly reduced from 598±19.3pg/ml (means ± S.E.M.) and 612±29.7pg/ml in control and sham-pinx groups to 96±6.5pg/ml in pinx fish (Fig.1.). Photophase levels for control, sham-pinx and pinx groups did not differ significantly having means of 63.3±4.5pg/ml, 70.3±3.2pg/ml and 64.2±1.3pg/ml respectively. The enucleation study produced lower overall levels of melatonin in all groups due to seasonal fluctuations (Randall *et. al.* 1995). Day and night-time levels of melatonin in control and pinx groups were significantly different (36.0±5.5 and 38.55±3.5 pg/ml during the photophase with 289.1±31.5 and 36.71±6.1 pg/ml during the scotophase). The enucleation+pinx group revealed significantly lower levels of melatonin during both light and dark phases with 16.92±1.0 and 19.48±1.9 pg/ml respectively

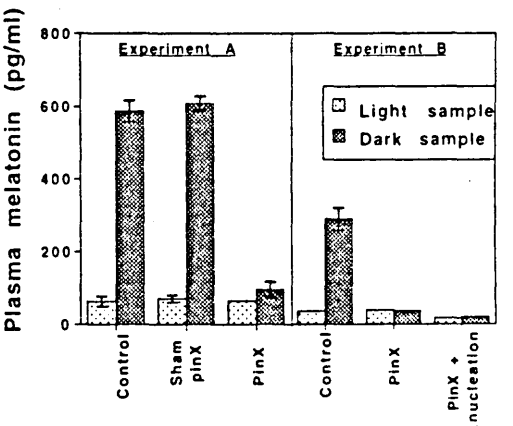

Fig.1. Plasma melatonin levels in pinX, sham-pinX, pinX+enucleated and control groups of Atlantic salmon taken during both phases of the light cycle.

Discussion

These results confirm that the pinealectomy procedure described here is an effective method of reducing nocturnal melatonin levels in young salmon. Further reductions were produced by enucleation, however, the pineal remains the principal source of circulating night-time melatonin. The origin of the remaining melatonin is as yet unknown, however, Huether (1993) identified the gastrointestinal tract as a possible site of melatonin synthesis in higher vertebrates.

Summary

Nocturnal plasma melatonin levels showed a significant decrease in pinealectomised Atlantic salmon parr in comparison with intact control and sham operated fish. Enucleation plus pinealectomy further reduced scotophase melatonin levels.

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(Supported by a BBSRC award to M.P. & N.E.R.C. grant to N.B. & C.R)

Journal of Fish Biology (1996) **48**, 1011–1013

The effect of pineal removal on circulating melatonin levels in Atlantic salmon parr

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(*Received 10 May 1995, Accepted 3 August 1995*)

Nocturnal melatonin levels showed a significant decrease in pinealectomized Atlantic salmon parr in comparison with intact control and sham-operated fish.

Key words: pinealectomy; Atlantic salmon; melatonin.

As in other vertebrates the pineal gland in salmonids is thought to use photoperiodic information to synchronize both daily and seasonal behavioural and physiological events. However, it is not yet clear whether this is achieved through neural or endocrine pathways. The production of melatonin by the pineal exhibits a distinct diel rhythm with elevated levels during the hours of darkness (Randall *et al.*, 1995). As both smoltification and maturation in Atlantic salmon *Salmo salar* L., are known to be under the influence of photoperiod a simple and effective method of pineal removal was developed for future long-term experiments.

In this study 30 1-year-old salmon parr (Otter Ferry/Mowi cross) were maintained under a constant LD 12 : 12 photoperiod and ambient temperature in 1-m diameter circular tanks. The fish weighed an average of 55·3 g at the time of surgery. Groups of 10 fish were pinealectomized (pinx), sham-pinealectomized (sham-pinx) or left intact (control). The pinx and sham-pinx fish were anaesthetized in a solution of 2-phenoxyethanol (0·05% v/v; Sigma Chemical Company Ltd. Poole, U.K.). A 5-mm horizontal incision was made posterior to the pineal window which is situated within the cranial bone and clearly visible in young salmonids through the skin. A flap of tissue was then lifted anteriorly to reveal the pineal. In the pinx group the pineal stalk was cut at the point of attachment to the diencephalon, the pineal removed with forceps and the area cleaned by suction using a pipette. The pineal was left intact in sham-pinx fish. The overlying tissue was then replaced and a 3 : 1 mixture of orahesive powder (Squibb, Hounslow, U.K.) and cicatrin antibiotic (The Wellcome Foundation, London, U.K.) applied over the incision. Subsequent experiments on larger fish used sutures to hold the flap of skin in place before application of the orahesive powder.

Further work on large numbers of parr revealed a 7% mortality rate for pinealectomized fish and less than 1% mortalities in the control and sham-pinx groups. After an interval of 3 months external differentiation of the operated groups was impossible. Twelve weeks after the operation a 0·5 ml blood sample was taken from the caudal aorta of each fish 3 h after lights out. Samples were taken under a dim red light (wavelength 650–800 nm) using 1-ml heparinized syringes (ammonium heparin salt: Sigma Chemical Company) and 23-G needles. To allow complete recovery and a return to ambient stress levels from the first blood sample the parr were left for 14 days before this procedure was repeated 3 h into the light phase. Samples were stored at −70° C prior to assay for melatonin as described by Randall *et al.* (1995). The absence of the pineal was confirmed by dissection at autopsy 15 months after the operation.

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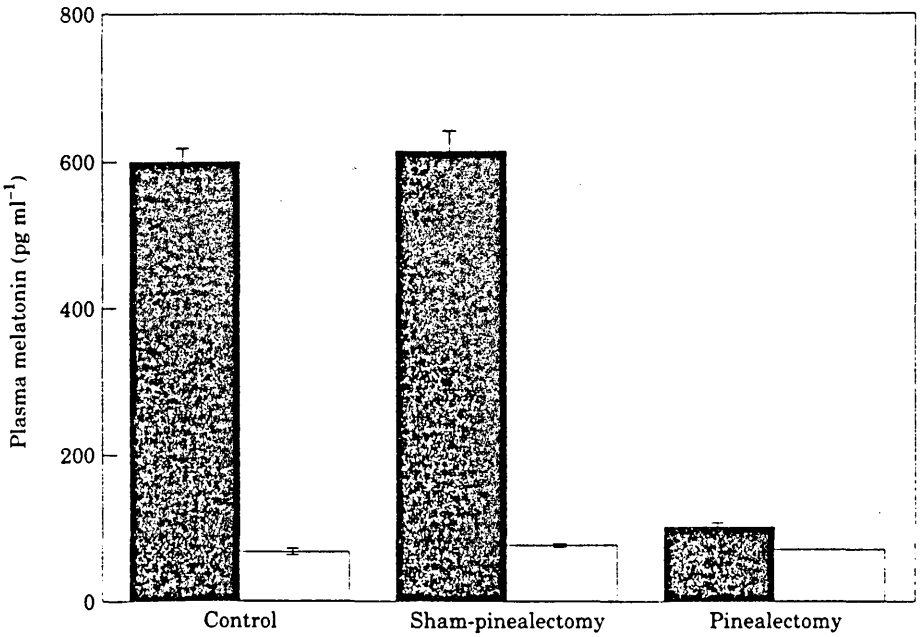

FIG. 1. Circulating plasma melatonin levels recorded during the light (□) and dark (■) phase from pinealectomized, sham-pinealectomized and intact *Salmo salar* parr.

Control and sham-pinealectomy groups exhibited diel fluctuations in melatonin levels (Fig. 1) similar in amplitude to those reported by Randall *et al.* (1995). However, after removal of the pineal gland, night-time levels of circulating melatonin were reduced significantly (Kruskal–Wallis Test, Dunn's test: $P<0{\cdot}001$) from means (± S.E.M.) of $598 \pm 19{\cdot}3$ pg ml^{-1} and $612 \pm 29{\cdot}7$ pg ml^{-1} in control and sham-pinx groups to $96 \pm 6{\cdot}5$ pg ml^{-1} in pinx fish. Similar levels of circulating melatonin were observed in the goldfish *Carassius auratus* L., by Kezuka *et al.* (1992).

Photophase levels of control, sham-pinx and pinx groups did not differ significantly (Kruskal–Wallis test) having means of $63{\cdot}3 \pm 4{\cdot}5$ pg ml^{-1}, $70{\cdot}3 \pm 3{\cdot}2$ pg ml^{-1} and $64{\cdot}2 \pm 1{\cdot}3$ pg ml^{-1} respectively. Interestingly, a significant variation (Dunn's test: $P<0{\cdot}01$) between day- and night-time levels of melatonin remained following pinealectomy; this was also reported by Gern *et al.* (1978) for pinealectomized rainbow trout *Oncorhynchus mykiss* (Walbaum).

The origin of the remaining melatonin is unknown as yet. However, Huether (1993) identified the retina and gastrointestinal tract as possible sources of extra-pineal melatonin in higher vertebrates. Rainbow trout retinae are known to produce melatonin (Gern & Karn, 1983) and Zachmann *et al.* (1992) also showed the retina to be an area of day- and night-time melatonin production in brook trout *Salvelinus fontinalis* Mitchill with maximum levels occurring during the photophase. This also agrees with findings by Falcon & Collin (1991) on the retina of the pike *Esox lucius* L. Consequently, enucleation studies are currently being undertaken to evaluate the importance of the retina as a source of circulating melatonin in Atlantic salmon.

In conclusion, these results confirm that the nocturnal increase in circulating melatonin in the Atlantic salmon is derived principally from the pineal and that the pinealectomy procedure described here is an effective method of reducing night-time melatonin levels in young salmon.

This was supported by a B.B.S.R.C. studentship to M.P. and a N.E.R.C. Research Grant to N.B. and C.R.

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